Contributors

Jack C. Bartley
Duane F. Brobst
George H. Cardinet III
Embert H. Coles
Charles E. Cornelius
W. Jean Dodds
Lars-Eric Edqvist
Delmar R. Finco
William E. Hornbuckle
Jiro J. Kaneko
John W. Kramer
Victor Perman
Mogens G. Simesen
George H. Stabenfeldt
Julia Stephens-Orvis
John B. Tasker
Bud C. Tennant
John S. Wilkinson

Clinical Biochemistry of Domestic Animals

Third Edition

Edited by

JIRO J. KANEKO

Department of Clinical Pathology
School of Veterinary Medicine
University of California
Davis, California

1980

ACADEMIC PRESS
A Subsidiary of Harcourt Brace Jovanovich, Publishers
New York London Toronto Sydney San Francisco

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data

Kaneko, Jiro J.
Clinical biochemistry of domestic animals.

Includes bibliographies and index.
1. Veterinary pathology. 2. Veterinary physiology.
3. Biological chemistry. I. Title.
SF769.K35 636.089'607 79-8873
ISBN 0-12-396350-8

PRINTED IN THE UNITED STATES OF AMERICA

80 81 82 83 9 8 7 6 5 4 3 2 1

List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Jack C. Bartley (53), Peralta Cancer Research Institute, Oakland, California 94609

Duane F. Brobst (259), Department of Veterinary Clinical Medicine and Surgery, Washington State University, Pullman, Washington 99164

George H. Cardinet III (545), Department of Anatomy, School of Veterinary Medicine, University of California, Davis, California 95616

Embert H. Coles (719), Department of Laboratory Medicine, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506

Charles E. Cornelius (201), College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610

W. Jean Dodds (671), Division of Laboratories and Research, New York State Department of Health, Albany, New York 12237

Lars-Eric Edqvist (513), Department of Clinical Chemistry, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, S-750 07 Uppsala, Sweden

Delmar R. Finco (337), Department of Physiology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602

William E. Hornbuckle (283), Department of Clinical Sciences, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Jiro J. Kaneko (1, 97, 119, 491, 649, 785), Department of Clinical Pathology, School of Veterinary Medicine, University of California, Davis, California 95616

John W. Kramer (175), Department of Veterinary Clinical Medicine and Surgery, Washington State University, Pullman, Washington 99164

Victor Perman (749), Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55108

Mogens G. Simesen (575), Institute of Internal Medicine, Royal Veterinary and Agricultural University, DK 1870 Copenhagen V, Denmark

George H. Stabenfeldt (513), Department of Reproduction, School of Veterinary Medicine, University of California, Davis, California 95616

Julia Stephens-Orvis (545), Department of Anatomy, School of Veterinary Medicine, University of California, Davis, California 95616

John B. Tasker (401), School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803

Bud C. Tennant (283), Department of Clinical Sciences, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

John S. Wilkinson (447), Veterinary Clinical Centre, University of Melbourne, Werribee 3030, Victoria, Australia

Preface

Since the publication of the first edition of "Clinical Biochemistry of Domestic Animals," veterinary clinical biochemistry has enjoyed a virtual explosion of new knowledge commensurate with the increased importance of companion animals, the livestock industry, and experimental animals. This third edition brings together some of the most important areas of clinical biochemistry pertinent to these sectors.

For this purpose, new chapters on the reproductive hormones and clinical enzymology have been added, in addition to a rewriting of the chapters on renal function and plasma proteins and extensive revisions of all other chapters. A series of appendixes designed to promote the transition from conventional units to the use of the International System of Units (SI) units has also been added. While the units used throughout the volume are conventional, they can be changed to SI units by the use of the conversion factors in the appendixes. The tables of normal values in the appendixes are given in conventional units in anticipation that their use is not likely to be drastically changed throughout the 1980s. While I support the conversion to the International System of Units, the realities of the situation dictate the pragmatic view that a major international conversion of this type is a long and slow process. Indeed, there is a controversy in the scientific community, as well as others, as to the value of a change if it is only for change's sake and when a rationale for a change has not been adequately promulgated. Nevertheless, it is hoped that these tables of normal values will be of assistance to users of this volume.

The assembly of a multiauthored volume of this nature is a formidable task, which could not have been completed without the dedicated cooperation of all the contributors who have made diligent efforts to meet deadlines. For their patience and fortitude, I would like to extend my thanks. To the staff at Academic Press my sincere appreciation. For her diligence and perseverance with my involvement in this volume, I express my thanks to my administrative assistant, Ms. Joan Cameron.

In summary, this third edition represents a major revision of the previous editions. It has been my pleasure and privilege to assume sole editorial responsibility for this third edition after having worked with my close friend and colleague, Charles E. Cornelius, in the first two editions. His imprint is retained in this edition by the contribution of his classic monograph on liver function in animals. Finally, I express thanks to my family and friends who have supported and encouraged me in this endeavor.

Jiro J. Kaneko

1

Carbohydrate Metabolism and Its Disorders

JIRO J. KANEKO

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

I. INTRODUCTION

The sustenance of animal life is dependent upon the availability of chemical energy in the form of foodstuffs. The ultimate source of this energy is the sun, and the transformation of solar energy to chemical energy in a form usable by animals is dependent upon the chlorophyll-containing plants. The photosynthetic process leading to the reduction of CO_2 to carbohydrates may be summarized as follows:

$$CO_2 + H_2O \xrightarrow[\text{chlorophyll}]{\text{light}} (CH_2O)_x + O_2\uparrow$$

The principal carbohydrate synthesized by plants and utilized by animals is starch. The large amounts of indigestible cellulose synthesized by plants are utilized by the herbivorous animals, which depend upon the cellulolytic action of the microbial flora in their digestive tracts.

The biochemical mechanisms by which the chemical energy of foodstuffs are made available to the animal are collectively described as metabolism. Thus, the description of the metabolism of a foodstuff encompasses the biochemical events which occur from the moment of ingestion to its final breakdown and excretion. It is convenient to retain the classical division of metabolism into the three major foodstuffs: carbohydrates, lipids, and proteins. The metabolism of lipids and proteins is discussed in other chapters.

The major function of the ingested carbohydrate is to serve as a source of energy, and its storage function is relatively minor. Carbohydrates also function as precursors of essential intermediates for use in synthetic processes. When the metabolic machinery of an animal is disrupted, a disease state prevails, e.g., diabetes. Presently, there is a voluminous literature describing the biochemistry of metabolism and disease in intricate detail. This chapter is not presented as an exhaustive treatise on the subject of carbohydrate biochemistry but rather as a basis for the better understanding of the disorders associated with carbohydrate metabolism.

II. DIGESTION

The digestion of carbohydrates in the animal organism begins with the initial contact of these foodstuffs with the enzymes of salivary juice. Starch of plant foods and glycogen of meat are split into their constituent monosaccharides by the action of amylase and maltase. This activity ceases as the food matter passes into the stomach, where the enzymatic action is destroyed by hydrochloric acid. Within the stomach, acid hydrolysis may occur, but the stomach empties too rapidly for complete hydrolysis to take place. Thus, only a small portion of the ingested carbohydrate is hydrolyzed prior to entrance into the small intestine. In the small intestine, digestion of carbohydrates takes place quickly by the carbohydrate-splitting enzymes contained in the copious quantities of pancreatic juice and in the succus entericus. Starch and glycogen are hydrolyzed to glucose by amylase and maltase; lactose to glucose and galactose by lactase; and sucrose to glucose and fructose by sucrase. The monosaccharide products of enzymatic hydrolysis—glucose, fructose, and galactose—are the principal forms in which absorption occurs.

III. ABSORPTION

The monosaccharides are almost completely absorbed through the mucosa of the small intestine and appear in the portal circulation as free sugars. Absorption occurs by two methods: (1) simple diffusion and (2) sodium-dependent active transport. Glucose and galactose are absorbed rapidly and by both methods. Fructose is absorbed at about half the rate of glucose, with a portion being converted to glucose in the process. Other monosaccharides, e.g., mannose, are absorbed slowly at a rate consistent with a simple diffusion process. The active absorption of glucose across the intestinal mucosa is thought to be by phosphorylation in the mucosal cell. The phosphorylated sugars are then transferred across the mucosal cell and rehydrolyzed, and free glucose appears in the portal circulation for transport to the liver.

IV. METABOLISM OF ABSORBED CARBOHYDRATES

A. General

Liver cells are freely permeable to absorbed carbohydrates. Within the liver, there are several general pathways by which the immediate fate of absorbed hexose is determined. Glucose, fructose, and galactose first enter the general metabolic scheme through a series of complex reactions to form glucose phosphates (Fig. 1). The enzyme galactose-1-P uridyltransferase, which catalyzes the reaction galactose-1-P + UDP-glucose→UDP-galactose + glucose-1-P, is blocked or deficient in congenital galactosemia of man (Isselbacher, 1959). The glucose phosphates are then converted to and stored as glycogen, catabolized to CO_2 and water, or, as free glucose, return to the general circulation.

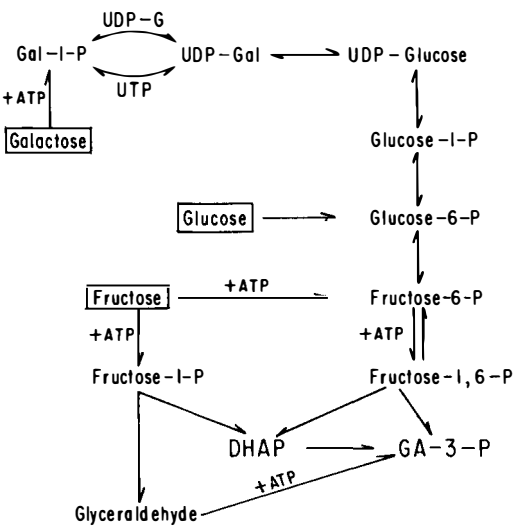


Fig. 1 Pathways for hexose metabolism. Abbreviations: ATP, adenosine triphosphate; UTP, uridine triphosphate; UDP-G, uridine diphosphate-glucose; DHAP, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde 3-phosphate.

Essentially, intermediate carbohydrate metabolism of animals evolves about the metabolism of glucose, and the liver is the organ of prime importance.

B. Storage as Glycogen

Glycogen is the chief storage form of carbohydrate in animals and is analogous to starch in plants. It is found primarily in the liver and in the muscle, where it occurs at about 3–6% and about 0.5%, respectively (Table I).

Glycogen is comprised solely of α-D-glucose units linked together through carbon atoms 1 and 4 or 1 and 6. Straight chains of glucose units are formed by the 1–4 links, and these are cross-linked by the 1–6 links. The result is a complex ramification of chains of glucosyl units with branch points at the site of the 1–6 links (Fig. 2). The internal chains of the glycogen molecule have an average length of four glucosyl units. The external chains beyond the last 1–6 link are longer and contain between 7 and 10 glucose units (Cori, 1954). The molecular weights may be as high as 4×10^6 and contain about 20,000 glucosyl units.

In Table II, the amount of carbohydrate available to meet the theoretical requirements of a hypothetical dog is shown. The amount present is sufficient for about half a day. It is apparent that the needs of the body which must be continually met are satisfied by alternate means and not solely dependent upon continuous ingestion of carbohydrates. During and after feeding (postprandial), absorbed hexoses are converted to glucose by the liver and enter the general circulation. Excesses are stored as glycogen or as fat. During the fasting or postabsorptive state, glucose is supplied by the conversion of protein (gluconeogenesis) and by the breakdown of glycogen (glycogenolysis). The continued rapid synthesis and breakdown of glycogen, i.e., turnover, is well illustrated by the finding that the biological half-time of glycogen is about 1 day.

C. Glycogen Metabolism

The process of glycogen synthesis, glycogenesis, and of breakdown, glycogenolysis, is now well known (Leloir and Cardini, 1957; Leloir *et al.*, 1959) to proceed by two separate pathways.

1. Glycogenesis

The initial reaction required for the entrance of glucose into the series of metabolic reactions which culminate in the synthesis of glycogen is the phosphorylation of glucose at

TABLE I

Liver Glycogen Content of Animals

Species	Glycogen in liver (%)	Reference
Dog	6.1	Lusk (1928)
Sheep	3.8	Roderick *et al.* (1933)
Cow (lactating)	1.0	Kronfeld *et al.* (1960)
Cow (nonlactating)	3.0	Kronfeld *et al.* (1960)
Baby pig	5.2	Morrill (1952)
Baby pig (newborn)	14.8	Swiatek *et al.* (1968)

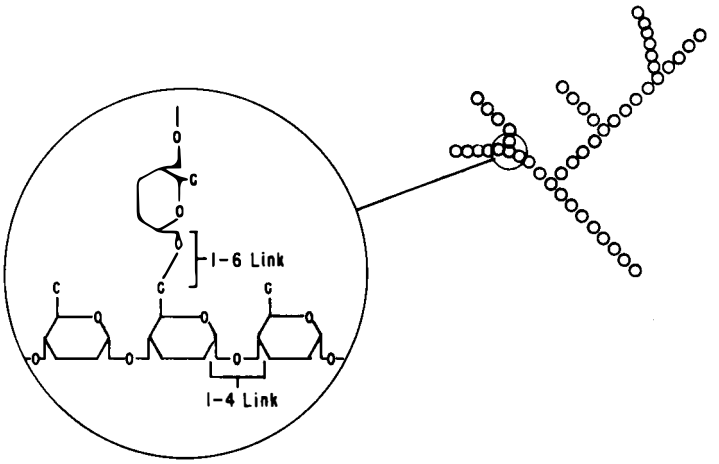


Fig. 2 Glycogen structure. (Adapted from Cori, 1954.)

the C-6 position. Glucose is phosphorylated by adenosine triphosphate (ATP) in the liver by an irreversible enzymatic reaction which is catalyzed by a specific glucokinase:

$$\text{Glucose} + \text{ATP} \xrightarrow[\text{(hexokinase)}]{\text{glucokinase}} \text{glucose-6-P} + \text{ADP} \qquad (1)$$

Liver contains both a nonspecific hexokinase and a glucose-specific glucokinase. Glucokinase is found only in the liver and has a high Michaelis constant ($K_m = 10^{-2}\ M$ glucose), indicating a low affinity for glucose. The rate of the phosphorylation reaction catalyzed by glucokinase is therefore controlled by the glucose concentration. The activity of this enzyme is increased by glucose feeding and insulin and is decreased during fasting and in diabetes. The nonspecific hexokinase, which is found in liver, muscle, nerve, and all tissues, has a low Michaelis constant ($K_m = 10^{-5}\ M$ glucose), indicating a high affinity for glucose. Hexokinase-catalyzed phosphorylation in all tissues, therefore, is not controlled by glucose concentration. The activity of this enzyme is not affected by fasting or carbohydrate feeding, by diabetes, or by insulin.

This unidirectional phosphorylation permits the accumulation of glucose in the liver

TABLE II

Carbohydrate Content of a Dog[a]

Muscle glycogen (0.5%)	25.0 gm
Liver glycogen (6%)	18.0 gm
Carbohydrate in fluids (100 mg %)	2.2 gm
	45.2 gm
Caloric value (45.2 × 4 kcal/gm) = 181 kcal	
Caloric requirement (70 $kg^{3/4}$ = 70 × 5.6) = 392 kcal/day	
$\frac{181}{392} \times 24$ hours = 11 hours	

[a] Body weight, 10 kg; liver weight, 300 gm; muscle weight, 5 kg; volume of blood and extracellular fluid, 2.2 liters.

cells since the phosphorylated sugars do not pass freely in and out of the cell in contrast to the readily diffusible free sugars. The glucose-6-phosphate (G-6-P) trapped in the cell next undergoes a mutation in which the phosphate grouping is transferred to the C-1 position of the glucose molecule. This reaction is catalyzed by the enzyme phosphoglucomutase and involves glucose-1,6-diphosphate:

$$\text{Glucose-6-P} \rightarrow \text{glucose-1-P} \tag{2}$$

Glycogen is synthesized from this glucose 1-phosphate (G-1-P) through reactions involving the formation of uridine derivatives. Uridine diphosphoglucose (UDP-G) is synthesized by the transfer of glucose from G-1-P to uridine triphosphate (UTP). This reaction is catalyzed by the enzyme UDP-G-pyrophosphorylase:

$$\text{UTP} + \text{G-1-P} \rightarrow \text{UDP-G} + \text{PP} \tag{3}$$

In the presence of a polysaccharide primer and the enzyme UDPG-glycogentransglucosidase (glycogen synthase), the glucose moiety of UDP-G is linked to the polysaccharide by an α-1-4 link:

$$\text{UDP-G} + (\text{glucose } 1\text{-}4)_n \xrightarrow[\text{transglucosidase}]{\text{UDPG-glycogen}} (\text{glucose } 1\text{-}4)_{n+1} + \text{UDP} \tag{4}$$

Through repeated transfers of glucose, the polysaccharide chain is lengthened. When the chain length of the polysaccharide reaches a critical level of between 7 and 21 glucose units, the brancher enzyme, amylo-1,4: 1,6-transglucosidase, transfers the terminal portion from an α-1-4 linkage to an α-1-6 linkage. The newly established 1-6 linkage thus becomes a branch point in the glycogen molecule. Approximately 7% of the glucose units comprising a glycogen molecule are involved in these branch points (Illingworth and Cori, 1952).

2. Glycogenolysis

The breakdown of liver glycogen to glucose takes place via a separate pathway. In the presence of inorganic phosphate, the predominant 1-4 linkages of glycogen are successively broken by active phosphorylases. This reaction cleaves the 1-4 link by the addition of orthophosphate in a manner analogous to a hydrolytic cleavage with water—hence, the term "phosphorolysis." Phosphate is added to the C-1 position of one glucose moiety while H^+ is added to the C-4 position of the other. Extensive investigations of Sutherland and co-workers (Rall *et al.*, 1956; Sutherland and Wosilait, 1956) have clarified the influence of glucagon and epinephrine in the phosphorolytic breakdown of glycogen. The phosphorolytic enzyme exists in liver in two forms: an active form designated liver phosphorylase (LP), which contains phosphate, and an inactive form designated dephosphophosphorylase (dephospho-LP), in which phosphate has been removed. The transformations between the active and inactive forms are catalyzed by specific kinase enzymes.

Normally, the level of LP is low, and epinephrine and glucagon shift the equilibirum toward a higher level of LP. The net result is an increase in the phosphorolytic breakdown of glycogen to glucose, a fact reflected in the well-known hyperglycemia observed after injection of these hormones. The mechanism of action of glucagon and epinephrine is thought to be through their stimulation of liver cell adenylate cyclase (Robinson *et al.*, 1968) to form 3′,5′-adenosine monophosphate (cyclic AMP) from ATP (Rall *et al.*, 1957;

Rall and Sutherland, 1958). The cyclic AMP stimulates a protein kinase which increases liver phosphorylase activity (Cherrington and Exton, 1976).

Cyclic AMP is also a key regulating factor in cellular processes in addition to liver phosphorylase activation. It is required for the conversion of inactive muscle phosphorylase b to active muscle phosphorylase a (Anstall, 1968). The actions of other hormones known to be mediated by increasing cyclic AMP include ACTH, LH, TSH, MSH, T_3, and insulin (Robison *et al.*, 1968). From these findings, a general concept of hormone action has evolved in which the hormone elaborated by the endocrine organ is the first messenger and cyclic AMP within the target cell is the second messenger.

The action of glucagon on glycogen appears to be confined to liver, whereas epinephrine acts on both liver and muscle glycogen. In liver, glucagon promotes the formation and release of glucose by increasing glycogenolysis and decreasing glycogenesis. An additional factor promoting hyperglycemia is the stimulation of hepatic gluconeogenesis by glucagon. With muscle glycogen, however, since the enzyme glucose-6-phosphatase (G-6-Pase) is absent from muscle, glycogen breakdown in muscle results in the production of pyruvate and lactate rather than glucose.

The continued action of LP on the 1–4 linkages results in the sequential release of G-1-P units until a branch point in the glycogen molecule is reached. The residue is a limit dextrin. The debrancher enzyme, amylo-1,6-glucosidase, then cleaves the 1–6 linkage, releasing free glucose. The remaining 1–4-linked chain of the molecule is again open to attack by LP until another limit dextrin is formed. Thus, by the combined action of LP and the debrancher enzyme, the glycogen molecule is successively reduced to G-1-P and free glucose.

Glucose-1-P is converted to G-6-P by the reversible reaction catalyzed by phosphoglucomutase (PGM) [Section IV,C,1, reaction (II)]. The G-6-P is then irreversibly cleaved to free glucose and phosphate by the enzyme G-6-Pase, which is found in liver and kidney. Unlike its phosphorylated intermediates, the free glucose formed can leave the hepatic cell to enter the general circulation, thereby contributing to the blood glucose. In muscle tissue, there is no G-6-Pase, and muscle glycogen cannot supply glucose directly by glycogenolysis. Muscle glycogen contributes to blood glucose indirectly via the lactic acid cycle (Section IV,D). The series of reactions described are illustrated schematically in Fig. 3.

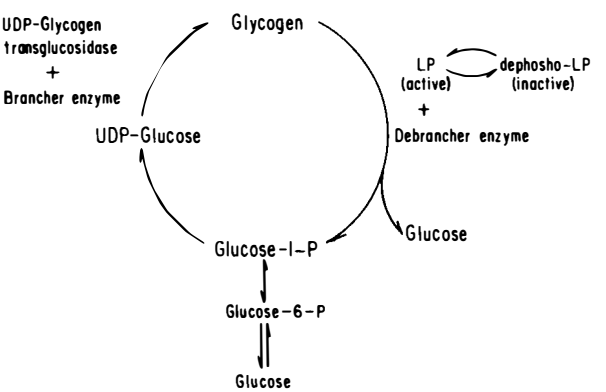


Fig. 3. Summary of liver glycogen metabolism. In muscle, phosphorylase a is the active form and phosphorylase b is the inactive form. Abbreviations: UDP, uridine diphosphate; LP, liver phosphorylase.

3. *Hormonal Influences upon Glycogen Metabolism*

The biochemical basis for the glycogenolytic and hyperglycemic action of glucagon and epinephrine has been discussed in Section IV,C,2. These effects of the hormones are the bases for the epinephrine and glucagon tolerance tests employed to assess the availability of liver glycogen and the sensitivity of the carbohydrate metabolic mechanisms. Many other hormones are known to have similar effects upon carbohydrate metabolism, indicating that metabolism should be considered an integrated concept.

One of the results of successful insulin therapy is a restoration of the depleted glycogen reserve. The mechanism of insulin action upon carbohydrate metabolism continues to be a subject of debate and is discussed more fully in Section VI. Briefly, the primary role of insulin is to promote glucose entry into peripheral cells and to enhance glucose utilization in liver cells by its effect on enzyme systems. In either event, glucose utilization toward glycogen synthesis or oxidation would be favored.

Promotion of liver glycogen storage is also one of the effects of the glucocorticoids. This effect may be attributed to their enhancement of gluconeogenesis, hyperglycemia, decreased glycogenolysis, and decreased glucose oxidations. A tendency toward a mild hyperglycemia is also present in hyperthyroid states, as the result of factors influencing carbohydrate metabolism. Thyroxine is thought to render the liver more sensitive to the action of epinephrine, thus resulting in increased glycogenolysis. Increased glycogenolysis and gluconeogenesis may also be the compensatory result of the increased rate of tissue metabolism. Freedland *et al.* (1968) reported that hepatic G-6-Pase activities increased markedly in rats made hyperthyroid; this finding is consistent with the viewpoint that hepatic glucose production is increased in hyperthyroid states. An additional factor is the stimulation of glucose absorption by the gastrointestinal tract.

4. *Glycogen in Disease*

In disease, alterations in glycogen levels are generally observed as decreases. Depletion of liver glycogen stores is seen in diabetes mellitus, bovine ketosis, and ovine pregnancy toxemia. Pathological increases in liver glycogen occur in the rare glycogen storage diseases (GSD). No clearly established counterpart of these diseases has yet been reported in domestic animals. A von Gierke-like syndrome in the dog, however, has been reported (Bardens, 1966). It will become apparent that the ultimate diagnosis of these conditions depends upon biochemical studies of the tissues involved, and procedures for their analysis are available (Illingworth and Cori, 1952). This group of disease entities has been classified into eight types on the basis of clinical and biochemical findings (Howell, 1978).

Liver glycogen is found in high concentration in classical von Gierke's disease, or Type 1 according to the Cori classification (Cori, 1954), which leads to a hepatomegaly. The response of blood glucose to the epinephrine tolerance test is minimal or absent. The liver glycogen in this form of GSD has been shown to have a normal structure and the defect to lie in a deficiency of the enzyme G-6-Pase. Enzyme deficiencies also account for the abnormal structures of glycogen found in other types (except Type 2) of GSD. Glycogen analyses have indicated that in Type 3 the debrancher enzyme is deficient. The glucose chains are shorter, and the glycogen molecule has an increased number of branch points. In Type 4, with a brancher enzyme deficiency, the chains are longer, and fewer branch points are present. Muscle and liver phosphorylase are absent in Types 5 and 6, respec-

tively. Type 7 is deficient in phosphofructokinase activity, and Type 8 is deficient in leukocyte phosphorylase b kinase.

D. Catabolism of Glucose

Carbohydrate in the form of glucose is the principal source of energy for the life processes of the mammalian cell. All cells require a constant supply of this indispensable nutrient, and only relatively small changes may be tolerated without adverse effects upon the health of the animal. Glucose is not oxidized directly to CO_2 and H_2O but rather through a series of stepwise reactions involving phosphorylated intermediates. The chemical energy of glucose is "stored" through the synthesis of "high-energy" phosphate bonds during the course of these reactions and used in other metabolic reactions. The subject of carbohydrate metabolism continues to be extensively studied, and a voluminous literature is available. The pathways of glucose catabolism have been elucidated, but the emphasis here is on the interrelationships of the pathways rather than on the details of the individual reactions. Detailed discussions of the individual reactions involved can be found in the many excellent texts of biochemistry presently available.

1. Pathways of Glucose 6-Phosphate Metabolism

The fundamental conversion required to initiate the oxidation of glucose by the cell is a phosphorylation to form G-6-P. This reaction has been described in Section IV,C,1. The G-6-P formed as a result of the glucokinase (or hexokinase) reaction is central to glucose metabolism. There are at least five different pathways which may be followed by G-6-P: free glucose, glycogenesis, glycolysis, pentose phosphate, and glucuronate pathway.

a. Free Glucose. The simplest pathway is the separate reverse reaction by which G-6-P is cleaved to form free glucose and inorganic phosphate. This reaction is catalyzed by the enzyme G-6-Pase:

$$\text{Glucose-6-PO}_4 \xrightarrow{\text{G-6-Pase}} \text{glucose} + P_i$$

This is essentially an irreversible reaction and opposes the previously described unidirectional glucokinase reaction. These two opposing and independently catalyzed enzyme reactions offer a site of metabolic control whereby the activities of the enzymes can play a regulatory role. Significant amounts of G-6-Pase are found only in the liver and to a lesser extent in the kidney. This is in accord with the well-known function of the liver as the principal source of glucose for the maintenance of blood glucose concentration.

Muscle tissue, due to the absence of G-6-Pase, cannot contribute glucose to blood directly from muscle glycogen breakdown. Muscle glycogen does, however, contribute indirectly via a pathway designated the lactic acid or Cori cycle. Lactic acid formed in muscle via muscle glycolysis is transported to the liver, where it is resynthesized to glucose and its precursors, as outlined in Fig. 4.

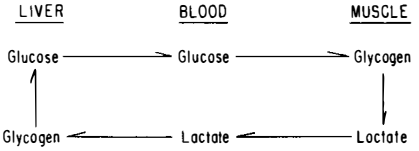


Fig. 4. Lactic acid cycle. Muscle glycogen indirectly contributes to blood glucose by this pathway.

b. Glycogenesis. This pathway for G-6-P leading to the synthesis of glycogen has been discussed in Section IV,C,1.

c. Glycolysis. One of the three oxidative pathways of G-6-P is the classical anaerobic Embden–Meyerhof (EM) glycolytic pathway. The intermediate steps involved in this pathway of breakdown of G-6-P to three-carbon compounds are summarized in Fig. 5. One mole of ATP is used in the phosphorylation of fructose 6-phosphate (F-6-P) to form fructose 1,6-diphosphate (F-1,6-P). This phosphorylation is also an irreversible reaction catalyzed by a specific kinase, phosphofructokinase (PFK). The opposing unidirectional reaction is catalyzed by a specific phosphatase, fructose-1,6-diphosphatase (F-1,6-Pase). The opposing PFK and F-1,6-Pase-catalyzed reactions offer a second site of metabolic control regulated by the activities of the two enzymes. It should be noted that, starting from glucose, a total of two high-energy phosphates from ATP have been donated to form 1 mole of F-1,6-P.

The F-1,6-P is then cleaved to form two three-carbon compounds, as shown in Fig. 5. Next, an oxidative step occurs in the presence of glyceraldehyde-3-phosphate dehydrogenase (GA-3-PD) with oxidized nicotinamide adenine dinucleotide (NAD^+) (or diphosphopyridine nucleotide, DPN^+) as the hydrogen acceptor. During the process, the molecule is phosphorylated. In the succeeding steps, the molecule is dephosphorylated at the points indicated, and 1 mole of ATP generated at each point.

A third site of control of glycolysis is the unidirectional formation of pyruvate by the pyruvate kinase (PK) reaction. In its reverse direction, it is circumvented by two enzymatic reactions. Pyruvate carboxylase (PC) catalyzes the carboxylation of pyruvate to

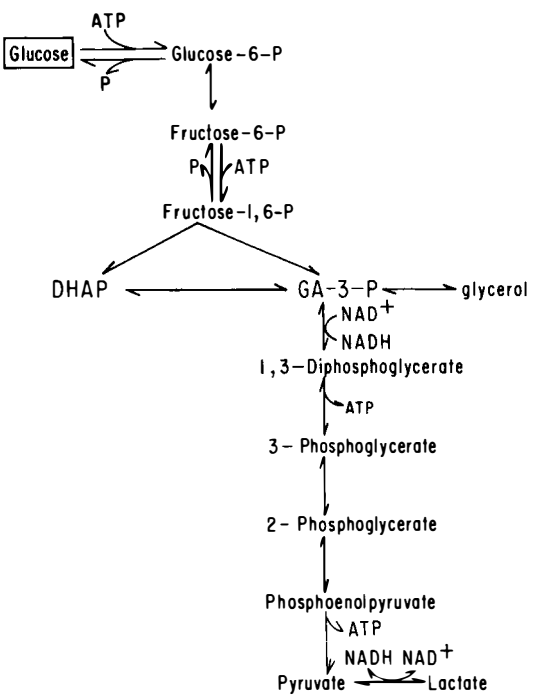


Fig. 5. Glycolytic or classical Embden–Meyerhof (EM) pathway.

oxaloacetate (OAA), and the OAA is converted to phosphoenolpyruvate (PEP) by the enzyme PEP-carboxykinase (PEP-CK) (Figs. 5 and 8). Thus, the overall conversion of 1 mole of glucose to 2 moles of pyruvate requires 2 moles of ATP for the initial phosphorylations, and a total of 4 moles of ATP are generated in the subsequent dephosphorylations. This net gain of 2 moles of ATP represents the useful energy of glycolysis.

For repeated functioning of the glycolytic pathway, a supply of NAD^+ (DPN^+) must be available for use in the oxidative step. In the presence of molecular O_2, reduced NADH (DPNH) is reoxidized via the cytochrome system:

$$H^+ + NADH + \frac{1}{2}O_2 \xrightarrow[\text{system}]{\text{cytochrome}} NAD^+ + H_2O$$

In the absence of O_2, i.e., anaerobiosis, NADH (DPNH) is reoxidized to NAD^+ (DPN^+) in a reaction catalyzed by lactate dehydrogenase whereby pyruvate is reduced to lactate. Thus, by this "coupling" of enzymatic reactions, glucose breakdown may continue in periods of anaerobiosis.

d. Pentose Phosphate Pathway. This alternate route of G-6-P oxidation has been referred to variously as the PPP, pentose cycle, direct oxidative pathway, Warburg-Dickens scheme, or the hexose monophosphate "shunt." The initial step involves the oxidation of G-6-P at the C-1 position to form 6-phosphogluconate (6-PG), as summarized in Fig. 6. The reaction is catalyzed by G-6-PD, and, in this pathway, oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) (triphosphopyridine nucleotide, TPN^+) serves as the hydrogen acceptor. In the second oxidative step, 6-PG is oxidatively decarboxylated by G-PG-dehydrogenase (6-PGD) to yield a pentose phosphate, ribulose 5-phosphate (Rib-5-P), again in the presence of $NADP^+$ (TPN^+). Thus, in the initial reactions, which are essentially irreversible, 2 moles of NADPH (TPNH) are formed. It should be noted that in this pathway only the C-1 carbon atom of the glucose molecule is evolved as CO_2. In contrast, glucose catabolism via the glycolytic scheme results in the loss of both the C-1 and C-6 carbon atoms when pyruvate is oxidatively decarboxylated to form acetyl-CoA. This phenomenon has been employed in a number of studies in which

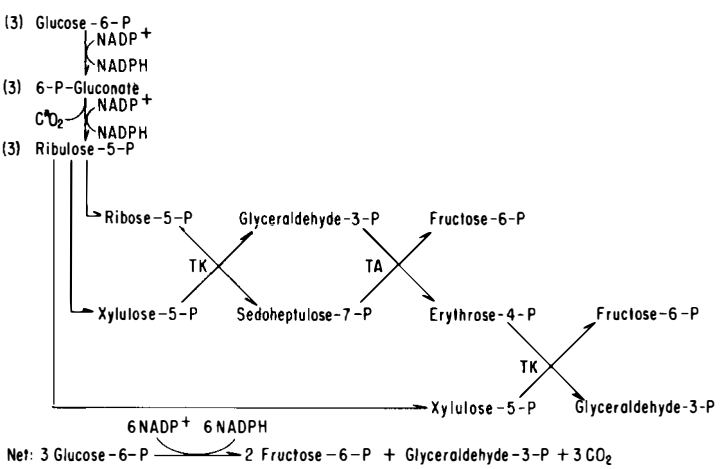


Fig. 6. Pentose cycle or hexose monophosphate shunt pathway. Abbreviations: $NADP^+$, nicotinamide adenine dinucleotide phosphate; TK, transketolase; TA, transaldolase; C^*O_2, derived from C-1 of glucose.

the relative contributions of the EM pathway and the PP pathway have been assessed in domestic animals (Black *et al.*, 1957). The subsequent metabolism of the Rib-5-P formed is also shown in Fig. 6. As a result of the series of transformations, F-6-P and GA-3-P are formed and serve as recycling links into the EM pathway.

For continued functioning of the PP pathway, a supply of $NADP^+$ (TPN^+) must be available as the hydrogen acceptor. $NADP^+$ (TPN^+) is regenerated from NADPH (TPNH) via the cytochrome system in the presence of O_2, and thus the PP pathway is an aerobic pathway of glucose oxidation. Reduced NADPH (TPNH) is also required as a hydrogen donor in the synthesis of fatty acids, and in this way carbohydrate metabolism is linked to that of fat (Siperstein and Fagan, 1957). Accordingly, the availability of NADPH (TPNH) generated in the PP pathway is essential for use in the synthesis of fat. Generally, the PP pathway is the major source of the NADPH (TPNH) required to maintain the reducing environment in all biosynthetic processes using NADPH (TPNH) as cofactor.

e. Glucuronate Pathway. This is an alternate pathway of G-6-P oxidation which has been termed the uronate pathway, glucuronate pathway, or C_6 oxidative pathway. It is shown in Fig. 7. Important contributions to its clarification, particularly in relation to L-xylulose metabolism and ascorbic acid synthesis, can be found in the studies of Touster *et al.* (1957) and Burns and Evans (1956). The initial steps of this pathway involve the formation of uridine diphosphoglucose (UDPG), an intermediate in glycogenesis. Glucose-6-P is first converted to G-1-P, which then reacts with UTP to form UDPG. This product is then oxidized at the C-6 position of the glucose moiety, in contrast to the PP pathway. This reaction requires NAD^+ (DPN^+) as a cofactor, and the products of the reaction are uridinediphosphoglucuronic acid (UDPGA) and NADH (DPNH). This UDPGA is involved in a large number of important conjugation reactions in animals, e.g., bilirubin diglucuronide formation and the synthesis of mucopolysaccharides (chondroitin sulfate) which contain glucuronic acid.

D-Glucuronic acid is next reduced to L-gulonic acid in the presence of the enzyme gulonic dehydrogenase, with NADPH (TPNH) as the hydrogen donor. The L-gulonic acid may follow a pathway leading to the synthesis of a pentose, L-xylulose, the compound found in the urine in xyloketosuria of man. In this reaction, the C-6 carbon of glucose is evolved as CO_2. As shown in Fig. 7, further reactions of L-xylulose lead to the formation of D-xylulose-5-P, and a cyclic pathway including the PP pathway may occur. L-Gulonic acid is also converted in an enzyme-catalyzed reaction to L-ascorbic acid in those species

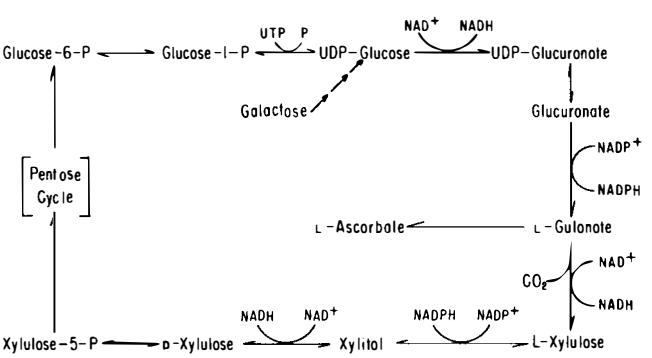


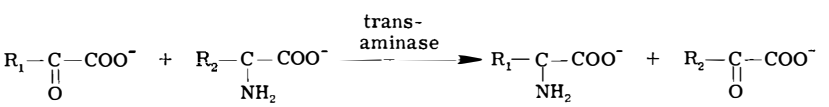
Fig. 7. Glucuronate pathway.

which can synthesize their own vitamin C, i.e., all domestic animals. The enzyme is lacking in man, nonhuman primates, and the guinea pig (Grollman and Lehninger, 1957). The enzyme is present only in the liver in the mouse, rat, pig, cow, and dog. In the dog, the liver enzyme activity is relatively low and the ascorbate destruction activity is rather high, so dogs may have an additional need for vitamin C in times of stress. For the synthesis of L-ascorbic acid, evidence has been presented (Evans *et al.*, 1959) that D-galactose may be an even better precursor than D-glucose. This pathway is also included in Fig. 7.

2. Terminal Oxidation

The metabolic pathways described thus far have been those followed essentially by carbohydrates alone. Similarly, the breakdown of fats and proteins follows independent pathways leading to the formation of organic acids. Among the organic acids formed are acetate from β-oxidation of fatty acids, and pyruvate, oxaloacetate, and α-ketoglutarate from transamination of their corresponding α-amino acids. These intermediate metabolites are indistinguishable in their subsequent interconversions. Thus, the breakdown of the three major dietary constituents converges into a pathway common to all, which then serves as a site for the interconversions between them.

a. Pyruvate Metabolism. The pathway for the breakdown of glucose to pyruvate has been described in Section IV,D,1. Pyruvate is then oxidatively decarboxylated in a complex enzymatic system requiring the presence of lipoic acid, thiamine pyrophosphate, coenzyme A, NAD^+ (DPN^+), and pyruvic oxidase to form acetyl-CoA and NADH (DPNH). Pyruvate may follow a number of pathways, as outlined in Fig. 8. The conversion of pyruvate to lactate has been described in Section IV,D,1. By the mechanism of transamination, pyruvate can be reversibly converted to alanine. The general reaction for transamination can be written



where the amino group of an amino acid is transferred to the α position of an α-keto acid, and, as a result, the amino acid is converted to its corresponding α-keto acid. This reaction requires the presence of vitamin B_6 as pyridoxal phosphate and is catalyzed by a transaminase specific for the reaction. Serum levels of these enzymes have been particularly useful in the assessment of liver and muscle disorders and are discussed more fully in later chapters.

The energetics of the reaction from phosphoenolpyruvate to form pyruvate are such that this is essentially an irreversible process, as is the breakdown of pyruvate to acetyl-CoA. An alternate pathway to effect a reversal of this process is available. Through a carbon dioxide fixation reaction in the presence of $NADP^+$ (TPN^+)-linked malate dehydrogenase, malate is formed from pyruvate. Malate is then oxidized to oxaloacetate in the presence of NAD^+ (DPN^+)-linked malate dehydrogenase. Oxaloacetate may also be formed directly from pyruvate by the reaction catalyzed by pyruvate carboxylase. Oxaloacetate formed by either route may then be phosphorylated and decarboxylated to form PEP in a reaction catalyzed by PEP-carboxykinase. Thus, a pathway which bypasses the direct reversal of the PK reaction is available for gluconeogenesis from lower intermediates. These pathways

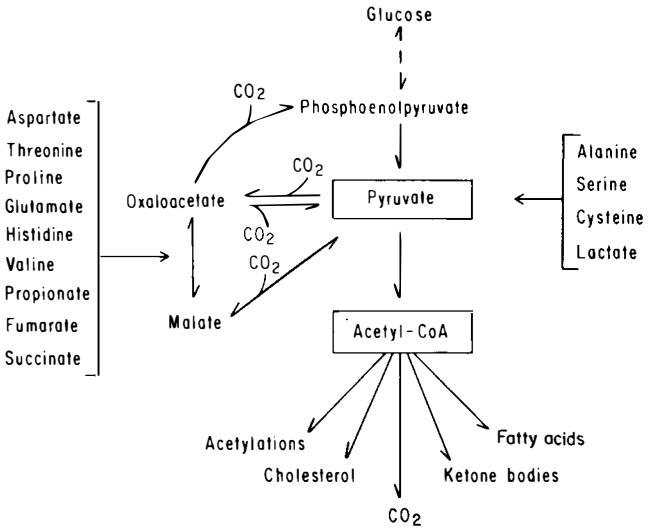


Fig. 8. Pathways of acetate and pyruvate metabolism.

for pyruvate metabolism are also outlined in Fig. 8, which includes the dicarboxylic acid cycle.

b. Tricarboxylic Acid Cycle. Acetyl-CoA (AcCoA) formed as a result of oxidative decarboxylation of pyruvate also has a number of metabolic routes available. This compound occupies a central position in synthetic as well as oxidative pathways, as shown in Fig. 8. The oxidative pathway leading to the breakdown of AcCoA to CO_2 and H_2O follows a cyclic pathway called the tricarboxylic acid (TCA) cycle, citric acid cycle, or Kreb's cycle. The major steps involved are given in Fig. 9. In a single turn of the cycle, 2 moles of CO_2 are evolved, and 1 mole of oxaloacetate is regenerated. The regenerated OAA may then condense with another mole of AcCoA, and the cycle continues. It should be noted that citric acid is a symmetric molecule which behaves asymmetrically, as shown in Fig. 9. The CO_2 is also not derived directly from that portion of the molecule contributed by AcCoA. The CO_2 is derived from the portion of the molecule contributed by OAA during each turn of the cycle. The expected distribution of isotopically labeled carbon atoms in one turn of the cycle is also given in Fig. 9. During one turn of the cycle, a randomization of label occurs at the succinate level such that CO_2 derived from the carboxyl group of acetate will be evolved during the next turn of the cycle.

In the process, 3 moles of NAD^+ (DPN^+) and 1 mole of a flavin nucleotide (FAD) are reduced, and 1 mole of ATP is generated, as noted in Fig. 9. In animal tissues, there is also a $NADP^+$ (TPN^+)-linked isocitric dehydrogenase (ICD), which is cytoplasmic and not associated with the mitochondria as is the NAD^+ (DPN^+)-linked ICD and the other enzymes of the citric acid cycle. The $NADP^+$ (TPN^+)-ICD is also used as an aid in the diagnosis of liver disorders (Cornelius, 1961).

3. Carbon Dioxide Fixation in Animals

According to Fig. 9, the TCA cycle is a repetitive process based upon the regeneration of OAA at each turn. Other metabolic paths, however, are also available for intermediates

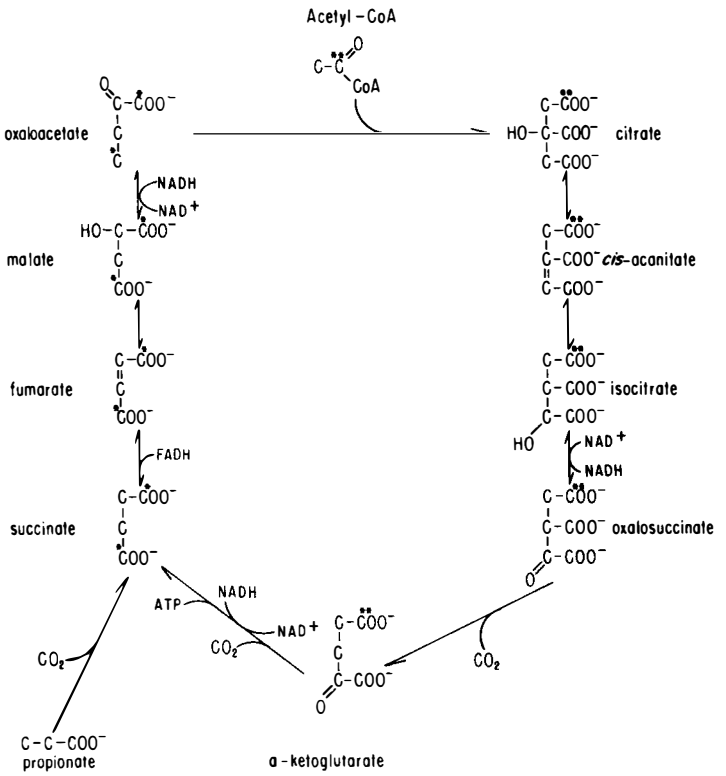


Fig. 9. Tricarboxylic acid cycle. The pathway for the entry of propionate into the metabolic scheme is also included. The asterisks show the expected distribution of isotopically labeled carbon in a single turn of the cycle starting with acetyl-CoA.

in the cycle. Reversal of the transamination reactions previously described would result in a withdrawal of OAA and α-ketoglutarate (αKG) from the cycle. By decarboxylation, OAA may form PEP, and malate may form pyruvate and then other glycolytic intermediates, as shown in Fig. 8. Continued losses of these intermediates into other metabolic pathways would theoretically result in a decrease in the rate of operation of the cycle. A number of metabolic pathways are known whereby the losses of cycle intermediates can be balanced by replacement from other sources and are shown in Fig. 8. The amino acids, aspartic and glutamic, may function as sources of supply as well as routes for withdrawal. The CO_2 fixation reactions, which are the reversal of the reactions previously described,

$$\text{Phosphoenolpyruvate} + CO_2 \rightarrow \text{oxaloacetate}$$
$$\text{Pyruvate} + CO_2 \rightarrow \text{malate}$$
$$\text{Pyruvate} + CO_2 \rightarrow \text{oxaloacetate}$$

may also function as important sources of supply. A fourth CO_2-fixing reaction is known

$$\text{Propionate} + CO_2 \rightarrow \text{succinate}$$

which is of special importance in ruminant metabolism for maintaining the supply of intermediates in the TCA cycle. Propionate is one of the three major fatty acids involved in ruminant metabolism.

4. Energy Relationships in Carbohydrate Metabolism

The energy of carbohydrate breakdown must be converted to high-energy phosphate compounds to be useful to the organism; otherwise, it is dissipated as heat. The total available chemical energy in the reaction

$$\text{Glucose} \rightarrow 2 \text{ lactate}$$

is about 50 kcal/mole, or about 7% of the 690 kcal/mole which is available from the complete oxidation of glucose to CO_2 and water. In glycolysis, it has been noted that the useful energy is represented by the net gain of 2 moles of ATP, the available energy of each being about 7 kcal. Thus, the efficiency of glycolysis starting from glucose is about 28%.

The major portion of the energy of oxidation is generated in the further aerobic oxidation of pyruvate to CO_2 and H_2O. In the oxidative or dehydrogenation steps, NADH (DPNH) or NADPH (TPNH) (FAD in the succinate step) is formed. In the presence of molecular O_2, these compounds are reoxidized to NAD^+ (DPN^+) or $NADP^+$ (TPN^+) in the cytochrome system. During the sequence of reactions comprising this system, 3 moles of ATP are formed per mole of NADH (DPNH) or NADPH (TPNH) oxidized to NAD^+ (DPN^+) or $NADP^+$ (TPN^+). This phenomenon is known as oxidative phosphorylation. The yield of high-energy phosphate bonds in the form of ATP in the system per atom of oxygen consumed ($\frac{1}{2}O_2$) is conventionally referred to as the P:O ratio, in this case, 3.

In Table III, a balance sheet of the ATP's formed in the various steps is given. The complete oxidation of 1 mole of glucose to CO_2 and water yields about 690 kcal; the net gain of 38 ATP's in the biological scheme represents about 266 kcal, or an overall efficiency of 38%.

V. INTERRELATIONSHIPS OF CARBOHYDRATE, LIPID, AND PROTEIN METABOLISM

The pathways by which the breakdown products of lipids and proteins enter the common metabolic pathway have been described in previous sections. The principal points at

TABLE III

ATP Yield in Glucose Oxidation

	ATP
Glucose	
↓ ← ATP (2×)	−2
fructose 1,6-diphosphate	
↓ → NADH (DPNH) → 3 ATP (2×)	+6
↓ → ATP (4×)	+4
2 pyruvate	
↓ → NADH (DPNH) → 3 ATP (2×)	+6
2 acetyl-CoA	
↓ → NADH (DPNH) → 3 ATP (6×)	+18
↓ → ATP (2×)	+2
↓ → FADH → 2 ATP (2×)	+4
4 CO_2	
Net: Glucose → 6 CO_2	+38 ATP

which carbohydrate carbon may be interconverted between amino acids and fatty acids are outlined in Fig. 10. Thus, certain amino acids (glucogenic) can serve as precursors of carbohydrate, and, by reversal of the reactions involved, carbohydrates can contribute to the synthesis of amino acids.

The relationship between carbohydrate and lipid metabolism deserves special mention, for the carbohydrate economy and the status of glucose oxidation strongly influence lipid metabolism. A brief description of lipid metabolism follows, and greater detail can be found in Chapter 2.

A. Lipid Metabolism

1. Oxidation of Fatty Acids

Cellular fatty acids are either synthesized in the cytoplasm or taken up as free fatty acids. Fatty acid oxidation begins in the cytoplasm with the activation of fatty acids to form fatty acyl-CoA. The activated fatty acyl-CoA is bound to carnitine for transport across the mitochondrial membrane into the mitochondria, where fatty acyl-CoA is released for intramitochondrial oxidation (Fritz, 1967).

The classical β-oxidation scheme for the breakdown of fatty acids whereby two-carbon units are successively removed is firmly established. This scheme is a repetitive process involving four successive reactions. After the initial activation to form a CoA derivative, there is (1) a dehydrogenation, (2) a hydration, and (3) a second dehydrogenation, which is followed by (4) a cleavage. The result is the formation of acetyl-CoA and a fatty acid shorter by two carbon atoms which can reenter the cycle. In the case of odd-chain fatty acids, propionyl-CoA is formed in the final cleavage reaction. The hydrogen acceptors in the oxidative steps are NAD^+ (DPN^+) and FAD. The further oxidation of acetyl-CoA to CO_2 and water proceeds in the common pathway of the TCA cycle. In the process, 2 moles of CO_2 are evolved per mole of ACCoA entering the cycle. Therefore, fatty acids could not theoretically lead to a net synthesis of carbohydrate. Net synthesis of carbohydrate from fatty acids would require the direct conversion of AcCoA into some glucose precursor, i.e., pyruvate. However, the reaction

$$\text{Pyruvate} \dashrightarrow \text{acetyl-CoA} + CO_2$$

is irreversible; the only route by which fatty acid carbon could theoretically appear in carbohydrate is through the TCA cycle intermediates, and this occurs without a net synthesis.

2. Synthesis of Fatty Acids

It had long been assumed that the pathway for lipogenesis was a direct reversal of the β-oxidation scheme. It is now apparent that the pathway of lipogenosis diverges from that of oxidation. The first point of divergence involves the initial condensation reaction. Carbon dioxide is a requirement, yet there is no evidence of the incorporation of the CO_2 into fatty acid (Porter *et al.*, 1957; Gibson, *et al.*, 1958). This has suggested a pathway involving the initial synthesis of malonyl-CoA. Malonyl-CoA is condensed with an aldehyde (Brady, 1958) or AcCoA (Wakil, 1958), and, in subsequent reactions, the original CO_2 moiety is cleaved from the condensation product. This malonyl-CoA pathway of extramitochondrial cytoplasmic synthesis of fatty acid is now well established.

The second point of divergence involves the requirements for NADPH (TPNH) as the

hydrogen donor rather than NADH (DPNH) or FADH. NADPH (TPNH) is generated significantly when glucose is oxidized in the pentose phosphate pathway. NADPH is also high in the extramitochondrial cytoplasm of tissues such as liver and adipose where PPP activity is high. The availability of this NADPH (TPNH) forms the basis for the linkage of carbohydrate oxidation to lipogenesis (Siperstein, 1959).

3. Synthesis of Cholesterol and Ketone Bodies

Acetyl-CoA is also the precursor of cholesterol and the ketone bodies: acetoacetate, β-OH-butyrate, and acetone. The synthesis of cholesterol proceeds through a complicated series of reactions starting with the stepwise condensation of 3 moles of AcCoA to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). Details of the metabolic pathway can be found in Chapter 2. As shown in Fig. 10, HMG-CoA is a common intermediate for the synthesis of both cholesterol and ketone bodies, which occurs in the liver. In liver, a deacylating enzyme is present which cleaves HMG-CoA to yield AcCoA and free acetoacetate (AcAc). This is termed the HMG-CoA cycle. The free AcAc then diffuses from the cell and enters the general circulation. For further oxidations to occur, acetoacetate is "reactivated" with CoA in extrahepatic tissues (muscle) by the transfer of CoA from succinyl-CoA to form AcAcCoA. Increased ketogenesis and ketonemia are the net result of alterations in metabolic pathways and/or enzymes that favor the accumulation of AcAcCoA.

The increased mobilization and utilization of fatty acids is a well-known requisite for ketogenesis under conditions of starvation and diabetes. Under these conditions, lipogenesis from AcCoA is also depressed. The net effect of either or both of these alterations favors the accumulation of AcAcCoA and thus ketogenesis (Fritz, 1961).

Intrahepatic processes are now recognized as also very important in ketogenesis. According to Krebs (1966), increased ketogenesis is always associated with an increased rate of gluconeogenesis, which is in turn associated with increases in the key gluconeogenic enzyme, PEP-carboxykinase. The increased rate of gluconeogenesis depletes OAA. Wieland (1968) also concluded that a depletion of OAA occurs but that it is the result of an

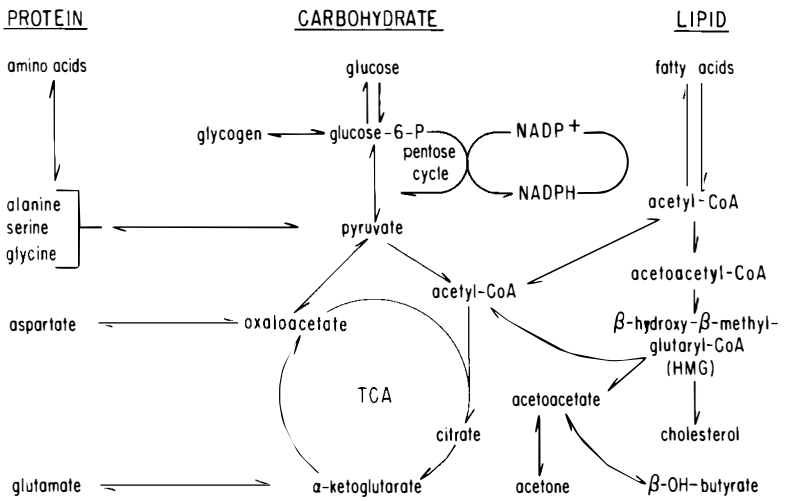


Fig. 10. Interrelation of carbohydrate, protein, and lipid metabolism.

increase in the reductive environment of the cell which is required for synthetic purposes, i.e., gluconeogenesis. The increased NADH/NAD ratio is cited as evidence for the conversion of OAA to malate, thereby depleting OAA. By either mechanism, the area of agreement is the depletion of OAA associated with increased gluconeogenesis, which is in turn associated with an absolute or relative deficiency of carbohydrate. Without OAA, AcCoA would be diverted to ketogenesis.

Hepatic ketogenesis is regulated by the rate of transfer of fatty acids across the mitochondrial membrane, the rate-limiting step. Carnitine acyltransferase, the enzyme system responsible for the mitochondrial uptake of fatty acids, is increased in diabetes.

B. Influence of Glucose Oxidation on Lipid Metabolism

In addition to the separation of metabolic pathways, an anatomical separation of lipid metabolism is also present. The liver is closely associated with fatty acid oxidation, and the major site of lipogenesis in animals is the adipose tissue. The rate of fat synthesis by a liverless animal is comparable to that of an intact animal (Masoro *et al.*, 1949), and *in vitro* adipose tissue converts glucose to fatty acids even faster than does liver tissue.

It is well known that, with excessive carbohydrate intake, fat depots in the body increase. Fasting an animal, on the other hand, depresses the respiratory quotient, an indication that the animal is utilizing body fat as an energy source. During fasting, unesterified fatty acids in plasma also increase, and, when carbohydrate is supplied, they decrease. The presence of glucose has been shown to both stimulate lipogenesis (Hirsch *et al.*, 1954) and have a sparing effect on fatty acid oxidation (Lossow and Chaikoff, 1955). In a condition with relative lack of carbohydrate, i.e., diabetes, and an inability to utilize glucose, depression of lipogenesis is a characteristic finding. With adequate glucose oxidation, the balance of lipid metabolism shifts toward lipogenesis from acetate in adipose tissue.

In conditions of decreased glucose use or availability, e.g., diabetes, starvation, exercise, there is an increased release of glucose precursors (amino acids) from muscle and free fatty acids (FFA) from adipose tissues mediated by activated hormone-sensitive lipasis (Khoo *et al.*, 1973). The amino acids and FFA are transported to the liver, where hepatic gluconeogenic pathways are activated and FFA metabolism is partitioned toward degradation and ketogenesis. Glucagon is thought to be responsible for the activation of hepatic ketogenic mechanisms. In addition, there is a large body of evidence that an underutilization of ketones in the peripheral tissues of dogs occurs (Balasse and Havel, 1971; McGarry and Foster, 1976). The net result is an overproduction of glucose and ketones in liver and an underutilization of both in the peripheral tissues.

VI. INSULIN AND CARBOHYDRATE METABOLISM

The internal secretions of the anterior pituitary, adrenal cortex and medulla, and pancrease are closely associated with carbohydrate metabolism. The pituitary and adrenal factors have been discussed, together with glucagon, in Section IV,C. Since the successful extraction of insulin by Banting and Best in 1921, a vast amount of literature has accumulated on its role in carbohydrate metabolism. Although the intricate details of insulin action are still being studied extensively, presently there is an underlying understanding of the major biochemical events which occur in animals with and without insulin.

A. Proinsulin and Insulin

The elucidation of the structure of insulin by Sanger (1959) was soon followed by the discovery of its precursor, proinsulin (Steiner and Oyer, 1967), and the elucidation of its structure (Chance and Ellis, 1969). It has been the subject of many reviews (Young, 1963), most recently by Kitabchi (1977). Proinsulin is now known to be a single-chain looped polypeptide linked by disulfide bridges (Fig. 11). It varies in length from 78 amino acid residues in the dog to 86 in man, horse, and rat. Its molecular weight is nearly 5000 daltons. Proinsulin is synthesized in the pancreatic β cells on the rough endoplasmic reticulum and transported to the Golgi apparatus. There, the central connecting polypeptide, or C-peptide, is cleaved from the chain by proteolytic enzymes, leaving the two linked end fragments which make up the insulin molecule. As the insulin is released from the proinsulin, it crystallizes with zinc for storage in the β granules. The dense central inclusion of these insulin secretory granules consists mainly of crystalline insulin (Greider *et al.*, 1969).

The A chain is made up of 21 amino acids, and the B chain is made up of 30 animo acid residues (Fig. 11). The relative molecular mass is about 6000, and it is the smallest unit possessing biological activity. Under physiological conditions, two molecules are probably linked together to form a dimer, or four to form a tetramer. Insulin obtained from various species differs in amino acid composition in chain A or chain B or both (Sanger, 1959) (Table IV). Differences occur within species also, since rats (Clark and Steiner, 1969) and mice (Markussen, 1971) have been shown to have two nonallelic insulins. These structural differences among the various species of animals are not located at a critical site, however, since they do not affect the biological activity. They do, however, affect their immunological behavior.

The amount of insulin stored in the pancreas by various species differs (Marks and Young, 1940). The dog stores about 3.3 units per gram of pancreas, which amounts to about 75 units in a 10-kg dog, an amount which, if suddenly released, would be fatal.

Insulin release is effected by glucose, mannose, leucine, other amino acids, ketone bodies, and fatty acids. This release is mediated by glucagon, a hormone which increases cAMP and potentiates the insulin response. The sulfonylureas are effective as pharmacological agents in releasing insulin, the basis for their therapeutic use.

Blood glucose is the primary regulator of both insulin release and its biosynthesis. This is a highly selective process because other peptides are not released to any extent, and it is a very rapid process. The insulin response curve to a glucose load (intravenous glucose tolerance test) exhibits two peaks in man, the early 5-minute peak representing release and

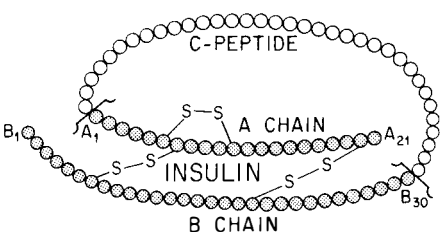


Fig. 11. Insulin and proinsulin. Proinsulin is the coiled polypeptide. When the connecting C-peptide (○) is removed, the insulin molecule (◎) is released.

TABLE IV

Species Variation in Amino Acid Sequences of Insulin of Animals[a]

Species	Position[b]			
	A chain			
	A_8	A_9	A_{10}	B chain
Human	Threonine	Serine	Isoleucine	Threonine
Dog	Threonine	Serine	Isoleucine	Alanine
Pig	Threonine	Serine	Isoleucine	Alanine
Sperm whale	Threonine	Serine	Isoleucine	Alanine
Rabbit	Threonine	Serine	Isoleucine	Serine
Horse	Threonine	Glycine	Isoleucine	Alanine
Cow	Alanine	Serine	Valine	Alanine
Sheep	Alanine	Glycine	Valine	Alanine
Sei whale	Alanine	Serine	Threonine	Alanine

[a] From Renold and Cahill (1966).
[b] These are the principal sites of variation.

the second 10- to 30-minute peak representing *de novo* insulin synthesis. This bimodal curve is not clear in dogs (Fig. 14).

During proinsulin hydrolysis, C peptide also accumulates in the granules. Therefore, when the granule contents are released by glucose stimulation, insulin, C-peptide, and proinsulin all appear in normal plasma and can be measured by radioimmunoassay. Currently, diabetes studies in man have focused on all three, but, in animals, the focus has been on insulin, and little is known of proinsulin or C-peptide in health or disease.

The influence of the various gastrointestinal hormones or insulin secretion has been of considerable interest since the demonstration by McIntyre *et al.* (1965) that plasma insulin levels were higher after an intraintestinal glucose load than an intravenous one. Currently, there are a number of gastrointestinal hormones known to influence insulin secretion to various degrees and sufficient to form a basis for a postulated enteroinsular axis (Sharkey, 1968; Buchanan, 1975). The hormones implicated are secretin (Unger *et al.*, 1967), cholecystokinin-pancreozymin (CCK-PZ) (Meade *et al.*, 1967), gastrin (Unger and Eisentraut, 1969), glucagon-like activity of the gut, and gastric inhibitory peptide (GIP). Except for GIP, the evidence for insulin control is tenuous. Gastric inhibitory peptide is now known to be a powerful stimulator of insulin secretion in man and dog, and this is associated with changes in blood glucose levels (Ross *et al.*, 1974). Thus, GIP is most likely to be central in any enteroinsular axis.

Once in the general circulation, insulin is transported to responsive tissues bound to a β-globulin. At the responsive tissue, insulin is probably bound to the tissue. All tissues, mainly liver and kidney, are able to inactivate insulin by reductive cleavage of the disulfide bonds. Liver inactivates about 50% of the insulin.

B. Mechanism of Insulin Action

It is well established that the principal site of insulin action is at the initial reaction of glucose metabolism. In order for extracellular glucose to gain entry into the metabolic

pathways, it must first enter the cell and then be phosphorylated. It is now known that insulin facilitates glucose entry into peripheral tissue cells such as muscle and adipose by a membrane transport mechanism. This insulin-sensitive membrane transport is a facilitated diffusion, independent of glucose phosphorylation and non-energy requiring. There is also a high degree of stereospecificity because D-glucose is transported but L-glucose is not. With increased accumulation of glucose in the cells, subsequent reactions in the metabolism of glucose are accelerated.

Insulin also influences the metabolism of glucose by the liver, the central organ of glucose homeostasis. The liver cell is freely permeable to glucose, and therefore the action of insulin on liver is beyond the transport step. The initial phosphorylation of glucose, catalyzed by glucokinase (GK), is rate limiting, and GK activity is influenced by insulin. In addition, the effect of insulin on other key directional phosphorylative steps directs glucose metabolism toward utilization and fatty acid synthesis. These are described in Section VII,C. Thus, there is a dual role for insulin, affecting membrane transport in muscle and adipose tissue and phosphorylation via GK in liver (Cahill *et al.*, 1959).

C. Effects of Insulin

The principal effects of administering insulin to an animal are summarized in Table V. The most characteristic finding following insulin administration is a hypoglycemia. This

TABLE V

Effects of Insulin on Animals

Tissue	Increase	Decrease
Whole animal	Anabolic	
	Food intake	
	Respiratory quotient	
Blood		Glucose
		Ketones
		Fatty acids
		Phosphate
		Potassium
Enzymes	Glucokinase	Glucose-6-phosphatase
	Phosphofructokinase	Fructose-1,6-phosphatase
	Pyruvate kinase	Pyruvate carboxylase
		PEP-Carboxykinase
	AcCoA-Carboxylase	Carnitine acyltransferase
		Serine dehydratase
Liver	Glucose oxidation	Glucose production
	Glycogen synthesis	Ketogenesis
	Lipid synthesis	
	Protein synthesis	
Muscle	Glucose uptake	
(skeletal/heart)	Glucose oxidation	
	Glycogen synthesis	
	Amino acid uptake	
	Protein synthesis	
Adipose	Glucose uptake	
	Glucose oxidation	
	Lipid synthesis	

occurs regardless of the nutritional state, age, etc., of the animal and is a net result of the increased removal of glucose from the plasma into the tissues. The respiratory quotient increases toward unity, indicating that the animal is now primarily utilizing carbohydrate. The consequences of this increased utilization of glucose follows a pattern of an increase in those constituents derived from glucose and a decrease in those which are influenced by increased glucose oxidation. The conversion of glucose to glycogen, fat, and protein is enhanced, whereas gluconeogenesis and ketogenesis are inhibited. The decreases in serum phosphate and potassium levels, which parallel those of blood glucose, are presumably due to their involvement in the phosphorylating mechanisms.

VII. BLOOD GLUCOSE CONCENTRATION AND ITS REGULATION

A. General

The blood glucose concentration depends upon a wide variety of factors, and the concentration at any time is the net result of the rates of entry and removal of glucose into the circulation. As such, all the factors that influence entry or removal become important in the regulation of blood glucose concentration. Furthermore, when the renal reabsorptive capacity for glucose is exceeded (renal threshold), urinary loss of glucose becomes an additional factor to be considered in the maintenance of the blood glucose concentration. The blood glucose levels at which this occurs vary among species and are listed in Table VI.

B. Glucose Supply and Removal

Glucose can be supplied by intestinal absorption of dietary glucose, hepatic production from other dietary carbohydrates such as fructose and galactose, from amino acids (gluconeogenesis), or from glycogen. The dietary sources of supply are especially variable. The absorptive process itself may vary with the degree of thyroid activity. In the postabsorptive state, hepatic production is the major source of supply for maintaining blood glucose. The hormonal influences (epinephrine, glucagon) involved in the release of glucose from glycogen have been described in Section IV,C,2. The influence of glucocorticoids is probably related to its effect upon gluconeogenesis.

Removal of glucose is also governed by a variety of factors, most of which ultimately relate to the rate of utilization of glucose by the animal. All tissues constantly utilize

TABLE VI

Renal Thresholds for Glucose in Domestic Animals

Species	Threshold (mg/dl)	Reference
Dog	175–220	Shannon *et al.* (1941)
Horse	180–200	Stewart and Holman (1940)
Cow	98–102	Bell and Jones (1945)
Sheep	160–200	McCandless *et al.* (1948)
Goat	70–130	Cutler (1934)

glucose either for energy purposes or for conversion to other products (glycogen, pentoses, lipids, amino acids). As such, an outflow governed by the rate of utilization occurs at all times. The level of blood glucose itself partially governs the rate of utilization, and therefore, in a sense, is autoregulatory. At high levels, the rate of glucose uptake by tissues such as muscle and liver increases due to a mass action effect. The presence of insulin increases the rate of utilization, either by increased transport (muscle, adipose) or increased phosphorylation (liver). The action of insulin is opposed by the diabetogenic factors, growth hormone, glucagon, and cortisol.

The liver supplies as well as removes glucose and therefore occupies a central position in the regulatory mechanism for blood glucose concentration. The major direction of liver metabolism, however, is directed toward supply rather than utilization of glucose. It has been estimated in liver slices that about 25% of glucose is oxidized to lactate or CO_2 and that the remainder goes to glycogen or to free (Cahill *et al.*, 1959). Muscle, on the other hand, does not contain G-6-Pase and is therefore primarily a glucose-utilizing tissue.

C. Role of the Liver

The membrane transfer system is rate limiting in peripheral tissues, which are sensitive to insulin (muscle, adipose). In the liver, however, this mechanism is not rate limiting because glucose diffuses freely across the liver cell membrane (Cahill *et al.*, 1958). It has been demonstrated (Soskin *et al.*, 1938; Cahill *et al.*, 1958) that at a blood glucose level of approximately 150 mg/dl, the liver ceases to take up or supply glucose to the circulation. This level might then be termed the "steady state" or the "glucostatic level" at which those mechanisms of normal supply and removal are operating at equal rates. Above 150 mg/dl, glucose removal is greater than mean supply, and, below 150 mg/dl, supply is greater than removal. Since the fasting blood glucose level in most animals is about 80 mg/dl, the liver supplies glucose throughout the day, except for the few postprandial periods. Insulin decreases liver glucose output and glycogenolysis and increases glucose uptake by the liver (Madison, 1969). This directional control is the result of the action of insulin on the key enzymes of glucose phosphorylation.

Directional control for glucose production or utilization is governed by opposing and irreversible enzyme reactions at three control points of glucose metabolism. These "key enzyme" couples are GK/G-6-Pase, PFK/F-1,6-Pase, and PK/PEP-CK, PC. The kinases direct metabolism toward glucose utilization, and the opposing enzymes are gluconeogenic.

The sensitivity of the rate-limiting GK reaction to insulin in the intact liver cell is readily demonstrated (Chernick and Chaikoff, 1951). The opposing G-6-Pase reaction increases during fasting (Ashmore *et al.*, 1954), a change favoring the production of glucose by the liver. An even greater increase is found in diabetes, in spite of the hyperglycemia. Increases in the other key enzymes of gluconeogenesis, F-1,6-Pase, PEP-CK, and PC, are also observed in diabetes (Ashmore and Weber, 1968). The increases in activity of G-6-Pase and other gluconeogenic enzymes then provide the directional control resulting in excessive production of glucose by the diabetic liver.

The amelioration of diabetes in an experimental animal by hypophysectomy (Houssay animal) is well established. The pituitary factor, which opposes the action of insulin, is associated with growth hormone. The influence of the glucocorticoids is exerted through

their effect upon increasing gluconeogenesis and thereby the intracellular concentration of G-6-P. An increase also results from the glycogenolytic action of epinephrine and glucagon, and the equilibrium is shifted in favor of glucose production. Therefore, the balance of the hormones that directly (insulin) or indirectly exert their influence upon glucose metabolism sets the homeostatic or glucostatic level of glucose, which controls hepatic glucose uptake or production.

D. Glucose Tolerance

The regulatory events which occur in response to changes in blood glucose concentration can be summarized by a description of the consequences of the ingestion of a test dose of glucose. When glucose is administered orally to a normal animal, a typical change in blood glucose concentration with time is observed, as shown in Fig. 12. During the absorptive phase, phase I, the rate of entry of glucose into the circulation exceeds that of removal, and the blood glucose level rises. As the blood glucose level rises, hepatic glucose output is inhibited, and the release of insulin is stimulated. This release of insulin is the result of glucose stimulation and the insulin-releasing effect of secretin, pancreozymin, gastrin, and glucagon. In 30–60 minutes, a peak level is reached, after which the blood glucose level begins to fall. During this phase of falling blood glucose concentration, phase II, the rates of removal exceed those of entry, and the regulatory mechanisms directed toward removal of glucose are operating maximally. The increased glucose utilization is enhanced by a decrease in hepatic output of glucose, and the blood glucose level falls rapidly. When the glucose level reaches the original level, it continues to fall to a minimal level and then returns to the original level. This hypoglycemic phase (phase III) is considered to be due to the inertia of the regulatory mechanisms because, in general, the higher the glycemia, the greater the subsequent hypoglycemia.

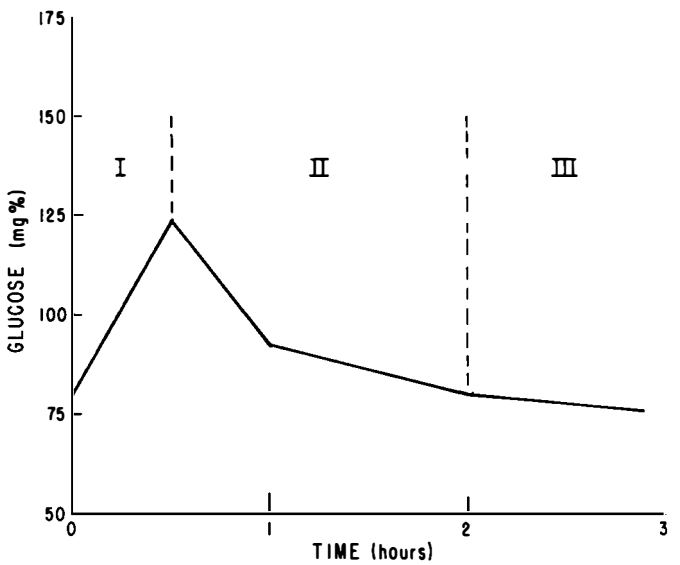


Fig. 12. Oral glucose tolerance in the dog. I, II, and III are phases of the curve. (For discussion, see text.)

VIII. METHODOLOGY

A large number of tests have been devised to assess the status of the carbohydrate economy of animals. Currently, the *o*-toluidine, hexokinase, and glucose oxidase methods are most widely used.

A. Blood Glucose

1. Methods

All blood glucose methods that were commonly employed earlier are based upon the reducing properties of glucose. These methods employ an alkaline copper solution containing cupric (Cu^{2+}) copper, which is reduced to the cuprous (Cu^{+}) form. The reduced copper than reacts with a reagent, phosphomolybdate or arsenomolybdate, to form a color. Another color reaction employs the reduction of ferricyanide to ferrocyanide to form Prussian blue. This is the basis for a micro method (Folin and Malmros, 1929) which employs 0.05 ml of blood. The colored solutions obtained in all these methods are then compared with standard glucose solutions in a colorimeter or photometer. These methods have been replaced by simpler and highly glucose specific methods.

The *o*-toluidine method is now widely used in unitized blood chemistry systems and in some automated analyzers. *o*-Toluidine condenses with glucose in glacial acetic acid to form a blue-green color. It is specific for aldose sugars, galactose, and mannose and, since blood contains no significant amounts of aldoses other than glucose, the values are taken as "true glucose."

Currently, three enzyme methods are in use: the glucose oxidase, hexokinase, and glucose dehydrogenase methods. The glucose oxidase method is specific for blood glucose and employs the enzymes glucose oxidase and peroxidase and a dye. Glucose oxidase catalyzes the conversion of glucose in the filtrate to gluconic acid:

$$\text{Glucose} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

The hydrogen peroxide formed in the reaction, in the presence of peroxidase, oxidizes a dye to form a colored product. The same principle is employed in the glucose-specific paper strips in common use for the estimation of urine glucose levels.

The hexokinase method is a glucose-specific method also available in kit form and in automated systems. Hexokinase catalyzes the phosphorylation of glucose, and the reaction is coupled to another reaction, such as G-6-P dehydrogenase, for measurement:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-P dehydrogenase}} \text{6-PG} + \text{NADPH}$$

A third enzyme-catalyzed reaction is the glucose dehydrogenase method, also available in kit form and in automated systems (Banauch *et al.*, 1975):

$$\text{Glucose} + \text{NAD} \xrightarrow{\text{glucose dehydrogenase}} \text{gluconolactone} + \text{NADH}$$

These enzymatic methods are also specific, reliable, and accurate for quantitative urinary glucose measurements.

No matter how accurate a method for blood glucose is, it cannot compensate for loss of

TABLE VII

Blood Glucose Concentration in Domestic Animals[a]

Horse	Cow	Sheep	Goat	Pig	Dog	Cat	Monkey (*Macaca* sp.)
75–115 (95 ± 8)	45–75 (57 ± 7)	50–80 (68 ± 6)	50–75 (63 ± 7)	85–150 (119 ± 17)	65–118 (91 ± 8)	50–75 (63 ± 7)	85–131 (107 ± 13)

[a] Milligrams per deciliter, plasma or serum, glucose oxidase method, adult animals.

glucose in an improperly handled blood sample. Glucose breakdown, i.e., glycolysis, by red blood cells takes place very rapidly, about 10% per hour loss, at room temperature and occurs even more rapidly if the sample is contaminated by microorganisms. For these reasons, plasma or serum must be separated from the red blood cells as soon as possible. If this is not possible, the glucose in the blood sample must be protected from glycolysis. This can best be accomplished by the use of vials containing sodium fluoride (10 mg per milliliter blood) and by refrigeration. The sodium fluoride acts both as an anticoagulant and as a glucose preservative. Sodium fluoride may also be added to the blood sample vial containing an anticoagulant.

2. Blood Glucose Levels in Animals

The normal ranges for blood glucose levels are given in Table VII. From the preceding sections, it is apparent that a standard procedure for sampling must be employed to minimize variations in blood glucose, especially those due to diet. This is accomplished in the nonruminant and in the young ruminant by a standard 16- to 24-hour fast prior to sampling. This is not necessary in the older ruminant, for it has been shown that carbohydrate given orally elicits no blood glucose response (Bell and Jones, 1945).

B. Tolerance Tests

1. Glucose Tolerance Tests

The term ''tolerance'' in its original usage referred to the amount of glucose that could be ingested by an animal without producing a glucosuria—hence, tolerance for glucose. Since in a normal animal the absence of a glycosuria indicates only a limited rise in blood glucose concentration when the renal threshold is not exceeded, the term ''glucose tolerance'' now refers to the blood glucose level following its administration. Accordingly, an animal with an increased glucose tolerance is one which shows a limited rise in blood glucose, whereas an animal with a decreased tolerance shows an excessive rise.

It is important to ascertain the nature of the animal's diet, especially with omnivores and carnivores, prior to performance of this test. A diabetic type of glucose tolerance curve is obtained when dogs are placed on a diet of horse meat alone for 1 week (Hill and Chaikoff, 1956). These dietary variations should be obviated by placing the dog on a high-carbohydrate diet (100–200 gm//day) for 3 days prior to performance of the test. The tolerance curve may also be affected by the status of the intestinal absorptive process, i.e., inflammation, increased motility, thyroxine. The variations due to absorption and the excitement often attending intubation can be avoided by use of the intravenous test.

a. Oral Glucose Tolerance Test (OGTT). In Section VII,D, the blood glucose curve following the oral administration of a test dose of glucose was described. The OGTT is ineffective in the ruminant because the ingested carbohydrate is almost totally fermented by the rumen microflora. The OGTT has been used in dogs by feeding of a test meal consisting of 4 gm glucose per kilogram body weight mixed with a few grams of horse meat. A fasting blood sample is taken, the test meal given, and blood samples taken at 30-minute intervals for 3 hours. The glucose tolerance curves in dogs receiving either glucose or galactose together with meat in their daily diet exhibited normal curves, as described in Section VII,D. The maximal level, 120–140 mg/dl was reached at 1 hour and returned to the fasting level, 65–95 mg/dl, in 2-3 hours. The OGTT may be simplified to the taking of a single sample at 2 hours after the glucose is given, i.e., 2-hour postprandial glucose. A normal blood glucose level 2 hours postprandially indicates that diabetes is unlikely. A persistent hyperglycemia at this time is indicative of a diabetic curve and should be confirmed with the complete GTT.

b. Intravenous Glucose Tolerance Test (IVGTT) and the Insulin Response. The standardization of the IVGTT in animals has recently been described and is recommended (Kaneko *et al.*, 1978b). After a standard 16- to 24-hour fast (except for an adult ruminant) a zero-time heparinized blood sample is taken. Timing is begun, and 0.5 gm glucose per kilogram body weight is infused IV as a sterile 50% solution in exactly 30 seconds. In large animals, it is given within 2–3 minutes, or more quickly if possible. Subsequent blood samples are taken at 5, 15, 25, 35, 45, and 60 minutes. The results of the IVGTT are plotted on semilogarithmic coordinates, from which the half time ($T_{\frac{1}{2}}$), the time required for the glucose concentration to fall by one-half, is graphically estimated or calculated between 15 and 45 minutes postinfusion. From the $T_{\frac{1}{2}}$; the fractional turnover rate, k, can also be calculated:

$$k = \frac{0.693}{T_{1/2}} \times 100 = \%/\text{minute}$$

The fractional turnover rate has been variously expressed as the glucose turnover rate, the glucose disappearance rate, the glucose disappearance coefficient, or simply the k value. The normal $T_{\frac{1}{2}}$ and k values in dogs are 25 ± 8 minutes and 2.76 ± 0.91 %/minute, respectively (Kaneko *et al.*, 1977). A diabetic animal with decreased glucose tolerance has a longer $T_{\frac{1}{2}}$ and lower k value.

The method is equally applicable and the only practical method in large animals. The k value in a spontaneously diabetic cow was 0.38 %/minute ($T_{\frac{1}{2}}$ = 182 minutes), compared to a normal of 1.98 %/minute [$T_{\frac{1}{2}}$ = 35 minutes (Kaneko and Rhode, 1964)] and was comparable to the k values obtained using [^{14}C]glucose (Kaneko *et al.*, 1966).

Standardization of the IVGTT, as described, has the following advantages: Adequate insulin response is provoked, the influence of urinary glucose loss is minimized, and more reproducible clearance values are obtained (Fig. 13). Other areas of the IVGTT with diagnostic significance which should be noted are the 5-minute peak, which is inordinately high in diabetics, and the 60-minute value, which does not return to the preinfusion level in diabetes (Table VIII).

The curve representing insulin response to the glucose load is obtained from the same samples used for glucose (Fig. 14). In the normal response curve, the peak insulin response occurs at 5 minutes, followed by a return toward normal at 60 minutes (Kaneko

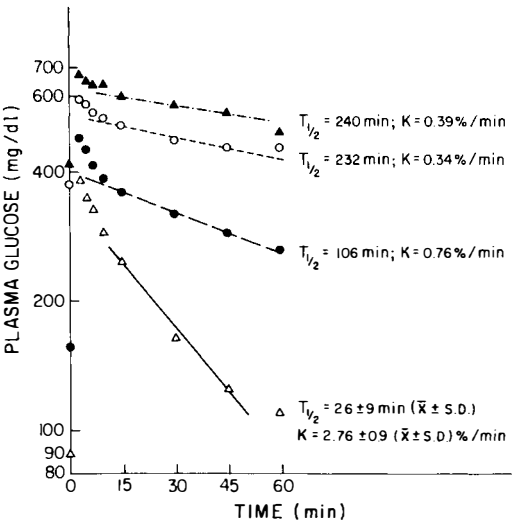


Fig. 13. Intravenous glucose tolerance test (IVGTT) in normal dogs and in dogs with various types of diabetes mellitus. Key: ▲, Type I; ○, Type II; ●, Type III; △, normal (Kaneko *et al.*, 1977.)

TABLE VIII

Diagnostic Criteria for Types of Diabetes Mellitus in Dogs

Diabetes type	IVGTT			Insulin[a]		
	G_0 (mg/dl)	$T_{1/2}$ (minutes)	k (%/minute)	I_0	I_p	$\Delta I/\Delta G$
I	>200	>70	<1.0	L	L	L
II	>200	>70	<1.0	N, H	L	L
III	110-200	>45	<1.5	N, H	N, D	L, N
Normal	70-110	15-45	>1.5	N	N	N

[a] Abbreviations: N, normal; L, low; H, high; D, delayed.

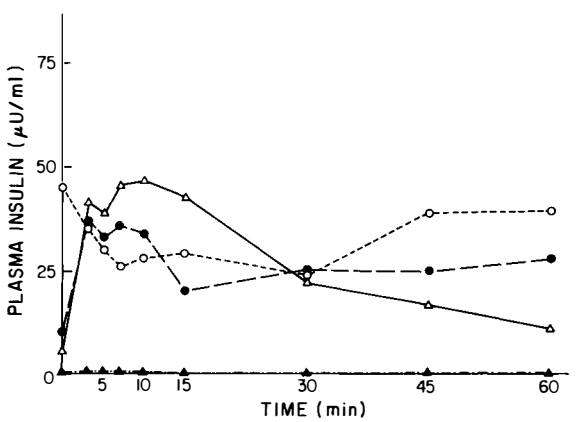


Fig. 14. Insulin response during the IVGTT in normal dogs and in dogs with various types of diabetes mellitus. Key: ▲, Type I; ○, Type II; ●, Type III; △, normal. (Kaneko *et al.*, 1977.)

et al., 1977). The early 5-minute peak is attributed to insulin release by the β cells due to the stimulation by glucose. In human beings, a second peak is described at 20–30 minutes which is attributed to the *de novo* synthesis of insulin by the β cells. This peak has not been experimentally discernible in dogs (Kaneko *et al.*, 1978a).

The glucose tolerance and insulin response test is of greatest value in the diagnosis and differentiation of types of diabetes, particularly those with a mild hyperglycemia and with no or intermittent glucosuria. Decreased tolerance is also observed, although less consistently in hyperthyroidism, hyperadrenalism, hyperpituitarism, and severe liver disease. An increased tolerance is observed in hypofunction of the thyroids, adrenals, and pituitary and in hyperinsulinism.

2. *Insulin Tolerance Test*

The blood sugar concentration of a normal animal after the administration of a test dose of insulin exhibits a characteristic response, as shown in Fig. 15. After a fasting blood sample is obtained, 0.1 unit of crystalline zinc insulin per kilogram body weight is injected intramuscularly or subcutaneously, and blood samples are taken every 30 minutes for 3 hours. The test measures (1) the sensitivity of the blood glucose level to a test dose of insulin and (2) the response of the animal to insulin-induced hypoglycemia. Normally, the blood glucose level falls to 50% of its fasting level in 20–30 minutes and returns to its fasting level in 1.5–2 hours. Two types of abnormal response are seen. If the blood glucose level does not fall to the 50% level or requires longer than 30 minutes to reach the maximal hypoglycemic level, the response is described as "insulin insensitive" or "insulin resistant." Insulin resistance is found, although inconsistently, in hyperfunction of the pituitary and adrenals.

If the hypoglycemia is prolonged and fails to return to the fasting level in 2 hours, the response is described as "hypoglycemia unresponsiveness." This type of response may be observed in hyperinsulinism, hypopituitarism, and hypoadrenalism and is most often employed in suspected cases of the latter two conditions. When this test is performed,

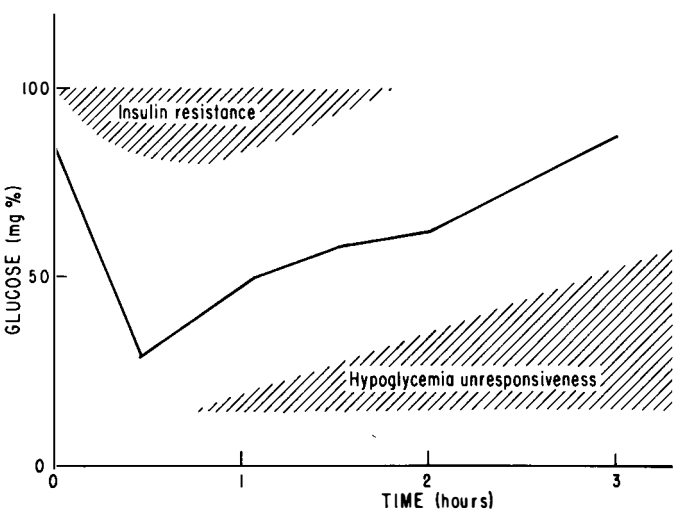


Fig. 15. Insulin tolerance in the dog.

since hypoglycemia is being induced, a glucose solution should be readily available for injection.

3. Glucagon Tolerance Test

As previously described, glucagon has a hyperglycemic effect, which in turn evokes an insulin response. The test is performed by the IV injection of 30 μg glucagon per kilogram body weight. A fasting blood glucose sample is obtained before injection and every 15–30 minutes for 2–3 hours. An exaggerated 15-minute insulin response and marked hypoglycemia at 2–3 hours or longer are expected in pancreatic islet cell tumors, although published reports are scarce (Johnson and Atkins, 1977).

4. Epinephrine Tolerance Test

The response of the blood glucose level following the injection of epinephrine is characteristic. The blood glucose level rises to a maximum of 50% above the fasting level in 40–60 minutes and returns to the original level in 1.5–2 hours. The test is performed by obtaining a fasting blood sample, injecting 1 ml of 1:1000 epinephrine-HCl (in the dog) IM, and obtaining blood samples every 30 minutes for 3 hours.

The characteristic increase in blood glucose has been used as an index of the availability of liver glycogen for the production of blood glucose. On the basis of a lowered response to epinephrine, Shaw (1943) concluded that liver glycogen in bovine ketosis was depleted, a finding later confirmed by direct measurement of glycogen in biopsy samples (Kronfeld *et al.*, 1960; Ford and Boyd, 1960). A lowered glycemic response is a characteristic response in the classical von Gierke's type of glycogen storage disease of man.

5. Leucine-Induced Hypoglycemia

The oral administration of L-leucine induces a marked and persistent hypoglycemia in hyperinsulinism due to pancreatic islet cell tumors. The hypoglycemia is associated with a rise in plasma insulin (Yalow and Berson, 1960) as a result of its release by tumorous islet cells. The test is performed by the oral administration (stomach tube) of 150 mg L-leucine per kilogram of body weight as an aqueous suspension to a fasting dog. A fasting blood glucose sample is taken before administration and every 30 minutes for 6 hours. A hypoglycemic effect is seen quickly at 0.5–1 hour and may persist for as long as 6 hours in hyperinsulinism. The normal dog exhibits no hypoglycemic effect.

6. Tolbutamide Test

The intravenous administration of tolbutamide, an oral hypoglycemic agent, results in the release of insulin from the pancreas and is utilized as a test of the available insulin in the pancreas. The blood glucose curve during the test parallels that obtained during the insulin tolerance test. This test has not received wide application in animals.

IX. DISORDERS OF CARBOHYDRATE METABOLISM

From the preceding sections, it is evident that alterations in blood glucose levels may occur in a variety of disease states and are of particular importance in endocrine disorders. Normal blood glucose levels are the result of a finely balanced system of hormonal interaction affecting the mechanisms of supply and removal. When imbalance occurs, a

new equilibrium is established. Whether this equilibrium is clinically evident as a persistent hypoglycemia or hyperglycemia depends upon the total interaction of the hormonal influences on carbohydrate metabolism. Further discussions concerning the disorders of the pituitary, adrenals, and thyroids can be found in Chapter 12. The following sections discuss the conditions in which the principal manifestations are closely related to derangements in carbohydrate metabolism.

A. Diabetes Mellitus

Although diabetes mellitus has been reported in virtually all laboratory animals (gerbils, guinea pigs, hamsters, mice, rats, nonhuman primates) and in horses, cattle, sheep, and pigs, it is most frequently found in dogs and cats. Estimates of the incidence of diabetes range as high as 1 : 152 for dogs (Krook *et al.*, 1960) and 1 : 800 for cats (Meier, 1960). Diabetes mellitus in animals has been frequently reviewed (Wilkinson, 1957, 1958; Krook *et al.*, 1960; Meier, 1960; Wilkinson, 1960; Dixon and Sanford, 1961; Berkow and Ricketts, 1965; Teunissen and Blok-Shuring, 1966; Cotton *et al.*, 1971; Foster, 1975; Ling *et al.*, 1977). The classic and still the most detailed study of diabetes in the dog and cat was that by Hjarre (1927).

The disease in dogs occurs most frequently in the mature or older female, often in association with estrus and in all breeds. It is frequently associated with obesity (Krook *et al.*, 1960). In contrast, male cats appear to be more commonly affected than females. Little is known of the genetic aspects of diabetes in animals as compared to man, in which the hereditary predisposition is well known. Diabetes has, however, been reported in the offspring of diabetic dogs (Roberts, 1954; Gershwin, 1975; Kaneko *et al.*, 1977). Kramer (1977) reported his observations on hereditary diabetes in a family of Keeshonds.

Furthermore, it has been suggested on the basis of serum insulin response patterns during the IVGTT that diabetes mellitus of dogs can be divided into three types: I, II, and III (Table VIII) (Kaneko *et al.*, 1977, 1979). Type I dogs are characterized by a very low initial insulin (I_0) level and no I response to the glucose load similar to the juvenile form of diabetes in children. Type II is characterized by a normal or high I_0 and, again, no increment of I response to the glucose, which are features of the maturity onset form of diabetes in human beings. Type III is characterized by a normal I_0, a normal or delayed I response to the glucose, and a delayed return of I to normal at 60 minutes, as seen in chemical diabetes. Since all these forms exhibit glucose intolerance, separation of diabetes into types can be done only by using the I response patterns. The importance of defining the types lies in the likelihood that Type III is the subject most amenable to successful oral hypoglycemic therapy and/or dietary therapy. It is also likely that Type III (chemical) precedes the development of the two clinically overt types of diabetes depending on the etiological nature of the insulin deficiency—absolute (Type I), of hereditary origin; or relative (Type II), due to pancreatic damage. Contributory factors to the onset of the disease most often mentioned are pancreatitis, obesity, infection, stress, and estrum. The possibility of a viral etiology has also been reviewed (Steinke and Taylor, 1974).

Autoimmunity has also been investigated as a possible cause of diabetes mellitus. An immune response is suggested because lymphocytic infiltration is frequently associated with immune processes and lymphocytic infiltration is found in spontaneous diabetes of cattle (Kaneko and Rhode, 1964) and man. It is also observed in cattle (Le Compte *et al.*, 1966) and or rabbits (Grodsky *et al.*, 1966) immunized with bovine insulin.

The high estimates of the incidence of the disease provide an indication of its importance as a clinical consideration. Furthermore, the similarities of the clinical picture of diabetes with that of other wasting diseases showing polyuria and polydypsia attest to the importance of laboratory examinations in the early and accurate diagnosis of diabetes. In no other disease is an understanding of the metabolic alterations so important in diagnosis and proper treatment.

The fundamental defect in diabetes mellitus is an absolute or relative lack of insulin, resulting in an inability to utilize glucose. The lack of insulin has been demonstrated in the spontaneously diabetic cow by the failure of a large test load of glucose to elicit a serum insulin response (Kaneko and Rhode, 1964). More recently, by the use of the standard IVGTT and the serum insulin response, spontaneous diabetes mellitus in dogs has been classified into Types I, II, and III, as described above. In all types, the inability to utilize glucose is demonstrated by the GTT. In the absence of insulin, the inability of the diabetic animal to utilize glucose is clearly shown in its inability to convert [^{14}C]glucose to $^{14}CO_2$ (Kaneko *et al.*, 1966; Phillips *et al.*, 1971). This inability was corrected by insulin. The inability of the animal to utilize glucose is reflected in the clinical signs of diabetes, loss of weight, polyuria, polydypsia, and, in the advanced states, ketoacidosis.

Several reports have suggested that the development of diabetes mellitus is the result of the interaction of several hormones, principally insulin and glucagon (Unger and Orci, 1975, 1976). Although excess glucagon is a pattern of diabetes and it can induce glucose intolerance or changes in diabetic control, it can do so only as long as insulin deficiency is present and pharmacological levels of glucagon are induced (Felig *et al.*, 1976). Thus, it is concluded that insulin deficiency is the primary cause of diabetes and that, although glucagon may modify the consequences, it is neither necessary nor sufficient for the development of diabetes (Felig *et al.*, 1976).

1. Hyperglycemia

The finding of a fasting hyperglycemia is one of the most important diagnostic criteria of diabetes mellitus. As previously stated, the homeostatic level of blood glucose is maintained in the normal animal as a result of an equilibrium established between glucose supply and removal. The endocrine balance is particularly important in establishing this equilibrium. The effect of insulin tends to lower the blood glucose level, whereas the opposing effects of the anterior pituitary, glucagon, and adrenal cortical factors tend to raise it. In the diabetic animal, with a real or relative lack of insulin, the equilibrium is shifted to a higher level of blood glucose. Peripheral glucose utilization is lowered and hepatic glucose production is increased due to increases in the gluconeogenic enzymes.

In the diabetic animal, the hyperglycemia itself tends to compensate in part for the decrease in peripheral utilization. This occurs partially as a mass action effect and results in an inflow of glucose into the tissues. Thus, the diabetic animal continues to utilize glucose in the absence of insulin but only at the expense of increased glucose production and hyperglycemia. As the deficiency of insulin progressively becomes more severe, the equilibrium level of blood glucose is established at higher and higher levels, and equilibrium may never be established. Blood glucose values in canine diabetics have been reported to be as high as 1250 mg/dl (Wilkinson, 1960). When the renal threshold (200 mg/dl) for glucose is exceeded, the diabetic animal is also faced with excessive loss of glucose, for which further compensation must be made.

In the uncomplicated diabetic, the blood glucose level is exquisitely sensitive to insulin

and has remained the mainstay for monitoring the success of insulin therapy. A glycohemoglobin fraction of hemoglobin, Hb_{1ac}, has been reported to reflect the average blood glucose level over several weeks and shows promise of being an effective means of monitoring insulin therapy (Rahbar, 1968; Gabbay *et al.*, 1977; Bunn *et al.*, 1978).

2. Glucose Tolerance and the Insulin Response

The glucose tolerance test is the most important test of carbohydrate function and is of particular value in those cases of diabetes in which the fasting blood glucose level is only moderately elevated and the diagnosis is equivocal. The blood glucose curve in diabetes characteristically shows a decreased tolerance for glucose (Fig. 12), as evidenced by the long $T_{\frac{1}{2}}$ or low k value, which reflects the inability of the animal to dispose of a test dose of glucose. The insulin response curve in Type I diabetes clearly demonstrates the inability of the pancreas to release insulin in response to the glucose load. The absence of an insulin response plays the primary role in the failure of the diabetic animal to utilize the added glucose. An equally important contributory factor is the overproduction of glucose by the diabetic liver in the presence of hyperglycemia. The test dose of glucose is in effect added to the burden of an already existing oversupply of glucose. Since the steady-state level at which the liver ceases to supply or remove glucose is elevated in diabetes, the liver continues to supply glucose, which contributes to the delayed return of the tolerance curve to its original level.

In Types II and III diabetes (see below), there is also glucose intolerance, but this occurs in the presence of a normal to elevated immunoreactive insulin (IRI) level. This would mean that the IRI in the plasma of these types is ineffective due to insulin resistance, binding to antibodies or other proteins, or abnormal structure or that a cross-reacting insulin-like molecule such as C-peptide is being measured. Thus, the glucose tolerance curve reflects an absolute (Type I) or relative (Types II, III) lack of insulin.

3. Insulin and the Insulin Response

Plasma IRI is characteristically very low or absent in Type I (juvenile) diabetes, whereas it is normal or even very high in Type II or III. Thus, Type I can be readily differentiated on the basis of the fasting IRI level. On the other hand, about 40% of even severe diabetics have normal to very high IRI levels. The classification of these can be done only by the nature of the insulin response curve during the IVGTT. Type II (maturity onset) has a normal to high IRI with no increment of IRI response to the glucose load. Type III (chemical) also has a normal to high IRI; there may or may not be an IRI response, but the most significant finding is a delayed return to preinjection levels (Kaneko *et al.*, 1977).

The classification of diabetes into types has important therapeutic and prognostic implications for the dog. Insulin replacement therapy would be the only effective treatment for Types I and II. Type III dogs with even a small insulin reserve would be the most likely subjects for successful oral hypoglycemic therapy. The early detection of diabetes and treatment using oral drugs would be advantageous. Prognostically, the severity of the diabetes can be assessed by the degree of glucose intolerance together with the IRI response.

4. Ketonemia and Lipemia

As the utilization of glucose progressively decreases in the diabetic animal, the utilization of fatty acids for energy purposes progressively increases. The supply of fatty acids

for hepatic utilization is obtained by mobilization from the body fat depots. Mobilization progressively increases as insulin deficiency becomes more severe due to increases in hormone-sensitive lipase. This enzyme is different from the lipoprotein lipase.

In severe diabetes, hyperlipemias are often so marked that the blood has the appearance of "tomato soup." A cream layer may separate out on storage in the cold due to hyperchylomicronemia. The plasma is turbid due to the presence of lipoproteins (very low density lipoprotein pre-β-lipoproteins). On chemical analysis, total triglycerides and cholesterol (very low density lipoproteins) are elevated (Rogers *et al.*, 1975). Diabetic hyperlipemia appears to be caused by impaired lipolysis of chylomicra secondary to a deficiency of lipoprotein lipase rather than to overproduction of VLDL (Bagdade *et al.*, 1967).

Concurrently with increased fatty acid oxidation in liver, a progressive decrease in fatty acid synthesis occurs. The net effect of the alterations in hepatic fatty acid metabolism is that acetyl-CoA units are generated in excess in the liver. This is determined by the increased rate of fatty acid β-oxidation via increased carnitine acyltransferase. Fatty acyl-CoA resulting from fat mobilization is also a marked inhibitor of citrate synthase, which would remove another route for disposal of AcCoA. The accumulated AcCoA units are then diverted into alternature pathways as described in SectionV,B and, with the activation of ketogenic mechanisms, excessive synthesis of ketone bodies (Kreisberg, 1978) and cholesterol results. In the peripheral tissues it is now known that there is an underutilization of ketone bodies in the diabetic dog (Balasse and Havel, 1971). Ketosis thus occurs as a result of an overproduction of ketone bodies and underutilization by the peripheral (muscle) tissues.

It has been suggested that the development of ketosis requires both a deficiency of insulin and an excess of glucagon (McGarry *et al.*, 1975; McGarry and Foster, 1976). This is in keeping with the proposal (Dobbs *et al.*, 1975; Unger and Orci, 1975) that diabetes develops due to a bihormonal interaction of insulin and glucagon since glucagon levels are high in insulin deficiency. These findings have been extensively reviewed by Kreisberg (1978), who concludes that "insulin deficiency is the major, if not the sole, hormonal abnormality responsible for the metabolic abnormalities of diabetes mellitus and ketoacidosis," in keeping with Felig *et al.* (1976).

In the ketoacidotic state, marked cholesterolemias as high as 700 mg/dl have been observed in clinical diabetes of the dog. It has been pointed out that net gluconeogenesis from fatty acid does not occur and that the precursors for gluconeogenesis are primarily proteins. The relative excess of glucagon, adrenal, and pituitary factors in the diabetic animal also contributes to protein catabolism and gluconeogenesis. The cofactors that provide the reductive environment required for gluconeogenesis can be made available by the increased production of reduced cofactors during the increased fatty acid oxidation (Renold and Cahill, 1966). This increase in the reductive environment of the cell has been proposed (Wieland, 1968) as the mechanism that stimulates gluconeogenesis, a corollary to the development of ketoacidosis.

5. *Electrolyte Balance and Ketoacidosis*

A mild glycosuria of the order of a few grams of glucose loss per day does not in itself precipitate the acidotic state because a degree of compensation occurs. With continued and severe loss of glucose, however, all the attending phenomena in attempts to compensate are exaggerated. Liver glycogen stores are depleted, and replacement supplies of glucose are obtained by increased protein breakdown and gluconeogenesis. The oxidation

of fatty acids is accelerated and, with it, there is an overproduction of acetoacetate, β-OH-butyrate, and acetone in the plasma, which is primarily responsible for the acidosis. The vapor pressure of acetone (bp 56.5°C) is high at body temperature, and thus this violatile compound is often detected in the breath of the severely ketotic animal. The ketone bodies AcAc and β-hydroxy butyrate are acidic anions which increase the "anion gap"; this increase is followed by a reduction in HCO_3^-, Cl^-, Na^+, and K^+. Acidosis ensues when the base is significantly reduced and respiratory compensation is inadequate. In addition, the decreased peripheral utilization of ketone bodies on starvation (Garber *et al.*, 1974) suggests that a similar underutilization of ketone bodies occurs in diabetes (Sherwin *et al.*, 1976).

In hyperketonemia, large amounts of ketones are wasted in the urine, together with losses of water and base. The acidic ketones are largely buffered by ammonium synthesized from glutamine in the renal tubules. However, excessive amounts of ketones are ultimately lost with Na^+ and K^+ in the urine. Even without ketonuria, the loss of electrolytes in the polyuria of diabetes may be considerable. Thus, the acidosis of the diabetic is a primary base deficit fundamentally related to the ketonemia and to the loss of ketones and base in the urine.

Excess glucose in the glomerular filtrate provokes an osmotic diuresis, leading to loss of water and dehydration. The progressively severe loss of water and electrolytes together with the accompanying dehydration and ketoacidosis ultimately leads to collapse, coma, and death. The condition is aggravated by renal impairment, which fortunately is not a common finding in diabetes of the dog. Not all the extracellular sodium deficit is due to urinary loss, however, for as H^+ increases it enters the cells, K^+ leaves the intracellular compartment in exchange, and Na^+ enters the cells. As the dehydration progresses, extracellular K^+ concentration may be very high even though there may be a total body deficit. This is an important consideration in the insulin fluid and electrolyte replacement therapy of diabetic ketoacidosis, for without the addition of K^+ the rapid expansion of the extracellular fluid compartment and the reverse exchange of K^+ may result in hypokalemia. Further discussion of this acidosis and its treatment can be found in Chapter 10.

6. Urinalysis

Considering that the renal threshold for glucose in the dog is about 200 mg/dl, the detection of even trace amounts of glucose in the urine is an important finding and warrants further consideration. In a total of 56 cases which were tentatively diagnosed as diabetes mellitus on the basis of glycosuria alone, the diagnosis was later confirmed in all (Wilkinson, 1960). Renal diabetes, although always a consideration, is an extremely rare occurrence. The fluctuations in blood glucose levels following feeding have been discussed in Section VII,D and should be considered in the interpretation of the results of urine glucose determinations. Transient glycosurias may occur for 1–1½ hours after a heavy carbohydrate meal, but a 2-hour postprandial glycosuria, or a fasting glycosuria, is a strong indication of diabetes.

An elevated urinary specific gravity (SG) has been considered in the past to be a good index of the degree of glycosuria and, hence, of diabetes. Specific gravity is a measure of the concentration of solutes in the urine, principally the cations (Na^+, K^+, NH_4^+), anions (PO_4^{2-} SO_4^{2-}, HCO_3^-, Cl^-), and urea. The observed SG of urine is the result of the additive effect of the contributions of each (Price *et al.*, 1940). Albumin in urine increases the SG 0.003 unit for each gram per deciliter, whereas glucose increases it by 0.004 unit

for each gram per deciliter. Even though the presence of glucose does increase the SG linearly, a 4+ reaction (2.5 gm % glucose) would increase the SG by only 0.010 unit. Therefore, although SG is a valuable measure of renal function, it is of little value with respect to the glycosuria of diabetes. Conversely, by subtracting the contribution of albumin and glucose from the observed SG, a more accurate measure of renal function in diabetes can be obtained.

Proteinuria is a common sign of renal disease and is often observed in diabetes in dogs (Wilkinson, 1957). There is doubt whether this is associated with chronic nephritis so common in dogs or whether it is due to a renal failure as an aftermath of diabetes.

Diabetic nephropathies as a result of microangiopathies of the glomerular tufts and of basement membrane injuries are frequent and serious complications of the chronic, poorly controlled human diabetic. A degree of renal arteriosclerosis is common in diabetic dogs (Meier, 1960), but this lesion is not exactly comparable to the Kimmelstiel-Wilson lesion seen in human beings. Also, only 1 of 10 diabetic dogs at necropsy had a significant renal lesion, although most had some degree of nephritis (Cotton *et al.*, 1971). In a study of renal function in experimental streptozotocin diabetes (Kaneko *et al.*, 1978a) and in spontaneous diabetes (Kaneko *et al.*, 1979), the urea, creatinine, and phosphate clearances were normal. Also, blood UN and Cr were only slightly elevated, and it was concluded that chronic renal disease was not a significant complication in the dog.

The ketone bodies are low renal threshold substances (Schwab and Lotspeich, 1954), and their appearance in the urine is an early and significant sign of developing ketonemia. It is not, however, diagnostic of diabetes, for ketonuria is observed in starvation and is absent in the mild diabetic. Ketonuria of various degrees is, however, common in the more advanced diabetic state. Ketonuria is also a valuable sign of developing acidosis and useful for prognostication. Urinary pH is of little value in detecting acidosis, for only in extreme cases does the pH vary beyond normal limits.

7. Summary

The alterations in blood plasma that have been described are summarized in Fig. 16. In the diabetic state, the uptake and hence utilization of glucose by muscle and adipose tissue is depressed. In these tissues, protein and lipid breakdown are enhanced, and increased amounts of their constituent amino acids and fatty acids are released to the circulation and carried to the liver. Increased hepatic urea production results from the metabolism of the amino acids. Increases in the key gluconeogenic enzymes of the liver, G-6-Pase, PEP-CK, and PC, direct glucose metabolism to an overproduction of glucose. Simultaneously, lipogenesis is suppressed, which, together with the increased mobilization of fatty acids, promotes the accumulation of acetyl-CoA followed by increased cholesterolgenesis and ketogenesis. In the peripheral tissues there is an underutilization of ketones with a net increase in blood ketones and subsequent ketoacidosis. Thus, diabetes mellitus is characterized by a fundamental overproduction and underutilization of both glucose and ketones as the result of the absolute or relative deficiency of insulin.

B. Hyperinsulinism

Following the discovery of insulin, a clinical state showing marked similarities to insulin overdosage was recognized as a disease entity in man and termed hyperinsulinism. The disease is now known to be due to a persistent hyperactivity of the pancreas, usually

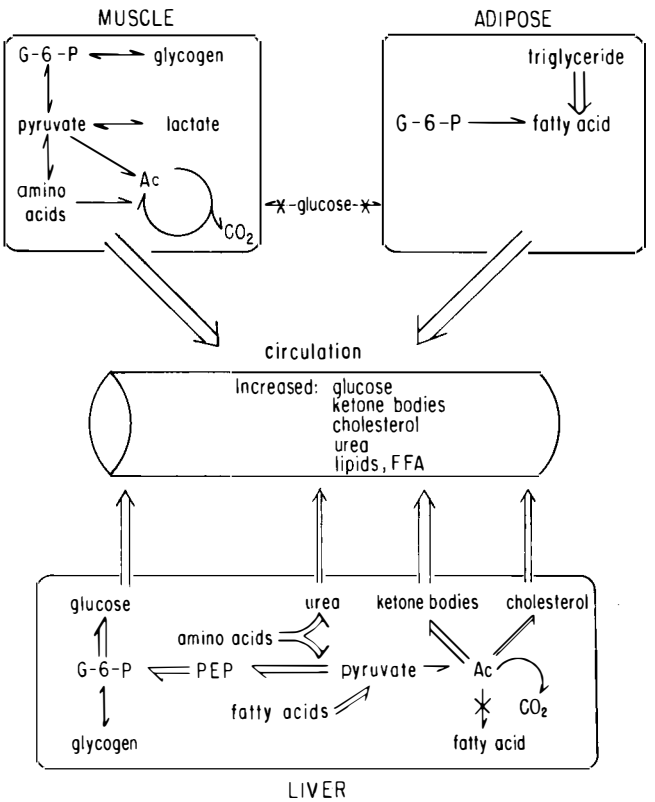


Fig. 16. Summary of metabolic alterations in tissues of major importance in the diabetic animal. Increased flow in the metabolic pathways is noted by larger arrows.

as a result of insulin-secreting islet cell tumors. In the classic case of Wilder *et al.* (1927), excess insulin was extracted from metastatic foci in liver as well as from the pancreatic tumor. The counterpart of this disease in dogs has been reported by Slye and Wells (1935), Hansen (1949), Cello and Kennedy (1957), Capen and Martin (1969), Teunissen *et al.* (1970), Spieth (1973), Hill *et al.* (1974), and Mattheeuws *et al.* (1976). Priester (1974) reported the results of an extensive survey of pancreatic islet cell tumors in domestic animals in the United States and Canada.

The disease as seen in dogs is characterized by a persistent hypoglycemia in association with periods of weakness, apathy, fainting, and, during hypoglycemic crisis, convulsions and coma. A history relating the attacks to periods after fasting or exercise provides a clinical basis for further investigations. Establishment of the diagnosis depends upon the finding of a significant hypoglycemia (below 50 mg/dl) at the time of occurrence of symptoms, and the symptoms are relieved by the administration of glucose. In mild cases, the fasting level may be normal, in which case diagnostic hypoglycemia may often be provoked by sequentially (1) placing the animal on a low-carbohydrate diet (meat only) for 1 week, (2) placing the animal on a 24-hour fast, and finally (3) moderately exercising the animal. Blood glucose levels are determined at the end of each step, and, if hypoglycemia is evident at any step, the provocation should be terminated.

The glucose tolerance test as performed in the conventional manner is of little value in

hyperinsulinism. The shape of the tolerance curve is markedly influenced by the previous carbohydrate intake of the dog (Hill and Chaikoff, 1956). A high-carbohydrate diet favors a low peak and, conversely, a low-carbohydrate diet shows a high peak in the tolerance curve. Diabetic (high) peaks are not unusual in states of hyperinsulin (disease or therapy). This has been interpreted by Somogyi (1959a,b) as being due to a transitory excess of insulin antagonists, the hormones of the anterior pituitary and adrenal cortex and epinephrine, which is provoked by the hypoglycemia and likened to "adrenaline diabetes."

The glucose tolerance curve is usually characteristic, however, if (1) the animal has been on a moderate carbohydrate diet for 3–4 days, (2) the intravenous test is used, and, most important, (3) blood sampling is continued for 6–8 hours. A prolongation of the hypoglycemic phase (phase III, Fig. 12) is the most significant portion of the curve. A curve of this type was observed in the dog, in which the hypoglycemic phase persisted for 7 hours (Cello and Kennedy, 1957).

An animal with a tendency toward persistent hypoglycemia is likely to show an abnormal response to the insulin tolerance test. The tolerance curve usually shows a minimal drop in blood glucose and remains below the original level for a prolonged length of time. Therefore, the curve shows "insulin resistance" and "hypoglycemia unresponsiveness." The use of this test, however, is not without risk, and, if used, glucose solution for intravenous administration should be at hand.

More recently, the hypoglycemia that follows oral administration of leucine in children (Cochrane *et al.*, 1956) was employed in studies of patients with islet cell tumors (Flanagan *et al.*, 1961). Marked hypoglycemia occurred within 30–60 minutes after L-leucine administration. It was also shown that leucine-induced hypoglycemia is associated with a rise in plasma insulin levels (Yalow and Berson, 1960). In the patients with islet cell tumors, leucine sensitivity disappeared after removal of the tumor, a finding which would indicate that the tumorous islet cells alone were being stimulated in these cases. This test has been employed in our laboratories in hypoglycemic dogs, and a description of its successful application in pancreatic islet cell tumors of dogs has been published (Bullock, 1965).

Currently, the most useful and straightforward test is the 24-hour fasting plasma immunoreactive insulin taken together with a 24-hour fasting plasma glucose. There is an inappropriately high level of IRI (2- to 10-fold above normal) in the presence of a hypoglycemia. The glucagon tolerance test has also been used when an exaggerated IRI response has been observed in insulinomas.

C. Hypoglycemia of Baby Pigs

Hypoglycemia of baby pigs was first observed by Graham *et al.* (1941), and our present knowledge of this condition is based largely upon the work of Sampson and associates (Sampson, 1958). The condition occurs during the first few days of life and is characterized by hypoglycemia (below 40 mg/dl), apathy, weakness, convulsions, coma, and finally death.

The newborn pig is particularly susceptible to hypoglycemia. At birth, the blood glucose level is high (103 mg/dl) and, unless the pig is fed, drops rapidly to hypoglycemic levels within 24–36 hours. The liver glycogen, which is high (14.8%) at birth, is almost totally absent at death (Morrill, 1952). In contrast, newborn lambs (Sampson *et al.*, 1955), calves, and foals (Goodwin, 1957) are able to resist starvation hypoglycemia for

more than 1 week. The ability of the baby pig to withstand starvation progressively increases from time of birth, and a 10-day-old pig can be starved for up to 3 weeks before symptoms of hypoglycemia occur (Hanawalt and Sampson, 1947).

These findings were confirmed by the studies of Swiatek *et al.* (1968), who concluded that gluconeogenesis was impaired in the newborn pig, which was associated with a decrease in plasma free fatty acids. These findings suggest that the gluconeogenic mechanisms of the baby pig are not fully developed at birth and are stimulated or induced by the initial feeding. A study of the hepatic gluconeogenic enzymes and their inducability by feeding would be of great value in understanding the mechanism of baby pig hypoglycemia.

The association of the condition with complete or partial starvation is shown by the findings that the stomachs are empty at necropsy and the syndrome itself is indistinguishable from experimental starvation of the newborn pig. Starvation of the newborn pig may occur due to factors relating to the sow (agalactia, metritis, etc.) or to the condition of the baby pig (anemia, infections, etc.), either case resulting in inadequate intake. If feeding is required to induce the hepatic gluconeogenic enzymes in the newborn pig, this would explain its inability to withstand starvation as can newborn lambs or foals.

X. DISORDERS OF RUMINANTS ASSOCIATED WITH HYPOGLYCEMIA

A. General

The principal disorders of domestic ruminants in which hypoglycemia is a salient feature are bovine ketosis and ovine pregnancy toxemia. Pregnancy toxemia characteristically is a widespread disease of high mortality occurring in the pregnant ewe just prior to term, which is the time when carbohydrate demands are highest, especially in those ewes carrying more than one fetus. Bovine ketosis, on the other hand, occurs in the high-producing dairy cow, characteristically during the early stages of lactation, when milk production is generally the highest. Abnormally high levels of ketone bodies, acetone, acetoacetate, β-OH-butyrate, and isopropanol appear in blood, urine, and milk. These alterations are accompanied by the clinical symptoms of the disorder: loss of appetite, weight, and milk production and nervous disturbances.

The energy metabolism of the ruminant is centered about the utilization of the volatile fatty acids produced by rumen fermentation rather than carbohydrate. The carbohydrate economy of the ruminant is significantly different from that of the nonruminant, and an appreciation of these differences is important for a clearer understanding of the alterations in these metabolic disorders of the ruminant.

B. Carbohydrate Balance

1. Glucose Requirements

The heavy demands for glucose in early lactation and in late pregnancy are well known. Kleiber (1959) calculated that about 60% of the lactating cow's daily glucose requirement is for the production of milk. The balance sheet (Table IX) indicates a total daily glucose requirement of 1140 gm, of which 700 gm appear in the milk. For sheep in late pregnancy, Kronfeld (1958) calculated from data of others that between one-third and one-half of the daily glucose turnover of 100 gm was utilized by the fetus.

TABLE IX

Carbohydrate Balance Sheet[a]

A. Cow's daily glucose flux	
1. *In 12.5 kg milk:*	*Carbohydrate carbon*
610 gm lactose	257 gm C/day
462 gm milk fat with 58 gm glycerol	23 gm C/day
Carbohydrate carbon in milk per day	280 gm C/day
2. *Daily glucose catabolism:*	
Cow produced daily 3288 liters CO_2 = 1762 gm C	
Transfer quotient plasma glucose → CO_2 is 0.1	
Thus, glucose to CO_2 per day	176 gm C/day
1 + 2 = daily flux of glucose	456 gm C/day
$\frac{180}{72} \times 456 = 1140$ gm glucose/day	
B. Cow's glucose sources	
Cow secreted daily in urine 34 gm N	
This indicates catabolism of 213 gm protein with	110 gm C/day
In urea	14 gm C/day
Maximum available for glucose synthesis from protein	96 gm C/day
Glucose flow in milk and respiration	456 gm C/day
Thus, glucose flow from nonprotein sources	360 gm C/day
$\frac{180}{72} \times 360 = 900$ gm glucose daily must have been supplied from nonprotein source	

[a] From Kleiber (1959).

An alternate approach to the assessment of the glucose requirements of an animal is to measure the rate at which glucose enters or leaves the circulation. This is best measured by the use of isotopically labeled glucose. In recent years, Kleiber's laboratory, using this technique, has reported estimates of the daily turnover or requirements for glucose by the lactating cow. Baxter *et al.* (1955) estimated a transfer out of the circulation of about 70 gm/hour or 1680 gm/day in a lactating cow, a figure that they realized may have overestimated the daily glucose turnover. A later report by this group gave an average estimate of 1440 gm/day (60 gm/hour) in four cows. For sheep, similar techniques gave an average turnover of about 144 gm/day in normal pregnant ewes just prior to term (Kronfeld and Simesen, 1961). It would appear that a reasonable estimate of the average daily glucose requirement would be about 50 gm/hour, or 1.20 kg/day, for a 1000-lb lactating cow and about one-tenth of this, or 120 gm/day, for a ewe in late pregnancy.

2. Glucose Sources

The large amounts of indigestible carbohydrates ingested by ruminants are fermented to volatile fatty acids by the rumen microflora. Little, if any, of the digestible carbohydrate (starch, glucose) in the diet escapes this fermentation. It has previously been emphasized that the oral route is ineffective for the performance of the glucose tolerance test in the mature ruminant. Thus, glucose absorption by the digestive tract accounts for little of the daily glucose requirement, in contrast to the case in nonruminants. It is known, however,

that the glucose which might have escaped rumen fermentation is readily absorbed (Larsen *et al.*, 1956), as in other species.

A possible source of blood glucose is ruminal lactic acid. Lactic acid is a product of many fermentation reactions, and blood lactate can be a source of blood glucose via the lactic acid cycle (see Fig. 4). Normally, blood lactate is derived principally from the breakdown of muscle glycogen. It has been demonstrated, however, that sodium lactate placed in the rumen results in increased blood lactate and glucose levels (Hueter *et al.*, 1956). Thus, some of the glucose requirement may be met from this source, but it is likely to be minimal since excesses of lactic acid in the rumen are toxic.

The carbohydrate balance sheet (Table IX) provides an indication of the contribution of protein as a source of carbohydrate for the lactating cow. Since glucose absorption in the ruminant is minimal, the balance sheet also illustrates the importance of an alternate nonprotein source of carbohydrate. These sources are the ruminal volatile fatty acids. It is now generally recognized that the principal products of rumen fermentation are the volatile fatty acids, acetic, propionic, and butyric acids, and that these acids are absorbed across the rumen wall and are the major source of nutriment for the ruminant. Various authors have used a variety of techniques to arrive at estimates of production and absorption of these acids. These fatty acids are found in the following approximate proportions: acetate, 65; propionate, 20; and butyrate, 10. Further details of fatty acid production and absorption by the ruminant may be found in Chapter 2.

According to established concepts, although carbon from acetic acid appears in carbohydrate (blood glucose, milk lactose) through the mechanism of the TCA cycle (Fig. 9), it cannot theoretically contribute to the net synthesis of carbohydrate. Numerous studies have shown that this is the case, and there is extensive evidence that acetate is not a glucogenic compound. The large amounts of acetate provided by rumen fermentation are utilized for energy purposes and for the synthesis of fat. A possible mechanism for the direct incorporation of acetate into a glucose precursor is the so-called glyoxylate pathway (Kornberg and Madsen, 1957), which occurs in plants but has not been demonstrated in animals.

Propionate, on the other hand, is a well-known precursor of carbohydrate (Kleiber *et al.*, 1953; Johnson, 1955; Armstrong and Blaxter, 1957). The pathway leading to a net synthesis of glucose from propionate is available via the reaction

$$\text{Propionate} + CO_2 \rightarrow \text{succinate}$$

as shown in Fig. 9. According to the scheme, 2 moles of propionate are theoretically required for the synthesis of 1 mole of glucose. A more recent refinement of the pathway for glucose production from propionate has been proposed by Ballard *et al.* (1968) which separates a mitochondrial pathway from the cytoplasmic. The overall reaction is, however, the same, and thus 1 gm of propionate theoretically can provide 1.23 gm of glucose. The amounts of propionate available from rumen fermentation can theoretically supply at least the glucose requiremets not accounted for by protein sources.

Butyrate, the third major fatty acid of rumen fermentation, influences glucogenesis (Kleiber *et al.*, 1954; Potter, 1952) but does not contribute carbon directly to glucose. Butyrate stimulates glucose production by liver by increasing phosphorylase (Phillips *et al.*, 1965) or by increasing gluconeogenesis. β-Oxidation of butyrate yields AcCoA, which has been shown to activate pyruvate carboxylase (Utter and Keech, 1963), a key gluconeogenic enzyme.

3. Utilization of Glucose

The overall utilization of glucose by the ruminant exhibits some significant differences from that observed in other animals. Reid (1950b), on the basis of carotid–jugular differences in glucose concentration, concluded that glucose was less important as an energy source for the sheep than for the nonruminant and that acetate oxidation plays the more important role in energy metabolism of the ruminant. The oxidation of glucose is also reflected in the excretion of its carbon atoms as respiratory CO_2. Using this technique with radioactive glucose, Baxter *et al.* (1955) estimated that only about 10% of the respiratory CO_2 arises from glucose oxidation, which is considerably less than the estimates ranging between 25 and 60% for the rat, dog, and man. The glucose tolerance of the cow (Holmes, 1951) and sheep (Reid, 1952) were reported to be decreased, but more recently (Kaneko and Rhode, 1964) the glucose tolerance of the cow was shown to be comparable to that of other animals. The plasma clearance $T_{\frac{1}{2}}$ was 33 minutes, which is similar to that observed in dogs (Kaneko *et al.*, 1977) and man.

Black *et al.* (1957) estimated that about 60% of the glucose oxidized in the mammary gland of the lactating cow occurred via the pentose cycle (Fig. 6). The same percentage of pentose cycle activity was observed in rat mammary gland (Abraham *et al.*, 1954). Pentose cycle activity in the mammary gland was also shown by measurement of the activities of G-6-P dehydrogenase and 6-P-G dehydrogenase in the sheep (McLean, 1958) and cow (Raggi *et al.*, 1961). Enzyme activities in sheep gland, however, were not as high as in rat gland. Thus, while the overall utilization of glucose may be lower in ruminants, the pathways, although not necessarily the proportions, by which glucose is catabolized are essentially similar to those of other animals.

The other major pathway for glucose oxidation is the classic Embden–Meyerhof pathway and the tricarboxylic acid cycle. The presence of TCA cycle activity in the lactating mammary gland of the cow was firmly established by Black and Kleiber (1957). Through this mechanism, carbon atoms from acetate, derived from any source, appear in milk products (Fig. 17). Thus, glucose carbon atoms may be given off as CO_2, appear in the amino acids of milk protein via transamination of oxaloacetate and α-ketoglutarate, or appear in milk fat. The shorter-chain fatty acids of butterfat are synthesized from acetate in the mammary gland in contrast to the higher-chain acids of butterfat, which are derived

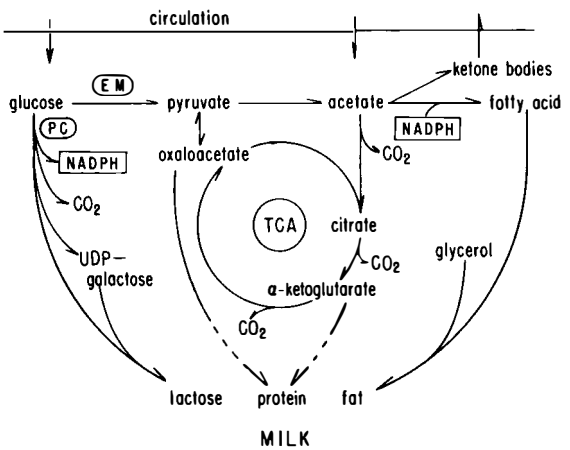


Fig. 17. Summary of some metabolic pathways in the mammary gland.

from blood lipids. The synthetic pathway for fatty acids in the gland appears to be the same as that in other animal tissues (see Section IX).

The major portion of glucose uptake by the mammary gland, however, provides for the biosynthesis of milk. The glucose and galactose moieties of lactose are probably derived solely from blood glucose. The rate of lactose synthesis is also constant over a wide range of blood glucose concentrations (20–80 mg/dl) (Storry and Rook, 1961), a finding which indicates that lactose synthesis is maximal even under hypoglycemic conditions. The mammary gland, therefore, is a glucose-utilizing tissue, principally for biosynthesis and less for oxidation. The principal metabolic pathways involved are summarized in Fig. 17.

Ruminant nervous tissue, i.e., brain, is similar to that of other animals in being an obligatory glucose-utilizing tissue (McClymont and Setchell, 1956). The glucokinase activity of sheep brain, however, is significantly lower than that of rat brain (Jarrett and Filsell, 1958; Gallagher and Buttery, 1959). These observations would suggest that, in spite of the glucose requirement, its utilization by this tissue is lower in the ruminant than in other animals. The same authors observed that the glucokinase activity of sheep intestine was similarly low when compared to that of rat intestine. Ruminant muscle also utilizes less glucose than muscle tissue of other species (Reid, 1950a,b).

Studies of the activities of ruminant liver enzymes, particularly with respect to gluconeogenesis and ketosis, have been conducted. While highest G-6-Pase activities were found in liver compared to other organs of sheep at slaughter, they were only about two-thirds of the activities found in rats (Raggi *et al.*, 1960). Hepatic G-6-Pase activities in older calves and lactating cows were slightly higher than G-6-Pase activities in rats and could be reduced by intraduodenal infusions of glucose (Bartley *et al.*, 1966). This is in general agreement with the concept that liver is a glucose-producing tissue and that increased production of glucose by liver is associated with increased G-6-Pase activity. It should be noted, however, that Ford (1961) observed that hepatic G-6-Pase activities remained relatively constant during early lactation, the period during which a cow's glucose requirement is even higher than during pregnancy (Brody *et al.*, 1948).

Ballard *et al.* (1968) and Baird *et al.* (1968) reported on their studies of a number of gluconeogenic enzymes of cow liver. Ballard *et al.* (1968) found that the activity of PEP-carboxykinase, a key gluconeogenic enzyme, of cow liver is already very high in comparison to that reported in rat liver (Krebs, 1966). This further supports the concept that the high-producing dairy cow that has been genetically selected for these qualities is already synthesizing glucose maximally under normal conditions.

To summarize, the ruminant appears to be an animal well adapted to a carbohydrate economy based upon the endogenous synthesis of glucose from noncarbohydrate sources (gluconeogenesis). The enzymatic mechanisms for gluconeogenesis are already operating at nearly maximal levels in the high-producing dairy cow. Glucose oxidation by individual tissues as well as by the intact animal is lower in ruminants than in nonruminants. Although overall oxidation may be different, the pathways by which this oxidation is accomplished are essentially similar to those of other animals (Fig. 17). Considering that the endocrine relationships of ruminants are also qualitatively similar to those of nonruminants, the normally low blood glucose concentration might also be considered to be a reflection of the degree of influence rather than kind. For example, the blood glucose response of the ruminant to insulin (Reid, 1951a,b; Jasper, 1953a,b) compared to that of the dog shows a slower rate of fall, i.e., insulin resistance.

C. Biochemical Alterations in Body Fluids

1. Hypoglycemia

The occurrence of a significant hypoglycemia in bovine ketosis and in ovine pregnancy toxemia has been repeatedly confirmed. Normal plasma glucose levels range between 45 and 75 mg/100 ml for cows and from 50 to 80 mg/100 ml for sheep. The importance placed upon this finding has led to suggestions that a better name for bovine ketosis would be "hypoglycemia." This hypoglycemia has played an important role in ketosis, not only as a rationale for therapy but as a basis for the concept of ketosis and pregnancy toxemia as manifestations of a carbohydrate deficiency which occurs under conditions of excessive and insurmountable demands.

2. Ketone Bodies

Ketosis is defined as a condition with an elevation of ketone bodies in the body fluids and is a characteristic of bovine ketosis and pregnancy toxemia of sheep. The ketone bodies in these animals are the same as those previously mentioned (Section V,A,3), acetoacetate, β-OH-butyrate, and acetone. A fourth compound, isopropanol, should also be included for the ruminant, and interconversions can occur between these ketone bodies (Thin *et al.*, 1959).

a. Site of Ketone Body Production. It has been previously mentioned (Section IX,A,3) that increased ketogenesis occurs under conditions that favor the accumulation of acetate. In the nonruminant, the liver is the principal, if not the sole, source of ketone bodies, and they appear in the body fluids when production exceeds the capacity for utilization. In the ruminant, the liver may not be the sole significant source of ketone bodies. It has been demonstrated that rumen epithelium (Pennington, 1952) and mammary gland (Kronfeld and Kleiber, 1959) can also be sources of ketone bodies. The extent of their contribution to the ketone bodies of the body fluids, however, is uncertain, although it could be considerable in the ketotic animal.

b. Hyperketonemia. Elevations of ketone body concentration in the body fluids may be influenced by a number of conditions which relate to the carbohydrate economy of the ruminant. Starvation is the most well known method of producing ketosis. Some degree of elevation of ketone levels is also often seen without detrimental effects in association with early lactation, late pregnancy, underfeeding, and high-fat diets. In these states, the continuing demands of the body for carbohydrate are not adequately met.

Elevated blood ketone levels are a consistent finding in bovine ketosis and pregnancy toxemia, although the degree of ketonemia does not necessarily parallel the severity of the clinical signs. Normally, total blood ketones in sheep or cows are less than 10 mg/100 ml (Thin and Robertson, 1953). Total ketone bodies as acetone can best be determined quantitatively by the salicylaldehyde method or modifications thereof (Adler, 1957; Thin and Robertson, 1952). These methods, however, do not lend themselves to routine use in the office laboratory. The measurement of ketone bodies in the blood has been essentially displaced in clinical practice by the rapid qualitative tests applied to urine and milk. The same rapid tests can be applied to serum diluted 1 : 1 with water and employed as screening tests for the detection of ketonemia.

c. Ketone Bodies in Urine and Milk. The modern, rapid qualitative stick tests for urinary ketones (see Chapter 9) may also be applied to milk, but care must be exercised in interpretation. The tests containing sodium nitroprusside are widely known as the Ross modification (Ross, 1931) of Rothera's test.

The results of the qualitative tests on urine and milk are often employed as indices of the degree of ketonemia. The correlation of these tests with blood ketone levels, however, is poor. Adler *et al.* (1957) studied the correlation of these tests on urine and milk with the degree of ketonemia. They employed a modification of the Ross test in which a flake of sodium hydroxide was substituted for ammonium hydroxide. Statistically, only three divisions for urine and two divisions of color intensity for milk were justifiable. The Ross test applied to urine is very sensitive, and false positives are not uncommon in the urine of normal cows. The test is not as sensitive when applied to milk, and a 1+ reaction is of much greater diagnostic significance than the same reaction with urine. For this reason, urine should be diluted 1 : 10 with water to take advantage of its sensitivity and increase its diagnostic accuracy.

Reagents

	For urine	*For milk*
Sodium nitroprusside	1 part	2 parts
Ammonium sulfate	99 parts	98 parts

Mix the dry reagents and store in a brown bottle.

Procedure For urine: Dissolve 1 gm of the dry mixture for urine in 5 ml of urine. Overlay the urine with 1 ml of concentrated ammonium hydroxide. Note the intensity of the purple color which develops at the interface in 5 minutes according to the following scale:

tr, trace purple
1+, slight purple
2+, moderate purple
3+, dark purple

For milk: Repeat the above procedure using 2.5 gm of the dry mixture for milk.

The tests that employ nitroprusside detect acetone and acetoacetate in the body fluids. These tests are much more sensitive to acetoacetate than to acetone, and therefore fresh samples, in which less breakdown of acetoacetate to acetone has occurred, usually give darker reactions. The presence of Bromsulphalein will give a false positive. Gerhardt's test, which uses $FeCl_3$, has also been employed clinically to detect ketone bodies. The $FeCl_3$ tests, however, detect only one ketone body, acetoacetate, and are less sensitive and generally less satisfactory than the nitroprusside tests.

D. Ruminant Ketosis

It has been repeatedly mentioned that the finely balanced carbohydrate economy of the ruminant plays an important role in the development of ketosis in cows and sheep. In the cow, large amounts of glucose must be produced by gluconeogenesis to meet the heavy demands for lactose, particularly in early lactation, when the demand is highest. There is also general agreement that, in sheep, a failure to meet the obligatory demands for hexoses

by the fetus is a precipitating cause of ketosis. The mechanisms whereby imbalances occur and are manifest as ketosis, however, are uncertain and have been the subject of numerous investigations.

The principal concept of a decade ago was that centered about a slowing of the TCA cycle and a deficiency of its intermediates, namely, oxaloacetate (Fig. 9). According to this hypothesis, the heavy demands of late pregnancy and early lactation for glucose and intermediates for biosynthesis result in a depletion of these intermediates of the TCA cycle, with a resulting slowing of the TCA cycle operation. The limiting factor is likely to be the TCA cycle intermediate, oxaloacetate, which occupies a central position in the metabolic scheme. Oxaloacetate may be withdrawn for amino acid synthesis and for gluconeogenesis, and it is also the condensing partner of AcCoA, which is required for operation of the TCA cycle. A deficiency of oxaloacetate would be expected to lead to a decrease in operation of the TCA cycle and hence a decrease in the oxidation of acetate to CO_2 by this pathway. This would favor the accumulation of acetate by removing a major pathway for its disposal, and its diversion to ketone body production would be enhanced.

This OAA deficiency theory, which had earlier been criticized and rejected (Shaw and Tapley, 1958; Krebs, 1961), has in recent years been revived and supported (Krebs, 1966; Wieland, 1968; Baird *et al.*, 1968). Only the mechanism by which mitochondrial OAA deficiency occurs has been modified. Under conditions of increased glucose demand (lactation, pregnancy), gluconeogenesis is increased and the major common precursor for glucose, OAA, is excessively drained and is likely to be depleted. Gluconeogenesis is increased either by an increase in activity of the gluconeogenic enzymes (Krebs, 1966) or by an increase in the "reducing pressure" (Wieland, 1968) as a result of increased fatty acid oxidation. It has been shown that gluconeogenesis is increased in bovine ketosis, as it is in alloxan-diabetic rats (Baird *et al.*, 1968). By either mechanism, a deficiency of mitochondrial OAA would be expected to lead to a diversion of AcCoA units to ketone body production.

The accumulation of acetate and hence increased ketogenesis would also be favored in conditions under which fatty acid oxidation were increased and its synthesis were decreased. It is well known that, in association with decreased glucose utilization, lipid mobilization and oxidation are increased and fatty acid synthesis is decreased. As previously discussed (Section V), in the presence of decreased glucose oxidation via the pentose cycle, heptic NADPH (TPNH) generation would be decreased and lipogenesis depressed. This proposal may be modified for the ketotic cow since, even though overall glucose utilization is normal, the pentose cycle activity has been shown to be depressed (Tombropoulos and Kleiber, 1960). As a consequence of decreased NADPH (TPNH) generation in the mammary gland, lipogenesis from acetate would be impaired, again enhancing ketone body production. This mechanism has been employed to explain the impaired lipogenesis from [^{14}C]acetate by the fasting ketotic cow (Simesen *et al.*, 1961). It has been suggested that, if a tissue such as mammary gland (Pearce, 1960) or a pathway for glucose oxidation, i.e., the pentose cycle (Tombropoulos and Kleiber, 1960), takes priority for available glucose, the metabolic alterations observed in ketotic cows can be explained. Such a mechanism would consider the mammary gland of the cow, in addition to the liver, as an important site of the biochemical events which culminate in the development of the ketotic state. The cow's gluconeogenic mechanisms in the liver are likely to be already operating at maximal levels to meet the heavy demands of lactation. This would then suggest an inability of the susceptible cow's enzymatic mechanisms to

respond to supply sufficient glucose for both lactose production and oxidative purposes during periods of excessive demand. In this respect, it is interesting that hepatic glucose-6-phosphatase activities of the cow remained constant during the period 60 days before to 60 days after calving (Ford, 1961). Furthermore, Baird *et al.* (1968) found no differences in gluconeogenic enzyme activity between normal and ketotic cows. Ballard *et al.* (1968) found that the activity of the key gluconeogenic enzyme, PEP-carboxykinase, in cow liver was already very high in comparison to rat liver and did not change. There was also no decrease in OAA, in contrast to the report by Baird *et al.* (1968). Thus, the ruminant is maximally gluconeogenic at the height of lactation (cow) or pregnancy (sheep). The ruminant cannot meet even a slight additional demand for glucose, becomes glucose deficient, and overproduces ketones, and ketosis ensues.

REFERENCES

Abraham, S., Hirsch, P. F., and Chaikoff, I. L. (1954). *J. Biol. Chem.* **211**, 31.

Adler, J. H. (1957). *Cornell Vet.* **47**, 354.

Adler, J. H., Roberts, S. J., and Steel, R. G. D. (1957). *Cornell Vet.* **47,** 101.

Anstall, H. B. (1968). *Am. J. Clin. Pathol.* **50**, 3.

Armstrong, D. G., and Blaxter, K. L. (1957). *Br. J. Nutr.* **11**, 247.

Ashmore, J., and Weber, G. (1968). *In* "Carbohydrate Metabolism and Its Disorders" (F. Dickens, P. J. Randle, and W. J. Whelan, eds.), Vol. 1, p. 336. Academic Press, New York.

Ashmore, J., Hastings, A. B., and Nesbett, F. B. (1954). *Proc. Natl. Acad. Sci. U.S.A.* **40,** 673.

Bagdade, J. D., Porte, D., and Bierman, E. L. (1967). *N. Engl. J. Med.* **276,** 427.

Baird, G. D., Hibbitt, K. G., Hunter, G. D., Lund, P., Stubbs, M., and Krebs, H. A. (1968). *Biochem. J.* **107**, 683.

Balasse, E. O., and Havel, R. J. (1971). *J. Clin. Invest.* **50**, 801–813.

Ballard, F. J., Hanson, R. W., Kronfeld, D. S., and Raggi, F. (1968). *J. Nutr.* **95**, 160.

Banauch, D., Brummer, W., Ebeling, W., Metz, H., Rindfrey, H., Leybold, K., and Rick, W. (1975). *Z. Klin. Chem.* **13**, 101.

Bardens, J. W. (1966). *Vet. Med.* **61**, 1174.

Bartley, J. C., Feedland, R. A., and Black, A. L. (1966). *Am. J. Vet. Res.* **27**, 1243.

Baxter, C. F., Kleiber, M., and Black, A. L. (1955). *Biochim. Biophys. Acta* **17**, 354.

Bell, F. R., and Jones, E. R. (1945). *J. Comp. Pathol. Ther.* **55**, 117.

Berkow, J. W., and Ricketts, R. L. (1965). *J. Am. Vet. Med. Assoc.* **146,** 1101.

Black, A. L., and Kleiber, M. (1957). *Biochim. Biophys. Acta* **23,** 59.

Black, A. L., Kleiber, M., Butterworth, E. M., Brubacher, G. B., and Kaneko, J. J. (1957). *J. Biol. Chem.* **227**, 537.

Brady, R. O. (1958). *Proc. Natl. Acad. Sci. U.S.A.* **44**, 993.

Brody, S., Worstell, D. M., Ragsdale, A. C., and Kibler, H. H. (1948). *Mo. Univ. Agric. Exp. Stn., Bull. No.* **412,** 1.

Buchanan, K. D. (1975). *In* "Diabetes: Its Physiological and Biochemical Bases" (J. Vallance-Owen, ed.), pp. 63-91. MTP Press, Lancaster, U.K.

Bullock, L. (1965). *Calif. Vet.* **19,** 14.

Bunn, H. F., Gabbay, K. H., and Gallop, P. M. (1978). *Science* **200**, 21.

Burns, J. J., and Evans, C. (1956). *J. Biol. Chem.* **223**, 897.

Cahill, G. F., Jr., Ashmore, J., Earle, A. S., and Zottu, S. (1958). *Am. J. Physiol.* **192**, 491.

Cahill, G. F., Jr., Asmore, J., Renold, A. E., and Hastings, A. B. (1959). *Am. J. Med.* **26**, 264.

Capen, C. C., and Martin, S. L. (1969). *Pathol. Vet.* **6**, 309.

Cello, R. M., and Kennedy, P. C. (1957). *Cornell Vet.* **47**, 538.

Chance, R. E., and Ellis, R. M. (1969). *Arch. Intern. Med. Symp.* **123**, 229.

Chernick, S. S., and Chaikoff, I. L. (1951). *J. Biol. Chem.* **188**, 389.

Cherrington, A. D., and Exton, J. H. (1976). *Metab. Clin. Exp.* **25,** 1351.

Clark, J. L., and Steiner, D. F. (1969). *Proc. Natl. Acad. Sci. U.S.A.* **62**, 278.

Cochrane, W. A., Payne, W. W., Simpkiss, M. J., and Woolf, L. I. (1956). *J. Clin. Invest.* **35**, 411.
Cori, G. T. (1954). *Harvey Lect.* **48**, 145.
Cornelius, C. E. (1961). *Cornell Vet.* **51**, 559.
Cotton, R. B., Cornelius, L. M., and Theran, P. (1971). *J. Am. Vet. Med. Assoc.* **159**, 863.
Cutler, J. T. (1934). *J. Biol. Chem.* **106**, 653.
Dixon, J. B., and Sanford, J. (1961). *J. Small Anim. Pract.* **2**, 9.
Dobbs, R., Sakurai, H., Sakai, H., Faloona, G., Valverde, I., Baetens, D., Orci, L., and Unger, R. (1975). *Science* **187**, 544.
Evans, C., Conney, A. H., Trousof, N., and Burns, J. J. (1959). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **18**, 223.
Felig, P., Wahren, J., Sherwin, R., and Hendler, R. (1976). *Diabetes* **25**, 1091.
Flanagan, G. C., Schwartz, T. B., and Ryan, W. G. (1961). *J. Clin. Endocrinol.* **21**, 401.
Folin, O., and Malmros, H. (1929). *J. Biol. Chem.* **83**, 115.
Ford, E. J. H. (1961). *J. Comp. Pathol. Ther.* **71**, 60.
Ford, E. J. H., and Boyd, J. W. (1960). *Res. Vet. Sci.* **1**, 232.
Foster, S. J. (1975). *J. Small Anim. Pract.* **16**, 295.
Freedland, R. A., Murad, S., and Hurvitz, A. I. (1968). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **27,** 1217.
Fritz, I. B. (1961). *Physiol. Rev.* **41**, 52.
Fritz, I. B. (1967). *Perspect. Biol. Med.* **10**, 643.
Gabbay, K. H., Hasty, K., Breslow, J. L., Ellison, R. C., Bunn, H. F., and Gallop, P. M. (1977). *J. Clin. Endocrinol. Metab.* **44**, 859.
Gallagher, C. H., and Buttery, S. H. (1959). *Biochem. J.* **72**, 575.
Garber, A. J., Menzel, P. H., Boden, G., and Owen, O. E. (1974). *J. Clin. Invest.* **54**, 981.
Gershwin, L. J. (1975). *J. Am. Vet. Med. Assoc.* **167**, 479.
Gibson, D. M., Titchener, E. B., and Wakil, S. J. (1958). *Biochim Biophys. Acta* **30**, 376.
Goodwin, R. F. W. (1957). *J. Comp. Pathol. Ther.* **67**, 289.
Graham, R., Sampson, J., and Hester, H. R. (1941). *Proc. Soc. Exp. Biol. Med.* **47**, 338.
Greider, M. H., Howell, S. L., and Lacy, P. E. (1969). *J. Cell Biol.* **41**, 162.
Grodsky, G. M., Feldman, R., Toreson, W. E., and Lee, J. C. (1966). *Diabetes* **15**, 579.
Grollman, A. P., and Lehninger, A. C. (1957). *Arch. Biochem. Biophys.* **69**, 458.
Hanawalt, V. M., and Sampson, J. (1947). *Am. J. Vet. Res.* **8**, 235.
Hansen, H. J. (1949). *Nord. Veterinaermed.* **1**, 363.
Hill, F. W. G., Pearson, H., Kelly, D. F., and Weaver, B. M. Q. (1974). *J. Small Anim. Pract.* **15**, 119.
Hill, R., and Chaikoff, I. L. (1956). *Proc. Soc. Exp. Biol. Med.* **91**, 265.
Hirsch, P. F., Baruch, H., and Chaikoff, I. L. (1954). *J. Biol. Chem.* **210**, 785.
Hjarre, A. (1927). *Arch. Wiss Prakt. Tierheilkd.* **57**, 1.
Holmes, J. R. (1951). *J. Comp. Pathol. Ther.* **61**, 15.
Howell, R. R. (1978). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wymgaarden, and D. S. Frederickson, eds.) 4th ed., p. 137. McGraw-Hill, New York.
Hueter, F. G., Shaw, J. C., and Doetsch, R. N. (1956). *J. Dairy Sci.* **39**, 1430.
Illingworth, B. A., and Cori, G. T. (1952). *J. Biol. Chem.* **199**, 653.
Isselbacher, K. J. (1959). *Am. J. Med.* **26**, 715.
Jarrett, I. G., and Filsell, O. H. (1958). *Aust. J. Exp. Biol.* **36**, 433.
Jasper, D. E. (1953a). *Am. J. Vet. Res.* **14**, 184.
Jasper, D. E. (1953b). *Am. J. Vet. Res.* **14**, 209.
Johnson, R. B. (1955). *Cornell Vet.* **45**, 273.
Johnson, R. K., and Atkins, C. E. (1977). *In* "Current Veterinary Therapy" (R. W. Kirk, ed.) Vol. 6, p. 1010.
Kaneko, J. J., and Rhode, E. A. (1964). *J. Am. Vet. Med. Assoc.* **144,** 367.
Kaneko, J. J., Luick, J. R., and Rhode, E. A. (1966). *Cornell Vet.* **56,** 401.
Kaneko, J. J., Mattheeuws, D., Rottiers, R. P., and Vermeulen, A. (1977). *J. Small Anim. Pract.* **18,** 85.
Kaneko, J. J., Mattheeuws, D., Rottiers, R. P., and Vermeulen, A. (1978a). *Am. J. Vet. Res.* **39,** 807.
Kaneko, J. J., Mattheeuws, D., Rottiers, R. P., Van Der Stock, J., and Vermeulen, A. (1978b). *Acta Endocrinol. (Copenhagen)* **87**, 133.
Kaneko, J. J., Mattheeuws, D., Rottiers, R. P., and Vermeulen, A. (1979). *Cornell Vet.*
Khoo, J. C., Steinberg, D., Thompson, D., and Mayer, S. E. (1973). *J. Biol. Chem.* **248,** 3823.
Kitabchi, A. E. (1977). *Metab. Clin. Exp.* **26,** 547.
Kleiber, M. (1959). *Proc. Interam. Symp. Peaceful Appl. Nucl. Energy, 2nd, Buenos Aires* p. 161.
Kleiber, M., Black, A. L., Brown, M. A., and Tolbert, B. M. (1953). *J. Biol. Chem.* **203,** 339.

Kleiber, M., Black, A. L., Brown, M. A., Luick, J., Baxter, C. F., and Tolbert, B. M. (1954). *J. Biol. Chem.* **210**, 239.
Kornberg, H. L., and Madsen, N. B. (1957). *Biochim. Biophys. Acta* **24**, 651.
Kramer, J. W. (1977). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36**, 279.
Krebs, H. A. (1961). *Arch. Intern. Med.* **107**, 51.
Krebs, H. A. (1966). *Vet. Rec.* **78,** 187.
Kreisberg, R. A. (1978). *Ann. Intern. Med.* **88**, 681.
Kronfeld, D. S. (1958). *Cornell Vet.* **48**, 394.
Kronfeld, D. S., and Kleiber, M. (1959). *J. Appl. Physiol.* **14**, 1033.
Kronfeld, D. S., and Simesen, M. G. (1961). *Cornell Vet.* **51**, 478.
Kronfeld, D. S., Simesen, M. G., and Dungworth, D. L. (1960). *Res. Vet. Sci.* **1,** 242.
Krook, L., Larsson, S., and Rooney, J. R. (1960). *Am. J. Vet. Res.* **21**, 120.
Larsen, H. J., Stoddard, G. E., Jacobson, N. L., and Allen, R. S. (1956). *J. Anim. Sci.* **15**, 473.
Le Compte, P. M., Steinke, J., Soeldner, J. S., and Renold, A. E. (1966). *Diabetes* **15**, 586.
Leloir, L. F., and Cardini, C. E. (1957). *J. Am. Chem. Soc.* **79**, 6340.
Leloir, L. F., Olavarria, J. M., Goldemberg, S. H., and Carminatti, H. (1959). *Arch. Biochem. Biophys.* **81**, 508.
Ling, G. C., Lowenstine, L. J., Pulley, L. T., and Kaneko, J. J. (1977). *J. Am. Vet. Med. Assoc.* **170**, 521.
Lossow, W. J., and Chaikoff, I. L. (1955). *Arch. Biochem. Biophys.* **57**, 23.
Lusk, G. (1928). "The Elements of the Science of Nutrition," 4th ed., p. 321. Saunders, Philadelphia, Pennsylvania.
McCandless, E. L., Woodward, B. A., and Dye, J. A. (1948). *Am. J. Physiol.* **154**, 94.
McClymont, G. L., and Setchell, B. P. (1956). *Aust. J. Biol. Sci.* **9**, 184.
McGarry, J. D., and Foster, D. W. (1976). *Am. J. Med.* **61**, 9.
McGarry, J. D., Wright, P. H., and Foster, D. W. (1975). *J. Clin. Invest.* **55**, 1202.
McIntyre, M., Holdsworth, C. D., and Turner, D. S. (1965). *J. Clin. Endocrinol.* **25**, 1317.
McLean, P. (1958). *Biochim. Biophys. Acta* **30**, 303.
Madison, L. L. (1969). *Arch. Intern. Med. Symp.* **123**, 284.
Marks, H. P., and Young, F. G. (1940). *Nature (London)* **146**, 31.
Markussen, J. (1971). *Int. J. Protein Res.* **3**, 149.
Masoro, E. J., Chaikoff, I. L., and Dauben, W. G. (1949). *J. Biol. Chem.* **179**, 1117.
Mattheeuws, D., Rottiers, R., Deijcke, J., DeRick, A., and DeSchepper, J. (1976). *J. Small Anim. Pract.* **7**, 313.
Meade, R. C., Kneubuhler, H. A., Schulte, W. J., and Barboriak, J. J. (1967). *Diabetes* **16**, 141.
Meier, H. (1960). *Diabetes* **9**, 485.
Morrill, C. C. (1952). *Am. J. Vet. Res.* **13**, 164.
Pearce, P. J. (1960). *Vet. Rev. Annot.* **6**, 53.
Pennington, R. J. (1952). *Biochem. J.* **51**, 251.
Phillips, R. W., Black, A. C., and Moeller, F. (1965). *Life Sci.* **4**, 521.
Phillips, R. W., Knox, K. L., Pierson, R. E., and Tasker, J. B. (1971) *Cornell Vet.* **61**, 114.
Porter, J. W., Wakil, S. J., Tietz, A., Jacob, M. I., and Gibson, D. M. (1957). *Biochim. Biophys. Acta* **25**, 35.
Potter, B. J. (1952). *Nature (London)* **170**, 541.
Price, J. W., Miller, M., and Hayman, J. M., Jr. (1940). *J. Clin. Invest.* **19**, 537.
Priester, W. A. (1974). *J. Natl. Cancer Inst.* **53**, 227.
Raggi, F., Kronfeld, D. S., and Kleiber, M. (1960). *Proc. Soc. Exp. Biol. Med.* **105**, 485.
Raggi, F., Hansson, E., Simesen, M. G., Kronfeld, D. S., and Luick, J. R. (1961). *Res. Vet. Sci.* **2**, 180.
Rahbar, S. (1968). *Clin. Chim. Acta* **22**, 296.
Rall, T. W., and Sutherland, E. W. (1958). *J. Biol. Chem.* **232**, 1065.
Rall, T. W., Sutherland, E. W., and Wosilait, W. D. (1956). *J. Biol. Chem.* **218**, 483.
Rall, T. W., Sutherland, E. W., and Berthet, J. (1957). *J. Biol. Chem.* **224**, 463.
Reid, R. L. (1950a). *Aust. J. Agric. Res.* **1**, 182.
Reid, R. L. (1950b). *Aust. J. Agric. Res.* **1**, 338.
Reid, R. L. (1951a). *Aust. J. Agric. Res.* **2**, 132.
Reid, R. L. (1951b). *Aust. J. Agric. Res.* **2**, 146.
Reid, R. L. (1952). *Aust. J. Agric. Res.* **3**, 160.
Renold, A. E., and Cahill, G. F., Jr. (1966). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Frederickson, eds.), 2nd ed., p. 69. McGraw-Hill, New York.

Roberts, I. M. (1954). *J. Am. Vet. Med. Assoc.* **124,** 443.
Robison, A., Butcher, R. W. and Sutherland, E. W. (1968). *Annu. Rev. Biochem.* **37**, 149.
Roderick, L. M., Harshfield, G. S., and Merchant, W. R. (1933). *Cornell Vet.* **23**, 348.
Rogers, W. A., Donovan, E. F., and Kociba, G. J. (1975). *J. Am. Vet. Med. Assoc.* **166**, 1092.
Ross, S. A., Brown, J. C., and Dupre, J. (1974). *Diabetologia* **10**, 384.
Ross, S. G. (1931). *J. Lab. Clin. Med.* **16**, 908.
Sampson, J. (1958). *In* "Disease of Swine" (H. W. Dunne, ed.), p. 521. Iowa State Univ. Press, Ames.
Sampson, J., Taylor, R. B., and Smith, J. C. (1955). *Cornell Vet.* **45**, 10.
Sanger, F. (1959). *Science* **129**, 1340.
Schwab, L., and Lotspeich, W. D. (1954). *Am. J. Physiol.* **176**, 195.
Shannon, J. A., Farber, S., and Troast, L. (1941). *Am. J. Physiol.* **133**, 752.
Sharkey, T. P. (1968). *J. Am. Diet. Assoc.* **52**, 108.
Shaw, J. C. (1943). *J. Dairy Sci.* **26**, 1079.
Shaw, W. V., and Tapley, D. F. (1958). *Biochim Biophys. Acta* **30**, 426.
Sherwin, R. S., Fisher, M., Hendler, R., and Felig, P. (1976). *N. Engl. J. Med.* **294**, 455.
Simesen, M. G., Luick, J. R., Kleiber, M., and Thin, C. (1961). *Acta Vet. Scand.* **2**, 214.
Siperstein, M. D. (1959). *Am. J. Med.* **26**, 685.
Siperstein, M. D., and Fagan, V. M. (1957). *Science* **126**, 1012.
Slye, M., and Wells, H. G. (1935). *AMA Arch. Pathol.* **19,** 537.
Somogyi, M. (1959a). *Am. J. Med.* **26**, 169.
Somogyi, M. (1959b). *Am. J. Med.* **26**, 192.
Soskin, S., Essex, H. E., Herrich, J. F., and Mann, F. C. (1938). *Am. J. Physiol.* **124**, 558.
Spieth, K. (1973). *Prakt. Tierarzt.* **54**, 292.
Steiner, D. F., and Oyer, P. E. (1967). *Proc. Natl. Acad. Sci. U.S.A.* **57**, 473.
Steinke, J., and Taylor, K. W. (1974). *Diabetes* **23**, 631.
Stewart, J., and Holman, H. H. (1940). *Vet. Rec.* **52**, 157.
Storry, J. E., and Rook, J. A. F. (1961). *Biochim. Biophys. Acta* **48**, 610.
Sutherland, E. W., and Wosilait, W. D. (1956). *J. Biol. Chem.* **218**, 459.
Swiatek, K. R., Kipnis, D. M., Mason, G., Chao, K., and Cornblath, M. (1968). *Am. J. Physiol.* **214**, 400.
Teunissen, G. H. B., Riznberk, A., Schotman, W., and Hackeng, W. H. L. (1970). *Kleintier-Prax.* **15**, 29.
Teunissen, J., and Blok-Shuring, P. (1966). *Schweiz. Arch. Tierheilkd.* **108**, 409.
Thin, C., and Robertson, A. (1952). *Biochem. J.* **51**, 218.
Thin, C., and Robertson, A. (1953). *J. Comp. Pathol. Ther.* **63**, 184.
Thin, C., Paver, H., and Robertson, A. (1959). *J. Comp. Pathol. Ther.* **69**, 45.
Tombropoulos, E. G., and Kleiber, M. (1960). *Biochem. J.* **80**, 414.
Touster, O., Mayberry, R. H., and McCormick, D. B. (1957). *Biochim Biophys. Acta* **25**, 196.
Unger, R. H., and Eisentraut, A. M. (1969). *Arch. Intern. Med.* **123**, 261.
Unger, R. H., and Orci, L. (1975). *Lancet* **1**, 14.
Unger, R. H., and Orci, L. (1976). *Physiol. Rev.* **56**, 778.
Unger, R. H., Kelterer, H., Dupre, J., and Ersentraut, A. M. (1967). *J. Clin. Invest.* **46**, 630.
Utter, M. F., and Keech, D. B. (1963). *J. Biol. Chem.* **238**, 2603.
Wakil, S. J. (1958). *J. Am. Chem. Soc.* **80**, 6465.
Wieland, O. (1968). *Adv. Metab. Dis.* **3**, 1.
Wilder, R. M., Allan, F. N., Power, M. H., and Robertson, H. E. (1927). *J. Am. Med. Assoc.* **89**, 348.
Wilkinson, J. S. (1957). *Vet. Rev. Annot.* **3**, 69.
Wilkinson, J. S. (1958). *Vet. Rev. Annot.* **4**, 93.
Wilkinson, J. S. (1960). *Vet. Rec.* **72**, 548.
Yalow, R. S., and Berson, S. A. (1960). *J. Clin. Invest.* **39**, 1157.
Young, F. G. (1963). *In* "Comparative Endocrinology" (U.S. Euler and H. Heller, eds.), p. 371. Academic Press, New York.

2

Lipid Metabolism and Its Disorders

JACK C. BARTLEY

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed

ISBN 0-12-396350-8

I. INTRODUCTION

Of the three basic organic foodstuffs, the chemical nature of common animal and vegetable lipids was known prior to similar basic knowledge of carbohydrates and proteins (Chevreul, 1823). Further studies, however, on the biochemical nature of lipid were inhibited by the lack of techniques for dealing with compounds insoluble in water. Hence, for many years the basic knowledge of lipid biochemistry developed more slowly than that of carbohydrate and protein. This situation no longer exists. The development of techniques, such as thin-layer and gas–liquid chromatography, specific for the separation of nonpolar compounds has resulted in a rapid accumulation of biochemical data on lipids. Similar rapid increases in our knowledge of the form in which lipid exists in the plasma have occurred since the introduction of methods for the isolation of lipoproteins by means of ultracentrifugation and gel electrophoresis. The intellectual fallout from these technical advances continues to propel the development of physiological chemistry of lipids at a rapid pace.

Lipids have special importance physiologically as the hydrophobic constituents of membranes and as the most concentrated source of energy (9 cal/gm) of any of the major foodstuffs. Lipids include compounds found in living organisms that are insoluble in water and soluble in fat solvents (diethyl ether, petroleum ether, chloroform, hot alcohol, benzene, carbon tetrachloride, and acetone) (Deuel, 1951). Such a definition is not all-inclusive; for example, lecithin is insoluble in acetone and yet is considered a lipid.

II. CLASSES OF LIPIDS

The broadness of the definition of lipids requires a further classification of the substances included. A modification of the classifications set forth by Masoro (1968) is useful. The three major classes are simple lipids, phospholipids, and sphingolipids.

A. Simple Lipids

Compounds that are not degraded by alkaline or acid hydrolysis or that upon hydrolysis yield only derived lipids, i.e., substances soluble in fat solvents, or derived lipids plus glycerol are all considered simple lipids. Thus, naturally occurring hydrocarbons (squalene), fatty acids, neutral glycerides, and lipid alcohols (cholesterol) and their esters are included in this category.

B. Phospholipids

Compounds that upon hydrolysis yield derived lipids plus inorganic phosphate, glycerol, and usually a third water-soluble product are classified as phospholipids. The

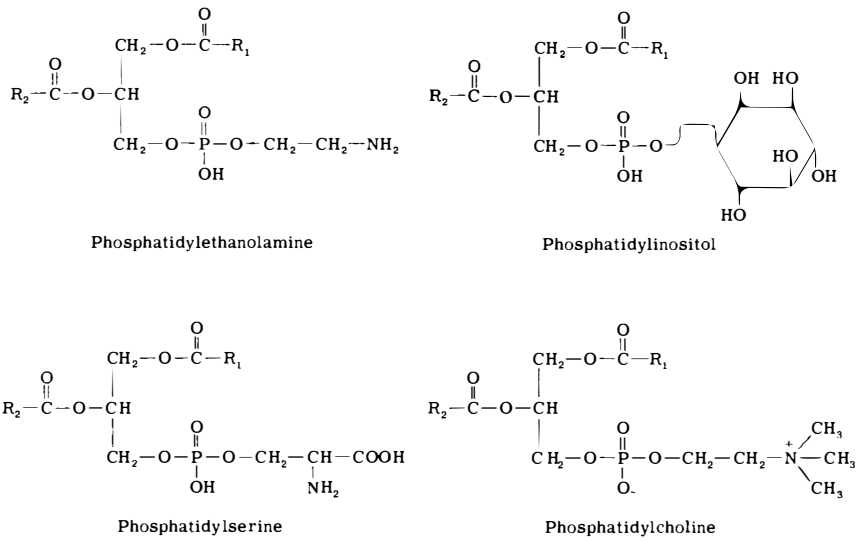


Fig. 1. Structural formulas of some common phospholipids.

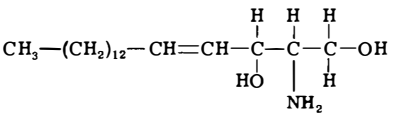
prototype of this category is phosphatidic acid. Other biologically important derivatives of phosphatidic acid are cardiolipin, in which two glycerides are linked via the phosphate ester, and a series of compounds in which the phosphate ester serves as a link to ethanolamine, inositol, serine, or choline (Fig. 1).

The naturally occurring phospholipids commonly contain one unsaturated fatty acid, usually esterified to the *sn*-2 position of glycerol, and one saturated fatty acid, usually esterified to the *sn*-1 position (Hanahan, 1954). Variations, of course, do occur. One variant, a lecithin containing two saturated fatty acids, deserves special mention. Clements *et al.* (1970; Clements, 1971) have shown that the surfactant secreted by certain cells in the lung (Mason *et al.*, 1977) is dipalmityl lecithin. This unique lecithin has been isolated from the lungs of many mammals, fowl, reptiles, and amphibians. Its presence is essential for normal respiratory function.

Not all phospholipids contain two acyl fatty acids. Lysolecithin is characterized by a single fatty acyl group, usually on the α or *sn*-1 carbon of glycerol. Some phospholipids contain linkages other than ester bonds to the alcohol group of glycerol. Plasmalogens contain a monovinyl ether as well as a mono-fatty acyl group (Rapport *et al.*, 1957). Upon hydrolysis this α,β-unsaturated ether yields a long-chain aldehyde, which accounts for Feulgen's (Feulgen *et al.*, 1929) detection of aldehydogenic material in phospholipid preparations. Although rare, phospholipids containing an ether group have been isolated in certain tissues, such as bovine red blood cells, and in certain tumors (Snyder, 1972).

C. Sphingolipids

Sphingolipids are characterized by the presence of sphingosine as one of their moieties:



Derived lipid and a water-soluble compound are the other products of hydrolysis. Sphingomyelin, common to most mammalian tissues, is a member of this group. In this case, an acyl fatty acid group is linked to sphingosine as an amide, and the primary alcoholic group forms a phosphate ester linkage with choline. Cerebrosides and cerebroside sulfates fulfill the requirements of the definition of sphingolipids. These compounds are found in high concentrations in brain tissues but are not limited to that tissue. Cerebrosides differ from sphingomyelin only in that a mono- or oligosaccharide is linked to the primary alcohol of sphingosine as a glycoside instead of choline via a phosphate ester. Gangliosides contain sphingosine with an acyl fatty acid or amido group linked with monosaccarides, hexosamines, and neuraminic acid. For example, a crystalline ganglioside containing equimolar amounts of sphingosine, stearic acid, glucose, galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid has been isolated from bovine brain (Kuhn and Egge, 1960). These compounds are found most commonly in the central nervous system, but gangliosides have been isolated from other tissues as well.

Decreased degradation, rather than increased synthesis, of sphingolipids has been incriminated in all of the lipid storage diseases of human beings. Several reviews discuss the metabolic, biochemical, and diagnostic aspects of these syndromes (e.g., Stanbury *et al.*, 1966; Shapiro, 1967; Brady, 1969, 1970; Gatt, 1970). The occurrence of some of these inborn errors of metabolism has also been noted in animals (Chrisp *et al.*, 1970; Baker *et al.*, 1971). Undoubtedly, more animal diseases will be reported in the future.

III. CHEMISTRY OF SOME LIPIDS

Although all types of lipid mentioned have physiological importance, the fatty acids, glycerides, and steroids require a more detailed description due to their wide distribution in mammalian tissue and broad physiological importance.

A. Classification of Fatty Acids

Naturally occurring fatty acids are straight-chain saturated or unsaturated monocarboxylic acids containing an even number of carbon atoms. Although the most common chain lengths in nature are 16–20 carbons, fatty acids of shorter lengths occur, notably in the milk of many species and in coconut oil. Fatty acids with an odd number of carbon atoms are found in small quantities throughout nature. The oil extracted from pelargoniums is rich in a saturated fatty acid containing 9 carbon atoms. Propionic acid is a fatty acid with an odd number of carbon atoms. The importance of this acid in ruminant nutrition and metabolism warrants its inclusion in a discussion of lipid metabolism of domestic animal (Section IX,A). Branched-chain and hydroxyl-containing fatty acids are found rarely in complex lipids of domestic animals.

The free form of a fatty acid rarely occurs in animal tissues. Usually, when the fatty acid content of a tissue or diet is referred to, the fatty acids are assumed to be in an esterified form. Therefore, specific reference to fatty acids in the free form (FFA) must be made.

The volatile fatty acids (VFA) also occur as the free acids but are distinct from FFA both chemically and metabolically. Volatile fatty acids are of short chain length (C_1 to

C_5), are readily soluble in water, and are steam-distillable. The metabolic differences between the short-chain VFA and the long-chain FFA will be discussed later.

The Geneva system of nomenclature has been used to designate fatty acids on the basis of their carbon chain length and their degree of saturation. A fatty acid is regarded as an aliphatic derivative of a hydrocarbon in which the terminal methyl group is replaced by a carboxyl group. The name of the acid is derived from the hydrocarbon, except that the terminal "e" is replaced by the suffix "oic." Thus, hexadecanoic acid (palmitic) is derived from hexadecane. This nomenclature also applies to the monounsatured fatty acids. In this case, the unsaturated hydrocarbon is designated by the suffix "ene" rather than "ane." Thus, the fatty acid is denoted by the ending "enoic", e.g., hexadecenoic acid (palmitoleic acid). When more than one unsaturated bond occurs, the combining form di, tri, etc., is inserted before the enoic to designate the appropriate number of unsaturated bonds.

Two systems are now in use to designate the site of the unsaturated bond within the molecule. The basis of the most common system is the number of carbon atoms away from the carboxyl group, considered C-1. In this system, palmitoleic acid is 9-hexadecenoic acid. In the same system, the presence of the unsaturated bond may be designated by δ and the site of the bond by superscripts. In the second system, the number of carbon atoms away from the terminal or ω-carbon of the fatty acid is used for designating the site of the unsaturated bond. In this case, palmitoleic acid is ω-7-hexadecenoic acid; oleic acid is ω-9-octadecenoic acid. The latter system is preferable because it indicates whether a biosynthetic relationship exists between the various naturally occurring unsaturated fatty acids. Usually, the naturally occurring unsaturated fatty acids are in the trans configuration. In general, the trivial name will be used here because of the general familiarity with the common fatty acids.

The polyunsaturated fatty acids, particularly linoleic acid, are required for normal function and growth of mammals, but they cannot be synthesized by the tissues of these animals. Therefore, these fatty acids must be provided in the diet and are referred to as essential fatty acids.

B. Chemical Reactions of Fatty Acids

Only selected chemical reactions of physiological and clinical importance will be discussed. The reader is referred to Sunderman and Sunderman (1960) and Kates (1972) for methods useful in clinical studies of lipid metabolism.

1. Solubility

The solubility of fatty acids is important from several viewpoints. Since physiological environments are aqueous, it is important to understand the mechanisms for maintaining solutions or emulsions of fatty acids. Some of these means are discussed in later sections. Analytically, the solubility of fatty acids is used to isolate and purify them.

The solubility of saturated fatty acids from C_6 to C_{18} in water at a variety of temperatures has been studied (Ralston and Hoerr, 1942). Solubility was inversely related to the length of the carbon chain at all temperatures, caproic acid being the most soluble, about 1 gm/100 gm water, and stearic acid being the least, 0.00018–0.0005 gm/100 gm water depending upon the temperature. The VFA, acetic, propionic, and butyric acids are

miscible with water in all proportions and 3.7 gm of valeric acid are soluble in 100 gm of water (Markley, 1947). Because of their water solubility, fatty acids of chain length less than eight carbons are not strictly lipids, but their metabolic fate is closely tied to lipid metabolism.

The solubility of fatty acids in various organic solvents at various temperatures has also been studied (Hoerr and Ralston, 1944; Hoerr *et al.*, 1946) and the results reviewed by Duell (1951). Accordingly, the solubility of fatty acids increases with decreasing chain length and increasing degree of unsaturation. The solubility of fatty acids increases almost linearly with increasing temperature in nonpolar solvents. The relationship between solubility and temperature is less predictable as the polarity of the solvent increases.

The sodium and potassium salts of fatty acids are soluble in water and insoluble in organic solvents. Thus, extraction of lipid samples with lipid solvents after saponification, but before acidification, separates the nonsaponifiable lipids, mainly steroids and hydrocarbons, from the saponifiable ones. The extraction repeated on the same sample after acidification separates the fatty acids from such a mixture. The types of fatty acids that are extractable from an acidified aqueous solution depend upon the organic solvent used. For example, Abraham and associates (1961) have shown that hexane at 20°C extracts mainly fatty acids with a carbon chain of C_8 or greater. Such a separation is valuable for the examination of fatty acids of tissues synthesizing short-chain fatty acids from acetate, e.g., mammary glands.

2. *Formation of Salts*

The FFA can react with bases to produce appropriate salts. Most of the naturally occurring fatty acids exist in the salt form as fatty acid anions at physiological pH. Soaps are the salts of long-chain fatty acids. Sodium and potassium form water-soluble soaps, but the alkaline earth metals, calcium and magnesium, form insoluble soaps. Formation of the sodium salts of FFA is the basis of the determination of FFA content of body fluids, particularly plasma.

3. *Formation of Fatty Acids Esters*

In the presence of H^+, fatty acids react with alcohol to yield esters and water:

$$R{-}COO^- + R_1OH \xrightarrow[H^+]{} R{-}\overset{\overset{O}{\|}}{C}{-}O{-}R_1 + H_2O \qquad (1)$$

The most common ester linkages in domestic animals are between fatty acids and glycerol to form glycerides and between fatty acids and cholesterol to yield cholesterol esters. Methyl esters of fatty acids are more volatile than the FFA; therefore, the esters are prepared for separation of fatty acids by gas–liquid chromatography.

4. *Hydrogenation and Halogenation*

Unsaturated fatty acids can be converted to saturated fatty acids by the addition of 1 mole of hydrogen at the site of each double bond (Deuel, 1951). In the presence of a metal catalyst, such as platinum, hydrogenation can be accomplished using hydrogen gas under either increased pressure, increased temperature, or both; this process is used extensively in the preparation of margarine from vegetable oils.

Halogenation is a comparable reaction except that 1 mole of a halogen is added at each unsaturated bond (Deuel, 1951). Iodine is the most common halogen used. Chlorine and

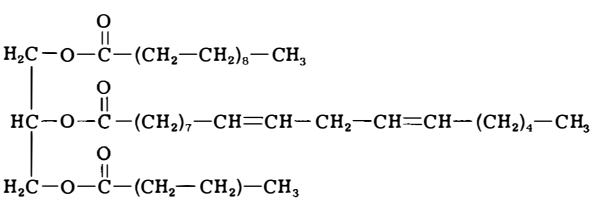
bromine are also effective. Since the degree of saturation of fatty acids is directly proportional to the amount of halogen consumed, this reaction has been used to determine the degree of unsaturation of fatty acids, namely, the iodine number of the lipid. The unsaturated fatty acids within glycerides and phospholipids can, of course, undergo the chemical reactions involving the double bond.

Halogenation is pertinent to clinical medicine. Fatty acids in glycerides used in absorption studies are often labeled with radioactive iodine, usually ^{125}I, by means of halogenation. Lipids labeled by this means are cheaper, more easily handled, and more easily assayed than comparable ^{14}C-labeled compounds. The specific tests using these compounds are discussed in Chapter 8. Whether fatty acids labeled with radioactive iodine are handled by the organism precisely as are natural fatty acids is open to question.

C. Chemistry of Neutral Glycerides

Of the neutral glycerides, triglycerides are by far the most common in nature. Triglycerides are molecules in which all three alcoholic groups are esterified with a fatty acid. When the carbons of glycerol are denoted relative to glyceraldehyde-3-P, diglycerides and monoglycerides have an acyl fatty acid on *sn*-2 and *sn*-1 carbons, respectively, of glycerol. In the case of the partial glycerides, any combination of the various alcoholic groups may be involved, but certain sites are favored over others, as will be discussed later.

The term "simple glycerides" refers to triglycerides containing only one type of fatty acid, e.g., tripalmitin. Mixed glycerides, then, contain more than one type of fatty acid. Naturally occurring glycerides are usually of the mixed variety. The site of the fatty acid within the molecule is designated by relating the name of the fatty acid to the site of esterification. The center carbon of glycerol is often referred to as the β-carbon or as *sn*-2, and the two terminal carbons as the α instead of *sn*-1 and *sn*-3. Thus, the following triglyceride could be referred to as β-linoleo-α-stearopalmitin or as *sn*-1-stearyl-2-linoleolyl-3-palmityl triacylglyceride:



The fatty acids comprising the triglyceride molecule are not distributed among the three alcoholic groups of glycerol in a random manner. The position is governed by chain length and the degree of unsaturation. The shorter and more unsaturated fatty acids tend to be found in the β position of the naturally occurring triglycerides (Desnuelle and Savary, 1963). An exception to this finding are the triglycerides of the domestic pig in that in this species palmitic acid is consistently found in the β position (Mattson *et al.*, 1964).

Neutral glycerides can be hydrolyzed either by acid or base to their constituent fatty acids and glycerol. Acid hydrolysis requires high temperature and pressure to complete the reaction; therefore, alkaline hydrolysis is more commonly used. Heating at 90°C for 90 minutes in an alcoholič solution of a strong alkali results in formation of salts of fatty acids along with glycerol.

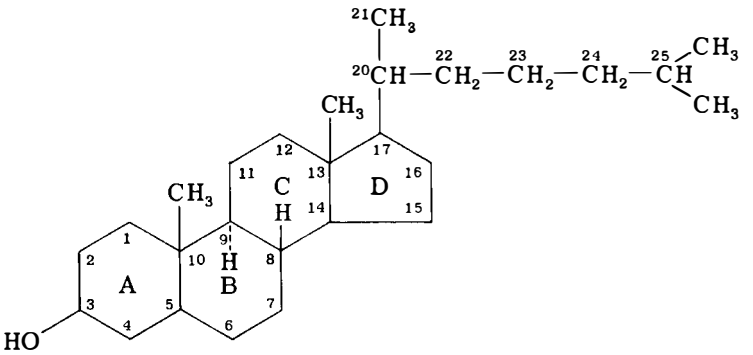


Fig. 2. Structural formula of cholesterol showing numbering of carbon atoms and designation of rings.

D. Chemistry of Steroids

The complicated chemical structure of these compounds will be covered only briefly to illustrate how the same basic structure can be varied to allow a wide variety of physiological roles. For a complete presentation the reader is referred to books by Shoppee (1958) and by Kritchevsky (1963). Revised tentative rules for the nomenclature of steroids have been published by the IUPAC Commission on the Nomenclature of Organic Chemistry and the IUPAC–IUB Commission on Biochemical Nomenclature.*

Steroids are compounds with a cyclopentanophenanthrene skeleton. The constituent carbons and the rings are designated as shown in Fig. 2. The structure of steroids is further complicated in that the rings may be fused in the trans or cis configuration. The orientation of the side chains depends on whether the rings are trans or cis. Two series of steroids are found in nature: the normal series, in which the relationship between ring A and ring B is cis and that between rings B and C and C and D is trans; and the allo series, in which all the rings are trans. Hence, in the normal series, the methyl group at C-10 and the hydrogen at C-5 are in the same configuration, designated β. In the allo series, the methyl group at C-10 is β, and the hydrogen at C-5 is α.

Superficially, it may be surprising that a group of compounds all with the same 17-carbon rings can vary so widely in their physiological roles. However, the basic structure can be varied by the following: (1) The configuration of the ring structures may be different, i.e., allo versus normal; (2) the ring structure may be broken at one bond, as in Vitamin D; (3) there may or may not be an alphatic side chain, and if present it may vary in its structure and functional groups; (4) the ring may contain double bonds in various positions; (5) the ring may contain various side groups of various sites; and (6) these side groups can be oriented in either the α or β position.

Certain sites on the basic cyclopentanophenanthrene ring seem to be favored in nature over others for the addition of methyl, alcoholic, ketonic, and aldehydic side chains. The potency of many physiologically active compounds commonly depends upon the type of group, and its orientation, at C-3, C-11, and C-17.

Cholesterol can be used as a typical steroid for discussion of the physical and chemical properties of compounds comprising this group. Cholesterol is the most common naturally

*Reprints are available from NAS–NRC Office of Biochemical Nomenclature, Dr. Waldo E. Cohn, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

occurring steroid, is implicated in vascular disease, and has diagnostic importance, e.g., hypothyroidism (Section VI,B).

Cholesterol has the basic steroid skeleton with a branched side chain at C-17, a secondary, β-oriented alcohol at C-3, and a double bond between C-5 and C-6 (Fig. 2). The hydroxyl group at C-3 is often in an ester linkage with the fatty acid. The esterification reaction is catalyzed by enzymes found in liver (Goodman *et al.*, 1964) and plasma (Glomset, 1968). The significance of the esters and reactions catalyzing their formation will be discussed later (Sections VI,B and VII,C).

Digitonin, a steroid saponin, reacts with a secondary alcohol group of cholesterol to form cholesterol digitonide. This complex is virtually insoluble in organic solvents. Thus, this reaction has been used extensively for the detection, isolation, and purification of cholesterol. The reaction is specific for β-oriented hydroxyls at C-3 so that the reaction is not limited to cholesterol. It does provide a means for separating free cholesterol from cholesterol esters.

Colorimetric reactions are used for the detection of cholesterol. The classic Libermann–Burchard reaction involves the conversion of a steroid to a polymeric unsaturated hydrocarbon. In this reaction, the sample is dissolved in acetic anhydride and then treated with sulfuric acid. The intensity of the green color is proportional to the amount of cholesterol present. This reaction is also not specific for cholesterol, as most unsaturated steroids undergo similar reactions.

Another colorimetric assay for cholesterol is related to the presence of the hydroxyl group as well as to the unsaturation. The basis of this assay is the color complex that results between cholesterol and $FeCl_3$ in the presence of sulfuric acid (Zak *et al.*, 1954; Rice and Lukasiewicz, 1957). Again, this reaction is not specific for cholesterol.

Since neither of these popular colorimetric methods is specific for cholesterol, if there is doubt about the quantity of compounds other than cholesterol in a given sample, cholesterol can be isolated either as the dibromide or by chromatography prior to the assay.

IV. DIGESTION AND ABSORPTION OF LIPIDS

The general discussion in this section applies to omnivores and carnivores only. A special section describes recent investigations of lipid digestion and absorption in ruminants because of the unique digestive system in this species (Section IX,A). The generalizations developed in studies with rodents and man should be applied with caution to herbivores.

The major portion of the lipid ingested is in the form of triglycerides, but some cholesterol, cholesterol ester, and phospholipid are also present in most diets. The digestion of lipid takes place primarily in the lumen of the small intestine. A lingual or pharyngeal lipase has been described in calves (Ramey and Yound, 1961), rats (Hamosh and Scow, 1973), and human beings (Hamosh *et al.*, 1975). This lipase may be of physiological importance, particularly in the newborn, when the secretion of pancreatic lipase is low (Zoppi *et al.*, 1972).

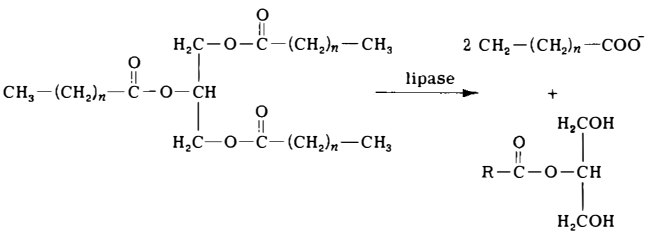
The manner in which lipids are absorbed was uncertain for many years (Deuel, 1955). Frazer (1938) presented the basic concept that triglycerides are only partially hydrolyzed to monoglycerides (MG) and FFA. Elaborating on his theory, Frazer (1946, 1952) suggested that, because of the surface properties of these compounds, they would tend to

form stable emulsions in the presence of bile acids, but the size of the lipid droplets formed would be small enough to allow absorption.

This concept has been verified, modified, and extended (Bergström and Borgström, 1955; Johnston, 1963; Senior, 1964). Digestion and absorption of lipids can be described as follows: (1) digestion of lipids, (2) formation of a micelle suitable for absorption, (3) entry into the mucosal cell and resynthesis of triglyceride and cholesterol ester and their incorporation into chylomicrons, and (4) release of chylomicrons into lymphatic circulation. The proximal jejunum is the major site where these events take place.

A. Digestion of Lipids

Hydrolysis of Triglyceride to MG and FFA



(2)

Pancreatic lipase acts specifically on the α-ester bond of the triglyceride, thereby releasing MG and FFA [Eq. (2)] (Desnuelle, 1961; Desnuelle and Savary, 1963). Any further hydrolysis takes place only after isomerization of the remaining fatty acid moiety of the MG to the α position, a slow reaction. Pancreatic lipase is also specific in that it acts most efficiently only at an oil–water interface. For example, triacetin, which is soluble in water, is not hydrolyzed at all by pancreatic lipase until the concentration of the lipid is increased to supersaturation so that an emulsion forms, i.e., oil droplets within the water phase (Desnuelle, 1961). This hydrolytic enzyme of the pancreas is so specific for the oil–water interphase that its kinetic properties must be expressed on the basis of interfacial area rather than on substrate concentration (Benzonana and Desnuelle, 1965).

Pancreatic lipase hydrolyzes all triglycerides with long-chain fatty acid moieties at about the same rate, if they are adequately and equally emulsified (Desnuelle and Savary, 1963). Fatty acids of short to medium chain lengths, i.e., containing 4–12 carbons, are hydrolyzed more quickly. This property of lipase is of critical importance in the treatment of pancreatic insufficiency. In spite of decreased secretion of pancreatic lipase in these cases, fatty acid digestion and absorption can be maintained by feeding triglyceride containing medium-length fatty acids (MCT). Coconut oil is a natural source of triglycerides containing a large proportion of fatty acids with a medium chain length (Deuel, 1951).

B. Formation of a Micelle Suitable for Absorption

Although bile salts aid in the formation of emulsions prior to hydrolysis of triglyceride, their most important role is the formation of lipid micelles suitable for absorption into the mucosal cell. The detergent properties of the bile salts promote the formation of molecular aggregates in aqueous solutions with the nonpolar portion directed inward and the polar group projecting outward (Roepke and Mason, 1940). Such aggregates are referred to as

micelles. Lipid molecules are readily trapped and concentrated in the micelle (Hofman and Borgström, 1962). The products of hydrolysis, MG and fatty acids, are also readily accumulated in the micelle. Furthermore, MG with an unsaturated fatty acid are more soluble in the micelle than saturated MG (Mattson and Volpenheim, 1963). Such MG are the most common ones formed by the action of pancreatic lipase because the naturally occurring triglycerides commonly have an unsaturated fatty acid at the β position. These mixed micelles are probably the form in which lipid is absorbed by the mucosal cell (Johnston and Borgström, 1964). Small amounts of di- and triglyceride may also enter the micelles (Hofman and Borgström, 1964). Quantitative studies with pig intestine reveal that the capacity for micellar absorption of lipid greatly exceeds the rate at which lipid is presented to the small intestine (Freeman *et al.*, 1968).

Although the formation of micelles by bile salts aids in the absorption of lipid, it is not indispensable for triglyceride absorption. Thirty to forty percent of the ingested triglyceride is absorbed in the absence of bile in dogs and rats (Annegers, 1954; Gallaher *et al.*, 1965). On the other hand, absorption of cholesterol and fat-soluble vitamins is totally dependent on biliary secretion. Hence, in pancreatic insufficiency there may be a complete inability to absorb triglyceride due to lack of hydrolysis, but the presence of micelles from bile salts allows absorption of vitamins A, B, D, and K, thus preventing deficiencies of fat-soluble vitamins in the syndrome (Dawson, 1967).

C. Entry into the Mucosal Cell

There is conflict between what is found biochemically and what is seen microscopically during the absorption of lipids. Studies have shown that MG and FFA are the products of digestion that are absorbed, yet electron micrographs reveal droplets larger than a micelle within the mucosal cell (Palay and Karlin, 1959). It now appears that the droplets are not quantitatively related to lipid absorption and, therefore, may represent an event taking place after actual absorption (Strauss, 1966). After absorption, the MG and FFA are again combined to form triglycerides. This step appears to be the rate-limiting step in lipid absorption (Shapiro, 1967). Triglyceride synthesis can proceed via two pathways: (1) the glyceride-glycerol of the monoglyceride is utilized as the backbone (Clark and Hübscher, 1961), or (2) a new glyceride backbone is formed by acylation of *sn*-glycerol 3-phosphate. The fatty acids are incorporated after being "activated" to acyl-CoA derivatives (Ailhaud *et al.*, 1962). The phosphatidic acid formed in pathway (2) is dephosphorylated and the resulting diglyceride acylated to form the final product (Coleman and Hübscher, 1962).

The lipase of the mucosal cells may be of significance as a control point for determining which pathway of triglyceride synthesis predominates and may thereby provide a means of balancing available glyceride-glycerol backbone with available FFA (Dawson, 1967). In contrast to pancreatic lipase, mucosal lipase is specific for MG (Senior and Isselbacher, 1963). Dawson (1967) postulated that, if MG is in excess over FFA available for esterification, this lipase could form more FFA from the MG. If FFA are in excess, the lipase would presumably be less likely to attack MG due to lack of available substrate. It should be noted that not all absorbed FFA is reesterified.

The enzymes of the mucosal cell responsible for activation and reesterification of the FFA prefer fatty acid with a carbon chain of 12 carbons or more (Brindley and Hübscher, 1966). Therefore, there can be a partitioning of fatty acids in the mucosal cell (Shapiro,

1967), the long-chain fatty acids being mainly incorporated into triglyceride, and the short-chain fatty acids (less than C_{10}) remaining mainly as FFA. The medium-chain fatty acids can go by either pathway. The majority of the FFA enter the portal system and are carried to the liver as albumin complexes. The absorption of triglyceride with medium and short-chain fatty acids is more rapid than that of triglyceride containing long-chain fatty acids because of more rapid hydrolysis, and the resulting FFA escapes the rate-limiting step of reesterification to triglyceride (Greenberger *et al.*, 1966). This observation is of clinical importance in any malabsorption syndrome, such as short-bowel syndrome, biliary obstruction, lymphatic obstruction, and, as mentioned, pancreatic insufficiency (Isselbacher, 1967).

1. Steroid Absorption

The cholesterol resulting from the hydrolysis of the cholesterol ester in the lumen of the intestine also enters the microvillus via the micelle. In the microvillus, the newly entering cholesterol displaces one already there, which migrates to the cytoplasm of the mucosal cell (David *et al.*, 1966). The cholesterol is then reesterified and transferred to the lymph as part of the chylomicron.

2. Phospholipid Absorption

Phospholipids in the diet are hydrolyzed to phosphoglyceride and free fatty acids in the lumen of the small intestine and absorbed in this form, and then the phosphoglyceride is reesterified in the mucosal cell to form various diacyl derivatives.

D. Formation of the Chylomicron

Within the mucosal cell, the resynthesized triglyceride, phospholipid, and cholesterol ester, along with some free cholesterol and small amounts of FFA and fat-soluble vitamins, are combined with a small quantity of protein to form a particle called a chylomicron. The formation of this particle is dependent upon protein synthesis in the mucosal cell (Isselbacher and Budz, 1963).

It is not clear how the chylomicron leaves the mucosal cell, but reverse pinocytosis has been suggested (Shapiro, 1967). In the extracellular space, the chylomicron diffuses through the lacteal membrane into lymph ducts, to the thoracic duct, and eventually to the circulatory system. The size of the chylomicron prevents its entrance into the capillaries of the portal system. Thus, long-chain fatty acids escape the initial filtration in the liver undergone by medium- and short-chain fatty acids and most other products of the absorptive process.

V. FATE OF DIETARY LIPIDS

A. Chylomicron Metabolism

Chylomicrons are rapidly removed from the circulation and their contents utilized by adipose tissue (Shapiro, 1965), cardiac muscle (Delcher *et al.*, 1965; Gousios *et al.*, 1963; Crass and Meng, 1966; Simpson-Morgan, 1968), liver (Belfrage *et al.*, 1965), and probably lung (Simpson-Morgan, 1968). The kinetics and the mechanism of chylomicron utilization have been extensively investigated (see Meng, 1964; Simpson-Morgan, 1967;

Hallberg, 1967). Triglyceride, the major component of chylomicrons, undergoes hydrolysis to glycerol and its constituent fatty acids. These components may be utilized for synthesis of new triglycerides and phospholipids or oxidized to CO_2. Whether the initial degradation takes place on the external plasma membrane (Green and Webb, 1964; Higgins and Green, 1966) or intracellularly (Shapiro, 1965; Belfrage *et al.*, 1965) is unsettled. There is evidence for both sites, and it is possible that some tissues take up the chylomicron intact whereas in others lipolysis occurs prior to entrance into the cells or that both mechanisms operate simultaneously.

Lipolysis of the chylomicron triglyceride is catalyzed by lipoprotein lipase. The inactive bound form of this enzyme is activated in the plasma by heparin. Because the hydrolysis of triglyceride to FFA clears the plasma, this enzymatic activity has been referred to as the plasma clearing factor. The anatomical site of binding of lipoprotein lipase is not known. The capillary endothelium has been suggested (Robinson, 1963). A congenital deficiency of lipoprotein lipase resulting in hyperlipemia of exogenous origin has been reported in a puppy (Baum *et al.*, 1969). Certain tissues contain lipoprotein lipase activity as well. Adipose tissue lipase is responsive to diet, increasing during feeding and decreasing during fasting (Schotz and Garfinkel, 1965). During lactation, this activity decreases, whereas the lipase associated with mammary gland increases (Zinder *et al.*, 1974).

The fate of FFA absorbed from the chylomicron varies with the tissue. In heart, it is mainly oxidized to CO_2; in adipose, it is mainly reesterified and stored as triglyceride; in liver, a portion may be oxidized, but another portion is reesterified and released back into the plasma in the form of very low density lipoprotein or pre-beta-lipoprotein (Section VI,C).

B. Medium- and Short-Chain Fatty Acids

The medium- and short-chain fatty acids are absorbed and transported to the liver via the portal system (Hashim *et al.*, 1964, 1965; Kiyasu *et al.*, 1952). The major portion is oxidized in the liver and does not enter the peripheral circulation (Kirschner and Harris, 1961). The fate of the large quantities of VFA absorbed into the portal system in ruminants varies with each compound and is discussed in Section IX,A.

C. Cholesterol and Cholesterol Ester

The dietary cholesterol and cholesterol ester in the chylomicron are utilized almost completely in the liver. The dietary cholesterol quickly mixes with the cholesterol that has been synthesized *de novo* in the liver. The total amount of cholesterol in mammals is under close homeostatic control. The rate of biosynthesis in the liver is indirectly proportional to the amount of cholesterol and cholesterol ester absorbed from the gut (Siperstein and Fagan, 1964; Bortz, 1967). The output of cholesterol is also variable, increasing when the intake increases, and decreasing when the intake is lessened. Cholesterol is lost from the animal in the form of bile acids and as free cholesterol and its derivatives in bile.

VI. TRANSPORT OF LIPIDS

None of the lipids normally found in the plasma are sufficiently soluble in water to circulate in the free form. In some cases they are bound to specific proteins, which keeps

them in suspension. Quantitatively, the transport of fatty acids and triglyceride is most important. Cholesterol and cholesterol ester are also important quantitatively in specific species (e.g., the chicken) and of clinical and diagnostic importance in all mammals. Most of the chylomicrons are transported in the plasma shortly after lipid absorption. The composition and fate of these particles has been discussed (Section V,A).

A. Free Fatty Acids

Only a small portion of the FFA are free in the plasma; most are bound to albumin (Goodman, 1958). This albumin–fatty acid complex is formed when fatty acids are released into the circulation. The mobilization of fatty acids from adipocytes is inhibited by a lack of albumin (Steinberg and Vaughan, 1965).

The FFA–albumin complex accounts for the major portion of lipid transported in the plasma. The concentration of FFA is lower than that of some other lipid components of plasma, but their turnover rate exceeds that of any other lipid fraction in plasma (Fredrickson and Gordon, 1958). The concentration of FFA in most species in the postabsorptive stage is 300–600 μEq per liter of plasma. The level of FFA increases three- to fourfold during prolonged fasting (Dole, 1956) or chronic nutritional stress (Annison, 1960).

B. Lipoproteins of Plasma

Lipoproteins are the protein-lipid complexes that carry triglyceride, cholesterol ester, cholesterol, and phospholipid in plasma. Major classes isolated by electrophoresis have been designated by a system analogous to that for other plasma proteins as α, β, and pre-β-lipoprotein. When separated on the basis of density by untracentrifugation, the classes have been named on the basis of their relative densities: high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low density lipoprotein (VLDL). The lipoproteins are spherical particles with a neutral lipid core (triglyceride and cholesterol ester) and a surface made up of the lipoprotein apoprotein in association with cholesterol and phospholipid (Hamilton and Kayden, 1974).

The structural basis of the lipid-protein complex is not clear, but the bonding is not primarily covalent (Fisher and Gurin, 1964). The following types of linkages have been suggested: electrovalent, hydrogen bonding, van der Waals, and the orientation of hydrophobic groups (Salem, 1962; Shen *et al.*, 1977; Segrist *et al.*, 1974). It is possible that any or all of these mechanisms operate. Which of these factors predominate in a given lipoprotein complex may depend upon the apoprotein present and the lipid complement (Scanu, 1978). For example, in LDL, apoprotein B is virtually the sole apoprotein in the presence of a mixture of neutral and charged lipids, whereas VLDL contains comparable amounts of apoproteins B and C with predominantly neutral lipid. Hence, in one particle, electrostatic interactions may prevail whereas, in another, hydrophobic forces may provide stability to the complex. Until the amino acid sequences are known and the secondary and tertiary structures can be predicted with certainty, how these complexes are maintained physically intact will be unclear. The clinical and physiological importance is that the forces are strong enough to allow isolation of the complex and yet weak enough to allow exchange of the lipid component among plasma lipoproteins themselves and between plasma and tissue.

The few studies of lipoproteins in domestic animals have made clear that species

variations exist (Evans, 1964; Evans *et al.*, 1961; Hillyard *et al.*, 1955; Puppione, 1969; Puppione *et al.*, 1972) and more recently that even within species, in this case equines, significant differences occur (Robie *et al.*, 1975a,b,c). Puppione's (1978) studies in the cow indicate that such investigation not only will increase our understanding of lipid metabolism in the species under examination but are likely to provide reagents and information clarifying our conceptualization of hypercholesterolemia and atherosclerosis (Section IX,B).

1. Methods of Isolation of Plasma Lipoproteins

The two most widely used techniques are electrophoresis and ultracentrifugation. The correspondence between the fractions isolated by each of these methods is clear, at least in human beings (Fredrickson *et al.*, 1968; Noble *et al.*, 1969; Puppione, 1978). The electrophoretic method of separation is the same as that for other plasma proteins except that lipid-specific stains are used for detection. Oil red O has been used routinely, but staining with Schiff's reagent after ozonization of the lipid has been used and gives a more intense color.* Counterstaining with any of the usual protein stains provides a means of identifying a lipid-carrying protein in relation to the other plasma proteins. The major lipoproteins are designated with the same Greek letters as the plasma globulins moving similarly in the electrophoretic field, i.e., α, β, and pre-β. Hatch and Lees (1968) have reviewed these techniques.

Separation of the plasma lipoproteins by ultracentrifugation is based on differences in density, mainly due to the lipid component of the lipoproteins. This method separates the lipoproteins into an almost limitless number of subgroups (Ewing *et al.*, 1965). By this means, the lipoproteins are commonly divided into four subfractions: HDL, LDL, VLDL, and chylomicron. As seen in Fig. 3, these divisions are arbitrary. The relative densities are expressed as Svedberg units (S_f) based on the flotation rate of the lipoprotein in a density gradient.

The detailed relationship between the electrophoretic and ultracentrifugal separations is shown in Fig. 3. The type and quantity of lipid found in each group are shown in Table I.

2. Formation of Lipoproteins

With the exception of chylomicrons from the intestine, the nascent lipoproteins are synthesized predominanly in the liver (Radding *et al.*, 1958; Marsh and Whereat, 1959; Windmueller and Levy, 1967; Kane, 1977; Schaefer *et al.*, 1978). Intestine also contributes to the synthesis of VLDL (Windmueller and Levy, 1968; Ockner *et al.*, 1969a,b). Low-density lipoprotein, being a product of VLDL catabolism, is not secreted by either intestine or liver (Sigurdsson *et al.*, 1975; Schaefer *et al.*, 1978). Nascent VLDL is modified in plasma by the addition of apoprotein C and HDL (Rubinstein and Rubinstein, 1972; Eisenberg *et al.*, 1973) to form the VLDL particle routinely isolated from plasma (Ockner *et al.*, 1969a; Zilversmit, 1969).

The site of synthesis of the apoproteins is less clear. Apoprotein B, the triglyceride carrier associated with VLDL and chylomicrons, is synthesized *de novo* in both liver and intestine (Windmueller *et al.*, 1973; Schaefer *et al.*, 1978). The site(s) of synthesis of the A apoproteins is still controversial. There is evidence for the liver as the site or the

*"Procedures, Techniques and Apparatus for Electrophoresis," p. 25. Gelman Instrument Co., Ann Arbor, Michigan.

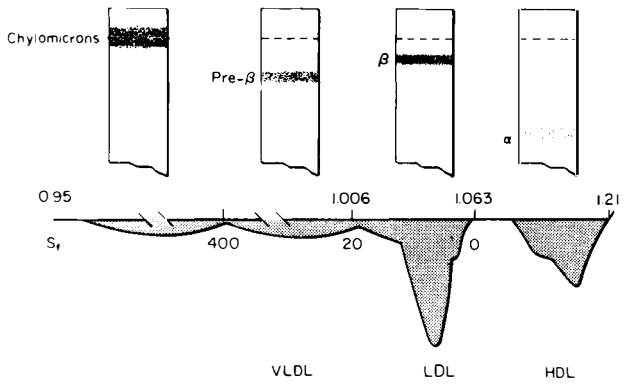


Fig. 3. Electrophoretic and ultracentrifugal patterns of the plasma lipoproteins of man. (After Fredrickson *et al.*, 1967.)

intestine, or both (see Kane, 1977; Schaefer *et al,* 1978). The site of synthesis of C apoproteins has been studied less, but, due to the lack of synthesis in the intestine, liver would appear to be the primary site of origin (Windmueller *et al.*, 1973). Since the other apoproteins (D and E or arginine-rich) are associated with HDL, the apparent site of their synthesis is the liver (Schaefer *et al.*, 1978).

3. Interrelationships among Lipoproteins

The α- and β-lipoproteins normally account for about 90% of the cholesterol and phospholipid of plasma. The change in concentration of these lipids with changing conditions in the animal is insignificant when compared to that of triglyceride. On the basis of this observation and others, Fredrickson *et al.* (1967) adopted a simplifying concept that conceives of lipoproteins with their constituent phospholipids and cholesterol as stable cargo vehicles for carrying glyceride from liver to other tissues. The triglyceride-loaded lipoprotein migrating as pre-β-lipoprotein with a density of VLDL is particulate and, thus, in high concentrations results in turbidity of the plasma. The term "endogenous lipid particle" denotes those particles containing triglycerides derived mainly from liver and chylomicrons, and the term "exogenous lipid particle," those particles with triglycerides arising from digestion and absorption.

Conceptually, this scheme is still valid, but great advances have been made in the

TABLE I

Lipid Composition of Serum Lipoproteins of Human Beings

Density class (gm/cm³)	Electrophoretic migration	Percent lipid composition[a,b]			
		TG	PL	CE	Chol
<1.006	Pre-β	63-75	16-25	13-16	4-6
1.006-1.063	β	15	26	46	13
1.063-1.21	α	8	48	40	4

[a] Estimated by Puppione (1969) from the data of Hatch and Lees (1968).
[b] TG, triglyceride; PL, phospholipid; CE, cholesterol ester; Chol, cholesterol.

isolation and characterization, including amino acid sequence in some cases, of the apoproteins of lipoproteins (for reviews of these studies, see Eisenberg and Levy, 1975; Jackson *et al.*, 1976; Havel, 1975; Kane, 1977; Morrisett *et al.*, 1977; Osborne and Brewer, 1977). Using the nomenclature introduced by Alaupovic *et al.* (1972), it appears that some apoproteins are solely concerned with lipid transport, but others act as activators, or coenzymes, for enzymes: apoprotein A-I for lecithin–cholesterol acyltransferase (LCAT) in plasma and apoprotein C-II for lipoprotein lipase in epithelial cells lining the capillaries of many tissues. These studies have provided evidence for interrelationships between apoproteins (Eisenberg and Levy, 1975; Kane, 1977; Schaefer *et al.*, 1978; Puppione, 1978). There is a dynamic interaction among the lipoproteins that regulates the transport, deposition, and tissue utilization of their components (Scanu, 1978).

When the triglyceride carriers, VLDL and chylomicrons, enter the plasma, their major protein is apo B. In the plasma, they interact with HDL to receive their complement of apo C and apo E. The addition of apo C alters the electrophoretic properties of VLDL and, more important metabolically, provides the activator of lipoprotein lipase. By this means, the utilization of VLDL is facilitated. About 80% of triglyceride is removed from VLDL during passage through heart, adipose tissue, or mammary gland. During lipolysis, apo C returns to HDL, to be utilized again by nascent VLDL. Apoprotein B remains with the remnant particle of intermediate density, or IDL, and eventually can be recovered with the LDL fraction (Eisenberg and Levy, 1975). Whether LDL is merely the final catabolic product of triglyceride utilization and transfer of excess phospholipid to HDL (Schaefer *et al.*, 1978; Kane, 1977) or is modified IDL by specific addition of cholesterol ester from HDL (Puppione, 1978) is not clear. The ultimate fate of IDL is removal by the liver (Eisenberg and Levy, 1975). In those species in which LDL is low, for example, cow and rat, presumably hepatic removal is very efficient (Puppione, 1978). In other species, notably man, in which LDL is high, the primary fate of LDL appears to be utilization in extrahepatic tissue (Sniderman *et al.*, 1974; Kane, 1977).

Human fibroblasts have a specific receptor for LDL (Goldstein and Brown, 1974, 1975, 1976). Binding of LDL to this receptor initiates a series of reactions resulting in adsorptive endocytosis of the LDL particle (Goldstein *et al.*, 1977). The cholesterol ester of LDL is hydrolyzed in the lysosomes to its component parts. The cholesterol is not only utilized by the cell for membrane synthesis but also inhibits the rate-limiting enzyme of cholesterol synthesis, HMG-CoA reductase (Siperstein and Guest, 1959) (Section VII,B), and stimulates the esterifying enzyme. Sufficient uptake of cholesterol by this process results in inhibition of synthesis of the specific LDL receptor. It is by this series of feedback mechanisms that the level of cellular cholesterol is normally controlled.

The role of LCAT and its protein activator, apo A, is not clear in the overall scheme of lipoprotein interactions. This enzyme catalyzes the esterification of cholesterol to cholesterol ester, with the acyl moiety coming from phospholipid, and phospholipid and cholesterol are transferred between lipoproteins. This makes it likely that the activity of LCAT is important in maintaining the proper lipid complement of the various lipoproteins. Some of the cholesterol taken up by HDL and converted to cholesterol ester may also come from cells (Bailey, 1965; Stein and Stein, 1973; Kane, 1977). Hence, the level and complement of lipid in HDL particles, i.e., their ability to accept tissue cholesterol, may be a factor in controlling excess cholesterol deposition in peripheral tissues (Glomset, 1968; Kane, 1977). In this regard, the possible importance of HDL in the normal hypercholesterolemia of lactating cows (Puppione, 1978) will be discussed later (Section IX,A).

C. Dynamics of Lipid Transport

The source, transport, and utilization of the exogenous lipid, chylomicrons, have been discussed in Section V,A. The source, mobilization, transport, and disposition of endogenous lipid will be discussed here. Adipose tissue is the main source of endogenous lipid. The lipid in adipose tissue comes from two sources: storage of lipid and synthesis in the tissue (O'Hea and Leveille, 1968). As with dietary lipid, most of this lipid is triglyceride (Vaughan and Steinberg, 1963).

Adipose tissue lipid is mobilized in the form of FFA, and glycerol is concomitantly released. Thus, hydrolysis of the glyceride easter bond is the first step in fat mobilization. This lipolysis is catalyzed by the lipase of adipose tissue, which differs from the lipoprotein lipase described earlier (Section V,A). This enzyme, which is sensitive to myriad hormones, catalyzes the release of one FFA leaving a diglyceride. Once formed, the diglyceride is rapidly degraded to FFA and free glycerol. This series of reactions is catalyzed by a second lipase that is not responsive to hormones (Biale *et al.*, 1965; Pope *et al.*, 1966; Kupiecki, 1966). Increased lipolysis does not ensure release of FFA from the tissue. If a source of *sn*-glycerol-3-P is available, the fatty acid can be reesterified and not be released. The latter situation is common during the absorptive state, when glucose delivered to adipose tissue is rapidly metabolized to phosphorylated derivatives including *sn*-glycerol-3-P. The glycerol released during lipolysis cannot be reutilized for esterification because adipose tissue lacks the enzyme glycerokinase, which catalyzes the formation of glycerol-3-P from glycerol. In summary, two factors enhance the mobilization of FFA: increased lipolysis and/or decreased esterification (the reverse reaction) (Steinberg and Vaughan, 1965).

The FFA released from adipose tissue cannot diffuse into the plasma unless albumin is available to solubilize them (Steinberg and Vaughan, 1965). Thus, a third factor is involved in the mobilization of FFA: the availability of albumin. This availability may vary with the albumin concentration in plasma and the rate of perfusion of adipose tissue.

The rate of mobilization of FFA is usually reflected in the plasma concentration of FFA; e.g., an increase in the plasma concentration of FFA usually indicates increased FFA release from adipose tissue. However, the rate of utilization of FFA also influences the plasma concentration. If an increased rate of release is matched by increased rate of utilization, the plasma concentration remains unchanged. The major site of FFA utilization is the liver, although most tissues can utilize these compounds for biosynthetic and oxidative purposes.

The FFA can be disposed of in the liver in a variety of ways:

1. It may be oxidized completely to CO_2 and H_2O. If there is a block in the utilization of acetyl-CoA derived from β-oxidation, complete oxidation cannot occur and the ketone bodies, β-OH-butyrate and acetoacetate, may be formed. These ketone bodies are readily released from the liver and can be catabolized by other tissues.
2. The FFA may be esterified to reform triglycerides. After incorporation into VLDL, they are released into the blood vascular system (Fredrickson *et al.*, 1967). The triglyceride portion of the VLDL is the same as that in chylomicrons; it may be utilized by other tissues or returned to the adipose tissue for storage.

The transport and fate of endogenous lipid are summarized diagramatically in Fig. 4.

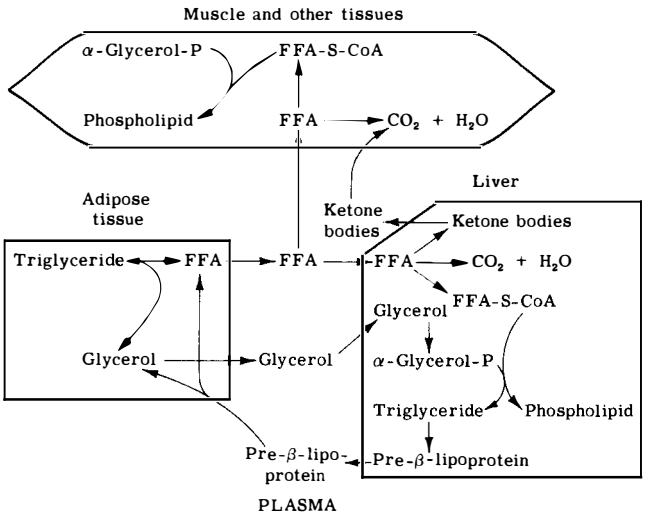


Fig. 4. Schematic presentation of the transport and fate of endogenous lipid.

D. Factors Affecting Fat Mobilization

1. Endocrine Factors

The catecholamines, whether secreted by the adrenal medulla or the endings of the postganglionic sympathetic nerves, markedly stimulate lipolysis in adipose tissue. The mechanism of their action involves an increase in the cellular level of cyclic AMP, which in turn activates the triglyceride lipase (Rizach, 1965). The catecholamines probably do not act during the normal FFA release in the postabsorbtive stage. Their role in mobilizing lipids is a part of the stress response. Glucocorticoids and thyroxine must be present in order for epinephrine and norepinephrine to exert their maximal effect on lipolysis (Steinberg and Vaughan, 1965).

Several peptides, including ACTH, TSH, MSH, and vasopressin, have lipolytic actions (Steinberg and Vaughan, 1965). The action of a given peptide is limited to certain species (Rudman *et al.*, 1965; Raben, 1965). In each case, the site of action is triglyceride lipase and involves cyclic AMP (Rizach, 1965). Another peptide, glucagon, acts similarly on adipose tissue (Kovacev and Scow, 1966). Growth hormone also stimulates lipolysis but by a different mechanism. This hormone also stimulates synthesis of the enzyme adenyl cyclase, which in turn catalyzes production of cyclic AMP (Fain, 1968). The physiological significance of these peptides and mobilization of lipid has yet to be established.

Thus far, only one hormone has been shown to inhibit the release of FFA from adipose tissue (Fain *et al.*, 1966). Insulin inhibits mobilization of FFA both directly and indirectly. Insulin has been shown to lower the level of cyclic AMP, which would decrease triglyceride lipase activity (Butcher *et al.*, 1966). Furthermore, increased glucose uptake by adipose tissue in the presence of insulin tends to enhance reesterification of FFA, thus decreasing their release (Section VI,C). Adipose tissue has been described as being exquisitely sensitive to insulin (Cahill, 1964). Thus, the action of insulin is of great importance in the control of lipid mobilization by adipose tissue during the feeding cycle, during high-carbohydrate feeding, and during fasting.

Prostaglandins appear to influence lipid mobilization by adipose tissue (Bergström and Samuelson, 1965). These compounds are synthesized in mammalian tissues from the essential fatty acids. Adipose tissue in the presence of prostaglandin releases more FFA than in their absence. In contrast, prostaglandin counteracts the stimulatory effects of catecholamine on lipolysis when both groups of compounds are present. The physiological importance of these observations has not been established.

2. Neural Factors

Electrical stimulation of the autonomic fibers of adipose tissue results in the release of FFA into the circulation (Correll, 1963). This reaction is presumably due to the release of norepinephrine from the sympathetic fibers in the neural network. Havel (1965) stressed the day-to-day importance of the sympathetic innervation of adipose tissue as a fundamental mechanism controlling the supply of nutrients to body tissues. In addition, the response of adipose tissue to sympathetic stimulation is also important in providing energy in stressful situations, such as fasting, cold exposure, and vigorous exercise (Havel, 1965). In this respect, lipolysis by adipose tissue and glycogenolysis by the liver are comparable responses to emergency situations and are elicited by similar mechanisms (see Sutherland, 1961; Raben, 1965).

E. Abnormalities of Plasma Lipoproteins

There have been too few studies in domestic animals to establish clearly that abnormalities and deficiencies described in man occur with significant regularity in other species. The extensive studies in man, however, provide an experimental model for conditions that might be observed in domestic animals. Recently, there has been a great deal of renewed interest in and excitement about human lipoproteins because HDL–cholesterol has been shown to be inversely correlated with and predictive of cardiac abnormalities (Rhoads *et al.*, 1976).

1. Inherited Lipoprotein Deficiencies

Two disorders of genetically determined origin have been described: the complete lack of β-lipoprotein, abetalipoproteinemia, and a lack of normal HDL in Tangier disease. There appears to be normal apoprotein synthesis in this disease, but the catabolism of apo A is abnormal and rapid, resulting in low levels (Schaefer *et al.*, 1976).

a. Abetalipoproteinemia. This lack of β-lipoprotein is manifest in infancy by retarded growth, steatorrhea, and abdominal distension. The acanthocytosis accompanying this syndrome was the first sign noticed (Bassen and Kornzweig, 1950). Later, the very low plasma cholesterol levels led to the demonstration of the absence of β-lipoproteins (Salt, 1960). Severe neurological defects manifest late in childhood with signs of degeneration of the posterolateral columns and the cerebellar tracts as well as retinal degeneration. The inability to absorb essential fatty acids and fat-soluble vitamins probably contributes to the clinical manifestations of this disease.

The electrophoretic pattern of the plasma lipoproteins from these patients indicates a complete lack of β-lipoprotein. The concentrations of cholesterol, phospholipid, and glycerides in plasma are the lowest recorded in any human disease. The lack of β-lipoprotein results in an inability to transport glyceride, either as VLDL or as chylomi-

crons. The ability to form chylomicrons means that lipids can be taken up by the mucosal cell but not released into the lymph. Blocking protein synthesis in rat intestinal mucosa produces a syndrome similar to this disease (Hatch *et al.*, 1963; Isselbacher and Budz, 1963).

When patients are fed diets high in glycerides, chylomicrons do not appear in the plasma. When fed high-carbohydrate diets, which normally result in the release of endogenous lipid into the plasma, there is no increase in the plasma glyceride concentration, nor is the presence of pre-β-lipoprotein observed (Levy *et al.*, 1966). These patients could digest and absorb MCT, but this diet could not overcome the lack of essential fatty acids. As will be discussed later, glyceride formed in the liver, as a result of either a high-carbohydrate diet or a diet containing MCT, can result in a fatty liver (Section VI,F). Glyceride would accumulate due to the lack of the protein complex to transport it to other tissues.

b. α-Lipoprotein Deficiency. Lack of α-lipoprotein has been called Tangier disease after the first cases found, namely, two children 5 and 6 years of age in the same family living on Tangier Island in Chesapeake Bay (Harbert *et al.*, 1978). As in abeta-lipoproteinemia the levels of plasma cholesterol and phospholipid are below normal. Glyceride concentrations in the plasma are high normal in the postabsorptive state. The deposition of cholesterol ester in all reticuloendothelial tissues provides a pathognomonic sign for this disease; the tonsils are grossly enlarged and have a unique orange color. This disease is not as serious as abeta-lipoproteinemia. There appears to be no malabsorption, and the ability to release endogenous lipid is unimpaired. All the clinical signs can be related to the abnormal deposition of lipid in the body tissues.

The electrophoretic pattern of the plasma lipoproteins reveals the complete lack of an α band and no distinct pre-β band.

2. Hyperlipoproteinemias

In contrast to the hypolipoproteinemias, these syndromes are more descriptive and less informative of the mechanism of lipid transport by the lipoprotein complex. Increased levels of major lipoprotein fractions are observed (1) secondary to systemic disease and (2) in primary lipoproteinemia, which often has a familial occurrence (Fredrickson, *et al.*, 1978). Before the advent of lipoprotein analysis, many of these conditions in both categories were referred to as hypercholesterolemia and hyperglyceridemia. These names are accurate, specific terms that are clinically valid in the absence of lipoprotein analysis.

On the basis of lipoprotein patterns and plasma lipid analyses, Fredrickson and his co-workers (1967, 1978) divided hyperlipoproteinemias into five categories. It is recommended for consistency that, initially, the findings of comparative studies in domestic animals be similarly categorized. A possible canine classification could be based on the canine classes of lipoproteins that have been identified: chylomicron, VLDL, LDL, HDL_1, and HDL_2 (Mahley and Weisgraber, 1974). Canine VLDL, LDL, and HDL_2 correspond to human VLDL, LDL, and HDL. Canine HDL has α mobility but is similar in size and density to LDL. Canine HDL contains almost as much cholesterol as LDL and may in fact be the major cholesterol carrier in the dog. The methods for sampling and analysis of the plasma have been described in considerable detail (Fredrickson *et al.*, 1967). In the future, as immunoassay methodologies become available for each apoprotein, the determination of their concentrations in specific disease states and in hyperlipoproteinemias

will add greatly to our understanding of lipid transport and deposition. It is likely that this new information will require modification of the current classification system for hyperlipoproteinemias.

Table II, adapted from Fredrickson *et al.* (1967), summarizes the characteristics of the plasma lipid in each category. The diseases of human beings in which such a pattern may occur secondarily are also listed. The latter information may be of value in recognizing and differentiating among such diseases in domestic animals. Some of this information is already in everyday use, e.g., hypercholesterolemia secondary to hypothyroidism.

For a detailed description of each type of hyperlipoproteinemia in the table, the reader is referred to Fredrickson *et al.* (1967, 1978). Type I is characterized by the presence of chylomicrons in the plasma in high concentrations at least 14 hours after a meal. The other lipoprotein fractions are low, in contrast to Type V, so that Type I is hyperchylomicronemia in almost pure form. Hyperchylomicronemia is characterized by the formulation of a cream layer on the plasma sample during storage in the cold. A familial disease in human beings included in Type I is a deficiency of lipoprotein lipase. In this disease, injection of heparin leads to no discernible change in the turbidity of the plasma. A similar disease was reported in a puppy (Baum *et al.*, 1969) and an idiopathic hyperchylomicronemia form in a dog (Rogers *et al.*, 1975a).

In severe diabetes, marked hyperlipemias occur frequently, and the blood often has the appearance of "tomato soup." A cream layer may separate out on storage, indicating hyperchylomicronemia as in Fredrickson's Type I. The plasma usually remains turbid, indicating the presence of VLDL also. On analysis, total triglycerides (TG) (chylomicrons) and cholesterol (VLDL) are elevated (Rogers *et al.*, 1975b). Diabetic hyperlipemia appears to be caused by impaired lipolysis of chylomicrons secondary to a deficiency of lipoprotein lipase rather than to overproduction of VLDL (Bagdale *et al.*, 1967).

Type II is characterized by an increase in the lipoproteins with β mobility on electrophoresis. In this type of hyperlipoproteinemia, the plasma cholesterol is markedly increased in the absence of a similar increase in the glyceride level. A lipoprotein pattern

TABLE II

Diseases Associated with the Hyperlipoproteinemias of Human Beings[a]

Type	Characteristic	Diseases associated with
I	Chylomicronemia	Diabetes; pancreatitis; acute alcoholism
II	Cholesterolemia	Hypothyroidism; obstructive hepatic disease; hypoproteinemia; familial xanthomatosis, etc.
III	β-Lipoproteinemia	Familial hyperglyceridemia plus cholesterolemia
IV	Pre-β-lipoproteinemia	Diabetes; pancreatitis; alcoholism; glycogen storage disease; hypothyroidism; nephrotic syndrome; dysglobulinemia; gestational hormones; familial disease
V	Pre-β-lipoproteinemia plus chylomicronemia	Not clear; may be a combination of I and IV

[a] From Fredrickson *et al.* (1967).

typical of Type III is commonly associated with hypothyroidism and obstructive liver disease, although Type IV is also seen. Type II occurs familially in human beings, the most common syndrome being the formation of xanthomas and atheromas. Type III varies from Type II only in that the plasma glycerides are also markedly elevated. Specific immunoassay of apo E has revealed abnormally high plasma levels of this protein in Type III (Currey *et al.*, 1976; Kushwaha *et al.*, 1977).

Type IV is distinguished from Types II and III by the fact that the hyperlipoproteinemia is due to endogenous lipid. Type IV can result from any of a variety of conditions in which the rate of release of glycerides into the plasma exceeds their rate of removal. In general, lack of control of carbohydrate metabolism or a caloric imbalance can result in Type IV hyperlipoproteinemia. Many diseases in which this is the case are also listed in Table II.

The electrophoretic pattern of the lipoproteins in Type IV is characterized by a marked increase in the pre-β band and an absence of chylomicrons. The triglyceride level is markedly increased and the cholesterol concentration moderately increased. When the triglyceride level becomes extremely high, there is marked tailing of the pre-β band, giving an indication of the presence of chylomicrons. Dilution of this plasma with saline prior to electrophoresis aids in the separation of the pre-β and chylomicron areas in samples where this occurs.

In human beings, Type IV seems to have a prevalence in certain families, especially in young adulthood. It often accompanies severe obesity and disappears when the patient returns to normal weight and avoids excessive carbohydrate intake. Type V hyperlipoproteinemia is characterized by an increase in circulating chylomicrons and pre-β-lipoproteins (VLDL) as well. It is not well distinguished from Type IV and may be a combination of abnormalities such as Types I and IV.

F. Plasma Lipoproteins and Fatty Liver

The relationship between liver triglycerides and plasma glycerides has been quantitated in the rat. In rats fed diets high in glucose, nearly all the triglyceride synthesized in the liver is disposed of as plasma lipoproteins, presumably as pre-β-lipoprotein (VLDL) (Baker and Schotz, 1964). Therefore, it is not surprising that any block in the synthesis of the apoproteins of the lipoproteins results in an accumulation of lipid, namely, glycerides, in the liver (Lombardi, 1966; Shapiro, 1965). Ethionine, CCl_4, puromycin, and orotic acid all appear to cause fatty livers by blocking synthesis of protein, particularly the apolipoproteins (Smuckler and Benditt, 1965; Robinson, 1964; Farber *et al.*, 1964; Lombardi and Uqazio, 1965; Venkataraman and Screenivasan, 1965). Similarly, choline deficiency results in fatty liver due to the lack of synthesis of phospholipid (Zilversmit and Diluzio, 1958), a necessary component of lipoprotein complex (Section VI,B). In all of these experimental disorders there is a fall in the level of plasma triglycerides and a corresponding decrease in lipoprotein concentration, particularly the VLDL.

Other disorders resulting in fatty livers are accompanied by hypertriglyceridemia. In these conditions, formation and release of triglycerides by the liver are both increased, e.g., ethanol ingestion (Isselbacher and Greenberger, 1964) or cortisone (Freidman *et al.*, 1965; Hill and Droke, 1963). The ultimate cause of the fatty liver and subsequent hypertriglyceridemia in these studies is presumably mobilization of FFA derived from adipose tissue (Jones *et al.*, 1965). The response to ethanol is complicated by the fact that

ethanol is also a ready precursor of acetyl units as well as a source of reducing power for fatty acid synthesis (Section VII,A). The effects of alcohol on lipid metabolism in the liver have been reviewed by Shapiro (1967).

VII. BIOSYNTHESIS OF LIPIDS

The pathways for the synthesis of phospholipids (Kennedy, 1961) and sphingolipids (Brady and Koval, 1958) are known, but factors altering their synthesis are not clear. Hence, in this section, emphasis is on fatty acid synthesis, glyceride synthesis and cholesterol synthesis and on the factors that influence these pathways.

A. Fatty Acid Synthesis

1. Tissues Involved in Fatty Acid Synthesis

The major sites of fatty acid synthesis in the animal are adipose tissue and liver. In the lactating animal, the mammary gland also synthesizes fatty acids. In mice, and presumably all mammals, adipose tissue contributes the major portion of fatty acid synthesized within the animal (Favarger, 1965). Adipose tissue is not simply a site for the passive storage of triglyceride, but rather it actively takes up, synthesizes, and releases fatty acids. In contrast to mammals, the liver of the chicken is the major site of fatty acid synthesis (O'Hea and Leveille, 1968).

2. Pathway of Fatty Acid Synthesis

In all systems studied, *Escherichia coli,* yeast, pigeon liver, rat liver, and rat mammary gland, the basic steps of fatty acid synthesis from acetyl-CoA are the same (Vagelos, 1964). The first step is the formation of malonyl-CoA by the addition of an active CO_2 to acetyl-CoA. This carboxylation is catalyzed by acetyl-CoA carboxylase, a biotin enzyme, and requires ATP [Eq. (3)].

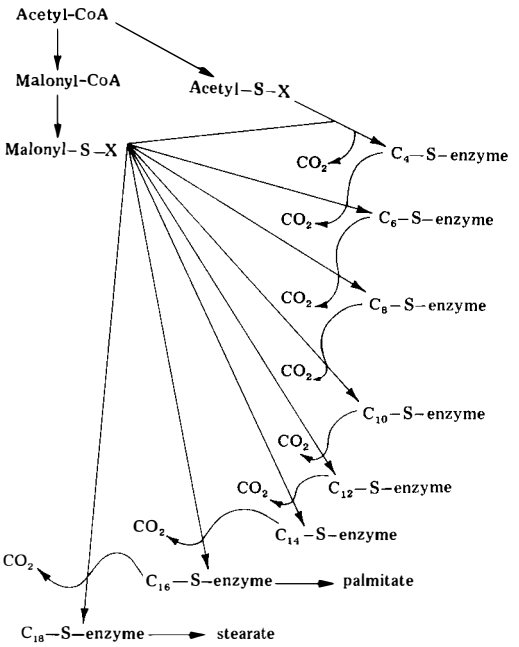
$$CH_3-\overset{\overset{O}{\|}}{C}-S-CoA + CO_2 + ATP \longrightarrow HOOC-CH_2-\overset{\overset{O}{\|}}{C}-S-CoA + ADP + P_i \qquad (3)$$

In step 2, catalyzed by a multienzyme complex, one acetyl-CoA is coupled stepwise with several malonyl-CoA molecules, depending upon the fatty acid formed, with the release of an equivalent number of moles of CO_2. After the addition of each two-carbon unit from malonyl-CoA, there is a reduction step, a dehydration step, followed by a final reduction. This multienzyme complex is called fatty acid synthetase. Studies in bacteria (Vagelos *et al.,* 1966) and in plants (Simoni *et al.,* 1967) have revealed that the substrates participate not as CoA derivatives but as an acyl derivative of 4-phosphopantatheine that is covalently bound to serine of the peptides (Vagelos *et al.,* 1966; Pugh and Wakil, 1965; Majerus, 1967). This peptide is referred to as acyl carrier protein (ACP).

Fatty acid synthetase has been isolated in pure form from pigeon liver (Hsu *et al.,* 1964), rat liver (Burton *et al.,* 1968), and mammary gland of lactating rats (Smith and Abraham, 1969) and cows (Knudsen, 1972; Kinsella *et al.,* 1975). In all cases, it has been impossible to isolate a peptide comparable to ACP of lower forms [designated X in Eq. (4)], but the multienzyme complex does contain 4-phosphopantatheine. Smith (1973) showed that the liver and mammary gland synthetases of rat appear to be identical

proteins, and yet their products are quite different; the gland produces fatty acid of medium chain length. Mammary gland of rats and rabbits, and probably of all species producing milk containing MCT, has a cytosolic thioesterase specific for MCT. This thioesterase limits the growth of the fatty acid carbon chain on the multienzyme complex (Knudsen *et al.*, 1976; Libertini and Smith, 1978).

The complete set of reactions taking place on the multienzyme complex is shown schematically in Eq. (4) (Smith, 1965).



(4)

3. *Cellular Compartmentalization and Fatty Acid Synthesis*

In all mammalian tissues examined, the enzumes of fatty acid synthesis are in the extramitochondrial, soluble portion of the cell, the cytosol. In most species, however, the major source of acetyl-CoA for fatty acid synthesis is the decarboxylation of pyruvate, which takes place within the mitochondria. The question arises: How does the acetyl-CoA reach the site of formation of fatty acids? Several possibilities have been suggested (Srere, 1965; Kornacker and Lowenstein, 1965). Early investigations indicated that, in liver, the acetyl unit was transferred as acetylcarnitine (Bressler and Katz, 1965) whereas, in mammary gland, citrate acted as the acetyl carrier (Bartley *et al.*, 1965). In the latter scheme, citrate cleavage enzyme (citrate lyase) plays a key role in catalyzing the release in the cytosol of the same acetyl unit condensed with oxaloacetate in the mitochondria, the reaction catalyzed by citrate condensing enzyme. In liver, as in mammary gland, citrate is the major carrier for acetyl units out of the mitochondria (Bressler and Brendel, 1966; Watson and Lowenstein, 1970).

4. *Factors Influencing Fatty Acid Synthesis*

It is well known that dietary changes are reflected in the rate of fatty acid synthesis in liver and adipose tissue (Masoro, 1962). All of these are changes that would tend to

maintain homeostasis. Fasting reduces lipogenesis in both liver (Lyon *et al.*, 1952) and adipose tissue (Hausberger and Milstein, 1955), whereas ingestion of large quantities of carbohydrate markedly increases lipogenesis in both tissues (Lyon *et al.*, 1952; Hausberger and Milstein, 1955). The carbohydrate effect in adipose tissue may be at least partly related to the circulating level of insulin. The stimulating effect of insulin on lipogenesis in adipose tissue is well documented (Winegrad *et al.*, 1964). On the other hand, hepatic lipogenic response to carbohydrate feeding requires exposure to the high levels of glucose in portal blood; hepatic autotransplants do not respond lipogenically to high-carbohydrate feeding (Bartley and Abraham, 1972a).

The lipogenic response to ingestion of large quantities of fat is not so clear. There appear to be differences in the response of liver and adipose tissue. Differences are also evident in the response elicited by rats as compared to mice. Hepatic lipogenesis in mice appears to be more sensitive to dietary fat than that in rats (Bartley and Abraham, 1972b). The degree of depression of hepatic lipogenesis is related to the linoleate, or at least to polyunsaturated fatty acid, content of the diet and not to the total lipid content (Allmann and Gibson, 1965; Allmann *et al.*, 1965; Bartley and Abraham, 1972b). The lipogenic response of adipose tissue to fat feeding has not been tested adequately in both species to detect any differences. Long-term feeding of diets high in lipid content appears to depress lipogenesis in adipose tissue. In summary, carbohydrate feeding elicits a greater response in adipose tissue than in liver whereas, in fat feeding, liver responds to a much greater extent. Because of the difference in the quantities of these two tissues, it is difficult to conclude which tissue is most influential in determining the lipogenic capacity of the whole animal. Studies on lipid feeding of mice and rats make it clear that comparisons of the lipogenic responses to high-fat and to linoleate feeding must be made with several species before any general conclusions can be reached.

It is not clear how dietary and hormonal changes influence the enzymatic activity of liver and adipose tissue. Several intracellular mediators of the dietary response have been suggested. Most of these are compounds known to alter the activity of acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis (Vagelos, 1964). Citrate (Vagelos, 1964), ATP, magnesium (Greenspan and Lowenstein, 1967), and palmitylcarnitine (Fritz and Hsu, 1967) have been shown to activate this enzyme. Palmityl-CoA has been shown to inhibit both the carboxylase (Bortz and Lynen, 1963) and the fatty acid synthetase (Porter and Long, 1958). These activities have been used to explain the extent of lipogenesis seen in various nutritional states. Furthermore, the ratio of palmitylcarnitine to palmityl-CoA in the cell has been used to explain lipogenic responses (Fritz and Hsu, 1967). None of these suggested effectors withstands the test of actual tissue measurements at the time of a given dietary condition. The actual measurements of the effectors either do not correlate with the lipogenic responses, or are always at inhibitory levels, or are too low to be effective activators. Cellular compartmentalization may explain some of these discrepancies, but the cellular effectors of lipogenic responses remain unknown.

B. Glyceride Synthesis

Glycerides, phospholipids, and neutral glycerides are rarely taken up by intact tissues. Therefore, the synthesis of these molecules is very important. Phospholipids are essential for cellular membranes. Triacylglycerides are important as sources of stored calories in the body and as the major lipid in milk.

Because neutral glycerides and phospholipids are synthesized, in part, by a common pathway, their synthesis will be described together. The glycerol moiety can be derived from either glycerol-3-P or dihydroxyacetone phosphate. The glycerol-3-P can be formed from glycerol and ATP via the glycerol kinase reaction or derived from glucose during glycolysis. Even in those tissues containing glycerol kinase, glycolysis is probably the major source of glycerol-3-P as an acyl acceptor (Hübscher, 1970).

Phosphatidic acid is the first intermediate on the pathway unique to glyceride synthesis. The acylation of glycerol-3-P takes place in two steps (Kornberg and Pricer, 1953), with the *sn*-1 position being filled first and *sn*-2 filled sequentially to produce phosphatidic acid (Numa and Yamashita, 1974; Tamai and Lands, 1974). There is positional specificity as well: Saturated fatty acids tend to fill the 1 position and unsaturated fatty acids, the 2 position (Van Deenan, 1965).

If dihydroxyacetone phosphate is the acyl acceptor, the acceptor is reduced following the addition of the first fatty acid moiety. The reduction requires NADPH and yields 1-acyl-*sn*-glycerol-3-P. Phosphatidic acid is then formed by a subsequent addition of a fatty acid at *sn*-2, as when glycerol-3-P is the initial acyl acceptor. The quantitative significance of the dihyroxyacetone phosphate pathway is not clear (Van Golde and Van den Bergh, 1977). The fact that it requires NADPH, rather than NADH, means that potentially it can function only in those tissues producing NADPH.

Two paths are open to phosphatidic acid. It can react with CTP to yield cytidine diphosphodiacylglycerol, the precursor of cardiolipin, phosphatidylglycerol, and phosphatidylinositol (Van Golde and Van den Bergh, 1977). The alternate path is dephosphorylation in the reaction catalyzed by phosphatidate phosphatase to yield 1,2-diacyl-*sn*-glycerol (Kates, 1955). Reaction of this diglyceride with cytidine derivatives of choline and ethanolamine results in the formation, respectively, of phosphatidylcholine (lecithin) and phosphatidylethanolamine. Triglycerides, or triacylglycerols, are formed by the addition of a third fatty acid to 1,2-diacyl-*sn*-glycerol. In contrast to positions 1 and 2, acylation of the *sn*-3 position of glycerol is not preferentially filled by a specific class of fatty acid, based either on chain length or degree of saturation (see Van Golde and Van den Bergh, 1977; Smith and Abraham, 1975). This position is filled with the fatty acid most readily available. Hence, in mammary glands making fatty acids of medium chain length, these medium-length fatty acids will be highest in position 3 because positions 1 and 2 are preferentially filled by long-chain fatty acids.

C. Cholesterol Synthesis

Cholesterol can be either absorbed from the intestine or synthesized by most tissues from acetate. Cholesterol is an important precursor of cholesterol ester, bile acids, and steroid hormones. The clinical chemistry of bile acids and steroid hormones will be covered elsewhere (Chapters 6 and 11, respectively). The absorption and movement of cholesterol and cholesterol ester have been covered in Sections V,C and VI,B. The pathway of cholesterol biosynthesis and its control are briefly discussed here.

1. Pathway of Cholesterol Synthesis

Cholesterol can be synthesized by many tissues of the body, but the liver is the primary endogenous site in most animals. Cholesterol is synthesized from acetate in discrete stages. Step 1 involves the stepwise conversion of acetyl-CoA to β-

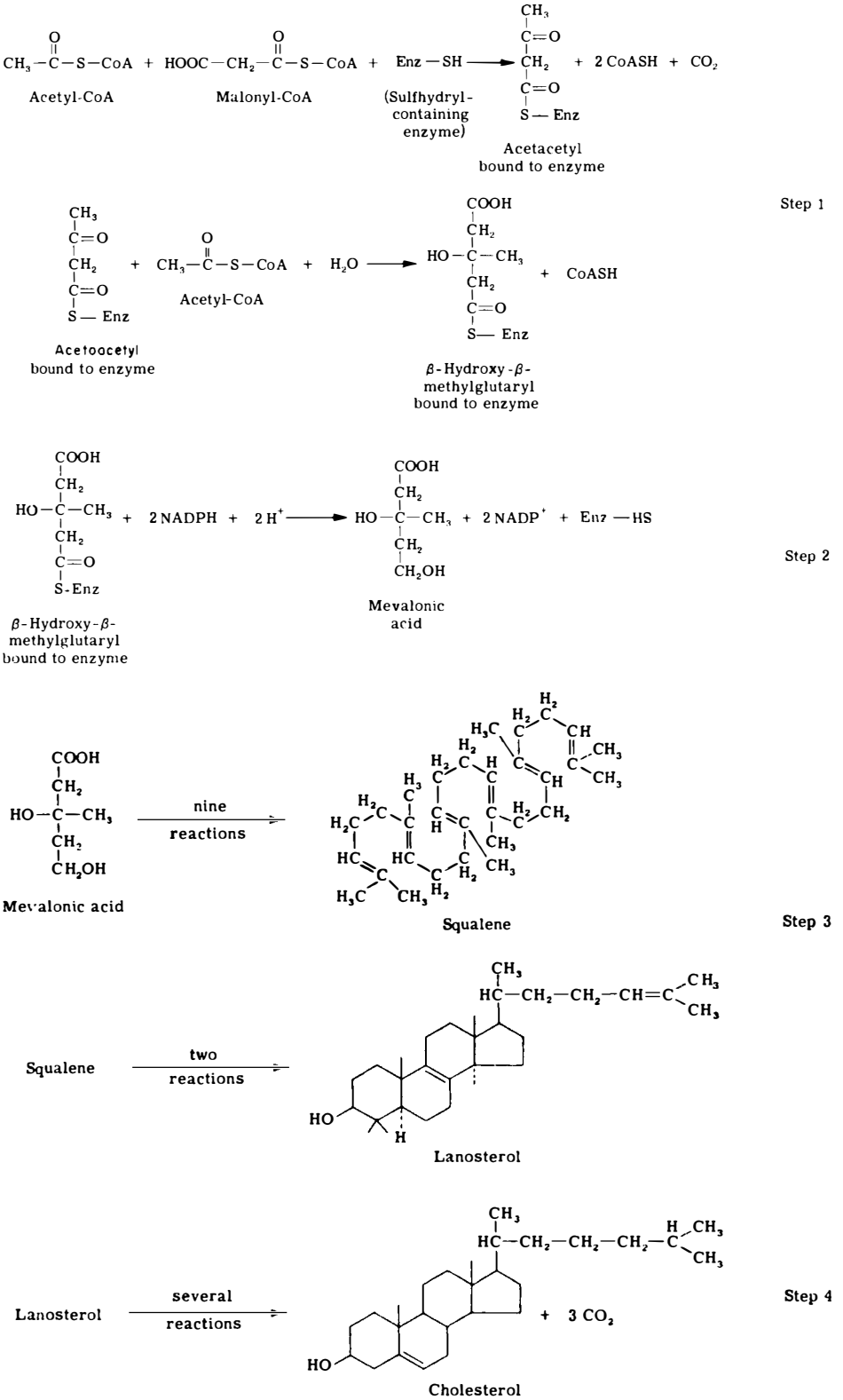


Fig. 5. Reactions involved in the synthesis of cholesterol.

OH-β-methylglutaryl-CoA (HMG-CoA). These reactions can take place either in mitochondria, where they are catalyzed by the same enzymes that form acetoacetate (Rudney, 1957), or extramitochondrially with the reactants bound to an enzyme complex (Brodie *et al.*, 1963). The next step, the conversion of HMG-CoA to mevalonic acid, is the rate-limiting step of cholesterol synthesis and the site of dietary control (Siperstein and Guest, 1959). Next, mevalonic acid is converted to squalene, which subsequently is cyclized to lanosterol. Last, lanosterol is converted to cholesterol. These reactions were reviewed by Bloch (1965) upon receiving the Nobel Prize and are shown schematically in Fig. 5.

2. Regulation of Cholesterol Synthesis

The extent of cholesterol synthesis by the liver is inversely proportional to the cholesterol content of the diet (Siperstein and Guest, 1959). The synthesis of cholesterol in other tissues, however, is not inhibited by high levels of cholesterol in the diet. In animals, e.g., human beings, where synthesis in the liver is not a major source of plasma cholesterol (Wilson and Lindsey, 1965), this feedback control has little effect on the total body cholesterol. In contrast, it may be significant in rats, where the liver is the major site of cholesterol biosynthesis. The importance of feedback control in domestic animals is unknown.

The reduction of HMG-CoA to mevalonate is the enzymatic reaction that responds to dietary control (Siperstein and Guest, 1959; Bucher *et al.*, 1959). This reaction is the first one that is unique to cholesterol synthesis and is virtually irreversible.

3. Synthesis of Cholesterol Esters

Cholesterol can be esterified with fatty acids by enzymes present in liver, adrenal cortex, intestinal mucosa, and even plasma. Most of the cholesterol in plasma, lymph, liver, and adrenal cortex is in the esterified form. Muscle, on the other hand, contains free cholesterol and almost no cholesterol esters. Hydrolysis of the cholesterol esters to free cholesterol and FFA takes place, not only in the intestinal lumen (Section IV,A), but in the liver and the adrenal gland.

The significance of whether cholesterol is in the free or esterified form in a tissue is not clear. It may be related to the structural characteristics of the membranes of a particular tissue.

An enzyme system, lecithin–cholesterol acyltransferase, has been described in plasma of human beings that transesterifies the fatty acid of lecithin to cholesterol, resulting in the formation of lysolecithin and cholesterol ester (Glomset, 1968). The reaction is slow, but significant amounts of cholesterol ester are formed in this manner. A lethal familial disease has been described in which LCAT is absent (Norum and Gjone, 1967). These patients have other plasma protein deficiencies which appear attributable to the absence of LCAT (Scanu, 1978).

VIII. OXIDATION OF FATTY ACIDS

The spiral path of long-chain fatty acids to acetyl-CoA during oxidation within the mitochondria is well known. A familiar outline is reproduced in Fig. 6. Perhaps less familiar is the form in which the fatty acid enters the mitochondria, a reaction shown to be

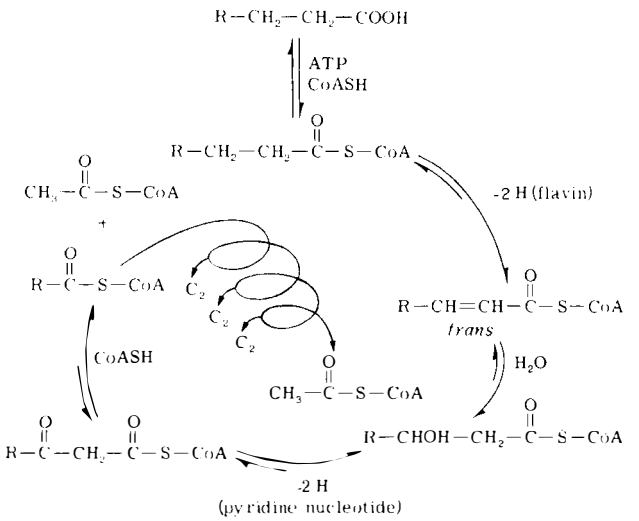


Fig. 6. Schematic presentation of β-oxidation of long-chain fatty acids to acetyl-CoA. (After Conn and Stumpf, 1966.)

the rate-limiting step in fatty acid oxidation (Fritz, 1968). The acyl-CoA derivative in the cytosol can arise from activation of FFA entering the cell (Section VI,C), from triglycerides and phospholipids being degraded within the cell, or from *de novo* synthesis in the case of liver and adipose tissue. In order for the fatty acid to enter the site of the oxidative spiral, it must first be converted to the acylcarnitine derivative (Fritz and Yue, 1963). The enzyme catalyzing this reaction appears to be a part of the inner mitochondrial membrane (Norum and Bremer, 1967). The activity of the palmityl-CoA–carnitine transferase correlates with the rate of fatty acid oxidation, both in a variety of tissues and in a variety of nutritional states (Fritz, 1968). The intracellular effectors of this key enzyme are unknown.

The activity of the palmityl-CoA–carnitine transferase has been evoked as a control point not only for fatty acid oxidation but for gluconeogenesis and ketogenesis (Fritz, 1968). Two acetyl-CoA pools within the mitochondria have been suggested: one derived from pyruvate, the other from long-chain fatty acids. Furthermore, the acetyl-CoA from fatty acids is produced in the same compartment containing pyruvate carboxylase and, hence, would have greater access to exert its stimulatory action on this enzyme than would acetyl-CoA from pyruvate (Utter and Keech, 1963). Thus, when FFA are readily available to the liver, as in fasting, the subsequent increase in acetyl-CoA derived from palmitylcarnitine could stimulate carboxylation of pyruvate to oxaloacetic acid, the reaction catalyzed by pyruvate carboxylase (Fritz, 1968), a rate-limiting step in gluconeogenesis (Krebs, 1964). Ketogenesis would result from the excessive production of acetyl-CoA in a situation in which the availability of oxaloacetic acid is limited (Krebs, 1966a,b) or from saturation of the citrate–oxaloacetate condensing reaction with acetyl-CoA derived from FFA (Fritz, 1968). The acetyl-CoA–carnitine transferase, suggested previously as a means of transferring acetyl units out of the mitochondria (Section VII,A) (Bressler and Katz, 1965), would act according to this hypothesis as an intramitochondrial shuttle for the two acetyl-CoA pools (Fritz, 1968).

IX. SPECIAL ASPECTS OF LIPID METABOLISM IN DOMESTIC ANIMALS

A. Lipid Metabolism in Ruminants

Five aspects of ruminant lipid metabolism are unique: (1) digestion and absorption, (2) lipoprotein complement and lipid transport, (3) transfer of lipid into milk, (4) unique features of lipogenesis, and (5) the use of the concentration of FFA in the plasma as a metabolic indicator.

1. Digestion and Absorption

The small intestine of the ruminant receives mainly saturated, free fatty acids rather than triglyceride because of the extensive lipolysis and hydrogenation of dietary lipid by the microorganisms in the rumen (Garton, 1960; Ward *et al.*, 1964). The ruminant normally receives a diet low in lipid, but it is becoming common to feed vegetable oil and animal tallow. These fats, when protected from microbial action in the rumen, can be incorporated in the diet up to 15% by weight (Pan *et al.*, 1972; Scott *et al.*, 1971; Sharma *et al.*, 1978). Therefore, the early studies by Leat and his co-workers (Leat and Hall, 1968; Leat and Cunningham, 1968; Leat and Harrison, 1967) are of more than comparative interest. If the ruminant intestine is exposed to triglyceride, is it capable of handling it?

Leat and Cunningham (1968) posed the question: If the small intestine of the ruminant naturally receives mostly free fatty acids, does the monoglyceride pathway operate? In studies with isolated segments of intestine from lambs and sheep, it was shown that, as in monogastric animals, the major pathway of triglyceride absorption is via the monoglyceride path (Leat and Harrison, 1967).

Studies of the lipid composition of plasma and lymph of cows indicate that the fate of lipid absorbed from the intestine and the composition of the chylomicrons are similar to those in monogastric animals (Leat and Hall, 1968). Hansen (1965a) showed that isolated intestinal mucosa of lambs readily incorporates fatty acids into triglyceride and, to a small extent, cholesterol ester. In contrast, his studies with rats revealed incorporation of fatty acids into both triglyceride and cholesterol ester. Differences between the two species were also noted in the fatty acids most readily esterified (Hansen, 1965a) and the position of glycerol preferentially esterified with a given fatty acid (Hansen, 1965b,c).

Although dietary fat can be digested and utilized in ruminant intestine, these diets result in depressed ruminal function. High amounts of dietary fat reduced the digestibility of other dietary components and depressed appetite (Brethour *et al.*, 1958; Brooks *et al.*, 1954; Dijkstra, 1969). These difficulties have been overcome by the development of fats that have been treated to minimize microbial digestion in the rumen. Fats so treated are commonly referred to as "protected" lipids (Pan *et al.*, 1972; Scott *et al.*, 1971). Studies utilizing protected lipids in the ration of lactating cows indicate that protected-fat diets increase milk fat content with little effect on other milk components (Bitman *et al.*, 1973; Plowman *et al.*, 1972; Mattos and Palmquist, 1974; Sharma *et al.*, 1978; Storry *et al.*, 1974). Because of the ruminant's dependence upon propionic acid for gluconeogenesis, one potentially detrimental effect is the reduced levels of this volatile fatty acid in the rumens of cows fed 15% protected tallow rations (Sharma *et al.*, 1978). As might be expected (Section IX,A), the fatty acid composition of the milk of cows fed protected tallow was modified to reflect the composition of dietary lipid (Sharma *et al.*, 1978).

The lactating cow is unique in its ability to maintain a high concentration of cholesterol in plasma with no detrimental effect (Maynard *et al.*, 1931). When protected tallow is fed, this already high level of blood cholesterol doubles (Sharma *et al.*, 1978). Whether a hypercholesterolemia exceeding 500 mg/100 ml can be tolerated over an extended period with no pathological consequences is unknown. The uniquely high levels of HDL, the cholesterol-carrying lipoprotein (Section VI,B), in the ruminant may help to protect this species from any detrimental effects of hypercholesterolemia (Puppione, 1978).

The major lipid-related material absorbed by the ruminant is the volatile fatty acid which enters the portal system. Of the major VFA, propionate and butyrate are utilized almost completely in the liver, but a large proportion of the acetate may pass on to the peripheral circulation for utilization by all tissues (Annison *et al.*, 1957). A major portion of the propionate is utilized for gluconeogenesis (Annison *et al.*, 1963). It now seems clear that butyrate does not contribute carbon directly to glucose production but may increase glycogenolysis in the liver of lambs (Phillips and Black, 1965), presumably by activation of phosphorylase (Phillips *et al.*, 1965). In other ruminants, the sole effect of butyrate on hepatic glucose output is via increased glyconeogenesis (Anand, 1967). Such an effect could be mediated by an increase in the level of acetyl-CoA derived from butyrate. It has been shown that acetyl-CoA activates pyruvate carboxylase (Utter and Keech, 1963), a key enzyme in gluconeogenesis (Krebs, 1964).

2. *Lipoprotein Complement and Lipid Transport*

As mentioned, cows normally develop hypercholesterolemia during lactation (Maynard *et al.*, 1931). This hypercholesterolemia is accompanied by an increase in the α-lipoproteins (Puppione *et al.*, 1972; Raphael *et al.*, 1973a,b). These α-lipoproteins contain both HDL and LDL, the latter accounting for about 50% of the increase in serum lipids occurring during lactation. Puppione (1978) suggested that the fraction with a bouyant density of LDL is HDL with a higher content of core lipid.

Puppione (1978) suggested three possible explanations for the hyper-α-lipoproteinemia of lactating cows: (1) adaptation to lactation by increasing the apo C reservoir, (2) increased VLDL utilization by mammary gland, and (3) increased nascent HDL synthesis and secretion by the liver in response to lactation. An increase in the level of apo C would result in increased availability of lipoprotein lipase activator (Section VI,B) and consequently enhance uptake of triglycerides by mammary gland. In the second and third explanations, a major role for LCAT is suggested. In one case, the utilization of core glyceride of VLDL by mammary gland will make cholesterol and lecithin from the surface available to LCAT, the action of which would produce core cholesterol ester for HDL. Puppione (1978) suggested that, if VLDL catabolism and transesterification were coupled, more cholesterol ester could be packaged into a single lipoprotein, yielding some α-migrating LDL, rather than into many smaller lipoproteins of greater density, HDL. The role of LCAT in the third case is the same as in the second except that, instead of loading circulating lipoprotein with cholesterol, the recipient of this core lipid is newly formed HDL. Isolation of pure subfractions of lipoproteins from the lactating cow and identification of the apoproteins moieties will help to resolve these possibilities.

The identity, fate, and relationship among remnant particles, IDL, and LDL is not clear (Section VI,B). If the LDL in the α-migrating lipoproteins is a catabolic product of an active mammary gland, then the cow provides an ideal subject for study. First, the normal exaggeration of this aspect of lipoprotein metabolism provides a model for investigating

the formation and fate of this particle. Second, it may be possible to isolate the HDL and LDL components separately and uncontaminated by other lipoproteins (D. L. Puppione, personal communication). These fractions would provide reagents to test in model systems, such as perfused liver and cells in culture, as well as sources for determining the apoprotein complement precisely.

If either the second or third mechanism is substantiated, it could have practical significance. In the second mechanism the degree of hyper-α-lipoproteinemia would be an indication of the capacity of mammary gland to form milk fat. The level of α-lipoprotein in the third scheme would be a gauge of the capacity of liver to produce lipoproteins essential to the utilization of plasma triglyceride for milk fat synthesis. Thus, lipoproteins in lactating cows might be a guide for predicting their milk-producing capabilities (Puppione, 1978).

3. Transfer of Plasma Lipid into Milk

A portion of the fatty acids in milk is synthesized in the mammary gland, but another portion, especially long-chain components, is derived from the blood (Folley and McNaught, 1961). On the basis of the arteriovenous differences across the mammary gland of lactating ruminants, it was determined that the fatty acids of triglycerides in chylomicrons and VLDL contribute essentially all of the fatty acids of milk derived from plasma (Barry *et al.*, 1963; Glascock *et al.*, 1966; West *et al.*, 1972; Gooden and Lascelles, 1973; Glascock and Welch, 1974). The plasma concentration of FFA was unchanged across the gland. Earlier work in both the lactating cow (Graham *et al.*, 1936) and the goat (Lintzel, 1934) had indicated that the mammary gland utilizes neutral lipids from the plasma. Studies with labeled fatty acids administered in the diet (Glascock *et al.*, 1956) or as isolated chylomicrons (Lascelles *et al.*, 1964) verified that plasma lipid is a precursor of milk lipid. Plasma lipids contribute 35–75% of the fatty acid in milk depending upon the nutritional state of the animal (Riis, 1964; Barry, 1966; Glascock *et al.*, 1966; Palmquist and Mattos, 1978). Palmquist and Mattos (1978) estimated from studies with labeled linoleate infused postruminally that 44% of the fatty acid in milk fat may be of dietary origin, an observation of great significance with the advent of feeding protected fat to ruminants. The preciseness of their estimate may be in doubt because linoleate is the favored substrate for bovine LCAT (Noble *et al.*, 1972). Hence, their tracer may not be truly representative of triglyceride fatty acid. Any difference, however, is likely to be of minor quantitative significance and would not alter the qualitative significance of the observation. As would be expected with such an efficient transfer of dietary lipid to milk fat, the fatty acid composition of milk fat will reflect that of the protected fat in the diet (Dunkley *et al.*, 1977; Macleod *et al.*, 1977; Sharma *et al.*, 1978; Storry *et al.*, 1974).

Annison and co-workers in both the intact lactating goat (Annison *et al.*, 1967) and the perfused caprine mammary gland (Linzell *et al.*, 1967) studied the utilization of labeled acetate, stearate, oleate, and β-OH-butyrate. They also measured the arteriovenous differences across the gland of FFA, neutral glycerides, and phospholipids. Free fatty acids are taken up by the gland, but a similar quantity is released so that the difference across the gland is unchanged. The FFA are incorporated into milk fat and are not extensively oxidized. These studies verified earlier investigations showing that (1) the plasma phospholipids contribute little to milk fat, (2) the fatty acids of milk with chain length of C_4 to C_{14} arise almost solely from acetate. Sixteen-carbon fatty acid is derived both from acetate and from plasma triglyceride, which is almost the sole precursor of oleate and stearate.

Studies with β-OH-butyrate indicated that some of this ketone body was incorporated into milk lipid as a C_4 unit. Similarly, labeled butyrate is incorporated into milk fat of lactating cows (Bines and Brown, 1968). There is evidence that the ruminant mammary gland forms large quantities of oleate from plasma stearate (Lauryssens *et al.*, 1961; West *et al.*, 1967; Annison *et al.*, 1967).

McClymont and Vallance (1962) suggested that the depression in milk fat concentration observed after administration of glucose to cows (Vallance and McClymont, 1959) is due to the decrease in lipid uptake by the gland under these conditions. Whether the depression in milk fat production brought about by feeding high-grain diets can be attributed to the changes in the concentration of circulating triglycerides (Storry and Rook, 1965) appears to be open to question. Varman and Shultz (1968) attribute the depression to changes in the availability of propionate and acetate from the rumen and to enzymatic changes in adipose tissue and mammary gland as observed by Opstvedt *et al.* (1967).

Palmquist (1976) reviewed the relationship among intestinal absorption of lipid, plasma lipid levels, and milk fat production in lactating cows. Although a correlation between the first two was strong, no clear relationship could be established between milk fat production and either one of the other two measurements. Continuous intravenous infusion of triglyceride for 48 hours increased the concentration of milk fat (Storry and Rook, 1964). Studies in which protected lipid was fed also support the concept of a relationship between the level of dietary and circulating lipid and the production of milk fat (Bitman *et al.*, 1973; Mattos and Palmquist, 1974; Plowman *et al.*, 1972; Dunkley *et al.*, 1977; Macleod *et al.*, 1977; Storry *et al.*, 1974; Sharma *et al.*, 1978). Palmquist and Mattos (1978) demonstrated that 44% of milk fat can be of direct dietary origin and implies that increased availability of dietary fat could result in increased milk fat synthesis. When protected fat is fed to lactating cows, a major limiting factor in the conversion of this lipid to milk triglyceride may be the capacity of the gland for triglyceride synthesis and secretion.

4. Lipogenesis

Early tracer studies by Kleiber's group (Kleiber *et al.*, 1952) revealed that glucose carbon is not incorporated into milk fat to any appreciable extent. The ready availability of acetate and butyrate as precursors of fatty acids sufficed to explain this finding. As the role of citrate cleavage enzyme in fatty acid synthesis became clear (Section VII,A), it was suggested that glucose carbon failed as a precursor of fatty acid due to low citrate cleavage enzyme activity in ruminant tissues (Hardwick, 1966). Direct enzymatic assays plus the extremely low incorporation of C-3 of aspartate into fatty acid verified the minimal operation of the citrate cleavage pathway (Hanson and Ballard, 1967) in cows.

The liver of the fetal ruminant contains citrate cleavage activity comparable to that of fetal rats, and isotope studies verified the operation of the citrate cleavage pathway (Hanson and Ballard, 1968). The activity of citrate cleavage enzyme is known to be very sensitive to the dietary and hormonal changes (Leveille and Hanson, 1966; Srere, 1965). Considering the ruminant as an animal on a high-fat, low-carbohydrate diet, it is not surprising that citrate cleavage activity is low when the rumen is functional. Malic enzyme, another adaptive enzyme related to lipogenesis, is also low in ruminants (Hanson and Ballard, 1968) as it is in monogastrics being fed high-fat, low-carbohydrate diets (Leveille and Hanson, 1966).

Conversely, when 50% glucose was infused into nonlactating cows continuously for 4 hours, enzyme assays of liver biopsies taken before and after the infusion revealed that the

activity of both citrate cleavage enzyme and malic enzyme increased 1.5-fold during the infusion (J. C. Bartley, unpublished observations). Longer-term carbohydrate loading studies will be required before the low activites of citrate cleavage and malic enzymes can be attributed solely to an adaptive response to the nutrients absorbed from the alimentary tract.

What is the clinical significance of the low activity of these two enzymes related to lipogenesis? Under normal conditions the enzymatic profile of ruminant liver, adipose tissue, and mammary gland would favor utilization of acetate for fatty acid synthesis (Ballard *et al.*, 1969) and thereby spare glucose for its indispensible roles, i.e., the central nervous system (McClymont and Setchell, 1956), lactose production (Kronfeld *et al.*, 1963), and support of the fetus (Kronfeld, 1958). Gluconeogenesis, mainly from propionate (Leng *et al.*, 1967), is essential for survival of the ruminant. The magnitude of the hepatic glucose formation is difficult to measure, but Bartley and Black (1966) and Ballard *et al.* (1969) suggested that in the ruminant the glucose entry must approximate the rate of gluconeogenesis due to the paucity of glucose from the gut. Hence, in the ruminant, most of the available oxaloacetic acid (OAA) would be channeled toward glucogenesis and very little toward lipogenesis. The enzymatic profile of ruminant liver and the incorporation of specific carbons into fatty acids by ruminant liver supports such a concept (Ballard *et al.*, 1969).

The ruminant, then, is more dependent upon gluconeogenesis than the monogastric animal, and the alteration of the availability of OAA by any factor has grave consequences. The availability of OAA could be decreased by increased demand for gluconeogenesis or by decreased supply. Krebs (1966a,b) suggested that ketosis is due to increased gluconeogenesis, when the utilization of OAA for glucose production by the liver is so extensive that there is not enough for condensation with acetyl-CoA in the tricarboxylic acid cycle. Baird *et al.* (1968) tested this hypothesis in ruminants and found no enzymatic evidence that would indicate an increase in gluconeogenesis in the ketotic ruminant. Nonetheless, they did find a decrease in the tissue level of OAA and its immediate metabolic derivatives in the liver of the same animals. This would mean that the rate of gluconeogenesis is at maximal capacity in ruminants, particularly those in pregnancy or lactation. Any factor which reduces the availability of OAA will precipitate hypoglycemia and ketonemia. Treatment of this syndrome, then, should be designed to replenish the tissue level of OAA without altering the rate of gluconeogenesis. The glucogenic amino acids, glycerol, and propionate are examples of compounds fulfilling these requirements.

The ketone bodies derived from the inability of the liver to oxidize acetyl-CoA at a rate commensurate with its formation can be utilized as sources of energy by other body tissues where the supply of OAA is not siphoned off by gluconeogensis (Williamson and Krebs, 1961). In ruminant ketosis, the utilization of ketone bodies equals or exceeds that in the normal animal up to certain limits (Bergman and Kon, 1964; Leng, 1965). As in nutritionally stressed monogastric animals, the major precursor of ketone bodies in the ruminant is FFA (Fig. 4), but formation of β-OH-butyrate in the ruminal epithelium contributes a portion, as it does in the normal ruminant.

5. *Significance of Plasma FFA in the Ruminant*

Kronfeld (1965) concluded that the response of plasma FFA in the cow resembles those in other species. Therefore, the plasma FFA level is a sensitive clinical index of fat

mobilization (Section VI,C). Kronfeld (1965) minimized the value of the plasma FFA level as such an index in bovine ketosis because cases of uncomplicated ketosis did not always have elevated plasma FFA levels. His studies of spontaneous bovine ketosis verified those of Adler *et al.* (1963). In contrast, measurements in pregnant sheep indicated that the level of FFA correlated well with the severity of ketonemia (Reid and Hinks, 1962; Bergman *et al.*, 1968). Although the discrepancy may be due to species differences, Radloff and Schultz (1967) found that, in the early stages of bovine ketosis, the FFA are indeed markedly increased.

The absolute levels of FFA observed by various workers in normal and ketotic cows, regardless of cause, do not agree, but the degree of increase does (see Kronfeld, 1965; Adler *et al.*, 1963). The differences in the absolute levels may be due to differences in the methods used to extract and determine the FFA content of plasma. Using the same method as Kronfeld [the Dole method, as modified by Trout *et al.* (1960)], we have found levels similar to those reported by him and concur that levels above 650 μEq/liter are an indication of unusually high mobilization of fatty acids, derived from the triglycerides of adipose tissue.

Russel and associates have suggested the use of the plasma concentration of FFA instead of body weight changes as an index to estimate maintenance requirements (Russel *et al.*, 1967; Doney and Russel, 1969). The rationale of such an index is that the food intake would be controlled so that the FFA of plasma would be stabilized at a level indicating neither high mobilization nor storage of lipid. Russel and Doney (1969) critically examined the use of FFA as a maintenance index and concluded that a relationship exists, but, as one might expect, it is not a simple one and breed differences exist between FFA levels reflecting an adequately maintained animal. Eventual application of such an index might aid in the prevention of ovine pregnancy toxemia and bovine ketosis.

B. Equine Lipemia

Lipemia in the equine has been observed accompanying maxillary myositis (Hadlow, 1962) and equine infectious anemia (Gainer *et al.*, 1966). When several ponies admitted to the Veterinary Medicine Teaching Hospital at the University of California exhibited lipemia, the question arose: Is the lipemia directly related to a specific equine disease or a general response to a stressor, such as inanition? To test the latter possibility, three ponies and three burros (and, later, horses) were fasted for up to 18 days (Bartley, 1970). Mature, nonlactating goats were included in the study for comparative purposes.

Fasting resulted in a marked increase in plasma triglycerides with lesser increases in total cholesterol and FFA in the equines, particularly ponies and burros. No hypertriglyceridemia was observed in the goats. Schotman and Wagenaar (1969) reported on a naturally occurring hypertriglyceridemia in various breeds of European ponies. The original observation was made 4 years earlier (Schotman, 1965), but unfortunately the contents of this thesis were not readily available at that time. Eriksen and Simesen (1970) confirmed these observations in pregnant ponies and pony mares that had just foaled. They (Erkisen and Simesen, 1970) attributed the initiation of the syndrome to inanition. This metabolic disorder is particularly prevalent and most serious in pregnant animals (Schotman and Wensing, 1977). It is accompanied by fatty infiltration of the liver, heart, and kidneys.

Since FFA are increased (Bartley, 1970; Baetz and Pearson, 1972; Wensing *et al.*,

1973a), we (Bartley, 1970) proposed that a metabolic stressor triggers mobilization of lipid from adipose tissue. Presumably because of a great capacity in liver for forming these mobilized FFA into triglyceride and their subsequent release as lipoprotein, the ponies develop lipemia, "fatty plasma," in addition to fatty liver. Other workers conceived the development of the syndrome in similar terms (Schotman and Wagenaar, 1969; Schotman and Wensing, 1977). Conceptually, the circulating lipid would be in the form of VLDL, and this has been confirmed (Morris *et al.*, 1972; Wensing *et al.*, 1973a). This is also consistent with the observations that lipemia is more serious in obese ponies (Eriksen and Simesen, 1970). In the one reported incidence of the natural occurrence of this disorder in which all treated animals survived, the ponies were not overweight (Breukink *et al.*, 1972).

Morris *et al.* (1972) suggested that the utilization of VLDL is impaired in this disorder. The reduction of the triglyceridemia by heparin infusions is compatible with this concept (Schotman and Wensing, 1977). Success has also been reported with therapy designed to reduce fat mobilization: infusion of insulin with oral glucose (Wensing *et al.*, 1973b). Therapy and prevention should be based on the rationale that this disorder is the result of any factor, particularly fasting, that results in mobilization of lipid stores more rapidly than the resulting VLDL can be utilized. Insulin and heparin therapies fit this category. Prevention obviously would involve avoiding factors resulting in fasting, such as transport and poor diet. The problem is particularly acute when the mobilization of fat is enhanced by obesity and when other metabolic stresses are present, such as pregnancy, lactation, extreme parasitism, or cold.

Another implication of the extreme capacity of the equine liver to form VLDL from mobilized lipid is that the lipoproteins in this species should be used to guage fat mobilization and the response to metabolic stress, as FFA are used in other species (Section VI,A).

Wensing *et al.* (1973a) examined the lipoprotein fractions of lipemic ponies ultracentrifugally and categorized the incidence in the Fredrickson system (Fredrickson *et al.*, 1967) (Section VI,B). One group of ponies coincided with Type IV, another with Type V hyperlipemia, but a third group was distinct from any class observed in human beings. Although the significance of this observation will have to await further study, it is clear that, as with ruminants, a wealth of untapped information is available from lipoprotein studies with domestic animals. Such investigations have a potential for increasing our understanding of normal lipoprotein metabolism and interactions as well as clarifying the nature of the metabolic lesions in abnormalities.

C. Canine Hyperlipemia

Lipemias in dogs are of relatively frequent occurrence but have only recently been studied. Baum *et al.* (1969) described a hyperlipemia in a puppy suspected of being deficient in lipoprotein lipase. Rogers *et al.* (1975a) described an abnormal lipoprotein pattern in miniature schnauzers with hyperlipoproteinemia which they suggested was a syndrome caused by an inherited defect of lipid metabolism. Mahley *et al.* (1974) characterized the lipoproteins of dogs in association with atherogenic and nonatherogenic hyperlipoproteinemia. Their hyperresponder class, which developed atherosclerosis, had cholesterol levels of 750 mg/dl, broad LDL (β) and VLDL (pre-β) bands, and a lowered HDL_2 level (α_1). Manning *et al.* (1973) described a familial hyperlipoproteinemia and

thyroid dysfunction in beagles. Rogers *et al.* (1975b) studied lipoproteins in dogs with hypothyroidism, diabetes mellitus, and acute pancreatitis. In hypothyroidism, lipoprotein values varied but the hypercholesterolemias were associated with increased HDL_1 (α_2) levels. Diabetic dogs also had hypercholesterolemia in association with increased HDL_1 (α_2) and LDL (β) levels. In acute pancreatitis, moderate increases in cholesterol and triglyceride were observed.

Hyperlipemias may also be secondary to other conditions, such as hepatitis, nephrotic syndrome, hypoalbuminemias, and starvation. Thus, lipoprotein profiles may be of great help in evaluating the hyperlipemias of either primary or secondary nature.

REFERENCES

Abraham, S., Matthes, K. J., and Chaikoff, I. L. (1961). *Biochim. Biophys. Acta* **49**, 268.

Adler, J. H., Wertheimer, E., Batana, U., and Flesh, J. (1963). *Vet. Rec.* **75**, 304.

Ailhaud, G., Sarda, L., and Desnuelle, P. (1962). *Biochim. Biophys. Acta* **59**, 261.

Alaupovic, P., Kostner, G., Lee, D. M., McConathy, W. J., and Magnani, H. (1972). *Expo. Annu. Biochim. Med.* **31**, 145.

Allmann, D. W., and Gibson, E. M. (1965). *J. Lipid Res.* **6**, 51.

Allmann, D. W., Hubbard, D. D., and Gibson, D. M. (1965). *J. Lipid Res.* **6**, 63.

Anand, R. (1967). Ph.D. Thesis, Univ. of California at Davis.

Annegers, J. H. (1954). *AMA Arch. Intern. Med.* **93**, 9.

Annison, E. F. (1960. *Aust. J. Agric. Res.* **11**, 58.

Annison, E. F., Hill, K. J., and Lewis, D. (1957). *Biochem. J.* **66**, 592.

Annison, E. F., Leng, R. A., Lindsay, D. B., and White, R. R. (1963). *Biochem. J.* **88**, 248.

Annison, E. F., Linzell, J. L., Fazakerley, S., and Nichols, B. W. (1967). *Biochem. J.* **102**, 637.

Baetz, A. L., and Pearson, J. E. (1972). *Am. J. Vet. Res.* **33**, 1941.

Bagdale, J. P., Porte, D., and Bierman, E. L. (1967). *N. Engl. J. Med.* **276**, 427.

Bailey, J. M. (1965). *Exp. Cell Res.* **37**, 175.

Baird, C. D., Hibbitt, K. G., Hunter, G. D., Lund, P., Stubbs, M., and Krebs, H. A. (1968). *Biochem. J.* **106**, 683.

Baker, H. J., Jr., Lindsey, J. R., McKhann, G. M., and Farrell, D. F. (1971). *Science* **174**, 838.

Baker, N., and Schotz, M. C. (1964). *J. Lipid Res.* **5**, 188.

Ballard, F. J., Hanson, R. W.,and Kronfeld, D. W. (1969). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **28**, 218.

Barry, J. M. (1966). *Outlook Agric.* **5**, 129.

Barry, J. M., Bartley, W., Linzell, J. L., and Robinson, D. S. (1963). *Biochem. J.* **89**, 6.

Bartley, J. C. (1970). *In* "Clinical Biochemistry of Domestic Animals" (J. J. Kaneko and C. E. Cornelius, eds.), 2nd ed., Vol. 1, pp. 88–92. Academic Press, New York.

Bartley, J. C., and Abraham, S. (1972a). *Biochim. Biophys. Acta* **260**, 169.

Bartley, J. C., and Abraham, S. (1972b). *Biochim. Biophys. Acta* **280**, 258.

Bartley, J. C., and Black, A. L. (1966). *J. Nutr.* **89**, 317.

Bartley, J. C., Abraham, S., and Chaikoff, I. L. (1965). *Biochem. Biophys. Res. Commun.* **19**, 770.

Bassen, F. A., and Kornzweig, A. L. (1950). *Blood* **5**, 831.

Baum, D. Schweid, A. I., Parde, D., and Bierman, E. L. (1969). *Proc. Soc. Exp. Biol. Med.* **131**, 183.

Belfrage, P., Elovson, J., and Olivecrona, T. (1965). *Biochim. Biophys. Acta* **106**, 45.

Benzonana, G., and Desnuelle, P. (1965). *Biochim. Biophys. Acta* **105**, 121.

Bergman, E. N., and Kon, K. (1964). *Am. J. Physiol.* **206**, 449.

Bergman, E. N., Starr, D. J., and Reulein, S. S.)1968). *Am. J. Physiol.* **215**, 874.

Berström, S., and Borgström, B. (1955). *Prog. Chem. Fats Lipids* **3**, 351.

Bergström, S., and Samuelson, B. (1965). *Ann. Rev. Biochem.* **34**, 101.

Biale, Y., Gorin, E., and Shafrir, E. (1965). *Isr. J. Chem.* **3**, 112.

Bines, J. A., and Brown, R. E. (1968). *J. Dairy Sci.* **51**, 698.

Bitman, J., Dryden, L. P., Goering, H. K., Wrenn, T. R., Yoncoskie, R. A., and Edmundson, L. F. (1973). *J. Am. Oil Chem. Soc.* **50**, 93.

Bloch, K. (1965). *Science* **150**, 19.

Bortz, W. M. (1967). *Biochim. Biophys. Acta* **135**, 533.
Bortz, W. M., and Lynen, F. (1963). *Biochem. Z.* **337**, 505.
Brady, R. O. (1969). *Med. Clin. North Am.* **53**, 827.
Brady, R. O. (1970). *Clin. Chem.* **16**, 811.
Brady, R. V., and Koval, G. J. (1958). *J. Biol. Chem.* **233**, 26.
Bressler, R., and Brendel, K. (1966). *J. Biol. Chem.* **241**, 4092.
Bressler, R., and Katz, I. B. (1965). *J. Biol. Chem.* **240**, 622.
Brethour, J. R., Sirny, R. J., and Tillman, A. D. (1958). *J. Anim. Sci.* **17**, 171.
Breukink, H. J., Wensing, T., and Blok, W. M. (1972). *Tijdschr. Diergeneeskd.* **97**, 1399.
Brindley, D. N., and Hübscher, G. (1966). *Biochim. Biophys. Acta* **125**, 92.
Brodie, J. D., Wasson, G., and Porter, J. W. (1963). *J. Biol. Chem.* **238**, 1294.
Brooks, C. C., Garner, G. B., Gehrke, C. W., and Muhrer, M. E. (1954). *J. Anim. Sci.* **13**, 758.
Bucher, N. L. R., McGarrahan, K., Gould, K., and Loud, A. V. (1959). *J. Biol. Chem.* **234,** 262.
Burton, D., Haavik, A. G., and Porter, J. W. (1968). *Arch. Biochem. Biophys.* **126,** 141.
Butcher, R. W., Sneyd, J. G. T., Park, C. R., and Sutherland, E. (1966). *J. Biol. Chem.* **241**, 1651.
Cahill, G. F. (1964). *In* "Fat as a Tissue" (K. Rodahl and B. Issekutz, eds.), pp. 169–183. McGraw-Hill, New York.
Chevreul, M. E. (1823). "Recherches chimique sur le corps gras d'origine animale." Levrant, Paris. Cited in Lawrie (1928, p. 17).
Chrisp, C. E., Ringler, D. H., Abrams, G. D., Radin, N. S., and Brenkert, A. (1970). *J. Am. Vet. Med. Assoc.* **156**, 616.
Clark, B., and Hübscher, G. (1961). *Biochim. Biophys. Acta* **46**, 479.
Clements, J. A. (1971). *Arch. Intern. Med.* **127**, 387.
Clements, J. A., Nellenbogen, J., and Trahan, H. J. (1970). *Science* **169**, 603.
Coleman, R., and Hübscher, G. (1962). *Biochim. Biophys. Acta* **56**, 479.
Conn, E. F., and Stumpf, P. K. (1966). "Outlines of Biochemistry," 2nd ed., p. 280. Wiley, New York.
Correll, J. W. (1963). *Science* **140**, 387.
Crass, M. F., and Meng, H. C. (1966). *Biochim. Biophys. Acta* **125**, 106.
Currey, M. D., McConathy, W. J., Alaupovic, P., Ledford, J. H., and Popovic, M. (1976). *Biochim. Biophys. Acta* **439**, 413.
David, J. S. K., Malathi, P., and Ganguly, J. (1966). *Biochem. J.* **98**, 662.
Dawson, A. M. (1967). *Br. Med. Bull.* **23**, 247.
Delcher, H. K., Fried, M., and Shipp, T. C. (1965). *Biochim. Biophys. Acta* **106**, 10.
Desnuelle, P. (1961). *Adv. Enzmol. Relat. Subj. Biochem.* **23**, 129.
Desnuelle, P., and Savary, P. (1963). *J. Lipid Res.* **4**, 369.
Deuel, H. J. (1951). "The Lipids," Vol. 1, pp. 2–3, 59–66, 148–155, 205. Wiley (Interscience), New York.
Deuel, H. J. (1955). "The Lipids," Vol. 2, pp. 5–6, 144–157. Wiley (Interscience), New York.
Dijkstra, N. D. (1969). *Neth. J. Agric. Sci.* **17**, 27.
Dole, V. P. (1956). *J. Clin. Invest.* **35**, 150.
Doney, J. M., and Russel, A. J. F. (1969). *J. Agric. Sci.* **71**, 343.
Dunkley, W. L., Smith, N. E., and Franke, A. A. (1977). *J. Dairy Sci.* **60**, 50.
Eisenberg, S., and Levy, R. I. (1975). *Adv. Lipid Res.* **13**, 1.
Eisenberg, S., Bilheimer, D. W., and Levy, R. I. (1973). *Biochim. Biophys. Acta* **326**, 361.
Eriksen, L., and Simesen, M. (1970). *Nord. Veterinaer med.* **22**, 273.
Evans, L. (1964). *J. Dairy Sci.* **47**, 46.
Evans, L., Patton, S., and McCarthy, R. D. (1961). *J. Dairy Sci.* **44**, 475.
Ewing, A. M., Freeman, N. K., and Lindgren, F. T. (1965). *Adv. Lipid Res.* **3**, 25.
Fain, J. N. (1968). *Endocrinology* **82**, 825.
Fain, J. N., Lovacev, V. P., and Scow, R. O. (1966). *Endocrinology* **78**, 773.
Farber, E., Shull, K. H., Villa-Trevino, S., Lombardi, B., and Thomas, M. (1964). *Nature (London)* **203,** 34.
Favarger, P. (1965). *In* "Handbook of physiology" (J. Field, ed.), Am. Physiological Soc., Sect. 5, pp. 19–23. Williams & Wilkins, Baltimore, Maryland.
Feulgen, R., Imhaüser, K., and Behrens, M. (1929). *Hoppe-Seyler's Z. Physiol. Chem.* **180**, 161.
Fisher, W., and Gurin, S. (1964). *Science* **143**, 362.
Folley, S. J., and McNaught, M. L. (1961). *In* "Milk: The Mammary Gland and Its Secretion" (S. K. Kon and A. T. Cowie, eds.), Vol. 1, p. 441. Academic Press, New York.
Frazer, A. C. (1938). *Analyst (London)* **63**, 308.

Frazer, A. C. (1946). *Physiol. Rev.* **26**, 103.
Frazer, A. C. (1952). *Biochem. Soc. Symp.* **9**, 5.
Fredrickson, D. S. (1969). *Trans. Assoc. Am. Physicians* **82**, 68.
Fredrickson, D. S., and Gordon, R. S. (1958). *J. Clin. Invest.* **37**, 1504.
Fredrickson, D. S., Levy, R. I., and Lees, R. S. (1967). *N. Engl. J. Med.* **276**, 32, 94, 148, 215, 273.
Fredrickson, D. S., Levy, R. I., and Lindgren, F. T. (1968). *J. Clin. Invest.* **47**, 2446.
Fredrickson, D. S., Goldstein, J. L., and Brown, M. S. (1978) *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds.), 4th ed., pp. 604–655. McGraw-Hill, New York.
Freeman, C. P., Noakes, D. E., Annison, E. F., and Hill, K. J. (1968). *Br. J. Nutr.* **22**, 739.
Freidman, M., Van den Bosch, J., Byers, S. O., and St. George, S. (1965). *Am. J. Physiol.* **208**, 94.
Fritz, I. B. (1968). *In* "Cellular Compartmentalization and Control of Fatty Acid Metabolism" (F. C. Gran, ed.), pp. 39–63. Academic Press, New York.
Fritz, I. B., and Hsu, M. P. (1967). *J. Biol. Chem.* **242**, 865.
Fritz, I. B., and Yue, K. T. N. (1963). *J. Lipid Res.* **4**, 279.
Gainer, J. H., Amster, R. L., Needham, J. W., and Schilling, K. F. (1966). *Am. J. Vet. Res.* **27**, 1611.
Gallagher, N., Webb, J., and Dawson, A. M. (1965). *Clin. Sci.* **29**, 73.
Garton, G. A. (1960). *Nutr. Abstr. Rev.* **30**, 1.
Gatt, S. (1970). *Chem. Phys. Lipids* **5**, 235.
Glascock, R. F., and Welch, V. A. (1974). *J. Dairy Sci.* **57**, 1364.
Glascock, R. F., Duncombe, W. G., and Reinius, L. R. (1956). *Biochem. J.* **62**, 535.
Glascock, R. F., Welch, V. A., Bishop, C., Davies, T., Wright, E. W., and Noble, R. C. (1966). *Biochem. J.* **98**, 149.
Glomset, J. (1968). *J. Lipid Res.* **9**, 155.
Goldstein, J. L., and Brown, M. S. (1974). *J. Biol. Chem.* **249**, 5153.
Goldstein, J. L., and Brown, M. S. (1975). *Arch. Pathol.* **99**, 181.
Goldstein, J. L., and Brown, M. S. (1976). *Curr. Top. Cell. Regul.* **11**, 147.
Goldstein, J. L., Brown, M. S., and Anderson, R. G. W. (1977). *In* "International Cell Biology" (B. R. Brinkley and K. R. Porter, eds.), pp. 639–648. Rockefeller Univ. Press, New York.
Gooden, J. M., and Lascelles, A. K. (1973). *Aust. J. Biol. Sci.* **26**, 1201.
Goodman, D. S. (1958). *J. Am. Chem. Soc.* **80**, 3892.
Goodman, D. S., Deykin, D., and Shiratori, T. (1964). *J. Biol. Chem.* **239**, 1335.
Gousios, A., Felts, J. M., and Havel, R. J. (1963). *Metab. Clin. Exp.* **12**, 75.
Graham, W. R., Jones, T. S. J., and Kay, H. D. (1936). *Proc. R. Soc. London, Ser. B* **120**, 330.
Green, C., and Webb, J. A. (1964). *Biochim. Biophys. Acta* **84**, 404.
Greenberger, N. J., Rodgers, J. B., and Isselbacher, K. J. (1966). *J. Clin. Invest.* **45**, 217.
Greenspan, M., and Lowenstein, J. M. (1967). *Arch. Biochem. Biophys.* **118**, 260.
Hadlow, W. J. (1962). *In* "Comparative Neuropathology" (J. R. M. Innes and L. Z. Saunders, eds.), p. 184. Academic Press, New York.
Hallberg, D. (1967). *U.S. Public Health Serv., Publ. No. 1742*, 194.
Hamilton, R. L., and Kayden, H. J. (1974). *In* "Biochemistry of Disease" (F. F. Becker, ed.), Vol. 5, pp. 531–572. Dekker, New York.
Hamosh, M., and Scow, R. O. (1973). *J. Clin. Invest.* **52**, 88.
Hamosh, M., Klaeveman, H. L., Wolf, R. O., and Scow, R. O. (1975). *J. Clin. Invest.* **55**, 908.
Hanahan, D. J. (1954) *J. Biol. Chem.* **211**, 313.
Hansen, I. A. (1965a). *Comp. Biochem. Physiol.* **15**, 27.
Hansen, I. A. (1965b). *Arch. Biochem. Biophys.* **109**, 98.
Hansen, I. A. (1965c). *Arch. Biochem. Biophys.* **111**, 238.
Hanson, R. W., and Ballard, F. J. (1967). *Biochem. J.* **105**, 529.
Hanson, R. W., and Ballard, F. J. (1968). *Biochem. J.* **108**, 705.
Hardwick, D. C. (1966). *Biochem. J.* **99**, 228.
Hashim, S. A., Bergen, S., Krell, K., and Van Itallie, T. B. (1964). *J. Clin. Invest.* **43**, 1238.
Hashim, S. A., Krell, K., Mao, P., and Van Itallie, T. B. (1965). *Nature (London)* **207**, 527.
Hatch, F. T., and Lees, R. S. (1968). *Adv. Lipid Res.* **6**, 1.
Hatch, F. T., Hagopian, L. M., Rubinstein, J. J., and Canellos, G. P. (1963). *Circulation* **28**, 659.
Hausberger, F. X., and Milstein, S. W. (1955). *J. Biol. Chem.* **214**, 483.

Havel, R. J. (1965). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiological Soc., Sect. 5, pp. 575–582. Williams & Wilkins, Baltimore, Maryland.
Havel, R. J. (1975). *Adv. Exp. Med. Biol.* **63**, 37.
Herbert, P. N., Gotto, A. M., and Fredrickson, D. S. (1978). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds.), 4th ed., pp. 544–588. McGraw-Hill, New York.
Higgins, J. A., and Green, C. (1966). *Biochem. J.* **99**, 631.
Hill, R. B., and Droke, W. A. (1963). *Proc. Soc. Exp. Biol. Med.* **114**, 766.
Hillyard, L. A., Entenman, C., Feinberg, H., and Chaikoff, I. L. (1955). *J. Biol. Chem.* **214**, 79.
Hoerr, C. W., and Ralston, A. W. (1944). *J. Org. Chem.* **9**, 329.
Hoerr, C. W., Sedgwick, R. S., and Ralston, A. W. (1946). *J. Org. Chem.* **11,** 603.
Hofman, A. F., and Borgström, B. (1962). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **21**, 43.
Hofman, A. F., and Borgström, B. (1964). *J. Clin. Invest.* **43**, 247.
Hsu, R. Y., Wasson, G., and Porter, J. W. (1964). *J. Biol. Chem.* **240,** 3736.
Hübscher, G. (1970). *In* "Lipid Metabolism" (S. J. Wakil, ed.).,ded.), pp. 279–370. Academic Press, New York.
Isselbacher, K. J. (1967). *U.S. Public Health Serv. Publ.* No. 1742, 30.
Isselbacher, K. J., and Budz, D. H. (1963). *Nature (LOndon)* **200**, 364.
Isselbacher, K. J., and Greenberger, N. J. (1964). *N. Engl. J. Med.* **270**, 351.
Jackson, R. L., Morrisett, J. D., and Gotto, A. M., Jr. (1976). *Physiol. Rev.* **56**, 259.
Johnston, J. M. (1963). *Adv. Lipid Res.* **1**, 105.
Johnston, J. M., and Borgström, B. (1964). *Biochim. Biophys. Acta* **84**, 812.
Jones, D. P., Perman, E. S., and Leiber, C. S. (1965). *J. Lab. Clin. Med.* **66**, 804.
Kane, J. (1977). *In*, "Lipid Metabolism in Mammals" (F. Snyder, ed.), Vol. 1, pp. 209–257. Plenum, New York.
Kates, M. (1955). *Can. J. Biochem.* **35**, 575.
Kates, M.)1972). "Techniques of Lipidology. Isolation, Analysis, and Identification of Lipids." Am. Elsevier, New York.
Kennedy, E. P. (1961). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **20**, 934.
Kinsella, J. E., Burns, D., and Infante, J. P. (1975). *Lipids,* **10**, 227.
Kirschner, S., and Harris, R. (1961). *J. Nutr.* **73**, 397.
Kiyasu, J. Y., Bloom, B., and Chaikoff, I. L. (1952). *J. Biol. Chem.* **199**, 415.
Kleiber, M., Smith, A. H., Black, A. L., Brown, M. A., and Tolbert, B. M. (1952). *J. Biol. Chem.* **197**, 371.
Knudsen, J. (1972). *Biochim. Biophys. Acta* **280**, 408.
Knudsen, J., Clark, S., and Dils, R. (1976). *Biochem. J.* **160,** 683.
Kornacker, M. S., and Lowenstein, J. M. (1965). *Biochem. J.* **94**, 209.
Kornberg, A., and Pricer, W. E. (1953). *J. Biol. Chem.* **204**, 345.
Kovacev, V. P., and Scow, R. O. (1966). *Am. J. Physiol.* **210,** 1199.
Krebs, H. A. (1964). *Proc. R. Soc. London, Ser. B* **159,** 545.
Krebs, H. A. (1966a). *Vet. Rec.* **78,** 187.
Krebs, H. A. (1966b). *Adv. Enzyme Regul.* **4,** 339.
Kritchevsky, D. (1963). *In* "Comprehensive Biochemistry" (M. Florkin and E. H. Stotz, eds.), Vol. 10, Part 2, pp. 1–22. Elsevier, Amsterdam.
Kronfeld, D. S. (1958). *Cornell Vet.* **48**, 394.
Kronfeld, D. S. (1965). *Vet. Rec.* **77**, 30.
Kronfeld, D. S., Mayer, G. P., Robertson, J. M., and Raggi, F. (1963). *J. Dairy Sci.* **46**, 559.
Kuhn, R., and Egge, H. (1960). *Angew. Chem.* **72,** 805.
Kupiecki, F. P. (1966). *J. Lipid Res.* **7,** 230.
Kushwaha, R. S., Hazzard, W. R., Wahl, P. W., and Hoover, J. J. (1977). *Ann. Intern. Med.* **87**, 509.
Lascelles, A. K., Hardwick, D. C., Linzell, J. L., and Mephan, T. B. (1964). *Biochem. J.* **92**, 36.
Lauryssens, M., Verbeke, R., and Peeters, G. (1961). *J. Lipid Res.* **2**, 383.
Lawrie, J. W. (1928). "Glycerol and the Glycols." Reinhold, New York.
Leat, W. M. F., and Cunningham, H. M. (1968). *Biochem. J.* **109**, 38.
Leat, W. M. F., and Hall, J. G. (1968). *J. Agric. Sci.* **71**, 189.
Leat, W. M. F., and Harrison, F. A. (1967). *Biochem. J.* **105**, 13.
Leng, R. A. (1965). *Res. Vet. Sci.* **6**, 433.

Leng, R. A., Steele, J. W., and Luick, J. R. (1967). *Biochem. J.* **103**, 785.
Leveille, G. A., and Hanson, R. W. (1966). *J. Lipid Res.* **7**, 46.
Levy, R. I., Lees, R. S., and Fredrickson, D. S. (1966). *J. Clin. Invest.* **45,** 531.
Libertini, L., and Smith, S. (1978). *J. Biol. Chem.* **253**, 1393.
Lintzel, W. (1934). *Lait* **14,** 1125.
Linzell, J. L., Annison, E. F., Fazakerley, S., and Leng, R. A. (1967). *Biochem. J.* **104**, 34.
Lombardi, B. (1966). "Biochemical Pathology," pp. 1-20. Williams & Wilkins, Baltimore, Maryland.
Lombardi, B., and Ugazio, G. (1965). *J. Lipid Res.* **6**, 498.
Lyon, I., Masri, M. S., and Chaikoff, I. L. (1952). *J. Biol. Chem.* **196**, 25.
Macleod, G. K., Yu, Y., and Schaeffer, L. R. (1977). *J. Dairy Sci.* **60**, 726.
McClymont, G. L., and Setchell, B. P. (1956). *Aust. J. Biol. Sci.* **9**, 184.
McClymont, G. L., and Vallance, S. (1962). *Proc. Nutr. Soc.* **21**, 41.
Mahley, R. W., and Weisgraber, K. H. (1974). *Circ. Res.* **35**, 713.
Mahley, R. W., Weisgraber, K. H., and Innerarity, T. (1974). *Cric. Res.* **35**, 722.
Majerus, P. W. (1967). *J. Biol. Chem.* **242**, 2325.
Manning, P. J., Corwin, L. A., and Middleton, C. C. (1973). *Exp. Mol. Pathol.* **9**, 378.
Markely, K. S. (1947). "Fatty Acids: Their Chemistry and Physical Properties," p. 177. Wiley (Interscience), New York.
Marsh, J. B., and Whereat, A. F. (1959). *J. Biol. Chem.* **234,** 3196.
Mason, R. J., Dobbs, L. G., Greenleaf, R. D., and Williams, M. C. (1977). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36**, 2697.
Masoro, E. J. (1962). *J. Lipid Res.* **3**, 149.
Masoro, E. J. (1968). "Physiological Chemistry of Lipids in Mammals." Saunders, Philadelphia, Pennsylvania.
Mattos, W., and Palmquist, D. L. (1974). *J. Dairy Sci.* **57**, 1050.
Mattson, F. H., and Volpenheim, R. A. (1963). *J. Lipid Res.* **4**, 392.
Mattson, F. H., Volpenheim, R. A., and Lutton, E. S. (1964). *J. Lipid Res.* **5**, 363.
Maynard, L. A., Harrison, E. S., and McCay, C. M. (1931). *J. Biol. Chem.* **92**, 263.
Meng, H. C., ed. (1964). "Lipid Transport." Thomas, Springfield, Illinois.
Morris, M. D., Zilversmit, D. R., and Hintz, H. F. (1972). *J. Lipid Res.* **13**, 383.
Morrisett, J. D., Jackson, R. L., and Gotto, A. M., Jr. (1977). *Biochim. Biophys. Acta* **472**, 93.
Noble, R. C., O'Kelly, J. C., and Moore, J. H. (1972). *Biochim. Biophys. Acta* **202**, 392.
Noble, R. P., Hatch, F. T., Mazrimas, J. A., Lindgren, F. T., Jensen, L. C., and Adamson, G. L. (1969). *Lipids* **4**, 55.
Norum, K. R., and Bremer, J. (1967). *J. Biol. Chem.* **242**, 407.
Norum, K. R., and Gjone, E. (1967). *Scand. J. Clin. Lab Invest.* **20**, 231.
Numa, S., and Yamashita, S. (1974). *Curr. Top. Cell. Regul.* **8**, 197.
Ockner, R. K., Hughes, F. B., an Isselbacher, K. J. (1969a). *J. Clin. Invest.* **48**, 2079.
Ockner, R. K., Hughes, F. B., and Isselbacher, K. J. (1969b). *J. Clin. Invest.* **48**, 2367.
O'Hea, K. E., and Leveille, G. A. (1968). *Comp. Biochem. Physiol.* **26**, 111.
Opstvedt, J., Baldwin, R. L., and Ronning, M. (1967). *J. Dairy Sci.* **50**, 18.
Osborne, J. C., Jr., and Brewer, H. B., Jr. (1977). *Adv. Protein Chem.* **31**, 253.
Palay, S. L., and Karlin, L. J. (1959). *J. Biophys. Biochem. Cytol.* **5**, 373.
Palmquist, D. L. (1976). *J. Dairy Sci.* **59,** 355.
Palmquist, D. L., and Mattos, W. (1978). *J. Dairy Sci.* **61,** 561.
Pan, Y. S., Cook, L. J., and Scott, T. W. (1972). *J. Dairy Res.* **39,** 203.
Phillips, R. W., and Black, A. L. (1965). *Comp. Biochem. Physiol.* **18**, 527.
Phillips, R. W., Black, A. L., and Moeller, F. (1965). *Life Sci.* **4**, 521.
Plowman, R. D., Bitman, J., Gordon, C. D., Dryden, L. P., Goering, H. K., Edmundson, L. F., Yoncoskie, R. A., and Douglas, F. W. (1972). *J. Dairy Sci.* **55,** 204.
Pope, J. L., McPherson, J. C., and Tidwell, N. C. (1966). *J. Biol. Chem.* **241**, 2306.
Porter, J. W., and Long, R. W. (1958). *J. Biol. Chem.* **233,** 20.
Pugh, E. L., and Wakil, S. J. (1965). *J. Biol. Chem.* **240**, 4727.
Puppione, D. L. (1969). Ph.D. Thesis, Univ. of California, Berkeley.
Puppione, D. L. (1978). *J. Dairy Sci.* **61**, 651.
Puppione, D. L., Raphael, B., McCarthy, R. D., and Dimick, P. S. (1972). *J. Dairy Sci.* **55**, 265.

Raben, M. S. (1965). *In* "Handbook of Physiology" (J. Field, ed.), Am. Phsyiolgoical Soc., Sect. 5, pp. 331-334. Williams & Wilkins, Baltimore, Maryland.
Radding, C. M., Bragdon, J. M., and Steinberg, D. (1958). *Biochim. Biophys. Acta* **30**, 443.
Radloff, H. D., and Schultz, L. H. (1967). *J. Dairy Sci.* **50**, 68.
Ralston, A. W., and Hoerr, C. W. (1942). *J. Org. Chem.* **7**, 546.
Ramey, H. A., and Young, J. W. (1961). *J. Dairy Sci.* **44**, 2227.
Raphael, B. C., Dimick, P. S., and Puppione, D. L. (1973a). *J. Dairy Sci.* **56**, 1025.
Raphael, B. C., Dimick, P. S., and Puppione, D. L. (1973b). *J. Dairy Sci.* **56**, 1411.
Rapport, M. M., Lerner, B., Alonzo, M., and Franzl, R. E. (1957). *J. Biol. Chem.* **225**, 859.
Reid, R. L., and Hinks, N. T. (1962). *Aust. J. Agric. Res.* **13**, 1112.
Rhoads, G. G., Gulbrandsen, C. L., and Kagan, A. (1976). *N. Engl. J. Med.* **294**, 293.
Rice, E. W., and Lukasiewiez, D. B. (1957). *Clin. Chem. (N.Y.)* **3**, 160.
Riis, P. H. (1964). Ph.D. Thesis, R. *Vet. Agric. Coll.*, Copenhagen.
Rizach, M. A.)1965). *In* "Handbook of Physiology" (J. Fields, ed.), *Am. Physiological Soc.*, Sect. 5 pp. 309-311. Williams & Wilkins, Baltimore, Maryland.
Robie, S., Jenson, C. H., Smith, S. C., and O'Connor, J. T., Jr. (1975a). *Am. J. Vet. Res.* **36**, 1705.
Robie, S., Smith, S. C., and O'Connor, J. T., Jr. (1975b). *Am. J. Vet. Res.* **36**, 1709.
Robie, S., Jenson, C. H., Smith, S. C., and O'Connor, T. J., Jr. (1975c). *Am. J. Vet. Res.* **36**, 1715.
Robinson, D. S. (1963). *Adv. Lipid Res.* **1**, 133.
Robinson, D. S. (1964). *In* "Lipid Transport" (H. C. Meng, ed.), pp. 194-201. Thomas, Springfield, Illinois.
Roepke, R. R., and Mason, H. L. (1940). *J. Biol. Chem.* **133,** 103.
Rogers, W. A., Donovan, E. F., and Kociba, G. F. (1975a). *J. Am. Vet. Med. Assoc.* **166**, 1087.
Rogers, W. A., Donovan, E. F., and Kociba, G. F. (1975b). *J. Am. Vet. Med. Assoc.* **166,** 1092.
Rubinstein, B., and Rubinstein, D. (1972). *J. Lipid Res.* **13**, 317.
Rudman, D., DiGirolamo, M., Malkin, M. F., and Garcia, L. A. (1965). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiological Soc., Sect. 5, pp. 535-540. Williams & Wilkins, Baltimore, Maryland.
Rudney, H. (1957). *J. Biol. Chem.* **227**, 363.
Russel, A. J. F., and Doney, J. M. (1969). *J. Agric. Sci.* **72**, 59.
Russel, A. J. F., Doney, J. M., and Reid, R. L. (1967). *J. Agric. Sci.* **68**, 351.
Salem, L. (1962). *Can. J. Biochem.* **40**, 1287.
Salt, H. B. (1960). *Lancet* **II**, 325.
Scanu, A. M. (1978). *Trends Biochem. Sci.* **3**, 202.
Schaefer, E. J., Blum, C. B., Levy, R. I., Goebel, R., Brewer, H. B., and Berman, M. (1976). *Circulation* **54**, Suppl. II, 27.
Schaefer, E. J., Eisenberg, S., and Levy, R. I. (1978). *J. Lipid Res.* **19**, 667.
Schotman, A. J. H. (1965). Hyperlipemia bij ponies. Thesis, Utrecht, The Netherlands.
Schotman, A. J. H., and Wagenaar, G. (1969). *Zentralbl. Veterinaermed.* **16**, 1.
Schotman, A. J. H., and Wensing, T. (1977). *Vet. Sci. Commun.* **1**, 337.
Schotz, M. C., and Garfinkel, A. S. (1965). *Biochim. Biophys. Acta* **106**, 202.
Scott, T. W., Cook, L. J., and Mills, S. C. (1971). *J. Am. Oil Chem. Soc.* **48**, 358.
Segrest, J. P., Jackson, R. L., Morrisett, J. D., and Gotto, A. M., Jr. (1974). *FEBS Lett.* **38**, 247.
Senior, J. R. (1964). *J. Lipid Res.* **5**, 495.
Senior, J. R., and Isselbacher, K. J. (1963). *J. Clin. Invest.* **42**, 187.
Shapiro, B. (1965). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiological Soc. Sect. 5, pp. 217-223. Williams & Wilkins, Baltimore, Maryland.
Shapiro, B. (1967). *Annu. Rev. Biochem.* **36**, 247.
Sharma, H. R., Ingalls, J. R., and McKirdy, J. A. (1978). *J. Dairy Sci.* **61**, 574.
Shen, B., Scanu, A. M., and Kezdy, F. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74**, 837.
Shoppee, C. W. (1958). "Chemistry of the Steroids." Academic Press, New York.
Sigurdsson, G., Nicoll, A., and Lewis, B. (1975). *J. Clin. Invest.* **56**, 1481.
Simoni, R. D., Criddle, R. S., and Stumpf, P. K. (1967). *J. Biol. Chem.* **242**, 573.
Simpson-Morgan, M. W. (1967). *U.S. Public Health Serv. Publ.* No. 1742, 200.
Simpson-Morgan, M. W. (1968). *J. Physiol. (London)* **199**, 37.
Siperstein, M. D., and Fagan, V. M. (1964). *Adv. Enzyme Regul.* **2**, 249.
Siperstein, M. D., and Guest, M. J. (1959). *J. Clin. Invest.* **38**, 1043.
Smith, S. (1965). Ph.D. Thesis, Univ. of Birmingham, Birminghan, England.

Smith, S. (1973). *Arch. Biochem. Biophys.* **156**, 751.
Smith, S., and Abraham, S. (1969). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **28**, 537.
Smith, S., and Abraham, S. (1975). *Adv. Lipid Res.* **13**, 195.
Smuckler, F. A., and Benditt, E. P. (1965). *Biochemistry* **4**, 671.
Sniderman, A. D., Carew, T. E., Chandler, J. G., and Steinberg, D. (1974). *Science* **183**, 526.
Snyder, F. (1972). *In* "Ether Lipids: Chemistry and Biology" (F. Snyder, ed.), pp. 273–295. Academic Press, New York.
Srere, P. A. (1965). *Nature (London)* **205**, 766.
Stein, O., and Stein, Y. (1973). *Biochim. Biophys. Acta* **326**, 232.
Steinberg, D., and Vaughan, M. (1965). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiological Soc. Sect. 5, pp. 335–347. Williams & Wilkins, Balitmore, Maryland.
Storry, J. E., and Rook, J. A. F. (1964). *Biochem. J.* **91,** 270.
Storry, J. E., and Rook, M. A. F. (1965). *Br. J. Nutr.* **19**, 101.
Storry, J. E., Brumby, P. E., Hall, A. J., and Johnson, V. W. (1974). *J. Dairy Res.* **41**, 165.
Strauss, E. W. (1966). *J. Lipid Res.* **7**, 307.
Sunderman, F. W., and Sunderman, F. W., Jr. (1960). "Lipids and the Steroid Hormones in Clinical Medicine." Lipincott, Philadelphia, Pennsylvania.
Sutherland, E. W. (1961). *Harvey Lect.* **57**, 17.
Tamai, Y., and Lands, W. E. M. (1974). *J. Biochem. (Tokyo)* **76**, 847.
Trout, D. L., Estes, E. H., and Freidberg, S. J. (1960). *J. Lipid Res.* **1**, 199.
Utter, M. F., and Keech, D. B. (1963). *J. Biol. Chem.* **238**, 2603.
Vagelos, P. R. (1964). *Annu. Rev. Biochem.* **33**, 139.
Vagelos, P. R., Majerus, P. W., Alberts, A. W., Larrabee, A. R., and Aihaud, G. P. (1966). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **25**, 1485.
Vallance, S., and McClymont, G. L. (1959). *Nature (London)* **183**, 466.
Van Deenan, L. L. M. (1965). *Prog. Chem. Fats Lipids* **8,** 1.
Van Golde, L. M. G., and Van den Bergh, S. G. (1977). *In* "Lipid Metabolism in Mammals" (F. Snyder, ed.), Vol. 1, pp. 1–33, 34–149. New York.
Varman, P. N., and Schultz, L. H. (1968). *J. Dairy Sci.* **51**, 1597.
Vaughan, M., and Steinberg, D. (1963). *J. Lipid Res.* **4**, 193.
Venkataraman, S., and Sreenivasan, A. (1965). *Indian J. Biochem.* **2**, 163.
Ward, P. F. V., Scott, T. W., and Dawson, R. M. C. (1964). *Biochem. J.* **92**, 60.
Watson, J. A., and Lowenstein, J. M. (1970). *J. Biol. Chem.* **245**, 5993.
Wensing, T., Schotman, A. J. H., and Kroneman, J. (1973a). *Tijdschr. Diergeneeskd.* **98**, 673.
Wensing, T., Schotman, A. J. H., and Kroneman, J. (1973b). *Neth. J. Vet. Sci.* **5**, 145.
West, C. E., Annison, E. F., and Linzell, J. L. (1967). *Biochem. J.* **102**, 23.
West, C. E., Bickerstaff, R., Annison, E. F., and Linzell, J. L. (1972). *Biochem. J.* **126**, 477.
Williamson, J. R., and Krebs, H. A. (1961). *Biochem. J.* **80**, 540.
Wilson, J. D., and Lindsey, C. A. (1965). *J. Clin. Invest.* **44**, 1805.
Windmueller, H. G., and Levy, R. I. (1967). *J. Biol. Chem.* **242**, 2246.
Windmueller, H. G., and Levy, R. I. (1968). *J. Biol. Chem.* **243**, 4878.
Windmueller, H. G., Herbert, P. N., and Levy, R. I. (1973). *J. Lipid Res.* **14**, 215.
Winegrad, A. T., Goto, Y., and Lukens, F. D. W. (1964). *In* "Fat as a Tissue" (K. Rodahl and B. Issekuts, eds.), pp. 344–361. McGraw-Hill, New York.
Zak, B., Dickenman, R. C., White, E. G., Burnett, H., and Cherny, P. J. (1954). *Am. J. Clin. Pathol.* **24**, 1307.
Zilversmit, D. B. (1969). *In* "Structural and Functional Aspects of Lipoproteins in Living Systems" (E. Tria and A. Scanu, eds.), pp. 329–368. Academic Press, New York.
Zilversmit, D. B., and Diluzio, N. R. (1958). *Am. J. Clin. Nutr.* **6**, 235.
Zinder, O., Hamosh, M., Fleck, T. R. C., and Scow, R. O. (1974). *Am. J. Physiol.* **226**, 744.
Zoppi, G., Andreotti, G., Pajno-Ferrara, F., Njai, D. M., and Gaburro, D. (1972). *Pediatr. Res.* **6**, 880.

3

Serum Proteins and the Dysproteinemias

JIRO J. KANEKO

I. INTRODUCTION

Reference to the nitrogenous compounds of blood plasma encompasses all the organic and inorganic nitrogen-containing compounds of blood. These include organic macromolecular compounds, such as proteins and nucleic acids; smaller molecular weight compounds, such as glutathione, urea, and creatinine; and inorganic compounds, such as nitrate. Nonprotein nitrogen (NPN) compounds are those grouped together as the fraction of N-containing compounds of plasma that are not removed by common protein-precipitating agents, such as trichloroacetic acid (TCA). The principal components of the NPN fraction are urea (50%) and amino acids (25%), which total about 50 mg N per deciliter plasma, in contrast to protein nitrogen, which totals more than 1 gm N per

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

deciliter plasma (or 6.25 gm protein per deciliter). This chapter deals primarily with the latter, significant fraction, which is classed collectively as the plasma or serum proteins. Emphasis is placed on the diagnostic and interpretive aspects. The reader is referred to the many excellent texts on biochemistry for the details of protein synthesis, structure, and function.

A. Structure

The fundamental units of protein structure in nature are the 22 natural amino acids. The indispensable amino acids of this group are those that are not synthesized by animals and hence must be supplied in the diet. The dispensable amino acids are those that are synthesized through transamination reactions from the carbon skeletons shown in the figures of Chapter 1. Thus, α-ketoglutarate of the TCA cycle is transaminated to become glutamic acid by transfer of the α-amino group of alanine, which then becomes pyruvate. This reaction is familiarly known as glutamic–pyruvate transaminase or as alanine aminotransferase.

The α-amino acids are linked together by the protein-synthesizing mechanisms within cells on the rough endoplasmic reticulum. The intricacies of the process by which genetic information is transmitted (transcription) and the mechanism by which this information is expressed in protein structure (translation) are summarized in the genetic code and the mechanisms of protein synthesis. Briefly, a precise sequence of nucleotides of DNA (chromosomal material) is replicated in ribosomal RNA, the template on which the polypeptide chains of proteins are synthesized. The nucleotides of the RNA, in a series of triplets, code for specific amino acids, and thus the sequence of triplets governs the amino acid sequence of a specific protein. Many replicates of the protein molecule can be synthesized on a single ribosomal RNA. When a cell divides, the genetic information is transmitted from mother to daughter cells via DNA so that identical proteins are synthesized by succeeding generations of cells. In certain disease states, a single cell or a family of genetically homogeneous cells (clone) might exhibit uncontrolled proliferation, e.g., myeloma, and excessively produce a single discreet species of protein, immunoglobulin M.

B. Function

The functions of proteins in the body are innumerable. They form the basis of structure of organs and tissues, operate as catalysts (enzymes) in biochemical reactions, are regulators (hormones), and are transport and carrier compounds for most of the constituents of plasma. The biological activity of proteins and polypeptides for these various functions is dependent upon their structure, from the primary amino acid sequence of the polypeptide hormones to the macromolecular fibers of the fibrin polymers which participate in clot formation.

The major proteins of plasma are globular, and only a few are fibrous, e.g., fibrinogen. The process of protein denaturation is the net effect of the alteration of the biological, chemical, and physical properties of a protein by disruption of its structure. It is therefore of great importance that proteins be handled in a way that prevents this structural change from occurring. Heat, ultraviolet light, surfactant detergents, and chemicals all have some effect on protein structure. If this effect is significant, the protein is denatured, its biologi-

cal and physical activity is irreversibly lost, and it cannot be accurately measured. A corollary to measurement of any protein is therefore the avoidance of denaturation by gentle handling and careful preservation at the appropriate temperature.

C. Identification

Albumin is a water-soluble, globular protein that is usually identifiable as a single, discreet molecular species. The globulins are also globular proteins but precipitate in water, so they require some salt to maintain their solubility. In contrast to albumin, one of the major features of globulins is that they are not a single, discreet species of protein. By virtue of their structure, they migrate in groups in an electric field or precipitate together as rather large families of proteins which are identifiable as the α-, β-, or γ-globulins. The separation of the various fractions by cellulose acetate electrophoresis is now the most commonly used first step in the investigation of protein dyscrasias. Electrophoresis has evolved into an extremely useful technique because aberrations are observed in so many disease states. There are, however, few diseases in which the electrophoretic pattern can be considered pathognomonic.

II. METHODOLOGY

A. Total Serum Protein

There are a number of methods for the determination of total protein in the serum or other body fluids. Classically, the N in the protein has been determined by the Kjeldahl analysis and multiplied by the factor 6.25 (16% N in protein). The technique is rather cumbersome and, for other reasons, is not amenable for use in the clinical laboratory. Other chemical or physical techniques are now widely used.

1. Chemical Methods

a. Biuret. This method is the most widely used colorimetric method for the determination of total protein in serum. The biuret reaction is highly specific for protein and is based upon the formation of a blue peptide–Cu complex in alkaline solution.

The method is highly accurate for the range of protein likely to be formed in serum (1–10 gm/dl), although not precise enough for the very low levels found normally in other body fluids, e.g., cerebrospinal fluid (CSF). It is the chemical method of choice for the clinical laboratory because of its simplicity, accuracy, and precision. It has been widely adapted for automated chemical analyzers as well as for unitized chemical analyzers for the office laboratory.

b. Phenol–Folin–Ciocalteau. This is an extremely sensitive method (Lowry *et al.*, 1951) and is the method of choice for dilute solutions such as CSF. It is based on the reaction of the phenolic group of tryptophan and tyrosine of proteins with the reagent to form a blue color. Automated as well as manual techniques are available.

c. Precipitation. Depending on their charges, proteins can be brought to their isoelectric points, where they precipitate, by the addition of either anions or cations.

Anionic precipitants, such as TCA, sulfosalicylic acid, and tungstic acid, combine with cationic proteins to cause precipitation. Barium and zinc ions are common cationic precipitants. These reagents are more generally used to prepare protein-free filtrates in the clinical laboratory but have been used for the determination of total protein, e.g., sulfosalicylic acid method for urine protein.

2. Physical Methods

a. Refractometric. Proteins in solution cause a change in refractive index of the solution which is proportional to the concentration of the protein. Properly controlled and used, this method can be quite accurate at the levels of protein found in serum or plasma. It is currently in widespread use as a screening method for the rapid determination of protein in serum, plasma, or other body fluids. Because of its rapidity and simplicity, it is well suited for the office or the emergency clinical laboratory. Due to its dependence on the transmission of light, it is important that the method be used only for clear, nonturbid, and nonlipsemic sera and fluids. A moderate degree of hemolysis or icterus does not interfere. The calibration of the refractometer should be frequently checked because this has been found to be a frequent source of error (Booij, 1972). The hand-held Goldberg refractometer* is the most useful and versatile refractometer for the veterinary clinical biochemical laboratory because it is scaled to read both total serum protein and urine specific gravity, its scale has finer divisions, and it is temperature-controlled.

b. Fibrinogen. Fibrinogen is a large protein of 300,000 relative molecular mass (Mr) units which constitutes about 5% of the total proteins of plasma. It is most simply and rapidly estimated by the heat precipitation–refractometer method (Kaneko and Smith, 1967), which takes advantage of the fact that fibrinogen precipitates on heating to 56°C. The total protein in a sample of plasma is first determined by refractometer. A second sample of plasma in a microhematocrit tube is heated at 56°C for 3 minutes in a water bath. The tube is then centrifuged, and the total protein in the clear supernate (serum) is determined. The difference between the serum and the plasma protein gives an estimation of the plasma fibrinogen. Alternately, the height of the precepitated fibrinogen column can also be measured. This method is now extensively used as a routine screening method, but, for more accurate results, fibrinogen is determined as the protein content of the clot (Davey *et al.*, 1972).

B. Fractionation of Serum Proteins

In order to determine the amounts of albumins and globulins which make up the total serum proteins, various fractionation techniques have been designed to separate and quantitate the serum proteins. Most fractionation techniques usually first require the determination of the total serum protein, after which the concentrations of the individual fractions are determined by calculations depending upon the fractionation method. In its simplest form, if either the albumin or globulin content is determined, the other can be obtained by subtraction from the total serum protein. Ultimately, the accuracy of any of the chemical methods is based upon comparisons with protein electrophoresis, the current standard of reference for serum protein fractionations.

*A. O. Spencer Refractometer.

1. Salt Fractionation

Salts added to proteins in solution dehydrate the proteins, causing them to precipitate. The most commonly used salts are those of sodium or ammonium sulfate. The fractionation technique is based on the differences in solubility of the various fractions at different salt concentrations. Albumin is soluble in water, and the various globulins can be precipitated independently by using different concentrations of salt. The principle of the technique has been applied for many years in clinical biochemistry in the form of the serum flocculation tests for liver function. Numerous tests have been devised and include the zinc sulfate, sodium sulfate, and ammonium sulfate turbidity tests. These are turbidometric tests in which the amounts of salts used have been adjusted for the normal concentrations of γ-globulins in human sera, and, in the presence of increased amounts of γ-globulins, flocculation occurs. Since the concentrations of globulins differ in animals, these tests are not directly applicable to animal sera. They can, however, be adapted to animal sera if the concentrations of salt are adjusted to the normal concentrations of γ-globulins in a particular species. This has been done in the calf (Pfeiffer *et al.*, 1977) and foal (Rumbaugh *et al.*, 1978a), where field tests for the detection of suckling have been evaluated using $NaSO_4$ turbidity. The test was found to provide acceptable results for total immunoglobulins in comparison to electrophoresis and other tests.

2. Dye Binding

The acid dye 2-(4′-hydroxyazobenzene)benzoic acid (HABA) was widely used in the past for the determination of serum albumin because of its adaptation for automated systems. It was quickly discovered that the dye bound so poorly to the albumins of domestic animals that the method was unacceptable. The HABA dye method has now been replaced by a method using bromcresol green (BCG). The BCG method, in comparison with electrophoresis, appears to be quite accurate within the normal limits of albumin concentrations in animals. However, its accuracy becomes progressively lower outside the normal limits and is usually unacceptable at very low levels.

3. Colorimetric Determination of Globulin

The reaction of glyoxylic acid with globulins forms a purple color complex which is proportional to the amount of globulin (Henry, 1964). The test has not gained wide acceptance in clinical biochemistry due to its variability. It has been adapted to some unitized blood chemistry systems, but it must be applied with caution, since its applicability to animal sera has not been established.

C. Electrophoretic Fractionation of Serum Proteins

The electrophoretic technique is the current standard of reference for the fractionation of serum proteins (SPE) in clinical biochemistry. The marked advances in technology of the past decade have made this previously elaborate technique a widely used, routine clinical biochemical test procedure. Its current widespread use is commensurate with its reflection of a variety of changes in serum protein patterns in disease. Although only a few changes in pattern can be considered diagnostic of a specific disease, the results of electrophoresis, properly interpreted, can be one of the most useful diagnostic aids available to the clinician. There are a large number of methods for serum protein electrophoresis which basically differ only in the type of support media used. The cellulose

acetate (CA) method is to be recommended, because of its relative simplicity and accuracy, as the test of choice. The principle of this method and the results of its use will be described more fully as the clinical laboratory method of choice for the electrophoretic fractionation of serum proteins.

1. Principle

The principle of the electrophoretic separation of serum proteins is based upon the migration of charged protein particles in an electric field. The direction and rate of migration of the particles are based upon the type of charge (positive or negative) on the protein, size of the protein, intensity of the electric field, and the support medium through which the protein particles are induced to migrate. It follows that, in order to compare results of SPE, it is important that the support medium, pH, buffer, and electric current be described.

When a support medium is used for electrophoresis, the process is called zone electrophoresis (as compared to free). The most commonly used support medium is CA, although methods using many other support media, such as agar gel, agarose, starch gel, or polyacrylamide gel, are available. Many of the latter yield greater separability of the serum proteins than does CA, but, in clinical biochemistry, CA remains the most useful method. It should be recalled that the globulins are comprised of myriad separate protein moieties---enzymes, carriers, antibodies, clotting factors—and that a specific serum protein can be identified only by additional special techniques.

2. Cellulose Acetate Electrophoresis

Depending upon factors already noted, particularly the charge and the medium, protein particles move toward the anode or the cathode of the electric field. Most serum proteins are negatively charged and, on CA in barbital buffer, pH 8.6, they migrate toward the anode. Albumin, with the strongest negativity, migrates farthest toward the anode. The globulins, with weaker negativities, migrate more slowly toward the anode, whereas the γ-globulins may not move or may move toward the cathode. The globulins move in groups classed as the α-, β-, and γ-globulins. Depending upon the species, there may normally be one or two α, one or two β, and one or two γ fractions (Table IV, Fig. 1).

A further advantage of CA is that, after the protein is stained, the CA membrane can be cleared and protein staining densities easily read by a recording densitometer. The recording provides a visual display (Fig. 1) of the relative amount of each protein class in the serum and is familiarly referred to as an electrophoretogram. When combined with an integrating unit, the intensity of staining of the different classes or bands of the electrophoretogram are translated into a percentage of the total protein. By multiplying this percentage times the total serum protein concentration, the absolute value of each of the separate fractions can be obtained.

A frequent difficulty in dealing with the sera of domestic animals is the difference in the normal pattern among species. This has created some confusion in the identification of a particular fraction of the electrophoretogram as α, β, or γ. As a first step toward standardization, a useful resolution is to identify the midpoint of the electrophoretogram, which lies between or very nearly between the α_2 and β_1 peaks (Fig. 1). This plus the knowledge of the normal number of peaks in a particular species is usually sufficient for identifying the individual peaks with confidence.

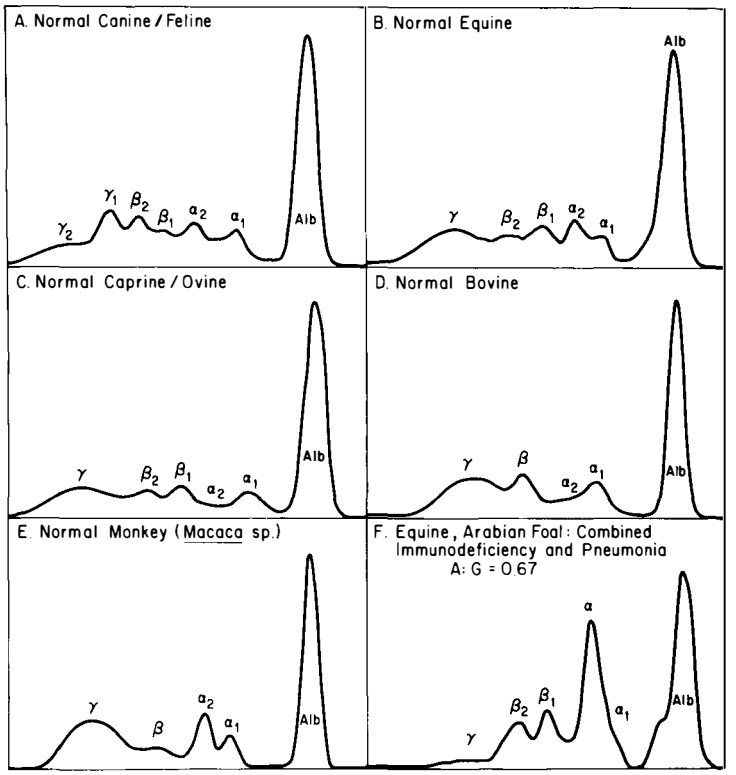


Fig. 1. Normal and abnormal cellulose acetate electrophoretic profiles of serum proteins of domestic animals.

3. Immunoelectrophoresis

This technique combines electrophoresis in one dimension with immune diffusion in a second dimension to obtain a more precise identification of the various proteins of serum. Quantitation of the specific immunoglobulins, G, M, A, and E, has also been performed in those species of animals in which specific antisera have been available. This field of immunology has undergone unprecedented growth in the last two decades, and the scope of its importance continues to increase. This is a highly specialized area with many specific texts and treatises, to which the reader is referred for further information.

III. NORMAL SERUM PROTEINS

There are well over 100 serum proteins described and quantitated in man and animals, many of which change markedly in disease and many of which change only subtly or not at all. Inasmuch as the proteins of an individual or a species are synthesized under genetic control, it would be expected that variations in proteins would occur among individuals and among species. These variations are reflected in the species differences of the normal SPE patterns. Thus, in ruminants such as the cow, the normal SPE pattern exhibits one albumin, one α, one β, and one γ fraction. Table I lists some of the important serum

TABLE I

Some Common Serum Proteins, Their Function, and Changes in Disease

Protein	Mr[a]	Function	Change in disease
1. Prealbumin	61,000	T_4 Transport	Not present in this region
2. Albumin	69,000	Osmotic pressure regulation Transport	Increase: only in dehydration Decrease: malnutrition, liver disease, renal disease, gastroenteropathies, blood loss, plasma loss
3. α-Globulins (α_1 and α_2)			
Thyroxine-binding globulin	50,000	T_4 Transport	
α_1-Fetoprotein	65,000		Increase: hepatoma?
α_1-Antitrypsin	45,000	Trypsin inhibitor	Decrease: chronic pulmonary disease Increase: acute inflammatory disease
α_1-Acid glycoprotein (orosomucoid, seromucoid)			Increase: acute inflammatory disease
α_1-Lipoprotein (HDL, α-lipoprotein)		Lipid transport	
α_2-Lipoprotein (VLDL, pre-β-lipoprotein)		Lipid transport	
α_2-Lipoprotein (LDL, β-lipoprotein)		Lipid transport	Nephrotic syndrome
α_2-Macroglobulin	820,000	Insulin binding Trypsin inhibitor	Increase: nephrotic syndrome, active liver disease, acute inflammatory disease
α_2-Globulin (prealbumin)		T_4 Transport	
Ceruloplasmin (α_2)	160,000	Copper transport ferrioxdase	Increase: acute inflammatory disease
Haptoglobin (α_2)	100,000	Hb binding	Decrease: hemolytic anemia, liver disease Increase: acute inflammatory disease
4. β-Globulins (β_1 and β_2)			
Transferrin	90,000	Iron transport	Decrease: chronic liver disease, iron storage disease Increase: acute liver disease, pregnancy, iron deficiency, anemia, nephrotic syndrome
Hemopexin	80,000	Heme binding	Decrease: hemolytic anemia, active liver disease
C_3 complement	75,000	Complement C_3 factor	Decrease: autoimmune disease Increase: acute inflammatory disease, atopic dermatitis
C_4 complement		Complement C_4 factor	Decrease: autoimmune disease
Plasminogen		Proenzyme of plasmin, fibrinolysis	Increase: disseminated intravascular coagulation (DIC)
Fibrinogen		Proform of fibrin, clotting	Decrease: DIC Increase: acute inflammatory disease

(*continued*)

TABLE I—*Continued*

Protein	Mr[a]	Function	Change in disease
5. γ-Globulins (γ_1 and γ_2)			
IgG	150,000	Antibodies to infectious agents, toxins	Decrease: agammaglobulinemia Increase: chronic disease, connective tissue disease, liver disease, myeloma, other monoclonal gammopathies
IgA	150,000	Antibodies, secretory antibodies in body fluids of the respiratory, gastrointestinal, and genitourinary tracts	Same as for IgG
IgE	200,000	Antibodies, allergic reactions	Decrease: agammaglobulinemia Increase: allergy, anaphylaxis
IgM	900,000	Antibodies, initiator of cell reaction	Decrease: agammaglobulinemia Increase: primary macroglobulinemia (Waldenstrom's), chronic disease
IgD	160,000	Unknown, not reported in animals	
Light chains (Bence-Jones)	30,000	Part of immunoglobulin structure	Increase: myeloma, monoclonal gammopathies

[a] Mr, relative molecular mass units.

proteins with their principal functions and conditions of alteration. Since there are significant differences in fractionation depending upon the method, reference is being made here only to CA electrophoresis. Normal values for total serum protein and its fractions are given in Appendix VI.

A. Prealbumin

This is the most rapidly migrating fraction in man, is usually not visualized, and may not exist as such in any domestic animal. However, a protein with the characteristics of prealbumin migrates in the α_2-globulin region of the SPE of dogs (Fex *et al.*, 1977). The only known function of prealbumin is thyroxine binding and transport (Oppenheimer *et al.*, 1965).

B. Albumin

1. Structure

Albumin is the most prominent of the serum proteins on SPE and, in animals, constitutes between 35 and 50% of the total serum proteins, in contrast to human beings and nonhuman primates, in which albumin accounts for 60–67% of the total. Its tertiary structure is globoid or ellipsoid, and it is the most homogenous fraction discernible on SPE. It is often described as the only discreet protein species which can be detected by SPE. However, even here, other methods, such as starch-gel electrophoresis, allow for the separation and detection of genetic polymorphisms of albumin.

It is also important to observe the sharpness of the albumin peak as a measure of the quality of the SPE fractionation. It is a useful guide for differentiating the sharp monoclonal globulin peaks from the polyclonal peaks. The horse frequently exhibits a minor postalbumin fraction, which appears as a shoulder on the cathodal side of the albumin peak. This shoulder becomes progressively more prominent with the severity of hypoalbuminemia or with acute inflammatory disease. Recently, a thyroxine binding function of this postalbumin fraction has been observed in horses (Blackmore, 1978).

2. Function

Albumin is synthesized by the liver, as are all plasma proteins except for the immunoglobulins, and is catabolized by all metabolically active tissues. Its rate of metabolism varies among species, and this is reflected in the half-times for disappearance (Table II). There appears to be a direct correlation between albumin turnover and body size, and it is of interest that clinically significant hypoalbuminemic edemas occur only in the larger animals. This would suggest that edema develops in those animals because of slow replacement. Albumin is a major storage reservoir of proteins and transporter of amino acids. It is the most osmotically active plasma protein due to its abundance and small size and accounts for about 75% of the osmotic activity of plasma. Another major function of albumin is as a general binding and transport protein. Virtually all constituents of plasma not bound and transported by a specific transport protein, and even many that are, e.g., thyroxine, are transported by albumin. Albumin binding solubilizes substances in plasma that are otherwise only sparingly soluble and permits their effective transport. Albumin binding also inhibits the loss of the constituents through the kidneys. The binding of unconjugated bilirubin (indirect bilirubin) or of fatty acids by albumin are examples of this function.

C. Globulins

1. α-Globulins

The α fraction is the most rapidly migrating of all the globulins, and, in most species except the ruminant, it migrates as an α_1 (fast) and an α_2 (slow) fraction. Most of the

TABLE II

Albumin Turnover in Animals

Species	$T_{1/2}$ (days)	Reference
Mouse	1.9	Allison (1960)
Rat	2.5	Allison (1960)
Guinea pig	2.8	Allison (1960)
Rabbit	5.7	Dixon *et al.* (1953)
Pig	8.2	Dick and Nielson (1963)
Dog	8.2	Dixon *et al.* (1953)
Sheep	14.28	Campbell *et al.* (1961)
Man	15	Dixon *et al.* (1953)
Baboon	16	Cohen (1956)
Cow	16.5	Cornelius *et al.* (1962)
Horse	19.4	Mattheeuws *et al.* (1966)

TABLE III

Markers of Acute Inflammatory Disease

α_1-Globulins
α_1-Antitrypsin
α_1-Acid glycoprotein (orosomucoid, seromucoid)
α_2-Globulins
α_2-Macroglobulin
Ceruloplasmin
Haptoglobin
β-Globulin
Fibrinogen

globulins of this fraction are synthesized by the liver, except for α_1-fetoprotein, which is synthesized by fetal liver cells. In general, the α_1-globulins are smaller than the α_2-globulins, but there appears to be no functional separation between the two fractions. Important proteins of this fraction are the α-lipoproteins [high-density lipoproteins (HDL)], which migrate as α_1, and the pre-β-lipoproteins [very low density lipoproteins (VLDL)], which migrate in the α_2 position.

The β-lipoproteins [low-density lipoproteins (LDL)] so named because they migrate in the β region on paper, also migrate in the α_2 region on CA. The latter two lipoproteins, together with α_2-macroglobulin, account for the increase in α_2-globulins seen in the nephrotic syndrome. α_2-Macroglobulin, haptoglobin, and ceruloplasmin are also diagnostically important markers of acute inflammatory disease (Table III).

2. *β-Globulins*

β-Globulins trail the α_2-globulins and similarly migrate as β_1 (fast) and β_2 (slow) fractions in most domestic animals except ruminants. Important proteins of this fraction are complement (C_3, C_4), hemopexin, and transferrin. Fibrinogen slightly trails the β_2-globulins and is another important marker of acute inflammatory disease. Some immunoglobulins, IgM and IgH, extend from the β_2 to the γ_2 regions. Therefore, in response to the antigenic stimulus of infectious agents, or in plasma cell malignancies, immunoglobulin can rise in the β_2 zone as well as in the γ_1 and γ_2 zones.

3. *γ-Globulins*

In most animals, the γ fraction is also observed as two fractions, a γ_1 (fast) and a γ_2 (slow). Of the immunoglobulins observed in animals, IgA, IgM, and IgE are found primarily in the γ_1 region, whereas IgG is found primarily in the γ_2 region. The specific identification and quantitation of the immunoglobulins requires the use of sophisticated immunochemical techniques (Iammarino, 1972; Mancini *et al.*, 1965). Immunochemical methods are widely used in protein research but are also rapidly evolving as valuable clinical methods. Only a brief description of immunological principles will be given here, as a basis for understanding the interpretation of dysproteinemias visualized on SPE.

a. Source. Antigens employed in immunochemical tests are of two types: complete antigens, which induce formation of specific antibodies, and incomplete antigens (haptens), which, although they react with antibodies, do not elicit an immune response. Complete antigens are usually of size 5000 Mr units or more and are proteins or carbohy-

drates. Haptens are low molecular weight compounds which, if coupled to a larger molecular weight compound such as protein, can elicit an antibody response. Most antibodies used in radioimmunoassay (RIA) have been produced in response to haptens coupled to albumin, e.g., thyroxine (T_4) antibody for T_4-RIA.

Antibodies produced in response to antigens are highly specific if only one antigenic determinant is involved. In nature, multiple antigenic determinants are usually more involved. Natural haptens are also usually multiple and elicit antibodies with multiple specificities or cross-reactivity.

Lymphocytes are now known to be the center of the immune system. There are two subpopulations, B lymphocytes (Bursa) and T lymphocytes (thymus), which can be identified by special immunological means.

The T cells are found in the peripheral blood and in the deep cortical areas and paracortical sinuses of lymph nodes. They are associated with cell-mediated immunity. The B cells were originally identified in the bursa of Fabricius of the chicken and are now thought to be differentiated in fetal liver cells. In the adult, they are found in the peripheral blood and the germinal centers of lymph nodes. The B cells respond to antigenic stimuli with the proliferation of plasma cells, which produce the specific antibody or immunoglobulin. Of the five known immunoglobulins, IgG, IgA, IgM, IgD, and IgE, four have been identified in dogs (lgG, lgA, IgM, and lgE) (Vaermans and Heremans, 1969) and three (IgG, IgM, and IgA) in cats (Gorham, 1971) and horses (McGuire *et al.*, 1975a). Under certain conditions, an excess of a portion of the immunoglobulin molecule, i.e., light chains, might also be produced. These light-chain fragments appear in the plasma and, because of their small size, appear in the urine as the Bence-Jones proteins.

A specific plasma cell population of defined genetic origin, a clone, produces a specific immunoglobulin. Uncontrolled growth of a specific B-cell clone (malignancy) results in the overproduction of a specific immunoglobulin species, which appears as a sharp "monoclonal" spike or gammopathy. A group of clones of different genetic origin can also overproduce a heterogeneous mix of immunoglobulins, which appear as a diffuse hyperglobulinemia or as a "polyclonal" gammopathy.

b. Immunoglobulins. The immunoglobulins are glycoproteins the basic structure of which is a monomer comprised of two heavy (H) and two light (L) chains linked together by disulfide bridges. Each H chain consists of 446 amino acids, and each L chain consists of 214 amino acids (Edelman, 1973). The structure of the H chain governs the class of immunoglobulin and is named by corresponding Greek letters: γ = G, μ = M, α = A, ϵ = E, δ = D. The structure of the L chain is either κ or λ and denotes type. Structural variations in the variable regimes of H or L chains provide a basis for further subdivision into subtypes and subclasses. To date, four subclasses of IgG have been identified: IgG_1, IgG_2, IgG_3, IgG_4; two subclasses of IgA—IgA_1 and IgA_2—have also been identified.

Immunoglobulin G, IgD, and lgE are monomers, IgA is a dimer, and IgM is a pentamer. Most viral, bacterial, and toxin antibodies are of the IgG type and are present in all animals. Immunoglobulin D has been reported only in human beings, where its function is unknown. Immunoglobulin E is involved in allergic and anaphylactic reactions and is present in dogs. Immunoglobulin A is a dimer of two basic units joined together by a secretory piece. It is found in the secretions of the respiratory, genitourinary, and gastrointestinal tracts. Immunoglobulin M is a pentamer of basic units arranged in a circle to form a high molecular weight unit. These are the macroglobulins or "M" components.

The Bence-Jones proteins are light-chain units, and their presence may reflect the asynchronous synthesis of H chains so that excess L chains appear. They are not detected on SPE, but, when immunochemical techniques are used, they are often found to accompany gammopathies (Solomon, 1976).

IV. INTERPRETATION OF SERUM PROTEIN PROFILES

The determination of serum proteins and the visualization of their SPE profiles have evolved into methods of great importance in clinical biochemistry. This has occurred even though a specific diagnosis can seldom be made with SPE. Abnormal serum protein profiles can now be identified with types of disease processes with some accuracy. Serum protein electrophoresis also provides the rationale for further in-depth studies. Another stimulus for its use has been the inclusion of total protein and albumin in automated systems, thereby providing the albumin/globulin ratio (A:G).

Serum protein electrophoresis provides the percentage of albumin and globulins and, since the total serum protein is known, the absolute value of each can be obtained by multiplication. It has been suggested by some that the A:G ratio is no longer useful and should be discarded in favor of absolute values. Although there is merit in the suggestion, the A:G ratio retains usefulness in interpretation of automated chemistry data. Changes in the A:G are often the first signal of a protein abnormality. (See Appendix VI for normal total serum protein and its fractions in domestic animals.)

A. Physiological Influences

Abnormalities of SPE must be interpreted in the light of many influences unassociated with disease. Normal physiological variations within an individual are relatively constant over a considerable period of time (Stockl and Zacherl, 1953), so even minor changes in the SPE profile can be of significance and warrant close scrutiny.

1. Influence of Age and Development

In the fetus, the concentration of total protein and albumin progressively increases with little change in globulins and an absence of γ-globulin. However, γ-globulin was detected in 25% of bovine fetal serum samples (Kniazeff *et al.*, 1967), which was attributed to transplacental transfer. After nursing and for up to 24 hours, baby pigs had large amounts of γ-globulin, which progressively decreased to 5% of the total serum protein by 4 weeks of age (Rutqvist, 1958; Rook *et al.*, 1951). In the calf, precolostral serum normally contains no γ-globulin (Pierce, 1955), but, within a few hours after ingestion, γ-globulin appears in serum and its absorption occurs up to 48 hours after birth (Ebel, 1953), after which gut permeability ceases. In colostrum-deprived calves, immunoglobulin increases minimally, the increase being due to external antigenic stimuli.

In all animals, there is a general increase in total protein, decrease in albumin, and increase in globulins with advancing age (Dimopoullos, 1961; Forstner, 1968; Tumbleson *et al.*, 1972), except in the very old, when proteins again decline. Similar changes due to age were also clearly shown in chickens by Tanaka and Aoki (1963), who periodically followed serum protein changes up to 210 days of age. Thus, age is an important consideration in the interpretation of SPE.

2. *Hormonal and Sexual Influences*

Hormonal effects on serum protein can be either anabolic or catabolic. Testosterone and estrogens are generally anabolic in all species. Diethylstilbestrol given to male calves and to chickens (Perk and Loebl, 1960) was found to increase total protein, decrease albumin, and increase globulins. Growth hormone is another well-known anabolic hormone with similar effects. On the other hand, thyroxine decreases total serum protein, most likely due to its catabolic effect (Sturkie, 1951). The glucocorticoids are characterized by gluconeogenic activity, but their effects on serum protein are not marked, since only small decreases in γ-globulin have been observed (Bjorneboe *et al.*, 1952). Thus, in general, hormonal effects of serum proteins are slight even though their effects on weight gain or body composition may be quite marked.

3. *Pregnancy and Lactation*

Generally during the course of gestation, albumins decrease and globulins increase. In ewes, albumin decreases to a minimum at midgestation and returns to nearly normal at term. Globulins and the total serum protein progressively decrease throughout gestation (Dunlap and Dickson, 1955). In cows, the total serum protein, γ_1-, and β_2-globulins begin to increase at 2 months before term, reach a maximum at 1 month, and then rapidly decline toward term (Larson and Kendall, 1957). These data suggest that the immunoglobulins rapidly leave the plasma during the last month of gestation, when colostrum is being formed in the mammary gland. Lactation and egg production impose further stresses on protein reserves and metabolism, and changes similar to those occurring in pregnancy also occur.

4. *Nutritional Influences*

Serum proteins are sensitive to nutritional influences, but in most cases the changes are subtle and difficult to detect and to interpret. A direct relationship between vitamin A and albumin has been observed in the cow (Erwin *et al.*, 1959), which can be corrected by the administration of carotene. Dietary protein depletion has been extensively studied and as expected manifests as a hypoproteinemia and hypoalbuminemia in rats (Weimer, 1961), chickens (Leveille and Sauberlich, 1961), and dogs (Allison, 1957). In human beings, kwashiorkor and marasmus, diseases of severe protein–calorie malnutrition, are also characterized by hypoproteinemia and hypoalbuminemia. Dietary protein depletion results in a decreased turnover of serum albumin in rats (Jeffay and Winzler, 1958). Immunoglobulins are affected only upon severe protein restriction (Benditt *et al.*, 1949), but the effects are reversible upon protein repletion (Wissler *et al.*, 1946).

5. *Stress and Fluid Loss*

Temperature stress, either febrile or cold, is associated with nitrogen loss, increased adrenal activity, and increased protein turnover, resulting in a decrease in total serum protein, a decrease in albumin, and often an increase in α_2-globulin. Similar findings are observed in crushing injuries, bone fractures, and extensive surgery. During tissue repair protein reserves are used and subsequent protein metabolism results in decreased albumin and increased α_2-globulin (Hoch-Ligeti *et al.*, 1953). In the inflammatory process, fluids and proteins move into tissue fluids, inducing edema and contributing to the decrease in albumin. Hemorrhage or exudation with losses of large amounts of plasma and the subsequent rapid movement of interstitial fluid into the plasma compartment result in

acute hypoproteinemia. Conversely, dehydration leads to hemoconcentration through reduction in fluid volume and consequent hyperproteinemia. During splenic contraction in the horse, a large mass of erythrocytes moves into the circulation with little or no change in the serum protein.

B. Dysproteinemias

The best method for the overall evaluation of protein status is SPE. Visualization of the SPE profile and evaluation of the absolute values provide an excellent basis for presumptive diagnoses and additional studies of the patient. Prior to the application of SPE, the derivation of the A:G ratio from the results of automated chemistry panels can be a useful basis for SPE.

There are several classifications of SPE profiles proposed (Kawai, 1973), all based on disease. Table IV, on the other hand, gives a classification of SPE based on the A:G ratio and the nature of the profile. This table provides a useful mechanism for alerting the clinical biochemist and the clinician to the underlying significance of the specific dysproteinemia.

1. Normal A:G—Normal Profile

a. Hyperproteinemia. Simple dehydration with loss of fluid is essentially the only instance when this form of hyperproteinemia occurs.

b. Hypoproteinemia. Overhydration through vigorous fluid therapy or excess water intake are common causes of simple hypoproteinemia. In the aftermath of acute blood loss, interstitial fluid moves rapidly into the plasma compartment, diluting the proteins. This dilution may be further intensified by the ingestion of water to satisfy the thirst commonly seen in acute blood loss. Similarly, in the early stages of acute plasma loss, whether internal or external by exudation or extravasation, simple hypoproteinemia results.

2. Decreased A:G—Abnormal Profile

a. Decreased Albumin. Decreases in albumin are a common form of dysproteinemia and, depending on the stage of the disease, can be associated with either slight hyper-, normo-, or, in its advanced stages, hypoproteinemia. Due to its small size and osmotic sensitivity to fluid movements, albumin is selectively lost in renal disease (Osborne and Vernier, 1973), gut disease (Meuten *et al.*, 1978; Kaneko *et al.*, 1965), and intestinal parasitism (Dobson, 1965). The hypoalbuminemia of intestinal parasitism is aggravated by increased albumin catabolism (Cornelius *et al.*, 1962; Holliday *et al.*, 1968; Holmes *et al.*, 1968). Furthermore, due to the sensitivity of albumin to nutritional influences, albumin loss impairs albumin synthesis and further compounds the hypoalbuminemia. Usually, decreased albumin precedes the development of generalized hypoproteinemia in dietary protein deficiencies. The liver is the sole site of albumin synthesis, and hypoalbuminemia is an important feature of chronic liver disease.

b. Increased Globulins

i. α-Globulins. α_1-Globulin increases are not of great diagnostic significance in animals, whereas α_2-globulin increases are a common finding. Several α_2-globulins have

TABLE IV

Classification of Dysproteinemias Based on the A : G Ratio and the SPE Profile

A. Normal A : G—normal profile
  1. Hyperproteinemia: dehydration
  2. Hypoproteinemia
    a. Overhydration
    b. Acute blood loss
    c. External plasma loss: extravasation from burns, abrasions; exudative lesions; exudative dermatopathies; parasites
    d. Internal plasma loss: gastroenteropathies; parasites
B. Decreased A : G—abnormal profile
  1. Decreased albumin
    a. Selective loss of albumin: glomerulonephritis; nephrosis; nephrotic syndrome; gastroenteropathies; parasites
    b. Decreased synthesis of albumin: chronic liver disease; malnutrition; chronic inflammatory disease
  2. Increased globulins
    a. Increased α_2-globulin
      i. Acute inflammatory disease: α_2-macroglobulin, haptoglobin
      ii. Severe active hepatitis: α_2-macroglobulin
      iii. Acute glomerulonephritis: α_2-macroglobulin
      iv. Nephrotic syndrome: α_2-macroglobulin, α_2-lipoprotein (LDL)
    b. Increased β-globulin
      i. Acute hepatitis: transferrin
      ii. Nephrotic syndrome: transferrin
      iii. Suppurative dermatopathies: IgM, C_3
    c. β-γ bridging
      i. Chronic active hepatitis: IgA, IgM
    d. Increased γ-globulin (broad increases)—polyclonal gammopathy: IgG, IgM, IgA
      i. Chronic inflammatory disease, infectious collagen disease
      ii. Chronic hepatitis
      iii. Hepatic abscess
      iv. Suppurative disease processes: Feline infectious peritonitis, TB
      v. Immunologically mediated diseases: AIHA, AITP, polyarthritis, equine infectious anemia, collagen diseases, Aleutian disease, lupus, allergies, auto immune disease, glomerulonephritis
      vi. Tumors of the RES: lymphosarcoma
    e. Increased γ-globulin (sharp increases)—monoclonal gammopathy
      i. Tumors of the RES: lymphosarcoma
      ii. Multiple myeloma
      iii. Macroglobulinemia
      iv. Aleutian disease of mink
C. Increased A : G—abnormal profile
  1. Increased albumin: Does not occur, except dehydration
  2. Decreased globulins
    a. Fetal serum
    b. Precolostral neonate
    c. Combined immunodeficiency of foals

been classed as markers of acute inflammatory disease (Table III). A rise in α_2-macroglobulin is commonly seen in acute inflammatory disease. This may or may not be accompanied by increases in ceruloplasma or haptoglobin and is readily visualized on SPE (Fig. 2). In the nephrotic syndrome, α_2-globulins increase due to the α_2-macroglobulin and the α_2-lipoprotein (LDL). Low-density lipoprotein is also named β-lipoprotein because it migrates in the β region on paper.

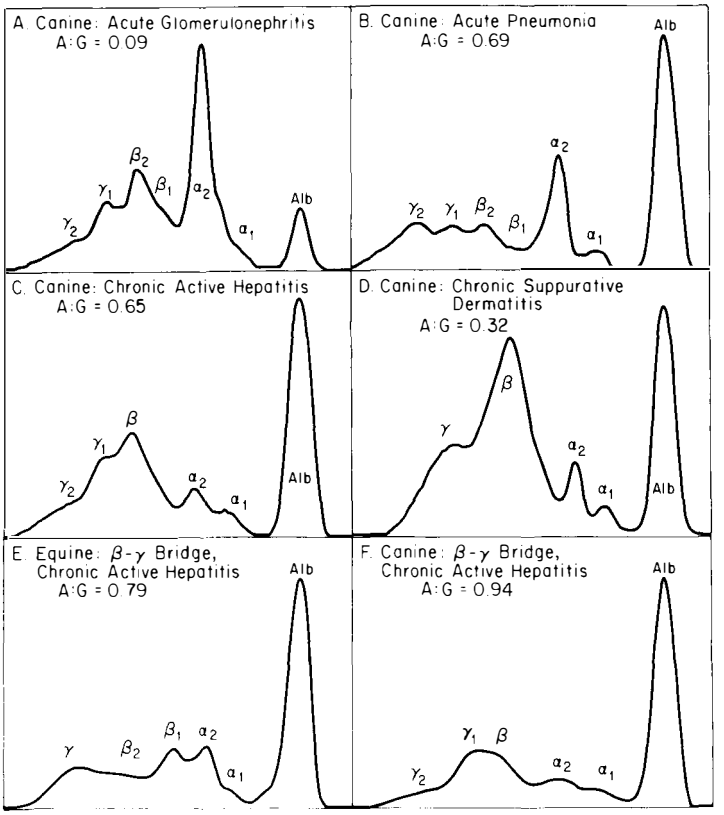


Fig. 2. Abnormal cellulose acetate electrophoretic profiles of serum proteins of domestic animals.

ii. β-Globulins. Increases in β-globulins alone are infrequent and are found in association only with active liver disease, suppurative dermatopathies, and occasionally the nephrotic syndrome. Transferrin appears to be the major component which rises in active liver disease together with hemopexin and complement. Immunoglobulin M can also rise in active liver disease in response to the antigenic stimulus of infectious agents. In the suppurative dermatopathies, a similar antigenic stimulus is thought to account for IgM and complement increases in the β fraction. In the nephrotic syndrome, increases in β-globulins are associated with increases in transferrin. Most increases in β-globulins are polyclonal, and only occasionally sharp monoclonal increases of multiple myeloma, Waldenstrom's macroglobulinemia, or lymphosarcoma are seen (Hurvitz *et al.*, 1977; MacEwen *et al.*, 1977).

iii. β–γ Bridging. The phenomenon of β–γ bridging is almost pathognomonic of chronic active hepatitis (Fig. 2). In this case, there is no clear separation between the β_2 and γ_1 fraction, which results from an increase of IgA, IgM, or both. Occasionally, a low-grade gammopathy of lymphosarcoma can result in a β–γ bridge.

iv. Increased γ-globulins (broad increase): polyclonal gammopathy. The diffuse or broad increases in γ-globulins which characterize polyclonal gammopathies (Fig. 3) are due to a variety of clones of plasma cells producing a heterogeneous mix of immuno-

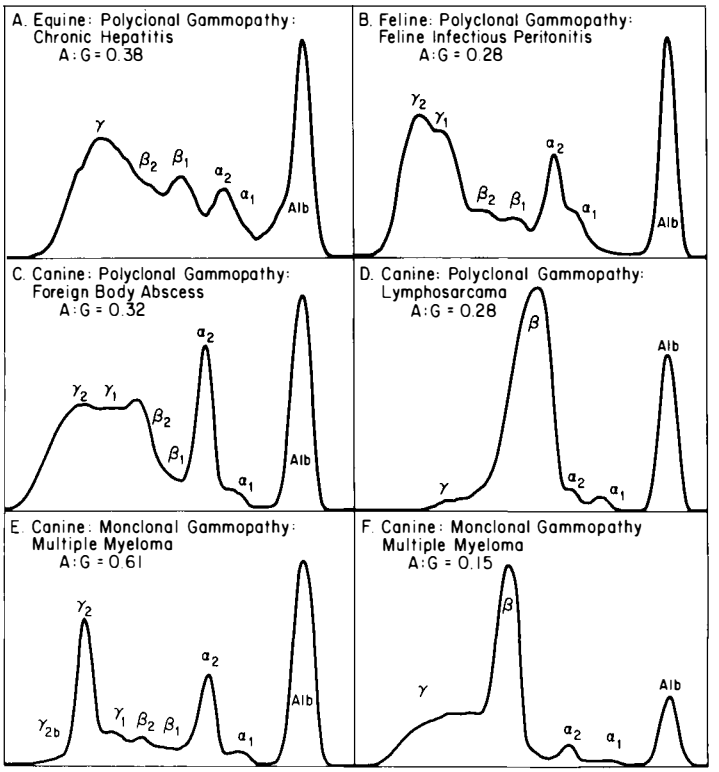


Fig. 3. Some abnormal cellulose acetate electrophoretic profiles of serum proteins of domestic animals.

globulins. Any one or all of the immunoglobulins IgM, IgG, and IgA can be present, but there is usually a preponderance of one.

The chronic inflammatory disease profile may be manifested by a variety of disease states, such as chronic infections, collagen diseases, and malignancies in general. There is a concomitant decrease in albumin as a result of decreased synthesis.

Chronic hepatitis, hepatic abscesses, and suppurative disease processes also exhibit changes characteristic of chronic disease (Rumbaugh *et al.*, 1978b). In these cases, the polyclonal increase is more marked and the hypoalbuminemia more severe than in chronic inflammatory disease. This phenomenon may merely be a reflection of the severity of the disease process and the more intense antigenic response generated.

Immunologically mediated disease processes are also characterized by polyclonal increases. These may take the form of immune processes directed against "self," i.e., autoimmune disease, or against external antigenic stimuli. In either case, multiple immunological response is elicited, one or more organs may be affected, and polyclonal increases are observed in the plasma. Immune complexes trapped in the glomeruli and reacting with antigens are thought to be involved in the glomerulonephritis often seen with heartworms, pyometra, and systemic lupus erythematosis (SLE). The latter is a multifaceted disease in the dog characterized by autoimmune hemolytic anemia (AIHA), thrombocytopenia (AITP), glomerulonephritis, and rheumatoid polyarthritis. The basic defect in SLE is the LE factor, an anti-DNA antibody, and the widespread dissemination of the

DNA–anti-DNA complex throughout highly vascular structures. The LE cell is a granulocyte which has phagocytized the DNA–anti-DNA complex.

Autoimmune hemolytic anemia is characterized by acute erythrocyte destruction, accelerated bone marrow response to the anemia, and the presence of autoantibodies against the patients's own erythrocytes. The antibodies may be of the warm or cold type but usually are warm. A prevailing view holds that the erythrocyte membrane is somehow altered to become a "foreign" antigen, which the body's immune mechanism now does not recognize as "self." Antierythrocyte antibodies are formed and coat the erythrocytes, and direct hemolysis, fragmentation, or phagocytosis occurs. Diagnosis of AIHA is made by demonstration of a positive direct Coomb's test.

Autoimmune thrombocytopenia is another form of autoimmune disease, in which the platelets have either absorbed viral or drug antigens on their surfaces and become coated with antibody or have adsorbed antigen–antibody complexes directly. These antibody-coated platelets are rapidly removed from the circulation (Wilkins *et al.*, 1973). Definitive diagnosis is made by use of the PF-3 test (Karpatkin and Siskind, 1969).

Rheumatoid arthritis is characterized by the development of rheumatoid factor, an autoantibody against IgG, and the immune complexes formed in the joints induce the chronic inflammatory lesion.

Tumors of the reticuloendothelial system (RES) exemplified by lymphosarcoma can elicit either a poly- or monoclonal response. The hyperglobulinemia peaks can occur anywhere between the β_1 and γ_2 regions and range from very broad, diffuse peaks to a very sharp monoclonal spike. The polyclonal peaks of lymphosarcoma are thought to be the result of a tumorous group of distantly related clones, in contrast to the single clones which give rise to the monoclonal spikes.

v. Increased γ-globulins (sharp increase): monoclonal gammopathy. The monoclonal forms are characterized by sharp "spikes" of immunoglobulin frequently but not limited to the γ region (Fig. 3). A useful interpretive guideline is to visually compare the sharpness of the peaks to the albumin peak. The monoclonal peak is as sharp or sharper than the albumin peak. The monoclonal spike is the result of a single clone producing a single class of immunoglobulin, usually abnormal in nature. These immunoglobulins have been described as "paraproteins" or as the "M" components because of the frequent occurrence of IgM. Waldenstrom's type of macroglobulinemia with hyperviscosity and IgM monoclonal spikes have been reported in dogs (Hurvitz *et al.*, 1970; MacEwen *et al.*, 1977). In multiple myelomas, Bence-Jones proteins (light chains) are detected in approximately 50% of the cases in human beings (Civantos *et al.*, 1973; Ritzmann *et al.*, 1972) and in dogs (Hurvitz, 1975). The characteristic monoclonal spike in the γ region, Bence-Jones proteinuria, and plasma cell tumors have been general findings in multiple myelomas reported in the horse (Cornelius *et al.*, 1959), dog (Osborne *et al.*, 1968; Shepard *et al.*, 1972), cat (Farrow and Penny, 1971), and rabbit (Pascal, 1961). Aleutian disease (AD) of mink, a valuable model of immunological disease, is also characterized by plasma cell infiltration, hypoalbuminemia, hyperproteinemia, and hyper-γ-globulinemia, frequently with a monoclonal spike (Leader *et al.*, 1963; Thompson and Aliferis, 1964). Light-chain disease, in which the "M" component was identified as L chains (Bence-Jones proteins) in the plasma and urine, was observed in dogs (Hurvitz *et al.*, 1971). Recently, an IgA monoclonal gammopathy in a dog without Bence-Jones proteinuria or plasma cell infiltration was also observed (Dewhirst *et al.*,

1977). Tumors of the RES, e.g., lymphosarcoma, frequently present as monoclonal spikes depending on the degree of cloning of the tumorous cells. The predominant "M" component was identified as IgM in a case of lymphocytic leukemia in a dog (Braund *et al.*, 1978).

Generally, the clinical characteristics of the monoclonal gammopathies are referrable to the magnitude of plasma cell proliferation, the extent of organ infiltration, and production of abnormal protein. Thus, immunologically associated diseases tend to be multifaceted and can present with bleeding tendencies, glomerulonephritis, polyarthritis, arteritis, hepatitis, and SLE. Treatment by cytotoxic drugs is largely ineffective, but symptomatic treatment by plasmapheresis is often followed by a period of clinical improvement. Infection is a common sequela and cause of death because of the suppression of normal antibody response.

3. Increased A:G—Abnormal Profile

a. Increased Albumin. True overproduction of albumin alone has not been known to occur in any animal. Any rise in albumin can be interpreted as dehydration.

b. Decreased Globulins. The absence of γ-globulins in fetal serum or in serum from precolostral or colostrum-deprived neonatal animals can be readily demonstrated on SPE (Fig. 1). Hypo-γ-globulinemia occurred in about 15% of foals less than 2 weeks of age and was attributed to failure of colostral transfer even though most had nursed (McGuire *et al.*, 1975b). Combined immunodeficiency of Arabian foals is thought to be an inherited autosomal recessive disease. The disease is characterized by lymphopenia, failure to synthesize IgG, IgM, and IgA, and early death (McGuire *et al.*, 1975a). Recently, a selective IgM deficiency was reported (Perryman *et al.*, 1977).

REFERENCES

Allison, A. C. (1960). *Nature (London)* **188,** 37.

Allison, J. B. (1957). *J. Am. Med. Assoc.* **164,** 283.

Benditt, E. P., Wissler, R. W., Woolridge, R. L., Rowley, D. A., and Steffee, C. H., Jr. (1949). *Proc. Soc. Exp. Biol. Med.* **70,** 240.

Bjorneboe, M., Raaschon, F., and Sondergard, T. (1952). *Acta Endocrinol. (Copenhagen)* **9,** 318.

Blackmore, D. (1978). Personal communication.

Booij, J. (1972). *Clin. Chim. Acta* **38,** 355.

Braund, K. G., Everett, R. M., and Albert, R. A. (1978). *J. Am. Vet. Med. Assoc.* **172,** 1407.

Campbell, R. M., Cuthbertson, D. P., Mackie, W., McFarlane, A. S., Phillipson, A. T., and Sudsaneh, S. (1961). *J. Physiol. (London)* **158,** 113.

Civantos, F., Dominguez, C. J., Rywlin, A. M., and DiBella, J. (1973). *Lab. Med.* **4,** 373.

Cohen, S. (1956). *Biochem. J.* **64,** 286.

Cornelius, C. E., Goodbary, R. F., and Kennedy, P. C. (1959). *Cornell Vet.* **49,** 478.

Cornelius, C. E., Baker, N. F., Kaneko, J. J., and Douglas, J. R. (1962). *Am. J. Vet. Res.* **23,** 837.

Davey, F. R., Carrington, C. E., and Nelson, D. A. (1972). *Clin. Chem. (N.Y.)* **18,** 1360.

Dewhirst, M. W., Stamp, G. L., and Hurvitz, A. I. (1977). *J. Am. Vet. Med. Assoc.* **170,** 1313.

Dick, J., and Nielson, K. (1963). *J. Comp. Med. Vet. Sci.* **27,** 269.

Dimopoullos, G. T. (1961). *Am. J. Vet. Res.* **22,** 986.

Dixon, F. J., Maurer, P. H., and Deechmiller, M. P. (1953). *Proc. Soc. Exp. Biol. Med.* **83,** 287.

Dobson, C. (1965). *Nature (London)* **207,** 1304.

Dunlap, J. S., and Dickson, W. M. (1955). *Am. J. Vet. Res.* **58,** 91.

Ebel, K. H. (1953). *Zentralbl. Veterinaermed.* **1,** 70.
Edelman, G. M. (1973). *Science* **180,** 830.
Erwin, E. S., Varnell, T. R., and Page, H. M. (1959). *Proc. Soc. Exp. Biol. Med.* **100,** 373.
Farrow, B. R. H., and Penny, R. (1971). *J. Am. Vet. Med. Assoc.* **158,** 606.
Fex, G., Laurell, C. B., and Thulin, E. (1977). *Eur. J. Biochem.* **75,** 181.
Forstner, M. J. (1968). *Zentralbl. Veterinaermed., Reihe A* **15,** 76.
Gorham, J. H. (1971). *J. Am. Vet. Med. Assoc.* **158,** 846.
Henry, R. J. (1964). "Clinical Chemistry," p. 197. Harper, New York.
Hoch-Ligeti, C., Irvine, K., and Sprinkle, E. P. (1953). *Proc. Soc. Exp. Biol. Med.* **84,** 707.
Holliday, G. J., Mulligan, W., and Dalton, R. G. (1968). *Res. Vet. Sci.* **9,** 224.
Holmes, P. H., Dargie, J. D., Maclean, J. M., and Mulligan, W. (1968). *Vet. Rec.* **83,** 227.
Hurvitz, A. I. (1975). *In* "Textbook of Veterinary Internal Medicine" (S. J. Ettinger, ed.), p. 1707. Saunders, Philadelphia, Pennsylvania.
Hurvitz, A. I., Haskins, S. C., and Fischer, C. A. (1970). *J. Am. Vet. Med. Assoc.* **157,** 455.
Hurvitz, A. I., Kehoe, J. M., Capra, J. D., and Prata, R. (1971). *J. Am. Vet. Med. Assoc.* **159,** 1112.
Hurvitz, A. I., MacEwen, E. G., Middaugh, C. R., and Litman, G. W. (1977). *J. Am. Vet. Med. Assoc.* **170,** 511.
Iammarino, R. M. (1972). *In* "Standard Methods of Clinical Chemistry" (G. R. Cooper, ed.), Vol. 7, p. 185, Academic Press, New York.
Jeffay, H., and Winzler, R. J. (1958). *J. Biol. Chem.* **231,** 111.
Kaneko, J. J., and Smith, R. (1967). *Calif. Vet.* **21,** 25.
Kaneko, J. J., Moulton, J. E., Brodey, R. S., and Perryman, V. D. (1965). *J. Am. Vet. Med. Assoc.* **146,** 463.
Karpatkin, S., and Siskind, G. W. (1969). *Blood* **33,** 795.
Kawai, T. (1973). "Clinical Aspects of the Plasma Proteins." Lippincott, Philadelphia, Pennsylvania.
Kniazeff, A. J., Rimer, V., and Gaeta, L. (1967). *Nature (London)* **214,** 805.
Larson, B. L., and Kendall, K. A. (1957). *J. Dairy Sci.* **40,** 659.
Leader, R. W., Wagner, B. M., Henson, J. B., and Gorham, J. H. (1963). *Am. J. Pathol.* **43,** 33.
Leveille, G. A., and Sauberlich, H. E. (1961). *J. Nutr.* **74,** 500.
Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265.
MacEwen, E. G., Hurvitz, A. I., and Hayes, A. (1977). *J. Am. Vet. Med. Assoc.* **170,** 1309.
McGuire, T. C., Banks, K. L., and Poppie, M. J. (1975a). *Am. J. Pathol.* **80,** 551.
McGuire, T. C., Poppie, M. J., and Banks, K. L. (1975b). *J. Am. Vet. Med. Assoc.* **166,** 71.
Mancini, G., Carbonara, A. O., and Heremans, J. R. (1965). *Immunochemistry* **2,** 235.
Mattheeuws, D. R. G., Kaneko, J. J., Loy, R. G., Cornelius, C. E., and Wheat, J. D. (1966). *Am. J. Vet Res.* **27,** 699.
Meuten, D. J., Butler, D. G., Thomson, G. W., and Lumsden, J. H. (1978). *J. Am. Vet. Med. Assoc.* **172,** 326.
Oppenheimer, J. H., Surks, M. I., Smith, J. C., and Squef, R. (1965). *J. Biol. Chem.* **240,** 173.
Osborne, C. A., and Vernier, R. L. (1973). *J. Am. Anim. Hosp. Assoc.* **9,** 101.
Osborne, C. A., Perman, V., Sautter, J. H., Stevens, J., and Hanlon, G. F. (1968). *J. Am. Vet. Med. Assoc.* **153,** 1300.
Pascal, R. R. (1961). *Cornell Vet.* **51,** 528.
Perk, K., and Loebl, K. (1960). *Refu. Vet.* **17,** 46.
Perryman, L. E., McGuire, T. C., and Hilbert, B. J. (1977). *J. Am. Vet. Med. Assoc.* **170,** 212.
Pfeiffer, N. E., McGuire, T. C., Bendel, R. B., and Weikel, J. M. (1977). *Am. J. Vet. Res.* **38,** 693.
Pierce, A. E. (1955). *J. Hyg.* **53,** 247.
Ritzmann, S. E., Daniels, J. C., and Lawrence, M. C. (1972). *Tex. Med.* **68,** 91.
Rook, J. A. F., Moustgaard, J., and Jacobsen, P. E. (1951). *K. Vet.- Landbohoejsk., Arsskr.* p. 81 [*Biol. Abstr.* **27,** 27237].
Rumbaugh, G. E., Ardans, A. A., Ginno, D., and Trommershausen-Smith, A. (1978a). *Am. J. Vet. Res.* **172,** 321.
Rumbaugh, G. E., Smith, B. P., and Carlson, G. P. (1978b). *J. Am. Vet. Med. Assoc.* **172,** 304.
Rutqvist, L. (1958). *Am. J. Vet. Res.* **19,** 25.
Shepard, V. J., Dodds-Laffin, W. J., and Laffin, R. J. (1972). *J. Am. Vet. Med. Assoc.* **160,** 1121.
Solomon, A. (1976). *N. Engl. J. Med.* **294,** 17.
Stockl, W., and Zacherl, M. K. (1953). *Hoppe-Seyler's Z. Physiol. Chem.* **293,** 278.
Sturkie, P. D. (1951). *Endocrinology* **49,** 565.

Tanaka, K., and Aoki, S. (1963). *Natl. Inst. Anim. Health Q.* **5,** 49.
Thompson, G. R., and Aliferis, P. (1964). *Arthritis Rheum.* **7,** 521.
Tumbleson, M. E., Burks, M. F., and Wingfield, W. E. (1972). *Cornell Vet.* **63,** 65.
Vaermans, J. P., and Heremans, J. F. (1969). *Immunochemistry* **6,** 779.
Weimer, H. E. (1961). *Ann. N.Y. Acad. Sci.* **94,** 225.
Wilkins, R. J., Hurvitz, A. I., and Dodds-Laffin, W. J. (1973). *J. Am. Vet. Med. Assoc.* **163,** 277.
Wissler, R. W., Woolridge, R. L., Steffee, C. H., Jr., and Cannon, P. R. C. (1946). *J. Immunol.* **52,** 267.

4

Porphyrin, Heme, and Erythrocyte Metabolism: The Porphyrias

JIRO J. KANEKO

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

I. INTRODUCTION

The metal-porphyrin complexes are found widespread in nature as constituents of compounds of fundamental importance in the metabolic processes of life. The photosynthetic pigment of plants, chlorophyll, is a magnesium-porphyrin. The iron-porphyrin complexes are found in combination with proteins, and these include the hemoglobins, myoglobins, cytochromes, and catalase. The porphyrins also exist in nature in the uncombined or free state, and it is this group which is associated with the porphyrias.

Present knowledge of the porphyrins has its basis in the classic studies of the German physician and chemist Hans Fischer, whose work on the porphyrins dates back to 1915. More recently, the development of more elegant methods of detection and identification of porphyrins, together with the use of isotopic tracer techniques, has resulted in the present understanding of the mechanisms of porphyrin biosynthesis and the biochemical bases for the disorders of porphyrin metabolism.

The metabolism of the erythrocyte has also received considerable attention in recent years (Bartlett and Marlow, 1951; Prankerd, 1961; Harris, 1963; Bishop, 1964; Valentine, 1971), due largely to interest in erythrocyte preservation and survival and to the discoveries of inherited erythrocyte enzyme deficiencies in man. Comparative aspects of erythrocyte metabolism have been reviewed (Kaneko, 1974).

II. ERYTHROPOIESIS*

The fundamental stimulus for erythropoiesis is tissue hypoxia, which in turn is controlled by the amount and ability of hemoglobin (Hb) to supply O_2 to the tissues. Thus, a wide variety of factors affecting Hb and its function secondarily affect erythropoiesis. These include the availability of iron, adequacy of circulation, ventilation, and oxygen dissociation.

A. Effective and Ineffective Proliferation

The normal proliferation sequence of the red blood cell (RBC) series is given in Fig. 1. The ordered progression of a single progenitor cell, the rubriblast, through four cell

*Abbreviations used throughout text and tables: EMP, Embden-Meyerhof pathway; PPP, pentose phosphate pathway; HK, hexokinase; GPI, glucose phosphate isomerase; PFK, phosphofructokinase; Aldo, aldolase; TPI, triosephosphate isomerase; GA3PD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; DPGAM, diphosphoglycerate mutase; DPGAP, diphosphoglycerate phosphatase; Enol, enolase; PK, pyruvate kinase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; LDH, lactate dehydrogenase; GOT, glutamic oxaloacetic transaminase; PGM, phosphoglucomutase; GR, glutathione reductase; GP_x, glutathione peroxidase; MR, methemoglobin reductase; Diaph, diaphorase; TK, transketolase; TA, transaldolase; GSSG, oxidized glutathione; PNP, purine nucleoside phosphorylase; ATPase, adenosine triphosphatase; Hex, Hexose; Gluc, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F16P, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; 1,3PGA, 1,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 3PGA, 3-phosphoglycerate; 2PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, Pyruvate; Lac, lactate; ADP, adenosene diphosphate; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide (DPN); NADH, reduced nicotinamide adenine dinucleotide (DPNH); NADP, nicotinamide adenine dinucleotide phosphate (TPN); Hb, hemoglobin; GSH, reduced glutathione; P_i, inorganic phosphate; RBC, red blood cell; MetHb, methemoglobin; NADPH, reduced nicotinamide adenine dinucleotide phosphate (TPNH); EP, erythropoietin; TCA, tricarboxylic acid; ALA, δ-aminolerulinic acid; PBG, porphobilinogen; CEP, congenital erythropoietic prophyria; EPP, erythropoietic protoporphyria.

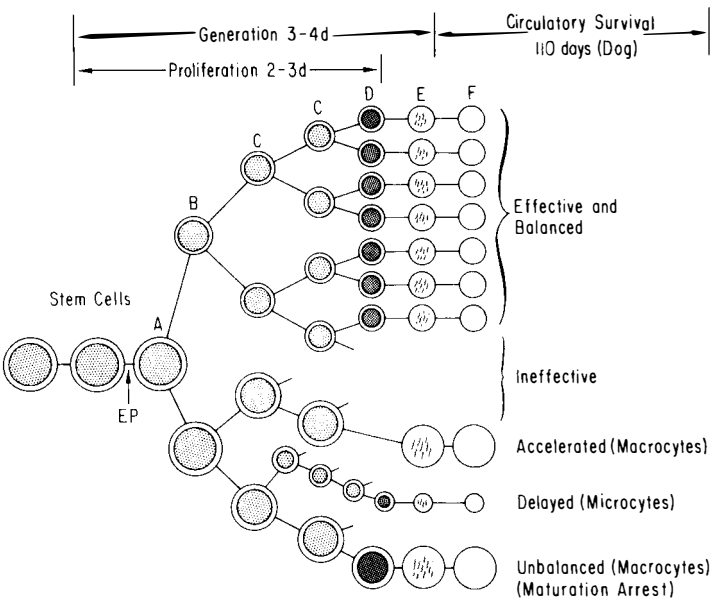


Fig. 1. Patterns of erythropoiesis. A, rubriblast; B, prorubricyte; C, rubricyte; D, metarubricyte; E, reticulocyte; F, red blood cell (see Fig. 2).

divisions results in the production of 16 (2^4) daughter metarubricytes (orthochromatic normoblasts). The metarubricytes then progressively differentiate and mature through reticulocytes to the mature cell. At the reticulocyte stage, cells are released from the bone marrow to the circulation, where they mature to the adult cell within a day and survive for a life span characteristic of the species.

The orderly progression of cell development has been termed effective erythropoiesis; however, not all rubriblasts proliferate to produce 16 daughter cells. A certain number of developmental cells normally undergo a form of destruction in the bone marrow with resulting ineffective erythropoiesis. Ineffective erythropoiesis is often increased under conditions of severe hemolytic anemia, e.g., in congenital erythropoietic porphyria of cattle (Kaneko, 1963), to the extent that even greater erythropoiesis must occur to compensate for and adequately replace lost red cells.

There are at least two major mechanisms by which the bone marrow can increase the rate of production of cells if one likens the schema to an industrial assembly line. One is to increase the number of progenitor (blast) cells, i.e., to multiply the number of assembly lines or, second, to increase the speed of each assembly line. There is no evidence that an increase in speed of erythrocyte proliferation occurs to a significant degree. Rather, more blast cells appear to be activated in order to increase erythropoiesis. The rate of increase must be rather prodigious considering that, in most cases, ineffective erythropoiesis is also increased during erythropoietic response to acute anemia.

The proliferative activities are controlled by a factor originating in the kidneys—erythropoietin. A profactor (β_2-globulin) is synthesized by the liver and transported to the kidney, where it is converted to active erythropoietin (EP) by the enzymatic action of a renal enzyme factor, renal erythropoietic factor, which is synthesized in the kidney (Zanjani *et al.*, 1969).

A proposed mechanism of action of EP in the normal physiological state, while not

uniformly accepted, is that it initiates differentiation of the committed marrow stem cell to the rubriblast. Under conditions of anemic stress and high EP concentrations, EP appears also to affect the cells of the maturation series. Early release of large reticulocytes may occur because of skipped terminal cell divisions, but the resulting macroreticulocytes have a reduced life span. It has also been proposed that EP induces hemoglobin synthesis and governs its rate of synthesis (Stohlman, 1968). When EP is in excess, Hb is rapidly synthesized, and, when a critical Hb concentration is reached in the cytosol, DNA synthesis is in turn shut off with consequent skipped terminal cell division and release of macrocytes. Conversely, Hb synthesis is delayed in iron deficiency. DNA synthesis is not shut off, more than the normal number of cell divisions can occur because the cells divide as soon as DNA is doubled, and microcytes are released to the circulation.

B. Balanced and Unbalanced Erythropoiesis

Another factor for consideration in erythropoiesis is the balanced, progressive development of the nucleus and the cytosol. As the nucleus passes through its series of proliferative mitotic phases, i.e., G_1, S, G_2, and M, a concomitant maturation of the cytosol occurs. This development can be readily observed by using light microscopy to detect progressive condensation of the nucleus and progressive increase in the Hb coloration of the cytosol. The changes in biochemical and metabolic activities as the RBC matures are depicted in Fig. 2. In the reticulocyte–metarubricyte, 95% of the synthesized

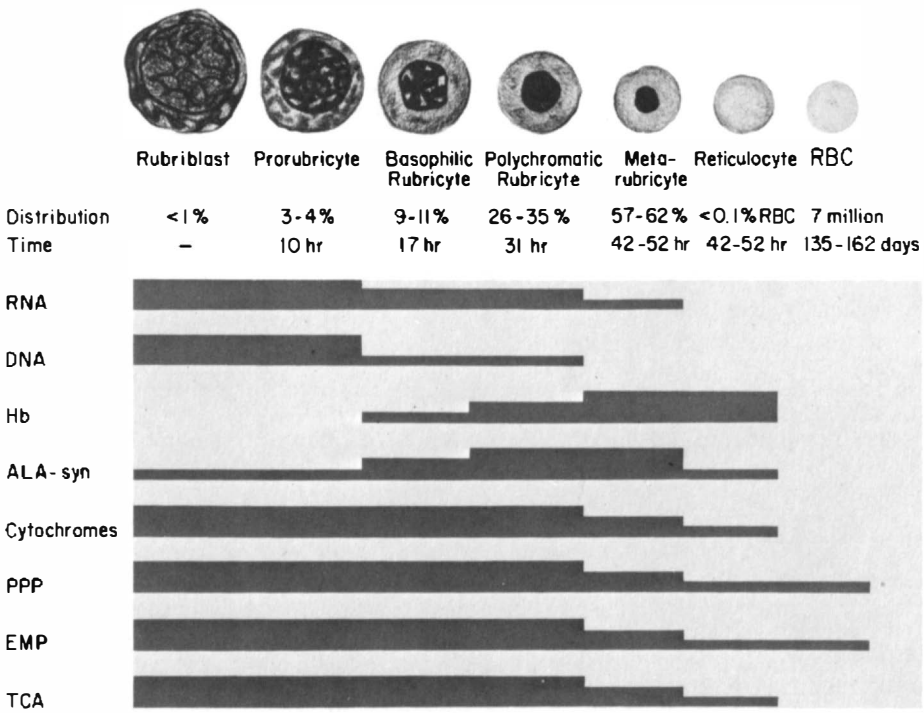


Fig. 2. Summary of metabolic activities of the erythrocytic maturation series. Maturation progresses from left to right. The time intervals indicated are for cows.

protein is Hb. The synthesis of DNA, the nuclear material which must be doubled for mitosis to occur, requires vitamin B_{12} and folic acid. It is now known that vitamin B_{12} is required for synthesis of tetrahydrofolic acid, which is required for DNA synthesis. In the presence of vitamin B_{12} and folic acid, ordered synthesis of DNA and mitosis can occur (Beck, 1964). Simultaneously, the protein synthetic machinery gradually shifts to the production of Hb.

Mammalian Hb is probably the most extensively studied protein, and studies of its synthesis have been the cornerstone of modern knowledge of protein structure. Genetic information in DNA is transcripted into messenger RNA according to a precise system of triplet nucleic acid base signals (genetic code). The coded RNA moves into the cytosol, where it combines with the polyribosomes to form the templates upon which the protein molecule is synthesized. Transfer RNA transports specified amino acids to polyribosomes to match the anticoded nucleic acid triplets (translation), and the amino acids are coupled together to form the specific polypeptide chains of the Hb of the species. The synthesis of Hb in animals has been reviewed by Kitchen (1969).

In a deficiency of vital factors, particularly vitamin B_{12} and folic acid, nuclear proliferation is delayed but protein synthesis itself progresses at its usual rate. Since cell division requires doubling of its chromosomes, i.e., DNA, the cell oversynthesizes protein while waiting for sufficient DNA to be synthesized. The common term "maturation arrest" is a morphological expression for this biochemical imbalance and describes the development of characteristic macrocytes and megaloblastic bone marrow of vitamin B_{12}–folate deficiency (Beck, 1964).

A deficiency of vitamin B_{12} through lack of nutrition is highly unlikely to occur in any animal, including man. Storage is generally adequate, turnover is slow, and absorption of B_{12} is generally adequate to forestall nutritional deficiency. Therefore, deficiency of B_{12} is more commonly seen in intestinal dysfunction, e.g., gastric failure with no intrinsic factor, (pernicious anemia, absorption failure). However, storage of folic acid is low, requirements for it are relatively high, and its turnover is rapid. Therefore, it is more likely that folate deficiency rather than B_{12} deficiency can occur. In either case, anemia due to insufficient erythropoiesis and characterized by macrocytosis and megaloblastic bone marrow is the salient feature of the deficiency.

III. THE DEVELOPING ERYTHROCYTE AND RETICULOCYTE

The developing erythrocyte is a cell comparable in all respects to cells of other tissues and is uniquely occupied with the synthesis of Hb. It contains a nucleus, cytoplasmic particles, and all the enzymatic machinery of other cells. During maturation, it respires actively, synthesizes protein and Hb, replicates, and differentiates. Near the end of maturation, it ceases replication and loses its nucleus to become a reticulocyte which continues to respire and synthesize Hb. Protein synthesis in the reticulocyte occurs on preformed RNA. When it loses its ribosomal reticulum (polyribosomes), it becomes a mature cell (Fig. 2).

A. Structure of the Porphyrins

The parent nucleus of the porphyrins is a cyclic tetrapyrrole, which consists of four pyrrole nuclei with their α-carbon atoms (adjacent to the N) linked together by methene

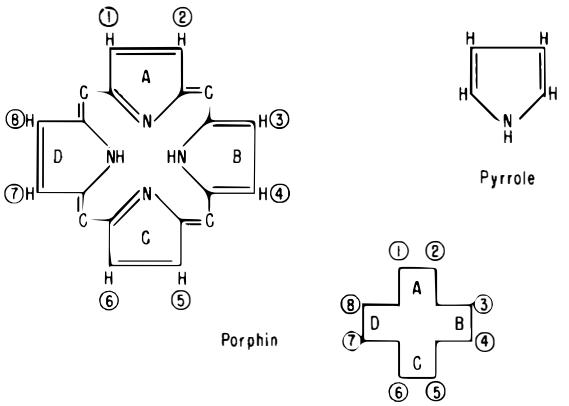


Fig. 3. Schematic representation of the porphin nucleus.

(—C═) bridges. This compound is called porphin and is shown in Fig. 3. The various synthetic and naturally occurring porphyrins are derivatives of porphin and are distinguished from each other by the type and position of the radicals substituted for the hydrogen atoms at positions 1 through 8. For convenience in discussing the substitutions, the simplified representation of the porphin nucleus, as shown in Fig. 3, is used.

The classification of the porphyrins is based upon the synthetic porphyrin etioporphyrin (ETIO), in which two different radicals are substituted. The substituted radicals are 4-methyl and 4-ethyl groups. The structural isomers possible with these eight radicals are the four shown at the top of Fig. 4. The porphyrins which occur in nature are only those in which the positioning of the substituted radicals corresponds to isomers I or III of etioporphyrin. This observation led to reference by Fischer of a "dualism" of porphyrins in nature, which is in essential agreement with present concepts of the biosynthesis of the porphyrin isomers as proceeding along parallel and independent paths.

The uroporphyrins also contain two different radicals, acetic acid and propionic acid, and four each of these are arranged to correspond to either isomer I or III (Fig. 4). Therefore, these are designated uroporphyrin I (URO I) or uroporphyrin III (URO III). Similarly, the coproporphyrins contain four methyl and four propionic acid groups and are designated coproporphyrin I (COPRO I) and coproporphyrin III (COPRO III). The protoporphyrin of heme (iron-protoporphyrin, the prosthetic group of hemoglobin) corresponds to the series III isomer. In this case, however, three different radicals, instead of two, are substituted. These consist of four methyl, two propionic acid, and two vinyl radicals. With three different radicals, a total of 15 isomers is possible, but the protoporphyrin of heme is the only naturally occurring isomer known. This isomer was designated protoporphyrin IX because it was ninth in the series of protoporphyrin isomers listed by Fischer. The arrangement of the methyl groups of this isomer as shown in Fig. 4 corresponds to that of a type III etioporphyrin isomer and is now termed protoporphyrin III (9) [PROTO III (9)].

Other naturally occurring or artificially prepared prophyrins are derivatives of PROTO III (9). If the two vinyl groups are hydrogenated to ethyl groups, the product is mesoporphyrin III (9). If the two vinyl groups are converted to hydroxyethyl groups, the product is hematoporphyrin III (9). Deuteroporphyrin III (9) results if the two vinyl groups are replaced by hydrogen atoms. It should be noted that protoporphyrin and deuteroporphyrin

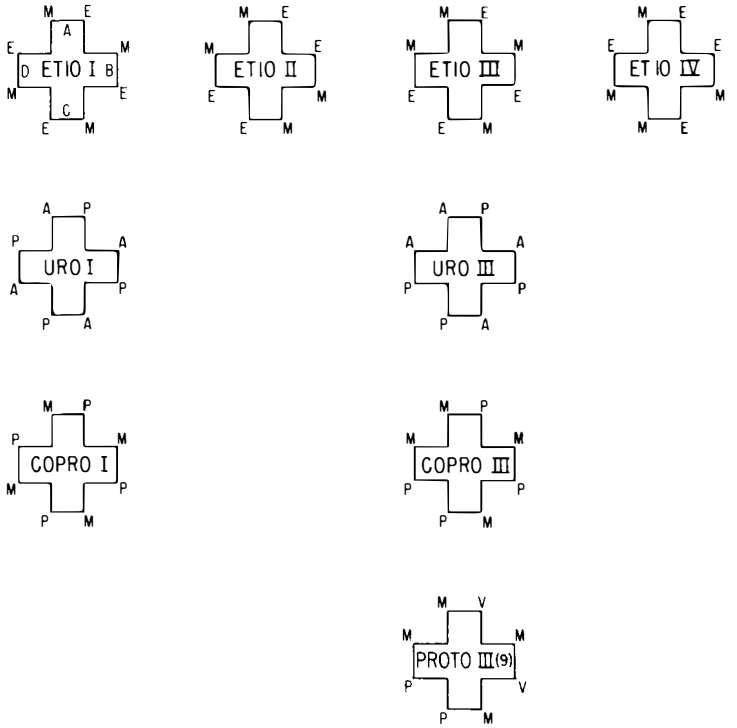


Fig. 4. The isomeric porphyrins (M, methyl; E, ethyl; A, acetic acid; P, propionic acid; V, vinyl).

occur in feces, but these are considered to be the result of intestinal bacterial action upon ingested meat.

B. Biosynthesis of the Porphyrins and Heme

Present knowledge of the pathway for heme biosynthesis has its basis in the demonstration by Shemin and Rittenberg (1946a) that the nitrogen atom of glycine is incorporated into the heme of hemoglobin. The concentration of the isotopic nitrogen (^{15}N) which was observed in heme indicated that the nitrogen atoms of glycine were direct precursors of the nitrogen atoms of the porphyrin ring. These findings were rapidly followed by extensive investigations which have almost completely elucidated the mechanisms of heme biosynthesis. The subject has been extensively reviewed, the most notable papers being those of Eales (1961), Tschudy (1965), Levere (1968), Schmid (1966), Marver and Schmid (1972), and Meyer and Schmid (1978).

It is now known that, in addition to contributing the nitrogen atoms, the methyl carbon atom (C-2) of glycine is also incorporated into the porphyrin ring. The methyl carbons supply 8 of the 34 carbon atoms of protoporphyrin: one for each of the four methene bridges and one for each of the pyrroles (Fig. 5). The carboxyl carbon atom of glycine is not incorporated into the molecule. The direct incorporation of the nitrogen atom and the methyl carbon atom of glycine into the heme of hemoglobin has been the basis for a useful technique with which to label the erythrocyte and measure its survival time. In their original studies, Shemin and Rittenberg (1946b) observed that after administering

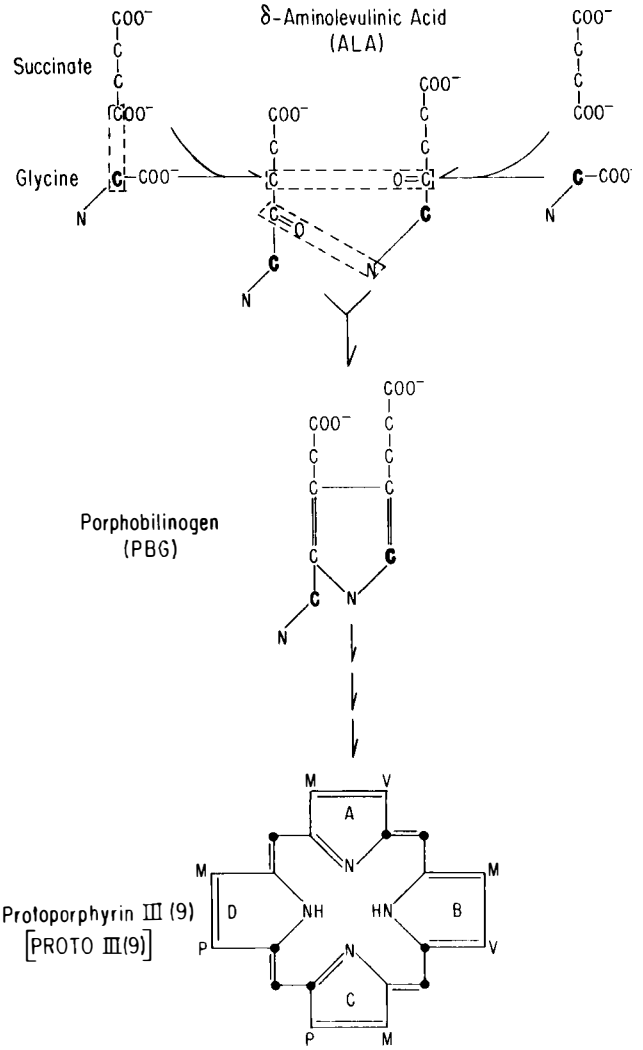


Fig. 5. The synthesis of protoporphyrin (M, methyl; V, vinyl).

[^{15}N]-glycine, the concentration of ^{15}N in the heme of hemoglobin rose rapidly, remained constant for a time, and then fell. Analysis of the data indicated a survival time of about 120 days for the human erythrocyte. On a similar basis, glycine labeled at the methyl carbon atom has been employed for studies of the survival time of the erythrocytes of a number of domestic animals (Table I).

The remaining carbon atoms of protoporphyrin are supplied by a tricarboxylic acid (TCA) cycle intermediate, succinyl-CoA. A schematic outline of the current concept of porphyrin biosynthesis is shown in Fig. 6.

1. δ-Aminolevulinic Acid

The initial step in the synthetic pathway involves the enzymatic condensation of glycine with succinyl-CoA (Figs. 5 and 6) to form δ-aminolevulinic acid (ALA). This reaction

TABLE I

Survival Time of Erythrocytes of Animals Determined Using [^{15}N] or [^{14}C]Glycine

Isotope	Species	Survival time (days)	Reference
^{15}N	Man	127	Shemin and Rittenberg (1946b)
	Rabbit	65–70	Neuberger and Niven (1951)
	Cat	77	Valentine *et al.* (1951)
^{14}C	Horse	140–150	Cornelius *et al.* (1960)
	Cow	135–162	Kaneko (1963)
	Sheep	64–118	Kaneko *et al.* (1961)
	Goat	125	Kaneko and Cornelius (1962)
	Guanaco	225	Cornelius and Kaneko (1962)
	Pig	62	Bush *et al.* (1955)
	Cat	66–79	Kaneko (1969a)
	Dog	86–106	Cline and Berlin (1963)
	Rat	64–68	Berlin *et al.* (1951); Berlin and Lotz (1951)
	Chicken	20	Brace and Altland (1956)
	Duck	39	Brace and Altland (1956)

requires the presence of vitamin B_6 as pyridoxal phosphate (Gibson *et al.*, 1958), and, in pyridoxine deficiency, anemia occurs. The pyridoxal-PO_4–glycine complex combines with succinyl-CoA (Kikuchi *et al.*, 1958). This condensing reaction, which is catalyzed by the enzyme ALA-synthetase (ALA-syn), occurs in the mitochondria. The ALA is then transferred to the cytoplasm (Sano and Granick, 1961). ALA-Synthetase is the rate-controlling enzyme for heme synthesis (Granick, 1966). Its formation is also known to be induced by the chemicals used in the experimental production of porphyria (Granick and Urata, 1963). The inducible synthesis of ALA-syn and its suppression by negative feed-

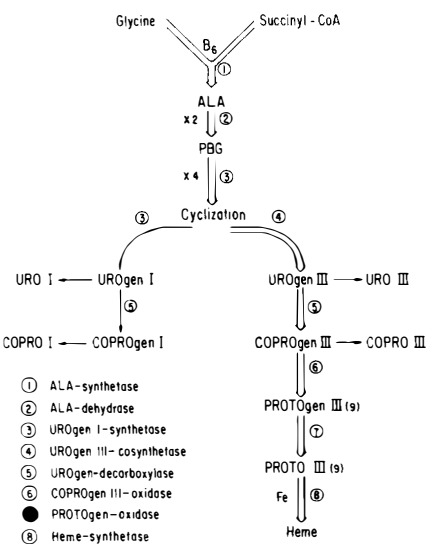


Fig. 6. Pathways of porphyrin biosynthesis. See text for abbreviations. Note that enzymes 3 and 4 are required to form the heme of hemoglobin.

back inhibition by heme is the basis for the mechanism controlling heme synthesis, as proposed by Granick and Levere (1964). Thus, the product of the reactions, heme, controls its own biosynthesis.

2. *Porphobilinogen*

The enzymatic condensation of two molecules of ALA (Figs. 5 and 6) to form the precursor pyrrole, porphobilinogen (PBG), is also known. This compound has been isolated from the urine of patients with the hepatic type of porphyria (Westall, 1952), and its structure (Fig. 5), as established by Cookson and Rimington (1953), is compatible with the mechanism shown in Fig. 6. This step and all those leading to coproporphrinogen (Fig. 6) occur in the cytoplasm of the cell. The enzyme catalyzing this reaction, ALA-dehydrase, has been reported to contain copper (Iodice *et al.*, 1958), and copper is a requirement for hemoglobin synthesis (Anderson and Tove, 1958). While the anemia of copper deficiency is well known, the mechanism by which copper contributes to hemoglobin synthesis remains unclear (Cartwright and Wintrobe, 1964). Studies of the anemia of copper deficiency in swine have indicated a close similarity to iron deficiency anemia and have led to the conclusion that copper deficiency affects iron metabolism in one or more ways (Bush *et al.*, 1956). The exact role of copper in heme biosynthesis continues to elude investigators.

3. *Porphyrinogens and Protoporphyrin*

The next intermediate is the cyclic tetrapyrrole uroporphyrinogen (UROgen). The gen forms are the reduced forms of the porphyrins on the pathway to heme formation. The oxidized forms, the uroporphyrins and coproporphyrins, are side-reaction products and do not participate in the pathway leading to heme formation (Fig. 6).

The mechanism of cyclization of the four molecules of PBG to form the UROgens remains obscure, although several hypotheses have been advanced (Bogorad and Granick, 1953; Shemin *et al.*, 1955; Granick and Mauzerall, 1958). The observations of Bogorad (1958a,b) on plant extracts provide an indication of the enzymatic complexity of this step. Uroporphyrinogen synthesis is the result of the combined action of at least two enzymes, porphobilinogen deaminase (PBG-D), now called uroporphyrinogen I synthetase (UROgen I-syn), and uroporphyrinogen isomerase (UROgen-Is), now called uroporphyrinogen III cosynthetase (UROgen III-cosyn). UROgen I-syn (PBG-D) first catalyzes the condensation of PBG to di- or tripyrroles, which are then cyclized in the presence of the second enzyme, UROgen III-cosyn (UROgen-Is), to yield UROgen III. If UROgen I-syn (PBG-D) is the only enzyme present in the system, type I uroporphyrinogen is formed. A system of this type has been demonstrated in animal tissues (Levin and Coleman, 1967). UROgen I-syn and UROgen III-cosyn were isolated from extracts of spleens from anemic mice, and, in the presence of both enzymes, UROgen III was produced. The exact mechanism of cyclization, however, is unknown.

The eight-carboxyl-group UROgens (I or III) are next progressively decarboxylated in a stepwise manner (de Viale and Grinstein, 1968), the principal product being the four-carboxyl-group COPROgen (I or III). The decarboxylating enzyme involved in this step is nonspecific, so that it catalyzes the decarboxylation of either the UROgen I or III isomer to the corresponding COPROgen. In the next step, however, the COPROgen-decarboxylating enzyme (COPROgen-oxidase) is specific for COPROgen III and does not decarboxylate COPROgen I. This enzyme catalyzes the transformation of two of the

propionic acid groups to two vinyl groups, and the resulting product is protoporphyrinogen III (PROTOgen III), a two-carboxyl porphyrinogen. The specificity of this decarboxylating enzyme for COPROgen III explains the occurrence of only type III isomers in nature. COPROgen I cannot be further oxidized and therefore cannot form heme (Fig. 6). Further oxidation of PROTOgen III then results in the formation of protoporphyrin III (9). Protoporphyrin III (9) combines with 4 moles of iron catalyzed by heme synthetase (ferrochelatase) to form the heme moiety of hemoglobin.

The scheme outlined in Figs. 6 and 7 portrays the current concept of heme biosynthesis, which places the uro- and coproporphyrins outside the mainstream of the synthetic pathway. The scheme in Fig. 6 indicates that the mechanism of cyclization remains to be clarified. It should also be noted that the mechanism of heme biosynthesis is an aerobic process associated with the mitochondria. The TCA cycle is an aerobic cycle, and therefore a lack of oxygen would preclude the synthesis of succinyl-CoA and hence of heme. The conversion of COPROgen III to PROTO III (9) and the incorporation of iron into PROTO III (9) to form heme are also oxygen-requiring systems.

Iron can be incorporated with relative ease by a nonenzymatic method into PROTO III (9). Conditions which help to maintain iron in its ferrous (2+) form, such as the presence of reducing agents (ascorbic acid, cysteine, glutathione) or anaerobiosis, enhance both enzymatic and nonenzymatic iron incorporation (Labbe and Hubbard, 1961). The enzymatic iron incorporation, however, is more than ten times that of the nonenzymatic incorporation. Heme synthetase (ferrochelatase) (Figs. 6 and 7) is localized in the mitochondria.

Mitochondria are a requirement for the initial synthesis of ALA and also for the final conversions of COPROgen to heme. The presence of mitochondria in the developmental stages and their absence in the mature, nonnucleated erythrocyte would account for the cessation of hemoglobin synthesis upon maturation. A control mechanism for heme biosynthesis has been proposed by Granick and Levere (1964) which is based upon the operon concepts of Jacob and Monod (1963). Heme is central to this mechanism because it can control its own synthesis by repressing the synthesis of ALA-syn and inhibit its

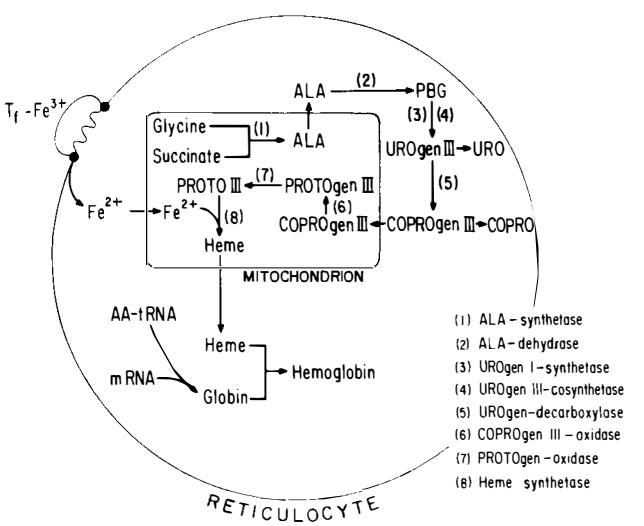


Fig. 7. Summary of hemoglobin synthetic mechanisms in the reticulocyte showing mitochondrial and cytoplasmic separation of activities.

activity by feedback inhibition. The overall mechanism and compartmentalization of hemoglobin synthesis in the developing erythrocyte are shown in Fig. 7.

C. Globin and Hemoglobin Synthesis

The requirements of mitochondria for heme synthesis and polyribosomes for globin synthesis preclude Hb synthesis in mature red cells, cells devoid of mitochondria or polyribosomes. Hemoglobin synthesis occurs in the anucleate reticulocyte, however, on residual polyribosomes. The mechanism for globin synthesis has been worked out in rather explicit detail, and descriptions can be found in most biochemistry or molecular biology texts. Briefly, polyribosomes in the cytoplasm contain the template RNA which has been coded by message from the DNA of the nucleus. ATP-Activated amino acids bound to transfer RNA bring the proper amino acid specified by the code into position, and they are then linked by a peptide bond. Since the RNA code specifies the amino acid sequence, the sequence is ultimately governed by the coding of genetic material, DNA. Completion of a single globin chain has been estimated to take 90 seconds (Itano, 1966).

The structure of Hb is also known in considerable detail, due largely to the work of Perutz (1965), who studied equine Hb and myoglobin. In general, all Hb's are globular proteins of four closely apposed globin chains, each chain containing a heme residue in a pocket. Human Hb is composed of two α chains each of 141 amino acids, two β chains each of 146 amino acids, and four associated hemes. The molecular weight is estimated to be 64,458 Mr. A shortened terminology is used in which a Greek letter specifies a polypeptide chain, a capital letter superscript denotes an Hb type, and a numerical subscript indicates the number of chains. Therefore, normal human Hb A is written $\alpha_2^A\beta_2^A$. Heme is held in a pocket of each chain by physical forces (hydrophobic) by linkage of the propionyl side chains of heme to lysyl residues of globin and by histidyl linkage to iron. The fifth coordinate bond of the Fe atom is linked to a histidyl residue at position 87 on the α chain and position 92 on the β chain. The sixth or remaining bond permits a loose association with molecular O_2 between it and a second histidyl residue in the chain. Transport of this O_2 bound to Hb does not require energy. The regulation of this binding of O_2 to Hb and its release, however, are strongly influenced by red cell metabolism and its metabolites, namely, 2,3-diphosphoglycerate (2,3-DPG).

The sequential amino acid structure attains additional metabolic significance because, of the total of six SH groups, two are readily accessible or "reactive." These "reactive" SH groups form mixed disulfides as a first step toward oxidative denaturation and Heinz body formation. The "nonreactive" groups lie buried deep within the molecule and are less accessible to oxidation. Presumably, oxidative denaturation of the Hb would have to have progressed far enough for the globular structure to be opened up to permit access to the buried SH groups.

D. Glutathione Synthesis

Glutathione (GSH) is a relatively simple compound synthesized not only by the immature but also by the mature RBC (Elder and Mortensen, 1956). The reactions of the GSH-synthetase system catalyzing the synthesis of this tripeptide are

$$\text{Glutamate} + \text{cysteine} \xrightarrow[\text{ATP, } Mg^{2+}]{\text{glut-cyst-syn}} \text{glutamylcysteine}$$

$$\text{Glycine} + \text{glutamylcysteine} \xrightarrow[\text{ATP, } Mg^{2+}]{\text{GSH-syn}} \text{glutathione}$$

The continued synthesis of GSH by the RBC even after maturation is an important mechanism for replenishing GSH since it has been shown that the oxidized form, GSSG, is readily transported out of the cell (Srivastava and Beutler, 1969).

E. Glucose Metabolism

The utilization rate of glucose by the nucleated RBC is estimated to be equal to that of any other nucleated metabolically active mammalian cell. Glucose utilization is comparable, and replication, protein synthesis, O_2 consumption, oxidative phosphorylation, and all synthetic and catabolic pathways, the pentose phosphate pathway (PPP), the Embdin-Meyerhof pathway (EMP), and Krebs TCA cycle are active. The only difference between the developing nucleated cell and other cells appears to be the extremely high content of 2,3-DPG in the former, as has been found in the bovine fetal RBC (Zinkl and Kaneko, 1973a,b). The definitions of pathways of carbohydrate metabolism common to all cells are available in standard works on biochemistry. Details of remaining pathways of the mature red cell are dealt with in later sections.

The developing red cell has a full complement of synthetic and metabolic mechanisms (Fig. 2). It respires, replicates, and synthesizes protein. In the course of its development, protein synthesis is gradually focused upon Hb, the main protein.

At the reticulocyte stage, Hb synthesis and respiration continue after loss of the nucleus, the former being dependent upon preformed RNA and residual cytochromes. These functions are lost within 12–48 hours after the nucleus disappears, after which the morphologically mature cell appears. The decrease in activity of the red cell enzymes begins at this time, and the mean adult degree of activity probably occurs at one-fourth to three-fourths of its life span. Near the end of the cell's life span, the critical loss of an enzyme(s), hexokinase (HK), occurs. The cell is unable to sustain itself and is removed from the circulation. The survival time of the RBC of a number of species is given in Table I.

IV. THE MATURE ERYTHROCYTE

The mammalian RBC is the most admirably suited cell for the study of comparative biology. It is among the most readily biopsied of tissues and easily separated from its surrounding medium (plasma) and associated cells [white blood cells (WBC), platelets]. The RBC can be obtained as a discrete fraction of a tissue more readily than any other cell. Furthermore, because of its ease of biopsy with relatively little trauma the RBC lends itself to time course studies, which are most important in the study of pathogenetic mechanisms.

The major physiological role of the RBC is the transport of O_2, the exchange of which is a process that does not require energy. It is now apparent that enclosure of the O_2 transport pigment hemoglobin within a membranous envelope imparts the following advantages to the RBC and to the organism which it supports (Harris and Kellermeyer, 1970):

1. Facilitates exchange of O_2 by bolus flow.
2. Decreases turbulence.
3. Permits survival of the hemoglobin for a period of months rather than the approximately 4–12 hours that free hemoglobin persists in plasma ($T_{1/2}$ 200 minutes).
4. Keeps hemoglobin in close proximity to the metabolically supported protective mechanisms.

5. Confines and maintains a solution of relatively high osmotic pressure. The osmotic pressure inside the red cell has been estimated to be five times greater than that of the plasma.

The mature RBC is devoid of nuclei and ribosomes and lacks a significant portion of the metabolic machinery characteristic of all nucleated cells. There is also no cytochrome system or Krebs cycle for terminal aerobic oxidation. Therefore, it synthesizes no proteins, maintains no oxidative phosphorylation, and consumes little O_2. It synthesizes only a few simple compounds, such as ATP, NAD, and GSH. The absence of a direct link to molecular O_2 (cytochromes) results in only a small but measurable O_2 uptake. Thus, the RBC is dependent mainly upon its preformed protein enzymes to permit survival of the red cell in a functional state for a life span characteristic for a species. The metabolic activities of the RBC are directed toward three major roles, all of which require energy:

1. The maintenance of the membrane and its deformability, which is vital to its ability to transverse the miles of capillary bed and, in particular, the splenic passages.
2. The maintenance of a critical ionic composition against a concentration gradient in most species, by use of an ATP-driven sodium pump.
3. The maintenance of an active reducing potential in the form of NADPH and NADH, which protects the hemoglobin and enzyme protein from oxidative denaturation.

The source of metabolic energy for these RBC-preserving mechanisms is the metabolism of glucose in all species with the possible exception of the domestic pig, which uses inosine. Glucose entry into the cell, while it does not require insulin, nevertheless is thought to be carried out through an active rather than a passive transfer mechanism. The focus of this section is the comparative metabolic fate of glucose within the RBC and the effects of this fate upon the ability of the RBC to carry out its vital O_2 transport function. The initial production, proliferation, maturation, and release are briefly described as a background for the understanding of the metabolic activities of the mature RBC. The human RBC has been reviewed frequently (Rorth, 1972; Beutler, 1972a; Kinoshita and Beutler, 1971). Intensive studies of the RBC began in the early 1940's with efforts to find better means of preserving human RBC for transfusion purposes. Further impetus came in the mid-1940's with the establishment of the University of Chicago–Army Medical Group to study antimalarial drugs. The group soon became aware of the strange hemolytic behavior of certain antimalarial drugs, mainly primaquine. The now classic studies (Carson *et al.*, 1956) initiated a virtual explosion of research on the intricate and active metabolic processes of the RBC. These studies established the nature of the hemolytic lesion as the result of an inherited deficiency of glucose-6-phosphate dehydrogenase (G6PD) in the RBC, now recognized as the most common RBC enzyme deficiency in human beings, affecting to various degrees 100,000,000 individuals. From this beginning, deficiencies of virtually all enzymes of the human RBC have been recorded. Paradoxically, parallel low activities of some of the same enzymes are known in domestic species; i.e., sheep have low G6PD activity but are inexplicably resistant to hemolysis by the same oxidant drugs known to readily precipitate hemolytic crises in human G6PD deficients. This could mean that the compensating metabolic activities of the red cells of the various animal species are unique to each species. Evidence of metabolic differences between RBC's is readily obtained since the RBC is easily obtainable as a discrete population of cells with similar metabolic pathways but with widely

differing metabolic activities, partition of metabolic pathways, and partition of metabolic energy. This would suggest that each species has evolved a unique pattern of RBC metabolism, the ultimate aim of which is the common denominator of O_2 transport.

The mature erythrocyte, lacking a nucleus, ribosomes, and mitochrondria, restricts its metabolism to anaerobic glycolysis (EMP) and PPP pathways. Heme and hemoglobin synthesis ceases, and there is no cytochrome or TCA cycle activity. The limited metabolism of the erythrocyte still permits it to survive for a life span characteristic of the species (Table II).

A. Overall Glucose Metabolism

The mature RBC requires energy to maintain its shape, structure, and internal ion composition and to synthesize some simple compounds such as GSH and NAD. Oxygen transport by Hb requires no expenditure of energy. A reducing potential, however, must be maintained within the RBC in order to maintain the vital enzymes and Hb in their functionally reduced state. Energy in the form of ATP and reducing potential in the form of NADH and NADPH are generated by the oxidation of glucose through a few residual pathways (Fig. 8).

The mature RBC has neither a cytochrome system nor a Krebs TCA cycle; therefore, there is a metabolic gap in its ability to utilize molecular O_2 for oxidative process as do other complete cells. This inability to use O_2 would impart certain advantages to the RBC since the oxidative pressure exerted by the high concentration of the O_2 in transport would be large. There is only a small, but measurable O_2 uptake by the RBC; it amounts to about 5% of that of a complete cell (Bishop, 1964). The little O_2 that is utilized is thought to be involved in the several direct oxidative reactions of the RBC:

$$\mathrm{Hb(Fe^{2+}) + O_2 \rightarrow H_2O_2 + MetHb(Fe^{3+})} \quad (1)$$

$$\mathrm{Hb(SH) + O_2 \rightarrow H_2O_2 + (HbS)} \rightarrow \begin{cases} \mathrm{HbSSHb_n} & \text{(Heinz body)} \\ \mathrm{HbSSG} & \text{(mixed disulfide)} \end{cases} \quad (2)$$

$$\mathrm{GSH + O_2 \rightarrow H_2O_2 + (GS)} \rightarrow \mathrm{GSSG} \quad (3)$$

$$\mathrm{Ascorbate + O_2 \rightarrow H_2O_2 + dehydroascorbate} \quad (4)$$

It is clear from Table III that the RBC's of all species utilize glucose. It is also readily apparent that the pig RBC is remarkable in its low or even virtual absence of glucose utilization (Engelhardt, 1930). The basis for this low glucose utilization is the inability of the pig RBC to transfer glucose (Kim and McManus, 1971). Once within the cell, however, it has the metabolic capability of utilizing glucose. It has been postulated that the pig uses the nucleoside inosine as an energy source.

$$\mathrm{Inosine + P_i \rightarrow ribose\text{-}1\text{-}P + hypoxanthine} \quad (5)$$

$$\mathrm{Ribose\text{-}1\text{-}P \rightarrow ribose\text{-}5\text{-}P} \rightarrow \mathrm{PPP \rightarrow EMP \rightarrow ATP} \quad (6)$$

This pathway is advantageous in that expenditure of ATP is not required for the generation of fructose 6-phosphate (F6P). The overall rates of glucose utilization among the various species also vary considerably and can be roughly grouped as very low (pig), low (cow, sheep), intermediate (dog, horse), and high (man, rabbit, rat) (Table II).

The partitioning of glucose metabolism by the human RBC is depicted graphically in Fig. 9. About 5–10% of the glucose is oxidized in the PPP, 30% by the unique DPG pathway of the red cell, and 65% via the EMP to lactate/pyruvate (Murphy, 1960).

TABLE II

Erythrocyte Glucose Utilization, Ion Concentrations, and Survival Times in Various Animal Species[a]

Species	RBC glucose utilization (μmoles/hour/ml)	RBC Na (mEq/liter)	Serum Na (mEq/liter)	RBC K (mEq/liter)	Serum K (mEq/liter)	RBC survival (days)
Man	1.18 ± 0.20 (6) 2.72 (9) 1.48 ± 0.11 (27)	21 (17)	142 (47)	155 (17)	5.5 (47)	120 (48)
Dog	2.24 ± 0.49 (16) 2.99 (9) 1.33 ± 0.12 (27)	107 (11) 135 (49)	146 (12)	9 (11) 10 (49)	5 (12)	97–133 (32) 86–106 (33)
Cat	0.94 ± 0.09 (27)	104 (11) 142 (49)	151 (12)	6 (11) 8 (49)	4.13 (12)	66–79 (34)
Pig	0.14 (9)	11 (11)	142 (12)	100 (11)	4.8 ± 0.4 (12)	86 ± 11.5 (35)
Nonhuman primate		Rh, 24 (49)	Rh, 148 ± 4.3 (36)	Rh, 145 (49)	Rh, 4.71 ± 0.53 (36)	98 (53)
Aquatic mammal	S, 1.59 ± 0.50 (6)	S, 147 ± 8 (6) D, 13.2 (50)	S, 161 ± 4 (6)	S, 8.3 ± 0.6 (6) D, 99.4 (50)	S, 4.2 ± 0.3 (6)	
Horse	0.64 ± 0.10 (27)	79 (11) 16 (49)	139 ± 3.5 (12)	88 (11) 140 (49)	3.51 ± 0.57 (12)	140–150 (37)
Cow	0.58 (9)	79 (11) 72 ± 8.8 (14)	142 (12)	22 (11) 24 ± 6.9 (14)	4.9 (16)	135–162 (38) 160 (39)
Sheep	1.06 (9) 0.69 ± 0.19 (23)	84 (11) 16 (11) 73.7 (52) 17.1 (52)	145 (12)	LK, 18 (11) HK, 64 (11) LK, 39.4 (52) HK, 98.7 (52)	4.8 (12)	64–94 (40) 131–157 (41)

Goat			150 ± 3.1 (12)		5.0 (12)	125 (45)
Rabbit	2.95 (9)	22 (49)	125.4 ± 9.7 (51)	142 (49)	5.1 ± 0.9 (51)	57 ± 0.2 (42)
Guinea pig	1.44 (9)		122.2 ± 10 (51)		4.87 ± 0.84 (51)	79 (44)
Rat	5.60 (9)	28 (49)	118.3 ± 11.1 (51)	135 (49)	2.70 ± 0.46 (51)	55 (43)
Mouse						20–30 (65)
Hamster						60–70 (64)

[a] All enzyme units are micromoles per minute per 100 gm Hb, except ATPase, given in nanomoles P_i per milligram membrane protein per hour. All chemical constituents are in micromoles per 100 ml RBC except where noted. All values have been recalculated to permit direct comparison between species. Standard deviation values are given where indicated. The figures in parentheses are the references cited below and apply to all tables. Abbreviations used are given in the footnote on p. 00. Abbreviations for animal species in the tables are Rh, rhesus; B, baboon; D, dolphin; P, porpoise; S, seal; R, rat; GP, guinea pig; H, hamster; M, mouse. LK = low potassium sheep, HK = high potassium sheep. Temperatures above 25°C are included in reference citations. References: (1) Harkness *et al.* (1969); (2) Harkness and Ponce (1969); (3) Kaneko (1973); (4) Zinkl and Kaneko (1973a,b); (5) Schecter *et al.* (1973); (6) Robin *et al.* (1971); (7) Proctor *et al.* (1971); (8) Cheun (1966); (9) Laris (1958); (10) Kaneko *et al.* (1969b); (11) Prankerd (1961); (12) Kaneko (1973); (13) Blunt *et al.* (1971); (14) Kaneko and Mills (1969c); (15) Smith *et al.* (1965); (16) Searcy *et al.* (1971); (17) Beutler and Srivastava (1972) (at 37°C); (18) Hutton (1972); (19) Sugarman *et al.* (1972); (20) Paniker and Beutler (1972) (at 37°C); (21) Oski *et al.* (1972) (at 30°C); (22) McManus (1967) (at 37°C); (23) Leng and Annison (1962); (24) Smith *et al.* (1972a) (at 37°C); (25) Passo *et al.* (1971); (26) Beutler (1975) (at 37°C); (27) Harvey and Kaneko (1976); (28) Smith *et al.* (1968) (at 37°C); (29) Salvidio *et al.* (1963); (30) Smith and Osburn (1967); (31) Kaneko and Smith (1964); (32) Weissman *et al.* (1960); (33) Cline and Berlin (1963); (34) Kaneko *et al.* (1969a); (35) Bush *et al.* (1955); (36) Robinson and Ziegler (1968); (37) Cornelius *et al.* (1960); (38) Kaneko *et al.* (1971); (39) Kaneko (1963); (40) Kaneko *et al.* (1961); (41) Judd and Matrone (1962); (42) Gower and Davidson (1963); (43) Forssberg and Tribukait (1962); (44) Edmondson and Wyburn (1963); (45) Kaneko and Cornelius (1962); (46) Bruns *et al.* (1958a,b); (47) Tietz (1976); (48) Garby (1962); (49) Bernstein (1954); (50) Eichelberger *et al.* (1940); (51) Burns and DeLannoy (1966); (52) Tucker and Ellory (1971); (53) Marvin *et al.* (1960); (54) Burr (1972); (55) Whaun and Oski (1969); (56) Bonting *et al.* (1961); (57) Chan *et al.* (1964); (58) Keeton and Kaneko (1972); (59) Dick *et al.* (1969); (60) Tosteson (1963); (61) Yunis and Arimura (1966); (62) Bartlett (1970); (63) Bunn (1971); (64) Rigby *et al.* (1961).

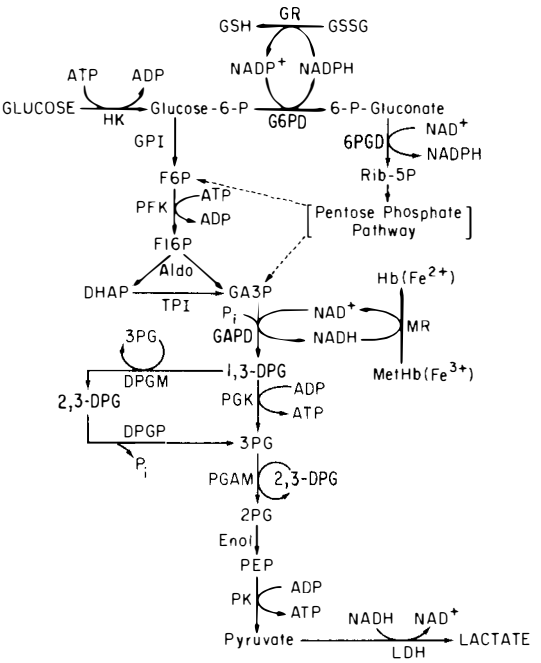


Fig. 8. Metabolic pathways of the mature RBC (for abbreviations, see footnote, p. 120).

Little is known about the exact mechanism of transfer of glucose across the red cell membrane (LeFevre, 1961). It does not require insulin, but it is likely to be more than a passive transfer of glucose. There are also marked species and age differences with respect to the ease of transfer across the RBC membrane. This cell of the adult cow is much less permeable to glucose than that of the human adult or infant (Bishop, 1964) or the newborn calf (Johnson and Stewart, 1967). The inability of the pig RBC to transfer glucose has already been alluded to.

B. Initial Phosphorylation of Glucose: Hexokinase

After glucose gains entry to the red cell, the first step is its phosphorylation to glucose 6-phosphate (G6P) catalyzed by the enzyme hexokinase. ATP is the phosphate donor, and Mg^{2+} is the essential cofactor, similar to most enzymatic phosphorylation reactions. Hexokinase is critical because of its position in the first enzymatic step (Fig. 8) in glucose utilization and its uniformly low activity in all species (Tables III and IV).

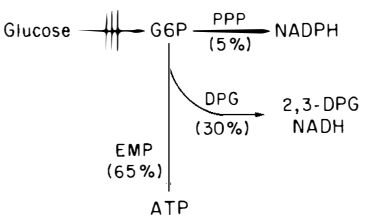


Fig. 9. Partitioning of glucose metabolism in the RBC (for abbreviations, see footnote, p. 120).

Hexokinase is generally regarded as the rate-limiting enzyme of RBC glycolysis (Rapoport, 1968). Low activity is regarded as evidence of rate limitation, and the data in Table IV indicate that HK is lowest in activity in all the species listed. Other lines of evidence also support this concept. The ratio of HK to glucose utilization is relatively constant; both decline in parallel with aging of RBC, and the pH optimum is the same, 8.1, as for glucose utilization (Chapman *et al.*, 1962). Furthermore, by thermodynamic analysis to compare reactant concentrations with the equilibrium constants, Minakami and Yoshikawa (1966) concluded that HK was a "controlled" reaction even though it might not be the only one. Zinkl and Kaneko (1973a,b) arrived at similar conclusions regarding bovine fetal, calf, and adult red cells on the basis of both enzymatic activities and thermodynamic analysis of glycolytic intermediate data (Tables V and VI).

In addition to glucose, mannose and fructose but not galactose may serve as substrates for HK. Beutler (1972b) gives the V_{max} for HK as 0.64 μmole of glucose per minute per gram Hb, which is about five times the rate of glucose utilization in the RBC. This also means that HK is saturated at the blood glucose level.

Hexokinase is strongly inhibited by its product, G6P, and P_i is reported (Rose *et al.*, 1964) to relieve this inhibition. This would explain in part the stimulatory effect of P_i on red cell glucose metabolism. ATP also stimulates the HK reaction. In addition, GSSG (Beutler and Teeple, 1969) and 2,3-DPG are also inhibitory to HK (Dische, 1964; Srivastava and Beutler, 1972). Two isoenzyme bands of HK have been identified electrophoretically (Malone *et al.*, 1968). The K_m (ATP) is 3 m*M* for HK according to Rapoport (1968) and 1 m*M* for HK according to Rose (1971).

Hexokinase activity is very high in young cells and declines with aging; it has been used as an index of red cell age. A hereditary deficiency of HK is a rare cause of nonspherocytic hemolytic anemia (Valentine, 1971).

C. Pentose Phosphate Pathway

A relatively small but extremely important percentage (5–10%) of the G6P is oxidized via the PPP. This is the only route by which CO_2 is generated in the mature RBC. The generation of NADPH in this process serves a vital function as a link in the reduction of GSSG to GSH. For greater detail of the PPP in red cells, the reader is referred to the work of Dische (1964).

1. G6PD and 6PGD

In the first step, G6P is oxidized to 6-phosphogluconate by the enzyme G6PD; in the process, NADP is reduced to NADPH. Since its establishment as the deficient enzyme in hereditary primaquine sensitivity, G6PD has been the most extensively studied RBC enzyme. Now, over 100 variants of this enzyme have been reported in human beings (Beutler, 1972b). Favism is a variant form of G6PD deficiency. It is sex-linked in man and in the horse (Trujillo *et al.*, 1965; Mathai *et al.*, 1966) and is likely to be so in all other species. A molecular weight of 105,000 has been reported (Ratozzi, 1968). The dimeric form is activated and stabilized by binding with NADP or NADPH, while the unbound form is an unstable monomer (Kirkman, 1962). The optimal pH is between 8 and 9 (Anstall and Trujillo, 1967). The K_m (G6P) of G6PD is 3.5×10^{-5} *M* (3.5 μmole/100 ml), and that of NADP is 4×10^{-6} *M* (Marks, 1964). The reported concentrations of 5.2 μmole G6P per 100 ml for the bovine RBC (Zinkl and Kaneko, 1973a) and 0.9 μmole

TABLE III

Erythrocyte Enzymes of Various Animal Species[a]

Enzyme	Man	Dog	Cat	Pig	Nonhuman primate	Aquatic mammal
HK	64 ± 27 (26) 35 ± 2 (27)	14 ± 1 (27)	10 ± 1 (27) 7.2 ± 1.8 (3)	19.2 ± 11 (22)		
GPI	2423 ± 20 (27) 2841 ± 687 (28)	1307 (15) 1701 ± 76 (27)	4441 ± 220 (27)	1643 (15) 9270 ± 480 (22)	Rh, 6693 (15)	
PFK	1260 ± 166 (26) 360 ± 38 (27)	31 ± 2 (27)	64 ± 3 (27)	125 ± 25 (22)		
Aldo	322 ± 49 (26)	126 (15)	190 (15) 80 ± 20 (22)	136 (15)		
TPI	144,000 ± 16,000 (26)			75,000 ± 6000 (22)		
GAPD	3858 (1) 5672 ± 597 (26)	3935 (1) 927 (15)	2006 (1)	1659 (1) 1194 (15) 4125 (22)	Rh, 1112 (15)	D, 810 (1)
PGK	3545 (1) 16,000 ± 952 (26)	815 (1)	992 (1)	1099 (1) 9600 ± 1875 (22)		D, 1347 (1)
PGAM	745 (1) 2230 ± 348 (26)	253 (1)	216 (1)	343 (1) 2785 ± 370 (22)		D, 499 (1)
PGM	308 ± 60 (26)					
GR	568 ± 152 (26) 346 ± 23 (27)	57.5 ± 19.9 (3) 137 ± 7 (27)	110 ± 48 (5) 405 ± 48 (27)	68.2 ± 9.2 (3)		
GP_x	532 ± 179 (26) 3497 ± 132 (27)	8921 ± 237 (27)	12,135 ± 616 (27)			

NADH-MR						
NADPH-diaph	134 ± 32 (20)	53 ± 3 (27)	39 ± 2 (2)			
	43 ± 2 (27)					
TK	18 ± 5 (27)	29 ± 4 (27)	35 ± 2 (27)	105 ± 20 (22)		
	23.5 (46)			20 (46)		
PNP	36,600 ± 3300 (21)	0 (21)	1800 ± 600 (21)	1830 ± 195 (22)	Rh, 27,000 ± 2100 (21)	
ATPase	558 ± 180 (55)	Nil (57)	Nil (56)			
	356 (57)		Nil (57)			
DPGM	68 (1)	35 (I)	2.7 (1)	19.7 (1)		D, 15.6 (1)
DPGP	0.71 (1)	0.33 (1)	0.18 (1)	0.23 (1)		D, 0.44 (1)
Enol	754 ± 137 (26)			875 ± 190 (22)		
PK	538 ± 167 (26)	359 ± 63 (3)	731 ± 197 (5)	152 ± 13 (3)		
		469 ± 86 (16)		1275 ± 140 (22)		
G6PD	427 ± 16 (27)	589 ± 127 (3)	969 ± 226 (5)	752 ± 81 (3)	Rh, 289 (15)	
	1355 ± 245 (26)	528 ± 129 (8)	682 ± 63 (27)	691 (15)	Rh, 509 ± 182 (8)	
	284 (28)	395 (15)		1765 ± 35 (22)		
	497 (29)	495 ± 16 (27)		580 (28)		
		442 (28)		542 (29)		
6PGD	860 ± 108 (26)	119 ± 4 (27)	173 ± 8 (27)	335 ± 40 (22)		
	200 ± 6 (27)	140 (29)		64 (28)		
	150 (28)			117 (29)		
	284 (29)					
LDH	14,000 ± 3500 (26)	1362 ± 348 (3)	1507 ± 218 (5)	728 ± 220 (3)	Rh, 2033 ± 460 (3)	
				2930 ± 175 (22)		
GOT	308 ± 58 (26)		113 ± 18.6 (3)			

[a] Given in micromoles per minute per 100 gm Hb. See footnote to Table II.

TABLE IV

Erythrocyte Enzymes of Various Animal Species[a]

Enzyme	Horse	Cow	Sheep	Goat	Rabbit	Mouse
G6PD	765 ± 192 (3)	540 ± 49 (4)	77 ± 23 (3)	133 ± 51 (3)	569 ± 73 (8)	1180 ± 60 (18)
	767 ± 83 (10)	244 ± 41 (14)	57.6 ± 18 (8)	95 ± 13 (8)	1296 (29)	R, 663 ± 57 (8)
	1005 ± 200 (15)	369 (15)	41.2 (15)	66 (28)	GP, 453 ± 57 (8)	
	2740 ± 127 (24)	335 (28)	38 (28)			
	890 (28)	345 (29)	59 (29)			
	798 (29)		82 ± 3 (31)			
6PGD	81.1 ± 9.86 (24)	83.6 ± 19.4 (4)	33 (28)	63 (28)		220 ± 20 (18)
	48.5 (28)	38 (28)	60 (29)			
	132 (29)	82 (29)				
LDH	507 ± 91 (3)	663 ± 132 (4)	516 ± 145 (3)	346 ± 76 (3)		12,200 ± 800 (18)
	345 ± 53 (10)					
	3230 ± 114 (24)					
GOT	71.5 ± 12.2 (3)	36.6 ± 15.2 (3)				
PGM		374 ± 14.7 (4)				
GR	33.3 ± 10.5 (3)	19.5 ± 3.9 (4)	34.3 ± 7.5 (3)	98 ± 16 (3)		420 ± 110 (18)
	18 ± 3.4 (10)					
	279 ± 31.4 (24)					
GP_x						
NADH-MR		183 ± 24 (4)				
NADPH-diaph	69.4 ± 5.0 (27)	45.1 ± 9.2 (20)				
TK	14.5 (46)	23 (46)			64 (46)	58.5 (46)
					GP, 52 (46)	R, 40.5 (46)

PNP					14,700 ± 3300 (21)	10,800 ± 120 (21)
ATPase		77.7 (58)	HK, 92 (59)		475 (61)	R, 570 (61)
			LK, 23 (59, 60)			R, 73 (57)
HK	243 ± 38 (24)	35.7 ± 14.4 (4)				99 ± 11 (18)
	48.4 ± 6.8 (3)	33.6 ± 7.9 (3)				
GPI	2680 (15)	1643 (15)				
	3130 ± 221 (24)	1695 ± 95 (4)	1383 (15)			3100 ± 500 (18)
PFK	352 ± 25 (24)	243 ± 126 (4)				440 ± 20 (18)
Aldo	818 (15)	73.1 (15)	62.1 (15)			115 ± 13 (18)
	271 ± 30.9 (24)	19.6 ± 9.9 (4)				
TPI						31,500 (18)
GAPD	2045 (1)	1582 (1)	1582 (15)	1003 (1)	2276 (1)	5100 ± 900 (18)
	1954 (15)	1434 (15)				
	5720 ± 448 (24)	242 ± 133 (4)				
PGK	1134 (1)	319 (1)		744 (1)	993 (1)	2800 ± 500 (18)
	6910 ± 627 (24)	1623 ± 727 (4)				
PGAM	194 (1)	1341 (1)				
		997 ± 445 (4)		365 (1)	156 (1)	610 ± 120 (18)
DPGM	20.4 (1)	14.3 (1)		1.4 (1)	26.5 (1)	
DPGP	0.21 (1)	1.07 (1)		0.33 (1)	0.27 (1)	
Enol	1360 ± 123 (24)	315 ± 98 (4)				1400 ± 100 (18)
PK	56.1 ± 17.8 (3)	572 ± 113 (4)	352 ± 106 (3)	205 ± 33 (3)		1760 ± 90 (18)
	151 ± 35 (10)					
	330 ± 29.1 (24)					

[a] Given in micromoles per minute per 100 gm Hb. See footnote to Table II.

TABLE V

Erythrocyte Chemical Constituents of Various Animal Species[a]

Constituent	Man	Dog	Cat	Pig	Primate	Aquatic mammal
Gluc	370 (17)	247 (3)	367 (3)	0 (9)		
G6P	0.9 (17)			0 (22)		
F6P	1.1 ± 0.25 (17)					
F16P	0.5 ± 0.09 (17)					
Lac	7.8 (17)					
ADP	20 (1)	17 (1)	8.2 (1)	21 (1)		D, 20 (1)
	20 ± 2.7 (17)					
ATP	135 ± 3.5 (17)	25 (1)	21 (1)	167 (1)		S, 41 ± 3 (6)
	90 (1)	57.4 ± 2.8 (54)	53 ± 2 (6)	90 (62)		D, 113 (1)
	150 ± 34 (6)	40 (62)	40 (62)			P, 90 (62)
	141 ± 22 (54)					
	90 (62)					
3PGA	4.8 ± 1.6 (17)					
2PGA	0.4 ± 0.15 (17)					
NAD	7 (1)	4.2 (1)	Nil (1)	3.6 (1)		D, 38 (1)
P_i	8 (1)	35 (1)	26 (1)	87 (1)		D, 27
GSH	195 ± 15 (17)	207 ± 36 (27)	197 ± 19 (27)			
	229 ± 27 (27)					
GSSG	0.36 ± 0.14 (17)					
2,3-DPG	400 (1)	427 (1)	50 (1)	520 (1)	B, 383 ± 100 (7)	S, 650 ± 53 (6)
	572 ± 67 (17)	432 ± 63 (25)	170 ± 24 (6)	1000 (62)	Rh, 510 (19)	D, 346 (1)
	630 ± 122 (6)	500 (62)	60 (62)			P, 500 (62)
	500 (62)	690 (63)	70 (63)			
	500 (63)					

[a] Given in micromoles per 100 ml RBC. See footnote to Table II.

TABLE VI

Erythrocyte Chemical Constituents of Various Animal Species[a]

Constituent	Horse	Cow	Sheep	Goat	Rabbit	Mouse
Gluc	325 (3)	122 (3)	67 (3)	89 (3)		
		55.7 ± 1.44 (4)	77 ± 8 (23)			
G6P		5.2 ± 1.5 (4)				
F6P		2.8 ± 1.1 (4)				
F16P		1.0 ± 1.2 (4)				
DHAP		3.5 ± 1.1 (4)				
GAP		1.7 ± 0.5 (4)				
3PGA		3.4 ± 1.2 (4)				
2PGA		0.9 ± 0.4 (4)				
PEP		1.9 ± 0.7 (4)				
Pyr		5.4 ± 2.4 (4)				
Lac		199 ± 76 (4)				
ADP	2.6 (1)	8.3 (1)		11 (1)	24 (1)	
		7.3 ± 2.2 (4)				
ATP	19 (1)	6.9 (1)	40 (62)	14 (1)	222 (1)	90 (62)
	20 (62)	63.3 ± 15.5 (4)			170 (62)	R, 40 (62)
		20 (62)			GP, 40 (62)	
NAD	4.4 (1)	Nil (1)		14 (1)	14 (1)	
P_i	21 (1)	40 (1)		85 (1)	39 (1)	
GSH		290 ± 50 (14)	339 ± 88 (30)			
			159 ± 10 (31)			
GSSG						
2,3-DPG	528 (1)	46 (1)	50 (62)	0 (1)	1000 (62)	
	500 (62)	1.34 ± 0.64 (4)	33 (13)	33 (13)	556 (1)	1000 (62)
	800 (63)	50 (62)	10 (63)	3 (2)	980 (63)	R, 1000 (62)
		10 (63)		10 (63)	GP, 500 (62)	R, 880 (63)
					GP, 610 (63)	

[a] Given in micromoles per 100 ml RBC. See footnote to Table II.

G6P per 100 ml for the human RBC (Beautler and Srivastava, 1972) indicate that this enzyme is subject to substrate control. Its V_{max} is 8.9 IU/gm Hb (Beutler, 1972b). It is highly specific for G6P. The enzyme is considerably more active in young than in older RBC's. The second enzyme in the initial oxidation step of the PPP is 6-phosphogluconate dehydrogenase (6PGD). This enzyme catalyzes the oxidative decarboxylation of C-1 of 6PG to CO_2 and ribulose-5-P. In the process, another NADP is reduced to NADPH. The V_{max} of this enzyme is 8.6 IU/gm Hb (Beutler, 1972b). Genetic variants of this enzyme have been reported in animals (Shih *et al.*, 1968). Generally, 6PGD activity is equal to or lower than that of G6PD (Tables IV and V). The oxidation of G6P to CO_2 in the PPP, therefore, takes place in two sequential steps. G6PD catalyzes the oxidation of G6P to 6PG with reduction of NADP to NADPH. Then, 6PGD oxidatively decarboxylates 6PG to CO_2, leaving ribulose-5-P, and reduces another NADP to NADPH. This is the only step by which G6P is oxidized to CO_2 in the RBC. These reactions are irreversible, and their product, ribulose-5-P, can be recycled back to G6P only by going through the PPP and forming F6P and glyceraldehyde 3-phosphate (GA3P).

The most important function of the PPP in the RBC is the generation of NADPH; in fact, it is the only source of NADPH. It serves as a crucial protector against the constant

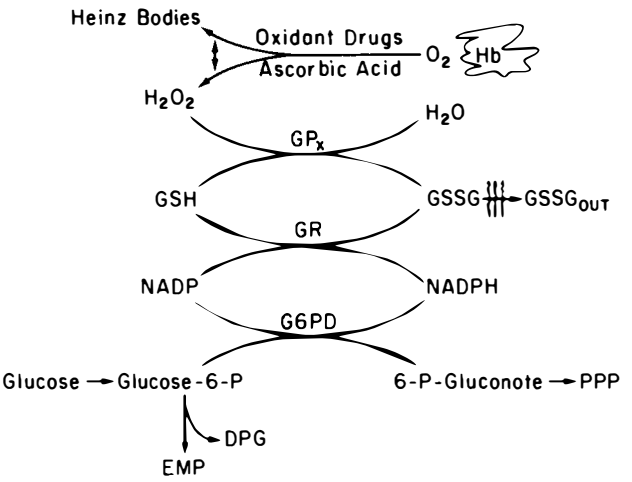


Fig. 10. Glutathione metabolism in the RBC (for abbreviations, see footnote, p. 120).

oxidative injury to Hb by a variety of sources, including the O_2 it transports. It serves primarily to reduce GSSG to GSH, which is vital in the antioxidant chain to detoxify H_2O_2 (Fig. 10).

It would seem logical that red cells deficient in G6PD would be more easily damaged than normal RBC's. This is true in the person who is G6PD deficient and who is unusually susceptible to hemolytic anemia caused by certain oxidant drugs such as primaquine. A wide variety of such drugs are now known (Keller, 1970). The high activity of G6PD in the young RBC and progressive lowering of activity with aging also explains the self-limiting nature of the lesion in man. When the mean cell age is older, the G6PD activity is low and the cell is unable to generate enough NADPH to combat oxidative injury. After acute drug-induced hemolysis, a large population of newly formed young RBC's with high G6PD activity is present, thus preventing oxidative injury by the drugs. With prevention of hemolysis, recovery from anemia can occur. Paradoxically, sheep and goats have G6PD activities within deficient ranges, but these species are not susceptible to primaquine injury (Smith, 1968).

2. Other PPP Enzymes

Other enzymes of the PPP are ribose phosphate isomerase, ribulose phosphate epimerase, transketolase (TK), and transaldolase (TA). Through interaction of these enzymes, F6P and GA3P are formed, which permit recycling of F6P through the PPP or by further oxidation via the EMP (Fig. 8). It is of interest that TK requires thiamine pyrophosphate for its action, and TK activity has been used as an index of thiamine deficiency (Wolfe *et al.*, 1958). The extent of G6P oxidation through the PPP and of recycling can have important comparative implications to explain the widely divergent oxidative patterns, varying levels of enzymes (Tables III and IV), and varying content of glycolytic intermediates (Tables V and VI) among animal species.

The NADPH generated in the PPP also serves as the major link between artificial electron acceptors, such as methylene blue and molecular O_2. The marked stimulation of RBC O_2 uptake and of the PPP by methylene blue (Brin and Yonemoto, 1958) is well

established and occurs in all species. In the presence of NADPH-diaphorase, methylene blue accepts an electron from NADPH and transfers it to molecular O_2, thereby increasing O_2 utilization. In the process, NADPH is rapidly oxidized to NADP, which in turn stimulates G6P oxidation through the PPP. This would mean that regulation of G6P flow through the PPP is also influenced by the availability of NADP.

3. *Metabolism of GSH*

Glutathione is a relatively simple compound synthesized not only by the immature, but also by the mature, RBC (Elder and Mortensen, 1956). The reactions of the GSH-synthetase system catalyzing the synthesis of this tripeptide are

$$\text{Glutamate} + \text{cysteine} \xrightarrow[\text{ATP, } Mg^{2+}]{\text{glut-cyst-syn}} \text{glutamylcysteine} \tag{7}$$

$$\text{Glycine} + \text{glutamylcysteine} \xrightarrow[\text{ATP, } Mg^{2+}]{\text{GSH-syn}} \text{glutathione} \tag{8}$$

The operation of the PPP serves as the vital link in GSH metabolism of the RBC (Fig. 10). Srivastava and Beutler (1969) studied RBC GSH metabolism extensively in various species. GSSG readily diffuses from the red cell. Therefore, maintenance of GSH in the reduced form is of great importance; otherwise, it would be lost to the plasma. As shown (Fig. 10), it serves as a vital link in the antioxidant function of glucose metabolism in the RBC. It also partly explains the deficiency and "instability" of GSH as observed in the early investigations of G6PD deficiency. Smith and Osburn (1967) observed a group of low-GSH mutant sheep which apparently suffered no clinical ill effects from this deficiency. Smith *et al.* (1973) have shown that the GSH deficiency in the Corriedale sheep is associated with a deficiency of the GSH synthetic enzyme glut-cyst-syn but not in another breed (Finnish Laudrace), which is also GSH deficient. Tucker and Ellory (1971) have studied the relation of low GSH activity to the high-K, low-Na mutant sheep.

Maintenance of GSH in reduced form is doubly important because it is necessary to stabilize protein SH groups, particularly those of SH enzymes and of Hb. At least one enzyme, GA3PD has GSH bound to it. It has also been found that "reactive" SH or the exposed SH groups of the Hb molecule must be maintained in their reduced state to remain functional. Preferential oxidation of GSH would protect the reactive SH group from oxidative formation of the mixed disulfides,

$$\text{Hb—SH} + \text{GSH} \rightarrow \text{HbS—SG} \tag{9}$$

which are probably the initial steps of oxidative injury to Hb. This reaction, however, is readily reversible in the presence of an active reducing potential, which, of course, depends on glucose oxidation through the PPP.

The link between NADPH and H_2O_2 requires both a GSH-reductase (GR) and a GSH-peroxidase (GP_x), as shown in Fig. 10. This GP_x reaction and catalase are both important to the RBC to detoxify H_2O_2 and thereby to protect the protein from oxidative injury by the H_2O_2 (Jacob *et al.*, 1964). The GP_x might be more important as the initial detoxifier at the early low concentrations of H_2O_2.

Glutathione reductase is a flavin enzyme which can utilize either NADPH or NADH as cofactor (Kaplan and Beutler, 1968). The K_m (NADPH) for GR is 9–16 μM, while that for NADH is between 170 and 310 μM (Icen, 1967; Waller, 1968). The K_m (GSSG) is 25–100 μM. Partial GR deficiency is now known to occur relatively frequently and, in

most instances, is related to riboflavin deficiency rather than to enzymatic deficiency (Beutler, 1971).

Glutathione peroxidase is now known to be a selenium-containing enzyme, with 4 moles of Se per mole of GP_x (Rotruck *et al.*, 1973; Flohe *et al.*, 1973). Studies with rats (Chow and Tappel, 1974; Hafeman *et al.*, 1974; Smith *et al.*, 1974), cows (Allen *et al.*, 1975), sheep (Oh *et al.*, 1974), and chicks (Noguchi *et al.*, 1973) have shown that the RBC GP_x activity is closely related to blood Se concentration and reflects dietary Se nutrition. Red blood cell GP_x activity is now widely used as a screening index of Se nutrition in cattle and sheep (Allen *et al.*, 1975; Wilson and Judson, 1976). Board and Peters (1976) developed a rapid spot test for RBC GP_x, which has been slightly modified by Segall *et al.* (1977).

Autosomally transmitted deficiency of GP_x has been reported (Necheles *et al.*, 1969). The activity of GP_x is also low in newborn human infants and has been used to explain the increased occurrence of Heinz body and hemolytic anemia during the first few days of life (Necheles *et al.*, 1968; Bracci *et al.*, 1969). Paglia and Valentine (1969) reported a K_m (H_2O_2) value of 25 μM and 10 μM for GSH. Flohe and Brand (1969) reported that in bovine RBC the K_m (H_2O_2) was 1 μM and that the enzyme displayed only first-order kinetics with respect to GSH. The very low K_m indicates that GP_x could indeed be a very important initial detoxifier of H_2O_2 at low concentrations.

D. Embden–Meyerhof Pathway

1. Generation of ATP

The oxidation of G6P to lactate through the EMP accounts for the greatest percentage of glucose utilized by the RBC. Between 90 and 95% of the glucose metabolized is initiated down this pathway, but the level of triose, an alternate DPG route is available (Fig. 8). On the basis of intermediate concentrations (Table V), it is estimated that about 30% of the G6P is diverted to 2,3-DPG. A phosphoglucomutase (PGM) catalyzing the formation of GIP is also present. Even though PGM activity is high, the RBC synthesizes no glycogen, and this path to glucose-1-phosphate (G1P) is a blind alley. The first step in EMP oxidation of G6P is conversion of G6P to F6P by glucose phosphate isomerase (GPI). The V_{max} of GPI is 42 IU/gm Hb (Beutler, 1972a). The K_m (G6P) is 0.6 μM (Arnold *et al.*, 1970). The activity is high, and analysis of intermediate concentrations (Zinkl and Kaneko, 1973a) indicates that this is not a "controlling" reaction in the EMP. The activity of GPI is higher in young than in old cells. Three electrophoretic variants of GPI have been detected.

The F6P is next phosphorylated to fructose 1,6-diphosphate (F16P) by phosphofructokinase (PFK) using ATP as the P donor. The V_{max} is 12.6 IU/gm Hb, and its K_m (F6P) is <0.1 mM (Rose, 1971). Phosphofructokinase is strongly stimulated by ADP and P_i and inhibited by 2,3-DPG (Srivastava and Beutler, 1972), 3-phosphoglycerate (3PGA), phosphoenolpyruvate (PEP), and ATP (Rose, 1971). The enzyme has been shown by thermodynamic analysis to be a rate-controlling enzyme in the bovine RBC (Zinkl and Kaneko, 1973a). The critical rate-controlling nature of this enzyme is also evidenced by the finding that it is responsible for glycolytic failure in stored red cells (Bartlett and Shafer, 1960). *In vivo,* its activity is found to be high in young and low in old red cells. Phosphofructokinase has the second lowest activity in the dog, cat, and pig and thus may

also be a controlling enzyme second to HK in these species. According to Rose (1971), PFK is the major enzyme reaction controlling the rate of G6P removal from the RBC; also, PFK regulates the HK reaction by its effect on G6P concentration. Increases in pH release PFK from ATP inhibition and thereby increase the glycolytic rate (Rorth, 1972). Aldolase next splits F16P into two trioses, dihydroxyacetone phosphate (DHAP), and GA3P. The V_{max} is 3.2 IU/gm Hb (Beutler, 1972b). Aldolase is relatively low enough in the horse, cow, mouse, and man to be a likely second controlling step. Aldolase in the bovine RBC, however, did not change with age, nor was it found to be a controlling enzyme (Zinkl and Kaneko, 1973a). Reports of aldolase change with age in the human RBC are conflicting (Bishop, 1964).

Triosephosphate isomerase (TPI) catalyzes the reversible conversion of DHAP to GA3P, which permits both "halves" of the F16P to be further oxidized down the EMP chain. The equilibrium actually favors DHAP, but the continued drain of GA3P shifts the equilibrium to GA3P. According to Table IV, TPI has the highest activity of all RBC enzymes.

Glyceraldehyde phosphate dehydrogenase (GAPD) is the first oxidative step in the EMP, and in this case NAD is the essential cofactor, which is reduced to NADH. GA3P is phosphorylated to 1,3-DPG in the process with P_i instead of ATP. A high-energy bond is thus formed without the expenditure of ATP. GAPD is a GSH-containing enzyme and, therefore, is another enzyme that requires protection against oxidative inactivation of its SH groups. Phosphoglycerate kinase (PGK) then catalyzes the transfer of high-energy phosphate to ADP, and the first ATP is generated. The V_{max} is 150 IU/gm Hb (Beutler, 1972a). Next, the 3PG is connected to 2PG by phosphoglycerate mutase (PGAM) using 2,3-DPG as a shuttle. Enolase then converts 2PG to PEP at a V_{max} of 4.7 IU/gm Hb (Beutler, 1972a). The next step, the pyruvate kinase (PK) reaction, is widely studied because it is the second most common hereditary RBC enzyme defect of human beings (Tanaka and Paglia, 1971). It is also the first RBC enzyme deficiency to be reported in dogs (Searcy *et al.*, 1971). Phosphoenolpyruvate is converted to pyruvate by PK, and the second ATP is formed. Thus, starting from glucose, 2 ATP are needed to phosphorylate and 4 ATP are generated for a net yield of 2 ATP per glucose molecule oxidized to pyruvate (Fig. 8). The reaction is essentially irreversible with a V_{max} of 7.7 IU/gm Hb (Beutler, 1972a). This is about 50 times greater than the maximal activity needed to account for the rate of lactate production. Thus, when glucose utilization increases, PK can easily catalyze the formation of pyruvate. Pyruvate kinase deficiency in man generally has a significant lowering of enzyme activity, but the reported defect in Basenji dogs (Searcy *et al.*, 1971) could be detected only by comparing enzyme activities of reticulocyte-rich dog RBC's. On the other hand, it has been reported (Standerfer *et al.*, 1974) that, while heterozygous Basenji dogs had about one-half the normal PK activity, homozygous dogs with the decrease had anomalously high PK activities. *In vitro* studies demonstrated that the PK activity of a homozygous dog was unstable on standing at room temperature. The authors proposed that the PK defect in Basenji dogs is a mutation giving rise to a PK molecule which is active but unstable.

Pyruvate kinase deficiency is also now known to occur in Beagle dogs (Prasse *et al.*, 1975; Harvey *et al.*, 1977), but it does appear to be an instability in this species. In order for the EMP to continue its function of generating ATP, there must be a source of NAD for the GAPD reaction. NAD appears to be readily oxidized directly by O_2. There is

TABLE VII

Partitioning of Glucose Metabolism in the Erythrocyte: Net Gain of Useful Product[a]

Pentose phosphate pathway[b] (5%)				
1. 3 Glu + 3 ATP + 6 NADP → 2 F6P + GAP + 3 CO_2 + 3 ADP + 6 NADPH		−3 ATP		+6 NADPH
Embden-Meyerhof pathway (65%)				
a. 2 F6P + 2 ATP + P_i + 4 NAD + 4 NADH + 8 ADP → 4 Lac + 4 NAD + 4 NADH + 8 ATP		+6 ATP		
b. GAP + NAD + P_i + NADH + 2 ADP → Lac + NADH + NAD + 2 ATP		+2 ATP		
2. 4 Glu + 8 ATP + P_i + 8 ADP + 8 NAD + 8 NADH → 8 Lac + 8 NADH + 8 NAD + 16 ATP		+8 ATP		
Diphosphoglycerate pathway (30%)				
3. 3 Gluc + 6 ATP + P_i + 6 NAD → 6 2,3-DPG + 6 ADP + 6 NADH	+6 2,3-DPG	−6 ATP	+6 NADH	
Net: 10 Glu + 7 ADP + 6 NAD + 6 NADP + P_i → 13 Lac + 3 CO_2	+6 2,3-DPG	+7 ATP	+6 NADH	+6 NADPH

[a] Ten moles are used to simplify the partitioning of glucose metabolism and to avoid the use of fractions.

[b] For abbreviations, see footnote, p. 120.

minimal synthesis, and nonphosphorylated compounds, such as pyruvate, readily diffuse out of the RBC. The RBC has high lactate dehydrogenase (LDH) activity, which catalyzes the reduction of pyruvate to lactate, and NADH is oxidized to NAD. This NAD can now "couple" with the GAPD reaction to produce a self-regenerating pathway with net ATP production (Fig. 8, Table VII). Even though LDH activity is high, lactate is not produced if pyruvate is lost from the RBC, and then NADH is not used in this reaction. The net NADH yield in the EMP can then be coupled to the NADH-methemoglobin reductase (MR) system (Fig. 8), to reduce the constantly produced MetHb (Jaffe and Hsieh, 1971).

2. *Functions of ATP*

About 15% of the available ATP would suffice to power the known energy requirements of the Na-K pump in the RBC (Harris and Kellermeyer, 1970). Furthermore, ATP is required by the RBC for synthetic activity, to maintain its shape and its deformability, and to power the Na-K pump. The relative importance of this pump in animals is certainly open to question considering the extremes of Na-K concentration among RBC's of various species (Table II, IV). Some species (dog, cat, seal) pump essentially no Na-K, some (horse, cow, sheep) are intermediate, and some (man, other primates, dolphin, rabbit, rat, pig, HK sheep) are moderate to very active "pumpers." ATPase is absent in the dog and cat and is of intermediate activity in the cow and sheep, corresponding to their abilities to pump Na. Conversely, those species that pump very few ions have either a low glucose utilization rate (Table III, sheep, cow, horse) or generate small amounts of ATP (Table VI, dog, cat, seal). The most extensive studies of RBC membrane phenomena in domestic animals have been those conducted by Tosteson (1963, 1969) on LK and HK sheep. Keeton and Kaneko (1972, 1973a,b) studied ATPase and the Na-K pump in cattle and found it to be as active as in other species.

3. *Functions of NADH*

The Hb of the mature red cell is constantly exposed to the oxidative pressure of the O_2 which it transports. A certain amount of direct oxidation of Hb (Fe^{2+}) to MetHb (Fe^{3+}) occurs [reaction(1)]. MetHb is unable to transport O_2, and a constant source of reducing "power" must be available to maintain Hb (Fe^{2+}). Although both an NADPH-dependent and an NADH-dependent MR are known, the NADH-MR appears to be the main physiologically active enzyme (Jaffe and Hsieh, 1971). Animals species vary widely in their ability to reduce MetHb (Smith and Beutler, 1966). The sheep, cow, and goat rapidly reduced MetHb, i.e., $T_{1/2} < 2$ minutes, while the pig had a $T_{1/2}$ of 7 minutes. It was concluded that those species which form MetHb most readily also reduce it most readily; i.e., the turnover rate is more rapid. Horse and man were intermediate, horse having a slightly slower turnover than man. MetHb regulation was also extensively studied in horses by Robin and Harley (1966, 1967). Rates of reduction varied, and no single explanation suffices for the variation among species.

As the metabolism of RBC slows, it might logically lose its ability to reduce MetHb, but methemoglobinemia has not been shown to be correlated with age of the cell. It is also paradoxical that species with the fastest turnover (cow, sheep, goat) are generally those with the lowest rate of glucose utilization. NADH-MR deficiency is now known to occur in dogs (Harvey *et al.*, 1974; Letchworth *et al.*, 1977), but its hereditary nature is unknown.

E. Diphosphoglycerate Pathway

The metabolism of 2,3-DPG and its high concentration in the RBC are unique features of the mammalian RBC. The diphosphoglycerate pathway, also called the Rapoport–Luebering cycle (Rapoport and Luebering, 1950), is an alternate pathway for 1,3-DPG. As shown in Fig. 8, the pathway bypasses the ATP-generating PGK step and, in this sense, is an energy-wasting pathway. However, ATP is generated from 2,3-DPG at the PK step when it completes its bypass. For this reason, 2,3-DPG has been considered to be an ATP store or "sink."

2,3-DPG is synthesized by phosphate transfer from 1,3-DPG to 3PG by DPG. It is an essentially irreversible reaction. The pH optimum is 7.5, its K_m (1,3-DPG) is 0.53 μM and K_m (3PG) is 1.5 μM, and it is quite sensitive to product inhibition (Rose, 1968).

The 2,3-DPG can be dephosphorylated by a specific phosphatase, DPGP, to 3PG. Its pH optimum is between 7 and 7.5, and K_m (2,3-DPG) is 0.4 μM (Harkness and Roth, 1969). The reaction is stimulated about tenfold by P_i. The enzyme has been shown to be a specific phosphatase rather than an activity of PGM as was previously thought (Rose, 1970). Harkness *et al.* (1969), in an extensive study of the DPG pathway and its intermediates, concluded that the low 2,3-DPG activity in the cat was the result of a low rate of synthesis (low DPGM, Table V), while that in the cow was explained by a very active DPGP (Table V) resulting in the catabolism of 2,3-DPG. However, the range of DPGP activity, 0.18–1.07, in all species is relatively narrow, and therefore it is possible that this is the limiting enzyme creating a metabolic block. As depicted in Fig. 8 and Table VII, 1 mole of NADH is generated per mole of 2,3-DPG formed. The NADH–MR couple could then be an equally important control mechanism and function of the 2,3-DPG. The lactate/pyruvate ratio is high in RBCs, and lactose production couples NADH to GAPD, so there is no net NADH production in the generation of ATP. An alternate supply of NADH for the NADH–MR couple could then be provided by the DPG pathway without accumulating ATP. Schröter and Winter (1966) proposed a similar relationship between the DPG pathway and MR. This system would parallel the coupling of H_2O_2 detoxification to NADPH in the PPP.

The amount of flux of glucose through the DPG pathway is not known, but a figure of 30% would seem to be reasonable considering the levels of intermediates involved (Tables V and VI). According to many (Harkness, 1971; Rose, 1970), the most important regulatory mechanism for 2,3-DPG synthesis is the "free" product inhibition of DPGM. 2,3-DPG preferentially binds to deoxyhemoglobin, and in periods of hypoxia, when there is more deoxyhemoglobin, "free" 2,3-DPG levels are low, DPGM inhibition is relieved, and 2,3-DPG synthesis can occur. This would also explain the increased RBC 2,3-DPG in anemia with its associated hypoxia. Slight increases in pH (0.1 unit) are also known to increase 2,3-DPG. Both pH and "free" 2,3-DPG are in turn affected by the degree of O_2 saturation, at least in human beings.

The almost simultaneous reports of Chanutin and Curnish (1967) and Benesch and Benesch (1967) that 2,3-DPG influenced Hb–O_2 dissociation curves has stimulated great interest in RBC metabolism. A mechanism for 2,3-DPG binding to hemoglobin has been described (Perutz, 1970). The high RBC concentration of 2,3-DPG in all species except the cat, cow, sheep, and goat is a singular feature of RBC metabolism. Greenwald (1925) originally observed high 2,3-DPG activity in the pig RBC, and since then its relatively high concentration has been observed in most species (Tables V and VI).

According to Fig. 11, increases in temperature, 2,3-DPG, and CO_2 or decreases in pH

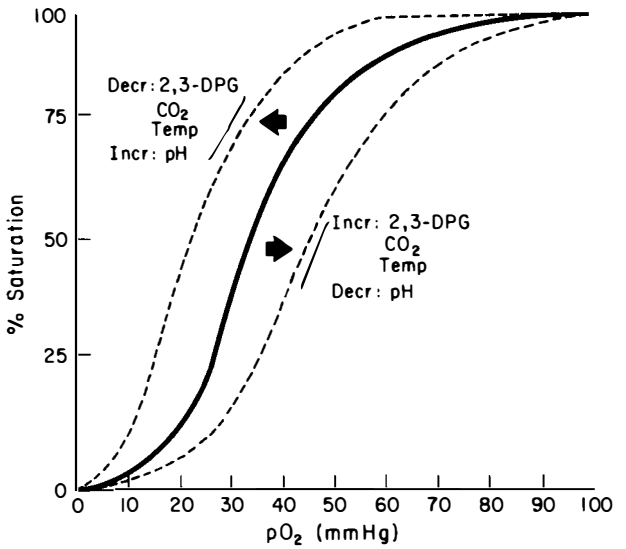


Fig. 11. The hemoglobin-oxygen dissociation curve and the factors influencing the position of the curve (for abbreviations, see footnote, p. 120).

all decrease O_2 affinity and "shift the dissociation curve" to the right. Decrease in affinity means greater ease of delivery of O_2 to the tissues, where 2,3-DPG apparently has its effect. Bunn (1971) reported the effects of 2,3-DPG on the Hb of some animals. The Hb of cat, cow, sheep, and goat, which are species with low 2,3-DPG activity, were found to have low O_2 affinities when "stripped" of 2,3-DPG. Addition of 2,3-DPG had no effect. In contrast, Hb of species with high levels of 2,3-DPG, i.e., man, horse, dog, rabbit, guinea pig, and rat, had decreased O_2 affinities when 2,3-DPG was added. Blunt *et al.* (1971) studied the effect of 2,3-DPG in newborn sheep and goats and could find no direct effect of 2,3-DPG on fetal Hb of goats. It has long been known that human fetal whole blood has a higher O_2 affinity than has maternal blood but that "stripped" Hb A and Hb F have identical O_2 affinities. This is explained by 2,3-DPG having an effect only on Hb A and very little on Hb F so that the 2,3-DPG, which is present equally in neonates and adults, preferentially decreases the O_2 affinity of adult RBC's with Hb A. Battaglia *et al.* (1970) observed a higher O_2 affinity of fetal sheep blood than of the adult and concluded from their study that 2,3-DPG decreased O_2 affinity by lowering pH in addition to interacting directly with hemoglobin. Zinkl and Kaneko (1973b) found very high 2,3-DPG values in fetal and newborn calves and very low levels in adult dams, which would indicate that 2,3-DPG is also unlikely to affect the O_2 affinities of bovine Hb F or Hb A. Therefore, it would appear that Hb of some species (man, horse, dog, rabbit, guinea pig, rat) is influenced by 2,3-DPG and that of others (sheep, cow, goat, cat) is influenced minimally or not at all. The fetal blood of a variety of animal species has higher O_2 affinity than has the maternal blood, although in the cat that of the fetus is equal to that of the mother (Novy and Parer, 1969).

F. Overall Partition of Glucose Metabolism

On the basis of intermediate and ion concentrations (Tables II, V, and VI) in various species, it has been estimated that most species, except the horse, have a balanced glucose

flux which approximates 5% PPP, 30% DPG, and 65% EMP (Fig 9; Table VII). Some species utilize significantly less glucose (cat, cow, sheep, goat) than others but still have a similar balanced flux of glucose catabolism. The horse appears to be a unique species the RBC's of which utilize glucose at a rate comparable to that of RBC's of ruminants; however, the horse metabolizes a high percentage of glucose through the PPP. The flow of glucose through the PPP is about 12% as determined by the [1-^{14}C]glucose incubation technique (Harvey and Kaneko, 1976). This would also mean that the total glucose flow through the PPP in the horse would approximate that of other animals and that the horse is not deficient in the PPP.

V. DETERMINANTS OF RBC SURVIVAL

Oxidative injury to the RBC may take a variety of forms, the major ones being those involving oxidative injury of enzyme and Hb proteins and damage to membranes. The RBC is constantly exposed to oxidative injury, but its metabolic activity is able to reverse the injury under normal conditions. With decreasing ability to reverse oxidative injury or increasing oxidative stress, irreversible damage to the RBC occurs, leading to its ultimate loss by hemolysis or by removal of the RBC or both.

A. Enzymatic Effects

Since a considerable number of enzymatic activities are high in the "young" and decline toward the "old" status of the RBC, it has generally been regarded that the RBC ages *because* its enzymatic activities decline. The most extensively studied enzyme in this respect is G6PD of deficient human beings, in whom it is known that RBC survival is shortened and primaquine shortens it even further. In horses with equine infectious anemia, G6PD was high in young RBC's following hemolytic episodes (Kaneko *et al.*, 1969b). Red cell survival was shortened in cases of equine infectious anemia (Obara and Nakajima, 1961). A similar pattern of enzyme rise and fall after hemolysis was observed in cases of bovine anaplasmosis (Smith *et al.*, 1972b), a disease with shortened RBC survival (Hansard and Foote, 1959); this was confirmed also for congenital erythropoietic porphyria of cattle (Kaneko *et al.*, 1971; Kaneko and Mills, 1969a). Human PK deficiency is another extensively studied enzyme deficiency disease characterized by shortened RBC survival. Pyruvate kinase deficiency in Basenji dogs, however, has not been established as having a shortened RBC survival time, but it is certainly to be expected.

Early cell death as a result of a specific metabolic enzyme attrition is a reasonable expectation considering the critical nature of each of the three major pathways of glucose catabolism of the RBC. However, it would be difficult to single out a specific determinant of death of the normal cell. An enzyme(s) in any of the three major paths could precipitate the chain of events leading to cell death. It has already been pointed out that HK is in a critical position as the initial enzyme of glucose metabolism and is probably the critical enzyme in RBC survival. An alternative possibility is that G6PD has the critical role in RBC survival.

B. Membrane Effects

Progressive oxidative injury to the membrane might also play a considerable role in cell survival. Cells deficient in G6PD become spherocytic, and their osmotic fragility in-

creases when they are incubated with primaquine drugs *in vitro* (George *et al.*, 1966). Mohler and Williams (1961) demonstrated ATP decreases in a similar system and thought that this was a reflection of increased demand for ATP by the pump as the damaged membrane leaked its sodium. An alternative possibility of membrane damage which is even more subtle and unclear is the influence of decreased ATP on cell deformability.

Membrane damage can potentially take at least two forms. First, the SH groups of the membrane protein are thought to form disulfides analagous to the mixed disulfides of Hb [reaction(9)]. Second, the high lipid content of the membrane provides the possibility that lipid peroxidation could also be a significant route of membrane injury. The shape and flexibility of the metabolically adequate membrane permit it to deform and pass through the minute passages of the spleen (Jacob, 1970). The spleen is thought to readily recognize and remove those nondeformable cells, which sequester in the splenic capillaries (Jacob, 1970). Whatever the mechanism, the membrane would be at least potentially susceptible to damage in the senescent cell.

C. Oxidative Effects: Heinz Bodies

It has already been noted that GSH of the normal RBC is likely to be the first line of protection against oxidative injury by itself being oxidized to GSSG. This reaction is readily reversed by GR (Fig. 10). The RBC is also protected from injury by the H_2O_2 which is constantly generated through the action of GP_x (Fig. 10). Moderate direct oxidation of the RBC also results in the formation of MetHb, which can be readily reduced by the NADH–MR system of the normal cell. Formation of MetHb does not in itself lead to hemolysis (Beutler *et al.*, 1954).

It has also been indicated that the "reactive" SH groups of hemoglobin are susceptible to oxidation to form mixed disulfides (Srivastava, 1971) [reaction(9)]. Mixed disulfides formed early may be reversible, but, with progressively more severe oxidative injury, irreversible oxidative injury occurs. Internal SH groups may become progressively oxidized, and "sulfhemoglobin" is formed. With further oxidation, denaturation progresses to the point where hemoglobin precipitates as spherical, refractile, inclusion bodies known as Heinz bodies (Allen and Jandl, 1961):

$$n(\mathrm{HbS}\cdot) \xrightarrow{(O)} \mathrm{HbSSHb}_n \quad \text{(Heinz body)} \tag{10}$$

The electron microscopic studies of Rifkin and Danon (1965) showed that, after their formation, the bodies coalesced and finally attached themselves to the RBC membrane. Attachment to this membrane is thought to be in the form of a mixed disulfide linkage of Heinz bodies with membrane SH. These Heinz-body-containing cells are rapidly removed by the spleen because of their rigidity (Jacob and Jandl, 1962). The splenic passages are very small and require extreme deformation of RBC's to pass through them. The rigid Heinz body forms a "sticking point," and the rest of the cell may break off or the entire cell may be destroyed (Jacob, 1970). In the cells undergoing oxidative injury, significant simultaneous cation leakage occurs, thus predisposing the RBC to osmotic swelling and lysis, spherocytosis, and removal by the spleen. Therefore, the cells undergoing Heinz body injury are placed in double jeopardy from membrane damage.

Heinz bodies were originally described in man, dog, rabbit, and frog (Heinz, 1890) and later identified in the cat as Schmauch bodies (Schmauch, 1899). Comparative studies indicate that Heinz and Schmauch bodies are identical (Beritic, 1965; Collins *et al.*, 1968). They are also identical to the "erythrocyte-refractile" bodies described in the

blood of cats (Schalm and Smith, 1963). Heinz bodies are known to occur commonly in the RBC of persons with enzymatic defects, e.g., G6PD, or with hemoglobinopathies, e.g., Hb Köln. As described earlier, an enzymatic defect in the PPP prevents the protective action of NADPH (Fig. 8) against oxidative injury to Hb. In the case of some abnormal Hb, e.g., Hb Köln, the structure of the Hb is such that it is "unstable" to heat at 50°C and its amino acid substitutions contribute to easy, direct oxidation of SH. In either event, a chain of oxidative events injurious to Hb ensues. Denatured Hb precipitates as Heinz bodies and attaches to the membrane, and the cells are more readily removed from the circulation by the spleen.

A large number of drugs, chemicals, and toxins are known to be associated with Heinz body production (Keller, 1970). The common feature of all these drugs is their oxidative property. Such drugs function as electron acceptors and serve as artificial links between cell constituents and the direct oxidative action of molecular O_2. Protection against such oxidation depends on NADPH and GSH, which in turn depend upon adequate function of the PPP. Since NADP is generated in the process, the oxidative drugs stimulate the PPP. An enzymatic system, NADPH-diaphorase, also couples redox dyes, such as methylene blue, and stimulates the PPP by this method.

The second type of Heinz body anemia associated with abnormalities of Hb structure such as Hb Köln has been reviewed by Jacob (1970). Structural mutations of the β chain of hemoglobin near the heme moiety are involved. The mutations appear to permit unusually easy loss of heme from the β chain. This phenomenon explains the characteristically high excretion of dipyrroles in the urine of Hb Köln patients. The loss of heme makes the β chain highly susceptible to oxidative denaturation and to Heinz body formation. This hypothesis is further supported by the finding that hemin added to Hb Köln preparations prevents the formation of Heinz bodies (Jacob, 1970).

The occurrence of Heinz bodies in a number of animals has been established. They are of frequent occurrence in the cat (Schalm and Smith, 1963) and readily induced in this species by methylene blue and drug preparations containing this dye (Schecter *et al.*, 1973). Heinz bodies occur as a result of phenothiazine toxicity in horses (Schalm *et al.*, 1963). Onion poisoning is known to induce Heinz body anemia in sheep (Van Kampen *et al.*, 1970), cattle (Koger, 1956), and horses (Thorp and Harshfield, 1939; Pierce *et al.*, 1972). The toxic compound of the wild onion is *n*-propyl disulfide, which induces Heinz body anemia when given experimentally to dogs (Gruhzit, 1931a,b). Curiously, sheep, which are known to be very low in G6PD and GSH (Kaneko and Smith, 1964), are insensitive to primaquine (Smith, 1968). Heinz body anemia has also been observed in severe postparturient hemoglobinuria of cows (Martinovich and Woodhouse, 1972).

Differences occur in susceptibility to Heinz body anemia among animal species, the cat being the most susceptible (Schecter *et al.*, 1973). It has been mentioned that human hemoglobin has two "reactive" SH groups. Taketa *et al.* (1967) demonstrated at least eight "reactive" SH groups in the Hb of cats. Thus, cat Hb is more easily oxidized and accounts for the observed variations in Heinz body susceptibilities.

D. Erythrocyte Survival

The RBC of each species survives for a period characteristic of the species (Table I, II). The determinant of RBC survival time in each species appears to be governed by the complement of links vital to its machinery for survival. The decrease in a variety of its

enzymes during agina has led to the concept that the enzyme mechanisms cease to function. The mature RBC has no capacity for synthesis and replenishment of its enzymes or other protein. It depends solely on its initial complement of preformed enzyme protein. The RBC depends on a balance of its metabolic needs and capabilities to meet these needs through glucose utilization. It survives until a critical need, resulting from stress or senescence, is unmet. The membrane may lose its flexibility, leak, and swell, or its hemoglobin and other proteins may become denatured, resulting in the cell's removal from the circulation. Imbalances of a variety of metabolic pathways already described lead to loss of viability. Oxidative stresses must be met and membrane integrity maintained through the residual metabolic pathways of the red cell by which energy and reducing potential are made available. The balance in the mechanism by which a variety of animal species meet their respective needs appears to be uniquely geared to that particular species.

Survival measurements are now sufficiently commonplace so that recommendations to attain uniformity have been published (International Committee for Standardization in Hematology, 1972). Red cell survival measurements are based on two major principles: cohort or pulse labeling and population labeling of RBC's. The pulse method involves labeling of a discrete population of RBC's during the period of their proliferation in the bone marrow and following their persistence and disappearance. A standard method is to label the iron (^{59}Fe) of heme or the heme with ^{14}C-labeled glycine. The survival of RBC's in domestic animals has been reviewed (Berlin, 1964). The RBC survival for a variety of species is given in Table I.

$Na^{51}CrO_3$ is the most common population label used for determining RBC survival; its relative ease facilitates clinical use. The rate of disappearance of ^{51}Cr-labeled RBC expressed as the $T_{1/2}$ reflects the *in vivo* survival.

VI. PORPHYRIAS OF ANIMALS

A. Classification

By convention, the term "porphyria" is used to define those disease states which have a hereditary basis and which exhibit increased urinary and/or fecal excretion of the uroporphyrins or their precursors. In addition, increased amounts of coproporphyrins are usually found in the urine and feces. The term "porphyrinuria" is used to define those acquired conditions in which the principal, if not the sole, porphyrins excreted are the coproporphyrins. The excretion of increased amounts of coproporphyrin has been observed in a wide variety of conditions in man, which include infections, hemolytic anemias, liver disease, and lead poisoning. The detection of coproporphyrinuria has been especially useful as a screening test for exposure to lead.

The classification of the porphyrias has undergone revision periodically and doubtless will continue to do so until the precise defects in the various forms are known. There is general agreement as to the use of the term "erythropoietic porphyria" to describe the hereditary conditions manifested by involvement of the erythropoietic tissue. The confusion exists in the classification of the disorders described as subgroups of the hepatic forms. These have been variously classified on the basis of clinical manifestations, genetics, and the principal prophyrin compound(s) excreted (Eales, 1961; Tschudy, 1965) (Table VIII).

TABLE VIII

Classification of the Porphyrias

I. Erythropoietic porphyrias
  A. Congenital erythropoietic porphyria (CEP)
  B. Erythropoietic protoporphyria (EPP)
II. Hepatic Porphyrias
  A. Acute intermittent porphyria (AIP) (Swedish porphyria)
  B. Mixed porphyria (variegate porphyria, South African porphyria, porphyria cutanea larda)
    1. Cutaneous manifestations
    2. Acute intermittent manifestations
    3. Combined
  C. Symptomatic porphyrias
    1. Idiosyncratic: Associated with alcoholism, liver disease, systemic disease, drugs, etc.
    2. Acquired: Hexachlorobenzene poisoning, hepatoma

Methods are presently available for the experimental production of the two major types. In lead or phenylhydrazine poisoning, a type of porphyrinuria is produced which exhibits some of the characteristics of the hereditary erythropoietic form of man and cattle (Schwartz *et al.*, 1952). A hepatic type may be produced by means of Sedormid (allylisopropylacetylcarbamide) (Schmid and Schwartz, 1952) or dihydrocollidine (Granick and Urata, 1963).

B. Methodology

The principal method now employed for the detection of porphyrins in biological materials in the clinical laboratory is based upon the characteristic red fluorescence observed when acidic solutions of the porphyrins are exposed to ultraviolet light. The color of the fluorescence cannot be used to distinguish between the uroporphyrins and the coproporphyrins, and therefore these must be separated prior to examination for fluorescence. The separation procedures take advantage of the solubility differences of the porphyrins in various organic solvents. In general, the following solubility properties are principally employed in the separation of the uroporphyrins from the coproporphyrins:

1. The coproporphyrins are soluble in diethyl ether, while the uroporphyrins are not, and therefore uroporphyrins remain in the aqueous phase.
2. Both uroporphyrins and coproporphyrins are soluble in strong acid (usually 1.5 *N* HCl). Coproporphyrins are therefore extracted from the organic phase with 1.5 *N* HCl. The uroporphyrins in the aqueous phase are absorbed with Al_2O_3 and subsequently eluted with 1.5 *N* HCl.

The acidic solutions of the porphyrins obtained are then observed visually for fluorescence or determined quantitatively in a sensitive fluorometer. The most suitable condition for the excitation of fluorescence is the use of ultraviolet light in the near-visible range using aqueous solutions of the porphyrins at pH 1–2. Further means of identification include spectrophotometric examination, melting points of the methyl esters, and paper chromatography. The methods for the quantitative determination of the porphyrins in

biological materials have been presented in detail (Schwartz *et al.*, 1960). The following simplified screening procedures are guides for further laboratory examinations:

1. Qualitative Test for Urinary Porphyrins

The urine for porphyrin examination should remain alkaline because in acidic urine, the porphyrins readily precipitate. Addition of 0.5 gm Na_2CO_3 to the collecting bottle for each 100 ml of urine will keep the urine alkaline. The alkalinized urine can be stored at 4°C for 2–3 days prior to examination. All contact of the urine with metal should be avoided, and unfiltered urine should be used. The following is a simplified procedure:

1. Place 5 ml urine in a 250-ml separatory funnel, and add 5 ml of acetate buffer (4 parts glacial acetic acid, 1 part saturated sodium acetate) and adjust to pH 4.6–5.0.
2. Add 15 ml cold distilled water.
3. Extract with two 50-ml aliquots of diethyl ether (or until the ether phases show no fluorescence) and pool. The coproporphyrin will go into the ether phase.
4. Many of the porphyrins present in urine are in the form of their nonfluorescent precursors. Storage of the urine for 24 hours will enhance their conversion to the fluorescent pigments. If fresh urine is being examined, the ether phase should be gently shaken with 5 ml of fresh 0.005% I_2 solution (prepared by diluting 0.5 ml of a stock 1% I_2 in alcohol solution to 100 ml with distilled water) to convert the precursors to the porphyrins.
5. Extract the pooled ether phases with 20 ml 5% HCl (1.5 *N*) and examine for fluorescence. Fluorescence indicates the presence of coproporphyrins.
6. Uroporphyrins are insoluble in ether. Therefore, fluorescence in the aqueous urinary phase indicates the presence of uroporphyrins.

2. Qualitative Test for Fecal Porphyrins

The qualitative test as described for urine may also be applied to fecal samples after prior extraction with strong acid. The following procedure is satisfactory (Sunderman and Sunderman, 1955). A 5-gm portion of a fecal sample is emulsified with 10 ml 95% alcohol. Twenty-five milliliters of concentrated HCl and 25 ml water are added to the emulsion and the mixture allowed to stand overnight at room temperature. It is then diluted to 200 ml with water and filtered, and the filtrate is examined for porphyrins as described for urine.

3. Qualitative Test for Porphobilinogen

The qualitative test for porphobilinogen developed by Watson and Schwartz (1941) is considered to be a reliable procedure since false positives are not commonly encountered. First, 3 ml of fresh urine and 3 ml of Ehrlich's aldehyde reagent (0.7 gm *p*-dimethylaminobenzaldehyde, 150 ml conc. HCl, and 100 ml H_2O) which is commonly used for the urine urobilinogen test, are mixed. Next, 6 ml of saturated sodium acetate are added and again mixed. Then, 5 ml chloroform are added, shaken vigorously, and allowed to separate. The porphobilinogen aldehyde formed in the test is insoluble in chloroform and will remain in the aqueous supernatant phase. If the pink color is due to urobilinogen, it will be extracted into the chloroform phase. Porphobilinogen is found characteristically in the urine of patients with the hepatic form of porphyria, which has not been reported in domestic animals.

C. Porphyria of Cattle

1. Bovine Congenital Erythropoietic Porphyria (CEP)

a. Introduction. One of the characteristic findings in bovine CEP is a reddish brown discoloration of the teeth and bones. Discolorations of this type have been observed in cattle after slaughter since the turn of the century, and these cattle are presumed to have had the disease. The first living cases were encountered in South Africa in a herd of grade Shorthorn cattle and the findings described by Fourie (1936) and Rimington (1936). Since that time, CEP has been reported in Denmark (Jorgensen and With, 1955), England (Amoroso *et al.*, 1957), the United States (Ellis *et al.*, 1958; Rhode and Cornelius, 1958), and Jamaica (Nestel, 1958). The disease is now known to occur in Holsteins and Jamaican cattle in addition to the Shorthorn breed.

The simple Mendelian recessive hereditary nature of the disease was established by study of the genealogy of the affected cattle and by breeding experiments (Fourie, 1939). The affected homozygous animals are characterized by discoloration of the teeth and urine, photosensitivity of the light areas of the skin, and generalized lack of condition and weakness. The condition is present at birth and severely affected calves must be kept out of sunlight if a reasonable state of health is to be maintained.

The predominating symptoms of teeth and urine discoloration and the photosensitization of the severely affected animal are readily apparent, and a tentative diagnosis can be confirmed by the red fluorescence of the teeth and urine when examined in the dark with Woods light. The symptoms of affected animals, however, may vary from minimal to severe and with age and time of year (Fourie and Roets, 1939). The discoloration of the teeth may vary in the same animal, usually being more pronounced in the young and less apparent in older animals. Since porphyrin deposits occur in the dentine, examination of the occlusal surface should also be included. The degree of photosensitization will vary with the extent of porphyrin deposition and exposure to sunlight and may be so slight as to escape recognition. At times, loss of condition may be the only outward symptom for which the veterinarian may be called upon to examine the animal. Marked variations in the urinary excretion of the porphyrins also occur. These may range from minimal to thousands of micrograms in the same animal. The variations observed in this condition provide an indication of the dynamic state of flux of porphyrin metabolism in the living animal, and the porphyrin deposits constitute a part of this dynamic state.

b. Distribution of Porphyrins. In Table IX are listed some normal values of porphyrin concentration in animals. It should be noted that these values are only approximations at best and are obtained from relatively few animals. The figures given, however, do provide an indication of the very low concentrations of the free porphyrins found normally in the body. As such, the finding of porphyrins in greater than trace amounts should not be ignored. Table X gives porphyrin concentrations in porphyric cows and calves (Kaneko and Mills, 1969, 1970).

i. Urine. It has been emphasized that porphyrin excretion may vary over wide limits. Jorgensen (1961a,b), in an extensive study of 52 cases, found values for urinary uroporphyrins between 6.3 and 3900 μg/dl and coproporphyrins between 2.1 and 8300 μg/dl. At concentrations of 100 μg/dl or more, a reddish discoloration is discernible in the urine. At 1000 μg/dl or more, intense red fluorescence of the urine is readily observed when exam-

TABLE IX

Normal Values of Porphyrin Concentration[a]

	Urine (μg/dl)		Feces (μg/gm)		RBC (μg/dl cells)		Plasma (μg/dl)		Bone marrow (μg/dl cells)		
Species	URO	COPRO	COPRO	PROTO	COPRO	PROTO	COPRO	PROTO	URO	COPRO	PROTO
Cattle	1.09 ± 0.92 0.80–1.60	4.06 ± 1.96 2.05–6.15	3.12 ± 0.96 1.11–4.28	0.75 ± 0.30 0.15–1.25	Trace	Trace	Trace	Trace	1	5	100
Swine		104[b]				118					
Rabbit	25[b]	41[b]	25[b]		2.6	83.3			Trace	4.5	87.5
Dog	50[b]			50[b]		35					

[a] References: Cattle: Jorgensen (1961b); Watson *et al.* (1959); Amoroso *et al.* (1957); Kaneko (1969). Swine: Cartwright and Wintrobe (1948). Rabbit: Schmid *et al.* (1952); Schwartz *et al.* (1952). Dogs: Schwartz *et al.* (1960).

[b] Micrograms per day.

TABLE X

Porphyrins in Blood and Excreta of Normal, Porphyric, and Porphyria Carrier Cattle[a]

Animals[b]	Red blood cells		Plasma		Urine		Feces	
	Copro-porphyrin	Protopor-phyrin	Copro-porphyrin	Protopor-phyrin	Copro-porphyrin	Uropor-phyrin	Copro-porphyrin	Protopor-phyrin
Normal mature cows (N = 10)	Trace	Trace	Trace	Trace	4.06 2.05-6.15	1.09 0.80-1.60	312 ± 96 (111-428)	75 ± 30 (15-125)
Mature porphyric cows								
1184	3.0	61	15.3	1.8	410	378	5670	46
652 (N = 3)	3.4	64	4.5	1.6	313	336	1900	12
2026 (N = 2)	3.1	457	8.9	1.5	498	487	2090	62
718	89.7	36	—	—	1450	1280	—	—
Mature porphyria carrier cows								
1140	Trace	Trace	Trace	Trace	Trace	Trace	292	88
1141	Trace	Trace	Trace	Trace	Trace	Trace	273	92
Porphyric calves (2-6 months old)								
1857 (N = 3)	2.9	104	1.5	Trace	13	70	796	144
1801 (N = 3)	7.8	252	38.4	2.6	1430	1144	12	72
1959	18.6	288	Trace	Trace	480	265	22	99
Porphyria carrier calf (5 months old)								
1802	Trace	Trace	Trace	Trace	Trace	Trace	495	40

[a] Values are expressed in micrograms per deciliter or micrograms per 100 gm; mean values plus or minus standard deviation (data in parentheses are minimal and maximal values) (Kaneko and Mills, 1969a, 1970).

[b] N denotes number of animals or number of determinations per animal.

ined in the dark with Woods light. The principal porphyrins excreted are URO I and COPRO I, and only a small fraction is of the type III isomers. The percentage of each appearing in the urine is not constant. In contrast to earlier reports, Jorgensen (1961b) observed a greater excretion of COPRO I than URO I.

Porphobilinogen is not characteristically present in bovine CEP urine, and earlier reports of its presence (Ellis *et al.*, 1958; Jorgensen and With, 1955) have not been confirmed (Jorgensen, 1961b). Normally colored, nonfluorescent urine of a CEP cow has consistently given a definite pink Ehrlich reaction, but, unlike porphobilinogen aldehyde, the pigment is soluble in chloroform (Kaneko, 1969). The nature of this pigment is unknown. Upon heating on a steam bath for 1 hour or standing at room temperature for several days, a definite red fluorescence is apparent upon exposure to ultraviolet light. Quantitative porphyrin determinations of this urine have yielded values of 135 and 87 μg/dl for uroporphyrin and coproporphyrin, respectively. Watson *et al.* (1959) also described a similar experience with bovine porphyria urine.

ii. Bile and feces. Bovine fecal porphyrins may be derived from two sources: the bile and chlorophyll of the food. The porphyrin derived from chlorophyll is, however, generally excluded by the usual analytical method. Essentially, the only porphyrin found in the bile and feces of CEP cattle is coproporphyrin I, and its concentration is similarly found to vary over wide limits (Table X). Fecal coproporphyrin varies between 1.9 and 11,800 μg/gm, and biliary coproporphyrin between 320 and 13,600 μg/dl (Jorgensen, 1961b). Only small amounts of COPRO III have been observed in feces. This preponderance of COPRO I in feces was also observed by Watson *et al.* (1959), who reported the presence of small amounts of URO I as well.

iii. Plasma and erythrocytes. Normally, only traces of free porphyrins are found in the plasma and in the RBC. In bovine CEP plasma, Watson *et al.* (1959) observed variable amounts, which were in general equally URO I (1–27 μg/dl) and COPRO I (4.2–25 μg/dl). A striking difference as compared to the human disease was the high level of free protoporphyrin in the erythrocytes of the CEP cow. The significance of this high level remains unclear. The isomer type of this protoporphyrin, although undetermined, is probably PROTO III (9) which was not used for heme synthesis. Elevated protoporphyrin levels are commonly found in iron deficiency and hemolytic anemias and in heavy-metal poisoning. Presumably, a real or relative lack of iron could account for the accumulation of free protoporphyrin in the erythrocyte (Watson, 1957). Serum iron levels, however, are normal or elevated in CEP (Watson *et al.*, 1959; Kaneko, 1963; Kaneko and Mattheeuws, 1966), and it would appear that something other than iron deficiency accounts for the high protoporphyrin level. Alternatively, the possibility has been considered (Schmid, 1966) that the protoporphyrin could be the type I isomer, a finding which would be a unique instance of its natural occurrence.

iv. Tissue. The range of concentration of porphyrins in various tissues of CEP cattle are given in Table XI. The deposition of porphyrins throughout the bones and soft tissues is readily apparent at postmortem of severe cases by the generalized discoloration. A reddish brown discoloration is most apparent in the teeth, bones, and bone marrow. The greatest discoloration of soft tissues occurs in the lungs and spleen, in which characteristic fluorescence may be observed with ultraviolet light. The high concentrations of porphy-

TABLE XI

Tissue Porphyrins in Bovine Erythropoietic Porphyria[a]

Tissue	URO	COPRO	PROTO	Total porphyrins
Bone marrow	Trace to 162	Trace to 1890	Trace to 394	Trace to 2396
Bones	6000	Tr	Tr	6000
Teeth	18,550	Tr	Tr	18,550
Spleen	0-10	Trace to 342	Trace to 60	7-400
Liver	0 to Trace	16-340	42-65	66-403
Lung	0-79	0-37	Trace to 20	20-130
Kidney	0	Trace to 117	5-16	5-133
Lymph node	0	0-40	1-7	1-49
Intestine	0	Trace to 65	7-77	18-104
Stomach	0	Trace to 58	12-82	12-111
Bile	0-690	112-12,205	0-856	112-13,750
Adrenal	0-6	Trace to 202	19-170	19-378
Ovary	0	65	1	66
Testes	0	0	9-14	9-14
Skin	0	0	0	0
Muscle	0	10	30	40
Brain and spinal cord	0	23	57	80

[a] Given in micrograms per 100 gram.

rins in splenic tissue are consistent with the hemolytic type of anemia observed in CEP. Discoloration of skin, muscle, heart, liver, and kidney is also observed, but only a part is due to porphyrins. The discoloration is presumably due to other porphyrin derivatives.

c. Hematological Findings. The hematological picture of the majority of reported cases is one of increased erythrogenesis in response to an anemic process of the type seen in hemolytic anemias. In general, the degree of response is related to the severity of the anemia associated with CEP. The anemia is normochromic and, depending on the degree of response, can be macrocytic. There is associated reticulocytosis, polychromasia, anisocytosis, basophilic strippling, and an increased number of metarubricytes. A consistent monocytosis has been observed (Rhode and Cornelius, 1958; Kaneko, 1963) but has remained unexplained. Bone marrow hyperplasia is shown by the markedly depressed myeloid:erythroid (M:E) cell ratio. As previously mentioned, bone marrow is also a principal site of porphyrin deposition. Watson *et al.* (1959) reported high concentrations of uroporphyrins in bone marrow of a CEP cow (Table XI).

The presence of porphyrins in the nucleated cells of the erythrocytic series is clearly evident by examination of unstained bone marrow smears with the fluorescent microscope. These have been designated as fluorocytes. This was originally observed by Schmid *et al.* (1955) in the bone marrow of a human patient and was an important contribution to the localization of the metabolic lesion in the metarubricyte [normoblast as used by Schmid *et al.* (1955)]. They also reported that fluorescence was observable in only one type of metarubricyte and that these were morphologically abnormal. The nuclei of the abnormal metarubricytes contained inclusions. Similar nuclear abnormalities were observed in bovine CEP marrow (Watson *et al.*, 1959). Schmid *et al.* (1955) concluded

that there were two separate populations of RBC, one normal and one which contains free porphyrins, but this has not been confirmed.

The presence of two populations of cells was reported in human beings but was attributed to the intermittent hemolytic crises which occurred (Gray *et al.*, 1950). Tschudy (1965) also pointed out that a single population of RBC is more likely to be present rather than two separate lines of cells. Runge and Watson (1969), in their studies of fluorescing bovine CEP bone marrow cells after bleeding, also concluded that their data were compatible with a single line of cells.

The hemogram of newborn CEP calves also have important differences from that seen in older CEP calves and cows. There is a striking erythrogenic response in the neonatal CEP calf which persists for the first 3 weeks of life. Nucleated erythrocyte counts during the first 24 hours of life have ranged from 5000 to 63,500 per microliter. Reticulocyte counts were lower than might have been expected (6.4%) and increased to a peak of only 12.5% at 4 days (Kaneko and Mills, 1969a). This may be a result of a postulated maturation defect as well as hemolysis (Smith and Kaneko, 1966; Rudolph and Kaneko, 1971).

d. Mechanism of the Anemia. An anemia with evidence of an erythropoietic response is a well-established occurrence in CEP. Morphologically, the anemia is compatible with that of a hemolytic process. The concentration of RBC porphyrins is high in CEP, and, if these erythrocytes were more susceptible to destruction, a shortening of life span would be expected. The RBC life span is indeed shortened in bovine (Kaneko, 1963) and in human (Gray *et al.*, 1950) CEP. There is also general agreement that this shortening is associated with hemolysis, but the mechanism of the hemolysis remains obscure.

In vivo ^{59}Fe metabolism studies are completely compatible with a hemolytic type of anemia, and a degree of bone marrow hemolysis, i.e., ineffective erythropoiesis, also occurs (Kaneko, 1963; Kaneko and Mattheeuws, 1966). Plasma iron turnover and transfer rates, RBC iron uptake, and organ uptakes were increased, in keeping with a hemolytic process. It has also been shown that erythrocyte survival in bovine CEP is inversely correlated with the RBC coproporphyrin concentration (Kaneko *et al.*, 1971). The shortest abnormal RBC survival time of 27 days was associated with the highest RBC coproporphyrin concentration. It would be reasonable to expect an association between one or more of the plasma or RBC porphyrins and RBC survival. The porphyrins are presumed in some way to alter the RBC and make it more susceptible to destruction or removal from the circulation.

The mechanism of cell alteration has also been studied in RBC s, reticulocytes, and developing RBC's. A number of RBC enzymes associated with RBC survival were determined, and only the GSH stability was found to be abnormal in CEP cows (Kaneko and Mills, 1969a,b).

The CEP reticulocyte has also been shown to have a biochemical defect. This defect is expressed as an increase in porphyrin synthesis, a marked decrease in heme synthesis, and a delay in the maturation of the reticulocyte (Smith and Kaneko, 1966). The $T_{1/2}$ for the maturation of the reticulocyte was 50 hours in comparison to the normal of 3–10 hours. This delay in reticulocyte maturation is thought to be the result of the defect in heme synthesis since the rate of heme synthesis controls the rate of maturation of the reticulocyte (Schulman, 1968).

A similar delay in maturation of the metarubricyte to reticulocyte was observed in the

bone marrow cells of CEP cows (Rudolph and Kaneko, 1971), but there was no effect on the younger cells of the erythrocytic series. Thus, it appears that the more mature cells of the erythrocytic series (metarubricyte, reticulocyte, RBC) are the ones noticeably affected by the high porphyrin content. This is not surprising since heme and hemoglobin synthesis are most active in the later stages (Fig. 1). Ultimately, the sum total of these changes may alter the cells, making them more susceptible to hemolysis, and this hemolysis, either intra- or extravascular, would occur with cells of the bone marrow or of the peripheral blood. Upon exposure to sunlight an enhancement of a photohemolysis of the type observed in erythropoietic protoporphyria (Harber *et al.*, 1964) might further aggravate the hemolysis.

This mechanism might also explain the striking erythrogenic response of the neonatal porphyric calf. If porphyrins do not cross the placental barrier (and this is not known), they would be stored in the fetus and exert a profound hemolytic effect in the fetus.

The newborn calf would therefore have a marked rate of erythrogenesis. At birth, the level of porphyrins is high and falls to a steady-state level in about 3 weeks in CEP calves. This is comparable to the rate of clearance of [^{14}C]porphyrin in urine, which fell to 0.1% of the initial concentration in 3 weeks (Kaneko, 1969). Furthermore, 3 weeks is also the time at which the erythrogenic response has stabilized at a steady-state level for the particular neonatal calf (Kaneko and Mills, 1970).

In summary, these findings suggest that a high porphyrin content induces a defect in heme synthesis. This biochemical defect is thought to be morphologically expressed as a maturation defect, and the defective and altered cell may be more susceptible to intra- or extravascular hemolysis. This would be compatible with the observed hemolytic type of anemia with shortened life span, the degree of which depends upon the severity of the porphyria.

e. Detection of the Carrier State. Bovine CEP is inherited as an autosomal recessive trait. In the past, carrier animals could be detected only by occurrence of disease in their progeny. Levin (1968a) developed an assay for the activity of UROgen III-cosyn. When it was used in hemolysates of porphyric cattle, its activity was found to be considerably less than in normal cattle (Levin, 1968b). Heterozygous cattle, which are clinically unaffected carriers of the porphyria gene, had UROgen III-cosyn activities intermediate between porphyrics and normals (Romeo *et al.*, 1970). Similar low UROgen III-cosyn activity is found in human CEP, but carriers are less readily detectable in human beings than in cattle (Romeo and Levin, 1969). These findings are also in keeping with the concept that the genetic defect in CEP is a deficiency of UROgen III-cosyn. Romeo (1977) has extensively reviewed the genetic aspects of all forms of hereditary porphyrias and concludes that the weight of evidence is conclusive for UROgen III-cosyn deficiency in CEP.

f. Metabolic Basis of Bovine Erythropoietic Porphyria. The mechanisms for heme biosynthesis and the biochemical nature of the porphyrins in the tissues and excreta provide a basis for an explanation of the metabolic defect in CEP. Certain features of the biosynthetic mechanism are particularly important in attempting to explain this porphyria in terms of enzymatic derangement. These can be briefly summarized as follows:

1. There appears to be an anatomical separation (or compartmentation) of the enzymes involved in heme biosynthesis. Some of the enzymes are found in the mitochondria and others are found in the cytosol.

2. The mitochondrial systems are involved in:
   a. The initial synthesis of ALA.
   b. The synthesis of protoporphyrin III (9) from COPROgen III.
   c. Heme formation, i.e., incorporation of iron into protoporphyrin III (9).
3. The enzymes of the cytosol catalyze the intervening steps which lead to the formation of PBG, UROgens, and COPROgens.
4. Mitochondria are present only in the immature erythroid cells, including the nucleated cells and the reticulocytes. The most active hemoglobin formation occurs in the metarubricyte and ceases when maturity is reached.

The metabolic defect is localized in the erythropoietic tissue of the CEP animal, and the probable site is within the developing erythroid cells. These are considered to be the mitochondria-containing cells, principally the metarubricytes, and would not include the mature, nonnucleated RBC.

The precise nature of the enzymatic defect awaits complete clarification of the steps involved in heme biosynthesis, mainly the mechanism for the cyclization of four molecules of PBG to form uroporphyrinogen. The action of the UROgen I-syn and UROgen III- cosyn systems in heme biosynthesis of animals would explain CEP. Accordingly, the formation of the type III isomer requires the combined action of both enzymes, and, if UROgen I-syn is acting alone, the type I isomer is formed. Thus, the activity of each of these enzymes would exercise a degree of control over which of the two pathways for isomer synthesis is traversed. In the homozygous state for CEP, there is a deficiency of UROgen III-cosyn and the formation of type I isomers is favored by the relative excess of UROgen I-syn. A decrease in UROgen III-cosyn in porphyric cows and human beings has been reported (Levin, 1968b; Romeo and Levin, 1969).

Total deficiency of UROgen III-cosyn is obviously incompatible with life. Also, there is wide variation in severity of the disease in animals, but the degree of severity is quite constant in each animal kept under standard conditions. A summary (Kaneko, 1970) of of a proposed metabolic basis for bovine CEP is given in Fig. 12. Central to this proposal is a genetically controlled UROgen III-cosyn deficiency and the resultant failure of heme feedback repression.

2. Bovine Erythropoietic Protoporphyria (EPP)

This disorder of porphyrin metabolism occurs in human beings and in cattle (Ruth *et al.*, 1977). It was first reported in 1961 (Magnus *et al.*, 1961) and is now well recognized in human beings (Redeker, 1963; Harber *et al.*, 1964). It is inherited as a dominant autosomal trait (Romeo, 1977). Patients do not have the major signs of CEP, such as anemia, urine porphyrin excretion, and discolored teeth. Photosensitivity of the skin appears to be the only clinical manifestation of the disease, and this is associated with high plasma protophorphyrin concentration. In the laboratory, the most striking findings are the high concentrations of PROTO III (9) in the erythrocytes and feces.

In cattle, in contrast to human beings, EPP has a pattern of recessive inheritance and may be sex-linked because to date it has only been seen in females. The photosensitivity also seems to diminish in adult life. Affected cattle also do not have anemia, urine porphyrin excretion, or discolored teeth. Levels of RBC and fecal protoporphyrin are very high in comparison to normals.

The fundamental enzymatic defect in bovine EPP has been shown to be a deficiency of ferrochelatase (heme synthetase) (Ruth *et al.*, 1977). Low ferrochelatase activity was

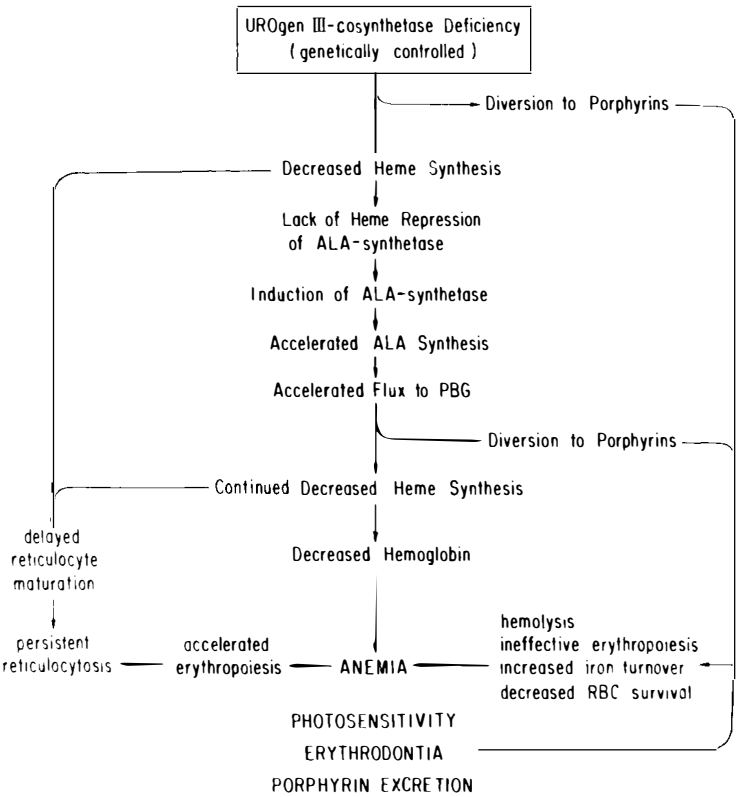


Fig. 12. Summary of the metabolic basis of bovine congenital erythropoietic porphyria predicated upon a deficiency of UROgen III-cosyn. Production of type I porphyrins accounts for most clinical features of the disease. Metabolically, a decrease in heme synthesis is the central biochemical defect.

found in all tissues of EPP calves examined, so the defect appears to be a total body defect. In the presence of low ferrochelatase activity, PROTO III (9) would then accumulate.

D. Porphyria of Swine

The occurrence of porphyria in swine was first recognized by Clare and Stephens (1944) in New Zealand. It was later recognized in Denmark, and a number of accounts of the studies conducted with these swine have been published (Jorgensen and With, 1955; Jorgensen, 1959).

In contrast to the bovine form, the disease in swine is inherited as a dominant characteristic. Except in very severe cases, there appears to be little or no effect upon the general health of the pig. Photosensitivity is not seen, even in white pigs. The predominant feature in the affected pig is a characteristic reddish discoloration of the teeth, which usually fluoresce when examined with ultraviolet light. Porphyrin deposition in the teeth of the newborn pig appears to be so consistent as to be pathognomic. On occasion, darkly discolored teeth may not fluoresce, but porphyrins may be extracted from these with 0.5 *N* HCl (With, 1955). Similar, although less apparent, deposition occurs in the bones. The

porphyrins, which have been found in concentrations of up to 200 μg/gm, are comprised principally of uroporphyrin I. The liver, spleen, lungs, kidneys, bones, and teeth also are discolored by another dark pigment, the nature of which is unknown (Jorgensen, 1959).

The urine is discolored only in the more severely affected pig. The 24-hour excretion of uroporphyrins ranged between 100 and 10,00 μg, and that of coproporphyrin was only 50 μg. These were both the type I isomers. Porphobilinogen is absent in the urine. Similarities in this pattern of porphyrin excretion close to that found in bovine porphyria are apparent, but the localization of the defect in erythropoietic tissue remains unestablished.

E. Porphyria of Cats

The occurrence of porphyria in cats was reported in a young male kitten (Tobias, 1964). One of its three littermates was affected, and kittens from a previous litter were also reported to have had the same unusually colored teeth. The kitten's teeth were brown and, under ultraviolet light, fluoresced red. It urine was amber-colored and was qualitatively positive for uroporphyrin, coproporphyrin, and porphobilinogen. There was no evidence of anemia or photosensitivity. These cats had been kept indoors all their lives. One was the propositus for a porphyric cat colony (Glenn *et al.*, 1968). Study of the inheritance of the porphyria in these cats indicates it to be of a simple Mendelian aurosomal dominant trait analagous to that seen in swine.

Detailed studies of CEP in another family of Siamese cats have been reported (Giddens *et al.*, 1975). Excessive accumulation of URO I, COPRO I, and protoporphyrins were observed in RBC's, urine, feces, and tissues. These cats had photosensitivity, severe anemia, and, in addition, severe renal disease. It is presumed that the principal defect is a deficiency in uroporphyrinogen III cosynthestase activity similar to human beings and cows.

F. Porphyria of Fox Squirrels

All fox squirrels *(Sciurus niger)* have a normal species characteristic which resembles human and bovine CEP (Flyger and Levin, 1977; Levin, 1974). The condition in squirrels is characterized by (1) a deficiency of UROgen III-cosyn; (2) increased concentrations of URO I in urine and feces; (3) accumulation of URO I in teeth, bones, spleen, and other soft tissues, giving a pink coloration; (4) fluorescence of the tissues under ultraviolet light; (5) increased erythropoiesis but no hemolytic anemia; and (6) no photosensitivity.

The deficiency in UROgen III-cosyn activity is found only in the fox squirrel and not in the closely related gray squirrel *(Sciurus carolinensis).* The urine porphyrin excretion in the fox squirrel is ten times greater than in the gray squirrel and is markedly increased when erythropoiesis is stimulated by bleeding. The UROgen III-cosyn of fox squirrel RBC's is very susceptible to heat, and it may indicate that CEP is due to increased lability of this enzyme.

G. Hepatic Porphyrias

This group of disorders has thus far been observed only in man and constitutes the most common type of porphyria seen. They are inherited as dominant traits. The salient features

of this group of porphyrias, together with those of erythropoietic porphyria and erythrpoietic protoporphyria, are summarized in Table XII. As the name implies, the metabolic defect in hepatic porphyria is involved with hepatic porphyrin metabolism. Further subdivisions are based upon the principal clinical manifestations of this disturbance.

In the intermittent acute type, paroxysmal attacks, which may be precipitated by barbiturates or alcohol, occur intermittently in an otherwise chronic condition. Attacks are manifested by extreme abdominal pain and/or nervous involvement, which are often the presenting symptoms. Photosensitivity is not a feature of this form. The principal urinary finding is the excretion of large amounts of ALA and PBG. An explanation for the biochemical defect in hepatic porphyria must explain this excess in ALA and PBG and increased hepatic ALA-syn activity. From experimental animal studies, it is known that ALA-syn is an inducible enzyme in liver (Granick and Urata, 1963) and also the rate-limiting enzyme in porphyrin synthesis. This would mean that when the ALA-syn level is high, excess ALA and PBG would be formed and excreted. This has been demonstrated in human hepatic porphyria (Tschudy, 1965). The genetic defect is now known to be a deficiency of UROgen I-syn activity in liver (Strand *et al.*, 1970). In view of the role of heme in the feedback regulation of ALA-syn, a block in heme synthesis would result in the observed induction of ALA-syn and increased ALA and PBG.

The cutaneous form has been subgrouped because photosensitivity is the predominant symptom. δ-Aminokvulinic acid and PBG may be present in the urine, but more commonly a mixture of porphyrins, both I and III, is found. The mixed or combined form, which is rarely seen, exhibits the symptoms of both the cutaneous and the intermittent acute forms.

TABLE XII

Summary of the Porphyrias

	Erythropoietic porphyrias		Hepatic porphyrias	
	Congenital erythropoietic porphyria	Erythropoietic protoporphyria	Acute intermittent porphyria	Mixed porphyria
Heredity				
Cattle	Recessive	Recessive		
Cat, pig	Dominant			
Man	Recessive	Dominant	Dominant	Dominant
Site of defect	Hematopoietic tissue	Hematopoietic tissue	Liver	Liver
Clinical anemia	Yes	No	No	No
Photosensitivity	Yes	Yes	No	Yes
Abdominal pain	No	No	Yes	Yes
Erythrodontia	Yes	No	No	No
Urine color	Reddish brown	Normal	Normal	Normal
Porphyrin	URO I, COPRO I	Normal	ALA, PBG	ALA, PBG, URO, COPRO
Liver	—	—	ALA, PBG	ALA, PBG, URO, COPRO
Bone marrow	URO I, COPRO I, PROTO	PROTO	Normal	Normal
RBC	COPRO I, PROTO	PROTO	Normal	Normal
Plasma	URO I, COPRO I	PROTO	Normal	Normal

REFERENCES

Allen, D. W., and Jandl, J. H. (1961). *J. Clin. Invest.* **40,** 454.

Allen, W. M., Ross, W. H., Anderson, P. H., Berrett, S, Bradley, R., and Patterson, D. S. P. (1975). *Vet. Rec.* **96,** 360–361.

Amoroso, E. C., Loosmore, R. M., Rimington, C., and Tooth, B. E. (1957). *Nature (London)* **180,** 230.

Anderson, R. L., and Tove, S. B. (1958). *Nature (London)* **182,** 315.

Anstall, H. B., and Trujillo, J. M. (1967). *Am. J. Clin. Pathol.* **47,** 296.

Arnold, H., Blume, K. G., Busch, D., Lenkeit, U., Löhr, G. W., and Lübs, E. (1970). *Klin. Wochenschr.* **48,** 1299–1308.

Bartlett, G. R. (1970). *Adv. Exp. Med. Biol.* **6,** 245–256.

Bartlett, G. R., and Marlow, A. A. (1951). *Bull. Scripps Metab. Clin.* **2,** 1.

Bartlett, G. R., and Shafer, A. W. (1960). *J. Clin. Invest.* **39,** 62.

Battaglia, F. C., McGaughey, H., Makowski, E. L., and Meschia, G. (1970). *Am. J. Physiol.* **219,** 217.

Beck, W. S. (1964). *Medicine (Baltimore)* **43,** 715–726.

Benesch, R., and Benesch, R. E. (1967). *Biochem. Biophys. Res. Commun.* **26,** 162.

Beritic, T. (1965). *Blood* **25,** 999–1008.

Berlin, N. I. (1964). *In* "The Red Blood Cell" (C. Bishop and D. M. Surgenor, eds.), pp. 423–450. Academic Press, New York.

Berlin, N. I., and Lotz, C. I. (1951). *Proc. Soc. Exp. Biol. Med.* **78,** 788.

Berlin, N. I., Meyer, L. M., and Lazarus, M. (1951). *Am. J. Physiol.* **166,** 565.

Bernstein, R. E. (1954). *Science* **120,** 459.

Beutler, E. (1971). *Semin. Hematol.* **8,** 311–347.

Beutler, E. (1972a). *Adv. Metab. Disord.* **6,** 131–160.

Beutler, E. (1972b). *In* "Hematology" (W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles, eds.), pp. 132–144. McGraw-Hill, New York.

Beutler, E. (1975). "Red Cell Metabolism: A Manual of Biochemical Methods." 2d ed. pp. 38–131. Grune and Stratton, New York.

Beutler, E., and Srivastava, S. K. (1972). *In* "Hematology" (W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles, eds.), pp. 94–100. McGraw-Hill, New York.

Beutler, E., and Teeple, L. (1969). *Acta Biol. Med. Ger.* **22,** 707.

Beutler, E., Dern, R. J., and Alving, A. S. (1954). *J. Lab. Clin. Med.* **44,** 177.

Bishop, C. (1964). *In* "The Red Blood Cell" (C. Bishop and D. M. Surgenor, eds.), pp. 147–188. Academic Press, New York.

Blunt, M. H., Kitchens, J. L., Mayson, S. M., and Huisman, H. J. (1971). *Proc. Soc. Exp. Biol. Med.* **138,** 800–803.

Board, P. G. and Peter, D. W. (1976). *Vet. Rec.* **99,** 144.

Bogorad, L. (1958a). *J. Biol. Chem.* **233,** 501.

Bogorad, L. (1958b). *J. Biol. Chem.* **233,** 510.

Bogorad, L., and Granick, S. (1953). *Proc. Natl. Acad. Sci. U.S.A.* **39,** 1176.

Bonting, S. L., Simon, K. A., and Hawkins, H. (1961). *Arch. Biochem. Biophys.* **95,** 416.

Bracci, R., Corvaglia, E., Princi, P., Bettini, F., and Dindinelli, C. (1969). *Ital. J. Biochem.* **18,** 100–113.

Brace, K. C., and Altland, P. D. (1956). *Proc. Soc. Exp. Biol. Med.* **92,** 615.

Brin, M., and Yonemoto, R. H. (1958). *J. Biol. Chem.* **230,** 307.

Bruns, F. H., Noltmann, E., and Valhauls, E. (1958a). *Biochem. Z.* **330,** 483.

Bruns, F. H., Noltmann, E., and Valhauls, E. (1958b). *Biochem. Z.* **330,** 497.

Bunn, H. F. (1971). *Science* **172,** 1049–1059.

Burns, K. F., and DeLannoy, C. U., Jr. (1966). *Toxicol. Appl. Pharmacol.* **8,** 429–437.

Burr, M. J. (1972). *Comp. Biochem. Physiol.* **418,** 687–694.

Bush, J. A., Berlin, N. I., Jensen, W. N., Brill, A. B., Cartwright, G. E., and Wintrobe, M. M. (1955). *J. Exp. Med.* **101,** 451–459.

Bush, J. A., Jensen, M. M., Athens, J. W., Ashenbrucher, H., Cartwright, G. E., and Wintrobe, M. M. (1956). *J. Exp. Med.* **103,** 701.

Carson, P. E., Flanagan, C. L., Ickes, C. E., and Alving, A. S. (1956). *Science* **124,** 484.

Cartwright, G. E., and Wintrobe, M. M. (1948). *J. Biol. Chem.* **172,** 557.

Cartwright, G. E., and Wintrobe, M. M. (1964). *Am. J. Clin. Nutr.* **14,** 224.

Chan, P. C., Calabrese, V., and Theil, L. S. (1964). *Biochim. Biophys. Acta* **79,** 424–426.
Chanutin, A., and Curnish, R. R. (1967). *Arch. Biochem. Biophys.* **121,** 96.
Chapman, R. G., Hennessey, M. A., Waltersdolph, A. M., Huennekens, F. A., and Gabrio, B. W. (1962). *J. Clin. Invest.* **41,** 1249–1256.
Cheun, L. H. (1966). *J. Clin. Pathol.* **19,** 614.
Chow, C. K., and Tappel, A. L. (1974). *J. Nutr.* **104,** 444.
Clare, H. T., and Stephens, E. H. (1944). *Nature (London)* **153,** 252.
Cline, J. J., and Berlin, N. I. (1963). *Am. J. Physiol.* **204,** 415.
Collins, J. D., Jain, N. C., Schalm, O. W., and Pangborn, J. (1968). *Am. J. Vet. Clin. Pathol.* **2,** 75–80.
Cookson, G. H., and Rimington, C. (1953). *Nature (London)* **171,** 875.
Cornelius, C. E., and Kaneko, J. J. (1962). *Science* **137,** 673.
Cornelius, C. E., Kaneko, J. J., Benson, D. C., and Wheat, J. D. (1960). *Am. J. Vet. Res.* **20,** 1123–1124.
de Viale, L. C. S. M., and Grinstein, M. (1968). *Biochim. Biophys. Acta* **158,** 79.
Dick, D. A. T., Dick, E. G., and Tosteson, D. C. (1969). *J. Gen. Physiol.* **54,** 123–133.
Dische, Z. (1964). *In* "The Red Blood Cell" (C. Bishop and D. M. Surgenor, eds.), pp. 189–209. Academic Press, New York.
Eales, L. (1961). *Annu. Rev. Med.* **12,** 251.
Edmondson, P. W., and Wyburn, J. R. (1963). *J. Exp. Pathol.* **44,** 72.
Eichelberger, L., Fletcher, E. S., Jr., Gerling, E. M. K., and Vos, B. J., Jr. (1940). *J. Biol. Chem.* **133,** 145.
Elder, H. A., and Mortensen, R. A. (1956). *J. Biol. Chem.* **218,** 261.
Ellis, D. J., Barner, R. D., Madden, D., Melcer, I., and Orten, J. M. (1958). *Mich. State Univ. Vet.* **18,** 89.
Engelhardt, W. A. (1930). *Biochem. Z.* **227,** 16.
Flohe, L., and Brand, I. (1969). *Biochim. Biophys. Acta* **191,** 541.
Flohe, L., Guenzler, W. A., and Schock, H. H. (1973). *FEBS Lett.* **32,** 132–134.
Flyger, V., and Levin, E. Y. (1977). *Am. J. Pathol.* **87,** 269–272.
Forssberg, A., and Tribukait, B. (1962). *Acta Physiol. Scand.* **54,** 152.
Fourie, P. J. J. (1936). *Onderstepoort J. Vet. Sci. Anim. Ind.* **7,** 535.
Fourie, P. J. J. (1939). *Onderstepoort J. Vet. Sci. Anim. Ind.* **13,** 383.
Fourie, P. J. J., and Roets, G. C. S. (1939). *Onderstepoort J. Vet. Sci. Anim. Ind.* **13,** 369.
Garby, L. (1962). *Br. J. Haematol.* **8,** 15.
George, J. M., O'Brien, R. L., Poooack, S., and Crosby, W. H. (1966). *J. Clin. Invest.* **45,** 1280.
Gibson, K. D., Laver, W. G., and Neuberger, A. (1958). *Biochem. J.* **70,** 71.
Giddens, W. E., Jr., Labbe, R. F., Swango, L. J., and Padgett, G. A. (1975). *Am. J. Pathol.* **80,** 367–386.
Glenn, B. L., Glenn, H. G., and Omtvedt, I. T. (1968). *Am. J. Vet. Res.* **29,** 1653.
Gower, D. B., and Davison, W. M. (1963). *Brit. J. Haematol.* **9,** 132.
Granick, S. (1966). *J. Biol. Chem.* **241,** 1359.
Granick, S., and Levere, R. D. (1964). *Prog. Hematol.* **4,** 1.
Granick, S., and Mauzerall, D. (1958). *J. Biol. Chem.* **232,** 1119.
Granick, S., and Urata, G. (1963). *J. Biol. Chem.* **238,** 821.
Gray, C. H., Muir, I. M. H., and Neuberger, A. (1950). *Biochem. J.* **47,** 542.
Greenwald, I. (1925). *J. Biol. Chem.* **63,** 339.
Gruhzit, O. M. (1931a). *Am. J. Med. Sci.* **181,** 812.
Gruhzit, O. M. (1931b). *Am. J. Med. Sci.* **181,** 815.
Hafeman, D. G., Sunde, R. A., and Hoekstra, W. G. (1974). *J. Nutr.* **104,** 580.
Hansard, S. L., and Foote, L. E. (1959). *Am. J. Physiol.* **197,** 711.
Harber, L. C., Fleischer, A. S., and Baer, R. L. (1964). *J. Am. Med. Assoc.* **189,** 191.
Harkness, D. R. (1971). *Adv. Intern. Med.* **17,** 189–214.
Harkness D. R., and Ponce, J. (1969). *Arch. Biochem. Biophys.* **134,** 113–119.
Harkness, D. R., and Roth, S. (1969). *Biochem. Biophys. Res. Commun.* **34,** 849.
Harkness, D. R., Ponce, J., and Grayson, V. (1969). *Comp. Biochem. Physiol.* **28,** 129–138.
Harris, J. W. (1963). "The Red Cell: Production, Metabolism and Destruction: Normal and Abnormal." Harvard Univ. Press, Cambridge, Massachusetts.
Harris, J. W., and Kellermeyer, R. W. (1970). *In* "The Red Blood Cell" (C. Bishop and D. M. Surgenor, eds.), p. 473. Academic Press, New York.
Harvey, J. W., and Kaneko, J. J. (1976). *J. Cell. Physiol.* **89,** 219–224.
Harvey, J. W., Ling, G. V., and Kaneko, J. J. (1974). *J. Am. Vet. Med. Assoc.* **164,** 1030–1033.
Harvey, J. W., Kaneko, J. J., and Hudson, E. B. (1977). *Vet. Clin. Pathol.* **6,** 13–17.

Heinz, R. (1890). *Arch. Pathol. Anat. Physiol. Klin. Med.* **122,** 112-116.
Hutton, J. J. (1972). *Blood,* **39,** 542-553.
Icen, A. (1967). *Scand. J. Clin. Lab. Invest.* **96,** 1.
International Committee for Standardization in Hematology (1972). *Blood* **38,** 378.
Iodice, A. A., Richet, D. A., and Schulman, M. P. (1958). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **17,** 248.
Itano, H. A. (1966). *J. Cell. Physiol.* **67,** 65.
Jacob, F., and Monod, J. (1963). *In* "Cytodifferentiation and Macromolecular Synthesis" (M. Locke, Ed.), p. 30. Academic Press, New York.
Jacob, H. S. (1970). *Semin. Hematol.* **7,** 341-354.
Jacob, H. S., and Jandl, J. H. (1962). *J. Clin. Invest.* **41,** 1514.
Jacob, H. S., Ingbar, S. H., and Jandl, J. H. (1964). *J. Clin. Invest.* **43,** 1259.
Jaffe, E. R., and Hsieh, H. S. (1971). *Semin. Hematol.* **8,** 417-437.
Johnson, R. B., and Stewart, W. E. (1967). *J. Dairy Sci.* **50,** 1828-1830.
Jorgensen, S. K. (1959). *Br. Vet. J.* **115,** 160.
Jorgensen, S. K. (1961a). *Br. Vet. J.* **117,** 1.
Jorgensen, S. K., and With, T. K. (1955). *Nature (London)* **176,** 156.
Judd, J. T., and Matrone, T. (1962). *J. Nutr.* **77,** 264.
Kaneko, J. J. (1963). *Ann. N.Y. Acad. Sci.* **104,** 689.
Kaneko, J. J. (1969). Unpublished observations.
Kaneko, J. J. (1970). *In* "Clinical Biochemistry of Domestic Animals" (J. J. Kaneko and C. E. Cornelius, eds.), Vol. 1, p. 152. Academic Press, New York.
Kaneko, J. J. (1973). "Standard Values in Domestic Animals." Univ. Of California, Davis.
Kaneko, J. J. (1974). *Adv. Vet. Sci. Comp. Med.* **8,** 117-153.
Kaneko, J. J., and Cornelius, C. E. (1962). *Am. J. Vet. Res.* **23,** 913.
Kaneko, J. J., and Mattheeuws, D. R. G. (1966). *Am. J. Vet. Res.* **27,** 923.
Kaneko, J. J. and Mills, R. (1969b). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **23,** 453.
Kaneko, J. J., and Mills, R. (1970). *Cornell Vet.* **60,** 52.
Kaneko, J. J., and Mills, R. (1969a). *Am. J. Vet. Res.* **30,** 1805-1810.
Kaneko, J. J., and Smith, J. E. (1964). *Am. J. Vet. Res.* **25,** 846-848.
Kaneko, J. J., Cornelius, C. E., and Heuschele, W. P. (1961). *Am. J. Vet. Res.* **22,** 683.
Kaneko, J. J., Green, R. A., and Mia, A. S. (1969a). *Proc. Soc. Exp. Biol. Med.* **123,** 783-784.
Kaneko, J. J., Tanaka, S., Nakajima, H., and Ushimi, C. (1969b). *Am. J. Vet. Res.* **30,** 543-549.
Kaneko, J. J., Zinkl, J. G., and Keeton, K. S. (1971). *Am. J. Vet. Res.* **32,** 1981-1985.
Kaplan, J., and Beutler, E. (1968). *Nature (London)* **217,** 256.
Keeton, K. S., and Kaneko, J. J. (1972). *Proc. Soc. Exp. Biol. Med.* **140,** 30-35.
Keeton, K. S., and Kaneko, J. J. (1973a). *Res. Vet. Sci.* **15,** 275.
Keeton, K. S., and Kaneko, J. J. (1973b). *Res. Vet. Sci.* **15,** 285.
Keller, D. F. (1970). *Crit. Rev. Clin. Lab. Sci.* **1,** 247-302.
Kikuchi, G., Kumar, A., Talmage, P., and Shemin, D. (1958). *J. Biol. Chem.* **233,** 1214.
Kim, H. D., and McManus, T. J. (1971). *Biochim. Biophys. Acta* **230,** 1-11.
Kinoshita, J. H., and Beutler, E. (1971). *Exp. Eye Res.* **11,** 261-428.
Kirkman, H. N. (1962). *J. Biol. Chem.* **237,** 2364.
Kitchen, H. (1969). *Adv. Vet. Sci. Comp. Med.* **13,** 247-330.
Koger, L. M. (1956). *J. Am. Vet. Med. Assoc.* **129,** 75.
Labbe, R. F., and Hubbard, H. (1961). *Biochim. Biophys. Acta* **52,** 130.
Laris, P. C. (1958). *J. Cell. Comp. Physiol.* **51,** 273-307.
LeFevre, P. G. (1961). *Pharmacol. Rev.* **13,** 19.
Leng, R. A., and Annison, E. F. (1962). *Aust. J. Agric. Res.* **13,** 31-43.
Letchworth, G. J., Bentinck-Smith, J., Bolton, G. R., Wootton, J. F., and Family, L. (1977). *J. Am. Anim. Hosp. Assoc.* **13,** 75-79.
Levere, R. D. (1968). *Adv. Clin. Chem.* **11,** 133.
Levin, E. Y. (1968a). *Biochemistry* **7,** 3781.
Levin, E. Y. (1968b). *Science* **161,** 907.
Levin, E. Y. (1974). *Ann. N.Y. Acad. Sci.* **241,** 347-359.
Levin, E. Y., and Coleman, D. L. (1967). *J. Biol. Chem.* **242,** 4248.
McManus, T. J. (1967). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **26,** 1821-1826.
Magnus, I. A., Jarrett, A., Prankerd, T. A. J., and Rimington, C. (1961). *Lancet* **ii,** 448.

Malone, J. I., Winegrad, A. I., Oski, F. A., and Holmes, E. W., Jr. (1968). *N. Engl. J. Med.* **279,** 1071.
Marks, P. A. (1964). *In* "The Red Blood Cell" (C. Bishop and D. M. Surgenor, eds.), pp. 211-237. Academic Press, New York.
Martinovich, D., and Woodhouse, D. A. (1971). *N. Z. Vet. J.* **19,** 259-263.
Marvin, H. N., Dinning, J. S., and Day, P. L. (1960). *Proc. Soc. Exp. Biol. Med.* **105,** 473-475.
Mathai, C. K., Ohno, S., and Beutler, E. (1966). *Nature (London)* **210,** 115-166.
Marver, H. S., and Schmid, R. (1972). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds.), 3rd Ed., pp. 1087-1140. McGraw-Hill, New York.
Meyer, U. A., and Schmid, R. (1978). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds.), 4th Ed., pp. 1166-1220. McGraw-Hill, New York.
Minakami, S., and Yoshikawa, H. (1966). *J. Biochem. (Tokyo)* **59,** 139-144.
Mohler, D. N., and Williams, W. J. (1961). *J. Clin. Invest.* **40,** 1735.
Murphy, J. R. (1960). *J. Lab. Clin. Med.* **55,** 286-302.
Necheles, T. F., Boles, T. A., and Allen, D. M. (1968). *J. Pediatr.* **72,** 319-324.
Necheles, T. F., Maldonado, N., Barquet-Chediok, A., and Allen, D. M. (1969). *Blood* **33,** 164.
Nestel, B. L. (1958). *Cornell Vet.* **48,** 430.
Neuberger, A., and Niven, J. S. F. (1951). *J. Physiol. (London)* **112,** 292.
Noguchi, T., Cantor, A. H., and Scott, M. L. (1973). *J. Nutr.* **103,** 1502.
Novy, M. J., and Parer, J. T. (1969). *Respir. Physiol.* **6,** 144-150.
Obara, J., and Nakajima, H. (1961). *Jpn. J. Vet. Sci.* **23,** 247.
Oh, S. H., Sunde, R. A., Pope, A. C., and Hoekstra, G. (1974). *J. Anim. Sci.* **39,** 247-248.
Oski, F. A., Sugerman, H. J., and Miller, L. D. (1972). *Blood,* **39,** 522:524.
Paglia, D. E., and Valentine, W. N. (1969). *J. Lab. Clin. Med.* **70,** 188.
Paniker, N. V., and Beutler, E. (1972). *J. Anim. Sci.* **34,** 75-76.
Passo, T., Kenson, D. A. T., and Manfredi, F. (1971). *J. Lab. Clin. Med.* **78,** 1010.
Perutz, M. F. (1965). *J. Mol. Biol.* **13,** 646.
Perutz, M. F. (1970). *Nature (London)* **228,** 726.
Pierce, K. R., Joyce, J. R., England, R. B., and Jones, L. P. (1972). *J. Am. Vet. Med. Assoc.* **160,** 323-327.
Prankerd, T. A. J. (1961). "The Red Cell." Blackwell, Oxford.
Prasse, K. W., Crouser, D., Beutler, E., Walder, M., and Schall, W. D. (1975). *J. Am. Vet. Med. Assoc.* **166,** 1170-1175.
Proctor, H. J., Lentz, T. R., and Johnson, G., Jr. (1971). *Ann. Surg.* **174,** 923-931.
Rapoport, S. (1968). *Essays Biochem.* **4,** 69-103.
Rapoport, S., and Luebering, J. (1950). *J. Biol. Chem.* **183,** 507.
Ratozzi, M. C. (1968). *Biochem. Biophys. Res. Commun.* **31,** 16.
Redeker, A. G. (1963). *J. Lab. Clin. Med.* **9,** 235.
Rhode, E. A., and Cornelius, C. E. (1958). *J. Am. Vet. Med. Assoc.* **132,** 112.
Rifkind, R. A., and Danon, D. (1965). *Blood* **25,** 885.
Rigby, P. G., Emerson, C. P., and Friedell, G. H. (1961). *Proc. Soc. Exp. Biol. Med.* **106,** 313.
Rimington, C. (1936). *Onderstepoort J. Vet. Sci. Anim. Ind.* **7,** 567.
Robin, E. D., Murdaugh, H. V., Jr., Cross, C. E., Smith, J., and Theodore, J. (1971). *Comp. Biochem. Physiol.* **39,** 807-821.
Robin, H., and Harley, J. D. (1966). *Aust. J. Exp. Biol. Med. Sci.* **44,** 519-526.
Robin, H., and Harley, J. D. (1967). *Aust. J. Exp. Biol. Med. Sci.* **45,** 77-88.
Robinson, F. R., and Ziegler, R. F. (1968). *Lab. Anim. Care* **18,** 50-57.
Romeo, G. (1977). *Hum. Genet.* **39,** 261-276.
Romeo, G., and Levin, E. Y. (1969). *Proc. Natl. Acad. Sci. U.S.A.* **63,** 856-863.
Romeo, G., Glenn, B. C., and Levin, E. Y. (1970). *Biochem. Genet.* **4,** 719-726.
Rorth, M. (1972). *Ser. Haematol.* **5,** 1-104.
Rose, I. A. (1971). *Exp. Eye Res.* **11,** 264-272.
Rose, I. A., Warms, J. V. B., and O'Connell, F. G. (1964). *Biochem. Biophys. Res. Commun.* **15,** 33-37.
Rose, Z. B. (1968). *J. Biol. Chem.* **243,** 4810.
Rose, Z. B. (1970). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **29,** 1105-1111.
Rotruck, J. T., Pope, A. G., Ganther, H. E., Swanson, A. B., Hafeman, D. G., and Hoekstra, W. G. (1973). *Science* **179,** 588.
Rudolph, W. G., and Kaneko, J. J. (1971). *Acta Haematol.* **45,** 330-335.
Runge, W., and Watson, C. J. (1969). *Blood* **32,** 119.

Ruth, G. R., Schwartz, S., and Stephenson, B. (1977). *Science* **198,** 199-201.
Salvidio, E., Pannaccuilli, I., and Tizianello, A. (1963). *Nature (London)* **200,** 372-373.
Sano, S., and Granick, S. (1961). *J. Biol. Chem.* **236,** 1173.
Schalm, O. W., and Smith, R. (1963). *Small Anim. Clin.* **3,** 311-322.
Schalm, O. W., Rhode, E. A., and Abrahams, J. W. (1963). *Calif. Vet.* **17,** 21-23.
Schecter, R. D., Schalm, O. W., and Kaneko, J. J. (1973). *J. Am. Vet. Med. Assoc.* **162,** 37-44.
Schmauch, G. (1899). *Arch. Pathol. Anat. Physiol. Klin. Med.* **156,** 201-244.
Schmid, R. (1966). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds), p. 939. McGraw-Hill, New York.
Schmid, R., and Schwartz, S. (1952). *Proc. Soc. Exp. Biol. Med.* **81,** 685.
Schmid, R., Hanson, B., and Schwartz, S. (1952). *Proc. Soc. Exp. Biol. Med.* **79,** 459.
Schmid, R., Schwartz, S., and Sundberg, R. D. (1955). *Blood* **10,** 416.
Schröter, W., and Winter, P. (1966). *Blut* **14,** 15-29.
Schulman, H. M. (1968). *Biochim. Biophys. Acta* **155,** 253.
Schwartz, S., Keprios, M., and Schmid, R. (1952). *Proc. Soc. Exp. Biol. Med.* **79,** 463.
Schwartz, S., Berg, M. H., Bossenmaier, I., and Dinsmore, H. (1960). *Methods Biochem. Anal.* **8,** 221.
Searcy, G. P., Miller, D. R., and Tasker, J. B. (1971). *Can. J. Comp. Med.* **35,** 67-70.
Segall, H. J., Siegel, D. M., Norman, B. B., and Oliver, M. N. (1977). *Calif. Vet.* **31**(12), 10-11.
Shemin, D., and Rittenberg, D. (1946a). *J. Biol. Chem.* **166,** 621.
Shemin, D., and Rittenberg, D. (1946b). *J. Biol. Chem.* **166,** 627.
Shemin, D., Russell, C. S., and Abramsky, T. (1955). *J. Biol. Chem.* **215,** 613.
Shih, L., Justice, P., and Hsia, D. Y. (1968). *Biochem. Genet.* **1,** 359.
Smith, J. E. (1968). *J. Lab. Clin. Med.* **71,** 826-833.
Smith, J. E., and Beutler, E. (1966). *Am. J. Physiol.* **210,** 347-350.
Smith, J. E., and Kaneko, J. J. (1966). *Am. J. Vet. Res.* **27,** 931.
Smith, J. E., and Osburn, B. I. (1967). *Science* **158,** 374-375.
Smith, J. E., Barnes, J. K., Kaneko, J. J., and Freedland, R. A. (1965). *Nature (London)* **205,** 298-299.
Smith, J. E., McCants, M., and Parks, P. (1968). *Comp. Biochem. Physiol.* **26,** 137-142.
Smith, J. E., Kiefer, S., and Lee, M. (1972a). *Comp. Biochem. Physiol. B* **43,** 413-417.
Smith, J. E., McCants, M., and Jones, E. W. (1972b). *Int. J. Biochem.* **3,** 345-350.
Smith, J. E., Lee, M. S., and Mia, A. S. (1973). *J. Lab. Clin. Med.* **82,** 713-718.
Smith, P. J., Tappel, A. C., and Chow, C. K. (1974). *Nature (London)* **247,** 392.
Srivastava, S. K. (1971). *Exp. Eye Res.* **11,** 294-305.
Srivastava, S. K., and Beutler, E. (1969). *Biochem. J.* **114,** 833-837.
Srivastava, S. K., and Beutler, E. (1972). *Arch. Biochem. Biophys.* **148,** 249-255.
Standerfer, R. J., Templeton, J. W., and Black, J. A. (1974). *Am. J. Vet. Res.* **35,** 1541-1543.
Stohlman, F., Jr. (1968). *N. Engl. J. Med.* **279,** 1437.
Strand, J. L., Felsher, B. F., Redeker, A. G., and Mawer, H. S. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 1315-1320.
Sugerman, H. J., Polloch, T. W., Rossato, E. F., Delivoria-Papadopoulos, M., Miller, L. D., and Oski, F. A. (1972). *Blood* **39,** 525.
Sunderman, F. W., Jr., and Sunderman, F. W. (1955). *Am. J. Clin. Pathol.* **25,** 1231.
Taketa, F., Smith, M. R., DiBona, F. J., and Lessard, J. L. (1967). *Biochemistry* **6,** 3809-3816.
Tanaka, K. K., and Paglia, D. E. (1971). *Semin. Hematol.* **8,** 367-396.
Thorp, F., Jr., and Harshfield, G. S. (1939). *J. Am. Vet. Med. Assoc.* **94,** 52-53.
Tietz, N. L. (1976). "Fundamentals of Clinical Chemistry." Saunders, Philadelphia, Pennsylvania.
Tobias, G. (1964). *J. Am. Vet. Med. Assoc.* **145,** 462.
Tosteson, D. C. (1963). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **22,** 19-26.
Tosteson, D. C. (1969). *In* "Red Cell Membrane Structure and Function" (G. A. Jameson and T. J. Greenwalt, eds.), pp. 291-308. Leppincott, Philadelphia.
Trujillo, J. M., Walden, B., O'Neill, P., and Anstall, H. B. (1965). *Science* **148,** 1603-1604.
Tschudy, D. P. (1965). *J. Am. Med. Assoc.* **191,** 718.
Tucker, E. M., and Ellory, J. C. (1971). *Res. Vet. Sci.* **12,** 600-602.
Valentine, W. N. (1971). *Semin. Hematol.* **8,** 348-366.
Valentine, W. N., Pearce, M. L., Riley, R. F., Richter, E., and Lawrence, J. S. (1951). *Proc. Soc. Exp. Biol. Med.* **77,** 244.
Van Kampen, K. R., James, L. F., and Johnson, A. E. (1970). *J. Am. Vet. Med. Assoc.* **156,** 328-332.

Waller, H. D. (1968). "Hereditary Disorders of Erythrocyte Metabolism," p. 185. Grune & Stratton, New York.
Watson, C. J. (1957). *AMA Arch. Intern. Med.* **99,** 323.
Watson, C. J., and Schwartz, S. (1941). *Proc. Soc. Exp. Biol. Med.* **47,** 393.
Watson, C. J., Perman, V., Spurell, F. A., Hoyt, H. H., and Schwartz, S. (1959). *AMA Arch. Intern. Med.* **103,** 436.
Weissman, S. M., Waldmann, T. A., and Berlin, N. I. (1960). *Am. J. Physiol.* **198,** 183.
Westall, R. G. (1952). *Nature (London)* **170,** 514.
Whaun J. M., and Oski, F. A. (1960). *Pediatr. Res.* **3,** 105–112.
Wilson, P. S., and Judson, G. J. (1976). *Br. Vet. J.* **132,** 428.
With, T. K. (1955). *Biochem. J.* **60,** 703.
Wolfe, S. J., Brin, M., and Davidson, C. S. (1958). *J. Clin. Invest.* **37,** 1476.
Yunis, A. A., and Arimura, G. K. (1966). *Proc. Soc. Exp. Biol. Med.* **121** 327–329.
Zanjani, E. D., Gordon, A. S., Wong, K. K., and McLaurin, W. D. (1969). *Proc. Soc. Exp. Biol. Med.* **131,** 1095.
Zinkl, J., and Kaneko, J. J. (1973a). *Comp. Biochem. Physiol. A* **45,** 463–476.
Zinkl, J., and Kaneko, J. J. (1973b). *Comp. Biochem. Physiol.A* **45,** 693-704.

5

Clinical Enzymology

JOHN W. KRAMER

I. INTRODUCTION

Each cell of an organ has a specific function and contains enzymes unique to that function. When the integrity of a cell is disrupted, enzymes escape into the surrounding

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

fluid compartment and into serum or cerebral spinal fluid (CSF), where their activity can be measured as a useful index of that cell's integrity.

An enzyme in serum, to be valuable as a clinical diagnostic aid, must be readily assayable; its assay must be economically feasible and reasonably reflect pathological change in a specific organ or group of organs. An example of assayability is the case of the three liver-specific enzymes, arginase (ARG), sorbitol dehydrogenase (SDH), and alanine aminotransferase (GPT). Arginase and SDH are liver-specific enzymes in many animals and are excellent markers of hepatocellular damage. Alanine aminotransferase is also liver specific in many species but is used in favor of the former two enzymes because the assay for GPT is simpler than that for ARG, SDH is not very stable, and a large body of clinical background data for GPT is available.

Other considerations are whether the activity of the enzyme is detectable after it has been released into the plasma or whether the enzyme remains in serum long enough to permit its detection. These considerations limit the organ-specific enzymes as markers of the site, magnitude, and type of disease affecting the patient (Bergmeyer, 1974; Schmidt and Schmidt, 1974).

Cells need not be necrotic to release their enzymes into plasma. Anoxia causes the cell membrane to lose its integrity, and soluble enzyme from the cytosol leaks into plasma (Hess and Raftopouls, 1957; Schmidt and Schmidt, 1967). This loss of integrity is often first observed microscopically as a swelling of the cell. An example of enzyme leak is in hepatic congestion, where the GPT activity in serum increases in the absence of frank hepatocytic necrosis. Another example is when a sample of serum is allowed to remain in contact with red blood cells (RBC) for a prolonged period of time; the anoxic RBC membrane leaks cytosolic LDH into plasma, causing a false increase in serum LDH.

When a serum enzyme lacks sufficient specificity for an organ, a second enzyme may be combined with the first to increase its diagnostic value. Serum alkaline phosphatase (AP) activity increases in bone and liver disorders, and, to assist in identifying the source of the increase, GPT or γ-glutamyltransferase (GGT) may be assayed as part of a hepatic profile.

II. BASIC ENZYMOLOGY

A. The Enzyme

An active enzyme is a complex referred to as a holoenzyme. The holoenzyme consists of a protein apoenzyme and a cofactor which is associated with but not always tightly bound to the apoenzyme. Not all enzymes have cofactors. The apoenzyme contains that portion of the enzyme which gives it its substrate specificity and may have a nonprotein moiety. The protein portion of an enzyme is subject to all physical and chemical denaturative processes including degradation *in vivo* by endogenous proteolytic enzymes of plasma. The cofactor portion of the holoenzyme is reversibly bound to the apoenzyme so that it may take the form of a cycling substrate.

Enzymes are present in tissues and in plasma in such low concentrations that it is generally impractical to determine enzymes directly on the basis of their quantity in plasma. Therefore, enzymes are measured indirectly on the basis of their activity *in vitro* under conditions at which their activity is proportional to enzyme concentration. Activity

is usually expressed as the rate at which an enzyme catalyzes the consumption of substrate or production of product. When the substrates or products of the enzyme reaction cannot readily be measured, the cofactors can often be measured.

Cofactors which can be measured conveniently and accurately are NADH and NADPH. They have a peak absorption at 340 nm and a molar extinction coefficient of 6.22×10^3. Since the rate of appearance or disappearance of NADH is stoichiometrically related to substrate consumption, it can be used for expressing the activity of enzyme.

In some reactions, no cofactor is required, nor can the substrate or the product be readily measured. In this case, the primary reaction can be coupled to a second reaction which utilizes a product of the primary reaction, and in this way a measurable cofactor or product is generated. An example of a coupled reaction in which three reactions are coupled together is that of creatine kinase (CK) [Eqs. (1)–(3)].

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP} \quad (1)$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose 6-phosphate} + \text{ADP} \quad (2)$$

$$\text{Glucose 6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6P-D}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+ \quad (3)$$

Substrate of the primary reaction, cofactors, and secondary enzymes are added in excess so that they are not limiting, and the only variable in the reaction sequence is the amount of enzyme, CK, in the sample being analyzed. The rate of generation of NADPH [Eq. (3)] is measured and is proportional to the amount of CK activity.

Plasma contains two groups of enzymes. In the first group are those endogenous enzymes which are involved in homeostasis—the proteolytic enzymes of the coagulation and complement systems. The second group consists of the exogenous enzymes which are derived from leaking cells as they complete their life span. These enzymes may either have no enzymatic activity *in vivo* or may occur as inactive zymogens in the plasma. Most of the endogenous plasma enzyme systems, such as the coagulation and complement systems, and some exogenous enzymes are present in serum as zymogens.

The digestive enzymes of the pancreas are released into plasma in acute pancreatitis. Pancreatic amylase and lipase are enzymes commonly used as indicators of pancreatitis. There are other proteolytic pancreatic enzymes, such as trypsin, which enter plasma as the zymogen, trypsinogen. There is no activation mechanism in plasma for the conversion of trypsinogen to trypsin, and no proteolytic activity takes place. In dogs and human beings with segmental pancreatic transplants in the abdominal cavity, the exocrine pancreatic secretions are discharged into the abdominal cavity with no known deleterious effects even though the activity of pancreatic enzymes, such as lipase and amylase, are 10- to 20-fold greater than normal when measured as enzymes *in vitro* under optimal conditions. Some forms of pancreatitis are known to produce severe local fat necrosis and calcium salts of fatty acids along with an inflammatory process. In these cases, there has been some activation of the zymogens, but the result of activation seldom becomes evident as a systemic lesion.

B. Enzyme Nomenclature

Enzyme nomenclature is based on the reactions enzymes catalyze. The International Union of Biochemistry (IUB) established a commission on enzyme nomenclature to

TABLE I

Enzymes Used in Veterinary Medicine

Abbreviation	Name	EC number
GOT (AST)	Aspartate aminotransferase	2.6.1.1
GPT (ALT)	Alanine aminotransferase	2.6.1.2
ARG	Arginase	3.5.3.1
CK (CPK)	Creatine kinase	2.7.3.2
ChE	Cholinesterase	3.1.1.8
AP	Alkaline phosphatase	3.1.3.1
Amyl	α-Amylase	3.2.1.1
SDH	Sorbitol dehydrogenase	1.1.1.14
LDH	Lactate dehydrogenase	1.1.1.27
OCT	Ornithine carbamoyltransferase	2.1.3.3
GGT	γ-Glutamyltransferase	2.3.2.2
PK	Pyruvate kinase	2.7.1.40
TK	Transketolase	
LIP	Lipase	3.1.1.3

systematize, categorize, and catalogue enzymes (Florkin and Stotz, 1973). The system is based on the type of reaction catalyzed; a formal name, a common name, initials, and a number are given to each enzyme. Lactate dehydrogenase (LDH) can be used to illustrate this system. Because this enzyme catalyzes a reversible reaction in which it participates in either an oxidation or reduction reaction, depending on the direction of the reaction, it is classified as an "oxidoreductase." If the enzyme incorporates a redox cofactor, such as NAD^+, it is referred to as a dehydrogenase. Therefore, the formal name for LDH is L-lactate : NAD oxidoreductase, its common name is lactate dehydrogenase, and its EC number is 1.1.1.27. Abbreviations, common name, and EC number of some enzymes are given in Table I.

Glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) are the former common names and abbreviations of enzymes frequently used in clinical medicine. The IUB commission recommends that these enzymes be classified as alanine aminotransferase (ALT) and asparate aminotransferase (AST), respectively. The formal names given by the IUB are undoubtedly more consistent, but for purposes of clarity the more conventional abbreviations GPT and GOT will be used.

C. Units of Enzyme Activity

Enzyme concentration in serum or tissue is expressed as the rate of the reaction catalyzed because enzyme concentrations are usually too low to be expressed as the amount of enzyme protein per unit volume of body fluid. For example, an enzyme such as GPT is in plasma at less than 0.10 mg/dl, whereas the total plasma protein concentration is about 7 gm/dl. The proportion of enzyme protein to total protein would be less than 1 in 7000, and the purification, isolation, and determination of enzymes under these conditions would not be feasible for clinical purposes.

The international unit (U) of enzyme activity has been defined by the IUB as the amount of enzyme that will catalyze the conversion of 1 μmole of a substrate or the production of 1 μmole of product per minute under specified conditions of time, temperature, pH, and

substrate concentrations (Appendix I). The unit specifies only the amount of substrate and time. The volume, temperature, and substrate in which the unit is expressed may vary and must be defined if a comparison is to be made among various methods. These variables play an essential role in the evaluation of results among laboratories, and for this reason laboratories should establish their own normal values. In order to avoid discrepancies in comparisons from various sources, discussions concerned with changes in enzyme activity will be made with reference to the magnitude of change from normal rather than in actual units.

D. Temperature

The temperature at which enzyme assays are conducted is usually either 37°, 30°, 25°, or 22°C. The higher the temperature, the more rapid the reaction, but also the greater the rate of heat denaturation of the enzyme. A reaction conducted at 37°C is approximately twice as fast as one carried out at 25°C (Appendix I).

E. Substrate

An enzyme is specific for the reaction it catalyzes. However, some enzymes are not specific for the substrate, i.e., the molecule on which the enzyme exerts catalytic action. Under standard conditions, the rate at which an enzyme reaction proceeds is assumed to be directly proportional to the enzyme's concentration. However, an enzyme's affinity varies from one substrate to another, and therefore, so does the reaction rate. This absence of substrate specificity *in vitro* has led to some confusion when comparing data from more than one laboratory where the assays are different, but the unit of expression is the international unit (U).

Confusion which can result from the lack of substrate specificity can be illustrated with serum AP, a collective term for a group of isoenzymes with a number of natural substrates remaining to be identified. For a number of years, synthetic substrates have been used to assay for serum AP with units of activity expressed as eponyms. With the advent of the international unit, investigators have begun to convert the eponym unit such as Bodansky and King-Armstrong to the U. This conversion has led to considerable confusion because investigators do not always state the substrate used in their assay systems, which in turn, makes comparisons or interconversions of data nearly impossible. Although some investigators have used conversion factors, it is more appropriate to compare values on the basis of magnitude of change from normal, rather than numerical units of activity.

F. Kinetics

Enzyme assays are performed under conditions for optimal enzyme activity. Concentrations of substrate, cofactors, activators, pH, and temperature are maintained constant so that the only variable is the enzyme. These are the conditions of zero-order kinetics, conditions at which the reaction rate is independent of initial substrate concentration. Thus, an enzyme as assayed in the laboratory may not have the same natural functional activity in the cell, where different conditions may exist.

The approximation of zero-order kinetics becomes limited when the rate of enzyme activity is very high. Reagents for an enzyme generally have only sufficient substrate

concentration to maintain zero-order kinetics for the time period of the assay or for magnitudes of enzyme activity two to three times greater than the activity normally occurring in the patient's serum. When one is performing an enzyme assay, it is imperative that the limitations of zero-order kinetics be understood. If the results of the assay exceed the limits of zero-order kinetics, the enzyme activity is in error and should be reported only as "greater than" that value established as the upper limit of zero-order kinetics. Alternatively, the assay can be repeated on a diluted sample, or the size of the sample can be reduced and appropriate dilution corrections made.

Determination of the maximal amount of enzyme activity that a procedure can measure requires knowledge of the equilibrium of the reaction and the concentration of substrate. An equilibrium constant (K_{eq}) of 100 indicates that there will be 100 times more product than substrate when the reaction has gone to completion. In this case, 99% of the substrate is converted to product. However, if $K_{eq} = 1$, as in the GPT reaction, only 50% of the substrate is converted to product at equilibrium and the reaction appears to stop. In some cases, if the reaction is reversible, the product may be reconverted to substrate as quickly as product is formed.

The K_{eq} may be altered by "trapping" the product or converting it to another form. In the reverse LDH reaction, lactate is converted to pyruvate by LDH, and, in order to drive the reaction to pyruvate, hydrazine is used to "trap" pyruvate. This form of "trapping" permits a greater amount of activity to be determined with the same amount of substrate than would be possible if no "trapping" reagent were used.

Enzyme assays in clinical biochemistry are generally carried out in one of two ways. The first is an "end point" procedure analogous to a colorimetric assay. The sample is added to the reaction mixture, and, after a suitable incubation period, the reaction is stopped by the addition of a reagent which destroys or inhibits the enzyme activity. The amount of substrate used or the amount of product produced during the incubation period is measured. This end point method is subject to a variety of errors as when the activity exceeds the limits of the substrate concentration, or if product inhibition occurs.

The second, "kinetic" assay procedure requires sequential reading either manually or with a constant recording device. A kinetic assay procedure is more sensitive, more accurate, and more easily controlled than end point assays, because the reaction rate can be visualized. The constant recording device is extremely useful for this purpose because the linearity of the reaction rate can be easily seen. The primary advantages of the kinetic method in clinical enzymology are as follows: (1) When high enzyme activities are encountered, the reaction rate can be determined before substrate is exhausted whereas, in an end point assay, it has to be repeated when the substrate is exhausted; and (2) if an activator is present, it can be detected on the graph (Fig. 1).

G. Specimen Requirements

Many enzymes require metallic ions for maximal activity, and therefore plasma with EDTA, citrate, or oxalate anticoagulants should not be used for enzyme assays which require metallic ions. Heparinized plasma can be used in some procedures, but, if there is doubt, serum should be used.

Some enzymes are very stable at room temperature, and AP may actually increase in activity when kept at room temperature for a few days (Table II; see also Appendix IV).

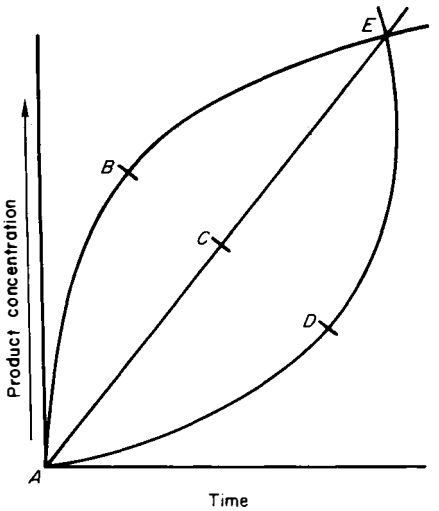


Fig. 1. Illustration of potential hazards of using a end-point enzyme assay. Line *ACE* is a zero-order reaction that permits accurate determination of enzyme activity for the entire reaction time. Curve *ABE* initially is a zero-order reaction of high rate followed by a reduction in rate, possibly due to exhaustion of substrate. Assay at point *E* would be in error. Curve *ADE* has an initial lag phase which also would be erroneous. (Reproduced with permission from J. B. Henry, 1963.)

Other enzymes are labile and will deteriorate rapidly even when frozen. For example, SDH is an important enzyme to equine and food animal practitioners because it is the only liver-specific enzyme readily available. However, SDH is unstable at room temperature and loses a great deal of activity at refrigeration temperatures within 8 hours, and freezing does not appreciably reduce the loss. Therefore, unless the specimen can be submitted to a laboratory within a day, the value of SDH becomes limited. A list of some enzymes and their storage characteristics are found in Table II (see also Appendix IV).

The glassware in which a specimen is obtained must be carefully cleaned, but whenever possible commercially available collection vials are recommended. Detergents interfere with many assays, and only thorough rinsing will reduce the detergent residue to a point which will not inhibit the enzymes. Serum must be separated from the cellular element of blood as soon as possible. Blood cells contain LDH and GOT and, when anoxic, may leak enzymes into serum even before hemolysis.

H. Quality Control

To maintain the quality of any enzyme assay, some form of control must be established which detects unpredicted variables as they arise. Potential unpredictable variables are numerous, ranging from the storage of reagents to selection of the correct wavelength for chromophore detection. Limitation of the number of variables serves to enhance the reliability of the procedure from the standpoint of both accuracy and precision. The accuracy of an assay refers to how closely the assay can measure the true value of the enzyme activity. The precision of the enzyme assay is a measure of the reproducibility of the assay itself and is characterized by the standard deviation (SD) and coefficient of

TABLE II

Enzyme Stability in Serum[a]

Enzyme	Storage	Time (days)	Activity (%)
α-Amylase (EC 3.2.1.1)	Room temperature	8	100
Cholinesterase (EC 3.1.1.8)	Room temperature	8	90
	0°–4°C	8	90
	Frozen	8	94
Creatine kinase[b] (EC 2.7.3.2)	Room temperature	1	25
	0°–4°C	1	32–65
	Frozen	8	25
Glutamate dehydrogenase (EC 1.4.1.2)	Room temperature	2	60
	0°–4°C	2	60–100
	Frozen	2	60
Aspartate aminotransferase (EC 2.6.1.1)	Room temperature	4	90
	0°–4°C	8	87
	Frozen	2	90
Alanine aminotransferase (EC 2.6.1.2)	Room temperature	4	75
	0°–4°C	8	78
	Frozen	8	31
Lactate dehydrogenase (EC 1.1.1.27)	Room temperature	8	74–88
	0°–4°C	8	81
	Frozen	8	81
Alkaline phosphatase (EC 3.1.3.1)	Room temperature	8	71
	0°–4°C	8	71
	Frozen	8	68
Sorbitol dehydrogenase (EC 1.1.1.14)	Room temperature	8	54
	0°–4°C	8	71
	Frozen	8	52

[a] Reproduced in part with permission from Bergmeyer (1974).
[b] An unactivated enzyme assay procedure was used to establish these values.

variation. Precision is of particular importance when one is assaying enzyme activity because many enzymes are unstable and control serums with known amounts of activity are not available for all enzymes.

Quality control (QC) programs in clinical enzymology should be conducted as for other clinical chemical procedures, however not all enzymes in commercial QC serums are stable. Each enzyme assay should be run in parallel with a QC serum sample of known activity previously determined by the same assay procedure. The results of the QC serum should be at least within 2 SD of the mean.

Assayed lyophilized QC serum with normal or abnormal activity can be purchased from commercial sources. Acceptable limits of the QC serum are within the 95% limits of confidence. Thus, when the test value of the control serum is within ± 2 SD of the mean of the control value, the assay of the unknown serum is accepted as reliable. When initially establishing a procedure, it may be desirable to extend the limit of reliability to 100% of the normal population, in which case a value of ± 3 SD of the mean would be acceptable.

When the value for the QC serum is established, the QC serum is used each time an assay is performed. A record should be maintained of the value obtained for the QC serum

for each assay and the value plotted on a graph. This visual record will indicate any trend or progressive change in the value of the QC serum, and the source of the trend can be found and corrected. It should be apparent that a QC program must be maintained in any clinical laboratory if reliable results are to be obtained.

III. SERUM ENZYMES OF CLINICAL DIAGNOSTIC IMPORTANCE

A. Alkaline Phosphatase

Alkaline phosphatase (EC 3.1.3.1.) was one of the first serum enzymes to be recognized as having clinical significance. In the 1920's, it was discovered that serum AP activity increased in various bone and liver diseases. Alkaline phosphatase has remained an important enzyme for characterizing bone and hepatic disorders.

Alkaline phosphatase is a nonspecific enzyme which hydrolyzes many types of phosphate esters and is present in multiple molecular forms, i.e., isoenzymes. The endogenous substrate of AP is unknown, but the enzyme catalyzes the dephosphorylation of ATP and is thought to be associated with energy-requiring membrane "pumps." The term "alkaline" refers to the optimal alkaline pH of this class of phosphatases *in vitro*. The pH optimum for AP is 10, a pH unlikely to occur in the body. It is possible that AP may have an entirely different activity in its natural environment (Pekarthy *et al.*, 1972; Bergmeyer, 1974).

Assays for AP use a great variety of synthetic substrates, which has led to confusion in the expression of its activity. Formerly, eponyms were used as units, such as Bodansky and King-Armstrong units, which were later converted to international units (see Appendix II). However, the conversion is of value for comparative studies only if substrate specificity and conditions of assay are taken into account. When one is converting data derived from different methods, it is satisfactory within the normal range, but, when outside the range, the activities are not usually comparable.

Serum AP is now known to be a group of isoenzymes. They are present in a large number of cells, but only in a few is the activity sufficient to be of clinical importance. In general, AP activity is associated with microvilli of secretory and absorptive cells, such as the epithelium of bile duct canaliculus, intestinal tract, renal tubular epithelium, and placenta. It is also found in liver cells and is associated with osteoblastic activity in bone. Bone probably contains more AP activity than any other tissue. Each of the isoenzymes of AP from bone, liver, intestine, placenta, and kidney has been isolated and characterized by electrophoresis, immunologically, by kinetic constants, with inhibitors, and by sensitivity to heat. Each of these criteria has been used to identify the isoenzymes under normal and abnormal conditions (Baetz, 1969; Pickrell *et al.*, 1974; Hoffman and Dorner, 1975, 1977; Hoffman *et al.*, 1977a,b; Saini and Saini, 1978a,b).

The major source of serum AP in young growing animals is bone. In all young animals the serum AP activity is greater than that found in the adult because of the high osteoblastic activity in the young. Serum AP activity is two or three times greater in puppies, foals (Fig. 2), and kittens than it is in adults (Pickrell *et al.*, 1974; Blackmore and Elton, 1975).

Liver AP contributes to serum AP activity throughout life, and, once bone growth has reached the rate of adult life, the liver becomes the primary source. In normal liver, bile duct epithelium has the most AP activity. When obstruction of the duct system occurs at

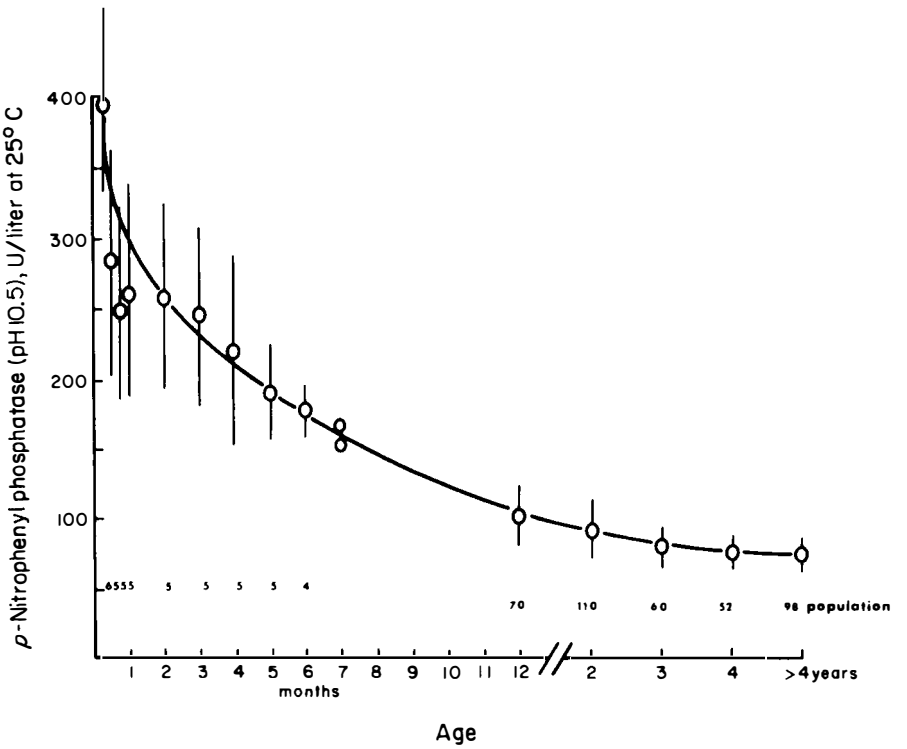


Fig. 2. Mean and standard deviation of serum alkaline phosphatase activity in thoroughbred horses in relation to age. (Reproduced with permission from Blackmore and Elton, 1975.)

any level, there is an increase in the hepatic AP activity in serum (Hoffman *et al.*, 1977a). Liver AP activity can increase in serum in association with hepatic fibrosis or obstruction, but the more common occurrence is its increase in association with hepatic lipidosis. Lipids are deposited in liver and serum AP activity increases in diabetes mellitus, hypothyroidism, hyperadrenocorticism, severe starvation, and late pregnancy. In addition, the therapeutic administration of steroids also causes increased liver AP activity in serum. The source within the liver of this steroid-induced AP is not known (Dorner *et al.*, 1974).

B. Creatine Kinase

Creatine kinase (EC 2.7.3.2), or creatine phosphokinase (CPK), is one of the most organ-specific serum enzymes in clinical use. It catalyzes the reversible phosphorylation of creatine by ATP to form creatine phosphate and ADP. Creatine phosphate is the major storage form of high-energy phosphate required by muscle for contraction.

Creatine kinase is a dimer consisting of two peptides, B (for brain) and M (for muscle), which make up the three isoenzymes, CK_1, CK_2, and CK_3, or BB, MB, and MM, respectively. Many cells contain CK, but only heart and skeletal muscle contain sufficient amounts of CK_2 and CK_3 to alter serum activity in organ-specific disorders. The isoenzyme CK_1 is found predominantly in the brain, and, although total serum CK activity

sometimes increases in people with cerebrovascular disorders, it is CK_3 which increases, not CK_1. Cardiac infarction in human beings is associated with an increase in CK_2 but an increase in CK_2 has not been demonstrated to be of particular importance in animals in which cardiac infarction is not a problem. On the other hand, increases in CK_3 are of diagnostic significance in skeletal muscle, and there is little need to differentiate between heart and skeletal muscle using CK isoenzymes. Muscle anoxia in patients in prolonged recumbency causes remarkable increases in CK. Animals postsurgically often have markedly increased CK activity. Shipping and excessive exercise also cause increases in CK activity. Creatine kinase has a short half-life in serum and returns to normal very quickly as the stress ceases.

A number of conditions and compounds inhibit CK in an assay system. Magnesium is required by CK for activity, but an excess is inhibitory. Creatine kinase is relatively unstable when stored at room, refrigerator, or freezing temperature. However, activity can be restored by incubating the serum with sulfhydryl activators, such as cysteine or glutathione. The reason for the poor stability and reactivation of the enzyme is not understood.

C. Alanine Aminotransferase

Alanine aminotransferase (ALT) (EC 2.6.1.2) and glutamic pyruvate transaminase (GPT) catalyze the reversible transamination of L-alanine and α-oxoglutarate to pyruvate and glutamate. Alanine aminotransferase is present in plasma and in cells. In the dog and cat, there is sufficient GPT activity in liver for the use of this enzyme as a liver-specific enzyme. Increases in plasma GPT activity in dogs and cats are associated with hepatocellular disorders. Acute hepatic diseases causing membrane damage or cell necrosis result in appreciable increases in plasma activity.

D. Aspartate Aminotransferase

Aspartate aminotransferase (AST) (EC 2.6.1.1), and glutamic oxaloacetate transaminase (GOT) catalyze the transamination of L-aspartate and α-oxoglutarate to oxaloacetate and glutamate. Aspartate aminotransferase is present in the mitochondria and cytosol of almost all cells and in plasma. The presence of GOT in so many tissues precludes the use of this enyzme as an organ-specific enzyme, but it is useful in conjunction with other enzymes as an index of hepatic or muscular cell damage.

The upper limit of normal GOT activity in horses is considerably greater than that observed in other species. Therefore, assay procedures established for other species are not usually satisfactory for the detection of abnormal increases in horse plasma GOT activity. In the horse, when the activity is high, it is best to dilute the sample one-half to one-third or reduce the volume of serum sample and correct for change in volume. The reason for the higher GOT activity in the horse is not known.

E. Sorbitol Dehydrogenase

Sorbitol dehydrogenase (EC 1.1.1.14) activity in plasma is very low, and its major source is the hepatocyte. Sorbitol dehydrogenase catalyzes the reversible oxidation of D-sorbitol to D-fructose with the cofactor NAD. The enzyme is very unstable, and at room

temperature appreciable amounts of activity are lost in a few hours (Table II). Heparinized plasma can be used, but serum is preferred. Metal-chelating anticoagulants, such as EDTA and oxalate, decrease the amount of SDH activity. This enzyme is liver specific in all species of animals tested, and hepatic injury appears to be the only source of increased SDH activity. It has been most useful in detecting acute hepatic disorders in cattle and horses.

F. Lactate Dehydrogenase

Lactate dehydrogenase (EC 1.1.1.27) catalyzes the reversible oxidation of L-lactate to pyruvate with the cofactor NAD. A large amount of LDH activity is found in all tissues. In blood there is as much as 150-fold greater LDH activity in the RBC than there is in plasma. Thus, even minimal hemolysis alters the plasma LDH activity appreciably. Anticoagulants, such as EDTA and oxalates, indirectly inhibit the enzyme; therefore, heparinized plasma or serum is the preferred sample.

Lactate dehydrogenase is a tetrapeptide made up of two types of peptides: H (heart) and M (muscle). The two types of peptides in various combinations make up five isoenzymes, LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5, which are designations for peptide combinations of HHHH, HHHM, HHMM, HMMM, and MMMM, respectively. Tissues contain various amounts of the LDH isoenzymes, and serum isoenzyme profiles have been used to identify specific tissue damage by electrophoretic separation (Prasse, 1969).

Lactate dehydrogenase isoenzyme profiles were the first isoenzyme profiles used by clinical laboratory medicine. Now a large number are available although not widely used in veterinary medicine (Moore and Feldman, 1974).

G. Cholinesterase

Serum cholinesterase is composed of two distinct cholinesterase (ChE) in the body. Their major substrate is acetylcholine, the neurotransmitter found at the myoneurojunction. Acetylcholinesterase (AChE) that is found at the myoneurojunction is so-called true ChE (EC 3.1.1.7) and is essential in hydrolyzing acetycholine so that the junction can be reestablished and prepared for additional signals. The myoneurojunction AChE is also found in RBC, mouse and pig brain, and rat liver. Only a small amount of AChE is found in plasma. The cholinesterase of plasma is a pseudocholinesterase, butyrylcholinesterase (ButChE) (EC 3.1.1.8), which hydrolyzes butyrylcholine four times faster than acetylcholine and is also located in white matter of the brain, liver, pancreas, and intestinal mucosa. Both AChE and ButChE have similar inhibitors and activators. Therefore, inhibition of ButChE reflects inhibition of AChE.

There are a number of inhibitors of ChE. They include drugs as well as natural occurring substrates. Currently, the most important inhibitors are organophosphate insecticides. The phosphoryl group of the organophosphates binds irreversibly with ChE, preventing hydrolysis and thereby inducing persistent nerve stimulation at the myoneurojunction. Decreases in ButChE have been reported in human beings with acute infection, pulmonary infection, muscular dystrophy, chronic renal disease, and pregnancy as well as insecticide intoxication.

The stability of ButChE is variable, lasting at least 6 hours at room temperature, many weeks at refrigerator temperature, and months at $-4\,^{\circ}C$.

H. Lipase

Serum lipase (EC 3.1.1.3) increases in activity in association with pancreatitis. It catalyzes the hydrolysis of triglycerides preferentially at the 1 and 3 positions, releasing two fatty acids and a 2′-monoglyceride. The enzyme is water soluble, but the substrate is not; therefore, the reaction takes place at the water–lipid interphase of the lipid micelle. Because the substrate is in suspension, the size of the micelle must be small and uniform in order for accurate results to be obtained. Bile salts aid in the emulsification of the substrate and increase the surface area exposed to the enzyme, but bile salts are not activators of the enzyme. Albumin and calcium enhance lipase activity. The means by which they do this is not clear, but it is believed that they combine with the fatty acid products and shift the equilibrium. In addition, albumin and bile salts may prevent denaturation of the enzyme.

The major source of lipase is the exocrine pancreas, with a smaller amount from the intestinal epithelial cells. Small amounts of the enzyme can be located in a few other cells but are not of diagnostic significance. Lipase in serum following pancreatitis has some properties which differ from those of the exocrine pancreatic lipase, but the reason is not clear. Normally, serum lipase activity is very low in dogs or cats, but in pancreatitis the activity increases significantly.

I. α-Amylase

α-Amylase (EC 3.2.1.1) is a calcium-dependent metalloenzyme which randomly catalyzes the hydrolysis of complex carbohydrates, e.g., glycogen, at α-1–4 linkages. The products of this action are maltose and limited dextrins. The enzyme's dependency on calcium requires the use of serum or heparinized plasma for assay.

Serum amylase activity in the dog and other animals is considerably greater than that in human beings. There are two isoenzymes of amylase. The primary isoenzyme present in the pancreas is $Amyl_1$, and the small intestine contains $Amyl_2$. In normal dogs, the serum contains $Amyl_2$. In experimental pancreatitis, there is initially an increase in $Amyl_2$ followed by an increase in $Amyl_1$, which ultimately accounts for the majority of the increase in serum activity (Stickel, 1978). In the dog, serum amylase is filtered by the kidney but reabsorbed by the renal tubules, with none appearing in urine (Eto *et al.*, 1969; Brobst *et al.*, 1970; Johnson *et al.*, 1977; Hudson and Strombeck, 1978). Therefore, urine amylase activity is of no diagnostic value in the dog. This is in contrast to human beings, in which amylase is cleared by the kidney and urine amylase activity can be used as an aid in diagnosing pancreatitis. There is no amylase in the saliva of dogs (Mendel and Underhill, 1907).

Serum amylase assay can be performed by either a saccharogenic or amyloclastic procedure. The saccharogenic procedure measures the rate of appearance of reducing substances from the starch substrate. The reducing substances are maltose and glucose. Dog plasma contains maltase as well as amylase, and thus the saccharogenic procedure incorporates the activities of both maltase and amylase. The amount of maltase activity in dog plasma varies and is not correlated with either amylase activity or pancreatic inflammation (Rapp, 1962). Therefore, the saccharogenic procedure is not acceptable for assay for amylase in dog serum.

Amyloclastic methods for serum amylase measure the rate of disappearance of the starch substrate. The natural and synthetic substrates used in amyloclastic procedures are

in fine suspension, so turbidimetric procedures are often used. Another procedure measures the rate of disappearance of the deep blue color of the starch–iodine complex. A recently developed procedure uses a dye linked to a starch substrate. The dye is bound to the second carbon of glucose of the starch. The glucose–dye or maltose–dye products are released, are soluble, and can be separated from the starch substrate by centrifugation or filtration. The rate of appearance of the soluble dye complexes in the supernatant is then measured spectrophotometrically. Even though the appearance of product is measured, this is an amyloclastic method because the dye complex is measured regardless of whether it is associated with glucose or maltose and the appearance of dye is a measure of starch disappearance. The dye–substrate amyloclastic procedure has been evaluated by comparison with the more classical procedures and has given comparable results.

The amyloclastic or dye–substrate procedures for amylase are available commercially but are designed for use in human beings. Normal canine serum amylase activity is five to six times greater than that in human beings, and the substrate concentrations in commercial kits may be limiting when used for animal serum. The procedure using these kits should be modified for use with dog serum by simply reducing the amount of serum or diluting it with saline.

J. γ-Glutamyltransferase

γ-Glutamyltransferase (EC 2.3.2.2) is a carboxypeptidase which cleaves C-terminal glutamyl groups and transfers them to peptides and other suitable acceptors. Glycylglycine is the most suitable. The physiological function of GGT is unknown, but it is speculated that the enzyme is associated with glutathione metabolism. Cell cytosol and membranes have GGT activity. Most cells have some GGT activity, ranging from the kidney with the highest and muscle the lowest. Serum contains GGT activity, most of which is derived from liver. Kidney GGT activity is detectable in serum, and urine contains only kidney GGT (Braun *et al.*, 1978).

Obstructive liver disease causes the greatest magnitude of increase in serum GGT activity in human beings (Lum and Gambino, 1972). Although there are very few studies of the value of GGT in animals, it may well be a more specific indicator of obstructive hepatic disorders than is AP (Ford, 1974; Johnson, 1976).

IV. ORGANS

A. Myopathies

Damage to muscle results in appreciable increases in serum LDH, aldolase (ALD), GOT, and, most specifically and spectacularly, CK. Although CK and ALD are regarded as specific for skeletal muscle, brain and cardiac muscle also contain appreciable amounts of CK. Currently, CK is accepted as the most specific marker of muscle damage available for clinical use.

A relatively small amount of muscle damage results in an increase in serum CK because of the high specific activity of CK in muscle. Serum CK may increase two- or threefold simply as a result of intramuscular injections and minimal exercise (Anderson *et al.*, 1976). Muscle ischemia from prolonged immobility, as during and after surgery, or the

"downer" cow syndrome results in a tenfold increase in serum CK. Cattle and horses after transportation frequently have elevated CK activity.

Serum CK activity in young dogs is twice that of dogs 6 months of age and older, and males have about 50% more activity than females. However, in horses, no relationship between age and CK activity was observed (Blackmore and Elton, 1975).

Exercise in horses results in a varied increase in serum CK activity. The magnitude of the increase correlates with the amount of exercise and degree of training the horse has previously received. Horses in good physical condition have a greater amount of CK at rest and have a lower increment of increase after exercise than do unconditioned horses (Anderson, 1975). These differences are thought to be the result of some form of modification of the muscle cell membrane which permits it to maintain its integrity during periods of prolonged work. Rats exercised on a treadmill also have four- to fivefold increases in CK activity. However, pretreatment of the rat with prednisolone decreases the increase to only two- to threefold (Wagner and Critz, 1968). It was postulated that the loss of CK from the muscle after exercise occurred because the steroid modified the cell membrane and there was less leakage of cytosol contents.

Hereditary muscular disorders occur in sheep, mink, dogs, chickens, hamsters, and mice (McGavin and Baynes, 1969; Hegreberg *et al.*, 1975; Wentink *et al.*, 1972; Kramer *et al.*, 1976; Asmundson and Julian, 1956; Homburger *et al.*, 1962; Michelson *et al.*, 1955). When active muscle degeneration has ceased, the serum CK values return to normal. In none of these disorders was the serum enzyme activity of value in detecting the carrier state.

The porcine stress syndrome (PSS) and porcine malignant hyperthermia (PMH) are two poorly defined, unrelated disorders in which, at slaughter, the pig has a pale, soft exudative musculature. These disorders are observed in the lean breeds of pigs, such as the Landrace and Pietrain. A high percentage of the pigs susceptible to PSS have normal resting serum CK and ALD activities. Pigs with PMH generally have nearly normal resting CK and ALD activities, but during halothane anesthesia they have hyperthermia, and within a few hours of recovery the serum CK and ALD activities are as much as tenfold greater than the preanesthetic activities. Screening tests for CK for both PSS and PMH have been used in selecting breeding gilts and boars (Allen *et al.*, 1970; Borgman *et al.*, 1978; Christian, 1975; van den Hende *et al.*, 1976; Nelson *et al.*, 1974; Woolf *et al.*, 1970).

Nutritional myopathies associated with vitamin E, selenium, and sulfur deficiencies are frequently referred to as "white muscle" disease and have been observed in cattle, dogs, horses, pigs, and chickens (Blincoe and Dye, 1958; Orstadius *et al.*, 1959; Owen *et al.*, 1977; Noguchi *et al.*, 1973; Manktelow, 1963; Van Vleet, 1975; Van Vleet *et al.*, 1975, 1977). These three factors are associated with the maintenance of cell membrane integrity. The leakage of enzymes into plasma occurs from a wide variety of cell types. However, because muscle constitutes the largest single mass of cells, the serum enzyme profile closely reflects muscle damage. The activity of all general intracellular enzymes, such as GOT and LDH, in addition to that of CK and ALD, increases.

In the selenium deficiency of white muscle disease there is a significant decrease in the erythrocyte glutathione peroxidase. Glutathione peroxidase (GP) is a selenium-containing enzyme. A decrease in GP activity in liver and RBC of selenium-deficient sheep (Oh *et al.*, 1974), cattle (Segall *et al.*, 1977; Allen *et al.*, 1975), and rats (Hafeman *et al.*, 1974) has been demonstrated to be an effective diagnostic test that is at least as sensitive as selenium

determination. The GP assay can be performed on whole blood by a spot screening test (Segall *et al.*, 1977). Specific therapy of a selenium-deficient patient with selenium results in normal serum CK activity within a matter of days.

When ischemia is experimentally induced in dogs, the classic changes in both LDH and CK isoenzymes occur, and these patterns are an appropriate indication of cardiac ischemia (Crawly and Sevenson, 1963; Hillis and Braunwald, 1977; Nydick *et al.*, 1957; Ruegsegger *et al.*, 1959; Siegel and Bing, 1956). Gerber (1968) and Hoffman (1967) reported increases in GPT, GOT, and CK and changes associated with LDH isoenzymes in horses with different forms of cardiac muscle dysfunction.

The skeletal muscle myopathies of horses, such as the "tying up" syndrome and myoglobinuria, also result in muscle necrosis and an increase in muscle-specific enzymes in serum. The pathogenesis of these disorders is not well defined but appears to be associated with a marked intramuscular lactic acidosis which results from the sudden demand for energy required by exercise. Serum CK and ALD activities increase dramatically, as do those of GOT and LDH. If the damage ceases and the lesion is not of a magnitude to cause infarction of another muscle, the CK activity will decrease appreciably within 2 days (Cardinet *et al.*, 1963; Cornelius *et al.*, 1963a; Crawly and Sevenson, 1963; Gerber, 1968). In the "tying up" syndrome, serum enzymes increase prior to the onset of clinical signs. This is in contrast to myoglobinuria, in which the increase occurs at the onset of clinical signs.

B. Osteopathies

Bone contains osteoblastic activity and a high AP activity, some of which leaks into serum. During normal bone growth in young animals, a large amount of AP is in serum, where it constitutes the primary fraction of the total AP. In 2-month-old dogs, AP activity is about twice that of normal dogs over 1 year of age. In 1-month-old horses, AP activity is nearly four times greater than that in horses 12 months of age (Blackmore and Elton, 1975) (Fig. 2). Therefore, age must be considered in the interpretation of AP activity.

Nutritional osteopathies, such as rickets and osteomalacia of dogs (Campbell, 1962; Hime, 1968), secondary hyperparathyroidism of horses (Krook and Love, 1964), and osteodystrophia fibrosa of New World primates (Hunt *et al.*, 1967), result in increased serum AP. Campbell (1962) reported normal serum AP values in puppies fed rations low in calcium and high in vitamin D. Increased serum AP can be the result of normal bone growth, an osteopathy, or a hepatic disorder, and the isoenzyme type can be determined by electrophoresis, inhibitors, or heat.

C. Pancreas

The pancreas contains high specific activity zymogens of proteases, phospholipase, lipase, and amylase. Their appearance in serum is a reliable indicator of pancreatic damage. Amylase and lipase are most commonly used to detect pancreatitis. Acute pancreatitis has been reported in dogs, cats, and horses, with the greatest incidence being in dogs (Baker, 1978; Finco and Stevens, 1969). The dog is also the species most commonly used to study experimental pancreatitis (Brobst *et al.*, 1970; Barnett *et al.*, 1976; Mia *et al.*, 1978). It is interesting that physical disruption of the pancreas by various mechanical and chemical methods does not routinely cause a very severe inflammatory process. Only

through the activation of the zymogens by trypsin is an experimental pancreatitis as severe as spontaneous pancreatitis in the dog (Brobst *et al.*, 1970).

Serum amylase has also been reported to increase during renal failure in the dog, as might be expected if renal tubular clearance were a primary mode of amylase clearance. Ligation of the renal artery in the dog and injection of pancreatic extract were used to demonstrate decreased disappearance rates of serum amylase and lipase (Hudson and Strombeck, 1978). However, ligation of the renal artery alone resulted in only minor increases in serum amylase.

Chronic pancreatitis results in a decrease in the amount of proteases in intestines. Decreases in amounts of fecal trypsin are observed in pancreatic deficiency.

D. Central Nervous System

Although the brain contains many different enzymes, the most specific enzyme for diagnosis of brain injury is CK. The specific activity of CK_1 in the brain is second only to those of CK_2 and CK_3 in muscle. These isoenzymes can be separated by electrophoresis, but seldom does CK_1 leak into serum or CSF. Increases in serum CK are sometimes observed in neurological disorders, but the activity is derived from muscle as a result of convulsions or muscle ischemia following prolonged immobility (Smith and Healy, 1968).

Cerebrospinal fluid CK activity is normally absent or very low. Increases in activity of CK and other enzymes have been associated with a number of disorders in dogs, cats, cattle, and horses (Wilson, 1977; Mayhew *et al.*, 1977), including demyelinating diseases and neoplasia.

Cerebrocortical necrosis (CN), polioencephalomalacia (PE), and focal symmetrical encephalomalacia (FSE) are three similar diseases of cattle and sheep in which serum CK changes have been reported (Edwin, 1970; Edwin and Jackman, 1974; Evans *et al.*, 1975; Smith and Healy, 1968). The serum CK and GOT activity increased only slightly in clinical cases of PE, markedly in FSE, and dramatically in control sheep with muscular dystrophy. Since total serum CK was reported, it is not clear whether the source of the activity was muscle or brain.

Thiamine deficiency is currently thought to be involved in the etiology of PE and CN. Thiaminase of rumen microflora may degrade thiamine in the diet of sheep and goats, which results in a deficiency of thiamine pyrophosphate (TPP) (Edwin and Lewis, 1971). Transketolase (TK) of tissues, including RBC, requires TPP as a cofactor. Red blood cell TK activity was noted to be low in calves with CN, and, when TPP was added to the assay system or thiamine fed to the calves, the TK activity quickly returned to normal (Pill, 1967). Thus, RBC TK activity and TPP activation may prove to be good diagnostic aids for this disorder.

E. Kidney

The kidney contains a large number of intracellular enzymes, of which AP and GGT occur with high specific activity (Robinson and Hesketh, 1976; Robinson and Gopinath, 1974; Bogen and Sommer, 1976; Braun *et al.*, 1978). The measurement of enzyme activity in urine requires some precautions. Since the rate of urine production varies with diet and time, 24-hour urine collections must be made. Urine also contains many salts and

metabolites which can denature, inhibit, or activate an enzyme and must be removed by dialysis. Thus, an enzyme of high specific activity in renal tissue may not be detectable in urine (Raab, 1972). Enzymes pass the glomerular filter but are almost totally reabsorbed in the lower nephron. In cases of proteinuria, some serum enzymes may enter urine, but they do not contribute greatly to the urine enzymes (Mattenheimaer, 1968).

Experimental nephrosis has been produced in sheep (Ford, 1974; Robinson and Hesketh, 1976; Shaw, 1976; Robinson and Tafford, 1977), dogs (Ellis and Price, 1973; Szczech *et al.*, 1974), and rats (Conzelman and Gribble, 1973) by the use of mercuric chloride, mycotoxins, and neomycin, respectively. In these studies AP, GGT, GOT, LDH, acid phosphatase, leucine aminopeptidase, β-glucuronidase, and isocitrate dehydrogenase were measured in urine and, in some studies, serum. Urine AP and GGT were the most consistent markers of epithelial cell damage. Urine enzyme activity increased in acute intoxications prior to the appearance of clinical signs, but no changes in serum enzyme activity correlated with the renal damage. In one study, AP activity remained elevated for the 3-day period during which mercuric chloride was given and then returned to normal (Ellis and Price, 1973).

F. Liver

Serum enzyme characterization of the liver has been well established for over 40 years, but new developments continue to improve the specificity of the diagnosis (Cornelius *et al.*, 1959; Freedland and Kramer, 1970). The urea cycle enzyme arginase has a high liver specificity and correlates well with acute hepatic necrosis for all species of ureotelic animals. Nevertheless, the complexity of the arginase assay and accumulated data on other enzymes such as GPT indicate that it is unlikely that arginase will be commonly used in clinical diagnostics for dogs and cats. However, Mia and Koger (1978) have introduced a new colorimetric procedure for arginase which is simpler than previous assays and may enhance the use of arginase in clinical medicine. Serum GPT is well established as a marker of acute hepatic damage in dogs (Cornelius *et al.*, 1959; Brobst and Schall, 1972) and cats (Cornelius and Kaneko, 1960; Wilkins and Hurvitz, 1975; Everett *et al.*, 1977a). In all species of animals SDH is a liver-specific enzyme for acute hepatocellular damage and is well established as the enzyme of choice for cattle and horses (Sova and Jicha, 1963b). On the other hand, SDH is unstable, and the assay must be performed quickly or the loss of activity negates the value of the test.

Chronic hepatic disorders result in increased serum AP in most animals studied (Armstrong and Banting, 1935; Freedland and Kramer, 1970; Pekarthy *et al.*, 1972; Tennant *et al.*, 1972; Young, 1974), and more recently GGT has been found to be liver specific (Ford *et al.*, 1968; Johnson, 1978). The magnitude of the increase is dependent on the specific activity of the hepatic AP in the species. Cats have a relatively low hepatic AP specific activity and low serum activity. Consequently, when bile duct obstruction occurs, there is little increase in serum AP activity as compared to dogs, but an increase does occur (Everett *et al.*, 1977b; Hoffman *et al.*, 1977b).

Various isoenzymes of AP have been identified, and most of the AP in serum in mature animals is the hepatic form. Hepatic AP is located in the cytomembranes of both bile duct epithelium and the hepatocyte (Reid, 1967). Serum liver AP may originate from normal cell death of hepatocytes, from regurgitation of bile duct AP, or by loss of integrity of the tight junctions between hepatocytes. The regurgitation process appears to partially ac-

count for the increased serum AP in bile duct obstruction. A second cause is *de novo* stimulation of hepatic AP.

Dorner *et al.* (1974) demonstrated that the well-known increase in serum AP seen in steroid therapy, Cushing's syndrome, and diabetes mellitus of dogs originated from liver. They had the same heat sensitivity and inhibitory characteristics but not the electrophoretic mobility of intestinal AP (Saini *et al.*, 1978). The intrahepatic source of the steroid-induced AP has not been determined. Dogs with intrahepatic bile duct obstruction have an increase in total serum AP which for the most part is hepatic AP with some steroid induced AP (Hoffman, 1976a,b).

γ-Glutamyltransferase is frequently used in human beings as a marker of hepatobiliary disease that is equally as sensitive as or more sensitive than AP (Lum and Gambino, 1972; Goldberg and Martin, 1975). Kidney has the highest GGT specific activity, followed by pancreas and liver. Nevertheless, experimental renal failure in sheep did not result in a significant change in serum GGT activity (Ford, 1974). Hepatobiliary disorders caused by experimental Senecio poisoning in cattle resulted in an increase in serum GGT (Johnson, 1978). Serum AP and GGT appear to increase in liver disorders, whereas only AP increases in bone disorders.

1. Dogs

Acute hepatic damage in dogs results in increased activity of serum GOT and GPT and many other enzymes. The degree of liver damage is reflected in the magnitude of increase in activity of these enzymes in serum (Freedland and Kramer, 1970). Infectious canine hepatitis is characterized by a marked increase in GOT and GPT activity. Moderate increases in serum GPT are observed when partial blood stasis occurs, as in heartworm infection and valvular insufficiencies of the heart.

Chronic hepatic fibrosis, hepatic lipidosis, and bile duct obstruction in dogs result in slight GOT and GPT increases, whereas serum AP increases more than any other enzyme of hepatic origin (Young, 1974). Hepatic lipidosis is associated with the highest increases in serum AP observed in clinical medicine. Hepatic lipidosis and increased AP are frequently seen in diabetes mellitus and Cushing's syndrome (Brobst and Schall, 1972; Hoffman *et al.*, 1977a,b; Ling *et al.*, 1977; Saini *et al.*, 1978). Treatment of dogs with cortisone, prednisone, prednisolone, dexamethasone, and triamcinolone induces an increase in serum AP activity which persists as long as 2½ weeks after treatment (Dorner *et al.*, 1974; Rogers and Ruebner, 1977). The increased AP in liver and serum is accounted for by steroid-induced AP not previously detected in serum or liver. Drugs, such as phenobarbital, diphenylhydantoin, and primidone, which cause increased smooth endoplasmic reticulum proliferation, also cause increases in hepatic and serum AP (Conning and Litchfield, 1971; Jennings *et al.*, 1974; Sturtevant *et al.*, 1977) (Table III).

2. Cats

The activity of serum GOT, GPT, and many other enzymes increases in acute hepatic inflammation of cats (Freedland and Kramer, 1970). Hepatobiliary disorders result in increased serum AP, but, in contrast to the dog, the magnitude of increase is not great (Everett *et al.*, 1977a,b; Hoffman *et al.*, 1977b). Diabetes mellitus in cats is also associated with increased serum AP. Steroid treatment alone results in only a small increase, and phenobarbital treatment in none (Hoffman *et al.*, 1977b; Schaer, 1977). The absence of a marked increase in serum AP may reflect the low specific activity of hepatic AP.

TABLE III

Drugs That Cause Changes in Serum Enzyme Activity in Dogs

Drug	Enzyme					Reference
	AP	GOT	GPT	LDH	ButChE	
Phenobarbital	+					Conning and Litchfield (1971); Sturtevant *et al.* (1977)
Primidone	+	–		–		Sturtevant *et al.* (1977); Jennings *et al.* (1974)
Diphenylhydantoin	+					Sturtevant *et al.* (1977)
ACTH	+					Sturtevant *et al.* (1977)
Prednisolone	+					Dorner *et al.* (1974); Rogers and Ruebner (1977)
Prednisone	+					Dorner *et al.* (1974); Rogers and Ruebner (1977)
Dexamethasone	+					Dorner *et al.* (1974); Rogers and Ruebner (1977)
Triamcinolone	+					Dorner *et al.* (1974); Rogers and Ruebner (1977)
Cortisone	+					Dorner *et al.* (1974); Rogers and Ruebner (1977)
Melengestrol/acetate (in high dose)	+	–	+	–		Goyings *et al.* (1977)
Dichlorvos					–	Seawright and Costigan (1977)

3. Horses

Markers of liver damage in horses are arginase, SDH, glutamate dehydogenase (GD), GOT, and isocitrate dehydrogenase (ICD) (Cornelius, 1961; Cornelius *et al.*, 1963b; Sova and Jicha, 1963a,b; Freedland *et al.*, 1965). Serum GPT is not specific for liver damage in the horse, and SDH has become the serum enzyme of choice as a marker of liver damage. As mentioned previously, SDH is not very stable and must be assayed soon after sampling. Serum GOT is useful when combined with CK to detect liver damage.

Gerber (1968) reported serum enzyme profiles for more than a hundred naturally occurring diseases of horses, and hepatic disease, tetanus, and azoturia had the most marked hepatic profiles. Acute hepatic diseases had increases in GOT, LDH, malate dehydrogenase, SDH, GPT, and GD activity. Hepatic cirrhosis and amyloidosis had little or no change. Hoffman (1967) indicated that the LDH isoenzymes were of value as markers of hepatic disorders. In serum hepatitis of the horse, the serum enzyme profile has a characteristic increase in GOT and GPT (Hjerpe, 1964; Thomselt, 1971).

Poisoning by pyrrolizidine alkaloid-containing plants, i.e., *Amsinkia intermedia*, induces a more chronic form of hepatobiliary disorder in horses, and AP, GOT, SDH, and GGT increase. It is of interest that in this disorder the change in GGT activity is a more sensitive marker of hepatic disease than the change in AP (Tennant *et al.*, 1972).

4. Cattle and Sheep

Hepatocellular degeneration in cattle and sheep has serum enzyme profiles similar to that in horses (Freedland and Kramer, 1970). Hepatic lipidosis and acetonemia are associated with increases in ICD and LDH. Hepatocytic or hepatobiliary damage has been

produced with ClC_4, bromide, sporidesmin, tansy ragwort, and bile duct ligation and results in increased serum activity of GGT, SDH, GD, ornithine carbamyltransferase (OCT), GOT, and LDH (Ford, 1965, 1967, 1974; Ford *et al.*, 1968; Johnson, 1978).

Serum AP activity has a wide range of normal values among individuals in both cattle and sheep and has not been found to be of clinical significance in hepatocellular necrosis (Boyd, 1962; Ford and Boyd, 1962). Serum GGT appears to be the enzyme of choice for diagnosis of hepatobiliary disorders, but additional data are needed.

5. *Pigs*

Nutritional hepatic dystrophy has significant increases in LDH, OCT, GPT, and GOT (Orstadius *et al.*, 1959; Michel *et al.*, 1969). Frequently there was a concurrent muscular dystrophy associated with the hepatic dystrophy so the source of LDH and GOT was obscured. The specific activity of GPT in pig liver is only moderate, and GPT cannot be considered a liver-specific enzyme (Cornelius *et al.*, 1959; Wretlind *et al.*, 1959). Ascarid larval migrans can induce increases in serum GPT and GOT activity (Andrews *et al.*, 1961).

G. Blood Cellular Elements

Enzymopathies of human blood cells are well documented, but only a few are recognized in veterinary medicine (see Chapter 4). The RBC enzymes of glycolysis and the pentose phosphate pathways differ in specific activity among animal species (Smith *et al.*, 1965). Also, RBC enzyme activity decreases with the age of the cell, with the reticulocyte having the highest enzyme activity (Tada *et al.*, 1961; Smith and Agar, 1975). The difference in enzyme activity in relation to age of the RBC makes it essential that the investigator use normal cells of matched age as controls when evaluating the enzyme activity of the cell. Even when reticulocytes are not present, the cell population is of various ages. Anemic horses do not readily release immature cell forms into the circulation. Horses with equine infectious anemia had a greater amount of LDH and glucose-6-phosphate dehydrogenase activity in their RBC's than did of normal horses. This increase in enzyme activity was attributed to a younger cell population in the anemic horse than in the normal horse (Kaneko *et al.*, 1969).

Genetic defects of various types have been reported in the RBC's of animals (Bannerman *et al.*, 1973). Searcy *et al.* (1971) characterized an inherited RBC pyruvate kinase (PK) deficiency hemolytic anemia in the Basenji dog which is now known to occur also in Beagle dogs (Harvey *et al.*, 1977; Prasse *et al.*, 1975). An NADH-methemoglobin reductase deficiency has also been reported in dogs (Harvey *et al.*, 1974; Letchworth *et al.*, 1977). Other investigators have studied the RBC of the inherited stomatocytic anemic, chondrodysplastic dwarf Alaskan malamute but have not identified an enzyme defect (Fletch *et al.*, 1975).

Merino, Corriedale, and Finnish Landrace sheep have an autosomal recessive disorder of the RBC in which the concentration of glutathione is lower than normal but no hemolytic anemia occurs. In the merino and Corriedale breeds and in their mixed breeds with low glutathione concentrations, there were lower amounts of γ-glutamylcysteine synthetase (Smith *et al.*, 1973) and glutathione peroxidase (Agar and Smith, 1973). No enzyme defect was located in the Finnish Landrace breed (Tucker and Kilgour, 1973).

Rumen bacterial thiaminase is associated with a deficiency of thiamine pyrophosphate

(TPP), which results in polioencephalomalacia (PE) of sheep and cattle. The RBC's of PE-affected sheep and cattle have a low TPP-dependent transketolase activity, which can be corrected *in vitro* by the addition of TPP (Pill, 1967).

White muscle disease of sheep and cattle is associated with selenium deficiency. Glutathione peroxidase is a selenoenzyme, and assay of RBC GP^x activity is as sensitive as selenium determination for the diagnosis of selenium deficiency (Kursa and Kroupova, 1976; Thompson *et al.*, 1976).

Lead poisoning in animals is associated with inhibition of aminolevulinate dehydrase and heme synthetase in developing RBC's and with the accumulation of free protoporphyrin in RBC's. Free RBC protoporphyrin concentration appears to be as useful as lead concentration in diagnosing lead poisoning of cattle (Osweiler and Ruhr, 1978).

REFERENCES

Agar, N. S., and Smith, J. E. (1973). *Anim. Blood Groups Biochem. Genet.* **4,** 133–140.

Allen, W. M., Berretts, S., Harding, J. D. J., and Patterson, D. S. P. (1970). *Vet. Rec.* **87,** 410–411.

Allen, W. M., Parr, W. H., Anderson, P. H., Berretts, S., Bradley, R., and Patterson, D. S. P. (1975). *Vet Rec.* **96,** 360–361.

Anderson, M. G. (1975). *Equine Vet. J.* **7,** 160–165.

Anderson, P. H., Berretts, S., and Patterson, D. S. P. (1976). *J. Comp. Pathol.* **86,** 531–538.

Andrews, M. F., McIwain, P. K., and Eveleth, D. F. (1961). *Am. J. Vet. Res.* **22,** 1026–1029.

Armstrong, A. R., and Banting, B. (1935). *Can. Med. Assoc., J.* **33,** 243–246.

Asmundson, V. S., and Julian, L. M. (1956). *J. Hered.* **47,** 248–252.

Baetz, A. L. (1969). *Proc. Technicon Int. Congr.*, pp. 4–6. Terrytown, New York.

Baker, R. H. (1978). *J. Am. Vet. Med. Assoc.* **172,** 269–270.

Bannerman, R. M., Edwards, J. A., and Pinkerton, P. H. (1973). *Prog. Hematol.* **8,** 131–176.

Barnett, A. M., Davidson, E. D., and Bradley, E. L. (1976). *J. Surg. Res.* **20,** 243–246.

Bergmeyer, V., ed. (1974). "Methods of Enzymatic Analysis," 2nd ed., Vol. 1. Academic Press, New York.

Blackmore, D. V., and Elton, D. (1975). *Equine Vet. J.* **7,** 34–38.

Blincoe, C., and Dye, W. B. (1958). *J. Anim. Sci.* **17,** 224–226.

Bogen, E., and Sommer, H. (1976). *Zentralbl. Veterinaermed., Reihe A* **23,** 394–400.

Borgman, R. F., Handlin, D. L., and Lightsey, S. F. (1978). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **37,** 330.

Boyd, V. W. (1962). *Res. Vet. Sci.* **3,** 256–268.

Braun, J. P., Rico, A. G., Benard, P., Thouvenot, J. P., and Bonnefis, M. J. (1978). *Res. Vet. Sci.* **25,** 37–40.

Brobst, D. F., and Schall, W. D. (1972). *J. Am. Vet. Med. Assoc.* **161,** 382–388.

Brobst, D. F., Ferguson, A. B., and Carter, J. M. (1970). *J. Am. Vet. Med. Assoc.* **157,** 1697–1702.

Campbell, J. R. (1962). *Vet. Rec.* **74,** 1340–1348.

Cardinet, G. H., Fowler, M. E., and Tyler, W. S. (1963). *Am. J. Vet. Res.* **24,** 980–984.

Christian, L. L. (1975). *Iowa Agric. Home Econ. Exp. Stn., Publ.* No. 410F.

Conning, D. M., and Litchfield, M. H. (1971). *J. Pathol.* **103,** xii.

Conzelman, G. M., and Gribble, D. H. (1973). *Toxicol. Appl. Pharmacol.* **26,** 158–160.

Cornelius, C. E. (1961). *Cornell Vet.* **51,** 559–568.

Cornelius, C. E., and Kaneko, J. J. (1960). *J. Am. Vet. Med. Assoc.* **137,** 62–66.

Cornelius, C. E., Bishop, J., Switzer, J., and Rhode, E. A. (1959). *Cornell Vet.* **49,** 116–126.

Cornelius, C. E., Burnham, L. G., and Hill, H. E. (1963a). *J. Am. Vet. Med. Assoc.* **142,** 639–642.

Cornelius, C. E., Douglas, G. M., Gronwall, R. R., and Freedland, R. A. (1963b). *Cornell Vet.* **53,** 181–191.

Crawley, G. J., and Sevenson, M. J. (1963). *Am. J. Vet. Res.* **24,** 1271–1270.

Dorner, J. L., Hoffmann, W. E., and Long, G. B. (1974). *Am. J. Vet. Res.* **35,** 1457–1458.

Edwin, E. E. (1970). *Vet. Rec.* **87,** 396–398.

Edwin, E. E., and Jackman, R. (1974). *J. Sci. Food Agric.* **25,** 357–368.

Edwin, E. E., and Lewis, G. (1971). *J. Diary Res.* **38,** 79–90.

Ellis, B. G., and Price, R. G. (1973). *Biochem. Soc. Trans.* **1,** 995–997.

Eto, K., Pairent, F. W., Appert, H. E., and Howard, J. M. (1969). *Arch. Surg. (Chicago)* **98,** 241–244.
Evans, W. C., Evans, A. I., Humphreys, D. J., Lewin, B., Davies, W. E. J., and Axford, R. F. E. (1975). *J. Comp. Pathol.* **85,** 253.
Everett, R. M., Duncan, J. R., and Prasse, K. W. (1977a). *Am. J. Vet. Res.* **38,** 963–966.
Everett, R. M., Duncan, J. R., and Prasse, K. W. (1977b). *Am. J. Vet. Res.* **38,** 1533–1538.
Finco, D. R., and Stevens, J. B. (1969). *J. Am. Vet. Med. Assoc.* **155,** 1686–1691.
Fletch, S. M., Pinkerton, R. H., and Brueckner, P. J. (1975). *J. Am. Anim. Hosp. Assoc.* **11,** 353–361.
Florkin, M., and Stotz, E. H. eds. (1973). "Enzyme Nomenclature. Recommendations (1972) of the Commission on Biochemical Nomenclature," 3rd ed. Elsevier, Amsterdam.
Ford, E. J. H. (1965). *J. Comp. Pathol.* **75,** 299–308.
Ford, E. J. H. (1967). *J. Comp. Pathol.* **77,** 405–411.
Ford, E. J. H. (1974). *J. Comp. Pathol.* **84,** 231–243.
Ford, E. J. H., and Boyd, J. W. (1962). *J. Pathol. Bacteriol.* **83,** 39–48.
Ford, E. J. H., Ritchie, H. E., and Thorpe, E. (1968). *J. Comp. Pathol.* **78,** 207–218.
Freedland, R. A., and Kramer, J. W. (1970). *Adv. Vet. Sci. Comp. Med.* **14,** 61–103.
Freedland, R. A., Hjerpe, C. A., and Cornelius, C. E. (1965). *Res. Vet. Sci.* **6,** 18–23.
Gerber, H. (1968). *Equine Vet. J.* **1,** 129–139.
Goldberg, D. M., and Martin, J. V. (1975). *Digestion* **12,** 232–246.
Goyings, L. S., Sokolowski, J. H., Zimbelmor R. G. and Greng, S. (1977). *Am. J. Vet. Res.* **38,** 1923–1931.
Hafeman, D. G., Sunde, R. A., Hoekstra, W. G. (1974). *J. Nutr.* **104,** 580–587.
Harvey, J. W., Ling, G. V., and Kaneko, J. J. (1974). *J. Am. Vet. Med. Assoc.* **164,** 1030–1033.
Harvey, J. W., Kaneko, J. J., and Hudson, J. B. (1977). *Vet. Clin. Pathol.* **6,** 13–17.
Hegreberg, G. A., Padjett, G. A., Prieur, D. J., and Johnson, M. I. (1975). *J. Hered.* **66,** 63–66.
Henry, J. B. (1963). *Postgrad. Med.* **33,** A64–A72.
Hess, B., and Raftopouls, R. (1957). *Dtsch. Arch. Klin. Med.* **204,** 97–106.
Hillis, L. D., and Braunwald, E. (1977). *N. Engl. J. Med.* **296,** 1034–1041.
Hime, J. M. (1968). *Symp. Zool. Soc. London* **21,** 2–9.
Hjerpe, C. A. (1964). *J. Am. Vet. Med. Assoc.* **144,** 734–740.
Hoffman, J. R. (1967). *Proc. Am. Assoc. Equine Pract.* **109–122,** New Orleans.
Hoffman, W. E. (1976a). *Gaines Vet. Symp., 26th,* 2–6, Columbus, Ohio.
Hoffman, W. E. (1976b). *J. Am. Anim. Hosp. Assoc.* **13,** 237–241.
Hoffman, W. E., and Dorner, J. L. (1975). *J. Am. Anim. Hosp. Assoc.* **11,** 283–285.
Hoffman, W. E., and Dorner, J. L. (1977). *Am. J. Vet. Res.* **38,** 1553–1556.
Hoffman, W. E., Renegar, W. E., and Dorner, J. L. (1977a). *Am. J. Vet. Res.* **38,** 1637–1639.
Hoffman, W. E., Renegar, W. E., and Dorner, J. L. (1977b). *Vet. Clin. Path.* **6,** 21–24.
Homburger, F., Baker, J. R., Nixon, C. W., and Wilgram, G. (1962). *Arch. Intern. Med.* **110,** 660–662.
Hudson, E. B., and Strombeck, D. R. (1978). *Am. J. Vet. Res.* **39,** 1316–1321.
Hunt, R. D., Garcia, F. G., and Hegsted, D. M. (1967). *Lab. Anim. Care* **17,** 222–234.
Jennings, P. B., Utter, W. F., and Fariss, B. L. (1974). *J. Am. Vet. Med. Assoc.* **164,** 1123–1125.
Johnson, A. E. (1976). *Am. J. Vet. Res.* **37,** 107–110.
Johnson, A. E. (1978). *Am. J. Vet. Res.* **39,** 1542.
Johnson, S. G., Ellis, C., and Levitt, M. D. (1977). *Clin. Res.* **25,** 313A.
Kaneko, J. J., Tanaka, S., Nakajima, H., and Ushimi, C. (1969). *Am. J. Vet. Res.* **30,** 543–549.
Kramer, J. W., Hegreberg, G. A., Bryan, G. M., Meyers, K., and Ott, R. L. (1976). *J. Am. Vet. Med. Assoc.* **169,** 817–820.
Krook, L., and Love, J. E. (1964). *Vet. Pathol.* **1,** 1–98.
Kursa, J., and Kroupova, V. (1976). *Res. Vet. Sci.* **20,** 97–98.
Letchworth G. J., Bentinck-Smith, J., Bolton, G. R., Wootton, J. F., and Family, L. (1977). *J. Am. Anim. Hosp. Assoc.* **13,** 75–79.
Ling, G. V., Lowenstine, L. J., Pulley, L. T., and Kaneko, J. J. (1977). *J. Am. Vet. Med. Assoc.* **170,** 521–530.
Lum, G., and Gambino, S. R. (1972). *Clin. Chem.* **18,** 358–362.
McGavin, M. D., and Baynes, I. D. (1969). *Vet. Pathol.* **6,** 513–524.
Manktelow, B. W. (1963). *N.Z. Vet. J.* **11,** 52–55.
Mattenheimaer, H. (1968). *Curr. Probl. Clin. Biochem.* **2,** 119–45.
Mayhew, I. G., Whitlock, R. H., and Tasker, J. B. (1977). *Am. J. Vet. Res.* **38,** 1271–1274.

Mendel, L. B., and Underhill, F. P. (1907). *J. Biol. Chem.* **3,** 135–143.
Mia, A. S., and Koger, H. D. (1978). *Am. J. Vet. Res.* **39,** 1381–1383.
Mia, A. S., Koger, H. D., and Tierney, M. M. (1978). *Am. J. Vet. Res.* **39,** 965–969.
Michel, R. L., Whitehair, C. K., and Keahey, K. K. (1969). *J. Am. Vet. Assoc.* **155,** 50–59.
Michelson, A. M., Russell, E. S., and Pinckney, J. A. (1955). *Proc. Natl. Acad. Sci. U.S.A.* **41,** 1079–1084.
Moore, W. E., and Feldman, B. F. (1974). *J. Am. Anim. Hosp. Assoc.* **10,** 420–429.
Nelson, T. E., Jones, E. W., Henrickson, R. L., Falk, S. N., and Kerr, D. D. (1974). *Am. J. Vet. Res.* **35,** 347–350.
Noguchi, T., Cantor, A. H., and Scott, M. C. (1973). *J. Nutr.* **103,** 1502–1511.
Nydick, J., Ruegsegger, P., Wroblewski, F., and La Due, J. S. (1957). *Circulation* **15,** 324–334.
Oh, S. H., Sunde, R. A., Pope, A. L., and Hoekstra, W. G. (1974). *J. Anim. Sci.* **39,** 247–248.
Orstadius, K., Wretlind, B., Lindberg, P., Dordstrom, G., and Lannek, N. (1959). *Zentralbl. Veterinaermed.* **6,** 971–980.
Osweiler, G. D., and Ruhr, L. P. (1978). *J. Am. Vet. Med. Assoc.* **172,** 498–500.
Owen, R., Moore, J. N., Hopkins, J. B., and Arthur, D. (1977). *J. Am. Vet. Med. Assoc.* **171,** 343–349.
Pekarthy, J., Short, J., Lansing, A. I., and Lieberman, I. (1972). *J. Biol. Chem.* **247,** 1767–1774.
Pickrell, J. A., Schluter, S. J., Belasich, J. J., Stewart, E. V., Meyer, J., Hobbs, C. H., and Jones, R. K. (1974). *Am. J. Vet. Res.* **35,** 897–903.
Pill, A. H. (1967). *Vet. Rec.* **81,** 178–180.
Prasse, K. W. (1969). *Am. J. Vet. Res.* **30,** 2181–2184.
Prasse, K. W., Crouser, D., Beutler, E., Walder, M., and Schall, W. D. (1975). *J. Am. Vet. Med. Assoc.* **166,** 1170–1175.
Raab, W. P. (1972). *Clin. Chem.* **18,** 5–25.
Rapp, J. (1962). *Am. J. Vet. Res.* **23,** 343–350.
Reid, E. (1967). *In* "Enzyme Cytology" (D. V. Roodyn, ed.), pp. 321–406. Academic Press, New York.
Robinson, M., and Gopinath, C. (1974). *Res. Vet. Sci.* **16,** 355–369.
Robinson, M., and Hesketh, A. (1976). *J. Comp. Pathol.* **86,** 307–318.
Robinson, M., and Tafford, J. (1977). *J. Comp. Pathol.* **87,** 275–280.
Rogers, W. A., and Ruebner, B. H. (1977). *J. Am. Vet. Med. Assoc.* **170,** 603–606.
Ruegsegger, P., Nydick, I., Freiman, A., and LaDue, J. S. (1959). *Circ. Res.* **7,** 4–10.
Saini, P. K., and Saini, S. K. (1978a). *Am. J. Vet. Res.* **39,** 1506–1509.
Saini, P. K., and Saini, S. K. (1978b). *Am. J. Vet. Res.* **39,** 1510–1513.
Saini, P. K., Peavy, G. M., Hauser, D. E., and Saini, S. K. (1978). *Am. J. Vet. Res.* **39,** 1514–1518.
Schaer, M. (1977). *J. Am. Anim. Hosp. Assoc.* **13,** 23–27.
Schmidt, E., and Schmidt, F. W. (1967). *Nature (London)* **213,** 1125–1126.
Schmidt, E., and Schmidt, F. W. (1974). *In* "Methods of Enzymatic Analysis" (H. U. Bergmeyer, ed.), 2nd ed., Vol. 1, pp. 6–69. Academic Press, New York.
Searcy, G. P., Miller, D. R., and Tasker, J. B. (1971). *Can. J. Comp. Med.* **35,** 67–70.
Seawright, A. A., and Costigan, P. (1977). *Aust. Vet. J.* **53,** 509–514.
Segall, H. J., Siegel, D. M., Norman, B. B., and Oliver, M. N. (1977). *Calif. Vet.* **31,** 12, 13.
Shaw, F. D. (1976). *Res. Vet. Sci.* **20,** 226–228.
Siegel, S., and Bing, R. J. (1956). *Proc. Soc. Exp. Biol. Med.* **91,** 604–607.
Smith, J. B., and Healy, P. J. (1968). *Clin. Chem. Acta* **21,** 295–296.
Smith, J. E., and Agar, N. S. (1975). *Res. Vet. Sci.* **18,** 231–236.
Smith, J. E., Barnes, J. K., Kaneko, J. J., and Freedland, R.A (1965). *Nature (London)* **205,** 298–299.
Smith, J. E., Lee, M. S., and Mia, A. S. (1973). *J. Lab. Clin. Med.* **82,** 713–718.
Sova, Z., and Jicha, J. (1963a). *Zentralbl. Veterinaermed. Reihe A* **10,** 293–304.
Sova, Z., and Jicha, J. (1963b). *Zentralbl. Veterinaermed. Reihe A,* **10,** 305–313.
Stickel, J. (1978). *Vet. Clin. Path.* **7,** 15.
Sturtevant, F., Hoffmann, W. E., and Dorner, J. L. (1977). *J. Am. Anim. Hosp. Assoc.* **13,** 754–757.
Szczech, G. M., Carlton, W. W., and Lund, J. E. (1974). *J. Am. Anim. Hosp. Assoc.* **10,** 171–174.
Tada, K., Watanabe, Y., and Fujiwara, T. (1961). *Tohoku J. Exp. Med.* **75,** 384–392.
Tennant, B. C., Evans, C. D., Kaneko, J. J., and Schalm, O. W. (1972). *Calif. Vet.* **26,** 15–18.
Thompson, R. H., McMurray, C. H., and Blanchflower, W. J. (1976). *Res. Vet. Sci.* **20,** 229–231.
Thomselt, L. R. (1971). *Equine Vet. J.* **3,** 15–19.
Tucker, E. M., and Kilgour, L. (1973). *Res. Vet. Sci.* **14,** 306–311.

van den Hende, C., Lister, D., Muylle, E., Ooms, L., and Oyaert, W. (1976). *Br. J. Anaesth.* **48,** 821–829.
Van Vleet, J. F. (1975). *J. Am. Vet. Med. Assoc.* **166,** 769–774.
Van Vleet, J. F., Meyer, K. B., Olander, H. J., and Ruth, G. R. (1975). *Am. J. Vet. Res.* **36,** 387–393.
Van Vleet, J. F., Crawley, R. R., and Amstutz, H. E. (1977). *J. Am. Vet. Med. Assoc.* **171,** 443–445.
Wagner, J. A., and Critz, J. B. (1968). *Proc. Soc. Exp. Biol. Med.* **128,** 716.
Wentink, G. H., Van der Linde-Sipmon, J. S., Meijer, A. E. F. H., Kamphuisen, H. A. C., Van Vorstenhosch, C. J. A. A. V., Hatmon, W., and Hendriks, H. J. (1972). *Vet. Pathol.* **9,** 328–349.
Wilkens, R. J., and Hurvitz, A. I. (1975). *J. Am. Anm. Hosp. Assoc.* **11,** 29–41.
Wilson, J. W. (1977). *J. Am. Vet. Med. Assoc.* **171,** 200–202.
Woolf, N., Hall, L., Thorne, C., Down, M., and Walker, R. (1970). *Br. Med. J.* **iii,** 386–387.
Wretlind, B., Olstodius, K., and Lindberg, P. (1959). *Zentralbl. Veterinaermed.* **6,** 963–970.
Young, J. T. (1974). *J. Am. Anim. Hosp. Assoc.* **10,** 415–419.

6

Liver Function

CHARLES E. CORNELIUS

I. INTRODUCTION

Prior to 1970, there were few substantive clinical reports describing the evaluation of liver function tests in specific hepatic disorders of animals, although considerable information was available on experimentally induced liver diseases. Recently, there has been

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

an increasing amount of literature on clinical cases, which has allowed for the recognition and evaluation of specific disease entities by modern biochemical techniques. Advances have come mainly from the collection and retrospective analysis of numerous cases from university hospitals and private practitioners and from basic biochemical studies on certain new tests and their applications to clinical veterinary medicine. This recent clinical evaluation of the many laboratory tests has long been overdue; ultimately, additional clinical studies will be necessary. The increasing use and number of liver function tests, coupled with our lack of knowledge concerning the pathogenesis of many liver diseases in animals, have created new diagnostic uncertainties for the practicing veterinarian. There is no question, however, that the state of the art has improved greatly in veterinary medicine with the recent advent of the use of serum enzymes and more information regarding the clinical interpretation of laboratory tests.

Only recently in human medicine has there been an attempt to measure the diagnostic effectiveness of a profile of biochemical tests by a computer technique utilizing discriminant analysis (Hamilton, 1977; Sher, 1977). Some hepatic tests may not correlate due to the *dissociation* of hepatic functions in various disorders. Such mathematical analyses in man, depending on the homogeneity of the diagnostic group, has allowed for better prediction of which biochemical tests are likely to be the best discriminators. In years to come, with similar techniques it may be possible to predict which hepatic tests in each species will be the most reliable for use by the veterinarian.

II. GENERAL TYPES OF LIVER DISEASE

The liver is composed of four anatomical units: the hepatic cells, the bile duct system, the vascular or hemodynamic system, and the reticuloendothelical cell system. Hepatic pathology involves primarily the hepatic cells, bile ducts, and vascular system. Function tests generally indicate either cell injury or the response to that injury. The majority of hepatic disorders are observed pathologically as (1) hepatic cell necrosis, (2) intrahepatic or extrahepatic bile duct obstruction (cholestasis), and (3) hepatic atrophy and/or fibrosis. In certain liver disorders, hepatic necrosis, cholestasis, and fibrosis may all be present to various degrees. Unfortunately, animals are nearly never presented to the clinician at the onset of the disease, and any combination of liver pathologies may be encountered. It is the fortunate veterinarian who observes a case with early hepatitis associated with an uncomplicated hepatic necrosis. Within a short time after this early necrotic phase, intrahepatic cholestasis of canaliculi may also occur with its characteristic elevations in serum enzyme activities associated with biliary obstruction. Following the initial necrotic insult, the liver may rapidly regenerate to the normal state or progress mysteriously to a chronic active hepatitis, which generally results in ductular hyperplasia and progressive fibrosis and/or atrophy. If common bile duct obstruction occurs and persists, it is followed by a secondary mild cellular necrosis with its characteristic enzyme release followed by hyperplasia and cholestasis of the smaller ductules throughout the liver. Animals with chronic hepatic lipid accumulations may also exhibit a secondary cellular necrosis and ultimately fibrosis. Any form of liver disease resulting in fibrosis markedly affects hemodynamics and hepatic perfusion by the formation of vascular anastomoses. These shunts allow for the bypass of the hepatic parenchyma by portal blood from the splanchnic

circulation. If congenital portosystemic shunts are present at birth, hepatic atrophy and decreased function will occur in addition to the usual signs of hepatoencephalopathy and coma.

Since the veterinary practitioner is confronted with the constant reaction of the liver to chronic injury, he must develop his diagnostic approach accepting this complexity. All interpretations of laboratory tests must be viewed in terms of short time intervals due to the rapid reactive nature of the organ, whether regenerative or degenerative. The *dissociation* of liver function has continually challenged the clinical investigator. No single liver function test is universally accepted since the organ in various disease states may lose specific biochemical functions in a different sequence. Lesions placed strategically may spare parenchymal metabolic processes but block biliary channels. Generalized or mixed lesions are most common and affect structural elements unevenly. To the clinician, the recognition of the *association* of liver functions is of prime importance. The unequal involvement of different functions cannot be attributed to a lack of sensitivity of different tests, but is due to the fact that certain functions are unduly sensitive and many times affected by pathological processes which are not primary hepatic.

It has been known for some time that there need be no strict correlation between function and structure of hepatic cells by light microscopy. More recent ultrastructural findings utilizing electron microscopy have allowed for better correlations; however, many biochemical functions can be affected before structural changes are observable by any modern technique. It is well known that some cholestatic syndromes resulting in decreased bile flow are due only to biochemical alterations with no observable structural canalicular abnormalities (Magnemat, 1973).

III. INDICATIONS FOR LIVER FUNCTION TESTS

The indications for liver function tests can be grouped as follows:

1. Primary liver disorders with or without icterus, such as infectious hepatitis; toxic necrosis; hepatic hemangioma; suppurative hepatitis; hepatoma, bile duct adenoma, or carcinoma; chronic active hepatitis; and copper storage disease.
2. Secondary liver disorders, such as the infiltrative lipidoses accompanying hypothyroidism, diabetes mellitus, pancreatic diseases, and starvation; chronic passive congestion in cardiac decompensation; and metastatic hepatic malignancies, secondary amyloidosis, and insufficiency due to congenital portocaval shunts
3. Differential diagnosis of icterus from hemolytic crisis, intrahepatic biliary cholestasis, and extrahepatic obstruction of the bile duct system
4. Anemias of undetermined origin, normocytic normochromic anemias from chronic fibrosis, anemias due to coagulation defects, etc.
5. Prognosis of hepatic diseases, including the evaluation of therapy, estimation of residual damage, and measuring hepatic function to evaluate the anesthetic risks in surgery
6. Specific research investigations, such as the testing of drugs for toxicity, measuring damage from parasitic migrations, and comparative physiological studies on various species

IV. CLASSIFICATION OF LIVER FUNCTION TESTS

Established tests can be grouped as (1) tests measuring the hepatic transport (uptake, conjugation, and excretion) of organic anions, (2) serum enzyme tests, (3) specific biochemical tests, and (4) liver biopsy and radiographic procedures.

A multitude of liver function tests have been experimentally studied in animals. Only the tests having undergone reasonable clinical evaluation or new tests of great promise are included in this brief chapter. Coagulation defects due to liver insufficiency are covered elsewhere (Chapter 17).

V. TESTS MEASURING THE HEPATIC TRANSPORT (UPTAKE, CONJUGATION, AND EXCRETION) OF ORGANIC ANIONS

A. Bile Pigments

1. Unconjugated and Conjugated Bilirubin in Serum

a. General. Open-chain tetrapyrrolic compounds played a central metabolic role in the early course of evolution (Lester and Troxler, 1969). Bile pigments in mammals are waste products and have a less central metabolic role than do those in unicellular and plant organisms. Bilirubin was first crystallized from ox gallstones in 1864, and Kuster later discovered its relationship to hemoglobin in 1899 (With, 1954). The great pathologist Virchow early recognized the relation of "hematoidin" in the tissues to degraded hemoglobin. As early as 1883, Ehrlich discovered that, after the addition of sulfanilic acid, hydrochloric acid, and sodium nitrite to serum containing bile pigments, a violet pigment of azobilirubin was formed (With, 1954). Van den Bergh and Müller (1916) concluded that two forms of bilirubin existed in the serum of some clinical cases of icterus since the addition of alcohol was not needed to produce the diazo reaction color in the "direct reaction." The "indirect reaction" required the presence of alcohol for color development and was found primarily in cases of hemolytic crisis. The present use of the van den Bergh test calls for the addition of alcohol to the mixture of acidic diazobenzenesulfonic acid and serum in order to measure the total bilirubin in the serum. Next, the "direct-reacting" pigment is measured at a 1-minute reaction interval. The calculated difference between the total and "direct-reacting" pigment gives an "indirect-reacting" bilirubin value. Normal values for the serum bilirubin concentration in various domestic animals are presented in Table I.

Prior to 1953, innumerable conflicting reports appeared concerning the interpretation of the van den Bergh reaction. Cole and co-workers (Cole and Lathe, 1953; Cole *et al.*, 1954), by means of reverse-phase partition chromatography, were able to separate the serum pigments giving the "direct" and "indirect reactions." Schmid (1956), with ascending paper chromatography, found that in obstructive icterus the "direct-reacting" pigment was a glucuronide conjugate of bilirubin. In hemolytic icterus the "indirect-reacting" serum bilirubin was in a free and unconjugated state. This free bilirubin was relatively insoluble and nonpolar at the pH of blood and therefore required alcohol in the reaction to render it soluble for diazotization. The use of the terms "conjugated" and "free bilirubin" in place of "direct-" and "indirect-reacting bilirubin," respectively, is

TABLE I

Normal Levels of Serum Bilirubin in Domestic Animals[a]

Species	Total bilirubin (mg/100 ml) Mean	σ^b	Range	Conjugated bilirubin (direct-reacting) (mg/100 ml) Mean	σ^b	Range	Reference
Dog			0.28–0.35			0.06–0.12	Karsai (1954)
	0.10		0–0.3	0.07		0–0.14	Klaus (1958)
	0.25	±0.10	0.07–0.61	0.14			Müller (1960)
	0.22		0.05–0.55				Van Vleet and Alberts (1968)
Cat			0.15–0.20				Lopez Garcia *et al.* (1943)
Horse	1.25	±0.07	0.81–2.07	0.37	±0.02	0.18–0.72	Muzzo (1949)
			1.1–1.2				Beijers *et al.* (1950)
			0.47–1.02				Benndorf (1955)
	1.10	±0.40		0.50	±0.20		Berger (1956)
		0.99	0.50–1.50	0.15		0.02–0.4	Klaus (1958)
	1.94			0.87			Eikmeier (1959)
Cow	0.14	±0.017	0.0–0.54				Muzzo (1949)
	0.31	±0.168	0–1.4				Garner (1953)
	0.21		0.01–0.47	0.18		0.04–0.44	Klaus (1958)
	0.19	±0.07	0.0–0.41			0.04–0.44	Hansen (1964)
Calf	0.7	±0.50		0.4	±0.30		Berger (1956)
Sheep	0.10	±0.007	0.0–0.18				Muzzo (1949)
	0.20	±0.2		0.10	±0.10		Berger (1956)
	0.19		0–0.39	0.12		0–0.27	Klaus (1958)
	0.23	±0.09	0.1–0.42				Hansen (1964)
			0–0.3				Roberts (1968)

[a] Conjugated bilirubin values may vary slightly according to the length of time (1.5 or 15 min) allowed by each author for the diazo reaction to proceed.

[b] σ = standard deviation.

presently recommended. So-called biphasic serum reactions were due to a mixture of conjugated and free bilirubin.

Grodsky and Carbone (1957), using liver homogenates, and Schmid *et al.* (1957), using hepatic microsomal preparations, observed that glucuronic acid was transferred to bilirubin in its activated form of uridine diphosphoglucuronic acid. In recent years, new research has produced a wealth of information on the biochemistry of bile pigments. The analysis of bile pigments by thin-layer chromatography has revealed a heterogeneity of bile pigment conjugates among animal species (Van Roy and Heirwegh, 1968). Although "direct-reacting" bilirubin is conjugated to a variety of glycosides in animals, it fortunately does not affect our current clinical interpretation of the serum van den Bergh test. It does suggest, however, that, if a hepatic conjugating deficiency should ever exist and result in elevated serum unconjugated bilirubin levels, a variety of hepatic glycosyltransferases would have to be measured in the biopsy sample.

Although studies have shown that the majority of bilirubin is conjugated to glucuronic acid in the mammalian liver, neutral glycosides such as glucose and xylose represent a substantial percentage of the total conjugating groups in the liver of cat, mouse, rabbit, horse, and dog (Fevery *et al.*, 1977). It was reported that horse bile contains bilirubin–glucose as the major conjugate (Cornelius *et al.*, 1975b). In man, dog, cat, and rat,

bilirubin is excreted primarily as diconjugated derivatives in contrast to monoconjugates in other species studied (Fevery *et al.*, 1977). The possible presence of disaccharide- and polypeptide-bilirubin conjugates has also been reported (Etter-Kjelsaas and Kuenzle, 1975)

Although mammals excrete primarily bilirubin conjugates, biliverdin has been observed in the bile of fowl, rabbits, nutria, and snakes. Tenhumen (1971) reported little or no biliverdin reductase activity in chicken tissues; this probably accounts for the high percentage of biliverdin in the bile of the chicken and certain other species.

The hepatocellular transport of organic anions such as bilirubin and sulfobromophthalcin (BSP) from plasma to bile can be simplified for clinical discussions if one considers three separate steps in the excretory process: (1) the *uptake* of anions by processes at the sinusoidal membrane, (2) the *binding* of anions by intracellular organelles, and (3) their terminal *excretion* into the canaliculus. Inherited clinical syndromes can therefore be classified as to whether the liver is biochemically defective in organic anion uptake (Southdown ovine mutants), conjugation (Gunn rats, Crigler–Najjar syndrome in human neonates, Gilbert's syndrome in man), or excretion (Dubin–Johnson syndrome) (Cornelius, 1970).

Gunn (1938), a Canadian geneticist, observed that a strain of rats developed jaundice and severe kernicterus shortly before birth. A similar syndrome of congenital nonhemolytic jaundice (Crigler and Najjar, 1952) was reported in human infants with serum free bilirubin levels of 25–45 mg/100 ml. Both syndromes are transmitted as simple autosomal recessives and are due to a deficiency of glucuronyltransferase in the liver. Another bile pigment disease, called Gilbert's syndrome or constitutional hepatic dysfunction, has been reported in human adults between 15 and 25 years with free bilirubin levels of between 1 and 6 mg/100 ml. Recent studies (Barrett *et al.*, 1968) indicate that a lower fractional transfer of bilirubin from plasma to liver exists in Gilbert's syndrome. A Southdown mutant sheep with a hepatic uptake defect for bilirubin has been studied (Cornelius and Gronwall, 1965) and in some respects resembles Gilbert's syndrome in man. Another syndrome has been reported in man and more closely resembles the Southdown mutants in that both bilirubin and BSP are not normally extracted and stored by the liver (Dhumeaux and Berthelot, 1975). Chronic idiopathic jaundice (Dubin and Johnson, 1954) in man is characterized by a tender liver, chronic intermittent bilirubinemia (both free and conjugated bilirubin), impaired BSP excretion, normal serum transaminase activity, and an unidentified melanin hepatic pigment. An identical syndrome in Corriedale sheep has been observed (Cornelius *et al.*, 1965a). Reduced transport maxima for bilirubin and BSP are characteristic signs of this disease.

Chromatographic analysis of bile pigment extracted from a gallstone in a calf revealed the presence of only free bilirubin (Cornelius *et al.*, 1960). Free bilirubin, which is nonpolar and relatively insoluble in alkaline bile, precipitates with biliary glycoproteins in man to form biliary calculi. Various cases of biliary lithiasis have been reported in many domestic animals (dogs, Schlotthauer, 1945; cows, Ford, 1955; horses Sastry, 1945).

b. Classification of Icterus. The various types of icterus can be classified as follows:

i. Prehepatic or hemolytic icterus. This condition is characterized by the presence of an indirect van den Bergh reaction due to the presence of an increased amount of free

bilirubin from excessive erythrocyte hemolysis or a lack of bilirubin clearance by the liver. Recent studies on the turnover of hepatic nonhemoglobin hemoproteins, such as catalase, peroxidase, tryptophan pyrrolase, and the mitochondrial cytochromes, suggest that their overproduction might rarely account for unexplained hyperbilirubinemias in certain disorders (Lester and Troxler, 1969).

ii. Hepatic icterus. This condition can be identified primarily by a van den Bergh "direct-reacting" pigment (bilirubin conjugates), which regurgitates into the serum from intrahepatic functional or mechanical obstruction of biliary canaliculi. In addition, some "indirect-reacting" pigment (free bilirubin) is present from decreased hepatic uptake of free bilirubin from the blood or increases in hepatic β-glucuronidase (Acocella *et al.*, 1968) due to hepatic cell insufficiency. A study of experimental hepatopathies (Gopinath and Ford, 1972) indicated that purely centrilobular lesions produce little hyperbilirubinemia, while moderate elevations occur if lesions include cells of the outer zones. Periportal lesions, however, that affect cell membrane integrity result in marked elevations in the serum bilirubin concentration.

iii. Obstructive icterus (extrahepatic). This condition is characterized by a direct reaction due to serum bilirubin conjugates. Bilirubin conjugates gain entry to the blood by extensive regurgitation via hepatic lymphatics from bile duct obstruction. Some unconjugated bilirubin is also present in the serum.

c. Van den Bergh Test. Since the biochemical clarification of the van den Bergh test in recent years, the clinican can now more rationally differentiate among the types of icterus present. The types of icterus and the enterohepatic circulation of bile pigments are given in Figs. 1–4.

The qualitative test is no longer used extensively as a practical diagnostic procedure.

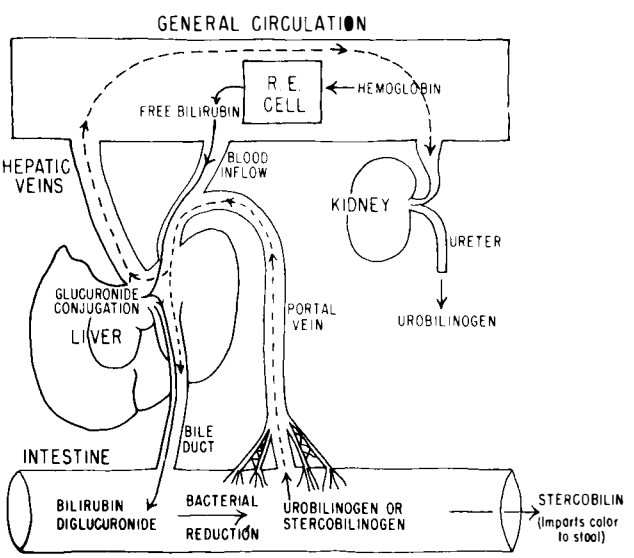


Fig. 1. Normal enterohepatic circulation of bile pigments.

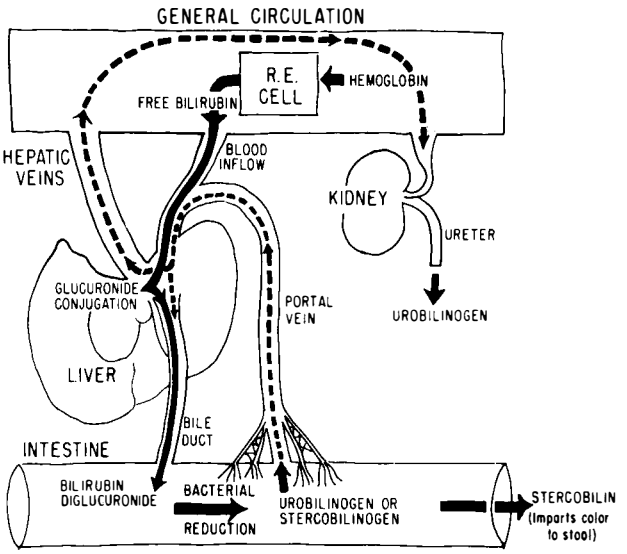


Fig. 2. Hemolytic crisis. Observe the increase in the quantity of free bilirubin in the serum (unable to pass the renal filter), stercobilin in the stool (imparts a darker color to the stool), and urinary urobilinogen. Increased urinary urobilinogen may be due partly to secondary liver damage (less reexcreted into the bile and hence lost to the serum and urine) in addition to the increased quantity of bile pigments metabolized owing to erythrocyte hemolysis. If secondary liver damage is extensive from hemosiderosis or bile pigment overload, some bilirubin glucuronide may be regurgitated and lost to the urine (not in diagram). RE, reticuloendothelical.

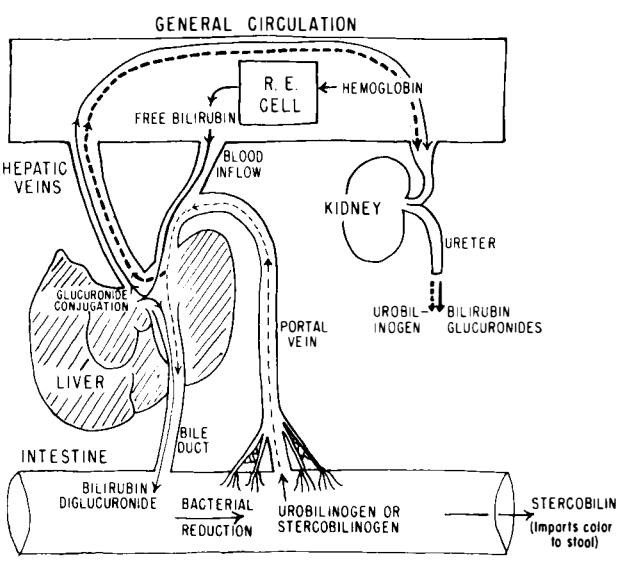


Fig. 3. Hepatocellular pathology. Observe the presence of bilirubin glucuronide and increased amounts of urobilinogen in the urine. Increased urinary urobilinogen is due to the inability of the altered hepatic cells to quantitatively reexcrete this pigment into the bile. Free bilirubin may also be elevated in the serum owing to a decreased uptake of the pigment.

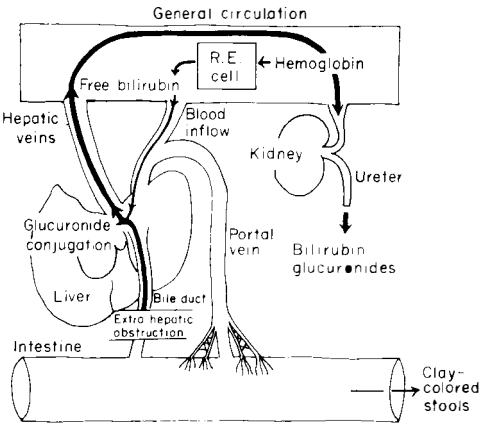


Fig. 4. Extrahepatic obstruction. Observe regurgitation to the serum and subsequently the urine of all bilirubin diglucuronide conjugated in the liver. Urinary urobilinogen and fecal stercobilin are absent.

The quantitative test, which measures the unconjugated (''indirect-reacting'') and conjugated (''direct-reacting'') bilirubin, may be carried out by routine methods (Malloy and Evelyn, 1937; Ducci and Watson, 1945).

A host of modifications of the 1-minute method of reading the direct reaction have been suggested in human medicine. A preponderance of unconjugated bilirubin indicates hemolytic icterus, but, if more than half is conjugated, the icterus may be either hepatocellular or obstructive.

d. Dog. The interpretation of the van den Bergh test in the dog more closely approximates that in man than that in other domestic animals. However, the low renal threshold (Rosenthal and Meier, 1921; Mills and Dragstedt, 1938) observed in the dog for bilirubin conjugates may be responsible in part for lower serum levels of bilirubin in intrahepatic cholestasis or extrahepatic obstruction of the biliary system. High levels of preponderantly free bilirubin are usually indicative of hemolytic diseases in the dog. If greater than 50% of the total bilirubin is of the conjugated variety, hepatocellular disease is most likely present. Extrahepatic obstruction of the bile duct system is accompanied by an even greater percentage of conjugated bilirubin in the serum, but minor amounts of unconjugated bilirubin are usually always present. The presence of unexplained amounts of serum unconjugated bilirubin in intrahepatic cholestasis may result from different net fractional clearance rates for bilirubin and its conjugates from serum during the process of active transport. This unconjugated bile pigment, however, may result from the lack of hepatic uptake of bilirubin by the diseased liver.

Because of the low renal threshold for the regurgitated bilirubin conjugate in the serum, slight elevations in concentration are quite indicative of hepatocellular disease. Needless to say, an elevated concentration in the urine is a sensitive and practical clinical test. Hoe and Harvey (1961b) also observed that dogs showing bilirubinuria were many times without hyperbilirubinemia. In cases of decreased renal function and bile duct obstruction, plasma bilirubin levels are clearly increased (Fulop and Brazeau, 1970).

Many recent clinical reports on hyperbilirubinemias in dogs have helped to clarify its use in diagnosis. In a detailed study of bilirubin in 300 canine sera, Hoe and Harvey

(1961a) found very low values in nearly all healthy sera, with 63% less than 0.2 mg/dl and 35% exhibiting no measurable bilirubin. A considerable range was reported for dogs with jaundice. Dogs with advanced cirrhosis exhibited serum levels ranging from 1 to 20 mg/dl, probably varying with the degree of renal insufficiency. Although serum bilirubin values can be quite elevated in prolonged extrahepatic obstruction or extensive hepatic cholestasis (Malherbe, 1959; Van Vleet and Alberts, 1968), most hepatocellular diseases are accompanied by serum bilirubin levels of less than 4 mg/dl. Due to the low renal threshold for bilirubin conjugates in the dog, plasma levels can be quite low in the presence of liver disease. Similar hepatic diseases in man would be accompanied by substantially higher serum bilirubin concentrations. Snell *et al.* (1925) early observed that only traces of bilirubin could be detected in canine serum 24 hours after common duct ligation with the gallbladder *in situ.* In 48–72 hours the serum contained 2–4 mg/dl, and it appeared in the urine. Bilirubin levels increased slowly and reached a plateau by 2 weeks. In dogs with cholecystectomy and common bile duct ligation, hyperbilirubinemia occurred within 30 minutes, increasing to 13.4 mg/dl by 17 days. Similar findings have been observed in cats.

Hyperbilirubinemias have been reported in a variety of diseases involving the liver. Examples are disseminated intravascular coagulation (Strombeck *et al.,* 1976a), cirrhosis (Grenn and Bulmer, 1972; Brobst and Schall, 1972), bile duct rupture (Bellinger, 1973; Robbins *et al.,* 1977); primary cholangiohepatitis (La Croix and Pulley, 1974), aflatoxicosis (Greene *et al.,* 1977), copper storage disease in Bedlington terriers (Hardy *et al.,* 1975), and babesiosis (Malherbe, 1965a).

Bilirubin toxicity. Kernicterus is only rarely observed in small animals (Tryphonas and Rozdilsky, 1970) but is a frequent problem in human neonates with hyperbilirubinemia. Since the fetal canine liver, unlike the human liver, contains hepatic bilirubin-conjugating enzymes *in utero,* there may well be fewer problems with this developmental disease in the dog. Kernicterus may, however, be a potential problem in any animal with a severe hyperbilirubinemia since animal albumins are capable of binding only one-half as much bilirubin as is human albumin. Serum levels of unconjugated bilirubin above 20 mg/dl result in nonbound bilirubin in infants which is capable of penetrating brain tissue if certain factors are present, such as acidosis, hypoxia, hypoglycemia, or drugs known to preferentially bind albumin (e.g., salicylates, sulfa drugs, hydrocortisone, digoxin, oxacillin, Valium, and Orinase).

e. Horse. The interpretation of the van den Bergh test (quantitative values of unconjugated and conjugated serum bilirubin) in the horse is quite different from that in other species. It is well known that the greater part of bilirubin found in the serum of horses with either hemolytic or hepatic icterus is free bilirubin (Eikmeier, 1959; Cornelius *et al.,* 1960; Benedek, 1961). It is possible to find serum levels up to 25 mg/100 ml of free bilirubin in equine hepatopathy with less than 2 mg/100 ml of "direct-reacting" pigment. The serum bilirubin from a series of horses with hepatic lesions was examined chromatographically, and the results confirmed the "indirect-reacting" pigment to be free bilirubin. Grassnickel (1959) and Eikmeier (1959) concluded that icterus of the equine scleral conjunctiva should not be used as a definitive symptom of damaged liver parenchyma or aberrant bilirubin metabolism. Since icterus and hyperbilirubinemia do not always coincide in time, it was concluded that the yellow coloration of the conjunctiva of a healthy

horse could be due to bilirubin storage. The measurement of conjugated and total bilirubin levels in horses with and without yellow coloration revealed no difference in their serum concentrations. The serum icteric index in the horse and the cow varies directly with the amounts of carotenoids and xanthophylls in the diet. The icteric index of the normal horse is usually between 7.5 and 20, and that of the cow between 5 and 25. For this reason, the use of the icteric indices in such herbivores for estimating the bilirubin level is quite unsatisfactory. Its use in sheep is feasible due to the lack of serum carotenoids; however, xanthophyll elevations in liver disease can be confusing (Roberts, 1968).

Elevations in the serum free bilirubin level may be observed secondarily to many environmental and pathological conditions in horses. Lopuchovsky and Jantosovic (1958) observed bilirubin levels to be elevated as follows: cardiac insufficiency, 2.76–6.27 mg/100 ml; constipation, 2.8–7.8 mg/100 ml; gangrenous pneumonia, 5.12 mg/100 ml; hemolytic diseases, 6.8–8.3 mg/100 ml; and primary hepatic disorders, 5.19–26.14 mg/100 ml. The majority of levels were over 20 mg/100 ml. Another investigator found serum bilirubin levels in horses with pneumonia and other infectious diseases to be between 10 and 50 mg/100 ml (Zenglein, 1930). In colic–constipation syndromes of horses, the serum free bilirubin is usually markedly elevated. The suggestion by many investigators that this is presumably due to occlusion of the biliary passages is questionable. Starvation of only a few days' duration is also accompanied by hyperbilirubinemia.

A study by Gronwall and Mia (1969) revealed that plasma bilirubin levels increased in the horse within 12 hours of last feeding and increased between 2 and 4 days to levels eight times prestarvation values. Infusion of bilirubin resulted in a more rapid rise in plasma unconjugated bilirubin levels in starved horses; in addition, a decreased removal capacity was present. Conjugated bilirubin increased little following starvation or bilirubin infusion. The fractional transfer of unconjugated [^{14}C]bilirubin from the "rapidly mixing pool" to the "storage pool" (primarily liver) was decreased in starved horses. Unconjugated hyperbilirubinemias occur in most animal species including man, but not to the extent observed in the horse. Such elevations are most likely due to defective hepatic uptake of bilirubin from the plasma rather than any overproduction of pigment by the liver or bone marrow.

Elevations in the serum free bilirubin were observed in a case of equine hemolytic anemia to be as high as 80 mg/dl (Bendixen and Carlström, 1928). In equine infectious anemia, serum bilirubin levels between 25 and 75 mg/100 ml are not uncommon (Zenglein, 1930). Icterus and hyperbilirubinemia are common findings in equine encephalomyelitis; however, degenerative changes are observed in the liver (Chernyak and Rozhov, 1953). Following experimental ligation of the bile duct in the horse, serum bilirubin was over 80% unconjugated during the first few days. During the remainder of the experiments, levels were below the initial serum concentrations and were approximately 50% unconjugated. This is in contrast to the lower concentrations of unconjugated pigment in the plasma of other domestic animals with ligated bile ducts (Ford and Gopinath, 1974; Cornelius *et al.*, 1965b). More recent studies on ligation in the donkey confirm these previous reports on the production of hyperbilirubinemia as well as the secondary hepatic necrosis and clinical neurological signs associated with elevated blood ammonia levels (Mandani and Adam, 1976). The observation of high serum bilirubin levels in a horse should be accompanied by other function tests which are more organ specific to determine whether a serious hepatopathy truly exists.

f. Cattle. Serum bilirubin levels are only slightly increased in diffuse and severe hepatic disease in the cow; they may be occasionally helpful but usually are not sensitive indicators of hepatic dysfunction. An extensive outbreak of severe hepatosis occurred in cattle from the ingestion of toxic herring meal in Norway (Hansen, 1964). All cattle with acute "toxic hepatosis" had livers with extensive centrilobular necrosis and damage to the hepatic veins. Chronic cases were typified by a nonportal fibrosis with obliterating changes in the central and sublobular veins. All cattle with the acute form had plasma values for total bilirubin higher than the "upper normal limit"; values for 15 cattle were recorded at 1.49 ± 0.86 mg/dl, as compared to levels of 0.19 ± 0.07 in normal animals. A study by Ford and Ritchie (1968) on ragwort intoxication in calves concluded that any interference with the liver's ability to remove bilirubin was a terminal change. The elevation of bilirubin levels in the serum of cattle, sheep, pigs, and dogs following conditions of starvation or near starvation was reported by Berger (1956) and Zink (1932). A more recent study by Hansen (1972) concluded that increases in "direct-reacting" pigment at times may be useful in diagnosing biliary obstructions; however, as a whole, the value of the test must be considered quite limited. Nineteen cattle with severe hepatic degeneration and 14 cattle following experimental administration of carbon tetrachloride had total bilirubin levels between 0.2 and 2.5 mg/dl, with no immediate "direct-reacting" bilirubin conjugates at a measurable concentration (Benedek, 1961). Similar hepatopathy in man would routinely be associated with levels greater than 10 mg/dl. Garner (1953) observed a positive correlation between the icteric indices and plasma carotenoid concentration. No correlation between the icteric indices and the serum bilirubin level was noted. More recent studies have confirmed the insensitivity of serum bilirubin levels in cattle with hepatic disease (Harvey and Obeid, 1974); however, slight, constant elevations can be observed in fascioliasis (Ehrlich *et al.*, 1960) and even marked elevations in terminal subacute fascioliasis (Sewell, 1966). A study on the adaptation of cattle to intensive feedlot conditions revealed decreased plasma clearance of injected bilirubin when the cattle were fed grain (Bide and Dorward, 1974). No hyperbilirubinemia was observed following halothane administration to calves (Gopinath and Ford, 1973). The levels of serum bilirubin shown in Table II were observed in bovine icterus and are characteristic of more recent observations (Garner, 1953).

Beijers (1923) observed a hyperbilirubinemia in newborn calves similar to that reported

TABLE II

Total Bilirubin in Bovine Icterus

Clinical description	No.	Total bilirubin (mg/dl)		
		Mean	Standard deviation	Range
Normal cattle	102	0.31	±0.17	0–1.4
Mild diffuse hepatic lesions	17	0.53	±0.03	0–2.4
Severe diffuse hepatic lesions	10	0.54	±0.03	0–1.2
Localized liver lesions	5	1.0	—	0–2.7
Biliary obstruction	2	0.3, 0.3[a]	—	—
Hemolytic icterus	2	2.3, 7.7[a]	—	—

[a] Two animals only.

in human infants but never reported visible icterus. In another study of 322 newborn calves, 24 were reported as icteric (Metzger, 1927). Few reports have confirmed these observations. The causes of neonatal jaundice are under extensive study today in many animal species. Species differences are great. The near-term fetal liver of the dog is relatively mature and contains a considerable amount of glucuronyltransferase for conjugation of bilirubin at birth. Unlike the canine placenta, the monkey placenta is a prime organ for bilirubin excretion into the maternal circulation, and hepatic glucuronyltransferase activities are low. Certainly the dependence on placental transfer and excretory immaturity in certain species are major contributory factors to the development of neonatal jaundice (Lester and Troxler, 1969). A thorough investigation of the development of the hepatic uptake, conjugation, and excretory mechanisms will be necessary to elucidate the causative factors in neonatal jaundice in each species in question.

g. Primates. Following the inoculation of squirrel monkeys with *Leptospira icterohaemorrhagiae* organisms, total serum bilirubin levels were elevated to 10.4 mg/dl by the tenth day, 50% of the bilirubin being "direct-reacting" (Minette and Shaffer, 1968). Studies by Deinhardt *et al.* (1967) on the transmission of human viral hepatitis to marmoset monkeys showed that hyperbilirubinemia occurred in about 20% of the affected animals and found it not to be a sensitive indicator of this experimental hepatic injury. A mild nutritional Laennec's cirrhosis in the rhesus monkey, characterized by lipidic infiltration and cirrhosis, was not accompanied by clinical jaundice, and serum bilirubin levels remained normal (Gaisford and Zuidema, 1965). No differences in bilirubin levels have been observed in morphine-addicted rhesus monkeys, as questionably reported to be present in man (Brooks *et al.*, 1963). Venoocclusive disease in monkeys receiving monocrotaline intraperitoneally was characterized by marked elevations of 8 of 18 experimental animals having serum bilirubin levels over 1.0 mg/dl (average of 2.8 mg/dl on the fourteenth day of the experiment). All monkeys which survived exhibited serum bilirubin levels below 1.0 mg/dl (Allen *et al.*, 1969).

Limited studies and observations on bilirubin metabolism in nonhuman primates to date suggest some similarity to humans in the interpretation of serum bilirubin levels in all types of icterus. Maximal rates of hepatic uptake, conjugation, and excretion of intravenously infused bilirubin have been determined in rhesus monkeys. Of great interest is the ability of this primate to concentrate bilirubin in bile at higher levels than in all other animals studied to date (Gartner *et al.*, 1971).

h. Other Species. The interpretation of the van den Bergh quantitative test is much the same in sheep, goats, and swine as it is in cattle. Major elevations in total bilirubin are usually found only in hemolytic crisis. Increased concentrations of bilirubin conjugates in the serum are indicative of severe hepatic involvement or extrahepatic obstruction. Lopez Garcia *et al.* (1943) observed that sheep, swine, and dogs did not tolerate complete biliary occlusion well and died as early as 3 weeks in some instances. Sheep receiving as many as 5000 metacercariae (fascioliasis) were ill by 4–9 weeks and died by the twelfth week. During the course of the disease, the bilirubin level was elevated only terminally to 1.0 mg/100 ml, but sera became discolored with a yellow-orange pigment postulated to be xanthophyll (Roberts, 1968). Hyperbilirubinemia is observed in the hepatic damage associated with acute lupinosis (Malherbe *et al.*, 1977). Hansen (1964) suggests that the diagnostic use of serum bilirubin levels in sheep must not be overlooked since, if eleva-

tions in concentration remain or increase, prognosis is grave. Complete biliary occlusion is well tolerated in man and monkey; reports have been published of case histories of over 300 days of complete bile duct obstruction without profound symptoms. Beijers (1923) reported hyperbilirubinemia in newborn lambs, pigs, and goats. A dose level of over 300 μg/kg of aflatoxins to young swine is highly toxic and results in progressive increases in the total serum bilirubin concentrations (Sisk *et al.*, 1968). Elevations in bilirubin also occur following starvation, pregnancy, parasitic obstruction of the bile ducts (*Ascaris lumbricoides* in pigs; *Thysanosoma actinioides* in lambs), ingestion of hepatotoxins, and infectious hepatopathy.

Two different lines of mutant sheep have been observed. Southdown mutants (Cornelius and Gronwall, 1968) with congenital photosensitivity and hyperbilirubinemia are characterized by elevations in serum bilirubin ranging from 0.5 to 1.9 mg/dl, of which over 60% is unconjugated. In addition, the plasma removal rate of bilirubin is greatly delayed. This Southdown mutant exhibits a hepatic uptake defect for bilirubin and other organic anions and resembles Gilbert's syndrome in man (Mia *et al.*, 1970). Only recently, it was observed that all indigo snakes uniquely exhibit high levels of unconjugated serum bilirubin, although they excrete 90% biliverdin into the bile as do other snake species (Cornelius *et al.*, 1977).

A Corriedale ovine mutant (Cornelius *et al.*, 1965a) possesses an organic anion excretory defect for bilirubin and certain other organic anions. This mutant is characterized by hyperbilirubinemias of 1.6 ± 0.1 mg/dl, of which over 60% is bilirubin glucuronide. Livers contain a melanin pigment in the pericanalicular dense bodies and are brownish-black. The condition in these sheep is nearly identical to that of human patients with Dubin–Johnson syndrome.

Bile in chickens, turkeys, snakes, and nutria contains over 90% biliverdin, with the remainder of the bilirubin conjugated to a variety of glycosides. Experimental obstruction of the bile ducts in the chicken resulted primarily during the first weeks in a hyperbilirubinemia with only trace amounts of biliverdinemia (Lind *et al.*, 1967); all chickens exhibited a marked hepatic fibrosis and ductal hyperplasia within 30 days. It has been postulated that little biliverdinemia results from cholestasis in chickens due to the extremely low renal threshold for this pigment which results in its rapid excretion into the urine.

2. *Bile Pigments in the Urine*

a. Bilirubin Conjugates

i. General. Since free bilirubin does not normally pass the renal filter in most species, only conjugated bilirubin moieties are present in the urine due to their glomerular filtration. The renal threshold for these bilirubin conjugates is directly related to their usefulness in clinical medicine. The low renal threshold for conjugated bilirubin in the dog allows for its use as an extremely sensitive test for early hepatocellular necrosis as well as bile duct obstruction. Naunyn (1868) early observed that simple inanition regularly caused bilirubinuria in the dog. The disappearance of bilirubinuria in dogs (with ligated bile ducts) in 24 hours following the administration of nephrotoxins ($HgCl_2$ and uranyl acetate) was reported by Nonnenbruch (1919). Bilirubinuria in hepatic diseases with accompanying renal malfunction may be difficult to interpret because of changes in renal function. Extrahepatic obstructions due to calculi, parasites, or tumors occluding the common bile

duct account for bilirubinurias of the greatest magnitude. Care should be exercised in interpreting its presence in canine urine since it may be present in a low concentration in any febrile state. The presence of a 1+ reaction when commercial pill tests are used is a common finding in many febrile diseases. A 2–3+ reaction is generally considered of diagnostic significance in the dog when specific gravity is between 1.020 and 1.035. The concentration of this bile pigment in the urine is directly proportional to the degree of biliary obstruction, whether intra- or extrahepatic in nature. Hoerlein and Greene (1950) observed that increased bilirubin concentrations in the urine of 16 dogs were proportional in most instances to the severity of BSP retention and elevation of the serum bilirubin level. Urinalysis on 11 canine cases of various types of liver disease without concomitant kidney damage revealed bilirubinurias of different degrees, somewhat predicted on the basis of the liver pathology (Hoe and O'Shea, 1965). Sixty percent of normal dogs excrete detectable bilirubin in their urine (Anonymous, 1959). Hoe and Harvey (1961b) observed, as expected, that many dogs with significant bilirubinuria many times show no bilirubinemia. Gardiner and Parr (1967) observed that the urinary bilirubin levels in two sheep with experimental lupinosis were significantly increased just following the first significant rise in plasma bilirubin levels.

In a group of 78 cattle with traumatic reticulitis, only 20 tested positively for bilirubinuria with the methylene blue test; two of three cattle with acetonemia and accompanying hepatic lipidosis were also positive for bilirubin in the urine (Heidrich, 1954). In the same study, only 60% of all cattle with established hepatopathy exhibited bilirubinuria. Bilirubinuria must be closely correlated with a careful clinical examination and other, more sensitive liver function tests for the cow (i.e., BSP clearance, serum arginase, γ-glutamyltransferase). Bilirubin concentration in the urine may be quite instructive concerning the quantitative regurgitation of bile pigments in obstructive processes.

Bilirubin conjugates are apparently not normally found in the urine of the horse, sheep, pig, and cat. Bierthen (1906) observed that bilirubin was nearly always absent from normal equine urine. Cases of equine hemolytic anemia with free serum bilirubin levels as high as 180 mg/dl have been observed without bilirubinuria (Beijers *et al.*, 1950). Two horses with extensive hepatopathy and total bilirubin levels of 19.6 mg/100 ml (8.8 mg/dl conjugated bilirubin) and 17.8 mg/100 ml (0.8 mg/dl of conjugated bilirubin), respectively, continuously excreted 4+ bilirubinurias when measured with the rapid diazo tablet test. Horses with other primary diseases routinely excreted 1+ bilirubinurias. Chronic hemolytic diseases with secondary hemosiderosis and/or hepatic necrosis from anemia are nearly always accompanied by bilirubinuria from hepatic regurgitation of bilirubin conjugates in all species. Recent studies (de Schepper and van der Stock, 1971, 1972) on the urinary excretion of bilirubin conjugates in the dog are of great interest. In hemolytic states when hemoglobin-binding capacity of the plasma haptoglobin is saturated, most dogs exhibit bilirubinuria without significant increases in the plasma conjugated bilirubin levels. This indicates that when excess hemoglobin is presented to the kidney, some is lost into the urine but a significant amount is degraded to bilirubin, conjugated by the renal tubules, and secreted into the urine. Bilirubinuria can therefore occur early in hemolytic disease in the dog and need not be due only to obstructive processes in the hepatobiliary system. Since male dogs excrete more bilirubin conjugates into the urine than do bitches when there is an excess of either hemoglobin or bilirubin in the plasma, it has been concluded that a sex-linked difference is present (de Schepper and van der Stock, 1971).

ii. Methods for the estimation of urinary bilirubin conjugates. The demonstration of conjugated bilirubin in urine is quite helpful in veterinary practice. Most methods used extensively in clinical laboratories are developed for both qualitative and semiquantitative determinations. If freshly voided urine is placed in a bottle and vigorously shaken, the presence of a greenish yellow foam is suggestive of bilirubinuria. This foam test is quite sensitive and easily performed; however, marked increases in the urinary urobilinogen level may give a false-positive test.

iii. Diazo tablet test. Rapid and sensitive tests are based on the use of a stable diazonium compound, which couples with bilirubin under specific conditions. One diagnostic tablet is composed of *p*-nitrobenzenediazonium-*p*-toluene sulfonate, sulfosalicylic acid, and sodium carbonate.

iv. Gmelin–Rosenbach test. In this test, nitric acid is added dropwise (two drops) to filter paper impregnated with urine. If bilirubin is present, the nitrous acid oxidizes the bile pigment to colored derivatives: biliverdin (green), bilicyanine (blue), and choletelin (yellow). A modification of this test calls for the addition of 10 ml of 10% barium chloride to 20 ml of urine. After the mixture stands for a few minutes, the precipitated bilirubin is filtered and tested with nitric acid as above. The positive reaction usually shows green on the periphery and then, in order toward the center, blue, violet, red, and yellow. The absence of green rules out the presence of bile pigment (Hepler, 1949). This test is often unsatisfactory in the cow due to the presence of false-positive reactions.

v. Harrison's spot test. After the precipitation of the urinary bilirubin conjugates with 10% barium chloride as described above, one or two drops of Fouchet's reagent (25 gm trichloroacetic acid, 100 ml distilled water, and 10 ml of 10% ferric chloride) are added. A positive reaction is indicated by a blue or green color (Hepler, 1949).

vi. Quantitative methylene blue test. According to Fellinger and Menkes (1933), this test provides an easily performed and accurate quantitative determination of urinary bilirubin. By measuring the number of drops of 0.2% methylene blue needed to change the initial green color to a definite blue, one can make a quantitative estimate. Two hundred drops of a 0.2% solution are needed for every milligram of bilirubin. The change of methylene blue to a green color in the presence of bilirubin may be from an admixture of pigments and not a specific chemical reaction with bilirubin. The test agrees well with other chemical tests for urinary bilirubin, and there is no reaction with urobilinogen. The presence of two drops of blood hemolyzed in 10 ml of water will, however, give a slightly positive test (Gradwohl, 1956). The test has worked well in hospital laboratories for domestic animals.

b. Urinary Urobilinogen.

i. General. This urinary pigment may represent a group of substances rather than a single chemical entity. The term "urobilinogen" in this chapter in most cases refers to all "urobilinoids" reacting positively with Ehrlich's reagent. Unless a specific chemical structure is noted (i.e., stercobilin), the reader should consider the general use of the term "urobilinogen" by various authors to refer to a mixture of pigments resulting from the

enteric reduction of bilirubin by the bacterial flora. The presence of the "urobilinogen group" in urine signifies the presence of an open bile duct with the simultaneous occurrence of an enterohepatic circulation of bile pigments. Members of this group are produced by bacterial reduction. Ten to 20% is partially absorbed into the portal circulation and recycled in part. The remainder of this reduced pigment is lost to the feces as "stercobilin," which imparts part of the normal color to the stool. Urobilinogen is also excreted into the bile intact and unaltered, in contrast to bilirubin, which must be conjugated to a polar derivative for excretion. The dipyrranes (bilifuscins and pentodyopent compounds) are also important degradation products of hemoglobin and account for much of the color of normal stool. These dipyrranes and possibly tissue cytochromes may be related chemically to the poorly identified "urochromes," which account for most of the color of urine. In addition, the colorless urobilinogen is oxidized by light to the highly colored urobilin by central bridge unsaturation, which imparts color to the urine. The presence of clay-colored stools is presumptive evidence for total biliary obstruction; however, in chronic, diffuse, and progressive fibrosis of the liver, clay-colored stools may also occasionally be present due to advanced intrahepatic obstruction in the presence of an open bile duct. A small portion of the "urobilinogen group," which is reabsorbed from the intestine, passes unchanged through the liver and enters the general circulation, where it is excreted into the urine. The absence of urinary urobilinogen at one sampling need not indicate bile duct closure since diurnal variations are common. Diuresis encountered in chronic renal diseases may excessively dilute the urobilinogen below the sensitivity of the test in urinalysis (Cornelius, 1957). Urine urobilinogen excretion partly depends on renal function, the state of hydration, and urine pH. It may be advisable in certain cases to carefully regulate hydration and the urinary pH.

In hepatocellular damage, there is a defect in the reexcretion into the bile of the urobilinogen from the portal blood; this results in the escape of a greater percentage of these pigments into the circulation and the urine. Royer (1943) found the blood concentration of urobilinogen, injected into the portal vein of dogs, to be five times as high as in hepatic vein blood. Since, in experimental hepatic necrosis, equal concentrations of urobilinogen in both the portal and hepatic veins were observed, the escape of large amounts of urobilinogen to the urine would be expected. In hemolytic anemias, greater amounts occur in the urine from both secondary hepatic insufficiency and a quantitative increase in the enterohepatic circulation of bile pigments. Stools are correspondingly darker owing to the increased concentration of stercobilin in hemolytic diseases from increased amounts of circulating pigments. Royer and Biasotti (1932) observed great increases in the concentration of urobilinogen in canine urine following acute hemolysis.

Watson (1959) studied the urobilin(ogen) group of pigments in urine, bile, and feces in normal human beings and in a variety of pathological circumstances, especially in hemolytic and hepatic states. The three important members of this group were *i*-urobilin, *d*-urobilin, and *l*-stercobilin. Optical activity measurement was of great value in confirming the presence of *d*-urobilin when accompanied by considerable amounts of *i*-urobilin. *i*-Urobilin was found to be preponderant in the urine of certain normal individuals, while *l*-stercobilin was observed in others. Similar findings were obtained in hemolytic and hepatic diseases. Fecal urobilin composion also varied greatly. Following the administration of broad-spectrum antibiotics, which greatly reduced the urine and fecal urobilinogen by suppression of enteric coliforms and clostridia (Sborov *et al.*, 1951), a great preponderance of *d*-urobilin is observed in the urine, feces, and bile (Watson, 1959). These

findings are compatible only with an enteric source of all three members of the urobilin(ogen) group.

Few studies are available concerning the metabolism of urobilinoids in man and animals. These have been extensively reviewed by With (1954). Berger (1956) reported to have isolated only stercobilin from the urine of herbivores (horse, cow) and found no urobilin present. In hepatic diseases, both bilirubin conjugates and urobilin were found in addition to the stercobilin. Another study on elevations in the levels of urinary urobilinoids in 100 slaughtered cattle revealed a 93% correlation with histopathological studies on hepatic tissues. In only 7 of 100 animals with elevated urobilinogen levels were no histopathological changes observed. The author concluded from extensive clinical studies in cattle, horses, and dogs that elevations in the urinary urobilinogen were good diagnostic signs of hepatopathy (Montemagno, 1954). Wester (1912) found only a slight increase in the urinary urobilinoid level following the injection of 3 liters of blood intraperitoneally into cows. This and other studies in dogs have shown that marked urobilinuria does not occur after hemorrhagic exudations and hematomas. Cattle have been reported to excrete 1.7 mg/day of fecal urobilinogen per kilogram of body weight, which agrees reasonably well with values predicted from the longevity of the red blood cells (Schwartz and Bracho, 1972).

Clinical reports concerning the use of the urinary urobilinogen test in hepatic disease in small animals have been conflicting. Early studies on normal and CCl_4-poisoned dogs revealed that various grades of hepatopathy resulted in the excretion of proportional increases in the urobilinogen of the urine. The elevation of urinary urobilinogen did not correlate directly with dye retention tests (phenoltetrachlorphthalein) and suggested a dissocation of specific liver functions (Wallace and Diamond, 1925). No marked abnormalities in the urinary urobilinogen were observed following mild hepatic damage in cats and rabbits experimentally poisoned with selenium (Smith *et al.*, 1940). Gornall and Bardawill (1952) reported normal levels of urinary urobilinogen in dogs of 0–2 mg/dl using Watson's technique. Following CCl_4 poisoning, urobilinogen levels both increased and decreased and led the authors to conclude that the test was of little use in the dog. Others observed that the time required after experimental liver damage (with xylidine) for the urine urobilinogen to increase varied considerably (Svirbely *et al.*, 1946). Most well-controlled studies in which fresh 24-hour urine volumes were available indicate that an absolute increase occurs in the urinary urobilinogen in domestic animals with hepatopathy. Since it is nearly impossible in clinical veterinary medicine to procure 24-hour urine aliquots for quantitative estimations, urinalysis is many times subject to semiquantitative estimates in measuring urobilinogen. Urobilinogen is rapidly oxidized to urobilin if urine samples are neglectfully left standing at room temperature and exposed to light. Since urobilin does not react in the Ehrlich test, clinical interpretations based on such samples may be misleading. Changes in the specific gravity of urine in the maintenance of water balance cause the urobilinogen concentration to vary considerably.

ii. Estimation of urinary urobilinogen. Either a commercial rapid strip test or a chemical procedure can be used. Urobilinogen and its colored oxidation product, urobilin, are of equal clinical significance, and the choice of tests for the qualitative determinations is a matter of convenience. Fresh urine samples contain primarily the colorless urobilinogen, which converts slowly to the brown urobilin upon standing. This change can be accelerated by oxidation of urobilinogen with iodine followed by spectroscopic examina-

tion for urobilin or by Schlesinger's test using zinc acetate (Todd *et al.*, 1953). Watson's quantitative method, which depends on the reduction of all urobilin to urobilinogen in a 24-hour sample with ferrous hydroxide, is quite accurate and used by most investigators (Watson *et al.*, 1944). In addition, the fresh specimen may be tested for urobilinogen with Ehrlich's aldehyde reagent, preferably followed by saturated sodium acetate to subdue interfering pigments.

Ehrlich's semiquantitative test is performed as follows:

1. To 2 ml of fresh urine in a test tube, add 2 ml of modified Ehrlich's reagent (0.7 gm *p*-dimethylaminobenzaldehyde, 150 ml concentrated HCl, and 100 ml distilled water).
2. Immediately mix, and add 4 ml of saturated sodium acetate after 15 and before 30 seconds.

The resulting cherry pink color, which becomes more prominent upon heating, can be reported semiquantitatively from 1 to 4+. Any pink color which results following the addition of Ehrlich's reagent suggests the presence of urobilinogen even though the final dilution with sodium acetate may excessively dilute the color. The color may be observed most sensitively by viewing down from the top of the test tube. A green color which develops immediately in icteric animals may result from the oxidation of bilirubin to biliverdin. Ehrlich's reaction is best performed in urine after precipitation and removal of excessive bilirubin by adding either a small amount of crystalline lead acetate or 1.0 ml of a 10% solution of calcium or barium chloride to 4 ml of urine. A rapid but not always successful method for detecting urobilinogen in the presence of excess bile pigments is the simple deletion of Ehrlich's reagent from the control tube using 2 ml of urine with saturated sodium acetate to volume. Excessive amounts of protein may also interfere by clouding the tube; these can be removed by filtering following protein precipitation with equal volumes of 0.3% sulfosalicylic acid.

Since porphobilinogen is found in the urine in cases of acute intermittent porphyria and also reacts with Ehrlich's reagent to produce a cherry red color, it must be differentiated from urobilinogen. A few milliliters of chloroform may be added and mixed thoroughly. The red aldehyde chromogen of porphobilinogen or indole is completely extracted into the chloroform.

3. Fecal Bile Pigments

a. Fecal Bilirubin. Bilirubin appears in the feces in conditions preventing its reduction to urobilinogen, such as in the diarrheas, in the newborn receiving milk, or from the suppression of bacterial action, i.e., the clinical use of broad-spectrum antibiotics. Little conjugated bilirubin is reabsorbed from the intestinal mass. The Gmelin or Harrison test may be used to test qualitatively for the presence of bilirubin. In the Gmelin test, a few drops of nitric acid (containing nitrous acid) are added to a smear of feces on a piece of white porcelain; the play of colors, green, blue, violet, red, and yellow, indicates a positive reaction. In stools with excessive chlorophyll or bacterial pigments, an aqueous extract can be tested. Fouchet's reagent (Harrison test) can be added to a 1 : 20 dilution of stool, and the presence of a blue color indicates the presence of bilirubin.

b. Fecal Stercobilin. Compounds of the urobilinoid group similar to those observed in the urine are found in the stool, with stercobilin(ogen) usually predominating. A daily

visual inspection for stool color of the icteric patient is quite important since stercobilin and various dipyrranes give the stool its characteristic color. In contrast to the situation in urine, most of the bile pigments are in their highly colored and oxidized form. They are elevated considerably following hemolytic processes and decreased in bile duct obstruction. Quantitative fecal stercobilin determinations cannot be recommended for routine practice since reproducible methods are quite lengthy. They should be used only in the investigation of cases of obscure icterus when one questions the presence of hemolytic manifestations.

The Schmidt test is carried out as follows. A pea-sized amount of fresh feces is rubbed into a petri dish and mixed with a 5% aqueous solution of mercuric chloride. The dish is covered and the mixture observed after several hours, preferably 24 hours. Urobilinoids give a pink color, while bilirubin is oxidized to a green biliverdin color. The absence of any color after 24 hours indicates the absence of bile pigments. Excessive lipids may be extracted with ether.

B. Foreign Dyes

Halogenated phthalein dyes were first used in the study of liver function by Rowntree *et al.* (1913), who measured the fecal excretion of phenoltetrachlorphthalein following its parenteral administration. Subsequently, it was shown by Rosenthal and White (1925) that intravenously administered BSP is removed almost exclusively by the liver. The uptake, conjugation, and excretion by the liver of foreign dyes are a measure of both hepatic biochemical integrity and blood flow. Owing to a number of rate-limiting steps in their excretion, these dyes are among the most sensitive indicators of hepatic dysfunction. Delay in their removal from the blood may be due to hepatic necrosis and/or fibrosis with a reduced parenchymal mass and subsequent depressed hepatic blood flow. Since competition for hepatic uptake between many foreign dyes and bilirubin occurs with subsequent delay of their clearance from the plasma, these tests offer certain problems of interpretation in the presence of icterus with high levels of serum bilirubin. Foreign dyes which have been used in veterinary medicine are BSP, rose bengal, indocyanine green, and phenoltetrachlorphthalein.

1. Sulfobromophthalein

The BSP clearance rate from plasma is widely used as an index of hepatic function in domestic animals. Wheeler and co-workers (1960a) demonstrated that the rate of hepatic uptake continues to increase in proportion to the plasma concentration, with the rate of excretion by the liver reaching a maximum when BSP is infused at high concentrations. Experimental data suggest that uptake, conjugation, and excretion of BSP are controlled by independent mechanisms. The major portion of BSP is conjugated in the liver with glutathione via the mercaptide linkage of the cysteine moiety (Grodsky *et al.*, 1959) and excreted into the bile. Philp *et al.* (1961) concluded that conjugation is one limiting step which affects the hepatic secretory rate of this dye; however, both unconjugated and conjugated BSP are excreted into the bile. A concentration maximum exists for BSP excreted in the bile of animals. The BSP excretory rate is therefore proportional to the bile flow rate when the concentration maximum for BSP in bile has been reached.

In a diagnostic procedure developed by Wheeler *et al.* (1960a,b), BSP is infused at three different rates, and both the hepatic storage (S) and transport maximum (T_m) for

BSP can be indirectly calculated from the analysis of multiple peripheral blood samples. This method allows for the independent calculation of S and T_m without any concern about hepatic blood flow or hepatic vein BSP concentrations. Many clinical disorders are characterized by differences in their BSP storage and T_m. The technique has already proven useful in the study of ovine mutants with hyperbilirubinemia (Cornelius *et al.*, 1956a). Numerous compounds known to interfere and compete with BSP uptake, such as cholic acid derivatives, bilirubin, and 17-substituted testosterones, should be avoided.

a. Dog. The usefulness of the BSP test in the dog was suggested early by Drill and Ivy (1944), Svirbely *et al.* (1946), and Hoerlein and Greene (1950). The rate of BSP disappearance, and hence percent retention, in dogs is independent of dosage between 5 and 20 mg BSP per kilogram (Moses *et al.*, 1948). The use of 5 mg/kg is generally accepted. The BSP retention test is particularly useful when a nonhemolytic jaundice is encountered in which no elevation in serum hepatic enzyme activities is present. In the case of cholestatic liver disease without necrosis, delayed BSP retention and elevations in the serum activities of the cholestatic enzymes (alkaline phosphatase and γ-glutamyltransferase) are diagnostic. Another specific diagnostic use of BSP is in the detection of portosystemic anastomoses or shunts which result in deficient hepatic circulation and hence abnormal BSP retention (Ewing *et al.*, 1974).

After the single injection of 5 mg BSP per kilogram, certain dogs may have less than 5% dye retention by 10–15 minutes, and the clinician can miss minor hepatic damage. When the Wheeler "indirect" technique was used for measuring BSP storage and T_m, average values of 25 mg BSP stored per milligram per 100 ml plasma per10 kg and 1.9 mg/minute per 10 kg, respectively, were found for the dog (Wheeler *et al.*, 1960b). This infusion technique has proven useful in dogs with specific biochemical transport defects (Strombeck and Qualls, 1978) but of little help in acute hepatic necrosis (Himes and Cornelius, 1973). It appears to be too complex for use in routine practice. The use of BSP in cats has been limited mainly to experimental studies, i.e., studies of the alteration of BSP retention in relation to morphological changes in the liver and bile passages following total biliary stasis (Cantarow and Stewart, 1935).

i. Method

1. Weigh the dog and divide its weight in pounds by the factor 22. This figure provides the number of milliliters of the BSP solution (50 mg/ml) to inject in order to give 5 mg/kg body weight.
2. Inject the dye solution into the cephalic vein slowly, taking care to prevent any perivascular infiltration, which may cause sloughing.
3. At 30 minutes after injection, remove 5 ml of heparinized blood from the opposite cephalic vein.
4. Centrifuge the blood, and pipet 0.5 ml of the unhemolyzed plasma into two cuvettes. Add 2.5 ml of distilled water to each tube.
5. Add 3 ml of 0.1 *N* HCl to the blank tube (colorless) and 3 ml of 0.1 *N* NaOH to the other tube to produce maximal BSP color.
6. Read unknown against the blank at 565 nm on a standard curve as recommended by Hepler (1949).
7. Percent retention can be calculated by multiplying the 30-minute concentration (milligrams per 100 ml) by the factor 10.

A comparator box with permanent standards can be used for rapid determinations in the veterinary medical hospital.

ii. Interpretation. Less than 5% BSP retention at 30 minutes is generally accepted to be the normal range for dogs. Most studies, however, agree that up to 10% retention at 30 minutes after injection will only rarely be found in dogs with no apparent hepatic injury.

Delayed BSP retention in dogs has been reported in the following diseases affecting the liver: leptospirosis (Hoerlein and Greene, 1950; Harvey, 1967; Van Vleet and Alberts, 1968); hepatic lipidoses with centrilobular necrosis, periportal fibrosis, focal hepatitis, carbon tetrachloride poisoning, infectious hepatitis (Larson and Morrill, 1960); diabetes mellitus with hepatic fibrosis, secondary hepatic degeneration associated with ascitis, ulcerative duodenitis, gastroentritis, coccidial hemorrhagic enteritis, thallium and tetrachloroethylene intoxication (Nielsen, 1952); and diseases of the uterus (Lettow, 1961). Larson and Morrill (1960) concluded in an extensive study on 85 dogs that, although BSP retention and hepatic damage were at times inconsistently related, BSP retention still remained one of the most sensitive tests for measuring acute viral and toxic hepatitis in the dog. In 63 bitches with hormonal uterine diseases, histological examination of the liver in nearly all cases showed some degree of degenerative or inflammatory changes; 52% of these bitches had severe secondary hepatic degeneration (Lettow, 1961). In addition, BSP retention has continued to be used routinely in chronic progressive hepatitis in Bedlington terriers (Hardy *et al.*, 1975), neoplastic diseases (Strombeck, 1978), cirrhosis (Brobst and Schall, 1972), chronic babesiosis (Malherbe, 1965a), and chronic active hepatitis (Strombeck *et al.*, 1976c). Delayed BSP clearance can be observed in conditions such as fever, cardiac decompensation, shock, and severe dehydration which cause a lowered hepatic blood flow and hence less BSP extraction.

Although the BSP test in the dog appears to be one of the most sensitive of all tests for the detection of latent liver damage, correlation of dye retention and hepatic lesions is not always consistent when either one is of a mild nature. In this test, the hepatic blood flow, the complex process of cellular BSP uptake, conjugation, and excretion, and the patency of the bile duct are all assessed.

b. Horse. Due to the rapid removal of injected BSP from equine blood, the fractional clearance K of BSP per minute has been proposed as the BSP method of choice for the horse (Cornelius and Wheat, 1957; Karsai, 1960). Others have proposed the use of methods in which the percentage of BSP retention at 15–30 minutes after injection is obtained (Aktan, 1954; Morgan, 1959a). Advantages of the BSP clearance test over the percent retention test are that sampling can be completed within 12 minutes after injection; plasma samples can be taken at any time between 5 and 12 minutes after injection; plasma volume can be calculated; and quantitative assessment of liver function can be made in terms of the kinetics of BSP disappearance from the plasma. Some disadvantages of any test which requires the calculation of percent retention are that the animal must be weighed; an exact quantity of dye must be injected; the plasma sample must be taken at one critical time only; and the influences of plasma volume and estimated hepatic blood flow are not assessable.

i. BSP clearance method. One gram of dye is used in large domestic species (Cornelius, 1958). It is injected intravenously. Following approximately a 5-minute

period, two heparinized blood samples are taken before 12 minutes, preferably about 4 minutes apart, i.e., 5 and 9 or 6 and 10 minutes postinjection. The BSP concentrations of the two samples are determined spectrophotometrically as follows: 2 ml plasma is transferred to 12 × 105 mm cuvettes containing 3 ml 0.1 *N* NaOH. The blank consists of 2 ml plasma, 3 ml 0.1 *N* HC, and 1 ml water. Any spectrophotometer with a wavelength of 565 nm can be used for the BSP determinations. Standard curves for BSP determinations should be made using 2 m equine plasma.

The BSP concentrations of the two samples (milligrams per 100 ml) are next plotted on semilog paper, and the $T_{1/2}$ for BSP clearance calculated. The $T_{1/2}$ is that time required for the BSP concentration to be halved in the plasma. The BSP clearance can be expressed clinically in $T_{1/2}$ units (minutes) (Fig. 5).

Fractional clearance K, or the percentage of dye cleared from the plasma per minute, can easily be calculated from the following formula:

$$K = \frac{\ln 2}{T_{1/2}} \quad \text{where } \ln 2 = 0.693$$

Fractional clearance K can also be calculated by using the following kinetic relationships:

$$K = \frac{c}{V} = 2.3 \frac{\log P_1 - \log P_2}{t_2 - t_1}$$

where c is clearance (units in milliliters plasma per minute); V is plasma volume; P is plasma BSP concentration (milligrams per deciliter); and t is time of sampling (minutes).

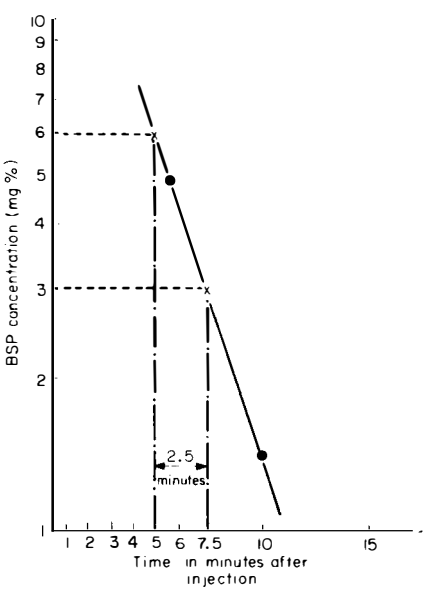


Fig. 5. Method of calculating $T_{\frac{1}{2}}$ values for the sulfobromophthalein (Bromsulphalein; BSP) clearance test in the horse (Cornelius, 1958). (1) Animal bled at 6 and 10 minutes after injection. (2) Points (●) of 4.9 and 1.4 mg % plotted. Line drawn through points. (3) Time observed, e.g., from 6 to 3 mg %. (4) $T_{\frac{1}{2}}$ = 2.5 minutes. Horse has normal liver. (Normals 2.81 ± 0.5 minutes.)

ii. Plasma volume. This can be readily determined since BSP is bound to albumin in the circulating blood. The two concentrations of BSP are plotted on semilog coordinates, and a line drawn through them to intersect the ordinate at 0 time. This ordinate intercept (X_0) represents the concentration of BSP at the time of injection in the plasma if mixing has been complete and instantaneous. Since the number of milliliters of BSP injected i and its concentration X_i are known, the plasma volume V can be accurately calculated from the BSP clearance test:

$$V = (X_i/X_\bullet - 1)i$$

Since normal horses have an average of 51 ml plasma per kilogram body weight (Dukes, 1946), the clinician can note whether hypovolemia exists. If dehydration and subsequent hypovolemia are present, the presence of a delayed BSP clearance should be expected due to subnormal hepatic plasma flow.

iii. Normal values. Average $T_{1/2}$ values for normal mature horses were 2.8 ± 0.5 minutes, with a range of 2–3.7 minutes. Average fractional clearance K was 0.25 ± 0.05 of dye removed per minute, with a range of 0.19–0.38 during the first 12 minutes. This exponential clearance was not altered in the same horse by the use of doses of BSP between 2 and 6.3 mg/kg. The same horse, tested at yearly intervals under different weight conditions, had nearly identical $T_{1/2}$ values (Cornelius and Wheat, 1957). Olsen and Phillips (1966) reported similar fractional BSP clearances for normal horses: 0.21 (0.1–0.47). Following the first 12 minutes after intravenous injection, a slower clearance may be observed when sufficient dye remains for detection. Morgan (1959a) reported that less than 10% retention at 15 minutes after injection is normally expected in horses of approximately 900 lb when 5 mg/kg of BSP is administered intravenously.

iv. Clinical observations. Delayed BSP excretion in the horse has been reported in hepatic fibrosis, chloroform poisoning (Aktan, 1954); hepatic hemosiderosis, extensive lipidosis, CCl_4 intoxication (Cornelius and Wheat, 1957); and cirrhosis and impaction (Petrovic, 1958; Morgan, 1959a). Following the oral administration of both phenothiazine and carbon disulfide to a group of horses, fractional BSP clearance was lower (0.15–0.223) but not associated with abnormal clinical symptoms (Olsen and Phillips, 1966). BSP clearance tests are quite useful in horses since a mild icterus is a common finding secondary to many clinical diseases. These tests can be useful in horses with symptoms of hepatoencephalopathy. Hepatic involvement can easily be distinguished from equine encephalomyelitis or "wobbles," if a severe hypovolemia from dehydration is not present. To determine whether delayed BSP clearance is from hepatic necrosis rather than fibrosis, the activities of serum enzymes such as arginase, glutamate dehydrogenase, or sorbitol dehydrogenase may be measured.

c. Cow. The BSP clearance technique is a sensitive test for detecting hepatic necrosis or fibrosis in cattle. Early studies on cattle, in which the percent retention of dye remaining at 45 minutes was calculated, were in disagreement. Vardiman (1953) reported that the BSP retention test was dependable only in the last stages of chronic fibrosis from chronic Senechio ingestion in cows. Jasper (1947) found greater dye retention in cows in late parturition and with ketosis. Extensive studies on BSP retention in cows by Freese (1952) indicated that the retention test was useful diagnostically in animals with advanced fascioliasis, bacterial septicemias, and "toxic" hemoglobinemias.

The advantages of the BSP clearance technique as compared to the dye retention method have been discussed in the previous section on the horse. The way in which the BSP clearance test is performed is identical to that described for the horse, by the intravenous injection of 1 gm BSP (Cornelius *et al.*, 1958a) or 1 mg/lb body weight (Mixner and Robertson, 1957).

The use of 1 gm BSP in cattle ranging in weight from 500 to 1200 lb results in a measurable concentration during the first 20 minutes in the plasma and eliminates the problem of ascertaining the weight of the animal. The veterinary practitioner should obtain at least two heparinized blood samples between 5 and 20 minutes after injection and plot the concentrations (ordinate) on semilog paper at each sampling time (abscissa). The $T_{1/2}$ values can be calculated rapidly.

i. Normal values. Average $T_{1/2}$ values for mature nonlactating dairy cows are 3.3 ± 0.5 minutes, with a range of 2.5–4.1 during the first 20 minutes after injection. Fractional clearance K is 0.22 ± 0.03/min of dye removed per minute, with a range of 0.17–0.28/min. The $T_{1/2}$ and K values for yearling steers and heifers were 4.5 ± 0.3 minutes and 0.15 ± 0.1/min per minute, respectively (Cornelius *et al.*, 1958a). Mixner and Robertson (1957) reported similar average K values for BSP in lactating cows (weighing on an average 558 kg): 0.19/min per minute, with a range of 0.16–0.24/min. Bull calves weighing 49 kg on average had mean BSP K values of 0.15 per minute, with a range of 0.11–0.20. Hunt and McCosker (1967) observed mean fractional clearance to be 0.166 ± 0.034/min in beef cattle weighing between 200 and 522 lb. No significant sex difference was observed. Differences in the fractional clearance of BSP between yearling and mature cattle may be attributable to developmental differences in gastrointestinal mass and the associated splanchnic blood flow. Decreased clearance has also been reported for 1 week after calving (Treacher and Sansom, 1969).

ii. Clinical observations. Extremely low BSP fractional clearance (increased $T_{1/2}$) was observed in two cows with suppurative hepatitis following acute coliform mastitis (0.07 and 0.13/min BSP cleared per minute), extensive fascioliasis with hepatic fibrosis (0.05 per minute), and hepatic abscesses (*Spherophorus necrophorus*) (0.14/min per minute). Hepatopathy was confirmed by biopsy and microscopic examination (Cornelius *et al.*, 1958a). The BSP clearance is markedly depressed in ketotic cows (average K = 0.09 per minute, with a range of 0.05–0.11/min) as compared to normal lactating cows, 1–4 weeks postpartum (Robertson *et al.*, 1957). Hansen (1964) observed a delayed clearance of BSP ($T_{1/2}$ = 11.7–69.3 minutes) in cattle in Norway with acute hepatosis from toxic herring meal. The use of BSP in differentiating bovine photosensitization due to photodynamic pigments from that due to the impaired excretion of phylloerythrin by the demaged liver has been reported (Caple and Vandergraaff, 1976). The authors concluded that the BSP clearance test was superior to the use of serum enzymes in the differentiation of liver damage caused by sporidesmin in pasture plants from primary photosensitization. In another study, it was observed that cows deprived of food and water for 4 days exhibited decreased BSP clearance rates (Cokala and Bieniek, 1975).

d. Sheep. Owing to the extremely rapid clearance of BSP from the plasma when injected at 5 mg/kg into sheep, the use of a percent retention method may be preferable (Cornelius *et al.*, 1958b; Arendarcik, 1959). The major disadvantage of the clearance

technique in sheep is that sampling must be performed quite rapidly since BSP disappears exponentially for only 7 minutes after dye injection. The percent retention of BSP in the serum of normal mature sheep at 10 minutes after injection is approximately 6 ± 2'. Mean fractional clearance K, determined using either 2 or 5 mg/kg BSP between 3 and 7 minutes after injection, was 0.35 ± 0.06/min per minute, with a range of 0.25–0.44/min. The mean $T_{1/2}$ value was 2 ± 0.3 minutes, with a range of 1.6–2.7 using 5 mg/kg body weight (Cornelius *et al.*, 1958b). Mean fractional BSP clearance in normal sheep on summer pasture was reported to be 0.31 ± 0.06 per minute, with a range of 0.195–0.484. Ewes on autumn pastures in England exhibited slightly lower clearance values (Forbes and Singleton, 1966).

In another investigation, it was suggested that the lower doses of 2 and 5 mg BSP per kilogram may be inadequate for plotting dependable BSP blood clearance rates. Mean $T_{1/2}$ values in sheep were approximately 3 minutes (Tucker *et al.*, 1971). A study on BSP clearance in goats (Sen *et al.*, 1976) revealed normal half-time values of 2.13 ± 0.19 minutes. The $T_{1/2}$ increased to over 4 minutes following the administration of a single dose of CCl_4 (1 ml/kg) and up to 34 minutes after bile duct ligation.

The $T_{1/2}$ value for BSP removal from the plasma between 15 and 30 minutes after injection in 12 ewes with severe ketosis was 20 ± 17 minutes, with a range between 7 and 62. These ewes had BSP retentions between 13 and 64% at 15 minutes after the injection of 2 mg BSP per kilogram. It was of interest that all ewes retaining over 20% BSP at 15 minutes after injection succumbed despite various therapeutic measures. The slow clearance of BSP by sheep with clinical ketosis is probably the result of both an impediment to hepatic blood flow from extensive lipidosis and the presence of biochemical lesions associated with cellular necrosis (Cornelius al., 1958b). Sheep with acute hepatosis from toxic herring meal revealed BSP half-time values ranging from 3.2 to 46.2 minutes (Hansen, 1964). The BSP fractional clearance test has also been useful in assessing the degree of hepatic damage (fascioliasis) from migrating metacercariae (Roberts, 1968).

Congenital photosensitivity and hyperbilirubinemia in Southdown sheep is characterized by an inherited defect in the hepatic uptake of phylloerythrin, bilirubin, and BSP. Mean BSP half-time values average 43.4 ± 5.8 minutes and are quite diagnostic since no structural changes are present in the liver. Hepatic storage S and transport maximum T_m in Southdown mutants, as calculated by the method of Wheeler *et al.* (1960b), are less than 10% of normal values. Hepatic S and T_m were 1.79 ± 0.86 mg/mg/100 ml/kg and 0.235 ± 0.019 mg/min/kg, respectively, in eight normal Southdown sheep (Cornelius and Gronwall, 1968; Gronwall, 1970).

Mutant Corriedale sheep (Dubin–Johnson syndrome) with a hepatic excretory defect for BSP exhibited normal hepatic S but hepatic T_m values were 19–25% of normal sheep. The BSP was retained in the plasma primarily as a glutathione conjugate in Corriedale mutants, whereas the sera of Southdown mutants contained only unconjugated BSP (Cornelius *et al.*, 1965a).

e. Swine. Prinz and Fiesel (1957) investigated BSP clearance in pigs in protein deficiency and following chloroform poisoning. The retention of BSP in normal swine weighing 30 kg was as follows: 5 minutes, 5.3 ± 2.0%; 10 minutes, 1.4 ± 0.9%; and 15 minutes, 0.6 ± 0.6%. Following 3–5 months in which the swine were on a protein-deficient diet, the following BSP retention ranges were observed after injection: 5 minutes, 10.5–16.7%; 10 minutes, 1.7–6.0%; and 15 minutes, 0.6–2.6%. The BSP retention

values returned to normal following protein supplementation. Chloroform narcosis increased the BSP retention three to four times in the protein-deficient syndrome.

The BSP half-times in four normal pigs were approximately 2 minutes before the administration of aflatoxin and increased to over 19 minutes by 6 hours following toxin introduction through gastric fistulas (Cysewski *et al.*, 1968). The retention of BSP in swine has also been reported by Pfeiffer (1970) using 6 mg/kg of body weight. At 15 minutes after injection, normal 100-kg pigs retain 3–4% of the dye in the serum.

f. Fowl. Methods for measuring BSP clearance in chickens and turkeys have been described; 20 mg/kg body weight has been the recommended dose. A sexual difference was observed in chickens. Males caponized by stilbesterol possessed a female type of clearance. Androgens caused a clear reversion to the male or more rapid clearance curve (Campbell, 1957). Studies in turkeys revealed that the rate of transfer of BSP between blood, liver, and bile can be predicted from a graph of the fall of plasma BSP alone. The BSP test appears to be quite a sensitive indicator of hepatopathy in the fowl (Clarkson, 1961). Additional studies by Clarkson and Richard (1967) calculated the transfer rates of BSP between plasma and liver in unanaesthetized turkeys by following the fall in plasma BSP concentration after a single injection.

g. Primates. Data concerning BSP clearance in various primate species are presented in Table III.

Increased BSP retention was reported by Deinhardt *et al.* (1962) in studies with human infectious hepatitis in chimpanzees. Retention of up to 15% at 40 minutes was observed. BSP studies on free-ranging howler monkeys with hepatic pigmentation did not suggest that an excretory defect for such organic anions exists as in Dubin–Johnson syndrome in man and Corriedale sheep. In two howler monkeys with pigmented livers, hepatic T_m and S values were observed to be within a range previously reported in normal man and dog (Katz *et al.*, 1968). Rhesus monkeys have been studied extensively using BSP to measure

TABLE III

Normal Values of BSP Clearance in Primates

Primate	Percent retention (5 mg/kg)	Fractional clearance (%/min)	Reference
Rhesus monkey		17.6 ± 4.2	Anderson (1966)
	<5/30 min		Allen and Carstens (1968)
	0.9–3.1/45 min		Gaisford and Zuidema (1965)
	0–3.6/30 min		Vogin *et al.* (1966)
Cebus monkey		81.3 ± 8.2/15 min	G. V. Mann *et al.* (1952)
African green monkey	5.7 ± 3.3 (1.5–19.7)/30 min		Pridgen (1967)
	5.5 ± 6.4 (1.3–33.0)/30 min[a]		
Howler monkey		27 (17–46)	Katz *et al.* (1968)
Chimpanzee	2–8/20 min		Deinhardt *et al.* (1962)
	0–3/30 min		
	21.1 ± 9.5/10 min		Wisecup *et al.* (1969)
	4.7 ± 2.6/20 min		

[a] Ten milligrams per kilogram, injected dose.

hepatic disorders; such conditions have included nutritional cirrhosis with retentions of up to 45% (Gaisford and Zuidema, 1965) and venoocclusive disease with up to 65% retention (Allen and Carstens, 1968).

2. *Rose Bengal*

This rose-colored dye was first used by Delprat (1923) to measure liver function in man. The dye is removed primarily by the liver (Glaser *et al.*, 1959) and excreted into the bile in an unconjugated state. The danger of photosensitivity following its use is one disadvantage. The pharmacokinetics of rose bengal has been reviewed by Klaassen (1976). Results with rose bengal are interpreted as being similar to those with BSP, although clearance rates are quite different. Shaw (1933) first proposed the use of rose bengal in sheep having extensive fascioliasis with fibrosis and hepatic lipidosis from ketosis. Garner (1952a) administered 2 mg rose bengal per kilogram body weight to cattle and questionably concluded that the test as performed was unreliable even in severe diffuse fibrosis and lipidosis. Since clearance procedures were not used for rose bengal in cattle as recommended in the section on BSP, the true value of rose bengal for cattle is not presently known. Svirbely and associates (1946) concluded that in the dog the rose bengal test was nearly as sensitive as the BSP test for assessing experimental hepatic necrosis from xylidine intoxication; 5 mg dye per kilogram body weight was injected, and the percent retention at 30 minutes was measured spectrophotometrically. The percent retention at 30 minutes in normal dogs was usually less than 10%, with values up to 73% reported in advanced cirrhosis.

Smith *et al.* (1940) intravenously injected 5 mg/kg rose bengal into a series of normal cats and cats with chronic selenium poisoning. Samples were taken at 15, 30, and 60 minutes after injection and the percent retention calculated following spectrophotometric measurements of plasma at 565 nm. The authors adopted the 30-minute plasma sample as the sample taken at the ideal time for comparative purposes. Normal cats had the following average rose bengal retentions: 15 minutes, 33%; 30 minutes, 8%; and 60 minutes, 3%. Percent retention of rose bengal in cats with chronic selenosis ranged between 9 and 58% at 30 minutes. Rose bengal clearance in sheep is closely correlated with hepatic damage induced with neptunium-237 (Wood *et al.*, 1968).

Holmes (1960) proposed the use of ^{131}I-labeled rose bengal to assess liver function in sheep. In this test 20 μCi of ^{131}I in 3 ml of 1% rose bengal were administered intravenously. A scintillation probe with a lead shield was connected to a rate meter and recorder operating at a rate of 6 in./hour. The probe was centered at right angles to the body on the right side, 3.5 in. behind and immediately above the point of the elbow. The crystal was directed at the hepatic tissue (which is slightly more dorsal) and not the gallbladder. Counts were recorded for 1.5–2 hours postinjection. Holmes (1960) observed maximal activity by 27 minutes on an average in seven experiments, with a range of 13–55 minutes. As much as 80 ml of CCl_4 were required per os to produce liver lesions in mature sheep observable by this technique.

3. *Indocyanine Green*

Fox and co-workers (1957) first introduced indocyanine green (ICG) for the measurement of blood flow and detection of cardiac malformation in man by indicator–dilution techniques. The test has not been used extensively in veterinary medicine due to the simplicity of the BSP test.

a. Dog. General. In the dog, the ICG removal rate is exponential for the first 15 minutes after injection. This is not true for BSP. Average fractional clearance, or the plasma removal rate per minute (K), was 7.6%/minute ($T_{1/2}$ = 9.1 minutes) following the administration of 1 mg/kg body weight (Hunton *et al.*, 1960). Individual dogs ranged from 5.5 to 9.8%/minute. Ketterer *et al.* (1960) reported fractional clearance of 6.9 ± 1.0%/minute at the 0.4–0.6 mg/kg injection level. Doses near 9.0 mg/kg body weight decrease the amount of dye removed per minute to approximately one-third the normal clearance. Three days following the administration of a single oral dose of 2.5 ml CCl_4 per kilogram, body weight, the plasma disappearance rate in a dog was reduced to 1.5%/minute (Hunton *et al.*, 1960). Van Vleet and Alberts (1968) preferred the BSP retention test over ICG clearance due to the simplicity of the BSP procedure.

b. Method (Ketterer *et al.*, 1960). The technique is summarized below for the dog and can be performed with any spectrophotometer with wavelengths in the infrared range.

1. Inject intravenously 0.5 mg ICG per kilogram body weight.
2. Take three blood samples from the opposite radial vein at any time between 3 and 15 minutes postinjection.
3. Centrifuge heparinized blood samples and read the plasma dye concentrations directly at a wavelength of 805 nm in a Beckman DU spectrophotometer. Since this dye is colorimetrically unstable in dilute aqueous solutions, small amounts of plasma must be added to all tubes in preparing the standard curve.
4. Plot the dye concentration on graph paper with semilogarithmic coordinates, as recommended in the section of BSP, and calculate the $T_{1/2}$ directly. The fractional clearance K, or percent removed per minute, can then be calculated from the equation

$$K = \frac{\ln 2}{T_{1/2}} \quad \text{where } \ln 2 = 0.693$$

The advantages of using ICG in the study of hepatic problems in the dog are quite obvious. The practitioner can obtain from this test, in addition to the removal rate of the dye, the plasma volume and an estimated hepatic blood flow by using the average extraction ratio of 18% observed by Ketterer and associates (1960).

c. Other Species. The normal plasma disappearance rates for ICG in rats and rabbits are greater than 30%/minute but are not single exponential functions.

Fractional clearances for ICG in unanesthetized rhesus monkeys are 36.5 ± 2.6%/minute in females and 52.9 ± 4.9%/minute in males. The slopes of clearance curves were exponentially linear for 12 minutes after injection. Pentabarbital anesthesia significantly delayed the clearance of dye (Vogin *et al.*, 1966). Mutant sheep with congenital photosensitivity and hyperbilirubinemia exhibit delayed ICG clearance ($T_{1/2}$ = 32.4 ± 5.0 minutes) as compared to normal Southdown sheep ($T_{1/2}$ = 4.8 ± 0.5 minutes) (Cornelius and Gronwall, 1968).

C. Bile Acids

One primary role of the liver is in the uptake, conjugation, and secretion of bile acids into the bile. It is well known that in liver dysfunction there is a decreased secretion of bile

acids into bile. In man, the levels of bile acids are elevated in acute and chronic liver diseases (Koplowitz *et al.*, 1973) and have been reported to be a better indicator of liver disease than are plasma bilirubin levels or transaminase activities (Frosch *et al.*, 1968). Plasma bile acid levels have also been used to quantify liver function in rabbits (Karbach and Rybak, 1973). Following the administration of CCl_4 to dogs, sheep, calves, and ponies, a significant rise in plasma bile acid concentration was associated with corresponding increases in serum sorbitol dehydrogenase and transaminase activities (Answer *et al.*, 1976). These studies suggest that plasma bile acid concentrations should be measured in a broad range of clinical hepatopathies in animals in order to determine their usefulness in veterinary medicine.

VI. SERUM ENZYME TESTS

Variations in the concentration of certain serum enzymes as measured by their biochemical activity occur primarily as a result of two processes involving the liver: (1) their elevation due to the escape of enzymes from disrupted hepatic parenchymal cells with necrosis or altered membrane permeability: alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT), arginase, isocitrate dehydrogenase (SICD), sorbitol dehydrogenase (SD), glutamate dehydrogenase (GD), ornithine carbamyltransferase (OCT), and lactate dehydrogenase (LDH); and (2) their elevation due to an overproduction of the enzyme in cholestasis of obstructive icterus: alkaline phosphatase (SAP). Elevations in serum activity provide little information regarding the reversibility, type of lesion, or functional state of the liver. One can, however, gain some quantitative estimate of the extent of the necrosis. The duration of elevations in the serum enzyme activity is dependent upon the enzyme's molecular size, its intracellular location, its rate of disappearance from the plasma, the concentration gradient across the plasma membrane, its rate of inactiviation, and its rate of production by the liver.

Enzymes which increase in concentration in the blood following hepatic necrosis (group 1) must be divided into two groups: (a) enzymes which are "liver specific" in that high concentrations are present primarily in hepatic tissue (SGPT in dogs, cats, and primates; SD and GD in sheep and cattle; SD in horses; arginase and OCT in all ureotelic animals); and (b) enzymes which are in high concentrations in other tissues in addition to liver (SGOT, LDH, and SICD). SGOT, total LDH, and ICD are not "liver specific," but may be of use diagnostically to measure the level of liver necrosis if all tissues other than liver are known to be free of pathology. The activities of the liver-specific enzymes are the most sensitive and reliable tests available for detecting mild to severe hepatic necrosis. They are excellent for prognostication and in the evaluation of therapy.

Enzymes which are elevated during cholestatic episodes, such as alkaline phosphatase and γ-glutamyltransferase, are useful in predicting the extent of hepatobiliary diseases resulting in intrahepatic ductal or extrahepatic duct obstructions. A prediction of which enzymes can serve as diagnostic agents is possible only through analytical studies on the enzyme composition of specific cells, their organelles, or tissues (Nagode *et al.*, 1966). The International Enzyme Commission recommends that serum levels of enzyme activity be expressed as micromoles per minute per liter. Unfortunately, much of the published work is expressed in other, more popular units.

A. Alanine Aminotransferase

1. Interpretation

The use of serum transaminase activities in the diagnosis of disease was pioneered by the investigations of Wroblewski and LaDue (1955, 1956a,b) in which they observed elevations in human cardiac infarction and hepatic necrosis. SGOT activity was early found to be elevated in experimental hepatic necrosis in the horse, cow, pig, dog (Cornelius *et al.*, 1959), and cat (Cornelius and Kaneko, 1960) but also elevated in diseases involving the cardiac (myocardial infarction) and skeletal systems (muscular dystrophy, azoturia) in these species. Considerable GOT activity was found in almost all tissues analyzed in mammals, while high GPT concentrations were observed only in canine, feline, and human hepatic parenchymal cells (Cornelius *et al.*, 1959). Since the livers of mature horses, cattle, sheep, and pigs do not contain significant levels of GPT, only very small elevations in SGPT occur from hepatic necrosis in these species. *Significant elevations in the SGPT activity are liver-specific only in small animals and primates.*

2. Methods

Normal values for SGPT activities in various species are presented in Table IV. Many times, SGPT and SGOT activities are determined by the methods of Wroblewski and Cabaud (1957) and Cabaud *et al.* (1956), respectively. Both methods give comparable results. In analytical techniques the assay for transaminase activity is based on the transfer of the α-amino group of either aspartic acid or alanine to α-ketoglutaric acid. The oxalacetate that results in the determination of GOT activity is converted to pyruvic acid by aniline citrate. In both procedures a pyruvate–dinitrophenylhydrazone is next prepared and measured colorimetrically at 490 nm without the extraction procedure (Sigma Chemical Co.). Units are usually expressed either as micrograms of pyruvic acid liberated in 20 minutes at 25°C per milliliter serum, or as Sigma–Frankel (S.F.) units. The Sigma–Frankel unit is defined as the enzyme activity necessary to produce a decrease in the optical density at 340 nm of 0.001/min per milliliter serum under the conditions of Karmen (1955) at 25°C/per centimeter light path.

3. Pathological Findings

Dal Santo (1959), Malherbe (1960), and Hoe (1961) observed significant elevations in SGPT activity in dogs with infectious hepatitis. Few or no such increases were observed in dogs with distemper, and this test appeared to be quite helpful in the differential diagnosis of certain cases in which clinical symptoms were questionable. Significant elevations in SGPT have been reported in the following primary and secondary hepatic diseases in the dog: intrahepatic cholestasis (Malherbe, 1959); complicated *Babesia canis* infections (Malherbe, 1960); hepatic neoplasia, fatty degeneration of the liver, leptospirosis (Hoe and Harvey, 1961a); CCl_4 poisoning (Kutas and Jarsai, 1961; Frankl and Merritt, 1959; Cornelius *et al.*, 1959); suppurative hepatic necrosis, severe anemia from a ruptured splenic hemangioma, arsenic poisoning, extensive lipidosis from hypothyroidism, hepatic necrosis secondary to pyometra, and extensive hepatic malignancies (Cornelius, 1957). More recent reports have attested to its usefulness in the diagnosis of early preclinical chronic progressive hepatitis in Bedlington terriers (Hardy *et al.*, 1975), glucocorticoid-induced hepatopathy (Rogers and Ruebner, 1977), hepatoma (Strombeck *et al.*, 1976c),

TABLE IV

Serum Alanine Aminotranferase (SGPT) and Aspartate Aminotransferase (SGOT) Activities in Normal Animals*

Species	Age or weight	SGPT	SGOT	Reference
Dog	9–12 months	21 ± 11	20 ± 7	Cornelius *et al.* (1959)
	>5 years	21.9 ± 5.6	22.4 ± 5.2	Crawley and Swenson (1965)
		20.5 (5–50)	22.8 (3–80)	Van Vleet and Alberts (1968)
		5–23	20–48	Hamilton *et al.* (1966)
		18.0 ± 2.3	27.0 ± 4.4	Hoe and Harvey (1961b)
Cat		15.6 ± 9.9	19.0 ± 4.8	Cornelius and Kaneko (1960)
Cow		43.2 ± 0.7	169.6 ± 3.2	Boyd (1962)
		24.1 ± 5.0	68.7 ± 17.5	Hansen (1964)
	2–10 years	16 ± 8	56 ± 14	Cornelius *et al.* (1959)
	7–27 days	2 ± 3	25 ± 6	Cornelius *et al.* (1959)
	1–97 weeks	18 ± 12	24 ± 17	Roussel and Stallcup (1966)
Horse	1–11 years	8 ± 6	158 ± 37	Cornelius *et al.* (1959)
	2.5 years		228 ± 66	Wolff *et al.* (1967)
		0.213 ± 0.264 (μmoles/100 ml/min)	6.14 ± 1.69	Freedland *et al.* (1965)
Baboon				
African		18 ± 12	34 ± 8.9	Pena and Goldzieher (1967)
Domestic		17 ± 7	30 ± 11	Pena and Goldzieher (1967)
Kenya		29 ± 11	10 ± 4	Pena and Goldzieher (1967)
Kenya	8.5–26.9 kg	33.2 ± 7.3	51 ± 5.5	Burns *et al.* (1967)

Monkey				
Rhesus	3.3–8.0 kg	37.9 ± 19.1	20.4 ± 9.9	Brooks *et al.* (1963)
		13.1 ± 5	27 ± 6.5	Anderson (1966)
Squirrel		117 ± 75	138 ± 62	Rosenblum and Cooper (1968)
Marmoset			98.7 (48–204)	Deinhardt and Deinhardt (1966)
	Mature	20.2 ± 8.3	143 ± 33	Burns *et al.* (1967)
			133 (62–106)	Holmes *et al.* (1967)
Howler		12–27	140–400	Maruffo *et al.* (1966)
		35 ± 8 (Karmen)	786 ± 10 (Karmen)	Katz *et al.* (1968)
African green		38 ± 9.2	61 ± 9.8	Pridgen (1967)
		47 ± 13.3	54 ± 9.9	Pridgen (1967)
Sheep		17.4 ± 5.1	74.7 ± 13.6	Hansen (1964)
	1 year (penned)		95.0 ± 6.1	Young *et al.* (1965)
	1 year (pastured)		122.3 ± 5.1	Young *et al.* (1965)
		23.2 ± 1.8	164 ± 23	Boyd (1962)
Goat		13 (mean)	63	Singh *et al.* (1972)
Swine	1.3 year	27 ± 8	31 ± 14	Cornelius *et al.* (1959)
			29	Cysewski *et al.* (1968)
Chicken	6 months	0	370 ± 186	Cornelius *et al.* (1959)
		4–16	177–260	McDaniel and Chute (1961)
Goose		3.1 ± 2.9 (IU/ml)	14.4 ± 5.0	Bokori and Karsai (1969)
Duck	1 day	5.1 ± 2.8 (IU/ml)	10.1 ± 4.2	Bokori and Karsai (1969)
	1 week	6.7 ± 1.94		Ahmed *et al.* (1975)
Chimpanzee	5–20 kg	36.9 ± 16.4	47.5 ± 13.8	Hartwell *et al.* (1968)
	2–12 years	16.9 ± 6.2	20.3 ± 4.9	Wisecup *et al.* (1969)
	Juvenile	30.1 ± 9.5	47.6 ± 15.1	Krushak and Hartwell (1968)

*units—see text for description.

aflatoxicosis (Himes and Cornelius, 1973), primary primary cholangiohepatitis (La Croix and Pulley, 1974), secondary liver damage following bile duct rupture (Borthwick *et al.*, 1973; Bellinger, 1973), and obstructive cholelithiasis (Schall *et al.*, 1973). No significant elevations are usually observed in distemper, uremia, chronic peritonitis, hypothyroidism, osteodystrophy, lymphatic leukemia, or pneumonias. Slight elevations can be observed in any highly febrile condition up to 100 S.F. units.

An extensive study by Hoe and Jabara (1967) concluded that the SGPT and SAP tests were more effective in diagnosing liver diseases in the dog than the SGOT, SICD, SLDH, and serum α-hydroxybutyrate dehydrogenase tests. Hoe and O'Shea (1965) were able to detect hepatic damage in 80–100% of the cases of severe fatty change, malignant neoplasia, hepatoma, cirrhosis, and hepatitis in dogs.

SGPT data as correlated with conventional microscopic examination of livers at autopsy can be *arbitrarily* interpreted as follows for the dog:

Normal values	10–50 units
Minor to moderate necrosis	50–400 units
Severe liver necrosis	400 units

Elevations above 1000 units are not uncommon in severe hepatic necrosis. Dogs should be tested daily for prognosis and for the evaluation of therapy. The use of the SGPT or the BSP test in conjunction with the SGPT test in dogs is useful. BSP clearance will be delayed in hepatic fibrosis or necrosis, whereas the SGPT activity will be elevated only in necrosis. The nature of the hepatic pathology, whether necrotic or cholestatic, can be determined by the simultaneous use of both SGPT and γ-glutamyltransferase (GGT) tests.

The use of the SGPT test should be confined to primates, dogs, cats, and smaller animal species since large animals contain little hepatic GPT activity (Cornelius, 1963). Elevated SGPT activity has been reported in many different conditions in primates: human viral hepatitis in marmosets (Deinhardt and Deinhardt, 1966) and chimpanzees (Deinhardt *et al.*, 1962); leptospiral infections in squirrel monkeys (Minette and Shaffer, 1968); hepatic hemangioma in the rhesus monkey (Woodruff and Johnson, 1968); venoocclusive disease in the rhesus monkey (Allen *et al.*, 1969); morphine addiction in the rhesus monkey (Brooks *et al.*, 1963); and malarial infections in baboons (Sadun *et al.*, 1966).

B. Aspartate Aminotransferase

Elevations in the activity of SGOT can be associated with alterations in cell necrosis of many tissues. Pathology involving the skeletal or cardiac muscle and/or the hepatic parenchyma allows for the leakage of large amounts of this enzyme into the blood.

Normal SGOT activities are presented in Table IV. Considerable SGOT activity was observed in nearly all tissues analyzed in the horse, cow, pig, dog, and chicken (Cornelius *et al.*, 1959). Since all major tissues contain high concentrations of GOT, the finding of significant elevations in SGOT need not indicate hepatic necrosis unless diseases of other large organ systems can be ruled out. SGOT, however, can be used successfully in large animals prognostically to evaluate the degree of liver necrosis once it has been established that nonhepatic diseases are absent. Since SGPT determinations are of little help in diagnosing hepatic necrosis in large domestic animals due to its absence in high concentrations in their livers, SGOT has been at times used in its place. It must be remembered,

however, that SGOT is not liver specific and that other enzymes, such as SD or GD, arginase or OCT, are far more liver specific in domestic animals.

No discussion of SGOT activities in liver disorders in the dog, cat, or primate will be attempted since the SGPT test is the test of choice in these species. Considerable information is available concerning elevations in the SGOT activity associated with liver diseases in species other than the dog. Elevations in SGOT activities have been observed in the following: postparturient state in the cow (Lupke, 1965), ragwort intoxication in calves (Ford and Ritchie, 1968), toxic hepatosis in cattle and sheep consuming contaminated herring meal (Hansen, 1964), extrahepatic bile duct obstruction in swine (Bicknell *et al.*, 1967); aflatoxicosis in swine (Cysewski *et al.*, 1968), halothane and chloroform anesthesia in the horse (Wolff *et al.*, 1967; Thorpe *et al.*, 1968), carbon tetrachloride poisoning in the horse (Freedland *et al.*, 1965), pasture conditions of sheep (Young *et al.*, 1965); sporidesmin intoxication in sheep (Mortimer, 1962), metacercariae (fascioliasis) migrations in sheep (Roberts, 1968), predicting copper poisoning in sheep (MacPherson and Hemingway, 1969), hyperlipemic syndrome in ponies (Schotman and Wagenaar, 1969), and metacercariae migrations of *Fasciola* infections in calves (Anderson *et al.*, 1977). No significant elevations in SGOT were observed in telangiectasis, in localized abscesses (Goetgheluck, 1970), or in the massive dilation of the intrahepatic portal capillary bed in bovine peliosis hepatitis (Seawright and Francis, 1971).

C. Arginase

Arginase is found in significant concentrations only in the liver of ureotelic mammals, such as man, dog, sheep, cattle, rat (Cornelius *et al.*, 1963), and pig (Dittrich *et al.*, 1974). The finding of extremely high arginase activities in the livers of various mammals as compared to all other tissues examined suggests that significant elevations in plasma arginase activity would indicate a necrotic process occurring within the liver per se. Normal values for serum arginase activity in various species are presented in Table V. A method using gel filtration for the determination of arginase activity in serum (Cornelius and Freedland, 1962) has been used. A more simple method is now available that does not require removal of plasma urea by gel filtration (Mia and Koger, 1978).

TABLE V

Serum Arginase Activities in Some Mature Mammals[a]

Species	Units/ml		
	Mean	σ	Range
Horse	0.64	±1.1	0–4.2
Nonlactating cow	0.34	±0.08	0–0.17
Lactating cow	0.50	±0.35	0.08–1.8
Wether	0.30	±0.57	0–2.7
Ewe	0.37	±0.29	0–1.1
Dog	0.03	±0.01	0–0.28
Rat	1.28	±0.98	0–4.2
Man	0.04	—	0–0.11

[a] Cornelius *et al.* (1963).

After the administration of a single oral dose of CCl_4 to the horse, calf, sheep, and dog, the serum arginase activity rose rapidly, followed by recovery to the normal range within 3–4 days. The SGPT (dog) and SGOT (horse, calf, sheep) activities remained significantly elevated for over 1 week in all animals, during the same CCl_4-poisoning experiments. There appeared to be a more rapid disappearance of the serum arginase, a mitochondrial-bound enzyme, as compared to the transaminases, which are in the hyaloplasmic fraction of cells. Preliminary clinical and experimental data suggest that, if both serum arginase and transaminase activities are continuously elevated, a progressive hepatic necrosis is most likely present. If normal serum arginase and elevated transaminase activities are observed following significant elevations of both enzymes, prognosis is favorable as hepatic necrosis is subsiding. This observation was more recently confirmed by Harvey and Hoe (1971). The determination of serum arginase activity is a good liver-specific enzyme test for active hepatic necrosis in all ureotelic species.

Significant elevations in serum arginase in hepatic necrosis were observed in horses following chloroform anesthesia, whereas halothane anesthesia produced only minor enzyme elevations (Wolff *et al.*, 1967). Cattle with *Fasciola gigantica* infestation, sheep with hepatic necrosis, and goats with extensive hepatic fatty degeneration all exhibit elevations in serum arginase activity (Adam *et al.*, 1974). It was reported that arginase was superior to alkaline phosphatase and bilirubin levels for diagnosing hepatic diseases in ruminants and camels in Sudan (Harvey and Obeid, 1974). The successful use of arginase in sheep has been reported in chronic copper poisoning (Ross, 1966), hexachorophene intoxication (Harvey and Hoe, 1971), leptospiral infections (Millar *et al.*, 1977) and progressive necrosis (Cornelius *et al.*, 1963).

D. Other Enzymes for the Measurement of Hepatic Necrosis

Due to the lack of GPT activity in the livers of large domestic animals, recent research has focused on finding other liver-specific enzymes for these species. Some of the more promising enzymes have been SD, GD, arginase, and OCT.

1. Sorbitol Dehydrogenase

Sorbitol dehydrogenase is highly concentrated in the livers of horses (Freedland *et al.*, 1965), dogs (Zinkl *et al.*, 1971), cattle (Treacher and Collis, 1977), and pigs (Baker *et al.*, 1974) (Table VI). Following CCl_4 poisoning, the sera of horses exhibited a 600-fold increase in SD activity and a 75-fold increase in GD activity. The usefulness of the SD test in most domestic animals has been confirmed by many investigators. One advantage of using SD to measure necrosis is that only one enzyme test is needed for all species. A disadvantage of measuring SD is that activity in serum declines appreciably after 24 hours, and serum samples should be analyzed within 12 hours.

Sorbitol dehydrogenase has been utilized experimentally and diagnostically in a variety of studies in the horse: hyperlipemias (Schotman and Wagenaar, 1969), CCl_4 intoxication (Ikeda *et al.*, 1976; Freedland *et al.*, 1965), and the repeated use of halothane (Gopinath *et al.*, 1970). Thiopentane–halothane anesthesia failed to increase serum SD activity, while the use of thiopentane–ether was followed by slight and transient increases (Lees *et al.*, 1973). Current data suggest that SD is a useful enzyme for measuring equine hepatic ncecrosis. It has also been utilized in studies on various liver diseases in ruminants (Boyd, 1962; Keller, 1971; Shaw, 1974; Ford and Gopinath, 1976), such as fascioliasis (Ander-

TABLE VI

Serum Sorbitol Dehydrogenase Activities in Domestic Animals

Species	Age	Unit	Mean ± σ	Range	Reference
Ponies		μmole/hr/ml	0.88 ± 0.22		Answer *et al.* (1976)
				<1.0 IU	Schotman and Wagenaar (1969)
Horses	Mature	μmole/min/dl	0.06 ± 0.06	0–0.18	Freedland *et al.* (1965)
Calves		μmole/hr/ml	0.88 ± 0.08		Answer *et al.* (1976)
		IU/liter		4.3–15.3	Finn and Tennant (1974)
Cattle	Mature	mU/ml	3.2 ± 1.0		Shaw (1974)
Sheep	Mature	μmole/hr/ml	0.99 ± 0.09		Answer *et al.* (1976)
Dogs	Mature	μmole/hr/ml	0.61 ± 0.18		Answer *et al.* (1976)
	Mature	μmole/min/ml	2.8 ± 2.0		Zinkl *et al.* (1971)

son *et al.*, 1977), and in the evaluation of fasciocidal drugs in sheep and cattle (Fowler, 1971; Harvey and Hoe, 1971; Alemu *et al.*, 1977). Only recently has SD been used both experimentally and clinically in the dog (Answer *et al.*, 1976; Strombeck *et al.*, 1976a).

2. *Glutamate Dehydrogenase*

Boyd (1962) and Keller (1971) have shown that GD is highly concentrated primarily in ovine and bovine liver and recommended its use in measuring hepatic necrosis in these species. Similarly, GD is present in high concentrations in equine (Freedland *et al.*, 1965) and canine liver (Zinkl *et al.*, 1971). It has become the enzyme of choice to date in measuring hepatic necrosis in ruminants. Normal serum values are listed in Table VII.

Glutamate dehydrogenase has been useful in studying experimental CCl_4 poisoning in cattle and sheep (Boyd, 1962; Fowler, 1971), measuring necrosis after hexachlorophene administration in sheep (Harvey and Hoe, 1971), calving (Treacher and Collis, 1977), and studying secondary effects on the liver in bile duct ligation in sheep (Ford and Gopinath, 1976).

3. *Ornithine Carbamyltransferase*

Ornithine carbamyltransferase can be considered quite similar to GD in all characteristics, being an excellent liver-specific enzyme for detecting necrosis in all species studied

TABLE VII

Serum Glutamate Dehydrogenase Activities in Domestic Animals

Species	Activity (μmole/hr/dl)	Reference
Horse	0.056 ± 0.042[a]	Freedland *et al.* (1965)
Cattle	18.5 ± 2.9	Boyd (1962)
Sheep	12.3 ± 2.9	Boyd (1962)
	15.6 ± 6.2	Harvey and Obeid (1974)
Dog	2.6 ± 2.5[a]	Zinkl *et al.* (1971)
Goat	20.2 ± 5.3	Harvey and Obeid (1974)
Camel	15.4 ± 4.7	Harvey and Obeid (1974)

[a] Micromoles per minute per deciliter.

to date (Treacher and Sansom, 1969). Cattle with hepatic lesions determined at slaughter exhibited levels as high as 2000 IU; a dog with hepatic carcinoma had a serum activity of 568 IU (Dotta and Abate, 1973). Markiewicz and co-workers (1975) reported a good correlation between serum OCT activity and the severity of bovine liver damage in fasciolosis. In cattle, OCT is almost totally confined to the liver (Treacher and Collis, 1977); it is also in high concentration in pig liver (Dittrich *et al.*, 1974) and has been recommended for use in swine (Wilson *et al.*, 1972). In the dog, both OCT and SGPT appear to be similar in sensitivity as diagnostic aids in the measurement of necrosis (Litchfield and Gartland, 1974). Average normal serum values are as follows: swine, 210 units (Cysewski *et al.*, 1968); cattle and sheep, 5.1 ± 2 and 4.0 ± 3.9 units, respectively (Hansen, 1964); and dog, 2.9 units (Dotta and Abate, 1973).

E. Alkaline Phosphatase

1. General Comments on the Use of Enzymes to Assess Cholestasis

A number of enzymes have been used to measure obstructive processes in the hepatobiliary system of man: SAP, GGT, leucine aminopeptidase (LAP), and 5′-nucleotidase (5′NT). γ-Glutamyltransferase, LAP, and 5′NT may have some distinct diagnostic advantages over SAP in that they originate in hepatobiliary tissue and their activities may be normal or only minimally elevated in the serum of animals with boney disorders. Although elevations in the SAP activity have long been used to measure intrahepatic and extrahepatic cholestasis in the dog, only recently have we known that this is due to an overproduction of the enzyme and not to its lack of excretion into the bile.

A great deal of confusion surrounds the word "cholestasis." Although a strict definition is "bile without motion," many interpretations are in the literature (Magnemat, 1973). Cholestasis can result from a variety of pathological conditions due to either biochemical or structural alterations. A reduction in bile volume can result from (1) altered bile salt concentrations, which affect hepatic membranes; (2) interference of drugs and abnormal bile acids with normal biliary micelle formation; (3) interference with sodium and water transport into the bile; or (4) increased intraluminal biliary pressure due to either intrahepatic or extrahepatic obstructions of ducts in various disease states. It must be understood that some cholestatic syndromes may result from decreased bile flow due to biochemical defects and thus may not be observable microscopically.

2. General

The alkaline phosphatases are enzymes of low substrate specificity which catalyze the hydrolysis of monophosphate esters at an alkaline pH. The hydrolysis of organic phosphates, such as those of glucose or glycerol, yields inorganic phosphate and the respective organic moiety. The Bodansky method estimates the phosphate liberated from glycerol phosphate, while the King-Armstrong method utilizes phenyl phosphate. Still another method, that of Bessey-Lowry, uses *p*-nitrophenol as a substrate. To relate the millimolar SAP unit of Bessey-Lowry to King-Armstrong units, the former can be multiplied by a factor of 2.5; however, the conversion of such units is not recommended. The Bodansky test is the most popular method in most clinical hospitals.

The various alkaline phosphatase isoenzymes are ubiquitous throughout the body and are in high concentrations primarily in liver, bone, intestine, kidney, placenta, and white

blood cells (Kaplan, 1972). By measuring the total SAP activity, it is impossible to ascertain the origin of the SAP activity and whether it is due to hepatic lesions. Separation of alkaline phosphatase isoenzymes can be achieved by electrophoretic, differential heat or urea sensitivity, or immunological and chromatographic methods; however, the procedures are complex and not easy to perform routinely in a diagnostic laboratory.

It is now well known that elevations in the SAP activity associated with either intrahepatic or extrahepatic biliary obstruction are due to overproduction of the enzyme by the liver (Kaplan and Righetti, 1970). There is evidence that liver alkaline phosphatase functions in hydrolyzing phosphorylcholine so that choline can be excreted in the bile. The stimulus for increased enzyme production by the liver may be increased concentrations of phosphorylcholine in the liver due to obstructive processes. The normal source of SAP may be organs other than the liver. The magnitude of increases in SAP due to increased osteoblastic activity or other pathological nonhepatic disorders is usually less than three times the normal serum levels. Values indicating cholestasis are usually well above the levels associated with nonhepatic disorders. The highest serum values are associated with obstruction of the extrahepatic ducts and metastatic carcinoma of the liver.

3. Dog and Cat

Normal values for SAP activity in the adult dog are <4 Bodansky units (Svirbely *et al.*, 1946), <10 King-Armstrong units (Hoe and O'Shea, 1965; Bloom, 1957), and <5 Sigma units/ml (Van Vleet and Alberts, 1968). Both Malherbe (1965b) and Hoe and Harvey (1961a) have stated that occasionally levels as high as 14 King-Armstrong units may be found in normal dogs. A comparison of mean values of SAP activity in the dog by four different methods has been reported by Harvey (1967). Normal values for SAP may be increased twofold in greyhounds under training conditions (Egan, 1978). The serum half-life of the hepatic isoenzyme has been estimated to be approximately 3 days in the dog. The serum of a normal dog contains SAP only of osseous origin. If hepatic cholestasis occurs, elevated SAP originates in hepatic tissue (Rogers, 1976).

Normal values for SAP activity in the cat have been reported to be <15 King-Armstrong units (Kelly *et al.*, 1975). Everett and associates (1977) reported SAP values in normal cats of <30 mU/ml, which were considerably lower than those observed in dogs. The AP excreted into the urine in cats with bile duct obstruction was independent of the SAP concentrations and most likely of renal origin.

The measurement of SAP has been a useful aid in measuring cholestatic disease in the dog. Elevations have been observed in extrahepatic obstruction (Armstrong *et al.*, 1934; Freeman *et al.*, 1938; Van Vleet and Alberts, 1968), in primary and metastatic neoplastic hepatic disease (Strombeck, 1978), in cholelithiasis (Schall *et al.*, 1973), following rupture of the bile duct (Bellinger, 1973; Borthwick *et al.*, 1973), in intrahepatic cholestasis (Dalton and Hill, 1972), in glucocorticoid-induced hepatopathy (Rogers and Ruebner, 1977), in chronic progressive hepatitis in Bedlington terriers (Hardy *et al.*, 1975), following the administration of anticonvulsive drugs which are microsomal enzyme inducers (Sturtevant *et al.*, 1977), in cirrhosis (Grenn and Bulmer, 1972), and following hepatic necrosis (Hoe and Harvey, 1961b). Significant elevations have also been reported in cats with inflammation of the bile ducts (Kelly *et al.*, 1975) and experimental hepatic necrosis and obstruction (Everett *et al.*, 1977). Both SAP and LAP activities are useful indicators of feline hepatic cholestasis.

4. Cattle and Sheep

The wide range of SAP activities in normal cattle and sheep prohibits the effective use of SAP as an indicator of cholestasis in these species. Although the enzyme activity of the serum of each cow and sheep remains fairly constant over extended periods of time, a great range of activities (cattle, 0.3–114.3 King-Armstrong units; sheep, 3–166 King-Armstrong units) is observed (Allcroft and Folley, 1941). In sheep, Ford (1958) reported ranges of 14–427 King-Armstrong units. An interesting genetic study of Agergaard and Larsen (1974) in Jersey cattle revealed that SAP activity was markedly age dependent and related to the J substance phenotype.

Leaver (1968) found that normal values for sheep ranged between 5 and 33 King-Armstrong units and reported no increase in SAP activity in either bile duct obstruction or sporodesmin poisoning which results in intrahepatic cholestasis. No reproducible trends in SAP activities have been observed in sheep following the administration of CCl_4 or hexachlorophene (Harvey and Hoe, 1971; Alexander and McDonald, 1960).

5. Other Species

Levels of SAP have been reported in primates as follows: rhesus monkey, 9.5 ± 5.1 Bessey-Lowry units (B-LU) (Anderson, 1966); squirrel monkey, 21.8 ± 10.7 B-LU (Rosenblum and Cooper, 1968); chimpanzee, 11.4 Bodansky units (Wisecup *et al.*, 1969); Tamarin marmoset monkey, 29.8 (11.7–75.9) B-LU and cotton-top marmoset monkey, 11.9 (3.6–39.9) B-LU (Holmes *et al.*, 1967); and African green monkey, 30 ± 14 Bodansky units (Pridgen, 1967).

In a study on CCl_4 poisoning in pigs, normal SAP levels were normally less than 5 Bodansky units, increasing to 20–40 units following the injection of CCl_4 into either the portal vein or hepatic artery (Van Leenhoff *et al.*, 1974). Other studies have indicated its usefulness in diagnosing bile duct obstruction in young pigs (Flukiger *et al.*, 1977). Schotman and Wagenaar (1969) reported normal SAP values to be between 1 and 4 B-LU in ponies and observed elevations as high as 50 B-LU from liver damage associated with fasting hyperlipemia. Elevations in serum arginase levels in horses following chloroform anesthesia were accompanied by normal SAP levels (Wolff *et al.*, 1967).

A considerable literature is available on the effects of age, sex, egg production, and inheritance on the SAP levels in chickens (Tanabe and Wilcox, 1960). Levels of SAP have also been reported in pheasants (Sova *et al.*, 1974), ducks and geese with and without liver dystrophy (Bokori and Karsai, 1969), and ducklings with viral hepatitis (Ahmed *et al.*, 1975). Elevations in SAP and certain other enzyme activities occurred regularly in all avian species with significant hepatopathy.

F. γ-Glutamyltransferase (γ-Glutamyl Transpeptidase)

Due to the elevations in SAP activity in a variety of nonhepatic diseases, a number of other enzymes have been recently evaluated and appear to be more specific indicators for measuring cholestasis. These enzymes are GGT, LAP, and 5′NT (Burke, 1975). Preliminary studies in animals suggest that GGT activity, unlike that of SAP, is not significantly elevated in early hepatic necrosis and that GGT may well be the enzyme of choice for measuring the degree of intrahepatic or extrahepatic cholestasis. Distinct advantages are apparent in the use of GGT in ruminant cholestasis, since SAP levels vary greatly and are

not useful in diagnosis. Since GGT is absent from skeletal tissues, animals with boney disorders nearly always exhibit normal values. In addition, GGT originates in hepatic biliary tissue and is not overproduced in hepatic diseases. In spite of the existence of high concentrations of GGT in the kidney, the enzyme as found in the plasma is of hepatic origin. Recent experimental studies in the rat suggest that the measurement of urinary GGT activities in nephritis is more sensitive than other kidney function tests (Braun *et al.*, 1977). γ-Glutamyltransferase activity is present primarily in renal, pancreatic, and hepatic tissue in cow, calf, swine, lamb, baboon, and macaque; however, no hepatic GGT was observed in the kitten, hamster, or mouse (Braun *et al.*, 1977). The successful use of another cholestatic enzyme, LAP, has been reported in extrahepatic obstruction in the cat (Everett *et al.*, 1977). Rarely, minor elevations of GGT can occur in pancreatic disease (Orlowski, 1963).

1. Dog

Normal plasma GGT activities are 0.92 (0–2.26) Sigma units/ml in the dog (Noonan and Meyer, 1979). Following the production of hepatic necrosis by the administration in CCl_4, SGPT, SGOT, SAP, SD, and arginase activities rose precipitously, while no elevation in GGT activity was present. After surgical ligation of the bile duct, mild elevations in SGPT and SD activities occurred and gradually declined. No increase occurred in the serum arginase levels, while GGT and SAP levels remained elevated throughout the study (Noonan and Meyer, 1979). These preliminary studies suggest that, by measuring the serum arginase and GGT activities, one can differentiate between necrosis and cholestasis under these experimental conditions. Additional clinical studies in the dog will be needed to confirm this promising research.

2. Cattle and Sheep

Mean normal plasma GGT activities have been reported in 2- to 3-month-old bull calves to be 16 mU/ml, ranging between 15 and 24 (Simesen *et al.*, 1973). Following infection with metacercariae of *Fasciola hepatica,* GGT activities rose to 20 times preinfection levels between 30 and 60 minutes. The measurement of GGT levels was superior to the measurement of SGOT in revealing the liver damage (Simesen *et al.*, 1973). Another study (Simesen and Nansen, 1974) revealed no serum GGT elevations in adult cattle with advanced hepatic fibrosis from fluke infestations. Rico and co-workers (1977a) reported plasma GGT activity in ten adult cows to be 17.7 ± 5.7 IU.

Normal mean serum GGT values for adult sheep are 22 mU/ml, with a range of 14–31 (Ford, 1974; Malherbe *et al.*, 1977). A rise in the activity of GGT in the serum was associated only with cholestasis and bile duct damage and not with experimental hepatocellular or renal necrosis (Ford, 1974). In another study, it was observed that levels of serum activity of GGT were more diagnostic than SGOT levels in evaluating low-grade acute intoxication and chronic liver involvement from lupinosis and *Phomopsis leptostromiformis* hepatotoxicosis (Malherbe *et al.*, 1977).

3. Other Species

Mean normal serum levels for adult horses and 100-kg pigs have been reported to be 12.3 ± 4.6 and 36 ± 14 IU/liter, respectively (Rico *et al.*, 1977b; Rico *et al.*, 1977a). In a study by Ikeda *et al.* (1976) in which horses were administered CCl_4, the use of GGT was superior to the use of SD and GD in assessing chronic liver diseases.

G. Other Enzyme Tests

1. Serum TPN-Linked Isocitrate Dehydrogenase

Elevations in the serum activity of this enzyme occur in hepatic necrosis as in the case of the transaminases. Normal serum values have been determined by Cornelius (1961). All tissues examined (liver, kidney, heart, and skeletal muscle) of cow, horse, sheep, dog, cat, chicken, and guinea pig contain considerable ICD activity. This suggests its distribution is similar to GOT activities. For this reason, elevations in SICD activity, as in the case of SGOT and LDH, may not indicate only the presence of liver necrosis. There are a considerable number of publications concerning SICD in a variety of species with liver necrosis: dog (Hoe and Jabara, 1967; Chaffee *et al.*, 1969), horse (Wolff *et al.*, 1967; Freedland *et al.*, 1965; Lees *et al.*, 1973), cow and sheep, (Boyd, 1962), and goose (Egyed *et al.*, 1974). Other serum enzymes, such as SGPT, SD, GD, and arginase, are liver specific for necrosis and should preferably be used.

2. Serum Lactate Dehydrogenase

Lactate dehydrogenases are found in high concentrations in a variety of tissues; therefore, the measurement of total LDH activity is not organic specific. Electrophoretic separation of the various LDH isoenzymes may be used diagnostically in man to confirm which organ is involved in a degenerative process. Specific isoenzyme profiles may occur in hepatic disease, specific malignancies, muscular damage, and nephrotoxicosis (Moore and Feldman, 1974). Due to the complexity of the test, its use is not recommended in hepatic disorders; however, it may be useful in the diagnosis of certain malignancies. Normal values have been reported for the dog (Zinkl *et al.*, 1971), ruminants (Boyd, 1962; Alemu *et al.*, 1977), horse (Yamaoka and Kameya, 1972; Schotman and Wagenaar, 1969), and chicken (McDaniel and Chute, 1961; Brown, 1965).

Many other serum enzymes have been studied in relation to their diagnostic use in hepatic disorders: malate dehydrogenase in sheep (Young *et al.*, 1965); LAP in the monkey (Anderson, 1966); cholinesterase in the dog, horse, and cow (Ferriera Neto, 1958; Hoe and Harvey, 1961a; Kutas and Karsai, 1961); procaine esterase in the horse (Humlicek and Kruska, 1960); and aldolase in the horse and sheep (Wurzer *et al.*, 1967; Trifonov, 1964).

VII. SPECIFIC BIOCHEMICAL TESTS

A. Carbohydrate Metabolism Tests

Hepatic physiologists have been stressing the importance of the liver in many biochemical processes ever since Claude Bernard first demonstrated the participation of the liver in carbohydrate metabolism. The ability of normal liver to metabolize increased amounts of glucose, galactose, fructose, and lactic and pyruvic acids in a consistent fashion has resulted in a number of liver function tests. These tests are not used extensively in veterinary medicine; however, their use in the liver profile may at times be interesting. Although the glucose tolerance test and the determination of blood lactic and pyruvic acid levels may be abnormal in hepatic insufficiency (Lichtman, 1953), these tests have little specific diagnostic value and have not been studied adequately in the domestic animals to

warrant their discussion. Experimental evidence, however, suggests that the galactose tolerance test may be successful in detecting hepatic alterations because only the liver can utilize galactose in significant amounts in certain species. The test, however, fails to reveal lesser degrees of liver damage and is quite time-consuming in execution and analysis.

1. Galactose Tolerance Test

The use of this sugar was first suggested as a test for man by Bauer (1906). Owing to a series of problems encountered in the oral test proposed by Bauer, the galactose tolerance test is now performed in man and animals by injecting galactose intravenously at an amount that the average normal liver can completely remove in approximately 1 hour. Any galactose above the expected values at 1 hour may imply liver pathology. Drill and Ivy (1944) suggested for the dog an intravenous injection of 1.0 ml of 50% galactose per kilogram body weight. The test is somewhat difficult to interpret in the dog; however, advanced hepatopathy can be detected. The test is not used often, as other tests are more sensitive and easier to perform.

Garner (1952b) administered 0.5 gm/kg body weight to cattle which had been fasted overnight. Blood samples were taken prior to and 0.5, 1, 1.5, and 2 hours after injection. Galactose was observed to persist in the blood for considerably longer periods of time in normal cattle than in human beings. The galactose disappearance curves in cattle with diffuse liver damage were quite elevated as compared to curves in control cattle. It was concluded that both the galactose tolerance and adrenaline response test with certain modifications could be applied to the detection of bovine hepatopathy (Garner, 1952b). Galactose and glucose tolerance, as well as pyruvic and lactic acid metabolism, have also been studied in normal horses and in equine hepatopathy (Forenbacher, 1957).

2. Fructose Tolerance Test

Since fructose is metabolized by both liver and muscle, it cannot be used as a specific index of liver disease. Stiebale (1942) studied fructose tolerance in normal dogs and following the administration of hepatotoxins (phosphorus and CCl_4). He concluded that the test showed diagnostic promise. Fructose was present only in the urine (Seliwanoff's reaction) in advanced liver disease. Blood glucose curves climbed to 30 mg/dl above the fasting level or remained elevated for over 4 hours following fructose administration to dogs with liver insufficiency. The test has not been used extensively owing to the availability of other simple and more specific function tests.

B. Protein Metabolism Tests

Although none of the alterations in the serum proteins are entirely specific for liver damage, the combination of an absolute low albumin and/or a high γ-globulin level is quite typical. Hyperplasia of the immunocytic variety of liver cells is associated with a marked increase in serum γ-globulin.

The concentrations of the plasma proteins depend upon a multitude of factors, including the extent, duration, severity, and primary nature of the damage, current rates of synthesis, catabolism, hepatic release, and distribution. In addition, each of these factors can be affected by circulatory, inflammatory, reparative, degenerative, metabolic, and regenerative processes occurring in the specific liver disease. It is therefore impossible to define a

typical plasma protein pattern in certain types of liver disease, but changes in certain types of patterns are characteristic of specific hepatopathies.

Paper electrophoresis or the rapid fractionation procedure of serum proteins by Wolfson *et al.* (1948) may be used. The original Tiselius (1930) free electrophoresis techniques were somewhat elaborate and not well suited for clinical application. However, the development of paper and other supporting media for electrophoresis has changed this situation, with the method now being a routine procedure in all laboratories. Since domestic animals may vary in their total serum protein concentration between 5 and 8 gm/dl owing to individual differences and variations in water balance, a differential fractionation is needed to allow for the detection of changes in the absolute quantities of certain specific serum proteins. Total serum protein values are rarely of aid in clinical interpretations unless values fall below 5 gm/dl; such values are usually observed only in late stages of disease processes.

1. Serum Albumin

The fall in serum albumin concentration from the failure of hepatic parenchymal synthesis is not an early change and therefore is found more commonly in chronic conditions, such as diffuse fibrosis or subacute hepatitis. Since only 12 and 5% of the serum albumin is synthesized per day in the dog and cow, respectively, a longer time lapse is required than for many tests to detect hepatic insufficiency. In portal fibrosis, the characteristic change is usually a diminution in the serum albumin level and an elevation in the γ-globulin level. As might be expected, the changes in albumin level are less conspicuous in acute hepatitis, but an elevation in the γ-globulins is a consistent finding. High β-lipoprotein levels have been observed primarily in biliary obstruction. Apart from liver disease, low albumin levels are found in domestic animals with nephritis, nephrosis, malnutrition, circulatory diseases, deficient protein digestion and absorption from pancreatic disorders, and a host of other chronic diseases resulting in cachexia.

2. Serum Globulins

Although the reasons for the fall in serum albumin level are well known in hepatic diseases, the elevations observed in the serum γ-globulins are only now being clarified. Serum globulin levels can be informative in conjunction with other tests, although they are of little value in detecting liver disease. It has been reported that human patients with liver disease frequently show elevated tiers of antibodies to a variety of gastrointestinal antigens (Trigger, 1976). Investigators have postulated that a greater antibody response may be elicited since antigens are not sequestered by the liver and consequently enter the general circulation. This enhanced antibody production and hyperglobulinemia in chronic liver disease may also partly result from the extrahepatic and intrahepatic shunting of antigens to the general circulation. Increased serum globulin levels have been reported shortly after the surgical production of portacaval anastomosis in animals. Elevated IgM, IgG, and IgA levels are observed in many human patients with biliary cirrhosis, chronic aggressive hepatitis, and alcoholic disease, respectively (Burke, 1975). Endotoxins from gastrointestinal bacteria are known to be absorbed by the liver and may be involved both in producing liver disorders as well as in greater antibody production.

A variety of newly discovered proteins have been measured and found to be elevated in serum of human beings with liver disease. These include the antinuclear, smooth muscle, and mitochondrial antibodies. Antimitochondrial antibodies are present in patients with

primary biliary cirrhosis but not in those with extrahepatic obstruction (Klatskin and Kantor, 1972). Antinuclear antibodies are elevated in the serum in many human disorders such as lupus erythrematosis, other collagen diseases, and chronic aggressive hepatitis (Partonetto, 1973). The radioimmunoassay for α_1-fetoprotein, a tumor marker for primary human hepatocellular carcinoma, has been useful in confirmation and prognostication (Chayvialle and Ganguli, 1973). α_1-Fetoprotein is normally synthesized by embryonal liver cells and secreted into fetal serum during gestation. Anaplastic hepatoma cells revert to secreting this embryonic protein. Another turmor protein, carcinoembryonic antigen, is also elevated in hepatoma and metastatic cancers of the colon (Gold *et al.*, 1973). Other new approaches to diagnosis are aimed at utilizing an immunoassay for the hepatic transport protein ligandin in both serum and urine as a measure of hepatocellular necrosis. Ligandin constitutes 5% of all soluble protein in the liver (Levi *et al.*, 1970). All of these intriguing possibilities must await confirmation through further study.

3. Flocculation Tests

A large number of empirical tests arising as the result of some serum protein abnormality have been extensively used in years past due to their simplicity in diagnosing human liver disease. Since the albumin/globulin ratios may be less than 1.0 in domestic animals, false-positive reactions may occur in these tests since they were developed for use on human sera with albumin/globulin ratios greater than 1.5. The chemical or electrophoretic fractionation of serum proteins is recommended as a more direct method to assess the status of the serum proteins in liver disorders in domestic animals.

4. Amino Acid Tolerance Tests

Deamination rates of injected amino acids such as tyrosine and arginine by the liver have been used as liver function tests in man and animals. In addition, increases in the amino acid concentration in the blood and urine may also be indicative of hepatic failure. Experimental studies have shown that approximately 85% of the effective hepatic parenchyma must be lost before these tests are of diagnostic significance. In infectious hepatitis, increased amounts of cystine in the plasma and urine seem to be quite a sensitive index of hepatic cell failure. Karsai (1954) noted in dogs that deamination was incomplete in hepatic diseases, and this resulted in increases in amino acids in the serum and urine. The presence of tyrosine and leucine crystals in the urine was indicative of both hepatic malfunction and extensive parenchymal destruction. Millon's reagent was used to detect the urinary tyrosine in this study. A positive test was observed only in dogs with severe hepatic dysfunction, and the test was best used to assess the severity of hepatic cell destruction.

Gornall and Bardawill (1952) performed both the tyrosine tolerance test (6 gm to fasted dogs, with postinjection samples hourly for 7 hours) and the arginine tolerance test (1.5 gm to fasted dogs). Fasting normal dogs had blood tyrosine and arginine levels of 1.1–1.9 and 1.6–2.2 mg/100 ml, respectively. Forty-eight hours after the administration of 5 ml of CCl_4, the fasting levels were above 2 mg/100 ml for tyrosine and within the normal range for arginine. The tolerance tests as performed gave little evidence of being useful. Others (Goettsch *et al.*, 1942; Doggart *et al.*, 1958) have confirmed the presence of increased blood and urine levels of various amino acids using paper chromatography in severe liver damage in dogs. Peredereev (1959) found similar results using a glycine tolerance test in cows.

The hippuric acid test is not presently used in canine veterinary medicine since any administered phenol derivatives such as benzoic acid are conjugated primarily by the kidney. Elevations in the serum "phenol body" concentration are diagnostic for renal insufficiency in the dog. A liver function test based on hippuric acid synthesis in cows following sodium benzoate administration was studied by Cerkasov (1959).

C. Blood Uric Acid

In the dog, hepatectomy is followed by an elevation in the level of blood and urine uric acid. The end product of purine metabolism is allantoin in all dogs except the Dalmation, whose incomplete renal tubular reabsorption of uric acid allows for its excretion. A membrane transport defect for this anion accounts for this difference. Since the dog converts uric acid to allantoin in the liver, the elevation of uric acid levels above 1 mg/100 ml in the blood has been recommended as an indicator of liver disease in this species. Normal blood values for the dog are between 0.1 and 1 mg/100 ml (Bloom, 1957). Since methods for accurate uric acid determinations are quite tedious (Brown, 1945), this test has not been used greatly and appears to be of doubtful value for the measurement of liver insufficiency in the dog. Ott (1956) indicated that blood uric acid levels were significantly elevated in hepatocellular jaundice but normal in hemolytic and obstructive jaundice. Malherbe (1959) and Morgan (1959b) concluded that the BSP test is much more sensitive than uric acid determinations in canine hepatopathy. Hoe and Harvey (1961a) showed in extensive studies with dogs that only 75% of clinical cases with the highest ranges of uric acid were dogs suspected of having liver dysfunction. While 100% of jaundiced canine cases showed slight elevations in AP activity, only 86% had elevated uric acid levels.

D. Blood Ammonia

The neuropsychiatric syndrome of hepatic encephalopathy (hepatic coma) is associated with any severe hepatocellular insufficiency or major circulatory bypass of the liver. Normally, NH_3 is absorbed after its production, primarily in the colon, and carried to the liver, where it is converted to urea. Hepatic coma is multifactoral in origin, and there are a number of current biochemical hypotheses regarding its etiology (Cornelius *et al.*, 1975a). It is well known that ammonia concentrations in the peripheral arterial or venous blood and cerebral spinal fluid are usually elevated in hepatic coma. It was observed by Pavlov as early as 1893 that Eck fistula dogs with a portocaval shunt diameter of at least 0.8 cm develop hyperammonemia and neurological signs after being fed meat for 10 days. The importance of blood ammonia in cerebral metabolism is well accepted; however, it cannot be considered the major or only factor involved in the pathogenesis of this complex entity. Hyperammonemia in hepatic coma is associated with decreased acetylcholine synthesis; elevations in inhibitory neurotransmitters, such as α-aminobutyric acid, and decreased α-ketoglutarate levels; accelerated glycolysis, and increased brain lactate levels; and increased bicarbonate flux into the cerebrospinal fluid. All of these may produce profound clinical effects (Gips *et al.*, 1973). Hyperammonemia can also occur due to a lack of urea cycle enzymes, such as a deficiency of hepatic arginosuccinate synthetase, which was recently reported in two dogs without liver disease and associated with blood NH_3 levels as high as 350 μg/dl (Strombeck *et al.*, 1975a).

1. Dog

The mean concentrations of venous and arterial ammonia in normal fasted dogs are 60 and 75 μg/dl, respectively. Blood ammonia values above 120 μg/dl are suspect; levels can commonly be found between 200 and 1000 μg/dl in clinical cases (Strombeck *et al.*, 1975b). Increased ammonia production can occur in a variety of conditions. In hemorrhagic shock, renal shutdown results in inefficient urea excretion; more urea finds its way to the intestine, where bacterial ureases convert it to ammonia. If vomiting has been excessive and results in hypokalemia and alkalosis, ammonia will exist in an ionized form, which is toxic due to more efficient cell penetration. Therapy has been aimed at low protein consumption and the use of neomycin to lower the number of urease-positive bacteria in the gut.

In a study of ten dogs with hepatoencephalopathy (Strombeck *et al.*, 1975b), six dogs had abnormal portosystemic shunts and four dogs had hepatic disease. Since advanced hepatic fibrosis and subsequent portal hypertension can result in shunting, it can either be congenital in younger dogs or secondary to liver disease, which is observed primarily in adult dogs. In young dogs with hepatic coma, probably from inherited shunts, it is common to observe all normal liver function tests except for BSP retention. High blood ammonia levels have been reported by a number of investigators in congenital anomalies of the portal vein (Ewing *et al.*, 1974; Simpson and Hribernik, 1976; Easley and Carpenter, 1975).

Meyer *et al.* (1978) studied the ammonia tolerance test in 20 normal dogs and 6 dogs with portosystemic shunts. The dogs with shunting exhibited a marked rise at 30 minutes in venous blood ammonia of 1049 ± 256 μg/dl as compared to fasting levels of 236 ± 116 μg/dl. Normal dogs exhibited increases at 30 minutes of 156 ± 71 μg/dl as compared to fasting levels of 80 ± 36 μg/dl. In the test, all dogs received 100 mg NH_4Cl per kilogram body weight, with a maximal dosage of 3 gm. The NH_4Cl was dissolved in 20–50 ml warm water and administered orally with a syringe. Fasting blood ammonia levels were not uniformly reliable in detecting which dogs contained portosystemic shunts.

2. Other Species

Hepatic insufficiency associated with central nervous system symptoms was early recognized in the horse (Stenius, 1941; Boiteux, 1958; Innes, 1953; Forenbacher *et al.*, 1959). Spongy degeneration of the central nervous system has been observed in horses, cattle, sheep, pigs, and goats with a variety of hepatic diseases, particularly associated with the ingestion of pyrrolizidine alkaloids (Hooper, 1975). Experimental studies on sheep which were infused intravenously with ammonium acetate confirmed a relationship between hyperammonemia and brain degeneration. It is well known that death can occur as early as 10–60 minutes when ruminants are given toxic amounts of urea (Austin and Briggs, 1966). It has been confirmed that ponies may also die from urea intoxication (450 gm), and this is consistent with a hypothesis that ammonia inhibits α-ketoglutarate decarboxylation. The primary site of bacterial urease activity in the horse is the cecum; it can contain 25% of the enzyme activity found in bovine rumen fluid (Hintz *et al.*, 1970). That hepatic coma and photosensitivity can be produced in the horse or donkey by ligation of the bile duct was confirmed by Cornelius *et al.* (1965b) and Mandani and Adam (1976). Blood levels were elevated to as high as 675 μg/dl (Cornelius *et al.*, 1965b) as compared to control values in three nonfasted normal horses of 113, 167, and 128 μg/dl. Normal

blood ammonia levels have been reported to be below 50 μg/dl in donkeys (Mandani and Adam, 1976).

E. Lipid Metabolism Tests

As early as 1862 (Flint, 1862), attempts were made to differentiate the types of icterus by the levels of cholesterol in the serum. The origin of cholesterol esters in the blood was a constant source of controversy. Thannhauser and Schaber (1926) reported that, in cases of liver disease in man, the serum cholesterol ester values were below those of the free cholesterol. In acute generalized hepatopathy, cholesterol esters levels were greatly depressed or absent. Adler and Lemmel (1928) evaluated this concept on an extensive scale in human subjects with liver disease. Their conclusions were that the esterification of cholesterol with fatty acids is primarily a function of the liver parenchyma. Increases in the serum total cholesterol level in obstructive jaundice may be due to overproduction by the liver (Byers *et al.*, 1951) and not retention of cholesterol normally excreted into the bile (see Chapter 2). Of greatest importance in hepatocellular damage is the ratio of free cholesterol to cholesterol esters in the serum. In both chronic and acute hepatocellular disease, esterification is significantly depressed, giving a higher free cholesterol to ester ratio than observed in the normal mammal.

The hypercholesterolemia of obstructive jaundice has been intensely studied (McIntyre *et al.*, 1975). The massive elevation of the low-density and very low density lipoproteins of the β-globulin fraction in chronic biliary obstruction is well known (Goffman *et al.*, 1954). That certain of these low-density lipoproteins are abnormal in composition was first established convincingly by Eder and co-workers (1955). It has subsequently been found that only one abnormal lipoprotein, named LP-X, makes a major contribution to the increased plasma free cholesterol of obstructive jaundice. In general, one can expect elevations in total cholesterol (primarily free), plasma triglycerides, and lipid phosphorus in the serum of animals with extrahepatic obstruction of the bile ducts. Unfortunately, LP-X is present in the serum in both intrahepatic and extrahepatic cholestasis and is of no value in their differentiation (Ritland, 1975). The test is simple to perform, however, and is reliable and sensitive in the demonstration or exclusion of cholestasis.

Limited data suggest that esterification is also depressed in liver disease of domestic animals (Romagnoli, 1954; Piccotin, 1956; Darraspen *et al.*, 1959; Done *et al.*, 1960). Normal dogs had total cholesterol and cholesterol ester values of 166 ± 32 and 115 ± 70 mg/100 ml, respectively (70 ± 8% as cholesterol ester). Dogs with liver diseases exhibited average total and esterified cholesterol values of 187 and 75 mg/100 ml, respectively (42% as cholesterol ester). This study showed a 40% drop in cholesterol ester in canine hepatopathy (Piccotin, 1956). Hoe and Harvey (1961a) found normal average total serum cholesterol values in kennel dogs and household pets to be 186 ± 14 and 258 ± 36 mg/100 ml, respectively. In both groups the percentage of ester was quite similar at 65 ± 12 and 62 ± 3%, respectively. The diet of household scraps containing a high concentration of cholesterol and other lipids most likely accounted for the higher levels in the household group. It was concluded in this study that, although a dog with a normal ester ratio (60–80%) would most likely not have liver involvement, the converse would not be true. High blood cholesterol levels also occur in such other diseases as hypothyroidism, diabetes mellitus, and advanced nephrosis. The ratio of blood cholesterol to phospholipids in dogs with hepatopathy was also reported by Darraspen *et al.* (1959). A study of

Bass *et al.* (1976) established canine lipid profiles in experimental pancreatitis and hepatic necrosis.

Romagnoli (1954) reported total average blood cholesterol levels of 50 mg/100 ml (range, 24–75 mg/100 ml) in healthy sheep. Sheep with livers affected moderately by *Echinococcus* and *Fasciola* infestations had blood cholesterol levels within this normal range. If livers were severely affected by these parasites, elevated cholesterol values averaged 76 mg/100 ml. Total cholesterol and cholesterol ester levels in 35 normal cattle were observed to be on average 140 and 103 mg/100 ml, respectively (73 ± 6% as cholesterol ester). Fifty-eight cattle with various liver diseases showed a significant decrease in the percentage of cholesterol ester. Total cholesterol and cholesterol ester levels in bovine hepatopathy averaged 161 and 85 mg/100 ml, respectively (54 ± 12% as cholesterol ester) (Piccotin, 1956). Hyperlipemia and hepatic lipidosis are a serious problem in foaling ponies refusing to eat; total serum lipid levels are elevated to 1000–7000 mg/dl as compared to normal levels of 130–990 mg/dl (Schotman and Wagenaar, 1969). The syndrome is often lethal and can be diagnosed many times by gross observation of the bluish hyperlipemic blood sample.

VIII. LIVER BIOPSY AND RADIOGRAPHIC PROCEDURES

A. Liver Biopsy and Laparoscopic Examination

Although liver biopsy is not usually referred to as a liver function test, its omission in this chapter would be a serious oversight. In the study of an animal with known liver disease, the diagnosis may still remain doubtful in spite of careful clinical and laboratory studies. Examination of liver biopsy samples in these cases may prove invaluable. This technique is usually not needed in the differential diagnosis of icterus since clinical history and laboratory tests will usually suffice. However, in certain cases, such as copper storage disease in the Bedlington terrier, biopsy and copper analysis are mandatory.

Liver biopsy is not without risk, particularly in unskilled hands. Complications may include biliary peritonitis from a punctured gallbladder or dilated bile duct; hemorrhage resulting from rupture of a large vessel or a low prothrombin level; hepatitis, pleuritis, or peritonitis from bacterial contamination; and puncture of other viscera. A small biopsy

TABLE VIII

Reference List for Liver Biopsy Methods in Domestic Animals

Species	References
Dog	Romagnoli (1958), Chapman (1965), Van Vleet and Alberts (1968), Dingwall *et al.* (1970), Feldman and Ettinger (1976)
Horse	Wittfogel (1939), Isaksson (1951), Konrad (1958), Wolff *et al.* (1967)
Swine	Jones *et al.* (1956), Kruif (1974)
Sheep	Dick (1952), Hamilton (1957), Phillippo (1973)
Fowl	Grueul (1959)
Cattle	Udall *et al.* (1952), Whitehair *et al.* (1952), Seghetti and Marsh (1953), Simesen and Moller (1959), Nikov (1960), Schultz *et al.* (1960)
Mink	Konrad *et al.* (1976)

sample may not reflect the status of the whole organ unless the lesions are uniformly of a diffuse nature. Laparoscopic examination has recently been used to either confirm pancreatic disorders or to obtain both visual observations and tissue from the liver (Dalton and Hill, 1972). The procedure is simple, and general anesthesia is not essential. This technique has certain advantages over biopsy and laparotomy and will no doubt be used more extensively in the future. References to be consulted for methods of liver biopsy in various domestic animals are presented Table VIII.

B. Radiographic Procedures

Angiographic studies on the liver and biliary system are becoming very important in veterinary medicine. No attempt is made to cover these important techniques in this chapter, and the reader should consult standard procedures described in veterinary radiological and X-ray tests. The recently observed existence of portosystemic shunts in dogs requires the use of special radiographic techniques for definitive diagnosis and rational therapy. In addition to abnormal BSP retention and high blood ammonia levels in these cases, angiographic and venographic techniques will identify which anomalous communication exists between the portal vein and the systemic circulation (L. M. Cornelius *et al.*, 1975; Ewing *et al.*, 1974).

REFERENCES

Acocella, G., Tenconi, L. T., Armas-Merino, R., Raia, S., and Billing, B. H. (1968). *Lancet* , 68.
Adam, S. E. I., Oberd, H. M. A., and Artour, G. (1974). *Acta Vet (Brno)* **43,** 225.
Adler, A., and Lemmel, H. (1928). *Dtsch. Arch. Klin. Med.* **158,** 173.
Agergaard, N., and Larsen, B. (1974). *Anim. Blood Groups Biochem. Genet.* **5,** 11.
Ahmed, A. A. S., El-Abdin, Y. Z., Hamza, S., and Saad, F. R. (1975). *Avian Dis.* **19,** 305.
Aktan, V. F. (1954). *Monatsh. Veterinaermed.* **9,** 97.
Alemu, P., Forsyth, G. W., and Searcy, G. P. (1977). *Can. J. Comp. Med.* **41,** 420.
Alexander, F., and McDonald, D. C. (1960). *Q. J. Exp. Physiol. Cogn. Med. Sci.* **45,** 12.
Allcroft, W. M., and Folley, S. J. (1941). *Biochem. J.* **35,** 254.
Allen, J. R., and Carstens, L. A. (1968). *Am. J. Vet. Res.* **29,** 1681.
Allen, J. R., Carstens, L. A., and Katagiri, G. J. (1969). *Arch. Pathol.* **87,** 279.
Anderson, D. R. (1966). *Am. J. Vet. Res.* **27,** 1484.
Anderson, P. H., Berrett, S., Brush, P. J., Hebert, C. N., Parfitt, J. W., and Patterson, D. S. P. (1977). *Vet. Rec.* **43,** 100.
Anonymous, Atlas Newsletter, Small Animal Digest (1959). *Vet. Med.* **54,** 307.
Answer, M. S., Engelking, L. R., Gronwall, R., and Klentz, R. D. (1976). *Res. Vet. Sci.* **20,** 127.
Arendarcik, J. (1959). *Vet. Cas.* **8,** 515.
Armstrong, A. R., King, E. J., and Harris, R. I. (1934). *Can. Med. Assoc. J.* **31,** 14.
Austin, J., and Briggs, M. H. (1966). *Agric. Vet. Chem.* **7,** 104.
Baker, P. R., Rigby, S. M., and Cuschieri, A. (1974). *Res. Vet. Sci.* **17,** 64.
Barrett, P. V. D., Berk, P. D., Menken, M., and Berlin, N. I. (1968). *Ann. Intern. Med.* **68,** 355.
Bass, D., Hoffman, W. E., and Dorner, J. L. (1976). *Am. J. Vet. Res.* **37,** 1355.
Bauer, R. (1906). *Wien. Med. Wochenschr.* **56,** 2537.
Beijers, J. A. (1923). Proefschrift, Univ. of Utrecht, Utrecht.
Beijers, J. A., Van Loghem, J. J., and Van der Hart, M. (1950). *Tijdschr. Diergeneeskd.* **75,** 955.
Bellinger, C. R. (1973). *Aust. Vet. J.* **49,** 298.
Bendixen, H. C., and Carlström, B. (1928). *Maanedsskr. Dyrlaeg.* **40,** 129.
Benedek, G. (1961). *Acta Vet. Acad. Sci. Hung.* **11,** 293.
Benndorf, E. (1955). Inaug. Diss., Univ. of Leipzig, Leipzig.

Berger, H. J. (1956). *Zentralbl. Veterinaermed.* **3,** 273.
Bicknell, E. J., Brooks, R. A., Osburn, J. A., and Whitehair, C. K. (1967). *Am. J. Vet. Res.* **28,** 943.
Bide, R. W., and Dorward, W. J. (1974). *Can. J. Anim. Sci.* **55,** 23.
Bierthen, E. (1906). Inaug. Diss., Univ. of Bern, Bern.
Bloom, F. (1957). *North Am. Vet.* **38,** 17.
Boiteux, R. D. (1958). *Rev. Mil. Remonta Vet. Rio de Janeiro* **17,** 13.
Bokori, J., and Karsai, F. (1969). *Acta Vet. Acad. Sci. Hung.* **19,** 269.
Borthwick, R., MacKenzie, C. P., Lewis, N. D., and Gregor, W. W. (1973). *Vet. Rec.* **92,** 356.
Boyd, J. W. (1962). *Res. Vet. Sci.* **3,** 256.
Braun, J. P., Rico, A. G., Benard, P., and Burgat-Sacage, V. (1977). *Ann. Biol. Clin.* **35,** 433.
Brobst, D. F., and Schall, W. D. (1972). *J. Am. Vet. Med. Assoc.* **161,** 382.
Brooks, F. P., Deneau, G. A., Potter, H. P., Reinhold, J. G., and Norris, R. F. (1963). *Am. J. Gastroenterol.* **44,** 287.
Brown, H. (1945). *J. Biol. Chem.* **158,** 601.
Brown, J. M. M. (1965). *S. Afr. Dep. Agric. Tech. Serv. Tech. Commun.* No. 35.
Burke, M. D. (1975). *Hum. Pathol.* **6,** 273.
Burns, K. F., Ferguson, F. G., and Hampton, S. H. (1967). *Am. J. Clin. Pathol.* **48,** 484.
Byers, S. O., Friedman, M., and Michaelis, F. (1951). *J. Biol. Chem.* **188,** 637.
Cabaud, P., Leeper, R., and Wroblewski, F. (1956). *Am. J. Clin. Pathol.* **26,** 1101.
Campbell, J. G. (1957). *J. Endocrinol.* **15,** 339.
Cantarow, A., and Stewart, L. (1935). *Am. J. Pathol.* **11,** 561.
Caple, I. W., and Vandergraaff, R. (1976). *Aust. Vet. J.* **52,** 192.
Cerkasov, D. P. (1959). *Sb. Cesk. Akad. Zemed. Ved, Vet. Med.* **4,** 97.
Chaffee, V. W., Edds., G. T., Himes, J. A., and Neal, F. C. (1969). *Am. J. Vet. Res.* **30,** 1737.
Chapman, W. L. (1965). *J. Am. Vet. Med. Assoc.* **146,** 126.
Chayvialle, J. A. P., and Ganguli, P. C. (1973). *Lancet* **I,** 1355.
Chernyak, N. Z., and Rozhov, D. I. (1953). *Sb. Tr. Leningr. Nauchno-Issled. Vet. Inst.* **5,** 81.
Clarkson, M. J. (1961). *Res. Vet. Sci.* **2,** 143.
Clarkson, M. J., and Richard, T. G. (1967). *Res. Vet. Sci.* **8,** 48.
Cokala, S., and Bieniek, K. (1975). *Zentralbl. Veterinaermed. Reihe A* **22,** 605.
Cole, P. G., and Lathe, G. H. (1953). *J. Clin. Pathol.* **6,** 99.
Cole, P. G., Lathe, G. H., and Billings, B. H. (1954). *Biochem. J.* **57,** 514.
Cornelius, C. E. (1957). *Gaines Symp., 7th, Kankakee, Ill.* p.20.
Cornelius, C. E. (1958). *Iowa State Coll. Vet.* **20**(3), 155.
Cornelius, C. E. (1961). *Cornell Vet.* **51,** 559.
Cornelius, C. E. (1963). *Nature (London)* **200,** 580.
Cornelius, C. E. (1970). "Progress in Liver Disease," vol. 3, Grune and Stratton, p. 419.
Cornelius, C. E., and Freedland, R. A. (1962). *Cornell Vet.* **52,** 344.
Cornelius, C. E., and Gronwall, R. R. (1968). *Am. J. Vet. Res.* **29,** 291.
Cornelius, C. E., and Kaneko, J. J. (1960). *J. Am. Vet. Med. Asoc.* **137,** 62.
Cornelius, C. E., and Wheat, J. D. (1957). *Am. J. Vet. Res.* **18,** 369.
Cornelius, C. E., Theilen, G. H., and Rhode, E. A. (1958a). *Am. J. Vet. Res.* **19,** 560.
Cornelius, C. E., Holm, L. W., and Jasper, D. E. (1958b). *Cornell Vet.* **48,** 305.
Cornelius, C. E., Bishop, J. A., Switzer, J., and Rhode, E. A. (1959). *Cornell Vet.* **49,** 116.
Cornelius, C. E., Kilgore, W. W., and Wheat, J. D. (1960). *Cornell Vet.* **50,** 47.
Cornelius, C. E., Douglas, G. M., Gronwall, R. R., and Freedland, R. A. (1963). *Cornell Vet.* **53,** 181.
Cornelius, C. E., Arias, I. M., and Osburn, B. I. (1965a). *J. Am. Vet. Med. Assoc.* **146,** 709.
Cornelius, C. E., Gazmuri, G., Gronwall, R. R., and Rhode, E. A. (1965b). *Cornell Vet.* **55,** 110.
Cornelius, C. E., Himes, J. A., and Gronwall, R. R. (1975a). *Methods Achie. Exp. Pathol.* **7,** 22.
Cornelius, C. E., Kelley, K. C., and Himes, J. A. (1975b). *Cornell Vet.* **65,** 90.
Cornelius, C. E., Olsen, G. A., and Noonan, N. E. (1977). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36,** 5.
Crawley, G. J., and Swenson, M. J. (1965). *Am. J. Vet. Res.* **26,** 1468.
Crigler, J. E., and Najjar, V. A. (1952). *Pediatrics* **10,** 169.
Cysewski, S. J., Pier, A. C., Engstrom, C. W., Richard, J. L., Dougherty, R. W., and Thurston, J. R. (1968). *Am. J. Vet. Res.* **29,** 1577.
Dal Santo, F. (1959). *Acta Med. Vet.* **5,** 579.

Dalton, J. R. F., and Hill, F. W. G. (1972). *J. Small Anim. Pract.* **13,** 527.
Darraspen, E., Floria, R., Cottereau, P., Lescure, F., and Campredon, G. (1959). *Rev. Pathol. Gen. Comp.* **59,** 1349.
Deinhardt, F., and Deinhardt, J. (1966). *Symp. Zool. Soc. London* **17,** 127.
Deinhardt, F., Courtois, G., Dherte, P., Osterrieth, P., Nenane, G., Henle, G., and Henle, W. (1962). *Am. J. Hyg.* **75,** 311.
Deinhardt, F., Holmes, A. W., Capps, R. B., and Popper, H. (1967). *J. Exp. Med.* **125,** 673.
Delprat, G. D. (1923). *AMA Arch. Intern. Med.* **32,** 401.
de Schepper, J., and van der Stock, J. (1971). *Experientia* **27,** 1264.
de Schepper, J., and van der Stock, J. (1972). *Arch. Int. Physiol. Biochim.* **80,** 279.
Dhumeaux, D., and Berthelot, P. (1975). *Gastroenterology* **69,** 988.
Dick, A. T. (1952). *Aust. Vet. J.* **28,** 234.
Dingwall, J. S., Boer, J., and Archibald, J. (1970). *J. Small Anim. Pract.* **7,** 429.
Dittrich, C., Stockl, W., and Desser, H. (1974). *Zentralbl. Veterinaermed., Reihe A* **21,** 165.
Doggart, J. R., McCredie, J. A., and Welbourn, R. B. (1958). *Vet. Rec.* **70,** 279.
Done, J., Mortimer, P. H., and Taylor, A. (1960). *Res. Vet. Sci.* **1,** 76.
Dotta, V., and Abate, O. (1973). *Nuova Vet.* **49,** 14.
Drill, V. A., and Ivy, A. C. (1944). *J. Clin. Invest.* **23,** 209.
Dubin, I. N., and Johnson, F. B. (1954). *Medicine (Baltimore)* **33,** 155.
Ducci, H., and Watson, C. J. (1945). *J. Lab. Clin. Med.* **30,** 293.
Dukes, H. H. (1946). "The Physiology of Domestic Animals." Cornell Univ. Press (Comstock), Ithaca, New York.
Easley, J. C., and Carpenter, J. L. (1975). *J. Am. Vet. Med. Assoc.* **166,** 167.
Eder, H. A., Russ, E. M., Rees Pritchett, R. A., Wilbur, M. M., and Barr, D. P. (1955). *J. Clin. Invest.* **34,** 1147.
Egan, P. S. A. J. (1978). *Ir. Vet. J.* **32,** 89.
Egyed, M. N., Malkinson, M., and Scholsberg, A. (1974). *Avian Pathol.* **3,** 79.
Ehrlich, I., Forenbacher, S., Rijavec, M., and Kurelac, B. (1960). *Vet. Arh.* **30,** 229.
Eikmeier, H. (1959). *Berl. Muench. Tieraerztl. Wochenschr.* **72,** 48.
Etter-Kjelsaas, H., and Kuenzle, C. C. (1975). *Biochim. Biophys. Acta* **400,** 83.
Everett, R. M., Duncan, J. R., and Prasse, K. W. (1977). *Am. J. Vet. Res.* **38,** 963.
Ewing, G. O., Suter, P. F., and Bailey, C. M. (1974). *J. Am. Anim. Hosp. Assoc.* **10,** 463.
Feldman, E. C., and Ettinger, S. J. (1976). *J. Am. Vet. Med. Assoc.* **169,** 805.
Fellinger, K., and Menkes, K. (1933). *Wien. Klin. Wochenschr.* **46,** 133.
Ferriera Neto, J. M. (1958). Thesis, Cornell Univ., Ithaca, New York.
Fevery, J., Van De Vijner, M., Michiels, R., and Heirwegh, K. (1977). *Biochem. J.* **164,** 737.
Finn, J. P., and Tennant, B. (1974). *Cornell Vet.* **64,** 136.
Flint, A. (1862). *Am. J. Med. Sci.* **44,** 305.
Flukiger, M., Althaus, V., and Strebell, H. M. (1977). *Zentralbl. Veterinaermed., Reihe A* **24,** 496.
Forbes, T. J., and Singleton, A. G. (1966). *Br. Vet. J.* **122,** 55.
Ford, E. J. H. (1955). *Vet. Rec.* **67,** 634.
Ford, E. J. H. (1958). *J. Anat.* **92,** 447.
Ford, E. J. H. (1974). *J. Comp. Pathol.* **84,** 231.
Ford, E. J. H., and Gopinath, C. (1974). *Res. Vet. Sci.* **16,** 186.
Ford, E. J. H., and Gopinath, C. (1976). *Res. Vet. Sci.* **21,** 12.
Ford, E. J. H., and Ritchie, H. E. (1968). *J. Comp. Pathol.* **78,** 207.
Forenbacher, S. (1957). *Vet. Arh.* **27,** 185.
Forenbacher, S., Marzan, B., and Topolnik, E. (1959). *Vet. Arh.* **29,** 322.
Fowler, J. S. L. (1971). *Br. Vet. J.* **237,** 304.
Fox, I. J., Brooker, L. G. S., Heseltine, D. W., Essex, H. E., and Wood, E. H. (1957). *Proc. Staff Meet. Mayo Clin.* **32,** 478.
Frankl, H. D., and Merritt, J. H. (1959). *Am. J. Gastroenterol.* **31,** 166.
Freedland, R. A., Hjerpe, C. A., and Cornelius, C. E. (1965). *Res. Vet. Sci.* **6,** 18.
Freeman, S., Chen, Y. P., and Ivy, A. C. (1938). *J. Biol. Chem.* **124,** 79.
Freese, U. (1952). Inaug. Diss., Tierärztliche Hochschule, Hannover.
Frosch, B., Wagener, H., and Immich, H. (1968). *Clin. Chim. Acta* **22,** 294.

Fulop, M., and Brazeau, P. (1970). *Am. J. Dig. Dis.* **15,** 1067.
Gaisford, W. D., and Zuidema, G. D. (1965). *J. Surg. Res.* **5,** 220.
Gardiner, M. R., and Parr, W. H. (1967). *J. Comp. Pathol.* **77,** 51.
Garner, R. J. (1952a). *J. Comp. Pathol. Ther.* **62,** 306.
Garner, R. J. (1952b). *J. Comp. Pathol. Ther.* **62,** 292.
Garner, R. J. (1953). *J. Comp. Pathol. Ther.* **63,** 247.
Gartner, L. M., Lane, D. L., and Cornelius, C. E. (1971). *Am. J. Physiol.* **220,** 1528.
Gips, C. H., Hendfelt, B., Holmin, T., and Kardel, T. (1973). *Scand. J. Gastroenterol.* **8,** 385.
Glaser, W., Gibbs, W. D., and Andrews, G. A. (1959). *J. Lab. Clin. Med.* **54,** 556.
Goetgheluck, J. C. (1970). Thesis, École Natl. Vet. Alfort,
Goettsch, E., Lyttle, J. D., Grim, W. M., and Dunbar, P. (1942). *J. Biol. Chem.* **144,** 121.
Goffman, J. W., Delalla, O., Glazier, F., Freeman, N. K., Lindgren, F. T., Nichols, A. V., Strisower, B., and Tamplin, A. R. (1954). *Plasma* **2,** 413.
Gold, P., Wilson, I., Romero, R., Shuster, J., and Freedman, S. O. (1973). Dis. Col. Rect. **16,** 358.
Gopinath, C., and Ford, E. J. H. (1972). *Vet. Pathol.* **9,** 99.
Gopinath, C., and Ford, E. J. H. (1973). *J. Pathol.* **110,** 333.
Gopinath, C., Jones, R. S., and Ford, E. J. H. (1970). *J. Pathol.* **102,** 107.
Gornall, A. G., and Bardawill, C. J. (1952). *Can. J. Med. Sci.* **30,** 256.
Gradwohl, R. B. H. (1956). "Clinical Laboratory Methods and Diagnosis, A Testbook on Laboratory Procedures, with Their Interpretation," 5th ed., 2 vols. Mosby, St. Louis, Missouri.
Grassnickel, V. H. (1959). *Berl. Muench. Tieraerztl. Wochenschr.* **72,** 444.
Greene, C. E., Barsanti, J. A., and Jones, B. D. (1977). *Cornell Vet.* **67,** 29.
Grenn, H. H., and Bulmer, W. S. (1972). *Can. Vet. J.* **13,** 285.
Grodsky, G. M., and Carbone, J. V. (1957). *J. Biol. Chem.* **226,** 449.
Grodsky, G. M., Carbone, J. V., and Fanska, R. (1959). *J. Clin. Invest.* **38,** 1981.
Gronwall, R. (1970). *Am. J. Vet. Res.* **31,** 2131.
Gronwall, R., and Mia, A. S. (1969). *Physiologist* **12,** 241.
Grueul, E. (1959). *Dtsch. Tieraerztl. Wochenschr.* **65,** 437.
Gunn, C. H. (1938). *J. Hered.* **29,** 137.
Hamilton, F. J. (1957). *Aust. Vet. J.* **33,** 273.
Hamilton, J. M., Cornwell, H. J. C., McCusker, H. B., Campbell, R. S. F., and Henderson, J. J. W. P. (1966). *Br. Vet. J.* **122,** 255.
Hamilton, M. (1977). *J. Clin. Pathol.* **30,** 454.
Hansen, M. A. (1964). *Nord. Veterinaermed.* **16,** 323.
Hansen, M. A. (1972). *Acta Vet. Scand.* **13,** 96.
Hardy, R. M., Stevens, J. B., and Stowe, C. M. (1975). *Minn. Vet.* **15,** 13.
Hartwell, W. V., Kimbrough, R. D, and Love, G. J. (1968). *Am. J. Vet. Res.* **29,** 1449.
Harvey, D. G. (1967). *J. Small Anim. Pract.* **8,** 557.
Harvey, D. G., and Hoe, C. M. (1971). *Vet. Rec.* **29,** 562.
Harvey, D. G., and Obeid, H. M. A. (1974). *Br. Vet. J.* **130,** 544.
Heidrich, K. M. (1954). Inaug. Diss., Tierärztliche Hochschule, Hannover.
Hepler, O. E. (1949). "Manual of Clinical Laboratory Methods," 4th ed., p. 110. Thomas, Springfield, Illinois.
Himes, J. A., and Cornelius, C. E. (1973). *Cornell Vet.* **63,** 424.
Hintz, H. F., Lowe, J. E., Clifford, A. J., and Visek, W. J. (1970). *J. Am. Vet. Med. Assoc.* **157,** 963.
Hoe, C. M. (1961). *Vet. Rec.* **73,** 153.
Hoe, C. M., and Harvey, D. G. (1961a). *J. Small Anim. Pract.* **2,** 22.
Hoe, C. M., and Harvey, D. G. (1961b). *J. Small Anim. Pract.* **2,** 109.
Hoe, C. M., and Jabara, A. G. (1967). *J. Comp. Pathol.* **77,** 245.
Hoe, C. M., and O'Shea, J. D. (1965). *Vet. Rec.* **77,** 1164.
Hoerlein, B. F., and Greene, J. E. (1950). *North Am. Vet.* **31,** 662.
Holmes, A. W., Passovoy, M., and Capps, R. B. (1967). *Lab. Anim. Care* **17,** 41.
Holmes, J. R. (1960). *Cornell Vet.* **50,** 308.
Hooper, P. T. (1975). *Acta Neuropathol.* **31,** 343.
Humlicek, O., and Kruska, K. (1960). *Sb. Vys. Sk. Zemed. Brne, Rada B* **8,** 16.
Hunt, S. E., and McCosker, P. J. (1967). *Clin. Chim. Acta* **18,** 133.

Hunton, D. B., Bollman, J. L., and Hoffman, H. N. (1960). *Proc. Staff Meet. Mayo Clin.* **35,** 752.
Ikeda, S., Yamaoka, S., Uratanable, H., Watanabe, H., and Kameya, T. (1976). *Exp. Rep. Equine Health Lab.* No. 13, 1.
Innes, J. R. M. (1953). *North Am. Vet.* **34,** 29.
Isaksson, A. (1951). *J. Am. Vet. Med. Assoc.* **118,** 320.
Jasper, D. E. (1947). Ph.D. Thesis, Univ. of Minnesota, Minneapolis.
Jones, E. W., Ulrey, D. E., and Gallup, W. D. (1956). *Cornell Vet.* **46,** 360.
Kaplan, M. (1972). *N. Engl. J. Med.* **286,** 200.
Kaplan, M., and Righetti, A. (1970). *J. Clin. Invest.* **49,** 508.
Karbach, Y. I., and Rybak, P. H. (1973). *Ukr. Biokhem. Zh.* **45,** 477.
Karmen, A. (1955). *J. Clin. Invest.* **34,** 131.
Karsai, F. (1954). *Magy. Allatorv. Lapja* **9,** 271.
Karsai, F. (1960). *Acta Vet. Acad. Sci. Hung.* **10,** 263.
Katz, S., Gilardoni, A., Genovese, N., Wikinski, R. W., Cornelius, C. E., and Malinow, M. R. (1968). *Lab. Anim. Care* **18,** 626.
Keller, P. (1971). *Schweiz. Arch. Tierheilkd.* **113,** 615.
Kelly, D. F., Baggott, D. G., and Gaskell, C. J. (1975). *J. Small Anim. Pract.* **16,** 163.
Ketterer, S. G., Wiegand, B. D., and Rapaport, E. (1960). *Am. J. Physiol.* **199,** 481.
Klaassen, C. D. (1976). *Toxicol. Appl. Pharmacol.* **38,** 85.
Klatskin, G., and Kantor, F. S. (1972). *Ann. Intern. Med.* **77,** 533.
Klaus, H. (1958). *Arch. Exp. Veterinaermed.* **12,** 725.
Konrad, J. (1958). *Veterinarstvi* **8,** 714.
Konrad, J., Hanak, J., and Mouka, J. (1976). *Kleintier-Prax.* **21,** 41.
Koplowitz, N., Kok, E., and Javitt, N. B. (1973). *J. Am. Med. Assoc.* **225,** 292.
Kruif, J. M. (1974). *Dtsch. Tieraerztl. Wochenschr.* **81,** 10.
Krushak, D. H., and Hartwell, W. V. (1968). *J. Am. Vet. Med. Assoc.* **153,** 866.
Kutas, F., and Karsai, F. (1961). *Acta Vet. Acad. Sci. Hung.* **11,** 277.
La Croix, J. A., and Pulley, L. T. (1974). *J. Am. Anim. Hosp. Assoc.* **10,** 55.
Larson, E. J., and Morrill, C. C. (1960). *Am. J. Vet. Res.* **21,** 949.
Leaver, D. D. (1968). *Res. Vet. Sci.* **9,** 265.
Lees, P., Mullen, P. A., and Tavenor, W. D. (1973). *Br. J. Anaesth.* **45,** 570.
Lester, R., and Troxler, R. F. (1969). *Gastroenterology* **56,** 143.
Lettow, E. (1961). *Zentralbl. Veterinaermed.* **8,** 353.
Levi, A. J., Gatmaitan, Z., and Arias, I. M. (1970). *N. Engl. J. Med.* **283,** 1136.
Lichtman, S. S. (1953). "Disease of the Liver, Gallbladder and Bile Ducts," Vol. 1. Lea & Febiger, Philadelphia, Pennsylvania.
Lind, C. W., Gronwall, R. R., and Cornelius, C. E. (1967). *Res. Vet. Sci.* **8,** 280.
Litchfield, M. H., and Gartland, C. J. (1974). *Toxicol. Appl. Pharmacol.* **30,** 117.
Lopez Garcia, A., Zelasco, J. F., and Pedace, E. A. (1943). *An. Inst. Invest. Fis. Apl. Pathol. Hum. (Buenos Aires)* **5,** 13.
Lopuchovsky, J., and Jantosovic, J. (1958). *Vet. Cas.* **7,** 327.
Lupke, H. (1965). *Nord. Veterinaermed.* **17,** 467.
McDaniel, L. S., and Chute, H. L. (1961). *Am. J. Vet. Res.* **22,** 99.
McIntyre, N., Harry, D. S., and Pearson, A. J. G. (1975). *Gut* **16,** 379.
MacPherson, A., and Hemingway, R. G. (1969). *Br. Vet. J.* **125,** 213.
Magnemat, P. (1973). *Helv. Med. Acta* **37,** 175.
Malherbe, W. D. (1959). *J. S. Afr. Vet. Med. Assoc.* **30,** 113.
Malherbe, W. D. (1960). *J. S. Afr. Vet. Med. Assoc.* **31,** 159.
Malherbe, W. D. (1965a). *J. S. Afr. Vet. Med. Assoc.* **36,** 569.
Malherbe, W. D. (1965b). *J. S. Afr. Vet. Med. Assoc.* **36,** 179.
Malherbe, W. D., Kellerman, T. S., Kriek, N. P. J., and Haupt, W. H. (1977). *Onderstepoort J. Vet. Res.* **44,** 29.
Malloy, H. T., and Evelyn, K. A. (1937). *J. Biol. Chem.* **119,** 481.
Mandani, M. O. K., and Adam, S. E. I. (1976). *J. Comp. Pathol.* **86,** 539.
Mann, G. V., Watson, P. L., and Adams, L. (1952). *J. Nutrition* **47,** 213.
Markiewicz, K., Kuleta, Z., and Romanrick, K. (1975). *Acta Parasitol. Pol.* **23,** 177.

Maruffo, C. A., Malinow, M. R., Depaoli, J. R., and Katz, S. (1966). *Am. J. Pathol.* **49,** 455.
Metzger, E. (1927). *Arch. Pathol. Anat. Physiol.* **263,** 703.
Meyer, D. J., Strombeck, D. R., Stone, E. A., Zenoble, R. D., and Buss, D. D. (1978). *J. Am. Vet. Med. Assoc.* **173,** 377.
Mia, A. S., and Koger, H. D. (1978). *Am. J. Vet. Res.* **39,** 1381.
Mia, A. S., Gronwall, R. R., and Cornelius, C. E. (1970). *Proc. Soc. Exp. Biol. Med.* **133,** 955.
Millar, K. R., Hodges, R. T., Sheppard, A. D., and Hammington, M. W. (1977). *N.Z. Vet. J.* **25,** 203.
Mills, M. A., and Dragstedt, C. A. (1938). *AMA Arch. Intern. Med.* **62,** 216.
Minette, H. P., and Shaffer, M. F. (1968). *Am. J. Trop. Med. Hyg.* **17,** 202.
Mixner, J. P., and Robertson, W. G.)1957). *J. Dairy Sci.* **40,** 914.
Montemagno, F. (1954). *Atti Soc. Ital. Sci. Vet.* **8,** 667.
Moore, W. E., and Feldman, B. F. (1974). *J. Am. Anim. Hosp. Assoc.* **10,** 420.
Morgan, H. C. (1959a). *J. Am. Vet. Med. Assoc.* **135,** 412.
Morgan, H. C. (1959b). *Am. J. Vet. Res.* **20,** 372.
Mortimer, P. H. (1962). *Res. Vet. Sci.* **3,** 269.
Moses, C., Critchfield, F. H., and Thomas, T. B. (1948). *J. Lab. Clin. Med.* **33,** 448.
Müller, L. F. (1960). *Zentralbl. Veterinaermed.* **7,** 183.
Muzzo, J. P. (1949). *Rev. Fac. Med. Vet. Univ. Nacl. Mayor San Marcos* **4,** 9.
Nagode, L. A., Frajola, W. J., and Loeb, W. F. (1966). *Am. J. Vet. Res.* **27,** 1385.
Naunyn, B. (1868). *Arch. Anat., Physiol. Wiss. Med.* p. 401.
Nielsen, I. M. (1952). *Nord. Veterinaermed.* **4,** 1192.
Nikov, S. I. (1960). *Monatsh. Veterinaermed.* **15,** 379.
Nonnenbruch, W. (1919). *Mitt. Grenzgeb. Med. Chir.* **31,** 470.
Noonan, N., and Meyer, D. (1979). Unpublished observations, Univ of Florida, Gainesville.
Olsen, R. E., and Phillips, T. E. (1966). *J. Am. Vet. Med. Assoc.* **149,** 400.
Orlowski, M. (1963). *Arch. Immunol. Ther. Exp.* **11,** 1.
Ott, R. L. (1956). *Mich. State Coll. Vet.* **16,** 169, 195.
Partonetto, F. (1973). *Postgrad. Med.* **53,** 156.
Pena, del al A., and Goldzieher, J. W. (1967). *In* "The Baboon in Medical Research" (H. Vagthorg, ed.), Vol. 2, pp. 379, Univ. of Texas Press, Austin.
Peredereev, N. I. (1959). *Tr. Mosk. Vet. Akad.* **25,** 98.
Petrovic, M. (1958). *Acta Vet. (Belgrade)* **8,** 101.
Pfeiffer, A. (1970). *Zentralbl. Veterinaermed. Reihe A* **17,** 157.
Phillippo, M. A. (1973). *J. Agric. Sci.* **80,** 313.
Philp, J. R., Grodsky, G. M., and Carbone, J. V. (1961). *Am. J. Physiol.* **200,** 545.
Piccotin, G. (1956). *Clin. Vet.* **79,** 129.
Pridgen, W. A. (1967). *Lab. Anim. Care* **17,** 463.
Prinz, W., and Fiesel, A. (1957). *Z. Tierernaehr. Futtermittelkd.* **12,** 170.
Rico, A. G., Braun, J. P., and Bernard, P. (1977a). *J. Dairy Sci.* **60,** 1283.
Rico, A. G., Braun, J. P., El Hassan, A. A., and Cazieux, A. (1977b). *Equine Vet. J.* **9,** 100.
Ritland, S. (1975). *Scand. J. Gastroenterol.* **10,** 785.
Robbins, G., Thornton, J., and Mills, J. (1977). *J. Am. Anim. Hosp. Assoc.* **13,** 55.
Roberts, H. E. (1968). *Br. Vet. J.* **124,** 433.
Robertson, W. G., Mixner, J. P., Bailey, W. W., and Lennon, H. D., Jr. (1957). *J. Dairy Sci.* **40,** 977.
Rogers, W. A. (1976). *J. Am. Vet. Med. Assoc.* **168,** 934.
Rogers, W. A., and Ruebner, B. H. (1977). *J. Am. Vet. Med. Assoc.* **170,** 603.
Romagnoli, A. (1954). *Atti Soc. Ital. Sci. Vet.* **8,** 685.
Romagnoli, A. (1958). *Atti Soc. Ital. Sci. Vet.* **12,** 715.
Rosenblum, L. A. and Cooper, R. W. (1968). "The Squirrel Monkey." Academic Press, New York.
Rosenthal, F., and Meier, K. (1921). *Arch. Exp. Pathol. Pharmakol.* **91,** 246.
Rosenthal, S. M., and White, E. C. (1925). *J. Am. Med. Assoc.* **84,** 1112.
Ross, D. B. (1966). *Br. Vet. J.* **122,** 279.
Roussel, J. D., and Stallcup, O. T. (1966). *Am. J. Vet. Res.* **27,** 1527.
Rowntree, L. G., Hurwitz, S. H., and Bloomfield, A. L. (1913). *Bull. Johns Hopkins Hosp.* **24,** 327.
Royer, M. (1943). "La Urobilina en el Estado Normal y Batologico," 2nd ed. El Ateneo, Buenos Aires.
Royer, M., and Biasotti, A. (1932). *C. R. Soc. Biol.* **111,** 409.

Sadun, E. H., Williams, J. S., and Martin, L. K. (1966). *Mil. Med.* **131,** 1094.
Sastry, M. R. S. (1945). *Indian Vet. J.* **21,** 350.
Sborov, V. M., Jay, A. R., and Watson, C. J. (1951). *J. Lab. Clin. Med.* **37,** 52.
Schall, D., Chapman, W. L., Finco, D. R., Griener, P., Mather, G. W., Rosin, E., and Welser, J. (1973). *J. Am. Vet. Med. Assoc.* **163,** 469.
Schlotthauer, C. F. (1945). *North Am. Vet.* **26,** 349.
Schmid, R. (1956). *Science* **124,** 76.
Schmid, R., Hammaker, L., and Axelrod, J. (1957). *Arch. Biochem. Biophys.* **70,** 285.
Schotman, A. J. H., and Wagenaar, G. (1969). *Zentralbl. Veterinaermed.* **16,** 1.
Schultz, J. A., Rossow, N., and Urbaneck, D. (1960). *Monatsh. Veterinaermed.* **15,** 257.
Schwartz, S., and Bracho, M. (1972). *Am. J. Vet. Res.* **33,** 2481.
Seawright, A. A., and Francis, J. (1971). *Aust. Vet. J.* **47,** 91.
Seghetti, L., and Marsh, H. (1953). *Am. J. Vet. Res.* **14,** 9.
Sen, M. M., Rahman, A., and Mia, A. S. (1976). *Indian Vet. J.* **53,** 504.
Sewell, M. M. H. (1966). *Vet. Rec.* **78,** 98.
Shaw, F. D. (1974). *Aust. Vet. J.* **50,** 277.
Shaw, J. N. (1933). *J. Am. Vet. Med. Assoc.* **82,** 199.
Sher, P. P. (1977). *Clin. Chem.* **23,** 627.
Simesen, M. G., and Moller, T. (1959). *Nord. Veterinaermed.* **11,** 787.
Simesen, M. G., and Nansen, P. (1974). *Acta Vet. Scand.* **15,** 239.
Simesen, M. G., Neilsen, K., and Nansen, P. (1973). *Res. Vet. Sci.* **15,** 32.
Simpson, S. T., and Hribernik, T. N. (1976). *J. Small Anim. Pract.* **17,** 163.
Singh, S. C., Joshi, H. C., Divivedi, S. K., and Singh, R. (1972). *Indian Vet. J.* **49,** 897.
Sisk, D. B., Carlton, W. W., and Curtin, T. J. (1968). *Am. J. Vet. Res.* **29,** 1591.
Smith, M. I., Westfall, B. B., and Stohlman, E. F. (1940). *Natl. Inst. Health Bull.* **174,** 21.
Snell, A. M., Greene, C. H., and Rowntree, L. G. (1925). *AMA Arch. Intern. Med.* **36,** 273.
Sova, Z., Trefny, D., Koudela, K., Janda, J., Pujman, V., and Hrdinova, A. (1974). *Zentralbl. Veterinaermed. Reihe A* **21,** 499.
Stenius, P. I. (1941). *Skand. Veterinaertidskr.* **31,** 193.
Stiebale, H. (1942). Inaug. Diss., Tierärztliche Hochschule, Hannover.
Strombeck, D. R. (1978). *J. Am. Vet. Med. Assoc.* **173,** 267.
Strombeck, D. R., and Qualls, C. (1978). *J. Am. Vet. Med. Assoc.* **172,** 1423.
Strombeck, D. R., Meyer, D. J., and Freedland, R. A. (1975a). *J. Am. Vet. Med. Assoc.* **166,** 1109.
Strombeck, D. R., Weiser, M. E., and Kaneko, J. J. (1975b). *J. Am. Vet. Med. Assoc.* **166,** 1105.
Strombeck, D. R., Rogers, W., and Gribble, D. (1976a). *J. Am. Vet. Med. Assoc.* **169,** 802.
Strombeck, D. R., Krum, S., Meyer, D., and Kappesser, R. M. (1976b). *J. Am. Vet. Med. Assoc.* **169,** 811.
Strombeck, D. R., Krum, S., and Rogers, Q. (1976c). *J. Am. Vet. Med. Assoc.* **169,** 813.
Sturtevant, F., Hoffman, W. E., and Dorner, J. L. (1977). *J. Am. Anim. Hosp. Assoc.* **13,** 754.
Svirbely, J. L., Monaco, A. R., and Alford, W. C. (1946). *J. Lab. Clin. Med.* **31,** 1133.
Tanabe, Y., and Wilcox, F. H. (1960). *Proc. Soc. Exp. Biol. Med.* **103,** 68.
Tenhunen, R. (1971). *Scand. J. Clin. Lab. Invest., Suppl.* **116,** 9.
Thannhauser, S. J., and Schaber, H. (1926). *Klin. Wochenschr.* **5,** 252.
Thorpe, E., Gopinath, C., Jones, R. S., and Ford, E. J. H. (1968). *J. Pathol.* **97,** 241.
Tiselius, A. (1930). *Nova Acta Regiae Soc. Sci. Ups.* **7**(4), 107.
Todd, J. C., Sanford, A. H., and Wells, B. B. (1953). "Clinical Diagnosis by Laboratory Methods," 12th ed. Saunders, Philadelphia, Pennsylvania.
Treacher, R. J., and Collis, K. A. (1977). *Res. Vet. Sci.* **22,** 101.
Treacher, R. J., and Sansom, B. F. (1969). *Res. Vet. Sci.* **10,** 461.
Trifonov, S. (1964). *Izv. Inst. Sravn. Patol. Zhitvotnykh* **10,** 227.
Trigger, D. R. (1976). *Gastroenterology* **71,** 162.
Tryphonas, L., and Rozdilsky, B. (1970). *J. Am. Vet. Med. Assoc.* **157,** 1084.
Tucker, R. E., Mitchell, G. E., and Little, C. O. (1971). *J. Anim. Sci.* **32,** 1190.
Udall, R. H., Warner, R. G., and Smith, S. E. (1952). *Cornell Vet.* **42,** 25.
van den Bergh, A. A., and Müller, P. (1916). *Biochem. Z.* **77,** 90.
Van Leenhoff, J. W., Hickman, R., Saunders, S. J., and Terblanche, J. (1974). *S. Afr. Med. J.* **12,** 1201.
Van Roy, F. P., and Heirwegh, K. P. M. (1968). *Biochem. J.* **107,** 507.

Van Vleet, J. F., and Alberts, J. O. (1968). *Am. J. Vet. Res.* **29,** 2119.
Vardiman, P. H. (1953). *Am. J. Vet. Res.* **51,** 175.
Vogin, E. E., Moreno, O. M., Brodie, D. A., and Mattis, P. A. (1966). *J. Appl. Physiol.* **21,** 1880.
Wallace, G. B., and Diamond, J. S. (1925). *AMA Arch. Intern. Med.* **35,** 698.
Watson, C. J. (1959). *J. Lab. Clin. Med.* **54,** 1.
Watson, C. J., Schwartz, S., Sborov, V., and Bertie, E. (1944). *Am. J. Clin. Pathol.* **14,** 605.
Wester, J. J. (1912). *Tijdschr. Veeartsenijk.* **39,** 817.
Wheeler, H. O., Epstein, R. M., Robinson, R. R., and Snell, E. S. (1960a). *J. Clin. Invest.* **39,** 236.
Wheeler, H. O., Meltzer, J. K., and Bradley, S. E. (1960b). *J. Clin. Invest.* **39,** 1131.
Whitehair, C. C., Peterson, D. R., Van Arsdell, W. J., and Thomas, O. O. (1952). *J. Am. Vet. Med. Assoc.* **121,** 285.
Wilson, G. D. A., Harvey, D. G., and Snook, C. R. (1972). *Br. Vet. J.* **128,** 596.
Wisecup, W. G., Hodsen, H. H., Hanley, W. C., and Felts, P. E. (1969). *Am. J. Vet. Res.* **30,** 955.
With, T. K. (1954). "Biology of Bile Pigments." Frost-Hansen, Copenhagen.
Wittfogel, H. (1939). *Dsch. Tieraerztl. Wochenschr.* **47,** 43.
Wolff, W. A., Lumb, W. V., and Ramsay, M. K. (1967). *Am. J. Vet. Res.* **28,** 1363.
Wolfson, W. G., Cohn, C., Calvary, E., and Ichiba, F. (1948). *Am. J. Clin. Pathol.* **18,** 723.
Wood, D. H., Murray, J. L., and Palotay, J. L. (1968). *Am. J. Vet. Res.* **29,** 1103.
Woodruff, J. M., and Johnson, D. K. (1968). *Pathol. Vet.* **5,** 327.
Wroblewski, F., and Cabaud, P. (1957). *Am. J. Clin. Pathol.* **27,** 235.
Wroblewski, F., and LaDue, J. S. (1955). *Ann. Intern. Med.* **43,** 345.
Wroblewski, F., and LaDue, J. S. (1956a). *Proc. Soc. Exp. Biol. Med.* **91,** 569.
Wroblewski, F., and LaDue, J. S. (1956b). *Ann. Intern. Med.* **45,** 782.
Wurzer, P., Leeman, W., and Weber, H. (1967). *Zentralbl. Veterinaermed, Reihe A* **14,** 208.
Yamaoka, S., and Kameya, T. (1972). *Exp. Rep. Equine Health Lab. No. 9,* 55.
Young, J. E., Younger, R. L., Radcliff, R. D., Hunt, L. M., and McLaran, J. K. (1965). *Am. J. Vet. Res.* **26,** 641.
Zenglein, G. (1930). Thesis, École d'Alfort.
Zink, B. (1932). Inaug. Diss., Tierärztliche Hochschule, Vienna.
Zinkl, J. G., Bush, R. M., Cornelius, C. E., and Freedland, R. A. (1971). *Res. Vet. Sci.* **12,** 211.

7

Pancreatic Function

DUANE F. BROBST

I. INTRODUCTION

The pancreas is composed of two organs, the endocrine and the exocrine portions, within one stroma. The functions of the endocrine pancreas are considered in Chapter 1, which deals with carbohydrate metabolism. Although the functions of the endocrine and exocrine portions of the pancreas may in some way be interrelated, the present chapter is concerned with the exocrine pancreas.

The symptoms of exocrine pancreatic disease are often nonspecific, and physical and roentgen examination are seldom diagnostic. Since the pancreas is so difficult to evaluate using these approaches, the clinician has come to rely on biochemical tests in the diagnosis of pancreatic disease. Certainly, when clinical findings and laboratory results are correlated, the clinical diagnosis can be established with more confidence. Correlation of the clinical and laboratory information and appreciation of pancreatic disease require a basic understanding of the anatomy and physiology of the pancreas and of the pathological processes which may occur in this organ.

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

II. PHYSIOLOGY OF THE PANCREAS

Pancreatic secretion is composed of a viscid, enzyme-rich portion and a watery portion containing electrolytes with a high concentration of bicarbonate (HCO_3^-). The enzymes are synthesized by the pancreatic acinar cells, which are arranged in clusters about the terminal pancreatic ductules. The epithelial cells lining the terminal and interlobular ducts, which carry pancreatic juice toward the duodenum, form the fluid and electrolyte portion of the pancreatic secretion.

A. Pancreatic Fluid and Electrolytes

Pure pancreatic juice of dogs has a pH between 8 and 8.3 and is iso-osmotic with that of plasma. The volume of secretion by the canine pancreas is approximately 67 ml/kg per 24 hours, and that by the pancreas of the normally fed sheep is 9.5 ml/kg per 24 hours (Taylor, 1962). In canine pancreatic juice, the HCO_3^- concentration ranges between 60 and 148 mEq/liter; in ovine pancreatic juice the HCO_3^- concentration is 15–30 mEq/liter. There is normally little fluctuation in the rate of pancreatic juice flow when a meal is eaten by a ruminant. This is because injesta normally flows almost continuously into the duodenum. When food is withheld for 48 hours, however, ruminant pancreatic secretion decreases by about 50% (Taylor, 1962). Taylor (1962) demonstrated that the pancreas of the dog secretes two or three times as much fluid per gram of tissue as does that of the sheep. The sheep pancreas, however, secretes only one-seventh the quantity of enzymes as the dog pancreas.

Of the ions in pancreatic secretion, bicarbonate and chloride are the principal anions, and their concentration is a function of rate of secretion. Bicarbonate concentration in pancreatic juice varies directly with rate of flow, while chloride varies inversely with bicarbonate concentration. The sum of bicarbonate and chloride tends to remain constant. Using micropuncture techniques, Lightwood and Reber (1977) studied pancreatic electrolyte secretion in the small intralobular and larger extralobular ducts of the cat pancreas. Pancreatic juice chloride concentration steadily decreased from 112 mEq/liter in the intralobular ducts to 46 mEq/liter in the main duct. There was no evidence of secretion of water and HCO_3^- by the main duct. Pancreatic secretion of water and electrolytes appeared to result from the admixture of a chloride-rich primary secretion originating from intralobular structures and a larger volume of bicarbonate-rich fluid originating from the smaller extralobular ducts. Bicarbonate and chloride exchange in these ducts accounts for the characteristic changes in concentration of bicarbonate and chloride as the flow rate varies. At highest rates of secretion there is thus least opportunity for exchange, and greatest opportunity for exchange at lowest rates of secretion.

Bicarbonate is believed to be secreted by the pancreatic ductal epithelial cells, with carbonic anhydrase catalyzing the reaction of CO_2 and H_2O to form H_2CO_3. At intracellular pH, H_2CO_3 dissociates to $H^+ + HCO_3^-$ (Janowitz and Dreiling, 1962). Carbonic anhydrase appears to be necessary for maximal secretion of HCO_3^-, with the maximal secretion being limited by the rate of transport of HCO_3^- from cell to duct (Rawls *et al.*, 1963). Swanson and Solomon (1972) demonstrated in the *in vitro* rabbit pancreas that it was not necessary for HCO_3^- to be in the perfusing fluid in order to have pancreatic juice formation and suggested that active transport of H^+ or OH^- was responsible for HCO_3^- secretion. It was speculated that a Na^+–H^+ exchange pump at the ductal epithelial cell

membrane moved Na^+ into the cell and H^+ out. Sodium entering the cell would preserve cellular electroneutrality and provide the required source of sodium for transport into ducts. When the transport of sodium into cells was inhibited, pancreatic juice was not formed.

Alterations in blood pH, plasma [HCO_3^-], and [CO_2] may result in certain changes in pancreatic juice. Rawls *et al.* (1963) demonstrated that dogs with metabolic acidosis secreted pancreatic juice containing less than normal concentrations of HCO_3^-. Dogs with metabolic alkalosis had an increased rate of flow of pancreatic juice containing increased concentration of HCO_3^-. Ordinarily metabolic alkalosis is a common feature of several upper intestinal disorders in the ruminant. Gingerich and Murdick (1975), however, observed in sheep that duodenal ligation anterior to the entrance of the common bile duct, into which the pancreatic duct enters, did not cause metabolic alkalosis. When ligation of the duodenum was made posterior to the common bile duct, alkalosis developed. It was concluded that secretion by the pancreas was greatly reduced when abomasal contents were prevented from entering the duodenum and that bicarbonate might have been absorbed by the intestine and contributed to the alkalosis.

The concentration of sodium and potassium in pancreatic secretion tends to parallel plasma concentrations. Calcium concentration in pancreatic juice of the dog, however, tends to be lower than in the plasma. Zimmerman *et al.* (1967) determined that the basal total pancreatic juice calcium of dogs ranged from 3.3 to 4.4 mEq/liter, with corresponding serum calcium in the range 4.0–5.5 mEq/liter. The studies by these investigators demonstrated that, under conditions in which pancreatic enzymes were being formed in large amounts, calcium output was greatest. They suggested that calcium was not secreted with the electrolyte components of pancreatic juice, but rather with the nonelectrolyte portion, and that calcium may be a part of the amylase molecule.

When furosemide was administered to human beings, pancreatic secretion increased and the output of bicarbonate, sodium, and chloride also increased. Furosemide had no effect on duodenal water transport. These observations indicated that furosemide stimulates pancreatic secretion of water and electroytes, possibly via inhibition of pancreatic ductal absorption of sodium (Thomas *et al.*, 1977).

B. Pancreatic Enzymes

The enzymes of pancreatic juice are capable of digesting all three major types of foodstuffs since the secretion contains proteolytic, lipolytic, and amylolytic enzymes.

1. Proteolytic Enzymes

The major proteolytic enzymes are secreted as inactive proenzymes and are trypsinogen, chymotrypsinogen, and procarboxypeptidase. Trypsinogen may spontaneously change to active trypsin in solution, but this change is accelerated by additional trypsin, calcium, and magnesium and by contact with the enzyme enterokinase from the intestinal mucosa. The spontaneous conversion to trypsin is suppressed by the presence of trypsin inhibitor in the pancreatic gland. A high concentration of calcium is required for activation of trypsinogen. Clemente and Meldolesi (1975) speculated that the low level of calcium in trypsinogen might be a mechanism working in synergism with trypsin inhibitor to prevent activation of proteolytic zymogen inside the acinar cell.

The activity of chymotrypsin is comparable to that of trypsin, but the two enzymes may

attack different linkages of the protein molecule. The pH optimum for both trypsin and chymotrypsin is between 8 and 9. The peptides resulting from protein digestion by trypsin and chymotrypsin are further digested by enzymes known as peptidases. Most of the peptidases are derived from intestinal mucosa, but the carboxypeptidases A and B are in pancreatic secretion. Procarboxypeptidases A and B, like chymotrypsinogen, are activated by trypsin. Pancreatic secretion contains other proteolytic enzymes, such as ribonuclease and deoxyribonuclease, which partially hydrolyze the corresponding nucleic acids into mononucleotides. Elastase and collagenase are also proteolytic enzymes in pancreatic secretion.

2. Lipases

Pancreatic lipase is secreted in an active form, but its activity is enhanced by bile salts. Bile salts enhance the efficiency of lipolysis by increasing the surface area of oil–water interfaces at which water-soluble lipase is effective. Colipase is a small protein, synthesized in the pancreas, which allows pancreatic lipase to function in spite of micellar concentrations of conjugated bile salts. Bile salts by themselves hinder lipase adsorption onto triglycerides by covering the whole water–substrate interface. Colipase tends to prevent this and acts as an anchor for lipase adsorption, thus allowing lipase to hydrolyze substrate (Vandermeers-Piret *et al.*, 1977). Calcium may be required for activation of lipase (Janowitz and Banks, 1976).

Pancreatic lipase exhibits optimal activity under alkaline conditions and hydrolyzes triglycerides to fatty acids and glycerol, but mono- and diglycerides are also end products. Pancreatic lipase has greater activity against short-chain than long-chain triglycerides (Cohen *et al.*, 1971).

Phospholipases A and B also are present in pancreatic juice. Phospholipase A splits off a fatty acid from lecithin or cephalin to form lysolecthin and lysocephalin. Phospholipase B is capable of splitting off a fatty acid and lysolecthin to form glycerylphosphorylcholine.

Pancreatic cholesterol ester hydrolase catalyzes the hydrolysis of dietary cholesterol esters in the intestinal lumen. The free cholesterol produced, together with free cholesterol in the diet and in the bile, is taken up by intestinal absorptive cells. Here it is reesterified to cholesterol esters, and these are transported into the lymph in chylomicrons (Gallo *et al.*, 1977). Elimination of pancreatic secretion from the intestine of dogs reduced the level of cholesterol ester in their lymph (Hernandez *et al.*, 1955).

3. Amylase

Pancreatic amylase is an α-amylase and splits the 1,4-glycosidic bond to hydrolyze starch and glycogen to the disaccharide maltose. Amylase requires chloride for activation, and calcium appears to be an integral portion of the enzyme (Janowitz and Banks, 1976).

One theory of pancreatic secretion of enzymes suggests that there is only one population of enzymes released from the zymogen granules of the acinar cells. This is termed "parallel secretion" and requires that the ratios of the enzymes and total protein bear a constant relation to each other regardless of the secretory stimulant. Scheele and Palade (1975) demonstrated that the guinea pig pancreas does discharge enzymes in parallel and in constant proportion. Nonparallel secretion of pancreatic enzymes has been described in the rabbit, rat, pig, and man. Sullivan *et al.*, (1974) demonstrated that parallel secretion

might be true for one stimulus but not for all. Working with swine, they found that vagal stimulation increased pancreatic secretion of amylase greater than it did the secretion of total protein or proteases. Dagorn *et al.* (1977) observed that in human beings pancreatic amylase secretion was proportionally less stimulated by cholecystokinin and pancreozymin than were chymotrypsin and lipase secretions.

Malaisse-Lagae *et al.* (1975) showed in the rat that the concentration of enzymes in acinar cells surrounding the islets of Langerhans (periinsular portion) differed from that of the rest, the teleinsular part of the pancreas. The concentration of amylase relative to that of lipase was invariably higher in the teleinsular than in the periinsular exocrine pancreas. Their findings indicate that the secretory partition of the exocrine pancreas could be responsible for modulation in the composition of pancreatic juice under different conditions. The periinsular acini may differ from the teleinsular tissue in the level of their secretory activity, their response to various secretagogues, and their relative content of different hydrolases. Functionally, the endocrine and exocrine cells of the pancreas may exert a mutual and direct control of their secretory activity. That is, insular hormones modify exocrine function, and exocrine factors may alter islet cell responsiveness to certain secretagogues. The high concentrations of insulin in the periinsular acinar cells may enhance protein synthesis in these cells.

The type of food ingested by some animals may, with time, alter the enzymatic composition of the pancreas. Lavau *et al.* (1974) found that rats on a diet high in starch or glucose and low in fat had increased concentration of amylase and decreased lipase in pancreatic tissue. The effect of glucose administered intravenously was similar to that of glucose given orally. Diets high in fat but low in carbohydrates raised pancreatic lipase levels and diminished amylase levels. Lipid given intravenously gave the same pancreatic enzyme response as lipid by mouth. An amino acid mixture given orally or intravenously, however, was without effect on pancreatic tissue enzyme composition other than to decrease lipase activity. These findings support the hypothesis that pancreatic lipase and amylase are induced by end products of digestion appearing in the blood, whereas proteins induce their respective enzymes by another mechanism.

Protein malnutrition may also be associated with alteration in the composition of pancreatic enzymes. Gyr *et al.* (1975) determined that moderate protein deficiency in monkeys caused a significant decrease in pancreatic secretion of amylase, lipase, and chymotrypsin. These animals also developed hypoalbuminemia after 11–24 weeks on a protein-deficient diet. Recovery of pancreatic function was noted in some animals after being fed a full protein diet. However, in some animals, exocrine pancreatic function was seriously impaired even with moderate protein deficiency.

Many antibiotics may mediate their effects by acting on cell membrane systems. As formation and secretion of pancreatic enzymes may involve membrane-associated functions, it is possible that antibiotics may interfere with enzyme formation or secretion. Singh *et al.* (1972) determined that tetracycline, valinomycin, and amphotericin B decreased secretion of pancreatic amylase without altering pancreatic tissue content of amylase. Streptomycin increased amylase secretion and decreased tissue content. Atropine did not alter secretion but reduced pancreatic tissue amylase content. It was considered that long-term administration of atropine could result in decreased pancreatic function by depriving the cells of basal acetylcholine stimulus.

Drieling *et al.* (1958) observed that glucagon administered to human beings caused a progressive fall in blood amylase and an elevation of blood sugar. On the other hand, they

found that intravenous insulin in man caused a decrease in blood amylase and glucose. Glucagon also decreased blood amylase in rabbits and amylase in their pancreatic tissue (Jarrett and Lacey, 1962). Blood amylase concentration varies with changes in carbohydrate metabolism and is depressed by conditions which increase the rate of carbohydrate utilization. Conversely, with decreasing glucose utilization, as following administration of epinephrine or cortisone, there is a rise in blood amylase (Drieling *et al.*, 1958).

The maintenance of blood amylase levels following removal of the pancreas, in some species, suggests that nonpancreatic sources of amylase are important in the regulation of normal blood amylase levels. Nothman and Callow (1971) observed that following pancreatectomy in dogs there was no decrease in serum amylase activity, but following pancreatectomy and hepatectomy there was. These results indicated that the amylase regularly present in the serum was not of pancreatic origin. Only those elevated amounts of amylase which appear in the blood under pathological conditions were considered to be from the pancreas. The liver was thought to be one of the sources of amylase in serum. Messer and Dean (1975), using immunochemical techniques, demonstrated that serum amylase and liver amylase of the rat were identical and very similar to parotid gland amylase. Pancreatic amylase was, however, quite distinct and had only some of the antigenic determinants present on parotid gland amylase. The Kupffer cells of the liver may be involved in the transport of amylase in and out of the normal hepatic cell. Hiatt *et al.* (1968) observed that, when Kupffer cells of the rat were blocked by injection of thorium dioxide, liver amylase increased 80% more than controls, and serum amylase decreased over 45%. A pancreatic influence on serum amylase in normal pigs, however, is present. When radioimmunoassay techniques specific for pancreatic amylase were used, the enzyme could not be detected in the serum of pigs 48 hours after pancreatectomy (Ryan *et al.*, 1975).

In some species pregnancy may also affect serum amylase levels. In both the pregnant woman and pregnant rat, serum amylase activity is greater than in nonpregnant controls. Ahlert *et al.* (1969) determined that the amniotic fluid of pregnant rats was high in amylase and that it had its origin in urine excreted by the fetus. Amniotic amylase was believed responsible for the increase in serum amylase.

C. Regulation of Pancreatic Secretion

A basal rate of pancreatic secretion exists in most animals, but, with the ingestion of food, a series of hormonal and neural mechanisms are set into action which activate and control the pancreatic secretion of water, electrolytes, and digestive enzymes. The dominant hormones are secretin, cholecystokinin (CCK), and pancreozymin (PZ). Secretin is released from the upper small intestine mucosa by the entry of H^+ ions into the duodenum and this stimulates the centroacinar and ductal cells of the pancreas to secrete the electrolyte-containing fluid. The HCO_3^- secreted by the pancreas neutralizes the HCl secreted by the stomach. Pancreozymin is also released from the duodenal mucosa by the products of protein digestion and is the major stimulant of enzyme secretion by the acinar cells. Cholecystokinin is released from the duodenal mucosal cells in response to products of protein digestion, and this hormone causes some secretion of pancreatic enzymes and contraction of the gallbladder. However, the distinction between electroyte- and enzyme-stimulating hormones is not absolute. Pancreozymin can stimulate some watery secretion, and secretin can cause some enzyme release. After a meal, the products of protein

digestion stimulate a wide area of upper intestinal mucosa to release large amounts of CCK-PZ, which potentiates the action of small amounts of secretin released in the proximal duodenum.

Plasma secretin levels were measured in dogs by radioimmunoassay (Llanos *et al.*, 1977). When the pH of the stomach content of dogs decreased to less than 3, the plasma secretin level increased to 43% above basal values, and the pancreatic secretion of HCO_3^- was near maximum. The threshold for release of secretin and HCO_3^- was pH 4.

In man, Ertan *et al.* (1971) determined that a mixture of amino acids perfusing the proximal jejunum caused a prompt increase in volume and HCO_3^- in pancreatic secretion. The amino acids also stimulated an increase in secretion of pancreatic enzymes. All pancreatic secretions reached peak output into the gut 30–45 minutes after the onset of stimulation and decreased sharply during the first 30 minutes after cessation of stimulation. In addition, amino acid perfusion of the jejunum produced a sustained increase in total bile salt output which was believed due to gallbladder contraction. The results suggest that amino acids in the jejunum cause the release of CCK-PZ, as well as secretin from the gut mucosa, and that they act as regulators of exocrine pancreatic and bilary function. In dogs the perfusion of phenylalanine and tryptophan through the upper intestine was effective in causing pancreatic secretion. Valine and leucine were less effective; methionine was ineffective.

Meyer and Jones (1974) determined that pancreatic output of bicarbonate and protein was, in part, controlled by fat and products of fat digestion in the intestine. Fatty acids of less than nine carbons in chain length did not stimulate pancreatic secretion, whereas those longer than nine carbons in chain length did. It was believed that fatty acids within the intestine stimulate the pancreas by releasing CCK from the gut.

Vasoactive intestinal peptide (VIP), which may be liberated from the intestine, shares a structural and functional homology with secretin. Each of these peptides inhibits gastric secretion, stimulates pancreatic secretion, increases adenylate cyclase activity in plasma membranes of the exocrine pancreas, and raises cellular adenosine 3′, 5′-monophosphate (cyclic AMP) content in the pancreas (Deschodt-Lanckman *et al.*, 1975). Konturek *et al.* (1977) determined that, in the cat, VIP is a secretin-like full agonist of pancreatic HCO_3^- secretion. The secretory effect of VIP on pancreatic HCO_3^- secretion is species dependent and, in dogs, can inhibit secretin-induced pancreatic secretion.

Deschodt-Lanckman *et al.* (1975) observed significant increases in cyclic AMP when rat pancreatic fragments were incubated with secretin or VIP. It was concluded that cyclic AMP is the intracellular messenger for both secretin and VIP in centroacinar cells. Pancreozymin inhibited the production of cyclic AMP when fragments of pancreas were incubated with secretin or VIP. This suggested that PZ was acting at a binding site different from that of secretin or VIP but that it still coupled with adenylate cyclase.

Although some investigators suggest that cellular accumulation of cyclic AMP is involved in stimulation of pancreatic enzyme secretion, others focus on changes in cellular membrane transport of cations as the key to pancreatic enzyme secretion. Electrical stimulation of pancreatic nerves and the action of PZ are believed to cause depolarization of pancreatic acinar cell membranes and, in so doing, make the membranes more permeable to various cations (Nishiyama and Petersen, 1974). The secretion of enzymes from the pancreatic cell occurs by a process of exocytosis, and this process is believed to require the presence of intra- and extracellular calcium. Gardner *et al.* (1975) have demonstrated that CCK causes a marked outflux of radioactive calcium from prelabeled

pancreatic tissue. The primary action of CCK-PZ may be to increase sodium influx, which consequently elicits a release of calcium from the endoplasmic reticulum, leading to a rise in calcium close to the structures involved in enzyme extrusion (Kanno *et al.*, 1977).

Intraduodenal calcium may also regulate pancreatic secretion. Holtermuller *et al.* (1976) determined that intraluminal $CaCl_2$ stimulated pancreatic enzyme secretion and gallbladder contraction. Induced hypercalcemia, however, had no effect on pancreatic or gallbladder function. It was hypothesized that gut endocrine cells were sensitive to extracellular calcium and that intraduodenal calcium directly induced release of CCK-PZ, which, in turn, stimulated the pancreas and gallbladder.

The hormone gastrin, produced by the mucosa of the pyloric antrum of the stomach, also may influence pancreatic secretion. Reber *et al.* (1977), working with hypergastremic rats with gastrojejunostomy and gastric antrum transplanted to the colon, observed a twofold increase in pancreatic fluid HCO_3^- and volume of secretion. Pancreatic weight also increased in these animals. It was concluded that endogenous hypergastremia produced functional hyperplasia of both pancreatic ducts and acinar cells.

Glucagon may be involved in the regulation of pancreatic secretion. Gyr *et al.* (1977) observed that glucagon decreased the tryptic activity of the pancreatic secretion of dogs fed a liquid test meal. The tryptic activity of pancreatic secretion of dogs was found to be greater than that of man, but, with the administration of glucagon, there was a significant decrease in volume of duodenal contents and enzyme concentration. Trypsin concentration decreased 59%, and chymotrypsin 53%. Dyck *et al.* (1969) observed that glucagon caused a greater degree of inhibition of pancreatic enzyme secretion than of volume and HCO_3^- output. The mechanism by which glucagon produces this effect is not known, although it is postulated that glucagon may have a protein catabolic effect, which would decrease the output of pancreatic enzymes. Glucagon may also reduce the blood flow to the pancreas, as it does to the stomach, and thereby diminish glandular activity. Epinephrine also is capable of decreasing pancreatic flow, HCO_3^- output, and enzyme secretion, an effect probably secondary to diminished blood flow to the organ and resulting from vasoconstriction (Dreiling, 1959).

Pancreatic secretion may be modified by antidiuretic hormone (ADH). In dogs in which pancreatic secretion was stimulated by intravenous injection of secretin plus ADH, the volume of secretion and bicarbonate concentration were less than if only secretin were administered (Banks *et al.*, 1968). It was theorized that ADH depressed the physiological activity of secreting cells of the pancreatic ducts or that ADH altered the permeability of these cells to various electrolytes, e.g., bicarbonate, in such a way that there would be a net movement of bicarbonate out of the duct system. It therefore appears that ADH acts as a fluid regulator in the pancreas as well as in the kidney. *Schapiro et al.* (1977) reported that the inflamed pancreas, however, was less sensitive to ADH inhibition than the normal pancreas.

The role of nervous regulation of the pancreas is less clearly understood than that of hormonal regulation. Vagal stimulation or parasympathomimetic drugs stimulate enzyme release, and atropine inhibits, to some degree, enzyme secretion. Sympathetic innervation may cause pancreatic inhibition, which, in turn, may be related to the effects of diminished blood flow. Vagotomy, in the dog, decreases pancreatic fluid volume and HCO_3^- response to duodenal acidification (Moreland and Johnson, 1971). It is believed

that vagal stimulation facilitates the release of secretin in response to low levels of duodenal acidification and results in an increased sensitivity of the pancreas to low levels of secretin.

III. PANCREATIC DISEASE

Pancreatic disease in its various forms is not uncommon in dogs and cats and occurs in other animals as well. The exocrine pancreas may be affected by acute or chronic inflammatory diseases; it may fail to develop to its proper size and thus be hypoplastic, or it may undergo atropic or neoplastic changes.

A. Acute Pancreatitis

1. Etiology

Acute pancreatitis occurs more commonly in dogs than in other animals. The intensity and duration of the illness range from a mild, subclinical, edematous form to acute, hemorrhagic pancreatitis, progressing to collapse and death. The etiology of the condition, however, is not well understood. Anderson (1968) points out that female dogs are more likely to acquire pancreatitis than male dogs and that obesity predisposes to the disease. Lean, active, young dogs apparently are less likely to acquire the disease. Goodhead (1971) also found that dogs on a poor plane of nutrition failed to develop as severe a degree of induced pancreatitis as well-nourished animals. Also from a nutritional standpoint, hemorrhagic pancreatitis was induced in mice on a choline-deficient diet but was prevented by supplementation of the diet with methionine (Rao *et al.*, 1976).

The reflux of bile from the bile duct to the pancreatic duct has been considered a likely means of initiating pancreatitis. This is anatomically unlikely in the dog but could conceivably occur in the cat because of the close association of the biliary and pancreatic ducts. In the dog, however, lymphatic communications do exist between the biliary tract and the pancreas, and Weiner *et al.* (1970) observed that, when experimental acute cholecystitis was produced in dogs, an acute pancreatitis developed. Conversely, lipase-induced pancreatitis resulted in a high incidence of acute cholecystitis. This indicates that the lymphatic system may be involved in the transmission of inflammatory disease from one organ to another in the pancreatobiliary system. In cats the combination of biliary tract infection and interstitial pancreatitis is not uncommon and is probably a sequel to ascending infection of the biliary and pancreatic ducts (Kelly *et al.*, 1975). Clinical disease in the cat associated with this type of involvement, however, is infrequent. In cats, the liver fluke *Amphimerus pseudofelineus* has been determined to be an agent causing inflammation of the biliary and pancreatic ducts. Toxoplasmosis in cats also has been associated with pancreatitis, with toxoplasma organisms present in the pancreatic and bile duct epithelial cells (Smart *et al.*, 1973). Traumatic hemorrhagic pancreatitis has been reported in cats which have survived falls from buildings (Suter and Olsson, 1969).

Acute hemorrhagic pancreatitis was described in bovine species, but whether the disease was primary or secondary to shock and toxemia was debatable (Little, 1963). Pancreatolithiasis occurs more frequently in cattle than in any other animal, and Verine (1973) reported a frequency of 1/2700 in slaughtered cattle. The associated pancreatic lesions,

however, were chronic in nature. Baker (1978) observed acute necrotizing pancreatitis in the horse and is of the opinion that many cases of acute pancreatitis in this species have gone undiagnosed.

Undoubtedly, no one factor alone is responsible for pancreatitis, and the distribution of etiological factors must vary in the different types of pancreatitis. As far as etiology is concerned, the bulk of cases of pancreatitis in animals at this time probably belong to the idiopathic group.

2. Pathogenesis

The pancreas is in reality a large sac of enzymes which under appropriate conditions are capable of digesting that organ and then creating a wide variety of secondary disorders. Thus, the clinician might look upon the pancreas as the potential "powder keg" of the abdomen.

The release of digestive enzymes into the parenchyma and interstitial tissue of the pancreas constitutes the most plausible mechanism in the pathogenesis of pancreatic necrosis and inflammation. The activation of trypsinogen by bile salts or other tissue juices may serve as the primary ignitor of pancreatic necrosis (Schmidt, 1970). Elastase, activated by trypsin, causes digestion of elastic fibers of pancreatic blood vessels, resulting in severe vascular damage and hemorrhage (Fig. 1). Phospholipase A, in the presence of small amounts of bile acids, converts cephalin and lecithin to their respective lyso compounds (lysocephalin and lysolecithin). These lyso compounds are capable of destroying the phospholipid layers of cell membranes and of producing pancreatic necrosis. Lipase, also in the presence of bile salts, is capable of causing fat necrosis in pancreatic and peripancreatic tissue.

Trypsin may also activate pancreatic kallikreinogen, with formation of vasoactive kallekrein and bradykinin. The effects of these substances are vasodilation, increased vascular permeability with edema developing in the pancreas, leukocyte infiltration, and pain (Fig. 1). The loss of body fluid through the process of edema formation in the pancreas and accumulation of fluid in the peritoneal cavity may lead to hypovolemia and shock. With pancreatic hypoperfusion, lysosomal disruption occurs in acinar cells of the pancreas. This is associated with release of a myocardial depressant factor (MDF) (Goldfarb and Weber, 1977), a peptide which depresses myocardial contractility and contributes to the lethality of circulatory shock. Spath *et al.* (1974) observed that maintenance of pancreatic blood flow at normal levels during hemorrhage in dogs limited formation of MDF and pancreatic necrosis. The kinin-forming activity of the ascitic fluid resulting from pancreatitis in dogs causes the release of additional kinin-forming enzymes from other tissues in the peritoneal cavity (Satake *et al.*, 1973). Thus, peritoneal dialysis during acute pancreatitis may improve the survival rate. It has also been shown that drugs interfering with the synthesis of proteins and nucleic acids tend to prevent pancreatic ascites. Korbova *et al.* (1977) demonstrated that cycloheximide administered to rats with experimental pancreatitis caused decreased production of abdominal fluid and that it lowered its lipase and amylase activities.

In experimental endotoxic shock in dogs, it was demonstrated that the duodenal mucosa releases less secretin and that the pancreas forms less HCO_3^-. Decreased pancreatic HCO_3^- secretion was associated with a decrease in neutralized hydrogen ions in the gut, and duodenal erosions occurred (Greenberg and Shimo-Takahara, 1976).

Evidence suggests that blood coagulation changes may occur in pancreatitis, leading to

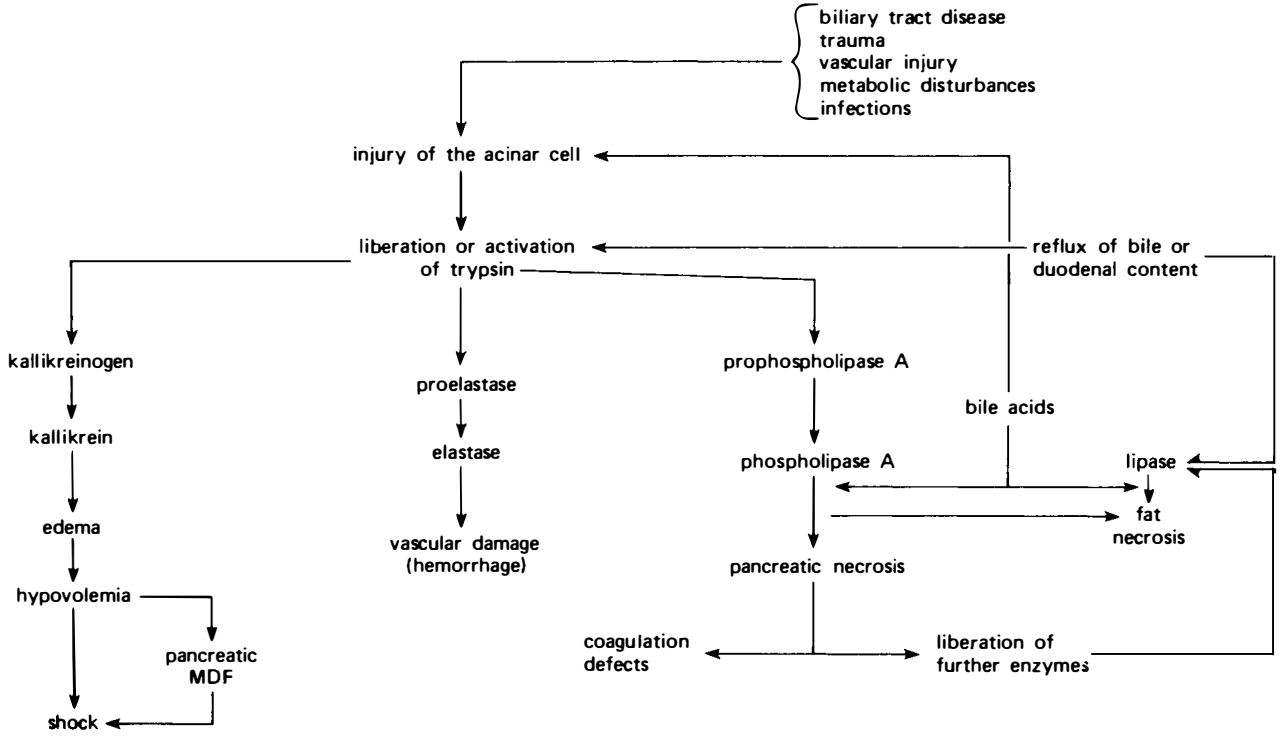


Fig. 1. Summary of etiological and pathogenetic factors in acute pancreatitis.

disseminated intravascular coagulation. The early intravascular consumption of coagulation factors correlate with increased pancreatic enzyme levels in the blood and indicate that enzyme-related intravascular coagulation may be a complication of acute pancreatitis (Ranson *et al.*, 1977). Lees *et al.* (1978) observed noncardiogenic pulmonary edema in a dog with acute pancreatitis. It has been proposed that free fatty acids liberated from lipids by circulating pancreatic lipase could mediate pulmonary injury or that pulmonary surfactant could be destroyed by pancreatic phospholipase A.

B. Chronic Pancreatitis

Chronic pancreatitis is the end result of repeated attacks of acute pancreatitis with progressive destruction of acinar tissue and replacement with fibrous connective tissue. The same etiological factors causing acute pancreatitis are thus involved in the development of chronic pancreatitis. The early clinical signs of chronic pancreatitis may resemble those of acute pancreatitis, with necrotizing process smoldering on asymptomatically. Chronic pancreatitis is commonly seen in canine species (Hardy and Stevens, 1975). Druffell (1975) observed that chronic and subacute pancreatitis is common in the cat.

With almost total destruction of the acinar tissue of the pancreas, pancreatic enzyme insufficiency occurs (Anderson, 1972). Without trypsin, the feces contains undigested meat fiber (creatorrhea), and there is nitrogen loss. In the absence of lipase, neutral fat is in the feces (steatorrhea). Without pancreatic amylase, starch is lost in the feces. However, this is of less importance than the resulting disturbed protein and fat digestion. The reserve capacity of the pancreas is great, and in man steatorrhea was not observed until lipase output was less than 10% of normal. Creatorrhea was observed also only when trypsin output was less than 10% of normal (Di Magno *et al.*, 1973).

Fatty food normally empties from the stomach more slowly than carbohydrate or protein foods. With pancreatic enzyme deficiency and steatorrhea in human beings, however, rapid gastric emptying of a fatty meal occurs. Studies indicate that fat must be digested or absorbed to inhibit gastric motility, and intestinal hormones released by fat appear to be the mediator of this inhibition (Long and Weiss, 1974). The addition of pancreatic enzymes to fatty meals slowed gastric emptying; however, the enzymes had no effect on the emptying of 5% glucose from the stomach. Below a certain level of pancreatic enzyme secretion, a vicious cycle may develop in which maldigestion leads to rapid gastric emptying, which, in turn, intensifies the digestive abnormality.

After total resection of the pancreas of dogs, the digestion and absorption of fat and protein are markedly disturbed, but this is not true of carbohydrates (Hamano, 1953). Yoshizawa *et al.* (1976) demonstrated that, with total resection of the pancreas of dogs, there was severely impaired absorption of olive oil by duodenal mucosa, and absorption of oleic acid and medium-chain triglycerides (MCT) was less affected. The absorption of fatty acids, after resection of the pancreas, was restored to normal by simultaneous administration of insulin and estrogen, although that of long- and medium-chain triglycerides was not. The disturbance of fat absorption was attributed to decreased activity in the gut epithelium of such enzymes as adenosine triphosphatase and alkaline phosphatase. The activities of these enzymes were restored by estrogen administration. The hydrolysis of MCT by pancreatic lipase is more rapid than that of long-chain triglycerides. The degree of steatorrhea in pancreatic insufficiency thus may be reduced by feeding MCT as the source of fat. Valdivieso (1972) demonstrated that rats with surgically induced pan-

creatic insufficiency absorb a substantial amount of MCT. By the use of isotope-labeled lipid, it was shown that MCT were not hydrolyzed in the jejunum but diffused intact into the gut epithelial cells. In the cell, MCT were partially hydrolyzed by intestinal lipase and then transferred to the portal blood partly as intact triglyceride and partly as fatty acid. In the cecum, MCT were hydrolyzed in the lumen by a hydrolytic system independent of the pancreas. The liberated fatty acids diffused through the cecal mucosa to the portal circulation. Hypertrophy of the cecum occurred in rat after induction of pancreatic atrophy.

The oral administration of pancreatic enzymes, either alone or with sodium bicarbonate, can be a useful therapy for patients with pancreatic insufficiency. Pancreatic enzyme therapy, in pancreatic insufficiency in man, increased trypsin in the duodenum to six to eight times and lipase three to five times pretreatment levels, although neither exceeded 2% of the normal concentration (Di Magno *et al.*, 1977). Duodenal pH in these patients was also less than that in health. Regan *et al.* (1977) demonstrated that the use of cimetidine (an inhibitor of gastric acid secretion) was a useful adjunct to oral pancreactic extract therapy in human beings with pancreatic insufficiency who failed to respond to pancreatic enzyme replacement alone.

C. Atrophy of the Pancreas

Anderson and Low (1965) reported several cases of primary atrophy in young dogs (juvenile atrophy). Although the dogs did have signs of digestive failure similar to that seen in chronic pancreatitis, the pancreases of these animals were without signs of inflammation. Also, these animals did not develop diabetes mellitus, which often complicates chronic pancreatitis.

In primary atrophy there is a progressive loss of cytoplasm and zymogen granules from the acinar cells. This form of atrophy is the result of malnutrition, especially protein depletion (Jubb and Kennedy, 1970). Atrophy of the acinar cells may also develop secondary to pancreatic fibrosis and ductal obstruction. A pancreas with secondary atrophy is often misshapen, coarsely nodular, and fibrous.

D. Neoplasia of the Pancreas

Carcinoma of the exocrine pancreas is observed rarely in dogs and cats and implies an origin in acinar or ductal cells. Ruwitch *et al.* (1964) reported finding adenocarcinoma of the pancreas of a dog which had occluded the pancreatic ducts, producing signs of acute pancreatitis.

IV. LABORATORY DIAGNOSTIC AIDS

Laboratory procedures used in detecting and measuring pancreatic disease may be considered to be of two types. One type demonstrates the presence or absence of an active pancreatic lesion and is used primarily in detecting the more acute phases of pancreatic disease. The other type is useful when the patient has recovered from the acute phase of the disease and one wishes to determine the degree of damage inflicted upon the pancreas or whether pancreatic insufficiency is present. For practical purposes the diagnostic aids can be considered tests for the acute and chronic phases of pancreatitis.

A. Acute Pancreatitis

1. Serum Amylase

a. Source. The measurement of serum amylase has been the single most important laboratory aid in the diagnosis of acute pancreatitis or acute exacerbations of chronic pancreatitis. In both veterinary and human medicine the pancreas has been considered the primary source of hyperamylasemia. Following ligation of the pancreatic ducts of dogs, Hiatt and Warner (1969) observed a marked rise in serum amylase and a subsequent fall to normal in 9 days. The accompanying pathological changes in the pancreas were minimal and believed not to be responsible for a marked hyperamylasemia. Similar results were observed by Gibbs and Ivy (1951). However, when the ligated pancreatic ducts were subjected to a pressure of 30 cm water, serum amylase levels rose twice as fast as in dogs with simple ligation. In those dogs in which the ducts were subjected to pressure, the pancreatic changes were of a more severe inflammatory nature than they were in dogs in which the pancreas was simply ligated.

In addition to the pancreas, other tissues of the body produce limited amounts of amylase, and thus hyperamylasemia may not necessarily indicate pancreatic involvement. Rats produce a salivary gland amylase, and salivary gland disease in this species may cause hyperamylasemia. The salivary glands of dogs produce no amylase. Some investigators believe that the intestinal mucosa of dogs is a source of amylase and that intestinal obstruction causes hyperamylasemia (Hiatt, 1959). Byrne and Boyd (1963) determined that small-bowel obstruction produced hyperamylasemia only in dogs which eat and then vomit. When total pancreatectomy was performed on dogs with high or low intestinal obstruction and these dogs were then allowed to eat and vomit, elevations of blood amylase did not occur. Duodenal perforation and intestinal infarction in guinea pigs have also been reported to cause hyperamylasemia (Orda *et al.*, 1976).

b. Significance. Janowitz and Dreiling (1959) were of the opinion that two pathological features within the pancreas influenced the extent to which serum amylase levels were elevated in pancreatic disease: (1) continued secretion against obstruction and (2) disruption of acinar cells and ductular system. Rapid accumulation of inflammatory exudate may increase intraductular pressure and cause a sudden elevation of serum amylase. This process may be self-limiting, however, since rapid glandular damage may lead to suppression of secretion. This would be in contrast to gradually increasing ductal obstruction, which would maintain elevation of serum enzyme over a longer period of time. Serum amylase values may thus be considered an index of the degree of ductal obstruction of the pancreas. The degree of amylase elevation, however, does not always correlate well with the severity of the pancreatitis.

Egdahl (1958), using dogs with bile-induced pancreatitis, determined that the initial rise in serum enzymes (1–3 hours) after injury was due to absorption of enzymes into the pancreatic venous blood. The later maintenance of the rise in enzymes was due primarily to lymphatic absorption of peritoneal fluid of high enzyme content. This fluid resulted from the passage of enzyme-containing fluid through the pancreatic capsule. Amylase levels in the serum of dogs with acute experimental pancreatitis peaked within 12–48 hours and subsided to normal within 8–14 days (Brobst *et al.*, 1970).

Stress and certain drugs may also affect serum amylase levels. Hiatt (1959) observed the fasting serum amylase level to vary greatly in the same dog from day to day and

considered increases in amylase in experimental dogs significant only if they exceeded preoperative levels by 50%. He related serum amylase levels to the dog's age, with higher levels in younger dogs. Finco and Stevens (1969) observed that the degree of stress induced in dogs by anesthesia and ovariohysterectomy had no significant effect on serum amylase concentration. Dogs ill with gastrointestinal disease, other than acute pancreatitis, likewise had no significant increase in serum amylase. In dogs with uremia, however, serum amylase activity was approximately two times normal, and, in some, concentrations of the enzyme were similar to those of dogs with acute pancreatitis. Thus, acute pancreatitis should be considered when, in the absence of uremia, serum amylase activity exceeds twice the normal value.

Serum amylase, formerly viewed as a single entity, is now considered to be composed of a number of molecular forms, termed isoenzymes, of amylase (isoamylases). Each isoamylase may originate from a different tissue. Berk *et al.* (1965) demonstrated that in the dog the majority of the amylase activity had an electrophoretic mobility similar to that of γ-globulin. A second peak of amylase activity was also in the α- and β-globulin areas. In pancreatectomized dogs the amylase activity in the γ-globulin zones was reduced to a greater degree than that in the α- and β-globulin zones.

c. Methodology. Amylase is a rather stable protein at 5°C. The enzyme requires calcium ion for activity, and thus plasma from blood samples with calcium-chelating agent present is not preferred for amylase determination (Hardy and Stevens, 1975).

The principle of serum amylase determination is the enzymatic hydrolysis of starch into its constituent maltose and glucose units. The rate of this hydrolysis is measured either by the rate of disappearance (amyloclastic) of starch or by the rate of appearance (saccharogenic) of reducing sugar, i.e., glucose and maltose, in the incubation mixture. A comparison of Somogyi's saccharogenic method and an amyloclastic method of determining serum amylase in dogs was made by Rapp (1962). He found that the determination of amylase by the saccharogenic method of Somogyi was invalid in the dog due to the presence of maltase in the serum which was additive to amylase activity. Loeb and Edge (1962), using an amyloclastic procedure, suggested 318–1050 Caraway units to be normal in healthy dogs. Saccharogenic methods give serum amylase values considerably greater than do the amyloclastic procedures in the dog (O'Donnell and McGeeney, 1975). These investigators were of the opinion that glucoamylase, as well as maltase, in canine serum contributed to the excess saccharogenic activity. A variation of Harding's amyloclastic modification of Somogyi's method (Perman and Stevens, 1969) for serum amylase determination has been used with good results. Values above 3200 Harding units are considered significant, and values of 6000–12,000 units are typical of acute pancreatitis. Lehane *et al.* (1977) assayed serum and urine specimens for amylase activity by an amyloclastic procedure (Caraway), a dyed-starch procedure (''Amylochrome''),* and a nephelometric procedure. They observed that the amyloclastic procedure was not as sensitive or as reliable as the other two methods. Using an Amylochrome kit, Traverso *et al.* (1976) found normal serum amylase concentration in the dog to be 870 dye units, with a standard deviation of 289 units. The Amylochrome method depends on amylase alone to solubilize the chromogenic amylase molecule and is not influenced by maltase activity.

*Roche Diagnostics, Nutley, New Jersey.

2. *Urine Amylase*

Human beings, guinea pigs, and baboons with acute pancreatitis have an elevated renal clearance of amylase relative to creatinine (Cam/Ccr), and this increased clearance makes urinary amylase a more sensitive indicator of pancreatitis than the serum level. High levels of urinary amylase have been found in acute pancreatitis after the normalization of serum amylase. To explain these findings, Johnson *et al.* (1976) concluded that there is either an increased glomerular permeability to amylase or a decreased tubular reabsorption of the filtered enzyme. Elevated serum levels of a amylase were produced in guinea pigs by experimentally inducing pancreatitis, duodenal perforation, and intestinal infarction. Renal clearance of amylase, expressed as a percentage of creatinine clearance, was found to be significantly increased in the animals with pancreatitis in comparison to animals with hyperamylasemia resulting from duodenal perforation or intestinal infarction (Orda *et al.*, 1976). Following the injection of baboon salivary and pancreatic amylase into baboons, the mean serum half-life was approximately 83 minutes (Duane *et al.*, 1971). Pancreatic amylase, however, was cleared more rapidly by the kidneys than was salivary amylase. Only 24% of the amylase cleared from the serum appeared in the urine, indicating that the majority of the amylase was removed from the serum by an extraurinary mechanism. Thus, the short half-life of amylase accounts for the transient nature of serum amylase elevations in pancreatitis, and the extraurinary removal of amylase prevents serum amylase levels from rising any higher than they do in uremia.

Dogs, which normally have a higher serum amylase concentration than do human beings, excrete very little amylase in their urine (Eto *et al.*, 1969) and have a very low Cam/Ccr ratio compared to man, guinea pigs, and baboons. This low Cam/Ccr ratio was found not to be due to a large unfilterable type of amylase since dog amylase was similar in size to that of man and guinea pig. Canine amylase was also rapidly cleared by the guinea pig kidney (Johnson *et al.*, 1977). The dogs' glomeruli were found to be permeable to amylase when the dogs serum amylase concentration was doubled by an infusion of guinea pig amylase. The Cam/Ccr then increased, and the increase was due to the heterologous amylase. Thus, the dogs' low Cam/Ccr ratio was considered to be due to an interaction between canine amylase and canine kidney, presumably in the tubule. When an inhibitor of renal Na-ATPase was administered intravenously to dogs, the Cam/Ccr ratio rose 20-fold. Urine lipase in dogs also has a low rate of clearance (Eto *et al.*, 1969).

3. *Serum Lipase*

The bulk of the digestive lipase is produced in the pancreas. A gastric lipase, however, is present in dogs, rats, and calves which originates from glands of the tongue and pharyngeal area (Hamosh *et al.*, 1975). Some are of the opinion that in acute pancreatitis serum lipase concentration becomes elevated for a longer period of time than amylase. Brobst *et al.* (1970), however, determined in dogs with experimentally induced pancreatitis that serum amylase and lipase concentrations tend to parallel each other. Kasahara *et al.* (1975) also observed that serum concentrations of lipase, amylase, and elastase, in dogs with experimentally induced pancreatitis, parallel each other. The concentrations of these enzymes were higher in the pancreatic vein than in the femoral vein, indicating that the enzymes did originate in the pancreas.

Hyperlipasemia, like hyperamylasemia, may be associated with conditions other than acute pancreatitis. In man, elevations of serum lipase have been reported in intestinal obstruction, peritonitis, and renal insufficiency (Bishop, 1968).

The determination of serum lipase is based on the hydrolysis of an olive oil emulsion into its constituent fatty acids. The fatty acids are titrated with NaOH, and the units of lipase are in direct relation to the number of milliliters of NaOH required to neutralize the fatty acids released per milliliter of serum. The Sigma–Tietz method requires a 6-hour hydrolysis period, and normal values for the dog are 0.0–1 unit (Small *et al.*, 1964). Brobst and Brester (1967), using a modification of a test (Roe and Byler, 1963) which employed only a 1-hour period of hydrolysis, reported normal serum lipase values in the dog of 0.8–12.0 Roe–Byler units. A rapid and highly reproducible turbidimetric method for the determination of serum pancreatic lipase in the dog has been described (Mia *et al.*, 1978). Serum lipase activities in normal dogs varied from 0 to 50 IU/liter, with a mean of 10 IU/liter ± 17 SD.

4. Serum Calcium

Hypocalcemia may occur in acute pancreatitis in the dog (Gage and Anderson, 1967). It was originally ascribed to deposition of calcium in areas of lipolysis; however, assay of intraabdominal calcium deposits demonstrated that the amount of calcium recovered was far short of that lost from the circulation (Edmondson *et al.*, 1952). The mechanisms by which the hypocalcemia of pancreatitis develops have not been established. It is known, however, that the total serum calcium concentration is dependent on serum protein concentration, with respect to both total serum calcium-carrying ability and the degree of ionization. Pigs with experimentally induced acute pancreatitis experienced an increase in hematocrit and a decrease in serum calcium, total protein, and albumin concentration. The rapid decrease in total protein confirmed a massive exudation of plasma from the circulation. The maintenance of normovolemia by the administration of protein-containing fluids prevented the development of hypocalcemia (Peoples *et al.*, 1977). Marenberg *et al.* (1978) observed significant decreases in ionized and total serum calcium in pigs with experimentally induced pancreatitis. Significant increases in serum inorganic phosphorus were also observed. The highest concentration of inorganic phosphorus and the lowest concentrations of both ionized and total calcium were seen 18 hours after the induction of pancreatitis. These findings suggested that parathormone was not being secreted in adequate amounts or that the target organs were unresponsive to parathormone.

Hypercalcemia may also precipitate pancreatic disease. Following the administration of excessive amounts of calcium to a dog with idiopathic hypocalcemia, pancreatic hemorrhage and necrosis suddenly developed (Neuman, 1975). It was believed that the hypercalcemia may have caused a precipitation of calcium carbonate from pancreatic juice within the ductal system, with this leading to obstruction and pancreatitis. Hypercalcemia also may have activated trypsinogen.

5. Serum Methemalbumin

Serum methemalbumin determinations have been made to differentiate acute hemorrhagic pancreatitis from acute edematous pancreatitis. Methemalbumin forms in acute pancreatitis from extravasation of blood in and around the pancreas. Pancreatic enzymes liberated locally as a result of the pancreatitis digest the surrounding hemoglobin, breaking it into two molecules: heme and globin. The enzymes oxidize the ferrous heme to ferric hematin, also known as metheme. Metheme is absorbed into the circulation and combines with albumin to form methemalbumin. Kelly *et al.* (1972) found that dogs with experimentally induced acute edematous pancreatitis had rises in serum amylase levels,

but serum methemalbumin concentrations remained normal. In contrast, dogs with hemorrhagic pancreatitis had increased concentrations of methemalbumin. Methemalbumin concentration was considered a good index for the diagnostic of hemorrhagic pancreatitis at the time when serum amylase levels had returned to normal. Anderson *et al.* (1969) reported that methemalbumin levels were elevated, not only in hemorrhagic pancreatitis, but also in other necrotizing diseases, such as intestinal infarction. The usefulness of the test in evaluating pancreatitis in veterinary medicine should be further explored.

6. Miscellaneous Procedures

Pathological hyperlipemia has been observed in some dogs with acute pancreatitis (Anderson and Straufuss, 1971). The lipemia is usually transient and may occur with exacerbations of acute pancreatitis. The most striking feature is an elevation of plasma triglyceride levels, which imparts a milky turbidity to the plasma. Kesseler *et al.* (1962) experimentally produced pancreatitis in rabbits, which resulted in an elevation of plasma triglycerides, with lipemia being present. Lipemic plasma from animals with pancreatitis inhibited the "lipemia-clearing factor," lipoprotein lipase of normal rabbits. There was a close correlation between the degree of inhibition of lipoprotein lipase and the level of plasma triglyceride. It was considered possible that inhibitors of lipoprotein lipase were released from necrotic pancreatic tissue.

Hyperlipemia may, in human beings, cause pancreatitis rather than be the result of it. In many people with hyperlipemia, serum triglyceride concentrations have been greater than normal (Greenberger, 1973). In the presence of hyperlipemia, it is theorized, lipase in pancreatic capillaries may cause hydrolysis of triglycerides in chylomicrons with release of fatty acids. The fatty acids might then cause capillary damage within the pancreas, resulting in ischemia and release of pancreatic enzymes.

Deoxyribonuclease activity has been reported to be elevated in the serum during the breakdown of pancreatic tissue. In an experimental study in dogs, Donahue *et al.* (1958) observed serum amylase to be elevated after ligation of the pancreatic ducts, but serum deoxyribonuclease underwent no significant change. However, after pancreatic cell necrosis was produced experimentally, there was a rise in serum deoxyribonuclease. The authors concluded that the rise in serum deoxyribonuclease was associated only with cell necrosis.

B. Chronic Pancreatitis

1. Serum Enzymes

Serum amylase and lipase levels may be elevated if acute exacerbations of pancreatitis occur or if chronic pancreatitis is associated with ductal obstruction. However, with complete destruction of acinar tissue and healing by fibrosis, serum enzyme elevations would not be expected.

2. Fecal Examination

When pancreatic insufficiency is under consideration, an examination of feces is important as a preliminary screening test. The gross and microscopic findings of a fecal examination may reflect the failure to digest and absorb fats and proteins as a result of pancreatic enzyme deficiencies. Pancreatic enzyme deficiencies may also be detected by the estimation of enzymes in feces.

Pancreatic enzymes undergo a rapid inactivation as they pass through the intestinal

tract. In the rat, diversion of pancreatic juice to the exterior of the body resulted in a rapid disappearance of enzymes from the small intestine, with virtually complete absence after 16 hours. In the rat intestine, pancreatic trypsin has a slower rate of inactivation than lipase (Pelot and Grossman, 1962). In human beings, there is a greater reduction in fecal tryptic activity than chymotryptic activity during intestinal transit. The content of chymotrypsin in the feces gave an excellent indication of the ability of the human pancreas to secrete chymotrypsin. The content of trypsin in the feces, however, less accurately reflected the amount secreted into the duodenum. When the content of trypsin in the feces was high, the pancreatic capacity to secrete trypsin was also high. The converse was not necessarily true, however, since low levels of tryptic activity in the feces were found in subjects whose capacity to secrete trypsin was normal. The content of trypsin in the feces alone cannot, therefore, be used as a reliable index of pancreatic enzyme secretory capacity (Sale *et al.*, 1974).

a. Gross and Microscopic Examination. With severe pancreatic insufficiency the stool is bulky or of a pale yellow or clay color; it may be glistening with neutral fat and often has a foul odor. Neutral fat in the feces may be stained with Sudan III or IV stain. The procedure as recommended by Masamune *et al.* (1977) required a saturated solution of Sudan III in 95% alcohol and a 37% acetic acid solution. Some fecal fat is present in the form of calcium soaps, which do not take the stains for neutral fat unless hydrolyzed with glacial acetic acid. A drop of Sudan III solution and acetic acid was added to small pieces of stool on a glass slide. After mixing, the specimen was heated and covered with a cover slip. The normal number of lipid droplets in human feces was less than 10 per field at a magnification of 100. When the number of fecal fat droplets was greater than normal, fecal fat, as determined chemically, was generally greater than normal. Lorenz (1976) was of the opinion that more than three or four fat droplets per high-power microscope field was indicative of pancreatic insufficiency in animals.

Fecal material may also be stained with Lugol's iodine, which stains starch granules a blue-black color. Excessive amounts of starch granules are compatible with pancreatic insufficiency. Relatively little amylase is required for the digestion of starch; thus, the tests for lipid in feces are better qualitative tests of pancreatic insufficiency. When fat, starch granules, or undigested muscle fibers are found in feces, the tests should be repeated at a later time to rule out possible dietary contribution. The results of these simple tests may provide information that can help to establish a diagnosis of pancreatic insufficiency, or they may indicate that other digestion or intestinal absorption tests are justified.

One of the most accurate methods of determining whether there is increased fat in the feces involves the quantitative determination of fecal fat residue. Likewise, fecal nitrogen determination by the macro-Kjeldahl method is one of the more accurate methods of measuring protein digestion. However, these determinations may not differentiate pancreatogenous from intestinal malassimilation and are more suitable for the research laboratory.

b. Fecal Trypsin. Two tests of fecal enzyme activity are suitable for the veterinary clinical laboratory (Jasper, 1954). The procedures are based on the incubation of gelatin with feces and the subsequent detection of proteolysis.

i. Tube test. This procedure utilizes the digestion of a gelatin solution by the test fecal sample and the detection of proteolysis by the failure of the gelatin solution to gel after incubation.

1. Bring 9 ml of water to 10 ml total volume by adding feces, and mix.
2. Warm 2 ml of a 7.5% gelatin solution to 37°C, and add 1 ml of 5% sodium bicarbonate and 1 ml of fecal dilution.
3. Incubate at 37°C for 1 hour (2.5 hours at room temperature).
4. Refrigerate for 20 minutes. Failure to gel indicates the presence of trypsin in the sample.

ii. Film test. This procedure utilizes digestion of the gelatin of exposed or unexposed X-ray film.

1. Bring 9 ml of 5% sodium bicarbonate solution to 10 ml total volume by adding feces, and mix.
2. Immerse a thin strip of X-ray film in the fecal dilution.
3. Incubate at 37°C for 1 hour (2.5 hours at room temperature).
4. Rinse off the film under tap water. A clearing of the submerged portion of the film strip indicates the presence of trypsin in the sample.

Of the two tests, the tube test is recommended as a more accurate procedure for the detection of fecal trypsin. The film test is less sensitive, with approximately 25% of the results being falsely negative. Thus, there is a need to show repeated negative results. If the film test is used, the tube test should be used for the confirmation of samples negative for trypsin. In either test, control samples using diluent only and a normal fecal sample should be run in conjunction with the suspected sample. Davies (1957) found that normal dog fecal material would digest gelatin on X-ray film in dilutions from 1 : 20 to 1 : 2000. Stools over 1 day old may give false-positive results due to bacterial action.

It should be remembered that the gelatin substrate is not specific for trypsin or chymotrypsin and may be hydrolyzed by proteolytic enzymes produced by intestinal bacteria or proteolytic enzymes of the succus entericus.

3. Pancreatic Chymotrypsin Secretion

The diagnosis of exocrine pancreatic insufficiency has been performed in animals by a procedure involving the oral administration of a chymotrypsin-labile peptide (*N*-benzoyl-L-tyrosyl) which contains *p*-aminobenzoic acid (PABA) as a tracer group. In the presence of chymotrypsin the peptide is split, and PABA is liberated. The PABA is absorbed from the gut, undergoes conjugation in the liver, and is excreted in the urine. The amount of PABA in the urine is used as an indirect measure of exocrine pancreatic function. The procedure has been shown to be reliable in detecting surgically induced pancreatic insufficiency in rats, swine, and dogs. Severe hepatic disease or severe renal disease, however, may interfere with the excretion of the PABA (Imondi *et al.*, 1972).

4. Absorption Tests

In veterinary medicine, absorption tests have been concerned primarily with the absorption of fats or vitamins in oil. The tests are based on the principle that dietary fats must be hydrolyzed to fatty acids and glycerol prior to absorption. It should be remembered that, in the presence of reduced ability to assimilate fats, the defect may lie in either a deficiency of pancreatic lipase or an inability of the small bowel to absorb properly digested fats.

a. Fat Absorption. This test is simple to perform, requires a minimum of equipment, and involves only visual comparison of the turbidity of the plasma (Brobst and Funk, 1972). A heparinized blood sample (5 ml) is drawn from the fasted animal and centrifuged. Then 2 ml peanut oil per kilogram body weight are added to a small quantity of food and fed to the dog. A second heparinized blood sample (5 ml) is drawn 2 hours after ingestion of the fat meal, and the sample centrifuged. The turbidities of the pre- and postfeeding samples are compared. Normally, the prefeeding sample has clear plasma, while the postfeeding sample has a creamy or turbid appearance (hyperlipemia). If the plasma samples are equally clear, one can assume that either pancreatic exocrine function is deficient or that the intestine is incapable of proper absorption. The two conditions can be differentiated by repeating the fat meal at a later date, this time supplemented with pancreatic extract as a source of lipase. A cloudy postfeeding sample indicates that absorption from the intestine was normal and that the pancreas was deficient in secretion of lipase. Enteritis may render the test unreliable because of false-negative results.

b. Labeled Fat Absorption. Measuring intestinal absorption of radioiodine-labeled triolein and radioiodine-labeled oleic acid has proved to be valuable in the differential diagnosis of pancreatic steatorrhea and intestinal malabsorption in dogs (Kaneko *et al.*, 1965). Kallfelz *et al.* (1968) demonstrated that in normal dogs approximately 13% of the oleic acid and 11% of the triolein was absorbed. In a dog with pancreatic fibrosis, the absorption of oleic acid was normal but that of triolein was less than 1%, indicating defective hydrolysis.

REFERENCES

Ahlert, G., Böhm, M., and Brüschke, G. (1969). *Experientia* **25,** 32.

Anderson, M. C., Toronto, I. R., Needleman, S. B., and Gramatica, L. (1969). *Arch. Surg.* **98,** 776.

Anderson, N. V. (1968). *In* "Current Veterinary Therapy III: Small Animal Practice" (R. W. Kirk, ed.), p. 526. Saunders, Philadelphia, Pennsylvania.

Anderson, N. V. (1972). *Vet. Clin. North Am.* **2,** 79.

Anderson, N. V., and Low, D. G. (1965). *Anim. Hosp.* **1,** 10.

Anderson, N. V., and Straufuss, A. C. (1971). *J. Am. Vet. Med. Assoc.* **159,** 885.

Baker, R. H. (1978). *J. Am. Vet. Med. Assoc.* **172,** 268.

Banks, P. A., Rudick, J., Dreiling, D. A., and Janowitz, H. D. (1968). *Am. J. Physiol.* **215,** 361.

Berk, J. E., Seercy, R. L., Hayashi, S., and Ugihira, I. (1965). *J. Am. Med. Assoc.* **192,** 389.

Bishop, R. P. (1968). *Am. J. Gastroenterol.* **49,** 112.

Brobst, D., and Brester, J. E. (1967). *J. Am. Vet. Med. Assoc.* **150,** 767.

Brobst, D. F., and Funk, A. (1972). *J. Am. Vet. Med. Assoc.* **161,** 1412.

Brobst, D., Ferguson, A., and Carter, J. M. (1970). *J. Am. Vet. Med.* **157,** 1697.

Byrne, J. J., and Boyd, T. F. (1963). *Am. J. Surg.* **105,** 720.

Clemente, F., and Meldolesi, J. (1975). *J. Cell Biol.* **65,** 88.

Cohen, M., Morgan, R., and Hoffman, A. F. (1971). *Gastroenterology* **60,** 1.

Dagorn, J. C., Sattel, J., and Sarles, H. (1977). *Gastroenterology* **73,** 42.

Davies, M. F. (1957). *Vet. Rec.* **69,** 1701.

Deschodt-Lanckman, M., Robberecht, P., De Neff, P., Labrie, F., and Christophe, J. (1975). *Gastroenterology* **68,** 318.

Di Magno, E., Go, V., and Summerskill, W. (1973). *N. Engl. J. Med.* **288,** 813.

Di Magno, E., Malagelada, J., Go, V., and Moertel, C. (1977). *N. Engl. J. Med.* **296,** 1318.

Donahue, J. P., Houck, J. C., and Coffey, R. J. (1958). *Surgery* **44,** 1070.

Drieling, D. (1959). *Am. J. Dig. Dis.* **4,** 581.

Drieling, D., Janowitz, H., Marshall, D., and Haemmerli, P. (1958). *Am. J. Dig. Dis.* **3,** 214.
Druffell, S. J. (1975). *J. Small Anim. Pract.* **16,** 365.
Duane, W., Frericks, R., and Levitt, M. (1971). *J. Clin. Invest.* **50,** 156.
Dyck, W. P., Rudick, J., Hoexter, B., and Janowitz, H. D. (1969). *Gastroenterology* **56,** 531.
Edmondson, H., Berne, C., Homann, R., and Wertman, M. (1952). *Am. J. Med.* **12,** 34.
Egdahl, R. H. (1958). *Ann. Surg.* **148,** 389.
Ertan, A., Brooks, F., Ostrow, D., Arvan, D., Williams, C., and Cerda, J. (1971). *Gastroenterology* **61,** 686.
Eto, K., Pairent, F., Appert, H., and Haword, J. (1969). *Arch. Surg.* **98,** 241.
Finco, D. R., and Stevens, J. B. (1969). *J. Am. Vet. Med. Assoc.* **155,** 1686.
Gage, E. D., and Anderson, N. V. (1967). *Anim. Hosp.* **3,** 151.
Gallo, L., Newbill, T., Hyun, J., Vahouny, G. V., and Treadwell, C. R. (1977). *Proc. Soc. Exp. Biol. Med.* **156,** 277.
Gardner, J., Conlon, T., Klaeveman, H., Adams, T., and Ondetti, M. (1975). *J. Clin. Invest.* **56,** 366.
Gibbs, G. E., and Ivy, A. C. (1951). *Proc. Soc. Exp. Biol. Med.* **77,** 251.
Gingerich, D. A., and Murdick, P. W. (1975). *Am. J. Vet. Res.* **36,** 663.
Goldfarb, R. D., and Weber, P. (1977). *Proc. Soc. Exp. Biol. Med.* **156,** 219.
Goodhead, B. (1971). *Arch. Surg.* **103,** 724.
Greenberg, L., and Shimo-Takahara, S. (1976). *J. Surg. Res.* **21,** 425.
Greenberger, N. J. (1973). *N. Engl. J. Med.* **289,** 586.
Gyr, K., Wolf, R., Imondi, A., and Felsenfeld, O. (1975). *Gastroenterology* **68,** 488.
Gyr, R. K., Kayasseh, L., Stalder, G. A., Rittmann, W., and Girard, J. (1977). *Am. J. Vet. Res.* **38,** 1949.
Hamano, K. (1953). *Arch. Jpn. Chir.* **22,** 500.
Hamosh, M., Klaeveman, H., Wolf, R., and Scow, R. (1975). *J. Clin. Invest.* **55,** 908.
Hardy, R. M., and Stevens, J. B. (1975). *In* "Textbook of Veterinary Internal Medicine" (S. J. Ettinger, ed.), Vol. 2, p. 1247–1269. Saunders, Philadelphia, Pennsylvania.
Hernandez, H. H., Chaikoff, L. L., and Kiyasu, J. Y. (1955). *Am. J. Physiol.* **181,** 523.
Hiatt, N. (1959). *Ann. Surg.* **149,** 77.
Hiatt, N., and Warner, N. E. (1969). *Am. Surg.* **35,** 30.
Hiatt, N., Bonorris, G., and Lanchantin, G. F. (1968). *RES, J. Reticuloendothel. Soc.* **5,** 8.
Holtermuller, K., Malagelada, J. R., McCall, J., and Go, W. (1976). *Gastroenterology* **70,** 693.
Imondi, A. R., Stradley, R. P., and Wolgemuth, R. (1972). *Gut* **13,** 726.
Janowitz, H. D., and Banks, P. A. (1976). *In* "Disorders of the Gastrointestinal Tract, Disorders of the Liver and Nutritional Disorders" (J. M. Dietschy, ed.), pp. 193–195. Grune & Stratton, New York.
Janowitz, H. D., and Dreiling, D. A. (1959). *Am. J. Med.* **27,** 924.
Janowitz, H. D., and Dreiling, D. A. (1962). *Ciba Found. Symp., Exocrine Pancreas* pp. 113–133.
Jarrett, L., and Lacey, P. (1962). *Endocrinology* **70,** 867.
Jasper, D. E. (1954). *North Am. Vet.* **35,** 523.
Johnson, S. G., Ellis, C., and Levitt, M. (1976). *N. Engl. J. Med.* **295,** 1214.
Johnson, S. G., Ellis, C., and Levitt, M. (1977). *Clin. Res.* **25,** 312A.
Jubb, K. V. F., and Kennedy, P. C. (1970). "Pathology of Domestic Animals," 2nd ed., Vol. 2, pp. 263–264. Academic Press, New York.
Kallfelz, F. A., Norrdin, R. W., and Neal, T. M. (1968). *J. Am. Vet. Med. Assoc.* **153,** 43.
Kaneko, J. J., Moulton, J. E., Brodey, R. S., and Perryman, V. D. (1965). *J. Am. Vet. Med. Assoc.* **146,** 463.
Kanno, T., Saito, A., and Sato, Y. (1977). *J. Physiol. (London)* **270,** 9.
Kasahara, K., Carballo, J., Takada, Y., Appert, H., and Howard, J. (1975). *Surg. Gynecol. Obstet.* **141,** 347.
Kelly, D., Baggott, D., and Gaskell, C. (1975). *J. Small Anim. Pract.* **16,** 163.
Kelly, T. R., Klein, R. L., Porquez, J. M., and Homer, G. M. (1972). *Ann. Surg.* **175,** 15.
Kessler, J., Finkel, M., Ho, P., and Janowitz, H. D. (1962). *Proc. Soc. Exp. Biol. Med.* **110,** 24.
Konturek, S., Domschke, S., Domschke, W., Wünsch, E., and Demling, L. (1977). *Am. J. Physiol.* **232,** E156.
Korbova, L., Kohout, J., Malis, F., Balas, V., Cizkova, J., Marek, J., and Cihak, A. (1977). *Gut* **18,** 913.
Lavau, M., Bazin, R., and Herzog, J. (1974). *J. Nutr.* **104,** 1432.
Lees, G. E., Suter, P. F., and Johnson, G. C. (1978). *J. Am. Vet. Med. Assoc.* **172,** 690.
Lehane, D. P., Wissert, P. J., Lum, G., and Levy, A. L. (1977). *Clin. Chem.* **23,** 1061.
Lightwood, R., and Reber, H. (1977). *Gastroenterology,* **72,** 61.
Little, P. B. (1963). *J. Am. Vet. Med. Assoc.* **143,** 874.

Llanos, O., Konturek, S., Rayford, P., and Thompson, J. (1977). *Am. J. Physiol.* **233,** E41.
Loeb, W. F., and Edge, L. T. (1962). *Am. J. Vet. Res.* **23,** 1117.
Long, W. B., and Weiss, J. B. (1974). *Gastroenterology* **67,** 920.
Lorenz, M. D. (1976). *In* "Veterinary Clinics of North America" (J. B. Tasker, ed.), Vol. 6, p. 663. Saunders, Philadelphia, Pennsylvania.
Malaisse-Lagae, F., Ravazzola, M., Robberecht, P., Vandermeers, A., Malaisse, W. J., and Orci, L. (1975). *Science* **190,** 795.
Marenberg, S. P., Lott, J. A., Pflug, B. K., Kibbey, W. E., and Carey, L. C. (1978). *Clin. Chem.* **24,** 881.
Masamune, O., Takahashi, T., Nagasaki, A., Iwabuchi, J., and Ishikawa, M. (1977). *Tohoku J. Exp. Med.* **122,** 397.
Messer, M., and Dean, R. T. (1975). *Biochem. J.* **151,** 17.
Meyer, J. H., and Jones, R. S. (1974). *Am. J. Physiol.* **226,** 1178.
Mia, A. S., Loger, H. D., and Tierney, M. M. (1978). *Am. J. Vet. Res.* **39,** 317.
Moreland, H. J., and Johnson, L. R. (1971). *Gastroenterology* **60,** 425.
Neuman, N. B. (1975). *J. Am. Vet. Med. Assoc.* **166,** 381.
Nishiyama, A., and Petersen, O. (1974). *J. Physiol. (London)* **238,** 145.
Nothman, M., and Callow, A. (1971). *Gastroenterology* **60,** 82.
O'Donnell, M. D., and McGeeney, K. F. (1975). *Comp. Biochem. Physiol. B* **50,** 269.
Orda, R., Orda, S., Wizniter, T., and Bawnik, J. (1976). *J. Surg. Res.* **21,** 457.
Pelot, D., and Grossman, M. (1962). *Am. J. Physiol.* **202,** 285.
Peoples, J., Eberlein, T., and Webster, M. W. (1977). *Am. J. Surg.* **133,** 42.
Perman, V., and Stevens, J. B. (1969). *J. Am. Vet. Med. Assoc.* **155,** 2053.
Ranson, J., Lackner, H., Berman, I. R., and Schinella, R. (1977). *Surgery* **81,** 502.
Rao, K. N., Tuma, J., and Lombardi, B. (1976). *Gastroenterology* **70,** 720.
Rapp, J. P. (1962). *Am. J. Vet. Res.* **23,** 343.
Rawls, J. A., Wistraad, P. J., and Maren, T. H. (1963). *Am. J. Physiol.* **205,** 651.
Reber, H. A., Johnson, F., Deveney, K., Montgomery, C., and Way, L. (1977). *J. Surg. Res.* **22,** 554.
Regan, P., Malagelada, J., DiMagno, E., Ghanzman, S., and Go, V. (1977). *N. Engl. J. Med.* **297,** 854.
Roe, J. H., and Byler, R. E. (1963). *Anal. Biochem.* **6,** 451.
Ruwitch, J., Bonertz, H. E., and Carlson, R. W. (1964). *J. Am. Vet. Med. Assoc.* **145,** 21.
Ryan, J., Appert, J., Carballo, J., and Davis, R. (1975). *Proc. Soc. Exp. Biol. Med.* **148,** 194.
Sale, J. K., Goldberg, D. M., Thjodleifsson, B., and Wormsley, K. (1974). *Gut* **15,** 132.
Satake, K., Rozmanith, J., Appert, H., Carballo, J., and Howard, J. M. (1973). *Ann. Surg.* **177,** 497.
Schapiro, H., McDougal, H. D., and Ludewig, R. M. (1977). *Am. Surg.* **43,** 802.
Scheele, G. A., and Palade, G. E. (1975). *J. Biol. Chem.* **250,** 2660.
Schmidt, C. W. (1970). *Scand. J. Gastroenterol., Suppl.* **6,** 471.
Singh, M., Black, O., and Webster, P. (1972). *Gastroenterology* **63,** 449.
Small, E., Olsen, R., and Fritz, T. (1964). *Vet. Med.* **59,** 627.
Smart, M., Downey, R., and Stockdale, P. (1973). *Can. Vet. J.* **14,** 313.
Spath, J. A., Gorczynski, R. J., and Lefer, A. M. (1974). *Am. J. Physiol.* **226,** 443.
Sullivan, J. F., Burch, R. E., and Magee, D. F. (1974). *Am. J. Physiol.* **226,** 1420.
Suter, P. F., and Olsson, S. (1969). *J. Am. Vet. Radiol. Soc.* **10,** 4.
Swanson, C. H., and Solomon, A. K. (1972). *Nature (London), New Biol.* **236,** 183.
Taylor, R. E. (1962). *Res. Vet. Sci.* **3,** 63.
Thomas, F. B., Sinar, D., Caldwell, J., Mekhjian, H., and Falko, J. (1977). *Gastroenterology* **73,** 221.
Traverso, W., Longmire, W., and Tompkins, R. (1976). *J. Surg. Res.* **21,** 247.
Valdivieso, V. (1972). *Am. J. Dig. Dis.* **17,** 129.
Vandermeers-Piret, M. C., Vandermeers, A., Wijns, W., Rathe, J., and Christophe, J. (1977). *Am. J. Physiol.* **232,** E131.
Vérine, H. (1973). *Comp. Pathol. Bull.* **5,** 3.
Weiner, S., Gramatica, L., Voegle, L., Hauman, R., and Anderson, M. (1970). *Am. J. Surg.* **119,** 55.
Yoshizawa, Y., Mizumoto, R., Nakase, A., and Honjo, I. (1976). *Am. J. Surg.* **131,** 689.
Zimmerman, M. J., Dreiling, D. A., Rosenberg, I. R., and Janowitz, H. D. (1967). *Gastroenterology* **52,** 865.

8

Gastrointestinal Function

BUD C. TENNANT AND
WILLIAM E. HORNBUCKLE

I. INTRODUCTION

The digestive system is composed of the gastrointestinal tract or alimentary canal, salivary glands, liver, and exocrine pancreas. The principal functions of the gastrointestinal tract are assimilation of nutrients and excretion of the waste products of digestion. Most nutrients are ingested in a form which is either too complex or insoluble for absorption. Within the gastrointestinal tract, these substances are solubilized and degraded enzymatically to simple molecules, sufficiently small in size and in a form which permits absorption across the mucosal epithelium. In the following section, the normal biochemi-

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

cal processes of intestinal secretion, digestion, and absorption are described. With these in perspective, we then discuss the mechanisms involved in the pathogenesis of the most important gastrointestinal diseases and the biochemical basis for diagnosis and treatment.

II. GASTROINTESTINAL SECRETION

A. Saliva

1. Mechanism of Secretion

Saliva is produced by three major pairs of salivary glands and by small glands distributed throughout the buccal mucosa and submucosa. Two types of secretory cells are found in the acinar portions of the salivary glands: (1) the *mucous cells,* which contain droplets of mucus, and (2) the *serous cells,* which contain multiple secretory granules. In those species which produce salivary amylase, the secretory granules are the zymogen precursors of this enzyme. A third cell type is found lining the striated ducts. The striations along the basal borders of these cells are caused by vertical infoldings of the cell membrane, a characteristic of epithelial cells involved in rapid movement of water and electrolytes. The primary secretion of the acinar cells is modified by active transport processes of the ductal epithelium.

The distribution of the different types of secretory cells in the salivary glands varies among species. The parotid glands of most animals are serous glands which produce a secretion of low specific gravity and osmolarity, containing electrolytes and proteins including certain hydrolytic enzymes. The mandibular (submaxillary) and sublingual glands are mixed salivary glands containing both mucous and serous types of cells and produce a viscous secretion which contains large amounts of mucus (Dukes, 1955).

2. Composition

a. Mucus. Mucus is an aqueous mixture of protein–polysaccharide complexes and glycoproteins (Gottschalk, 1972), which have relatively large amounts of carbohydrate bound to protein. The protein–polysaccharide complexes have long polysaccharide chains containing repeating units bound to a protein core. The glycoproteins contain numerous oligosaccharide residues distributed along the polypeptide chain.

One of the most completely studied glycoproteins is mucin from the submaxillary glands of ruminants. The carbohydrate portion is a disaccharide of *N*-acetylneuraminic acid (a sialic acid) and *N*-acetylgalactosamine. Approximately 800 such disaccharide molecules are present per molecule of mucin (Bhavanandan *et al.,* 1964; Bertolini and Pigman, 1967). An enzyme capable of linking protein with hexosamine was demonstrated in sheep submaxillary glands (McGuire and Roseman, 1967).

The physiological functions of mucin are closely related to its high viscosity. *N*-Acetylneuraminic acid is the component responsible for the formation of viscous aqueous solutions. At physiological pH, it causes expansion and stiffening of the mucin molecule (Gottschalk and Thomas, 1961). The resistance of mucin to enzymatic breakdown is also due to the presence of disaccharide residues. Removal of the terminal *N*-acetylneuraminic acid residues by neuraminidase significantly increases the susceptibility of peptide bonds to trypsin (Gottschalk and Fazekas de St. Groth, 1960).

b. Amylase. The saliva of most species contains the α-amylase *ptyalin*. This enzyme is said to be absent, however, in the saliva of dogs, cats, and horses (Dukes, 1955). Salivary amylase splits the α-1,4-glucosidic bonds of various polysaccharides. The salivary enzyme is similar in all major respects to pancreatic α-amylase, which is described below (Section II,D). Salivary amylase initiates digestion of starch and glycogen in the mouth of those species which secrete the enzyme. The optimal pH for amylase activity is approximately 7, and activity therefore terminates when the enzyme mixes with acidic gastric contents.

3. Functions of Saliva

Saliva bathes the oral cavity continuously, serving to protect the surface epithelium. Ingested food is moistened and lubricated by saliva, facilitating mastication and swallowing. The teeth also are protected from decay by saliva, which washes food particles from the surfaces of the teeth and, because of its buffering capacity, neutralizes the organic acids produced by bacteria normally present in the mouth.

Ruminants produce much greater quantities of saliva than simple-stomached animals, and the saliva has a higher pH and bicarbonate ion concentration. In ruminants, saliva serves several unique functions (Phillipson, 1977). It is required for maintenance of the composition of the contents of the rumen. The great buffering capacity is necessary to neutralize the large amounts of short-chain fatty acids which are the major end products of rumen fermentation. The urea in saliva can be utilized by rumen bacteria for protein synthesis. Protein synthesized in the rumen is then used to meet dietary protein requirements. In this way, urea nitrogen can be "recycled" through the amino acid pool of the body and in ruminants need not be considered an end stage in protein catabolism. The ability to reutilize urea has also been demonstrated in the horse and may be of particular benefit during periods of protein deficiency (Houpt and Houpt, 1971; Prior *et al.*, 1974).

B. Gastric Secretion

The stomach is divided into two main regions on the basis of secretory function (Grossman, 1958). The *oxyntic gland area* corresponds approximately to the body of the stomach in most species of domestic animals and also to the fundus in the dog and cat. The oxyntic glands contain (1) *oxyntic* or *parietal cells,* which are responsible for hydrochloric acid production, (b) *peptic* (zymogenic, chief) *cells,* which produce pepsinogen, and (c) *mucous cells*. The *pyloric gland area* contains the pyloric glands, which are slightly alkaline, and, in addition to mucus, contains the polypeptide hormone gastrin.

1. Control of Gastric Secretion

A variety of stimuli can initiate gastric secretion. The sight or smell of food or the presence of food within the mouth causes gastric secretion by a reflex mechanism involving the vagus nerve. The presence of certain foods within the stomach or distention of the stomach alone also can initiate both intrinsic and vagal nerve reflexes which cause secretion of gastric juice. In addition to neural reflexes, these stimuli cause release of the polypeptide hormone *gastrin* from the pyloric gland area, which enters the bloodstream, stimulating gastric secretion. The release of gastrin from the specific G cells responsible for synthesis is inhibited by excess hydrogen ion, and this negative feedback mechanism is

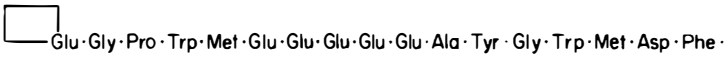


Fig. 1. Amino acid sequence of porcine gastrin I (Gregory, 1966). Gastrin II differs from gastrin I by the presence of a sulfate ester group on the single tyrosyl residue.

believed to be of physiological importance in the control of hydrochloric acid production.

Gastrin has been isolated in pure form from the antral mucosa of swine (Gregory *et al.*, 1964; Gregory and Tracy, 1964; Tracy and Gregory, 1964). When administered intravenously, the purified hormone causes the secretion of hydrochloric acid and pepsin. It also stimulates gastrointestinal motility and causes pancreatic secretion.

Two separate peptides have been obtained from porcine gastric mucosa and have been designated gastrin I and gastrin II. The structure of gastrin has been determined (Gregory *et al.*, 1964) and has been confirmed by synthesis (Anderson *et al.*, 1964). It is a heptadecapeptide amide, with a pyroglutamyl N-terminal residue and the amide of phenylalanine as the C-terminal residue (Fig. 1). In the center of the molecule is a sequence of five glutamyl residues, which give the molecule its acidic properties. Gastrin II differs from gastrin I only in the presence of a sulfate ester group linked to the single tyrosyl residue. The C-terminal tetrapeptide amide, Trp-Met-Asp-Phe-NH_2, is identical in all species so far studied (Gregory, 1967). The tetrapeptide has all of the activities of the natural hormone. It is not as potent as the parent molecule, but activity can be increased by lengthening the peptide chain.

Gastrin is the only hormone known to stimulate HCl secretion (Walsh and Grossman, 1975). As indicated above, gastrin is released in response to vagal stimulation by distention of the pyloric antrum and by direct luminal contact with food, particularly partially hydrolyzed protein (Walsh and Grossman, 1975). The exact mechanism of action is not known, but studies using isolated preparations of isolated parietal cells suggest that the effects of gastrin are not mediated by cyclic AMP (Soll, 1977).

Some of the other factors which are important in regulation of HCl secretion are summarized in Fig. 2 after Dousa and Dozois (1977). There is little doubt that histamine secreted locally within the mucosa has a major effect on the function of parietal cells (Soll

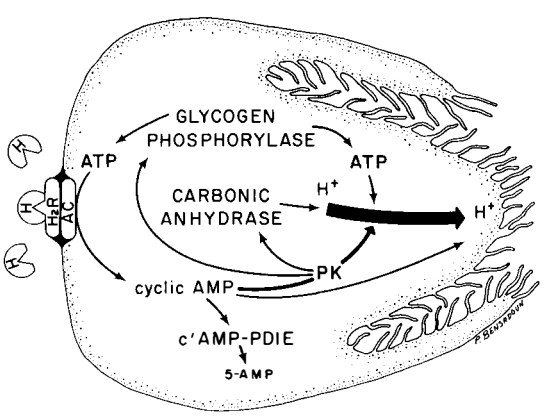


Fig. 2. Schematic representation of a parietal cell and proposed role of histamine in HCl secretion. Abbreviations: H_2R, H_2-receptor; H, histamine molecule; AC, adenylate cyclase; PK, protein kinase; cAMP-PDIE, cyclic AMP phosphodiesterase. (After Dousa and Dozois, 1977.)

and Grossman, 1978). Histamine has been recognized as a potent stimulant of HCl production for many years (Code, 1965). This effect, however, was not inhibited by traditional antihistaminic drugs (H_1 antagonists), and, until the demonstration of H_2 receptors in the stomach (the atrium and uterus) by Black *et al.* (1972), the physiological role of histamine in HCl secretion was controversial. Specific H_2 antagonists (burimamide, cimetidine, metiamide) now have been shown to inhibit the secretory response not only to histamine, but also to gastrin, to cholinergic stimuli, and to food (Grossman and Konturek, 1974).

Although there has been significant conflict in the published literature, current evidence suggests that histamine activates the adenylate cyclase of parietal cells (Dousa and Dozois, 1977), resulting in synthesis of cyclic AMP and ultimately in HCl secretion (Fig. 2). The controversy with regard to the role of cyclic AMP as a mediator of histamine action has come from observations that prostaglandins and secretin, both potent inhibitors of gastric HCl secretion, also stimulate adenylate cyclase (Thompson *et al.*, 1977). It is now believed that prostaglandins, in addition to inhibiting HCl secretion, act on a mucosal cell population which is different from parietal cells and that these cells secrete cytoprotective substances (mucin, glycosaminoglycans). The ulcerogenic effects of prostaglandin inhibitors (indomethacin, acetylsalicylic acid) apparently result from inhibition of this protective effect of endogenous prostaglandins.

2. *Composition of Gastric Secretion*

a. Basal versus Stimulated Secretion. Gastric juice is composed of two components. One is secreted continuously by the surface epithelial cells and other mucus-producing cells. The other component is produced by the oxyntic glands in response to various stimuli. The basal component is neutral or slightly alkaline. The electrolyte composition is similar to that of an ultrafiltrate of plasma (Table I) and contains large amounts of mucus, which protects the epithelium. The secretory component produced by the oxyntic glands in response to stimulation contains free hydrochloric acid and pepsinogen, the principal enzyme of gastric digestion.

The composition of gastric juice depends on the relative amounts of the two secretory components present, which in turn is a function of flow rate. In the dog, gastric juice is

TABLE I

Composition of Parietal and Nonparietal Secretions of Canine Gastric Mucosa

Component	Parietal secretion[a] (mEq/liter)	Nonparietal secretion[a] (mEq/liter)	Nonparietal secretion[b] (mEq/liter)
Na^+	—	155.0	138.0
H^+	159.0	—	—
K^+	7.4	7.4	4.0
Ca^{2+}	—	3.7	5.0
Cl^-	166.0	133.0	117.0
pH	<1.0	7.54[c]	7.42

[a] Determined *in vivo* using dogs with gastric fistulas (Gray and Bucher, 1941).
[b] Determined *in vitro* with isolated gastric mucosa (Altamirano, 1963).
[c] Calculated from bicarbonate concentration assuming CO_2 of 40 mm Hg.

produced in the resting state at a rate of approximately 5 ml/hour (Gray and Bucher, 1941), and the composition is similar to that of the basal component, containing practically no peptic activity or hydrochloric acid. When the flow of gastric juice is stimulated maximally, the dog may produce 80 ml or more per hour (Gray and Bucher, 1941), and this secretion contains large amounts of peptic activity and hydrochloric acid. Sodium, which is the principal cation in the basal secretion, is replaced to a large extent by hydrogen ion. The concentration of potassium is similar in both basal and stimulated secretions and therefore remains relatively constant at various rates of flow.

Hydrochloric acid and pepsinogen are secreted by separate mechanisms, but these appear to be closely linked under physiological conditions. Stimulation of the vagus nerve (Bachrach, 1953; Hirschowitz and Sachs, 1965) or intravenous injection of gastrin (Hirschowitz, 1966) increases pepsinogen and hydrochloric acid levels together. Other stimuli may affect the two processes differently. In the dog, for example, histamine infusion stimulates hydrochloric acid production maximally but inhibits pepsinogen secretion (Abrams and Brooks, 1960; Hirschowitz, 1966; Emas and Grossman, 1967).

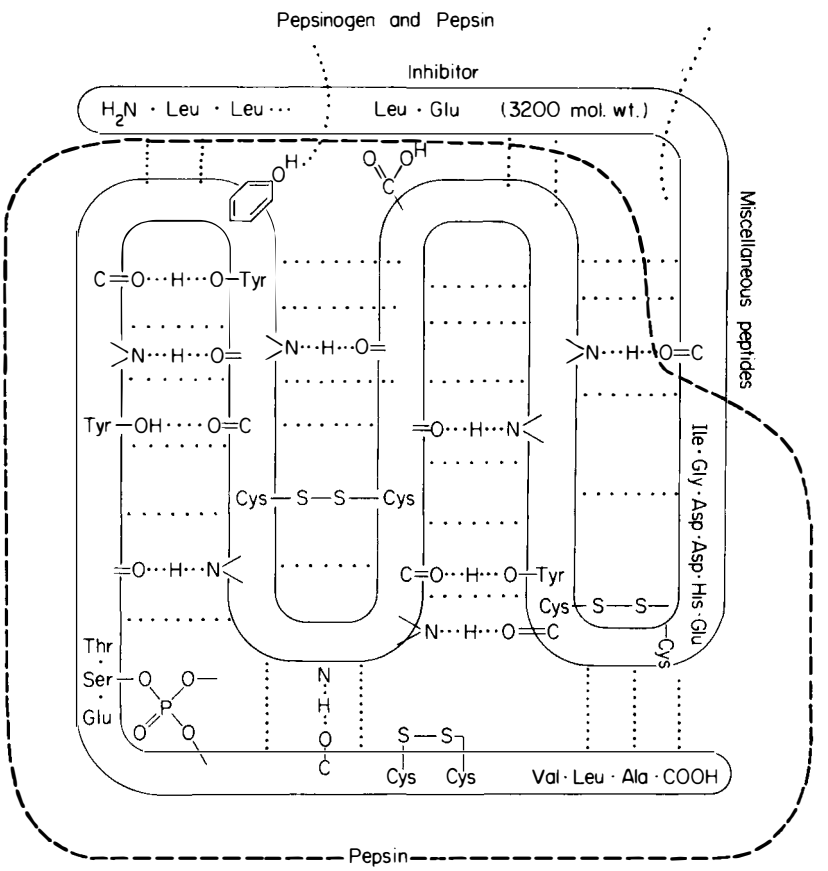


Fig. 3. Structure of pepsinogen–pepsin molecule showing position of inhibitor polypeptide, as proposed by Herriott (1962).

b. Pepsin. Pepsinogen is the zymogen, or inactive precursor, of pepsin, the principal proteolytic enzyme of gastric juice. Pepsinogen was first crystallized from the gastric mucosa of swine (Herriott, 1938), and several pepsinogens have been separated by Ryle (1965), Ryle and Porter (1959), and Ryle and Hamilton (1966). Porcine pepsinogen has a molecular weight of approximately 43,000 and is composed of the pepsin molecule and several smaller peptides (Fig. 3).

One of these peptides has a molecular weight of 3200 and is an inhibitor of peptic activity (Herriott, 1962). Activation of pepsin from pepsinogen occurs by selective cleavage of this small basic peptide from the parent pepsinogen (Neurath and Walsh, 1976). Autocatalytic conversion begins below pH 6.0. At pH 5.4, the inhibitor peptide dissociates from the parent molecule, and, at pH 3.5–4.0, the inhibitor is completely digested by pepsin (Taylor, 1968).

Pepsin has a very acidic isoelectric point, being stable in acidic solution below pH 6.0 but irreversibly denatured at pH 7.0 or above. In contrast, pepsinogen is stable in neutral or slightly alkaline solution. The optimal pH for peptic activity is generally between 1.6 and 2.5, but the effect of pH may vary with the substrate. Pepsin is capable of hydrolyzing peptide bonds of most proteins, mucin being one important exception. Pepsin splits bonds involving phenylalanine, tyrosine, and leucine most readily but can hydrolyze almost all other peptide bonds.

c. Rennin. Rennin is another proteolytic enzyme produced by the gastric mucosa and has some characteristics which are similar to those of pepsin. It has been separated from pepsin in preparations from the stomachs of newborn calves. Rennin splits a mucopeptide from casein to form paracasein, which then reacts with calcium ion to form an insoluble coagulum. The coagulated milk protein probably delays gastric emptying and increases the efficiency of protein digestion in young calves.

d. Hydrochloric Acid. Hydrochloric acid is produced by the oxyntic cells. When the normal mucosa is stimulated, both chloride and hydrogen ions are secreted together, but current evidence suggests that H^+ and Cl^- are secreted by separate, closely coupled pump mechanisms. Small amounts of Cl^- are secreted continuously by the unstimulated parietal cells in the absence of H^+ secretion, and this mechanism is responsible for the relative negative charge of the resting mucosal surface. Hydrogen ion and Cl^- secretory systems may also be differentiated *in vitro* by the demonstration of hydrogen ion secretion in the absence of Cl^-. A scheme for the secretion of hydrochloric acid is presented in Fig. 4. For every H^+ secreted, an electron is removed. The electron ultimately is accepted by oxygen to form OH^-, which is neutralized within the cell by H^+ from carbonic acid. The bicarbonate ion produced enters the venous blood, and this explains why the pH of gastric venous blood frequently is greater than that of arterial blood during hydrochloric acid secretion (Davenport, 1966).

Conversion of carbon dioxide and water to carbonic acid is catalyzed by carbonic anhydrase, which is present in high concentration within parietal cells. When the rate of acid secretion is high, this enzyme contributes to the secretory mechanism by maintaining normal intracellular pH. Carbonic anhydrase inhibitors, such as acetazolamide, interfere with hydrochloric acid production in high concentrations and when the rate of acid secretion is high (Janowitz *et al.*, 1952).

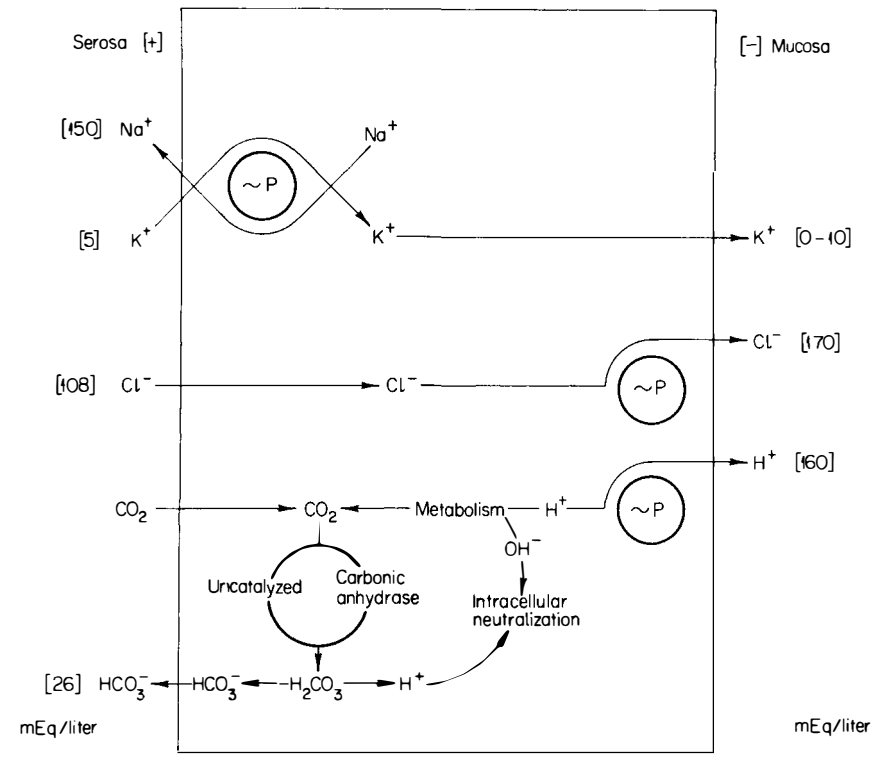


Fig. 4. Movement of ions in the parietal cell during secretion of hydrochloric acid. [Modified from Davenport (1966) and based on the data of Harris and Edelman (1964).]

C. Bile

Bile is secreted continuously by the hepatocytes into the bile canaliculi and is transported through a system of ducts to the gallbladder, where it is modified, concentrated, and stored. During digestion, bile is discharged into the lumen of the duodenum, where it aids in emulsification, hydrolysis, and solubilization of dietary lipids. The digestive functions of bile are accomplished almost exclusively by the detergent action of its major components, the bile salts and phospholipids.

1. Synthesis of Bile Acids

The primary bile acids are C^{24} carboxylic acids synthesized by the liver from cholesterol. Bile acid formation represents the major pathway for cholesterol metabolism (Danielsson, 1963). Cholic acid ($3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholanoic acid) and chenodeoxycholic acid ($3\alpha,7\alpha$-dihydroxy-5β-cholanoic acid) are the primary bile acids formed by most species of domestic animals. In swine, chenodeoxycholic acid is hydroxylated at the 6α position by the liver to yield hyocholic acid, which is a major primary bile acid in this species (Haslewood, 1964).

Bile acids are secreted as amino acid conjugates of either glycine or taurine. Taurine conjugates predominate in the dog, cat, and rat. In the rabbit, the conjugating enzyme system appears to be almost completely specific for glycine (Bremer, 1956). Both taurine and glycine conjugates are present in ruminants. In the newborn lamb, 90% of the bile

acids are conjugated with taurine. As the lamb matures, glycine conjugates increase, accounting for one-third of the total in mature sheep (Peric-Golia and Socic, 1968).

Under normal conditions, only conjugated bile acids are present in the bile and in the contents of the proximal small intestine. In the large intestine, the conjugated bile acids are hydrolyzed rapidly by bacterial enzymes so that, in the contents of the large intestine and in the feces, free or unconjugated bile acids predominate. Several genera of intestinal bacteria, including *Clostridium, Enterococcus, Bacteroides,* and *Lactobacillus* (Midtvedt and Norman, 1967), are capable of splitting the amide bonds of conjugated bile acids.

Intestinal bacteria also modify the basic structure of the bile acids. One such reaction is the removal of the α-hydroxyl group at the 7 position of cholic acid or chenodeoxycholic acid. These bacterial reactions yield the secondary bile acids, deoxycholic acid, and lithocholic acid, respectively (Gustafsson *et al.,* 1957). Lithocholic acid is relatively insoluble and is not reabsorbed to any great extent (Gustafsson and Norman, 1962). Deoxycholic acid is reabsorbed from the large intestine in significant quantities and is either rehydroxylated by the liver to cholic acid and excreted (Lindstedt and Samuelsson, 1959) or excreted as conjugated deoxycholic acid. The extent to which bacteria transform the primary bile acids depends on the nature of the diet, the composition of the intestinal microflora, and the influences which these and other factors have on intestinal motility (Gustafsson *et al.,* 1966; Gustafsson and Norman, 1969a,b).

2. *Detergent Properties of Bile*

The carboxyl group of the bile acids is completely ionized at the pH of bile and is neutralized by sodium ion, resulting in the formation of *bile salts.* The bile salts are effective detergents. They are amphipathic molecules, which have both hydrophobic and hydrophilic regions. In low concentrations, bile salts form molecular or ideal solutions, but, when their concentration increases above a certain critical level, they form polymolecular aggregates known as *micelles.* The concentration at which these molecules aggregate is called the *critical micellar concentration* (CMC).

Bile salt micelles are spherical and consist of a central nonpolar core and an external polar region. Fatty acids, monoglycerides, and other lipids are solubilized when they enter the central core of the micelle and are covered by the outside polar coat. Solubilization occurs only when the CMC is reached. For the bile salt–monoglyceride–fatty acid–water system present during normal fat digestion, the CMC is approximately 2 mM, which is ordinarily exceeded both in bile and in the contents of the upper small intestine (Hofmann, 1963). Phospholipids, principally lecithin, are also major components of bile. In the lumen of the small intestine, pancreatic phospholipase catalyzes the hydrolysis of lecithin, forming free fatty acid and lysolecithin. The latter compound also is a potent detergent which acts with the bile salts to disperse and solubilize lipids in the aqueous micellar phase.

3. *Enterohepatic Circulation of Bile Acids*

The enterohepatic circulation begins as conjugated bile acids near the duodenum and mix with the intestinal contents, forming emulsions and micellar solutions. The bile acids are not absorbed in significant amounts from the lumen of the proximal small intestine. Absorption occurs primarily in the ileum (Lack and Weiner, 1961, 1966; Weiner and Lack, 1962), where an active transport process has been demonstrated (Dietschy *et al.,* 1966). The conjugated bile acids pass unaltered into the portal circulation (Playoust and

Isselbacher, 1964) and return to the liver, where the cycle begins again. This arrangement provides optimal concentrations of bile acids in the proximal small intestine, where fat digestion and absorption occur, and then efficient absorption after these functions have been accomplished. Absorption of unconjugated bile acids from the large intestine accounts for 3–15% of the total enterohepatic circulation (Weiner and Lack, 1968).

In dogs, the total bile acid pool was estimated to be 1.1–1.2 gm. The half-life of the bile acids in the pool ranged between 1.3 and 2.3 days, and the rate of hepatic synthesis was 0.3–0.7 gm/day (Wollenweber *et al.*, 1965). The daily requirement for bile acids greatly exceeds the normal synthetic rate. This necessitates repeated reutilization of the bile acids, which is accomplished by means of the enterohepatic circulation. Under steady-state conditions, the entire bile acid pool passes through the enterohepatic circulation approximately ten times each day (Hofmann, 1966).

The size of the bile acid pool is dependent upon diet, the rate of hepatic synthesis, and the efficiency of the enterohepatic circulation. Surgical removal of the ileum in dogs interrupts the enterohepatic circulation, causing an increase in bile acid turnover rate and a reduction in the size of the bile acid pool (Playoust *et al.*, 1965). In diseases of the ileum, there may be defective bile salt absorption and bile salt deficiency. If severe, impaired utilization of dietary fat may occur, resulting in steatorrhea and impaired absorption of the fat-soluble vitamins.

D. Secretion of the Exocrine Pancreas

The exocrine pancreas is an acinous gland with the same general structure as the salivary glands. The cytoplasm of the secretory cells contains numerous zymogen granules, which vary in size and number depending on the activity of the gland. These granules contain the precursors of the hydrolytic enzymes responsible for digestion of the major dietary components. The cells of the terminal ducts probably secrete the bicarbonate ion responsible for neutralizing hydrochloric acid which enters the duodenum from the stomach.

1. Composition

a. Electrolyte Composition. The cation content of pancreatic secretion is similar to that of plasma. Sodium is the predominant cation, with smaller concentrations of potassium and calcium being present. A unique characteristic of pancreatic juice is its high bicarbonate ion concentration and alkaline pH. In the dog, the pH ranges from 7.4 to 8.3, depending on HCO_3^- content. The volume of pancreatic juice is directly related to HCO_3^- content and pH increase and the Cl^- concentration decreases. The sodium and potassium ion concentrations and osmolarity appear to be independent of secretory rate (Fig. 5).

b. α-Amylase. The amylase produced by the pancreas catalyzes the specific hydrolysis of α-1,4-glucosidic bonds, which are present in starch and glycogen (α-1,4-glycan-4-glycan hydrolase). Pancreatic amylase appears to be essentially identical to the amylase of saliva. It is a calcium-containing metalloenzyme (Vallee *et al.*, 1959). Removal of calcium by dialysis inactivates the enzyme and markedly reduces the stability of the apoenzyme. Pancreatic amylase has an optimal pH for activity of 6.7–7.2 and is activated by chloride ion.

Synthesis of pancreatic α-amylase occurs in the ribosomes. The enzyme is transferred

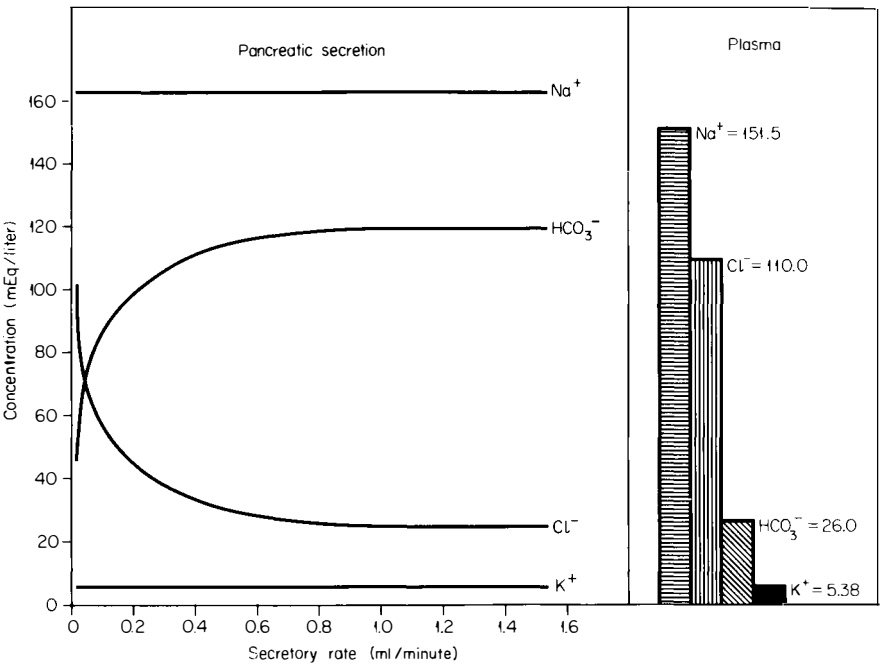


Fig. 5. Influence of secretory rate on the electrolyte composition of canine pancreatic juice. (From Bro-Rasmussen *et al.*, 1956.)

from the endoplasmic reticulum to cytoplasmic zymogen granules for storage (Redman *et al.*, 1966). It is secreted in active form upon stimulation of the acinar cells. Newborn calves (Huber *et al.*, 1961) and pigs (Walker, 1959) secrete amylase at a significantly lower rate than mature animals. The rate of synthesis is also influenced by diet. Animals fed a high-carbohydrate diet synthesize amylase at several times the rate of animals on a high-protein diet (Ben Abdeljlil and Desnuelle, 1974).

Unbranched α-1,4-glucosidic chains, such as those found in amylase, are hydrolyzed in two steps. The first is rapid and results in formation of the disaccaride maltose and maltotriose. The second step is slower and involves hydrolysis of maltotriose with formation of glucose and maltose. Polysaccharides such as amylopectin and glycogen contain branched chains with both α-1,4- and α-1,6-glucosidic linkages. When α-amylase attacks these compounds, the principal products are maltose (α-1,4-glycosidic bond), isomaltose (α-1,6-glucosidic bond), and small amounts of glucose. Final hydrolysis of the maltose and isomaltose occurs at the surface of the mucosal cell, where the enzymes maltase and isomaltase are integral parts of the microvillous membrane.

c. Proteolytic Enzymes. The proteolytic enzymes of the pancreas are responsible for the major portion of protein hydrolysis, which occurs within the lumen of the gastrointestinal tract. Two types of peptidases are secreted by the pancreas. Trypsin, chymotrypsin, and elastase are *endopeptidases,* which attack peptide bonds along the polypeptide chain, producing smaller peptides. The *exopeptidases* attack either the carboxy-terminal or amino-terminal peptide bonds, releasing single amino acids. The principal exopeptidases secreted by the pancreas are carboxypeptidases A and B. The endopeptidases and exopep-

TABLE II

Relationship between the Activities of Pancreatic Endopeptidases and Exopeptidases

Enzyme	Type	Activity
Trypsin	Endopeptidase	Produces peptides with C-terminal basic amino acids
Carboxypeptidase B	Exopeptidase	Removes C-terminal basic amino acids
Chymotrypsin	Endopeptidase	Produces peptides with C-terminal aromatic amino acids
Elastase	Endopeptidase	Produces peptides with C-terminal nonpolar amino acids
Carboxypeptidase A	Exopeptidase	Removes C-terminal aromatic and nonpolar amino acids

tidases act in complementary fashion (Table II), producing free amino acids, which are absorbed directly, or small peptides, which are further hydrolyzed by the aminopeptidases of the intestinal mucosa (see Section III,C).

The pancreatic peptidases are secreted as inactive proenzymes or zymogens termed trypsinogen, chymotrypsinogen, and procarboxypeptidase A and B. Trypsinogen is converted to active trypsin in two ways. At alkaline pH, trypsinogen can be converted autocatalytically to trypsin, the activated enzyme converting more zymogen to active enzyme. Trypsinogen can also be activated by the enzyme *enterokinase,* which is produced by the duodenal mucosa. The latter reaction appears to be highly specific in that enterokinase will not activate chymotrypsinogen. Chymotrypsinogen, proelastase, and the procarboxypeptidases A and B are converted to active enzymes by the action of trypsin.

The amino acid sequences and other structural characteristics of bovine trypsinogen and chymotrypsinogen have been determined (Hartley *et al.,* 1965; Hartley and Kauffman, 1966; Brown and Hartley, 1966). The polypeptide chain of trypsinogen contains 229 amino acid residues. Activation of the proenzyme occurs with hydrolysis of a single peptide bond located in the 6 position between lysine and isoleucine. The C-terminal hexapeptide is released as enzyme activity appears. There is also substantial change in the helical structure of the parent molecule (Davie and Neurath, 1955; Neurath *et al.,* 1956). Chymotrypsinogen A is composed of 245 amino acid residues and has numerous structural similarities to trypsinogen. Activation of the chymotrypsinogen also occurs with cleavage of a single peptide bond. For a complete discussion of this subject, see the review by Keller (1968).

d. Lipase. The pancreas produces several lipolytic enzymes with different substrate specificities. The most important of these from a nutritional viewpoint is the lipase responsible for hydrolysis of dietary triglyceride. This enzyme has the unique property of requiring an oil–water interface for activity so that only emulsions can be effectively attacked (Sarda and Desnuelle, 1958). The principal products of lipolysis are glycerol, monoglycerides, and fatty acids. The monoglycerides and fatty acids accumulate at the oil–water interface and can inhibit enzyme activity. Their transfer from the interface to the aqueous phase is favored by the presence of sodium bicarbonate also secreted by the pancreas and by bile salts.

Mattson and Volpenhein (1966) described two other carboxylic ester hydrolases in pancreatic juice. Both enzymes have an absolute requirement for bile salts, in contrast to glycerol ester hydrolase, which is actually inhibited by bile salts at pH 8. One of these

enzymes is a sterol ester hydrolase responsible for hydrolysis of cholesterol esters. The other enzyme hydrolyzes various water-soluble esters. The two enzyme activities have been differentiated on the basis of stability and optimal pH.

The pancreas secretes a third lipolytic enzyme which hydrolyzes phospholids. Phospholipase A converts lecithin which is present in bile to lysolecithin, an effective detergent which aids in emulsification of dietary fat.

2. *Control of Secretion*

Pancreatic secretion is controlled and coordinated by neural and endocrine mechanisms. When ingesta or hydrochloric acid enters the duodenum, the hormone secretin is released into the circulation by the duodenal mucosa. Secretin increases the volume, pH, and HCO_3^- concentration of the pancreatic secretion.

Secretin is a polypeptide hormone which contains 27 amino acid residues. All 27 amino acids are required to maintain the helical structure of the molecule and its activity (Bodanszky *et al.*, 1969). The C-terminal amide is a property of other polypeptide hormones, such as gastrin and vasopressin, which act on the flow of water in biological systems (Mutt and Jorpes, 1967). In addition to its effects on the pancreas, secretin increases the rate of bile formation (Wheeler and Mancusi-Ungaro, 1966).

The pancreatic juice which results from stimulation by secretin is large in volume and has high bicarbonate concentration but is low in enzyme activity. Stimulation of the vagus nerve causes a significant rise in enzyme concentration. This type of response also is produced by *pancreozymin,* another polypeptide hormone secreted by the duodenal mucosa. Pancreozymin is now believed to be identical to cholecystokinin, an intestinal hormone which causes contraction of the gallbladder (Thompson, 1969). The C-terminal pentapeptide of pancreozymin–cholecystokinin is exactly the same as that of gastrin. This fascinating relationship suggests that gastrin and pancreozymin–cholecystokinin may participate in some unified but as yet poorly understood system of digestive control (Thompson, 1969).

E. Other Gastrointestinal Polypeptide Hormones

During the past several years, a large number of papers have been published on the endocrine function of the gastrointestinal mucosa and on several new polypeptides which are being classified as gut hormones (Table III). Many of these new substances have not met the rigid physiological requirements for true hormone status, including (1) biological action in very small concentration, (2) release into the bloodstream, and (3) normal serum levels comparable to those provided experimentally by exogenous administration. These criteria probably will be modified, particularly with regard to requirements for transport in the vascular system. A large class of peptides are under investigation which have paracrine rather than endocrine activities; that is, their actions are on cells and tissues in the immediate vicinity of the cells of origin.

Motilin is a polypeptide containing 22 amino acids that was originally isolated from porcine duodenal mucosa (Brown *et al.*, 1971). The amino acid composition and sequence have been described (Brown *et al.*, 1972, 1973). Immunoreactive motilin has been found in the enterochromaffin cells of the duodenum and jejunum of several species (Polak *et al.*, 1975) and, by means of radioimmunoassay, motilin has been identified in the plasma of dogs (Dryburgh and Brown, 1975). Motilin has been shown to stimulate pepsin output and motor activity of the stomach (Brown *et al.*, 1971) and to induce lower

TABLE III

Gastrointestinal Polypeptide Hormones[a]

Hormone	Source
Motilin	Small intestine (enterochromaffin cell); gastric fundus (enterochromaffin-like cell)
Glucagon	Gastric fundus, canine (A cells)
Enteroglucagon	Distal small intestine; colon
Vasoactive intestinal polypeptide	Intestine
Gastric inhibitory polypeptide	Small intestine
Somatostatin	Pyloric antrum; upper small intestine
Secretin	Small intestine (S cells)
Cholecystokinin-pancreozymin	Duodenum; jejunum

[a] From Pearse *et al.* (1977).

esophageal sphincter contractions (Jennewein *et al.*, 1975). Studies by Itoh *et al.*, (1978) suggest that motilin plays an important role in initiating interdigestive gastrointestinal contractions.

Somatostatin, which is named for its growth hormone release-inhibiting activity, was first purified from bovine hypothalamus (Brazlua and Guilleman, 1974). Somatostatin also has been demonstrated in the stomach, pancreas, and intestinal mucosa in concentrations higher than in the brain (Pearse *et al.*, 1977). Somatostatin is a potent inhibitor of insulin and glucagon release. It also inhibits gastrin release and gastric acid secretion (Barros D'Sa *et al.*, 1975; Bloom *et al.*, 1974), apparently acting independently on parietal cells and on G cells. These and a variety of other physiological effects suggest that somatostatin has important gastrointestinal regulatory functions (Pearse *et al.*, 1977).

Enteroglucagon is the hyperglycemic, glycogenolytic factor isolated from the intestinal mucosa. It occurs primarily in the distal small intestine and colon in at least two forms, one with a molecular mass of 3500 daltons and the other somewhat larger (Valverde *et al.*, 1970). Enteroglucagon differs from pancreatic glucagon biochemically, immunologically, and in its mode of release. The physiological function of enteroglucagon is not known, but its release from the mucosa following a meal and the associated increase in circulating blood levels have suggested a regulatory role on bowel function (Pearse *et al.*, 1977). Enteroglucagon also differs significantly from glucagon produced by the A cells of the gastric mucosa of the dog (Sasaki *et al.*, 1975). Canine gastric glucagon is biologically and immunochemically identical to pancreatic glucagon. Gastric glucagon appears to be unique to the dog, similar activity not being observed in the stomach of the pig or the abomasum of cattle and sheep (Sutherland and de Duve, 1948).

III. DIGESTION AND ABSORPTION

A. Water and Electrolyte Absorption

1. Mechanisms of Mucosal Transport

The microvillous membrane of the intestinal mucosa, like other cell membranes, is a lipid structure which acts as a barrier to water and water-soluble substances. Water and polar solutes penetrate in one of two ways. (1) They may pass through *pores* in the membrane, which are believed to be aqueous channels connecting the luminal surface of

the cell with the apical cytoplasm. The "effective" diameter of jejunal pores has been estimated to be approximately 0.4 nm (Lindemann and Solomon, 1962). (2) They may attach to *membrane carriers,* which facilitate passage through the lipid phase of the membrane.

Transport of water and water-soluble compounds is influenced by the permeability characteristics of the limiting membrane and by the nature of the driving forces which provide energy for transport. Passive movement occurs either by simple diffusion or as a result of gradients in concentration (activity), pH, osmotic pressure, or electrical potential which may be present across the membrane. The passive movement of an ion in the direction of an electrochemical gradient is referred to as *single-file* diffusion (Hladky, 1965). When a substance moves in a direction opposite that of an established electrochemical gradient, an *active transport* process is said to be responsible.

Most water-soluble compounds, such as monosaccharides and amino acids, cannot diffuse across the intestinal mucosal membrane at rates which are adequate to meet nutritional requirements. Transport of these substances is believed to be by means of membrane carriers. The nature of thse carriers is not well understood, but they are believed to be an integral part of the membrane and responsible for binding the transported substance in a rather specific way. Their existence is based primarily on kinetic evidence. Carrier-mediated transport systems can be saturated and are competitively inhibited by related compounds.

Three types of carrier transport mechanisms are recognized (Curran and Schultz, 1968). (1) *Active transport,* as stated previously, involves movement of electrolytes against an electrochemical gradient. In the case of nonelectrolytes, such as glucose, active transport is defined as movement against a concentration gradient. Active transport requires metabolic energy and is inhibited by various metabolic blocking agents or by low temperature. (2) *Facilitated diffusion* occurs when the passive movement of a substance is more rapid then can be accounted for by simple diffusion. Facilitated diffusion systems can increase the rate of movement across the membrane by two or three orders of magnitude. The carrier mechanism is similar to that involved in active transport in that it displays saturation kinetics, may be inhibited competitively, and is temperature dependent. However, transport does not occur against concentration or electrochemical gradients, and direct expenditure of energy is not required. (3) *Exchange diffusion* is a transfer mechanism similar to facilitated diffusion. It was postulated originally by Ussing (1947) to explain the rapid transfer of radioactive Na^+ across cell membranes. The mechanism does not give rise to net transport but contributes in a major way to unidirectional flux rates, which are measured with isotopic tracers.

In the intestine, net water absorption is the result of bulk flow through pores in the membrane. Diffusion in the usual sense plays no important role in a water movement (Section III,A,4). When bulk flow occurs, it is possible for solutes to move across the membrane in the direction of flow by a phenomenon called *solvent drag.* The effect of solvent drag on the transport of a given solute depends on the rate of volume flow and upon the *reflection* coefficient, which is an expression of the relationship between the pore radius and the radius of solute molecule being transported. A solute such as urea can be transported by the intestine against a concentration gradient by means of solvent drag (Hakim and Lifson, 1964).

2. *Sodium Chloride Absorption*

Studies with isotopic tracers have shown that transport of water and electrolytes by the intestinal mucosa is a dynamic process, with rapid unidirectional fluxes of the substances

occurring continuously in both directions. Net absorption occurs when the flow from lumen to plasma exceeds that in the opposite direction (Code *et al.*, 1960; Berger *et al.*, 1959; Hindle and Code, 1962).

Active transport of Na^+ can occur along the entire length of the intestine, but the rate of absorption is greatest in the ileum and colon, where most net sodium and water absorption occurs. Sodium transport is believed to be accomplished by an energy-requiring "sodium pump." The characteristics of this pump are not completely understood, but Skou (1965) presented evidence that the pump is intimately related to the activity of a Na^+-K^+-dependent adenosine triphosphatase located within the cell membrane. This enzyme is inhibited by cardiac glycosides, such as oubain, which also are effective inhibitors of Na^+ transport, and it has been suggested that this enzyme system may actually be the pump. In the jejunum, net absorption of sodium occurs slowly unless nonelectrolytes, such as glucose or amino acids, are absorbed simultaneously. In *in vivo* studies by Fordtran *et al.* (1968), jejunal absorption of sodium appeared to be explained, in part, by solvent drag which was associated with active glucose transport. In the ileum, Na^+ absorption was independent of glucose absorption. Water absorption in the jejunum also appears to be almost entirely dependent upon the absorption of glucose, while absorption from the ileum is unaffected by glucose (Barry *et al.*, 1961). The differential effect of glucose on absorption from the jejunum and ileum appears to be the result of fundamental metabolic differences between these two areas of the intestine (Curran, 1960; Gilman and Koelle, 1960).

As sodium is transported across the mucosal membrane, an equivalent amount of anion must be transported simultaneously to maintain electrical neutrality. A significant amount of chloride ion absorption can be accounted for on this basis. It is generally agreed that chloride transport in the intestine is a passive process (Clarkson *et al.*, 1961), although active secretion by the gastric mucosa seems well established. The intestinal mucosa can, under certain circumstances, absorb Cl^- independently of cation absorption and maintain electrical neutrality by exchange secretion of bicarbonate into the lumen (Ingraham and Visscher, 1936).

3. *Potassium Absorption*

Dietary potassium is absorbed almost entirely in the proximal small intestine. Absorption appears to be a passive process since movement across the mucosa occurs down a concentration gradient (high luminal concentration to a low concentration in plasma). The fluid which reaches the ileum from the jejunum has a potassium concentration and a sodium/potassium ratio which is similar to that of plasma. In the ileum and colon, the rate of sodium absorption is much greater than that of potassium so that, under normal conditions, the sodium/potassium ratio in the feces is much lower than that of plasma, approaching a ratio of 1.

4. *Water Absorption*

The absorption of water has been one of the most extensively studied aspects of intestinal transport. It is now generally agreed that water movement is the result of bulk flow through membranous pores and that simple diffusion plays only a minor role. The question of whether water is actively or passively transported has been the subject of considerable controversy, and the controversy itself points to the fundamental difficulties which arise in trying to establish a definition of active transport. Hypertonic saline solutions can be absorbed from canine intestine *in vivo* (Grim, 1962) and from canine

(Hakim *et al.*, 1963) and rat (Parsons and Wingate, 1961) intestine *in vitro*. These observations indicate that water absorption can occur against an activity gradient and that the process is dependent upon metabolic energy. This would suggest that an active transport process is involved. Curran (1965), however, presents an alternate interpretation which is now generally accepted. This view is that water transport occurs secondarily to active solute transport and is the result of local gradients established within the mucosal membrane. Water transport is then coupled to the energy-dependent process responsible for solute transport but is one step removed from it.

In the dog and probably other carnivores, the ileum is the main site of net sodium and water absorption. The colon accounts for no more than perhaps 20% of the total. In the case of herbivorous animals in which the large intestine is developed extensively, net secretion of water may occur in the ileum so that all net absorption of water must take place in the cecum and colon (Powell *et al.*, 1968; Argenzio, 1975).

B. Carbohydrate Digestion and Absorption

1. Polysaccharide Digestion

a. Starch and Glycogen. Carbohydrate is present in the diet primarily in the form of polysaccharides of glucose. The most common polysaccharides are starch, glycogen, and cellulose. Starch and glycogen are composed of long chains of glucose molecules linked together by repeating α-1,4-glucosidic bonds. Branching chains are linked by α-1,6-glucosidic bonds. In those species which secrete salivary amylase, digestion of starch and glycogen begins in the mouth when this enzyme mixes with food. The action of salivary amylase is interrupted in the stomach, however, because of the low pH of the gastric secretion.

Starch digestion begins again in the proximal small intestine with the action of pancreatic amylase. This enzyme catalyzes a series of stepwise hydrolytic reactions, resulting in formation of the principle end products of starch digestion, the disaccharides maltose and isomaltose, and small amounts of glucose. Glucose is absorbed directly by the intestinal mucosa and transported to the portal vein. The disaccharides are broken down further by hydrolytic enzymes of the brush border.

b. Cellulose. Cellulose, like starch, is a polysaccharide of glucose but differs from starch in that the glucose molecules are linked by β-1,4-glucosidic bonds. Starch can be utilized by all species, but cellulose is utilized as a source of energy only by animals which have extensive bacterial fermentation within the gastrointestinal tract. Ruminant species digest cellulose most efficiently, but other animals in which the large intestine is well developed also can utilized cellulose to some degree.

In ruminants, hydrolysis of cellulose is accomplished by cellulytic bacteria, which are part of the complex rumen microflora. The end products of cellulose fermentation are short-chain fatty acids—acetic, propionic, and butyric acids. These are absorbed directly from the rumen and serve as the major source of energy for ruminants. Propionic acid is the major precursor for synthesis of carbohydrate.

2. Disaccharide Digestion

Maltose and isomaltose are the disaccharides (glucose–glucose) produced as end products of starch digestion. The diet also contains lactose (galactose–glucose) and sucrose (fructose–glucose). It once was believed that disaccharides were hydrolyzed within the

TABLE IV

Enzymes of the Microvillous Membrane

Enzyme	Reference
Lactase	Alpers (1969), Forstner *et al.* (1968)
Sucrase	Eichholz (1967), Forstner *et al.* (1968)
Maltase	Eichholz (1967), Forstner *et al.* (1968)
Isomaltase	Eichholz (1967), Forstner *et al.* (1968)
Trehalase	Eichholz (1967), Forstner *et al.* (1968)
Cellobiase	Forstner *et al.* (1968)
Leucylnapthylamidase	Rhodes *et al.* (1967), Eichholz (1968)
Leucylglycine hydrolase	Rhodes *et al.* (1967), Eichholz (1968)
Cholesterol ester hydrolase	Malathi (1967)
Retinyl ester hydrolase	Malathi (1967)
Alkaline phosphatase	Eichholz (1967), Forstner *et al.* (1968)
ATPase	Eichholz (1967), Forstner *et al.* (1968)

lumen of the intestine by enzymes secreted by the mucosa. There is now general agreement, however, that disaccharide digestion is completed at the surface of the cell by disaccharidases (Gray, 1975), which are components of the brush border (Table IV). This is considered a form of intracellular digestion (Ugolev, 1965).

The disaccharidases have been solubilized from the brush border and partially purified. Two separate maltases have been isolated (Auricchio *et al.*, 1965). Isomaltase and sucrase have been separated and purified together as a two-enzyme complex (Kolinská and Semenza, 1967). The mucosa also contains two enzymes with lactase activity. One of these is a nonspecific β-galactosidase which hydrolyzes synthetic β-galactosides effectively but which hydrolyzes lactose at a slow rate. This enzyme has an optimal pH of 3 and is associated with the lysozomal fraction of the cell. The other lactase hydrolyzes lactose readily. It is associated with the brush border fraction of the cell and is the enzyme which is important in the digestive process (Alpers, 1969).

Maltase, isomaltase, and sucrase are almost completely absent from the intestine in newborn pigs (Hartman *et al.*, 1961; Dahlqvist, 1961) and calves (Huber *et al.*, 1961). The activity of these disaccharidases increases after birth and reaches adult levels during the first months of life. Lactase activity is highest at birth and decreases gradually during the neonatal period. The relatively high lactose activity seems to be an advantage to the newborn in utilizing the large quantities of lactose present in the diet. Bywater and Penhale (1969) demonstrated lactase deficiency following acute enteric infections and suggested that lactose utilization may be decreased in such cases.

3. Monosaccharide Transport

a. Specificity of Monosaccharide Transport. Regardless of whether monosaccharides originate in the lumen of the intestine or are formed at the surface of the mucosal cell, transport across the mucosa involves processes which have a high degree of chemical specificity. Glucose and galactose are absorbed from the intestine more rapidly than other monosaccharides. Fructose is absorbed at approximately one-half of the rate of

glucose, and mannose is absorbed at less than one-tenth the rate of glucose (Kohn *et al.*, 1965). Glucose and galactose can be absorbed against concentration gradients and are said, by definition, to be actively transported. Active absorption requires metabolic energy and can be inhibited by a variety of substances which block oxidative phosphorylation.

The monosaccharides that are transported most efficiently against concentration gradients have certain common structural characteristics, which were summarized by Wilson (1962). These include (1) the presence of a pyranose ring, (2) a carbon atom attached to C-5, and (3) a hydroxyl group at C-2 with the same stereoconfiguration as D-glucose. These features once were believed to be necessary for active monosaccharide transport, but recent observations suggest that they are not absolute requirements. Both D-xylose, which has no substituted carbon atom at C-5, and D-mannose, which lacks the appropriate hydroxyl configuration at C-2, can be transported against concentration gradients under proper experimental conditions (Csáky and Lassen, 1964; Csáky and Ho, 1966; Alvarado, 1966b).

b. Characteristics of the Membrane Carrier. Most current concepts imply that, during the initial phase of monosaccharide absorption, the monosaccharide molecule attaches to a mobile carrier located within the cell membrane (Crane, 1965). The evidence for such membrane carriers comes from kinetic studies of the overall transport process. The rate of glucose absorption is independent of luminal concentration over a rather wide range, but a maximal rate of absorption can be demonstrated at very high concentrations. This limitation of transport is believed to be due to saturation of binding sites on the membrane carrier.

Glucose transport is competitively inhibited by galactose (Cori, 1925; Fisher and Parsons, 1953) and by a variety of substituted hexoses, which compete with glucose for carrier binding sites. The glucoside phlorizin is a very potent inhibitor (Parsons *et al.*, 1958; Alvarado and Crane, 1962). Phlorizin also competes for binding sites but has a much higher affinity for these sites than does glucose.

The absorptive surface of the mucosal cell is the microvillous membrane, or brush border (Figs. 6a and 6b). It is through this part of the plasma membrane that glucose must pass during the initial phase of mucosal transport. Techniques have been developed for isolating highly purified preparations of microvillous membranes from mucosal homogenates (Eichholz and Crane, 1965; Forstner *et al.*, 1968). Faust *et al.* (1967) studied the binding of various sugars to these isolated membrane fractions. They found that D-glucose was bound by the membrane preferentially to L-glucose or to D-mannose and that glucose binding was completely inhibited by 0.1 m*M* phlorizin. The specificity of their observations suggested that binding represented an initial step in glucose transport, namely, attachment to a membrane carrier.

c. Sodium Requirement. The absorption of glucose and other monosaccharides is influenced significantly by sodium ion (Schultz and Curran, 1970; Kimmich, 1973). When sodium is present in the solution bathing the intestinal mucosa, glucose is absorbed rapidly, but, when sodium is removed and replaced by equimolar amounts of other cations, glucose absorption virtually stops (Riklis and Quastel, 1958; Csáky, 1961; Bihler and Crane, 1962; Bihler *et al.*, 1962). Glucose abosrption is inhibited by oubain, digitalis, and other cardiac glycosides which are also inhibitors of Na-K-dependent adenosine

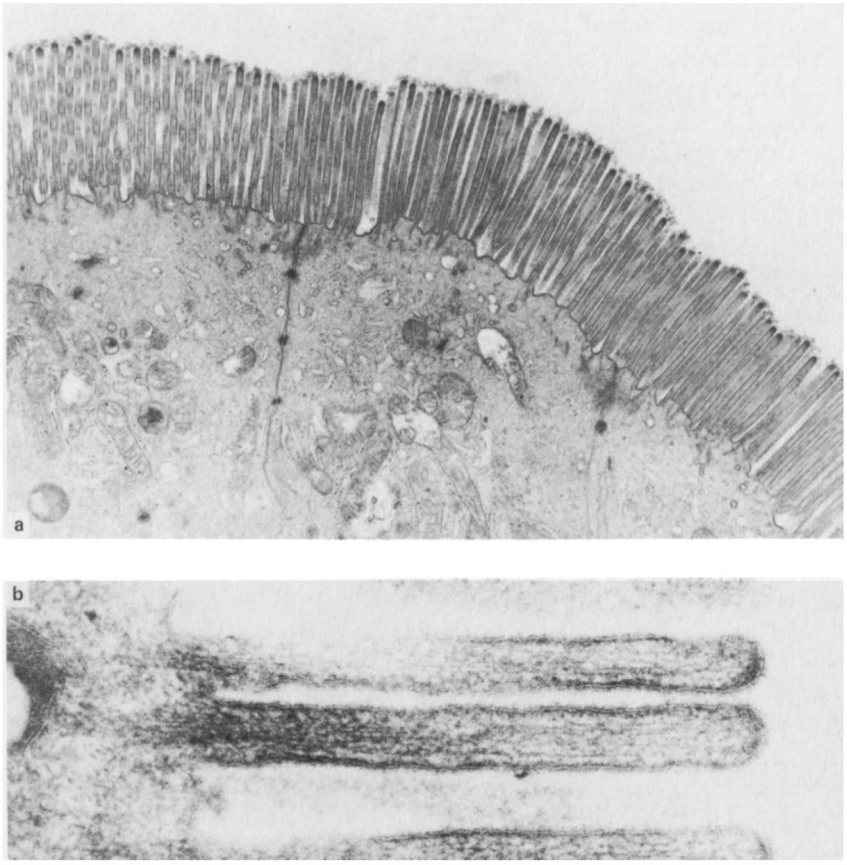


Fig. 6. (a) Absorptive surface of the mucosal epithelium showing the microvillous membrane and, below it, the apical cytoplasm (×8500). (b) Electron photomicrograph of microvilli (×51,500). (Courtesy of S. Lui and K. J. Isselbacher, Massachusetts General Hospital, Boston, Massachusetts.)

triphosphatase activity and sodium transport (Csáky and Hara, 1965; Schultz and Zalusky, 1964). These observations suggest a close relationship between the transport of glucose and sodium. On the basis of their own observations, Crane and co-workers (1965) suggested that sodium ion acts directly upon the membrane carrier to increase affinity of the carrier for glucose. Csáky (1963) interprets the apparent coupling of sodium transport to the transport of various nonelectrolytes as being due to the need to maintain a critical intracellular sodium concentration, which, in turn, is essential for conversion of metabolic energy (ATP, etc.) to energy for transport.

C. Protein Digestion and Absorption

1. Enzymatic Hydrolysis

The initial step in protein digestion is enzymatic hydrolysis of peptide bonds with formation of smaller peptides and amino acids. The *endopeptidases* (proteases) hydrolyze peptide bonds within the protein molecule and also hydrolyze certain model peptides.

Exopeptidases hydrolyze either the carboxy-terminal (carboxypeptidase) or the amino-terminal (aminopeptidase) amino acids of peptides and certain proteins.

Dietary proteins first come in contact with proteolytic enzymes in the stomach. The best known of the gastric proteases is the family of pepsins (Samloff, 1971), which attack most proteins with the exception of keratins, protamines, and mucins. Pepsins are relatively nonspecific endopeptidases and split peptide bonds involving many amino acids. The most readily hydrolyzed peptide bonds are those of leucine, phenylalanine, tyrosine, and glutamic acid (Ryle, 1965; Ryle and Hamilton, 1966; Meyer and Kelly, 1977).

The extent of proteolysis in the stomach depends on the nature of the dietary protein and the length of time spent in the stomach. The food bolus mixed with saliva has a neutral or slightly alkaline pH as it enters the stomach, and a certain period of time is necessary for it to mix with gastric secretions and become acidified. Proteolytic digestion begins when the pH of the gastric contents approaches 4 and occurs optimally in two pH ranges, 1.6–2.4 and 3.3–4.0 (Taylor, 1959a,b). Because of the relative lack of specificity of the pepsins, some peptide bonds of almost all dietary proteins are split during passage through the stomach. The gastric phase of protein digestion appears to have only a minor and probably dispensable role in overall protein assimilation (Freeman and Kim, 1978). The reservoir function of the stomach, however, contributes to the gradual release of nutrients, insuring more efficient utilization in the small intestine.

Partially digested protein passes from the stomach to the duodenum, where the acidic contents are neutralized by sodium bicarbonate secreted in the bile and pancreatic juices. Peptic activity persists in the duodenum only during the period required to raise the pH above 4.0. The major peptidase activity in the lumen of the small intestine comes from the pancreatic enzymes trypsin, chymotrypsin, elastase, and carboxypeptidases A and B. The action of these enzymes is integrated so that the endopeptidases produce peptides with C-terminal amino acids which are appropriate substrates for the exopeptidases. Trypsin produces peptides with basic C-terminal amino acids which are particularly suited for the action of carboxypeptidase B. Chymotrypsin produces peptides with aromatic amino acids in the C-terminal position, and elastase produces peptides with C-terminal amino acids which are nonpolar. Carboxypeptidase A hydrolyze both types of C-terminal peptide bonds (Table II).

The intestinal mucosa contains a broad range of aminopeptidases which complete the process of protein digestion (Heizer and Laster, 1969). Most of the aminopeptidase activity is found in the soluble fraction of the cell (Newey and Smyth, 1960), but a small fraction is tightly bound to the microvillous membrane and appears to serve a digestive function at the cell surface similar to that described for the disaccaridases (Rhodes *et al.*, 1967). An endopeptidase from the intestinal mucosa was studied by Hsu and Tappel (1965) using hemoglobin as substrate. Over 95% of the activity was located in the particulate fraction of the cell. The association with other acid hydrolases suggests that this is a lysozomal enzyme, and its relationship to the normal process of protein digestion is not known.

2. *Form in Which Products of Protein Hydrolysis Are Absorbed*

Despite the long interest in and controversy regarding the subject of this section, the relative amounts of the various types of protein digestion products, i.e., peptides and amino acids, which are actually absorbed by intestinal mucosal cells during normal digestion are still not known. It is a difficult process to investigate from a kinetic

standpoint because the products of proteolysis are absorbed rapidly after they are formed. Studies of luminal contents, therefore, give only an estimate of the overall rate of protein digestion. In addition, dietary protein is continually mixed with endogenous protein in the form of digestive secretions and extruded mucosal cells. Endogenous protein is hydrolyzed and the amino acids absorbed in a manner similar to that of dietary protein, and the two processes occur simultaneously. Endogenous protein accounts for a significant part of the amino acids of the intestinal contents (Nasset and Ju, 1961). Even when the dietary protein is labeled with a radioactive tracer, there is such rapid utilization that the tracer soon reenters the lumen in the form of endogenous protein secretion.

In adult mammals, protein is not absorbed from the intestine in quantities of nutritional significance without previous hydrolysis. Most neonatal animals absorb significant amounts of immunoglubin and other colostral protein, but this capacity is lost soon after birth (see Section III,A,4 below). The intestinal mucosa is not totally impermeable to large polypeptide molecules, however. The absorption of insulin (MW 5700) (Laskowski *et al.*, 1958; Danforth and Moore, 1959), ribonuclease (MW 13,700) (Alpers and Isselbacher, 1967), territin (Bockman and Winborn, 1966), and horseradish peroxidase (Cornell *et al.*, 1971) has been demonstrated. The intestine produces a part of the plasma β-globulin. This is believed to be the result of *de novo* synthesis of protein, however, presumably from individual amino acid precursors.

During the digestion of protein, the amino acid content of the portal blood increases rapidly. Attempts to demonstrate parallel increases in the level of peptides in the portal blood have not been successful (Levenson *et al.*, 1959). This has sometimes been taken as evidence that only amino acids can be absorbed by the intestinal mucosa and that the absorption of peptides does not occur. While it seems clear that a significant part of the dietary protein is absorbed in the form of free amino acids, peptides also may be taken up by the mucosal cell.

Evidence of the mucosal uptake of peptides came originally from experiments with isolated loops of intestine (Wiggans and Johnston, 1959; Newey and Smyth, 1959). Various peptides were placed in solutions bathing the mucosa and analyses made subsequently of the serosal fluid. With the exception of small amounts of glycylglycine, peptides were never found on the serosal side, but free amino acids were found in significant quantities.

The final steps to peptide digestion appear to be associated with mucosal epithelial cells. Almost all of the aminopeptidase activity is associated with the mucosa, and very little activity is present in luminal contents (Lindberg, 1966). As described above, mucosal aminopeptidase activity is located in the cytosol and in the brush border membrane fractions of the epithelial cell (Heizer and Laster, 1969; Kim *et al.*, 1972). These physically separate enzymes have remarkably different substrate specificities (Kim *et al.*, 1974). The brush border enzyme has more than 50% of the activity for tripeptides, yet less than 10% of the total activity for dipeptides relative to the cytosolic enzyme(s) (Peters, 1970; Kim *et al.*, 1972). Almost all activity for tetrapeptides is present in the brush border (Freeman and Kim, 1978). Proline-containing peptides are hydrolyzed almost exclusively by cytosolic peptidases, whereas leucine aminopeptidase activity is located primarily in the brush border.

From these studies, it appears that, in the intact animal, peptides are absorbed in physiologically important quantities by intestinal mucosal cells and hydrolyzed either at

the cell surface or intracellularly to constituent amino acids. The individual amino acids then are transported to the apical part of the cell and finally enter the portal circulation.

3. *Transport of Amino Acids*

Amino acids, like glucose and certain other monosaccharides, are absorbed and transferred to the portal circulation by active transport processes. The same type of saturation kinetics observed in studies of monosaccharide absorption are observed with amino acids, suggesting carrier transport mechanisms. Certain monosaccharides inhibit amino acid transport (Saunders and Isselbacher, 1965; Newey and Smyth, 1964). Inhibition generally has been of the noncompetitive type, but Alvarado (1966a) demonstrated competitive inhibition between galactose and cycloleucine, suggesting that some form of common carrier may be involved.

Most amino acids are transported against concentration and electrochemical gradients, and the overall transport process requires metabolic energy. The chemical specificity of these transport mechanisms is demonstrated by the observation that the natural *l* forms of various amino acids are absorbed more rapidly than the corresponding *d* forms, and only the *l*-amino acids appear to be actively transported. Sodium ion is necessary for absorption of amino acids as it is for a variety of other nonelectrolyte substances (Schultz and Curran, 1970; Gray and Cooper, 1971).

Separate transport systems appear to exist for different groups of amino acids. Each member of a group inhibits the transport of other members competitively, suggesting that they share the same binding site. There is some overlap between groups, indicating that, in the overall transport process, certain steps may be common to all amino acids and other steps more specific (Saunders and Isselbacher, 1966; Matthews and Laster, 1965; Wiseman, 1968). These groups are the following:

1. Monoaminomonocarboxylic (neutral) amino acids, including histidine. These amino acids show mutual competition for transport and have the greatest requirement for Na^+.
2. Monoaminodicarboxylic amino acids. Aspartic and glutamin acids are not transported against concentration gradients. Following uptake, they are transaminated, and, under physiological conditions, almost all of the aspartic and glutamic acid enters the portal blood as alanine.
3. Dibasic amino acids, including lysine, arginine, ornithine, and the neutral amino acid cystine. These amino acids are apparently transported by the same transport system.
4. Proline, hydroxyproline, the *N*-substituted glycine derivatives *N*-methylglycine (sarcosine), and *N*-dimethylglycine, and betaine. Proline and hydroxyproline also can be transported by the first mechanism but the affinity of both amino acids for the Na-dependent pathway is low.

The γ-glutamyl cycle has been proposed as a possible transport system for amino acids (Meister and Tate, 1976). γ-Glutamyltransferase (GGT) is a membrane-bound enzyme which is present in a number of mammalian tissues and catalyzes the initial step in glutathione degradation. The γ-glutamyl moiety of glutathione is transferred to amino acid (or peptide) receptors with the production of cysteinylglycine:

$$\text{Glutathione} + \text{amino acid} \xrightarrow{\text{GGT}} \gamma\text{-glutamyl-amino acid} + \text{Cys-Gly}$$

The highest GGT activity is present in tissues which are known to transport amino acids

actively, e.g., the jejunal villus and the proximal convoluted tubule of the kidney. Meister and his colleagues (1976) have suggested that GGT may function in translocation by interaction with extracellular amino acids and with intracellular glutathione. The hypothetical mechanism involves the noncovalent binding of extracellular amino acids to the plasma membrane, while intracellular glutathione interacts with GGT to yield a γ-glutamyl *enzyme*. When the γ-glutamyl moiety is transferred to the membrane-bound amino acid, a γ-glutamyl–amino acid complex is formed and, when released from the membrane binding site, moves into the cell. The γ-glutamyl–amino acid complex is split by the action of γ-glutamylcyclotransferase, an enzyme appropriately located in the cytosol. Glutathione is regenerated by means of the γ-glutamyl cycle, which are good substrates for GGT (Thompson and Meister, 1975).

The γ-glutamyl cycle does not require sodium, and the previously demonstrated sodium dependence for amino acid transport would not be explained by the cycle. The cycle is not considered to be the only amino acid transport system, and its quantitative significance in individual tissues is unknown. Certain nutrient cell types which are deficient in GGT have been shown to transport amino acids normally.

4. Neonatal Absorption of Immunoglobulin

At birth most domestic species, including the calf, foal, lamb, pig, kitten, pup, and infant, absorb significant quantities of colostral protein from the small intestine (Brambell, 1958; Walker and Isselbacher, 1974). γ-Globulin either is absent in the serum of these species at birth, or is at a low level. Within a few hours after ingestion of colostrum, the serum γ-globulin level rises. This is the principal mechanism by which the young of the above-listed species acquire maternal immunity. Under normal environmental conditions, ingestion of colostrum is an absolute requirement for the health of these species during the neonatal period (Fig. 7).

In the neonatal calf, immunoglobulin deficiency has a role in the pathogenesis of gram-negative bacterial septicemia (Smith, 1962; Gay, 1965; Roberts *et al.*, 1954). Most calves deprived of colostrum develop septicemia early in life but may develop diarrhea before death (Smith, 1962; Roberts *et al.*, 1954; Wood, 1955; Tennant *et al.*, 1975). Hypogammaglobulinemia is almost always demonstrable in calves dying of gram-negative bacterial septicemia (Fey, 1971), and hypogammaglobulinemia is believed to be due to insufficient immunoglobulin intake or to insufficient intestinal absorption. The factor in colostrum that protects against systemic infections is the IgM fraction (Penhale *et al.*, 1971).

Serum immunoglobulin values of neonatal calves vary, and a 10% incidence of hypogammaglobulinemia may occur in clinically normal calves (Tennant *et al.*, 1969a; House and Baker, 1968; Smith *et al.*, 1967; Thornton *et al.*, 1972; Braun *et al.*, 1973). Most hypogammaglobulinemic individuals probably had insufficient colostrum intake. Even when calves were given the opportunity to ingest colostrum, a surprising number were hypogammaglobulinemic. Some of the reasons for varying gammaglobulinemia values are recognized, but the relative importance of each reason is not known. The concentration of lactoglobulin, the volume consumed (Bush *et al.*, 1971; Selman *et al.*, 1971), the time elapsed from birth to ingestion of colostrum (Selman *et al.*, 1971), and the method of ingestion (natural suckling versus bucket feeding) may have an important influence on the serum γ-globulin (Smith *et al.*, 1967; McBeath *et al.*, 1971). Calves that suckle their dams usually attain serum γ-globulin concentrations that are higher than those

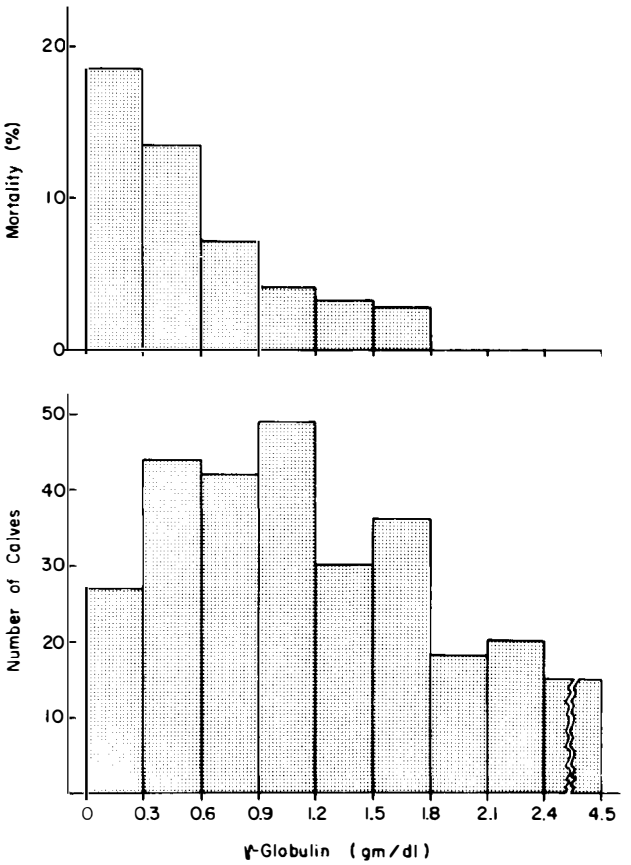


Fig. 7. Histogram showing distribution of serum γ-globulin levels in neonatal market calves and the relation of hypogammaglobulinemia to mortality (From Braun and Tennat, 1979).

attained by calves given colostrum from a bucket. The frequency of hypogammaglobulinemia may be influenced by seasons (Gay *et al.*, 1965b; McEwan *et al.*, 1970a), although this relationship has not always been observed (Smith *et al.*, 1967; Thornton *et al.*, 1972). Familial factors also influence hypogammaglobulinemia (Tennant *et al.*, 1969a).

Regardless of cause, the mortality of hypogammaglobulinemic calves is higher than that of calves with normal serum γ-globulin values (Gay, 1965; House and Baker, 1968; Thornton *et al.*, 1972; McEwan *et al.*, 1970a; Boyd, 1972; Naylor *et al.*, 1977). In addition to having more septicemic infections (Smith, 1962; Gay, 1965a; Roberts *et al.*, 1954; Wood, 1955; Fey, 1971; McEwan *et al.*, 1970a), hypogammaglobulinemic calves have a greater prevalence of acute diarrheal disease (Boyd, 1972; Naylor *et al.*, 1977; Penhale *et al.*, 1970; Gay *et al.*, 1965); the local protective effects of immunoglobulin in the intestine apparently are important (Fisher *et al.*, 1975; Logan and Penhale, 1971).

The prevalence of hypogammaglobulinemia and the high mortality associated with it has led to the development of several rapid tests for identification of hypogammaglobulinemic calves (McBeath *et al.*, 1971; Aschaffenburg, 1949; Fisher and McEwan, 1967a; Patterson, 1967; Stone and Gitter, 1969). The zinc sulfate turbidity test (Kunkel,

1947) was the first to be used for determination of serum immunoglobulin concentrations of neonatal calves (McEwan *et al.*, 1970). A close correlation has been established between test results and the amount of serum IgG and IgM (Fisher and McEwan, 1967a,b; McEwan *et al.*, 1970b; Penhale *et al.*, 1967).

The sodium sulfite turbidity test is similar to the zinc sulfate test and also has been used to identify hypogammaglobulinemic calves (Stone *et al.*, 1969; Pfeiffer and McGuire, 1977). Failure of turbidity to develop when serum is added to a saturated solution of sodium sulfite indicates immunoglobulin deficiency, and semiquantitative assessment of the immunoglobulin concentration may be made by grading the degree of turbidity (Stone and Gitter, 1969).

The refractometer is used as a rapid test for immunoglobulin deficiency (McBeath *et al.*, 1971; Boyd, 1972). The close relationship between the concentration of γ-globulin and that of total serum protein in neonatal calves was described previously (Tennant *et al.*, 1969a), and the wide variation in total protein concentration was due to differences in γ-globulin concentration. Direct linear correlation between the serum protein concentration (refractive index) and the immunoglobulin concentration also has been described (McBeath *et al.*, 1971). The equation for the regression line in that report was virtually identical to that observed recently (Tennant, *et al.*, 1978). The Y intercepts in our study and in that previously reported were identical (4 gm/dl). The refractometer has value as a rapid field instrument for the assessment of immunoglobulin status, but in cases of hemoconcentration it has limitations (Boyd, 1972).

The glutaraldehyde coagulation test was used originally for the detection of hypergammaglobulinemia in cattle, using whole blood (Sandholm, 1974). Glutaraldehyde reagent also has been used in a semiquantitative test to evaluate γ-globulin in canine (Sandholm and Kivisto, 1975) and human serum (Sandholm, 1976). We modified this procedure to detect hypogammaglobulinemic calves (Table V). Calves that had a negative test result (serum γ-globulin $\leq$0.4 gm/dl) had markedly higher mortality than did calves with positive results (Table VI) (Tennant, *et al.*, 1979), findings similar to those obtained by using the zinc sulfate turbidity test (Gay *et al.*, 1965a; McEwan *et al.*, 1970a). Many tests can be initiated at one time using the glutaraldehyde coagulation test, and all results can be evaluated rapidly without instrumentation (Tables V and VI).

Protein enters the absorptive cell by pinocytosis and passes across the cell to the lymphatics. The process is not selective because many proteins other than the immune globulins can be absorbed (Payne and Marsh, 1962a,b). The ability to absorb intact protein is lost by domestic species within 1 or 2 days following birth. In rodents, protein absorption normally continues for approximately 3 weeks. The mechanism of intestinal "closure" was studied by Lecce and co-workers (1964; Lecce, 1966; Lecce and Morgan,

TABLE V

Procedure for Detection of Hypogammaglobulinemia

Reagent	10% glutaraldehyde: 4 ml 25% glutaraldehyde 6 ml distilled water
Procedure	Mix 1 part reagent (50 μl), 10 parts serum (0.5 ml) Observe coagulation time by tilting tube; samples that do not coagulate within 1 hour can be classified as hypogammaglobulinemia

TABLE VI

Relationship between Results of the Glutaraldehyde Coagulation Test, Serum γ-Globulin Concentration, and Death Rate

Source of calves	No.	Glutaraldehyde reaction	Serum γ-globulin (gm/dl) Mean (±SD)	Serum γ-globulin (gm/dl) Extremes	Death rate (%)
Calves before ingestion of colostrum	10	Negative	0.18 (±0.06)	0.1–0.25	—[a]
Calves from production unit	60	Negative	0.35 (±0.13)	0.11–0.63	16.7[b]
	13	Incomplete	0.60 (±0.13)	0.42–0.85	7.7
	208	Positive	1.46 (±0.63)	0.42–4.4	3.4

[a] Samples of serum were obtained at birth, but no follow-up of calves was made.

[b] The death rate of calves that were test negative was significantly ($P < 0.01$) greater than that of test-positive calves, using t test for significance of differences between two percentages.

1962). They found that complete starvation of pigs lengthened the period of protein absorption to 4–5 days, whereas early feeding shortened the period. Feeding different fractions of colostrum including lactose and galactose resulted in loss of protein absorptive capacity. The route of feeding may not be the critical factor, however. Calves prevented from eating but which receive nutrients parenterally lose the ability to absorb protein at the same time as control calves (Deutsch and Smith, 1957).

D. Lipid Digestion and Absorption

1. Absorption of Fats

a. Luminal Phase. The fat present in the diet is primarily in the form of triglycerides of long-chain fatty acids. The initial step in utilization of triglycerides occurs in the lumen of the proximal small intestine, where hydrolysis is catalyzed by *pancreatic lipase.* This enzyme, which is secreted in active form, requires an oil–water interface for activity so that only emulsions are attacked (Sarda and Desnuelle, 1958). Enzyme activity is directly related to the surface area of the emulsion. The smaller the emulsion particle, the greater the total surface area of a given quantity of triglyceride and the greater the rate of hydrolysis (Benzonana and Desnuelle, 1965). Bile salts are not an absolute requirement, but they favor hydrolysis (1) by their detergent action, which causes formation of emulsions with small particle sizes, and (2) by stimulating lipase activity within the physiological pH range of the duodenum (Borgström, 1954, 1964a).

A colipase is present in the pancreatic secretion which facilitates the interaction of lipase with its triglyceride substrate and protects lipase from inactivation (Borgström and Erlanson, 1971).

Pancreatic lipase splits the ester bonds of triglycerides preferentially at the 1 and 3 positions (Sari *et al.,* 1966), so that the major end products of hydrolysis are 2-monoglycerides and nonesterified fatty acids (Mattson *et al.,* 1952; Mattson and Volpenhein, 1962, 1964). Both compounds are relatively insoluble in water but are brought rapidly into micellar solution by the detergent action of bile salts. The mixed micelles so formed have a diameter of approximately 2.0 nm (Borgström, 1964b; Laurent and Persson, 1965) and are believed to be the form in which the products of fat digestion are

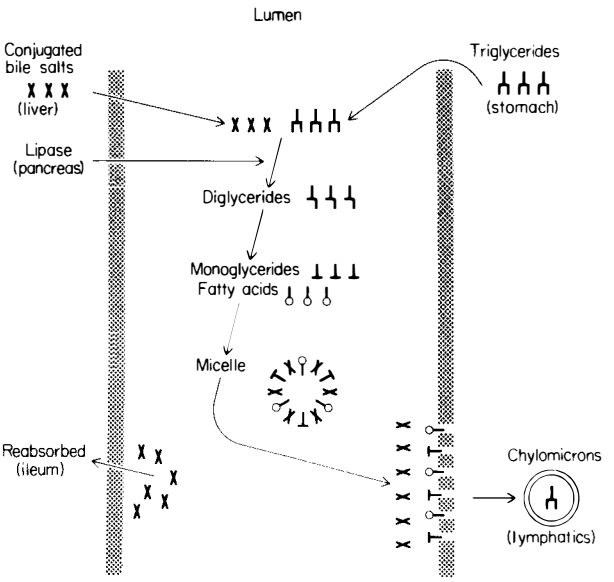


Fig. 8. Intraluminal events which occur during fat absorption. (From Isselbacher, 1967.)

actually taken up by the mucosal cell (Hofmann and Small, 1967). The intraluminal events which occur in fat absorption are schematically summarized in Fig. 8.

b. Mucosal Phase. The initial step in fat transport is the uptake of fatty acids and monoglycerides by the mucosal cell from micellar solution. Just how this occurs is not completely clear, but present evidence suggests that the lipid contents of the micelle are somehow discharged at the cell surface so that they enter the cell in molecular rather than micellar form (Isselbacher, 1967). The net effect is the absorption of the end products of lipolysis with the exclusion of bile salts, which are absorbed farther down the intestine, primarily in the ileum (Lack and Weiner, 1963). Uptake of fatty acids appears to be a passive process having no requirement for metabolic energy (Johnston and Borgström, 1964; Strauss, 1966).

Within the mucosal cell, the fatty acids are transported by a soluble binding protein to the endoplasmic reticulum, where the fatty acids and monoglycerides are rapidly reesterified to triglyceride (Ockner and Manning, 1974; Ockner and Isselbacher, 1974). The two biochemical pathways for triglyceride biosynthesis in the intestine are summarized in Fig. 9. Direct acylation of monoglyceride occurs in the intestine (Senior and Isselbacher, 1962) and probably is the major pathway for lipogenesis in the intestine during normal fat absorption (Kern and Borgström, 1965; Mattson and Volpenhein, 1964). The initial step in this series of reactions involves activation of fatty acids by acyl-CoA synthetase, a reaction which requires Mg^{2+}, ATP, and CoA (Dawson and Isselbacher, 1960; Clark and Hübscher, 1960, 1961; Brindley and Hübscher, 1965) and which has a marked specificity for long-chain fatty acids (Dawson and Isselbacher, 1960; Brindley and Hübscher, 1965). This specificity appears to explain the observation by Bloom *et al.* (1951) that medium- and short-chain fatty acids are not incorporated into triglycerides during intestinal transport but enter the portal circulation as nonesterified fatty acids. The activated fatty acids

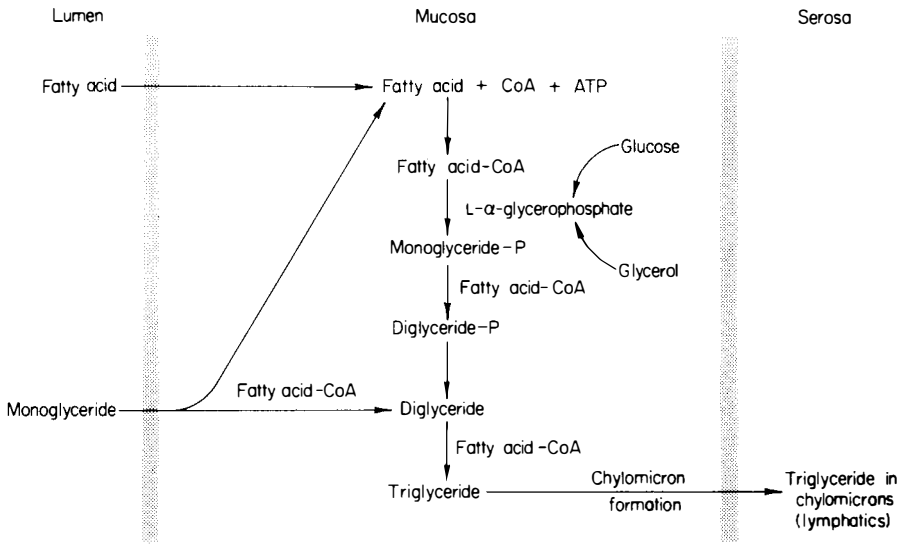


Fig. 9. Biochemical reactions involved in intestinal transport of long-chain fatty acids and monoglycerides. (From Isselbacher, 1966.)

then react sequentially with mono- and diglycerides to form triglycerides in steps catalyzed by mono- and diglyceride transacylases (Ailhaud *et al.*, 1964). The enzymes responsible for this series of reactions were partially purified by Rao and Johnston (1966) from the microsomal fraction of the cell. They observed that purification of the separate enzyme activities occurred simultaneously, suggesting that these enzymes occur together in the endoplasmic reticulum as a "triglyceride-synthetase" complex.

An alternate route which is available for fatty acid esterification involves L-α-glycerophosphate, which may be derived from glucose or from dietary glycerol by the action of intestinal glycerokinase (Haessler and Isselbacher, 1963; Clark and Hübscher, 1962). Activated fatty acid CoA derivatives react with L-α-glycerophosphate to form lysophosphatidic acid (monoglyceride phosphate), which by a second acylation forms phosphatidic acid (diglyceride phosphate). Phosphatidic acid phosphatase then hydrolyzes the phosphate ester bond, forming diglyceride, and by means of a transacylase step similar to that described in the previous paragraph, triglyceride can then be formed. Although this pathway appears to be one of minor importance for triglyceride synthesis in the intestine, Johnston (1968) pointed out the importance of certain of the intermediates in this sequence of reactions in the synthesis of phospholipids which are necessary for stabilization of the chylomicron.

The next step in fat transport is formation of chylomicrons within the endoplasmic reticulum. The chylomicron is composed primarily of triglyceride and has an outer membranous coating of cholesterol, phospholipid, and protein (Zilversmit, 1965). The β-lipoprotein component of the chylomicron is synthesized by the intestinal mucosal cell (Isselbacher and Budz, 1963; Hatch *et al.*, 1966; Windmueller and Levy, 1968). Inhibition of protein synthesis by puromycin or acetoxycycloheximide interferes with chylomicron formation and significantly reduces fat transport (Sabesin and Isselbacher, 1965).

The final step in fat absorption is extrusion of the chylomicron into the intercellular space opposite the basal lateral portion of the absorptive cell. This is accomplished by a

process which is essentially the reverse of pinocytosis (Palay and Karlin, 1959). From the intercellular space the chylomicron passes through the basement membrane and enters the lacteals through small pores. The chylomicron passes from the lacteal into lymph ducts and ultimately reaches the general circulation, having bypassed the liver completely during the initial phase of absorption.

2. *Absorption of Other Lipids*

a. Cholesterol. Dietary cholesterol is present in both free and esterified forms, but only nonesterified cholesterol is absorbed (Vahouny and Treadwell, 1964). Cholesterol esters are hydrolyzed within the lumen of the intestine by sterol esterase secreted by the pancreas. Bile salts are required both for the action of this enzyme (Vahouny *et al.*, 1965) and for the absorption of nonesterified cholesterol. In the mucosal cell, cholesterol is reesterified and transferred by way of the lymph to the general circulation. The type of triglyceride present in the diet significantly affects the absorption of cholesterol and its distribution in lymph lipids (Ockner *et al.*, 1969).

b. Vitamin A. The diet contains vitamin A activity in two principal forms: (1) as esters of preformed vitamin A alcohol (retinol) and fatty acids and (2) as provitamin A, primarily in the form of β-carotene. Vitamin A ester is hydrolyzed by a pancreatic esterase within the lumen (Murthy and Ganguly, 1962), and the free alcohol is absorbed in the upper small intestine by a process which apparently requires metabolic energy (Skála and Hrubá, 1964). Vitamin A alcohol is reesterified in the mucosa utilizing primarily palmitic acid (Mahadevan *et al.*, 1963). The vitamin A ester is absorbed by way of the lymph. After reaching the general circulation, it is rapidly cleared from the plasma and stored in the liver. In the postabsorptive state, vitamin A circulates as the free alcohol. This is also the form released from the liver as needed by the action of a specific hepatic retinylpalmitase esterase (Mahadevan *et al.*, 1966). The blood level of vitamin A is independent of the liver reserve, and, as long as a small amount of vitamin A is present in the liver, the blood level remains normal (Dowling and Wald, 1958).

In diets which lack animal fat, the carotenes, mainly β-carotene, serve as the major vitamin A precursors. The intestinal mucosa plays the primary role in conversion of provitamin A to the active vitamin, although conversion can occur to a limited degree in other tissues (Bieri and Pollard, 1954; Zachman and Olson, 1963). The exact mechanism involved in the conversion of β-carotene to vitamin A is not completely established, but studies by Olson (1961) suggest that there is central cleavage of β-carotene into two active vitamin A alcohol molecules, which are subsequently esterified and transported by the lymphatics as with the preformed vitamin.

Bile salts are required for the mucosal uptake of β-carotene and for the conversion of β-carotene to vitamin A. Uptake of carotene and release of vitamin A ester into the lymph appear to be rate-limiting steps. Cattle also absorb substantial amounts of carotene without prior conversion to vitamin A, and these pigments are responsible for much of the yellow color of the plasma. Most other species have no carotene in the plasma, and it has been suggested that extraintestinal conversion may be more efficient in these species than in cattle (Ganguly and Murthy, 1967).

c. Vitamin D. Vitamin D, like cholesterol, is a sterol which is absorbed from the intestine by way of the lymph (Schachter *et al.*, 1964). Intestinal absorption differs,

however, in that vitamin D is transported to the lymph in nonesterified form (Bell, 1966). The uptake of vitamin D by the mucosal cell is favored by the presence of bile salts. Simultaneous absorption of fat from micellar solutions increases transport out of the cell into the lymph, a step which appears to be rate limiting (Thompson *et al.*, 1969).

One of the major actions of vitamin D is to enhance the intestinal absorption of calcium ion. The mechanism of action of vitamin D has been described by Wasserman and co-workers (1968; Wasserman and Taylor, 1966, 1968). They have shown that vitamin D causes synthesis of a calcium-binding protein present in the soluble fraction of the intestinal mucosal cell. They have accumulated a substantial amount of evidence which suggests that this protein plays a central role in the active transport of calcium.

IV. DISTURBANCES OF GASTROINTESTINAL FUNCTION

A. Vomiting

Vomiting is a coordinated reflex act which results in rapid, forceful expulsion of gastric contents through the mouth. The reflex may be initiated by (1) local gastric irritation caused by a variety of toxic irritants or infectious agents, (2) foreign bodies, (3) gastric tumors, (4) obstruction of the pyloric canal or of the small intestine, or (5) drugs, such as apomorphine, or other toxic substances which act centrally on the "vomiting center" located in the medulla.

Severe vomiting produces loss of large quantities of water and of H^+ and Cl^- ions. These losses cause *dehydration, metabolic alkalosis* with elevated plasma bicarbonate concentration, and *hypochloremia*. Chronic vomiting may also be associated with loss of tissue K^+ and hypokalemia. The K^+ deficit is caused primarily by increased urinary excretion, which is the result of the existing alkalosis (Leaf and Santos, 1961). Gastric secretions contain significant quantities of K^+ (Section II,B), and losses in the vomitus also contribute to the K^+ deficiency. Potassium deficiency, which develops initially because of alkalosis, ultimately may perpetuate the alkalotic state by interfering with the ability of the kidney to conserve H^+ (Koch *et al.*, 1956; Darrow, 1964). Both potassium deficiency and the hypovolemia caused by dehydration may result in renal tubular damage and ultimately in renal failure (Haden and Orr, 1923, 1924).

Vomiting occurs frequently in the dog, cat, and pig but is an unusual sign in the horse, which has anatomical restrictions of the esophagus that interfere with expulsion of gastric contents. In cattle, sheep, and goats, the physiological process of rumination utilizes neuromuscular mechanisms similar to those involved in vomiting. Uncontrolled expulsion of ruminal contents is, however, an uncommon sign, occurring most frequently after ingestion of toxic materials. The contents of the abomasum are not expelled directly even when the pyloric canal is obstructed. A syndrome does occur in cattle with pyloric obstruction, however, which is similar metabolically to that observed in nonruminants. The syndrome has been observed in right-sided displacement of the abomasum with torsion (Espersen, 1961; Boucher and Abt, 1968). We have also observed the syndrome in cows with functional pyloric obstruction, the result of reticuloperitonitis (a variety of "vagal indigestion"). When the pylorus is obstructed, abomasal contents are retained, causing distention of the abomasum, which in turn stimulates further secretion and retention. Retained abomasal contents may be regurgitated into the large reservoir of the rumen

and there are sequestered from other fluid compartments of the body. The net result is loss of H^+ and Cl^- ions and development of metabolic alkalosis, hypochloremia, and hypokalemia (Espersen and Simesen, 1961; Svendsen, 1969).

Chronic hypertrophic gastritis has been demonstrated in the dog (van der Gagg *et al.*, 1976; Van Kruiningen, 1977; Happe and van der Gagg, 1977; Kipnis, 1978) which resembles Menetrier's disease in man. Van Kruiningen's series of cases were Basenjis which had concomitant lymphocytic–plasmocytic enteritis. Three unpublished cases were studied at the New York State College of Veterinary Medicine. Signs of illness usually involved chronic vomiting, weight loss, and occasionally diarrhea. Hypoalbuminemia was documented in most of these cases. In man, hyperchlorhydria or achlorhydria can occur. The morphological changes in the stomach wall (hypertrophic rugae) as well as some of the clinical features help to differentiate this disease from gastric neoplasia and canine Zollinger–Ellison syndrome.

Canine Zollinger–Ellison syndrome was reported in four dogs (Straus *et al.*, 1977; van der Gagg *et al.*, 1978). Vomiting, diarrhea, inappetance, and weight loss were reported. All of the dogs had pancreatic non-β islet cell tumors, resulting in hypergastrinemia, hyperchlorhydria, hypertrophic gastritis, peptic esophagitis, and duodenal ulcers.

B. Diarrhea

The term "diarrhea" is used loosely to describe the passage of abnormally fluid feces with increased frequency and/or with increased volume. The significance of diarrhea depends primarily on the underlying cause and on the secondary nutritional and metabolic disturbances which are caused by excessive fecal losses.

There are theoretically three factors which could act independently or in combination to produce diarrhea: (1) increased rate of intestinal transit, (2) decreased intestinal absorptive capacity, and (3) increased secretion into the intestinal lumen. An increase in the rate of intestinal transit has been considered to be important in various functional disorders of the gastrointestinal tract in which "hypermotility" has been considered the primary cause. Although increased intestinal motility may be a factor in certain types of diarrheal disease when the direction of motility has been investigated, diarrhea has actually been associated with decreased motility (Christiansen, 1972).

Decreased intestinal assimilation of nutrients may result from either (1) decreased intraluminal hydrolysis of nutrients, e.g., *maldigestion* (Kalser, 1964), due to pancreatic exocrine insufficiency or to bile salt deficiency or (2) defective mucosal transport of nutrients, *malabsorption,* which may be the result of various types of inflammatory bowel disease, intestinal lymphoma, or intrinsic biochemical defects in the mucosal cell which interfere with normal digestion and absorption. The role of increased intestinal secretion in the pathogenesis of certain types of acute diarrhea is now recognized.

Enteropathogenic strains of *Escherichia coli* have been shown to produce soluble enterotoxins (Smith and Halls, 1967; Kohler, 1968; Moon, 1978), which alter bidirectional sodium and water flux (Fig. 10). Rapid advances in understanding the pathogenesis of enterotoxin-induced diarrhea and the molecular basis of enterotoxin action have been made. The most extensively studied enterotoxin is that produced by *Vibrio cholerae*. This bacterium produces a large molecular weight heat-labile toxin (CT), one subunit of which has properties similar to those of heat-labile (LT) enterotoxin produced by certain strains of *E. coli* (Richards and Douglas, 1978). The mechanism of action of CT is believed to

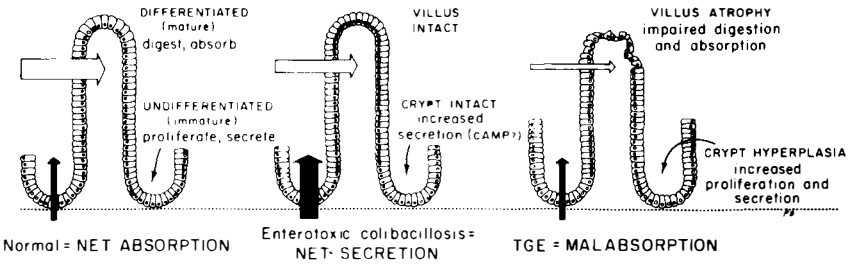


Fig. 10. Proposed pathogenesis of diarrhea caused by *E. coli* enterotoxin and by coronavirus. (After Moon, 1978.)

involve the activation of adenylate cyclase. This membrane-bound enzyme converts ATP to cyclic 3′,5′-adenosine monophosphate (cAMP), which is then responsible for the greatly increased secretion of water and electrolytes by the intestinal mucosa (Moon, 1978). Although species differences have been observed (Hamilton *et al.*, 1978a,b; Forsyth *et al.*, 1978), this mechanism appears to be important in the mode of action of *E. coli* LT as well (Richards and Douglas, 1978).

Additional extensive studies have centered on the molecular mechanism of action of CT. Under physiological conditions, adenylate cyclase is activated by the binding of guanosine triphosphate to the inactive enzyme. An associated GTPase inactivates the enzyme by converting enzyme-bound GTP to GDP and inorganic phosphate. This GTP-GDP system apparently plays a critical role in the regulation of adenylate cyclase. Cholera toxin is believed to bind to the adenyl cyclase in a way which inhibits hydrolysis of GTP, thereby maintaining the enzyme in an activated state (Levinson and Blume, 1977; Johnson *et al.*, 1978; Cassel and Pfeuffer, 1978) (Fig. 11).

Certain enteropathogenic strains of *E. coli* produce a low molecular weight heat-stable toxin (ST) alone or in addition to LT (Richards and Douglas, 1978; Moon, 1978; Hamilton *et al.*, 1978a). In most epidemiological studies of neonatal diarrheal diseases of calves, isolated strains of *E. coli* produce only ST (Moon *et al.*, 1976; Braaten and Myer, 1977; Larivier *et al.*, 1979). In contrast to LT and CT, which induce intestinal sodium and

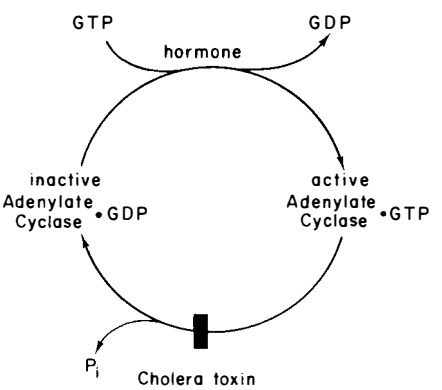


Fig. 11. Proposed mechanism of action of cholera toxin, which inhibits hydrolysis of GTP, thereby increasing adenylate cyclase activity. (After Cassel and Selinger, 1978.)

water secretion only after a lag phase of several hours, ST increases intestinal secretion at once. Recent evidence suggests that ST induces intestinal secretion by activating guanylate cyclase and that the mediator of intestinal secretion induced by ST is cyclic 3′,5′-guanosine monophosphate (Hughes *et al.*, 1978; Field *et al.*, 1978).

Such advances in our fundamental knowledge of the pathogenesis of enterotoxin-induced diarrheal disease have opened several avenues of investigation which may lead to pharmacological modification of intestinal secretion as a mode of therapy or prophylaxis. Enterotoxin-induced intestinal secretion has been shown to be effectively blocked by cycloheximide, inhibitor of protein synthesis (Serebro *et al.*, 1969). The lack of specificity and the toxicity of cycloheximide precluded its clinical use, but acetazolamide has been shown to inhibit intestinal fluid secretion (Norris *et al.*, 1969; Moore *et al.*, 1971), and ethacrynic acid, another potent diuretic, has been shown to inhibit enterotoxin-induced fluid secretion (Carpenter *et al.*, 1969). Unfortunately, the diuretic effects of these drugs preclude their clinical use, but an "intestinal-specific" derivative would have significant therapeutic potential. Adenosine analogues also have been shown in preliminary studies to inhibit cholera toxin-stimulated intestinal adenylate cyclase, but their potential as prophylactic or therapeutic agents is not known.

Prostaglandin E_1 and CT have a similar effect on electrolyte transport in rabbit ileum. Application of either to the mucosa inhibits sodium absorption and stimulates chloride secretion. One possible explanation for the effects of CT is that it stimulated release of prostaglandin, which then acted on adenylate cyclase, producing cAMP. To test this hypothesis, the effects of inhibitors of prostaglandin release on enterotoxin-stimulated intestinal secretion were investigated. Both indomethacin (Gots *et al.*, 1974) and acetylsalicylic acid (Farris *et al.*, 1976) were shown to be potent inhibitors of enterotoxin-induced intestinal secretion using laboratory animal models. Current evidence does not support the hypothesis that prostaglandins play a primary role in the pathogenesis of cholera or other enterotoxin-induced diarrheal diseases (Schwartz *et al.*, 1975), but the effects of these known prostaglandin inhibitors and other drugs on the intestinal secretory process warrant their evaluation as possible prophylactic and therapeutic agents. In preliminary studies, Jones and his colleagues demonstrated a positive therapeutic response to a new prostaglandin inhibitor (Jones *et al.*, 1977).

The autonomic nervous system has important effects on intestinal ion transport and water absorption (Tapper *et al.*, 1978). Catecholamines stimulate formation of cAMP in a variety of mammalian cells (Sutherland and Rall, 1960; Schultz *et al.*, 1975), apparently by activating the GTP–GDP system described above (Cassel and Selinger, 1978; Ciment and deVellis, 1978). Adrenergic blocking agents, such as chlorpromazine (Holmgren *et al.*, 1978) and propranolol (Donowitz *et al.*, 1979), have been shown to have significant inhibitory effects on enterotoxin-induced intestinal secretion. Although the mechanism of action of these two adrenergic blockers is not known, they represent still another class of drugs which may be of therapeutic benefit.

The intestinal "adsorbent" drug Pepto Bismol, a patented medication containing bismuth subsalicylate, and Attapulgite, a heat-treated silicate, have been shown to have antienterotoxic effects (Drucker *et al.*, 1977; Ericsson *et al.*, 1977; Gyles and Zigler, 1978). Controlled therapeutic trials with bismuth subsalicylate have demonstrated significant therapeutic benefit in certain large-volume diarrheal diseases in man suspected of being enterotoxigenic in origin (Portnoy *et al.*, 1976; DuPont *et al.*, 1977; DuPont, 1978). The mechanism of action in inhibiting intestinal secretion has not been determined, but the chemical relation of bismuth subsalicylate to other known prostaglandin inhibitors

is recognized. It is possible that such drugs, by decreasing endogenous production of prostaglandin, decrease the basal level of cyclic nucleotides, which in turn causes an increase in the threshold of response to enterotoxin. Recent evidence suggests that salicylates also may stimulate sodium chloride absorption (Powell *et al.*, 1979). These observations taken collectively suggest that new, innovative methods for therapy and control of acute clinical diarrheal disease may be developed in the not too distant future.

Acute diarrhea represents the leading cause of morbidity and mortality in neonatal calves and pigs. The pathogenesis of the neonatal enteric infection is complex, often involving nutritional or environmental factors as well as infectious agents, such as enteropathogenic strains of *E. coli,* the transmissible gastroenteritis virus (TGE), rota viruses, and other bacterial and viral pathogens. The severe clinical signs and frequently fatal outcome of acute diarrheal disease are often directly related to dehydration and to associated hydrogen ion and electrolyte disturbances (Dalton *et al.*, 1965; Fisher and McEwan, 1967b; Tennant *et al.*, 1972, 1978).

In acute diarrhea with watery stools of large volume, the fecal fluid originates primarily in the small intestine. The electrolyte composition of the stool in such cases is similar to that of the fluid found normally in the lumen of the small intestine, which in turn is similar to that of an ultrafiltrate of the plasma. The rapid dehydration which accompanies acute enteritis in the newborn soon produces hemoconcentration and ultimately hypovolemic shock. Such cases are characterized by metabolic acidosis (Dalton *et al.*, 1965; Phillips and Knox, 1969) caused by decreased excretion of H^+ due to renal failure and by increased production of organic acids, the result of decreased tissue oxygenation, which leads to excessive anaerobic glycolysis. Hyperkalemia also is observed characteristically in young, severely dehydrated animals. Hyperkalemia in such cases is the result of increased movement of cellular potassium into the extracellular fluid and to decreased renal excretion. Cardiac irregularities caused by hyperkalemia can be demonstrated with the electrocardiogram, and cardiac arrest related to hyperkalemia is believed to be a direct cause of death in calves with acute diarrhea (Fisher, 1965; Fisher and McEwan, 1967b). Marked hypoglycemia also has been observed occasionally prior to death in calves with acute enteric infections. Hypoglycemia is believed to be due to decreased gluconeogenesis and increased anaerobic glycolysis, the result of hypovolemic shock (Tennant *et al.*, 1968). The sequence of metabolic changes which occur during acute neonatal diarrhea is summarized in Fig. 12.

In chronic forms of diarrheal disease, excessive fecal losses of electrolyte and fluid may be compensated in part by renal conservation mechanisms and by oral ingestion. If water is consumed without adequate ingestion of electrolytes, hyponatremia and hypokalemia may develop (Tasker, 1967; Patterson *et al.*, 1968). In such cases, the osmolarity of the plasma is significantly decreased and *hypotonic* dehydration occurs. In longer-standing cases of chronic diarrhea, the plasma K^+ concentration may become dangerously low. It is imperative, in this situation, that intravenous fluids contain sufficient K^+ to prevent further reduction in plasma concentration. If they do not, additional cardiac irregularities or cardiac arrest may result.

C. Intestinal Malabsorption

Decreased assimilation of nutrients may occur either as a result of defective intraluminal digestion (maldigestion) (Kalser, 1964), or because of defects in mucosal transport (Jeffries, *et al.*, 1969; Floch, 1969; Wilson and Dietchy, 1971). Intestinal malabsorption

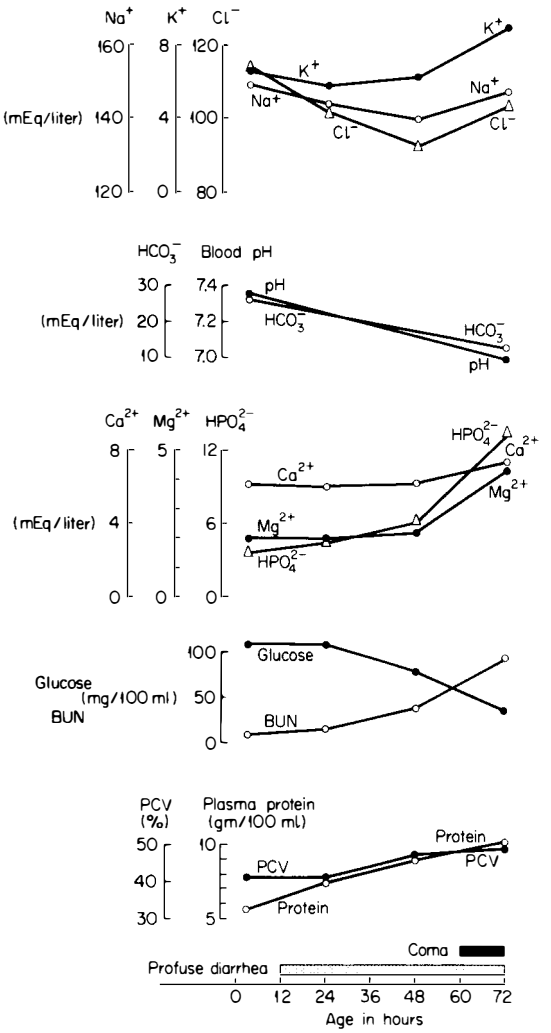


Fig. 12. Metabolic alterations during the course of fatal enteric infection in a neonatal calf (From Tennant *et al.*, 1972.)

or the malabsorption syndrome is observed in several types of intestinal disease, including chronic intestinal granulomatous diseases such as Johne's disease, intestinal parasitic infections, and lymphoma of the intestine. Primary clinical signs include persistent or recurrent diarrhea, nutrient loss in the feces (e.g., steatorrhea), and weight loss. Mucosal cell-enzymatic defects may be accompanied by chronic inflammation, villous atrophy, or cellular infiltrations of the lamina propria of the intestine.

Early reports of primary or idiopathic intestinal malabsorption in dogs (Miller, 1960; Vernon, 1962; Kaneko *et al.*, 1965) were compared to nontropical sprue (adult celiac disease, gluten induced enteropathy) of man, but no convincing association to with gluten sensitivity was demonstrated. Subsequent reports of malabsorption syndromes in the dog have described a variety of causes (Van Kruiningen, 1968; Ewing, 1971; Van Kruiningen

and Hayden, 1972; Hill, 1972; Hill and Kelly, 1974; Schall, 1974; Anderson, 1975, 1977; Burrows *et al.*, 1979), which must be distinguished from the maldigestion caused by pancreatic insufficiency (Anderson and Low, 1965a,b) (juvenile pancreatic atrophy, chronic pancreatitis) and from certain forms of hepatic or gastric disease. Intestinal malabsorption can occur with protozoal enteritis (giardiasis, coccidiosis), lactase deficiency, eosinophilic gastroenteritis, lymphangiectasis, villus atrophy, lymphocytic–plasmacytic enteritis, histoplasmosis, chronic "bacterial" enteritis, malignant lymphoma, and intestinal amyloidosis of the bowel. Some authors (Anderson, 1977; Hayden and Van Kruiningen, 1973; Arrick and Kleine, 1978) described malabsorption and pseudoobstruction secondary to hypoplasia of the tunica muscularis of the jejunum in a dog.

Intestinal malabsorption is reported less frequently in the cat than in the dog (Theran and Carpenter, 1968; Wilkinson, 1969).Malabsorption syndromes similar to those recognized in dogs are being recognized with increased frequency in farm animals (Blood *et al.*, 1979). Meuten *et al.* (1978), Cimprich (1974), and Merritt *et al.*, (1976) have reported malabsorption in the horse secondary to chronic granulomatous enteritis and specific amino acid malabsorption has been reported in Johne's disease (Patterson and Berret, 1969).

1. Malabsorption of Fat

Steatorrhea, the presence of excessive amounts of fat in the feces, is a prominent sign of intestinal malabsorption in dogs. The stools are bulky, gray or tan, and, grossly, may have an oily appearance. The normal dog excretes 3–5 gm of fat in the stool each day. This level of fecal fat is quite constant and is independent of dietary fat intake over a wide range of 15 to 48 gm/day (Heersma and Annegers, 1948). In intestinal malabsorption, the ability to absorb fat is decreased and fecal fat excretion increases significantly. Under these conditions, the amount of fecal fat excreted becomes proportional to dietary intake.

Merritt *et al.* (1979) reported that body weight is an important factor in fat output. Small dogs (i.e., less than 10–15 kg body weight) with intestinal malabsorption had fecal fat outputs lower than or equal to published normal values. Fecal fat excretion for normal dogs was 0.24 ± 0.01 gm/kg body weight per day.

Steatorrhea can be documented qualitatively by staining the fresh stool with a lipophilic stain, such as Sudan III, and observing increased numbers of oil droplets under the light microscope. In experienced hands, this method is a reliable diagnostic procedure (Drummey *et al.*, 1961). The following methods can be used to demonstrate neutral and split fats. For *neutral fat,* two drops of water are added to a stool sample on a glass slide and mixed. Two drops of 95% ethanol are then added and mixed followed by several drops of a saturated solution of Sudan III in 95% ethanol. A coverslip is applied to the mixture, which is then examined for yellow or pale orange refractile globules of fat, particularly at the edges of the coverslip. Normally, two or three fat droplets per high-power field are present. A large number of neutral fat droplets suggests a lack of pancreatic lipase activity, i.e., exocrine pancreatic insufficiency.

For *free fatty acids,* several drops of 36% acetic acid are added to a stool sample on a glass slide and mixed. Several drops of Sudan III solution are then added and mixed. A coverslip is applied, and the slide gently heated over an alcohol burner until it begins to boil. The slide is air-cooled and then quickly heated again, this procedure is repeated two or three times. The warm slide is examined for stained free fatty acid droplets, which, when warm, appear as deep orange fat droplets from which spicules and soaps, resem-

bling the pinna of the ear, form as the preparation cools. Normal stools may contain many tiny droplets of fatty acids (up to 100 per high-power field). With increasing amounts of split fats, the droplets become larger and more numerous, which suggests an abnormality in fat absorption.

Quantitation of fecal fat is the most accurate method of assessing steatorrhea (Burrows *et al.*, 1979) with dietary fat balance being determined for a period of 48–72 hours. Fecal fat is analyzed using a modification of the technique of van de Kamer *et al.* (1949), which employs ether extraction of fecal lipid and titration of fatty acids. The results are expressed as grams of neutral fat excreted per 24 hours. Merritt *et al.* (1979) have suggested that dogs be fed 50 gm fat per kilogram per day for two to three days prior to fecal collection. Analysis of a 24-hour collection of stool when this is done is believed to be as accurate as a 72-hour stool collection. Results are expressed as fat excretion in grams per kilogram body weight.

2. *Malabsorption of Other Nutrients*

In addition to malabsorption of fat, the canine malabsorption syndrome is associated with decreased absorption of other nutrients. These defects in absorption are responsible for the progressive malnutrition which is a cardinal feature of the disease. There may be malabsorption of vitamin D and/or calcium, resulting in osteomalacia. The anemia sometimes observed may be the result of malabsorption of iron or of the B vitamins, which are required for normal erythropoiesis. Malabsorption of vitamin K can result in hypoprothrombinemia. Glucose malabsorption has been clearly documented by Kaneko *et al.* (1965), and it is likely that amino acids, which are absorbed at a similar level of the small intestine, are also malabsorbed. Carbohydrate and fat malabsorption unquestionably contributes to the calorie deficit which results in weight loss. Amino acid malabsorption may contribute to the development of hypoproteinemia, although this is thought to be due primarily to increased intestinal loss of plasma protein (see Section IV,C).

3. *Differential Diagnostic Considerations*

The diagnosis of idiopathic canine malabsorption can be made only after appropriate diagnostic procedures have ruled out the presence of (1) other primary inflammatory, neoplastic, or parasitic diseases of the intestine and (2) the diseases of the pancreas, liver, or stomach which result in defective intraluminal digestion. The presence of parasitic infection is determined by examining the feces for parasite ova. Other inflammatory or neoplastic diseases of the intestine may be suggested on the basis of clinical or radiological examination, but a definitive diagnosis usually depends on histopathological examination of an intestinal biopsy specimen.

Both primary and secondary intestinal malabsorption must be differentiated from those diseases in which there is decreased intraluminal hydrolysis of nutrients. The latter are due most frequently to pancreatic exocrine insufficiency, the result of such diseases as chronic pancreatitis or juvenile atrophy. In these diseases, degradation of the major dietary constituents is reduced because of a primary lack of pancreatic enzymes. Intraluminal hydrolysis of fat may also be decreased because of a deficiency of bile salts caused either by decreased hepatic secretion or by bile duct obstruction. Under certain experimental conditions, diversion of bile flow in the dog actually has a quantitatively small effect on fat absorption (Wells *et al.*, has a quantitatively small effect on fat absorption (Wells *et al.*, 1955; Hill and Kidder, 1972a).

The problems of pancreatic exocrine deficiency are discussed in detail elsewhere in this text (Chapter 7). The most simple and perhaps most widely used test to differentiate intestinal malabsorption from pancreatic exocrine insufficiency is that described by Jasper (1954). The test is employed to detect reduction in trypsin-like activity in the feces of dogs with decreased pancreatic exocrine secretion (Grossman, 1962). There is wide variation in normal activity, making interpretation of the test difficult (Frankland, 1969; Hill and Kidder, 1970; Burrows *et al.*, 1979). The test reveals only the presence or absence of hydrolysis of gelatin and does not differentiate between gelatinase activity produced by intestinal bacteria from that secreted by the pancreas. There is evidence in some species that trypsin is almost completely destroyed by bacteria during its passage through the intestine and that the proteinase activity of the feces is primarily of bacterial origin (Borgström *et al.*, 1959). Despite these theoretical objections, the test has been of clinical diagnostic value in our hands. Fecal gelatinase activity has been detected consistently in cases of intestinal malabsorption and is almost always absent when severe pancreatic exocrine insufficiency is present. Burrows *et al.* (1979) reported that the mean 24-hour trypsin output in dogs with pancreatic insufficiency was significantly lower, and in dogs with malabsorption significantly higher, than clinically normal dogs.

An indirect method to test chymotrypsin activity has been described (Strombeck, 1978). A synthetic peptide, *n*-benzoyltyrosine/*p*-aminobenzoic acid, is administered to test dogs orally. If chymotrypsin is present in the duodenum, hydrolysis of this peptide occurs and *p*-aminobenzoic acid (PABA) is released in a free form, which is absorbed and subsequently excreted in the urine within 6 hours. The urine is analyzed for PABA. Less than 43% PABA excretion identifies dogs with suspected pancreatic exocrine insufficiency.

4. Tests of Intestinal Absorption

a. Oleic Acid and Triolein Absorption. Several tests have been developed for the clinical evaluation of intestinal absorptive capacity. The absorption of ^{131}I-labeled oleic acid and ^{131}I-labeled triolein has been studied extensively in normal dogs (Turner, 1958; Michaelson *et al.*, 1960), and Kaneko *et al.* (1965) used this test to study dogs with intestinal malabsorption. The day before administration of the ^{131}I-labeled compound, a small amount of Lugol's iodine solution is administered to block thyroidal uptake of the isotope. Tracer amounts of the test substances are mixed with nonradioactive carrier and are administered orally. Absorption is determined by measuring the radioactivity of the plasma at intervals following administration can calculating the percentage of the dose absorbed based on plasma volume.

It is possible to use the results of these two tests, when performed in sequence, to differentiate between steatorrhea caused by a deficiency of pancreatic enzymes and that caused by a primary defect in absorption (Kallfelz *et al.*, 1968). If steatorrhea is caused by a lack of pancreatic lipase, oleic acid absorption will be normal, whereas that of triolein, which requires lipolysis for absorption, will be significantly reduced. The absorption of both compounds is reduced in intestinal malabsorption (Fig. 13a,b). The results of this test also may vary depending on the rate of intestinal motility (Tennant *et al.*, 1969b).

b. Vitamin A Absorption. The vitamin A absorption test measures intestinal lipid absorption (Hayden and Van Kruiningen, 1976). Vitamin A absorption requires normal secretion of bile and pancreatic enzymes. Following oral administration of 200,000 units

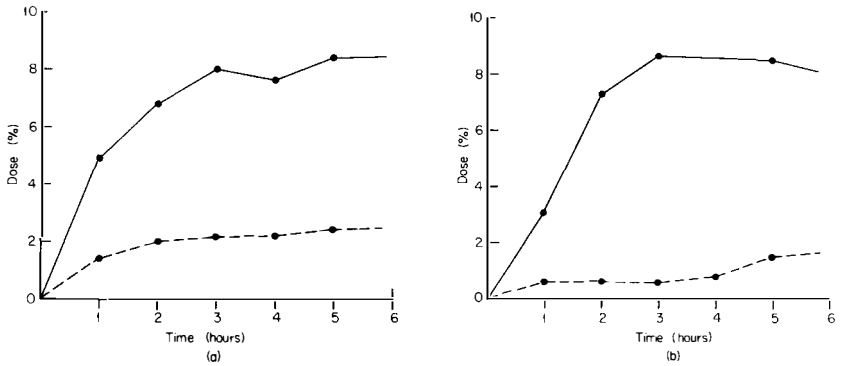


Fig. 13. (a) Absorption of ^{131}I-Labeled oleic acid in normal dogs (3) and dogs with intestinal malabsorption (2). Absorption is expressed as percentage of the dose present in the plasma at intervals following administration. (———) normal; (– – –) intestinal malabsorption. (From Kaneko *et al.*, 1965.) (b) Absorption of ^{131}I-labeled in normal dogs (3) and in dogs with intestinal malabsorption (2). Absorption is expressed as percentage of the dose present in the plasma at intervals following administration. (———) normal; (– – –) intestinal malabsorption. (From Kaneko *et al.*, 1965.)

of vitamin A, mean serum vitamin A concentrations peak at 6–8 hours, with values ranging between three and five times fasting serum levels in normal dogs. Breed differences and delayed gastric emptying will alter results.

c. Glucose Absorption. The absorption of glucose can be measured by means of an oral glucose tolerance test in which a test dose of glucose is given by mouth and the blood glucose level measured at intervals for 3–4 hours following administration. The test has been used in canine malabsorption in which the normal rise in blood glucose level is reduced (Kaneko *et al.*, 1965). The test also has been reported for use in the horse (Roberts and Hill, 1973). Dogs with pancreatic exocrine deficiency may, however, have "diabetic" tolerance curves (Hill and Kidder, 1972b). The major disadvantage of relying on this test alone is that it does not differentiate between decreased intestinal absorption and increased tissue uptake following absorption. This problem can be minimized by comparing results of the oral glucose tolerance test with those obtained with the intravenous tolerance test. The results of this test, however, must be interpreted carefully and in relation to other clinical and laboratory findings. Hill and Kidder (1972) reported that dogs on low-carbohydrate diets can have "diabetic" tolerance curves; test dogs should be on a high-carbohydrate diet 3–5 days before testing.

d. D-Xylose Absorption. The absorption of D-xylose also can be used to evaluate intestinal function. D-Xylose is not metabolized by the body to any significant degree, and the problems of evaluating tissue utilization which occur with glucose are eliminated. Because of the large amounts of D-xylose used in the test, absorption is independent of active transport processes, and the rate of absorption is proportional to luminal concentration.

A D-xylose absorption test for dogs has been described by Van Kruiningen (1968). In this procedure, a standard 25-gm dose of D-xylose is administered by stomach tube. During the 5-hour period following administration, the dog is confined in a metabolism

cage, and urine is collected quantitatively. At the end of the 5-hour test period, the urine remaining in the bladder is removed by catheter, and the total quantity excreted in 5 hours is determined. Normal dogs excreted an average of 12.2 gm during the test period, with a range of 9.1–16.5 gm. The results obtained by this method are dependent not only on the rate of intestinal absorption, but also on the rate of renal excretion, and it is necessary, therefore, to know that kidney function is normal.

The oral xylose tolerance test has received most clinical use (Hill *et al.*, 1970; Hayden and Van Kruiningen, 1973). Dogs are fasted overnight, a blood sample is obtained, and D-xylose is administered by stomach tube at the rate of 0.5 gm/kg. A control test is performed on a normal dog simultaneously with each dog with signs of intestinal malabsorption. The first blood sample is obtained one-half hour after administration. The second sample is obtained 1 hour following administration, and additional samples are taken at hourly intervals for 5 hours. The xylose concentration in the blood is determined by the method of Roe and Rice (1948). Maximal blood levels almost always are reached at 1 hour after administration of the test dose; Hill expects a xylose level of at least 45 mg/dl within 60–90 minutes in a normal dog. In preliminary studies of four dogs with the malabsorption syndrome, maximal blood xylose levels averaged 58% of corresponding control values. In dogs with pancreatic exocrine insufficiency with normal intestinal mucosa, there should be a normal xylose response test. The D-xylose absorption test also has been described for use in differential diagnosis of equine diarrheal diseases (Roberts, 1974). Bolton *et al.* (1976) reported that a dosage of 0.5 gm xylose per kilogram body weight was useful in detecting horses that absorbed the pentose abnormally. Gastrointestinal lesions associated with abnormal results were classified as (1) villous atrophy, (2) edema of the lamina propria, or (3) necrosis of the lamina propria. At this dosage in normal horses, the mean peak plasma concentration is less than one-third that seen in normal dogs given xylose (normal dogs: 60–70 mg % at 60 minutes).

D. Protein-Losing Enteropathy

Albumin, γ-globulin, and other plasma proteins are present in normal gastrointestinal secretions. Because protein usually undergoes complete degradation within the intestinal lumen, it has been suggested that the gastrointestinal tract must have a physiological role in the catabolism of plasma proteins. The relative significance of this pathway, however, has been the subject of considerable controversy. Some investigators have concluded that as much as 50% or more of the normal catabolism of albumin (Glenert *et al.*, 1961, 1962; Campbell *et al.*, 1961; Wetterfors, 1964, 1965; Wetterfors *et al.*, 1965) and γ-globulin (Andersen *et al.*, 1963) occurs in the gastrointestinal tract. Others believed that the physiological role of the intestine in plasma protein catabolism is far less significant, accounting for only about 10% of the total catabolism (Waldmann *et al.*, 1967, 1969; Katz *et al.*, 1960; Franks *et al.*, 1963a,b).

Regardless of the questions concerning the relative importance of the gastrointestinal tract in plasma catabolism, it is well established that normal intestinal losses are increased significantly in a variety of gastrointestinal diseases, which are referred to collectively as *protein-losing enteropathies*. The increased loss causes hypoproteinemia (especially hypoalbuminemia), which may be observed in various types of chronic enteric diseases. The excessive losses are produced by ulcerations or other mucosal changes which alter

permeability or by obstruction of lymphatic drainage from the intestine. If severe, hypoalbuminemia may result in retention of fluid with development of ascites and subcutaneous edema of pendant areas.

Excessive plasma protein loss has now been demonstrated in swine with chronic ileitis (Nielsen, 1966), in calves with acute enteric infections (Marsh *et al.*, 1969), in cattle with parasitic or other inflammatory abomasal disease (Nielsen and Nansen, 1967; Halliday *et al.*, 1968; Murray, 1969), and in Johne's disease (Patterson *et al.*, 1967; Nielsen and Andersen, 1967; Patterson and Berret, 1969). In addition to the classic mucosal and submucosal lesions of Johne's disease, Nielsen and Andersen (1967) demonstrated the presence of secondary intestinal lymphangiectasia. Meuten *et al.*, (1978) described protein-losing granulomatous enteritis in two horses and discussed a comparative overview of diseases causing malabsorption in the horse, cow, dog, pig, and man.

Protein-losing enteropathy has been seen with some frequency in the dog (Campbell *et al.*, 1968; Farrow and Penny, 1969; Hill, 1972; Finco *et al.*, 1973; Hayden and Van Kruiningen, 1973; Mattheeus, *et al.;* Hill and Kelly, 1974; Milstein and Sanford, 1977; Barton *et al.*, 1978; Olson and Zimmer, 1978). Intestinal lymphangiectasia was commonly reported. The dog described by Milstein and Sanford (1977) was not hypoproteinemic because the rate of albumin synthesis by the liver was greater than protein loss into the intestine. Protein loss has also been documented in dogs with chronic hypertrophic gastritis (Section IV,A).

Increased intestinal protein loss is the most likely explanation for the hypoalbuminemia associated with certain other enteric diseases, including intestinal malabsorption and lymphoma of the intestine. Munro (1974) demonstrated that protein loss in dogs with experimentally induced protein-losing gastropathy occurs by an intercellular route. Isotope-labeled polyvinylpyrolidine (^{131}I-PVP), ^{67}Cr-labeled ceruloplasmin, and ^{51}Cr-labeled albumin have been used to evaluate enteric protein loss in the dog (Finco *et al.*, 1973; Barton *et al.*, 1978; Hill and Kelly, 1974; van der Gagg *et al.*, 1976; Olson and Zimmer, 1978).

E. Canine Ulcerative Colitis

Canine ulcerative colitis was described originally in the report of Cello (1964). Since that time, ulcerative colitis and its variant form, granulomatous colitis of Boxer dogs, has been reported by several investigators (Van Kruiningen *et al.*, 1965; Kennedy and Cello, 1966; Koch and Skelley, 1967; Sander and Langham, 1968; Ewing and Gomez, 1973; Gomez *et al.*, 1977; Russell *et al.*, 1971). The etiology is generally unknown. Ewing and Aldrete (1973) reported a case of canine giardiasis presenting as chronic ulcerative colitis and cases of ulcerative colitis in dogs have been attributed to trichuriasis, balantidiasis, protothecosis, histoplasmosis, eosinophilic ulcerative colitis, or neoplasia (Lorenz, 1975). Rarely, severe ulcerative colitis is seen in the cat. In some of these cases, feline leukemia virus is demonstrated. Shindel *et al.* (1978) described colonic lesions in cats caused by feline panleukopenia.

Histopathologically, periodic acid–Schiff-positive macrophages are pathognomonic for the granulomatous colitis of boxer dogs. The disease causes chronic, intractable diarrhea, which is often hemorrhagic. In addition, afflicted dogs may vomit and are often emaciated. Fever is usually not present.

Biochemical manifestations of ulcerative colitis depend upon duration and severity of

TABLE VII

Serum Proteins of Dogs with Ulcerative Colitis[a]

Protein	Mean[b] (gm/dl)	Range[b] (gm/dl)	Normal values (gm/dl)
Total serum protein	5.98	4.40–9.10	5.90–6.70
Albumin	2.44	1.14–4.83	3.00–3.70
α-Globulin	0.92	0.39–1.50	0.86
β-Globulin	1.08	0.60–1.86	1.30
γ-Globulin	1.53	0.46–4.60	0.60–0.90
Albumin/globulin ratio	1.00	0.17–2.45	1.00

[a] Ewing and Gomez (1973).
[b] Thirty-six observations on 29 affected dogs.

illness, degree of colorectal involvement, and the presence of systemic complications. In severe cases of long duration with extensive colorectal involvement, hypoalbuminemia and hypergammaglobulinemia (Table VII) are sometimes observed. The pathogenesis of hypoalbuminemia probably involves increased loss of plasma through the denuded and inflammed colorectal mucosa. Hypergammaglobulinemia is probably an associated response to chronic inflammation.

F. Disturbances of Rumen Function

The digestive process of ruminants differs from that of other animals because of microbial digestion and metabolism in the rumen which occurs prior to other normal digestive processes. The short-chain fatty acids (acetic, propionic, and butyric acids) are the primary end products of rumen fermentation and represent the chief dietary source of energy for ruminants (Hungate *et al.*, 1961). The polysaccharide cellulose, which undergoes only very limited digestion in most simple-stomached animals, is readily utilized by ruminants because of the activity of cellulytic bacteria. Significant quantities of nonprotein nitrogen also can be utilized by ruminal bacteria for protein synthesis, and this bacterial protein subsequently can be utilized to meet the protein requirements of the animal.

Bacterial production of vitamins may also meet essentially all the requirements of ruminants. Maintenance of bacterial fermentation within the rumen also presents certain unusual hazards to ruminant animals. When rapid changes in dietary intake occur, the products of fermentation can be released more rapidly than they can be removed. Acute rumen tympany, acute indigestion of D-lacticacidosis, and urea poisoning are diseases which result from such abrupt changes in diet (Hungate, 1966, 1968).

1. Acute Rumen Engorgement (Rumen Overload, Lacticacidosis, Acid Indigestion)

Acute rumen indigestion occurs in sheep or cattle on a high-roughage diet when they inadvertently are allowed access to large amounts of readily fermentable carbohydrate, e.g. grain and apples (Dunlop, 1972). *Streptococcus bovis* is the rumen microorganism believed to be chiefly responsible for rapid fermentation and for production of large quantities of lactic acid (Hungate *et al.*, 1952; Krogh, 1963a,b).

As lactic acid accumulates more rapidly than absorption, the rumen pH falls and rumen

atony results. Rumen bacteria produce a racemic mixture of lactic acid. Some L-lactate may be metabolized by the liver and other tissues, but D-lactate cannot be and contributes significantly to the acid load of the body. The excessive lactic acid production results in metabolic acidosis, which is characterized by reduced blood pH and bicarbonate concentration and by a fall in urine pH from a normal value of 8.01 -8.0 to as low as 5.0. Fluid accumulates in the rumen because of increased osmolarity of its contents, causing hemoconcentration, which may lead to hypovolemic shock and death (Hyldgaard-Jensen and Simesen, 1966). If affected animals survive the initial period of explosive fermentation, a chemical rumenitis, caused by lactic acid, may develop. Secondary mycotic rumenitis may also occur and be fatal. Hepatic abscesses also may result from severe rumenitis.

2. Acute Rumen Tympany (Bloat)

The rumen of mature cattle can produce 1.2-2.0 liters gas per minute (Hungate *et al.*, 1965). The gas is composed primarily of carbon dioxide and methane, which are products of rumen fermentation. Carbon dioxide is also released when salivary bicarbonate is acted upon by organic acids within the rumen. Under normal conditions, these large amounts of gas are continually removed by eructation.

Any factor which interferes with eructation can produce acute tympany of the rumen (bloat), leading to rapid death. Interruption of the normal eructation reflex or mechanical obstruction of the esophagus typically results in free-gas bloat. The most important form of bloat, however, is seen in cattle consuming large quantities of legumes or in feedlot cattle on high-concentrate diets. The primary factor in these more common types of bloat is a change in the ruminal contents to a foamy or frothy character. Because of altered surface tension, gas is trapped in small bubbles with the rumen and cannot be eliminated by eructation (Clarke and Reid, 1974).

The chemical changes which cause foam to form within the rumen are not completely clear. Some reports (Nichols, 1966; Nichols and Deese, 1966) suggest that plant pectin and pectin methyl esterase, an enzyme system also from plants, are critical factors. The enzyme acts on pectin to release pectic and galacturonic acids, which greatly increase the viscosity of the rumen fluid, resulting in formation of a highly stable foam. Slime-producing bacteria also have been incriminated in the pathogenesis of frothy bloat. These microorganisms produce an extracellular polysaccharide, which results in stable foam formation.

Effective medical treatment and control are directed toward decreasing or preventing foam formation. This has been accomplished with certain nonionic detergents with surfactant properties which break up or prevent formation of foam within the rumen (Bartley, 1965). Another approach has been the prophylactic administration of sodium alkyl sulfonate, which inhibits pectin methyl esterase activity, preventing foam formation by eliminating the products of this enzyme reaction (Nichols, 1963). Much effort is now being directed toward genetic selection of cattle which are less susceptible to rumen tympany and to varieties of legumes which are less likely to produce bloat (Howarth, 1975).

3. Urea Poisoning

Unlike monogastric species, ruminants can effectively use nonprotein nitrogen to meet dietary protein requirements. Urea, biuret (Oltjen *et al.*, 1969) and ammonium salts

(Webb *et al.*, 1972) all can serve as dietary supplements. Urea, which is the most frequently used, is hydrolyzed by bacterial urease within the rumen and the free ammonia formed is incorporated into amino acids by microorganisms within the rumen. The bacterial protein so produced is digested and absorbed in the small intestine along with protein from the diet.

Signs of urea poisoning typically develop within minutes after consumption of food containing toxic amounts of urea. Clinical manifestations are the result of excessive ammonia production (Word *et al.*, 1969; Elmer and Barclay, 1971) and are due to the encephalotoxic effects of free ammonia absorbed from the rumen. Tolerance to urea may be significantly increased by gradually elevating the amounts of urea in the diet or by adding readily fermentable carbohydrate. It has actually been possible for ruminants to adapt and thrive on a diet in which urea was the sole source of dietary nitrogen. However, if urea is fed at a level of more than 3% in the diet of unadapted animals, toxic effects are likely. Poisoning may occur when, by accident, animals obtain access to large amounts of urea-containing dietary supplement or in animals receiving bulk feed when there has been an error in formulation or when the urea-containing additive is incompletely mixed. Oral administration of acetic acid has been shown to reduce acute urea toxicity, apparently by decreasing absorption of free ammonia from the rumen. Acetic acid also has been used clinically for the treatment of urea poisoning but under experimental conditions it has more value prophylactically than in animals with frank signs of poisoning (Word *et al.*, 1969).

REFERENCES

Abrams, R., and Brooks, F. P. (1960). *Proc. Soc. Exp. Biol. Med.* **104,** 278.

Ailhaud, G., Samuel, D., Lazdunski, M., and Desnuelle, P. (1964). *Biochim. Biophys. Acta* **84,** 643.

Alpers, D. H. (1969). *J. Biol. Chem.* **244,** 1238.

Alpers, D. H., and Isselbacher, K. J. (1967). *J. Biol. Chem.* **242,** 5617.

Altamirano, M. (1963). *J. Physiol. (London)* **168,** 787.

Alvarado, F. (1966a). *Science* **151,** 1010.

Alvarado, F. (1966b). *Biochim. Biophys. Acta* **112,** 292.

Alvarado, F., and Crane, R. K. (1962). *Biochim. Biophys. Acta* **56,** 170.

Andersen, S. B., Glenert, J., and Wallevik, K. (1963). *J. Clin. Invest.* **42,** 1873.

Anderson, J. C., Barton, M. A., Gregory, R. A., Hardy, P. M., Kenner, G. W., Macleod, J. K., Preston, J., Sheppard, R. C., and Morley, J. S. (1964). *Nature (London)* **204,** 933.

Anderson, N. V. (1975). *In* "Textbook of Veterinary Internal Medicine—Diseases of the Dog and Cat" (S. J. Ettinger, ed.), Vol. 2, p. 1150. Saunders, Philadelphia, Pennsylvania.

Anderson, N. V. (1977). *In* "Current Veterinary Therapy" (R. W. Kirk, ed.) 6th ed., p. 942. Saunders, Philadelphia, Pennsylvania.

Anderson, N. V., and Low, D. G. (1965a). *J. Am. Anim. Hosp. Assoc.* **1,** 101.

Anderson, N. V., and Low, D. G. (1965b). *J. Am. Anim. Hosp. Assoc.* **1,** 189.

Argenzio, R. (1975). *Cornell Vet.* **65,** 303.

Arrick, R. H., and Kleine, L. J. (1978). *J. Am. Vet. Med. Assoc.* **172,** 1201.

Aschaffenburg, R. (1949). *Br. J. Nutr.* **3,** 200.

Auricchio, S., Semenza, G., and Rubino, A. (1965). *Biochim. Biophys. Acta* **96,** 498.

Bachrach, W. H. (1953). *Physiol. Rev.* **33,** 566.

Barros D'Sa, A. A. J., Bloom, S. R., and Baron, J. H. (1975). *Lancet* **I,** 886.

Barry, B. A., Matthews, J., and Smyth, D. H. (1961). *J. Physiol. (London)* **157,** 279.

Bartley, E. E. (1965). *J. Am. Vet. Med. Assoc.* **147,** 1397.

Barton, C. L., Smith, C., Troy, G., Hightower, D., and Hood, D. (1978). *J. Am. Anim. Hosp. Assoc.* **14,** 85.

Bell, N. H. (1966). *Proc. Soc. Exp. Biol. Med.* **123,** 529.

Ben Abdeljlil, A., and Desnuelle, P. (1964). *Biochim. Biophys. Acta* **81,** 136.
Benzonana, G., and Desnuelle, P. (1965). *Biochim. Biophys. Acta* **105,** 121.
Berger, E. Y., Kanzaki, G., Homer, M. A., and Steele, J. M. (1959). *Am. J. Physiol.* **196,** 74.
Bertolini, M., and Pigman, W. (1967). *J. Biol. Chem.* **242,** 3776.
Bhavanandan, V. P., Buddecke, E., Carubelli, R., and Gottschalk, A. (1964). *Biochem. Biophys. Res. Commun.* **16,** 353.
Bieri, J. G., and Pollard, C. J. (1954). *Br. J. Nutr.* **8,** 32.
Bihler, I., and Crane, R. K. (1962). *Biochim. Biophys. Acta* **59,** 78.
Bihler, I., Hawkins, K. A., and Crane, R. K. (1962). *Biochim. Biophys. Acta* **59,** 94.
Black, J. W., Duncan, W. A., Durant, C. J., Ganellin, C. R., and Parsons, E. M. (1972). *Nature (London)* **236,** 385.
Blood, D. C., Henderson, J. A., and Radostits (1979). "Veterinary Medicine," p. 101, 132, 535. Lea & Febiger, Philadelphia, Pennsylvania.
Bloom, B., Chaikoff, I. L., and Reinhardt, W. O. (1951). *Am. J. Physiol.* **166,** 451.
Bloom, S. R., Mortimer, C. H., and Thornen, M. G. (1974). *Lancet ii,* 1106.
Bockman, D. E., and Winborn, W. B. (1966). *Anat. Rec.* **155,** 603.
Bodanszky, A., Ondetti, M. A., Mutt, V., and Bodanszky, M. (1969). *J. Am. Chem. Soc.* **91,** 944.
Bolton, J. R., Merritt, A. M., Cimprich, R. E., Ramberg, C. F., and Street, W. (1976). *Cornell Vet.* **66,** 183.
Borgström, B. (1954). *Biochim. Biophys. Acta* **13,** 149.
Borgström, B. (1964a). *J. Lipid Res.* **5,** 522.
Borgström, B. (1964b). *Biochim. Biophys. Acta* **106,** 171.
Borgström, B., and Erlanson, C. (1971). *Biochim. Biophys. Acta* **242,** 509.
Borgström, B., Dahlqvist, A., Gustafsson, B. E., Lundh, G., and Malmquist, J. (1959). *Proc. Soc. Exp. Biol. Med.* **102,** 154.
Boucher, W. B., and Abt, D. (1968). *J. Am. Vet. Med. Assoc.* **153,** 76.
Boyd, J. W. (1972). *Vet. Rec.* **90,** 645.
Braaten, B. A., and Myers, L. L. (1977). *Am. J. Vet. Res.* **38,** 1989–1991.
Brambell, F. W. R. (1958). *Biol. Rev. Cambridge Philos. Soc.* **33,** 488.
Braun, R. K., Norcross, M. L., and Campbell, S. G.(1973). *J. Am. Vet. Med. Assoc.* **163,** 1188.
Brazlua, P., and Guilleman, R. (1974). *N. Engl. J. Med.* **290,** 963.
Bremer, J. (1956). *Biochem. J.* **63,** 507.
Braun, R. K., and Tennant, B. (1980). Unpublished observations.
Brindley, D. N., and Hübscher, G. (1965). *Biochim. Biophys. Acta* **106,** 495.
Bro-Rasmussen, F., Killmann, S.-A., and Thaysen, J. H. (1956). *Acta Physiol. Scand.* **37,** 97.
Brown, J. C., Mutt, V., and Dryburgh, J. R. (1971). *Can. J. Physiol. Pharmcol.* **49,** 399.
Brown, J. C., Cook, M. A., and Dryburgh, J. R. (1972). *Gastroenterology* **62,** 401.
Brown, J. C., Cook, M. A., and Dryburgh, J. R. (1973). *Can. J. Biochem.* **51,** 533.
Brown, J. R., and Hartley, B. S.(1966). *Biochem. J.* **101,** 214.
Burrows, C. F., Merritt, A. M., and Chiapella, A. M. (1979). *J. Am. Vet. Med. Assoc.* **174,** 62.
Bush, L. J., Aguilera, M. A., Adams, G. D., and Jones, E. W. (1971). *J. Dairy Sci.* **54,** 1547.
Bywater, R. J., and Penhale, W. J. (1969). *Res. Vet. Sci.* **10,** 591.
Campbell, R. M., Cuthbertson, D. P., Mackie, W., McFarlane, A. S., Phillipson, A. T., and Sudsaneh, S. (1961). *J. Physiol. (London)* **158,** 113.
Campbell, R. S. F., Brobst, D. F., and Bisgard, G. (1968). *J. Am. Vet. Med. Assoc.* **153,** 1050.
Carpenter, C. C. J., Curlin, G. T., and Greenough, W. B. (1969). *J. Infect. Dis.* **120,** 332–338.
Cassel, D., and Pfeuffer, T. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2669–2673.
Cassel, D., and Selinger, Z. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 4155–4159.
Cello, R. M. (1964). *Mod. Vet. Pract.* **45,** 35.
Christensen, J., Weisbrodt, N. W. and Hauser, R. L. (1972). *Gastroenterology,* **62,** 1167–1173.
Ciment, G., and deVellis, J. (1978). *Science* **202,** 765–768.
Cimprich, R. E. (1974). *Vet. Pathol.* **11,** 535.
Clark, B., and Hübscher, G. (1960). *Nature (London)* **185,** 35.
Clark, B., and Hübscher, G. (1961). *Biochim. Biophys. Acta* **46,** 479.
Clark, B., and Hübscher, G. (1962). *Nature (London)* **195,** 599.
Clarke, R. T. J., and Reid, C. S. W. (1974). *J. Dairy Sci.* **57,** 753.
Clarkson, T. W., Cross, A. C., and Toole, S. R. (1961). *Am. J. Physiol.* **200,** 1233.

Code, C. F. (1965). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **24,** 1311.
Code, C. F., Bass, P., McClary, G. B., Newnum, R. L., and Orvis, A. L. (1960). *Am. J. Physiol.* **199,** 281.
Cori, C. F. (1925). *J. Biol. Chem.* **66,** 691.
Cornell, R., Walker, W. A., and Isselbacher, K. J. (1971). *Lab. Invest.* **25,** 42.
Crane, R. K. (1965). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **24,** 1000.
Crane, R. K., Forstner, G., and Eichholz, A. (1965). *Biochim. Biophys. Acta* **109,** 467.
Csáky, T. Z. (1961). *Am. J. Physiol.* **201,** 999.
Csáky, T. Z. (1963). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **22,** 3.
Csáky, T. Z., and Hara, Y. (1965). *Am. J. Physiol.* **209,** 467.
Csáky, T. Z., and Ho, P. M. (1966). *Life Sci.* **5,** 1025.
Csáky, T. Z., and Lassen, U. V. (1964). *Biochim. Biophys. Acta* **82,** 215.
Curran, P. F. (1960). *J. Gen. Physiol.* **43,** 1137.
Curran, P. F. (1965). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **24,** 993.
Curran, P. F., and Schultz, S. G. (1968). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiol. Soc., Sect. 6, Vol. III, p. 1217. Williams & Wilkins, Baltimore, Maryland.
Dahlqvist, A. (1961). *Nature (London)* **190,** 31.
Dalton, R. G., Fisher, E. W., and McIntyre, W. I. M. (1965). *Br. Vet. J.* **121,** 34.
Danforth, E., and Moore, R. O. (1959). *Endocrinology* **65,** 118.
Danielsson, H. (1963). *Adv. Lipid Res.* **1,** 335.
Darrow, D. C. (1964). "A Guide to Learning Fluid Therapy." Thomas, Springfield, Illinois.
Davenport, H. W. (1966). "Physiology of the Digestive Tract," 2nd ed. Yearbook Publ., Chicago, Illinois.
Davie, E. W., and Neurath, H. (1955). *J. Biol. Chem.* **212,** 515.
Dawson, A. M., and Isselbacher, K. J. (1960). *J. Clin. Invest.* **39,** 150.
Deutsch, H. F., and Smith, V. R. (1957). *Am. J. Physiol.* **191,** 271.
Dietschy, J. M., Salomon, H. S., and Siperstein, M. D. (1966). *J. Clin. Invest.* **45,** 832.
Donowitz, M., Charney, A. N., and Hynes, R. (1979). *Gastroenterology,* **76,** 482-491.
Dousa, T. P., and Dozois, R. R. (1977). *Gastroenterology* **73,** 904-912.
Dowling, J. E., and Wald, G. (1958). *Proc. Natl. Acad. Sci. U.S.A.* **44,** 648.
Drucker, M. M., Ogra, P. L., Goldhar, J., and Neter, E. (1977). *Infection* **5,** 211-213.
Drummey, G. D., Benson, J. A., and Jones, C. M. (1961). *N. Engl. J. Med.* **264,** 85.
Dryburgh, J. R., and Brown, J. C. (1975). *Gastroenterology* **68,** 1169.
Dukes, H. H. (1955). "The Physiology of Domestic Animals," 7th ed. Cornell Univ. Press (Comstock), Ithaca, New York.
Dunlop, R. H. (1972). *Adv. Vet. Sci. Comp. Med.* **16,** 259.
DuPont, H. L. (1978). *J. Am. Vet. Med. Assoc.* **173,** 649-653.
DuPont, H. L., Sullivan, P., Pickering, L. K., Haynes, G., and Ackerman, P. B. (1977). *Gastroenterology* **73,** 713-715.
Eichholz, A. (1967). *Biochim. Biophys. Acta* **135,** 475.
Eichholz, A. (1968). *Biochim. Biophys. Acta* **163,** 101.
Eichholz, A., and Crane, R. K. (1965). *J. Cell Biol.* **26,** 687.
Emas, S., and Grossman, M. I. (1967). *Gastroenterology* **52,** 29.
Ericsson, C. D., Evans, D. G., DuPont, H. L., Evans, D. J., and Pickering, L. K. (1977). *J. Infect. Dis.* **136,** 693-696.
Espersen, G. (1961). *Nord. Veterinaermed., Supp..* **1,** 7.
Espersen, G., and Simesen, M. G. (1961). *Nord. Veterinaermed.* **13,** 147.
Ewing, G. O. (1971). *In* "Current Veterinary Therapy" (R. W. Kirk, ed.), 4th ed., p. 551. Saunders, Philadelphia, Pennsylvania.
Ewing, G. O., and Aldrete, A. V. (1973). *J. Am. Anim. Hosp. Assoc.* **173,** 52.
Ewing, G. O., and Gomez, J. A. (1973). *J. Am. Anim. Hosp. Assoc.* **9,** 395.
Farris, R. K., Tapper, E. J., Powell, D. W., and Morris, S. M. (1976). *J. Clin. Invest.* **57,** 916-924.
Farrow, B. R. H., and Penny, R. (1969). *J. Small Anim. Pract.* **10,** 513.
Faust, R. G., Wu, S. L., and Faggard, M. L. (1967). *Science* **155,** 1261.
Fey, H. (1971). *Ann. N.Y. Acad. Sci.* **176,** 49.
Field, M., Graf, L. H., Laird, W. J., and Smith, P. L. (1978). *Proc. Natl. Acad. Sci.* **75,** 2800-2804.
Finco, D. R., Schall, W. B., Hooper, B. E., Chandler, F. W., and Keating, K. A. (1973). *J. Am. Vet. Med. Assoc.* **163,** 262.

Fisher, E. W. (1965). *Br. Vet. J.* **121,** 132.
Fisher, E. W., and McEwan, A. D. (1967a). *Vet. Rec.* **80,** 290.
Fisher, E. W., and McEwan, A. D. (1967b). *Br. Vet. J.* **123,** 4.
Fisher, E. W., Martinez, A. A., Trainin, Z., and Meirom, R. (1975). *Br. Vet. J.* **131,** 402.
Fisher, R. B., and Parsons, D. S. (1953). *J. Physiol. (London)* **119,** 224.
Floch, M. H. (1969). *Am. J. Clin. Nutr.* **22,** 327.
Fordtran, J. S., Rector, F. C., and Carter, N. W. (1968). *J. Clin. Invest.* **47,** 884.
Forstner, C. G., Sabesin, S. M., and Isselbacher, K. J. (1968). *Biochem. J.* **106,** 381.
Forsyth, G. W., Hamilton, D. L., Goertz, K. E., and Johnson, M. R. (1978). *Infect. Immun.* **21,** 373–380.
Frankland, A. L. (1969). *J. Small Anim. Pract.* **10,** 531.
Franks, J. J., Mosser, E. L., and Anstadt, G. L. (1963a). *J. Gen. Physiol.* **46,** 415.
Franks, J. J., Edwards, K. W., Lackey, W. W., and Fitzgerald, J. B. (1963b). *J. Gen. Physiol.* **46,** 427.
Freeman, H. J., and Kim, Y. S. (1978). *Annu. Rev. Med.* **29,** 99.
Ganguly, J., and Murthy, S. K. (1967). *In* "The Vitamins" (W. H. Sebrell, Jr. and R. S. Harris, eds.), 2nd ed., Vol. 1, p. 125. Academic Press, New York.
Gay, C. C. (1965). *Bacteriol. Rev.* **29,** 75.
Gay, C. C., Anderson, N., Fisher, F. W., and McEwan, A. D. (1965a). *Vet. Rec.* **77,** 148.
Gay, C. C., Fisher, E. W., and McEwan, A. D. (1965b). *Vet. Rec.* **77,** 994.
Gilman, A., and Koelle, E. S. (1960). *Am. J. Physiol.* **199,** 1025.
Glenert, J., Jarnum, S., and Riemer, S. (1961). *Acta Chir. Scand.* **121,** 242.
Glenert, J., Jarnum, S., and Riemer, S. (1962). *Acta Chir. Scand.* **124,** 63.
Gomez, J. A., Russell, S. W., Trowbridge, J., and Lee, J. (1977). *Am. J. Dig. Dis.* **22,** 485.
Gots, R. E., Formal, S. B., and Giannella, R. A. (1974). *J. Infect. Dis.* **130,** 280–284.
Gottschalk, A., ed. (1972). "Glycoproteins: Composition, Structure and Function," 2nd ed. Elsevier, Amsterdam.
Gottschalk, A., and Fazekas de St. Groth, S. (1960). *Biochim. Biophys. Acta* **43,** 513.
Gottschalk, A., and Thomas, M. A. W. (1961). *Biochim. Biophys. Acta* **46,** 91.
Gray, G. M. (1975). *N. Engl. J. Med.* **292,** 1225.
Gray, G. M., and Cooper, H. L. (1971). *Gastroenterology* **61,** 535.
Gray, J. S., and Bucher, G. R. (1941). *Am. J. Physiol.* **133,** 542.
Gregory, R. A. (1966). *Gastroenterology* **51,** 953.
Gregory, R. A. (1967). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiol. Soc., Sect. 6, Vol. II, p. 827. Williams & Wilkins, Baltimore, Maryland.
Gregory, R. A., and Tracy, H. J. (1964). *Gut* **5,** 103.
Gregory, R. A., Hardy, P. M., Jones, D. S., Kenner, G. W., and Sheppard, R. C. (1964). *Nature (London)* **204,** 931.
Grim, E. (1962). *Am. J. Dig. Dis.* **7,** 17.
Grossman, M. I. (1958). *Gastroenterology* **34,** 1159.
Grossman, M. I. (1962). *Proc. Soc. Exp. Biol. Med.* **110,** 41.
Grossman, M. I. and Konturek, S. J. (1974). *Gastroenterology* **66,** 517.
Gustafsson, B. E., and Norman, A. (1962). *Proc. Soc. Exp. Biol. Med.* **110,** 387.
Gustafsson, B. E., and Norman, A. (1969a). *Br. J. Nutr.* **23,** 429.
Gustafsson, B. E., and Norman, A. (1969b). *Br. J. Nutr.* **23,** 627.
Gustafsson, B. E., Bergstrom, S., Lindstedt, S., and Norman, A. (1957). *Proc. Soc. Exp. Biol. Med.* **94,** 467.
Gustafsson, B. E., Midtvedt, T., and Norman, A. (1966). *J. Exp. Med.* **123,** 413.
Gyles, C. L., and Zigler, M. (1978). *Can. J. Comp. Med. Vet. Sci.* **42,** 260–268.
Haden, R. L., and Orr, T. G. (1923). *J. Exp. Med.* **37,** 377.
Haden, R. L., and Orr, T. G. (1924). *J. Exp. Med.* **38,** 55.
Haessler, H. A., and Isselbacher, K. J. (1963). *Biochim. Biophys. Acta* **73,** 427.
Hakim, A. A., and Lifson, N. (1964). *Am. J. Physiol.* **206,** 1315.
Hakim, A., Lester, R. G., and Lifson, N. (1963). *J. Appl. Physiol.* **18,** 409.
Halliday, G. J., Mulligan, W., and Dalton, R. G. (1968). *Res. Vet. Sci.* **9,** 224.
Hamilton, D. L., Forsyth, G. W., Roe, W. E., and Nielsen, N. O. (1978). *Can. J. Comp. Med. Vet. Sci.* **42,** 316–321.
Hamilton, D. L., Johnson, M. R., Forsyth, G. W., Roe, W. E., and Nielsen, N. O. (1978). *Can. J. Comp. Med. Vet. Sci.* **42,** 327–331.

Happe, R. P., and van der Gagg, I. (1977). *J. Small Anim. Pract.* **18,** 179.
Harris, J. B., and Edelman, I. S. (1964). *Am. J. Physiol.* **206,** 769.
Hartley, B. S., and Kauffman, D. L. (1966). *Biochem. J.* **101,** 229.
Hartley, B. S., Brown, J. R., Kauffman, D. L., and Smillie, L. B. (1965). *Nature (London)* **207,** 1157.
Hartman, P. A., Hays, V. W., Baker, R. O., Neagle, L. H., and Catron, D. V. (1961). *J. Anim. Sci.* **20,** 114.
Haslewood, G. A. D. (1964). *Biol. Rev. Cambridge Philos. Soc.* **39,** 537.
Hatch, F. T., Aso, Y., Hagopian, L. M., and Rubenstein, J. J. (1966). *J. Biol. Chem.* **241,** 1655.
Hayden, D. W., and Van Kruiningen, H. J. (1973). *J. Am. Vet. Med. Assoc.* **162,** 379.
Hayden, D. W., and Van Kruiningen, H. J. (1976). *J. Am. Anim. Hosp. Assoc.* **12,** 31.
Heersma, J. R., and Annegers, J. H. (1948). *Am. J. Physiol.* **153,** 143.
Heizer, W. D., and Laster, L. (1969). *Biochim. Biophys. Acta* **185,** 409.
Helmer, L. G., and Bartley, E. E. (1971). *J. Dairy Sci.* **54,** 25–31.
Herriott, R. M. (1938). *J. Gen. Physiol.* **21,** 501.
Herriott, R. M. (1962). *J. Gen. Physiol.* **45,** 57.
Hill, F. W. G. (1972). *J. Small Anim. Pract.* **13,** 575.
Hill, F. W. G., and Kelly, D. F. (1974). *Am. J. Dig. Dis.* **19,** 649.
Hill, F. W. G., and Kidder, D. E. (1970). *J. Small Anim. Pract.* **11,** 191.
Hill, F. W. G., and Kidder, D. E. (1972a). *J. Small Anim. Pract.* **13,** 23.
Hill, F. W. G., and Kidder, D. E. (1972b). *Br. Vet. J.* **128,** 207.
Hill, F. W. G., Kidder, D. E., and Frew, J. (1970). *Vet. Rec.* **87,** 250.
Hindle, W., and Code, C. F. (1962). *Am. J. Physiol.* **203,** 215.
Hirschowitz, B. I. (1966). *Am. J. Dig. Dis.* **11,** 183.
Hirschowitz, B. I., and Sachs, G. (1965). *Am. J. Physiol.* **209,** 452.
Hladky, S. B. (1965). *Bull. Math. Biophys.* **27,** 79.
Hofmann, A. F. (1963). *Biochem. J.* **89,** 57.
Hofmann, A. F. (1966). *Gastroenterology* **50,** 56.
Hofmann, A. F., and Small, D. M. (1967). *Annu. Rev. Med.* **18,** 333.
Holmgren, J., Lange, S., and Lonnroth, I. (1978). *Gastroenterology* **75,** 1103–1108.
Houpt. T. R., and Houpt, K. A. (1971). *Am. J. Vet. Res.* **32,** 579.
House, J. A., and Baker, J. A. (1968). *J. Am. Vet. Med. Assoc.* **152,** 893.
Howarth, R. E. (1975). *Can. Vet. J.* **16,** 281.
Hsu, L., and Tappel, A. L. (1965). *Biochim. Biophys. Acta* **101,** 83.
Huber, J. T., Jacobson, N. L., Allen, R. S., and Hartman, P. A. (1961). *J. Dairy Sci.* **44,** 1494.
Hughes, J. M., Murad, F., Chang, B., and Guerrant, R. L. (1978). *Nature (London)* **271,** 755–756.
Hungate, R. E. (1966). "The Rumen and its Microbes." Academic Press, New York.
Hungate, R. E. (1968). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiol. Soc., Sect. 6, Vol. V, p. 2725. Williams & Wilkins, Baltimore, Maryland.
Hungate, R. E., Doughterty, R. W., Bryant, M. P., and Cello, R. M. (1952). *Cornell Vet.* **42,** 423.
Hungate, R. E., Mah, R. A., and Simesen, M. (1961). *Appl Micribiol.* **9,** 554.
Hungate, R. E., Fletcher, D. W., Dougherty, R. W., and Barrentine, B. F. (1965). *Appl. Microbiol.* **13,** 161.
Hyldgaard-Jensen, J., and Simesen, M. G. (1966). *Nord. Veterinaermed.* **18,** 73.
Ingraham, R. C., and Visscher, M. B. (1936). *Am. J. Physiol.* **114,** 676.
Isselbacher, K. J. (1966). *Gastroenterology* **50,** 78.
Isselbacher, K. J. (1967). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **26,** 1420.
Isselbacher, K. J., and Budz, D. M. (1963). *Nature (London)* **200,** 364.
Itoh, Z., Takeuchi, S., Aizawa, I., Mori, K., Taminato, T., Seino, Y., Imura, H., and Yanaihara, N. (1978). *Am. J. Dig. Dis.* **23,** 929.
Janowitz, H. D., Colcher, H., and Hollander, F. (1952). *Am. J. Physiol.* **171,** 325.
Jasper, D. E. (1954). *North Am. Vet.* **35,** 523.
Jeffries, G. H., Weser, E., and Sleisenger, M. H. (1969). *Gastroenterology* **56,** 777.
Jennewein, H. M., Hummelt, H., Siewert, R., and Waldeck, F. (1975). *Digestion* **13,** 246.
Johnson, G. L., Kaslow, H. R., and Bourne, H. R. (1978). *Proc. Natl. Acad. Sci. U.S.A* **75,** 3113–3117.
Johnston, J. M. (1968). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiol. Soc., Sect. 6, Vol. III, p. 1353. Williams & Wilkins, Baltimore, Maryland.
Johnston, J. M., and Borgström, B. (1964). *Biochim. Biophys. Acta* **84,** 412.
Jones, E. W., Hamm, D., and Bush, L. (1977). *Bovine Pract.* **10,** 48–54.

Kallfelz, F. A., Norrdin, R. W., and Neal, T. M. (1968). *J. Am. Vet. Med. Assoc.* **153,** 43.
Kalser, M. H. (1964). *In* "Gastroenterology" (H. L. Bockus, ed.), Vol. 2, p. 423. Saunders, Philadelphia, Pennsylvania.
Kaneko, J. J., Moulton, J. E., Brodey, R. S., and Perryman, V. D. (1965). *J. Am. Vet. Med. Assoc.* **146,** 463.
Katz, J., Rosenfeld, S., and Sellers, A. L. (1960). *Am. J. Physiol.* **200,** 1301.
Keller, P. J. (1968). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiol. Soc., Sect. 6, Vol. V, p. 2605. Williams & Wilkins, Baltimore, Maryland.
Kennedy, P. C., and Cello, R. M. (1966). *Gastroenterology* **51,** 926.
Kern, F., and Borgström, B. (1965). *Biochim. Biophys. Acta* **98,** 520.
Kim, Y. S., Birtwhistle, W., and Kim, Y. W. (1972). *J. Clin. Invest.* **51,** 1419.
Kim, Y. S., Kim, Y. W., and Sleisenger, M. H. (1974). *Biochim. Biophys. Acta* **370,** 283.
Kimmich, G. A. (1973). *Biochim. Biophys. Acta* **300,** 31.
Kipnis, R. M. (1978). *J. Am. Vet. Med. Assoc.* **173,** 182.
Koch, A. R., Brazeau, P., and Gilman, A. (1956). *Am. J. Physiol.* **186,** 350.
Koch, S. A., and Skelley, J. F. (1967). *J. Am. Vet. Med. Assoc.* **150,** 22.
Kohler, E. M. (1968). *Am. J. Vet. Res.* **29,** 2263.
Kohn, P., Dawes, E. D., and Duke, J. W. (1965). *Biochim. Biophys. Acta* **107,** 358.
Kolínská, J., and Semenza, G. (1967). *Biochim. Biophys. Acta* **146,** 181.
Krogh, N. (1963a). *Acta Vet. Scand.* **4,** 27.
Krogh, N. (1963b). *Acta Vet. Scand.* **4,** 41.
Kunkel, H. G. (1947). *Proc. Soc. Exp. Biol. Med.* **66,** 217.
Lack, L., and Weiner, I. M. (1961). *Am. J. Physiol.* **200,** 313.
Lack, L., and Weiner, I. M. (1963). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **22,** 1334.
Lack, L., and Weiner, I. M. (1966). *Am. J. Physiol.* **210,** 1142.
Larivier, S., Lallier, R., and Moria, M. (1979). *Am. J. Vet. Res.* **40,** 130-134.
Laskowski, M., Jr., Haessler, H. A., Miech, R. P., Peanasky, R. J., and Laskowski, M. (1958). *Science* **127,** 1115.
Laurent, T. C., and Persson, H. (1965). *Biochim. Biophys. Acta* **106,** 616.
Leaf, A., and Santos, R. F. (1961). *N. Engl. J. Med.* **264,** 335.
Lecce, J. G. (1966). *Biol. Neonat.* **9,** 50.
Lecce, J. G., and Morgan, D. O. (1962). *J. Nutr.* **78,** 263.
Lecce, J. G., Morgan, D. O., and Matrone, G. (1964). *J. Nutr.* **84,** 43.
Levenson, S. M., Rosen, H., and Upjohn, H. L. (1959). *Proc. Soc. Exp. Biol. Med.* **101,** 178.
Levinson, S. L., and Blume, A. J. (1977). *J. Biol. Chem.* **252,** 3766-3774.
Lindberg, T. (1966). *Acta Physiol. Scand., Suppl.* **285.**
Lindemann, B., and Solomon, A. K. (1962). *J. Gen. Physiol.* **45,** 801.
Lindstedt, S., and Samuelsson, B. (1959). *J. Biol. Chem.* **234,** 2026.
Logan, E. F., and Penhale, W. J. (1971). *Vet. Rec.* **89,** 628.
Lorenz, M. D. (1975). *In* "Textbook of Veterinary Internal Medicine—Diseases of the Dog and Cat" (S. J. Ettinger, ed.), Vol. 2, p. 1203. Saunders, Philadelphia, Pennsylvania.
McBeath, D. G., Penhale, W. J., and Logan, E. F. (1971). *Vet. Rec.* **88,** 266.
McEwan, A. D., Fisher, E. W., and Selman, I. E. (1970a). *J. Comp. Pathol.* **80,** 259.
McEwan, A. D., Fisher, E. W., Selman, I. E., ano Penhale, W. J. (1970b). *Clin. Chim. Acta* **27,** 155.
MeGuire, E. J., and Roseman, S. (1967). *J. Biol. Chem.* **242,** 3745.
Mahadevan, S., Sastry, P. S., and Ganguly, J. (1963). *Biochem. J.* **88,** 531.
Mahadevan, S., Ayyoub, N. I., and Roels, O. A. (1966). *J. Biol. Chem.* **241,** 57.
Malathi, P. (1967). *Gastroenterology* **52,** 1106.
Marsh, C. L., Mebus, C. A., and Underdahl, N. R. (1969). *Am. J. Vet. Res.* **30,** 163.
Mattheeuws, A., DeRick, H., Thoonen, H., and Van der Stock, J. (1974). *J. Small Anim. Pract.* **15,** 757.
Matthews, D. M., and Laster, L. (1965). *Gut* **6,** 411.
Mattson, F. H., and Volpenhein, R. A. (1962). *J. Lipid Res.* **3,** 281.
Mattson, F. H., and Volpenhein, R. A. (1964). *J. Biol. Chem.* **239,** 2772.
Mattson, F. H., and Volpenhein, R. A. (1966). *J. Lipid Res.* **7,** 536.
Mattson, F. H., Benedict, J. H., Martin, J. B., and Beck, L. W. (1952). *J. Nutr.* **48,** 335.
Meister, A. (1973). *Science* **180,** 33.
Meister, A., and Tate, S. S. (1976). *Annu. Rev. Biochem.* **45,** 559.

Merritt, A. M., Cimprich, R. E., and Beech, J. (1976). *J. Am. Vet. Med. Assoc.* **169,** 603.
Merritt, A. M., Burrows, C. F., and Cowgill, L. (1979). *J. Am. Vet. Med. Assoc.* **174,** 59.
Meuten, D. J., Butler, D. G., Thomsen, G. W., and Lumsden, J. H. (1978). *J. Am. Vet. Med. Assoc.* **172,** 326.
Meyer, J. H., and Kelly, G. A. (1977). *Am. J. Physiol.* **231,** 682.
Michaelson, S. M., El-Tamami, M. Y., Thomson, R. A. E., and Howland, J. W. (1960). *Am. J. Vet. Res.* **21,** 364.
Midtvedt, T., and Norman, A. (1967). *Acta Pathol. Microbiol. Scand.* **71,** 629.
Miller, R. M. (1960). *Mod. Vet. Pract.* **41,** 34.
Milstein, M., and Sanford, S. E. (1977). *Can. Vet. J.* **18,** 127.
Moon, H. W. (1978). *J. Am. Vet. Met. Assoc.* **172,** 443–447.
Moon, H. W., Whipp, S. C., and Skartvedt, S. M. (1976). *Am. J. Vet. Res.* **37,** 1025–1029.
Moore, W. L., Jr. Bieberdorf, F. A., Morawski, S. G., Finkelstein, R. A., and Fordtran, J. S. (1971). *J. Clin. Invest.* **50,** 312–318.
Munro, D. R. (1974). *Gastroenterology* **66,** 960.
Murry, M. (1969). *Gastroenterology* **56,** 763–772.
Murthy, S. K., and Ganguly, J. (1962). *Biochem. J.* **83,** 460.
Mutt, V., and Jorpes, J. E. (1967). *Recent Prog. Horm. Res.* **23,** 483.
Nasset, E. S., and Ju, J. S. (1961). *J. Nutr.* **74,** 461.
Naylor, J. M., Kronfeld, D. S., Bech-Nielsen, S., and Bartholomew, R. C. (1977). *J. Am. Vet. Med. Assoc.* **171,** 635.
Neurath, H., and Walsh, K. A. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 3825.
Neurath, H., Rupley, J. A., and Dreyer, W. J. (1956). *Arch. Biochem. Biophys.* **65,** 243.
Newey, H., and Smyth, D. H. (1959). *J. Physiol. (London)* **145,** 48.
Newey, H., and Smyth, D. H. (1960). *J. Physiol. (London)* **152,** 367.
Newey, H., and Smyth, D. H. (1964). *Nature (London)* **202,** 400.
Nichols, R. E. (1963). *J. Am. Vet. Med. Assoc.* **143,** 998.
Nichols, R. E. (1966). *Am. J. Vet. Res.* **27,** 369.
Nichols, R. E., and Deese, D. (1966). *Am. J. Vet. Res.* **27,** 623.
Nielsen, K. (1966). *Acta Vet. Scand.* **7,** 321.
Nielsen, K., and Andersen, S. (1967). *Nord. Veterinaermed.* **19,** 31.
Neilsen, K., and Nansen, P. (1967). *Can. J. Comp. Med. Vet. Sci.* **31,** 106.
Norris, H. T., Curran, P. F., and Schultz, S. G. (1969). *J. Infect. Dis.* **119,** 117–125.
Ockner, R. K., and Isselbacher, K. J. (1974). *Rev. Physiol. Biochem. Pharmacol.* **71,** 107.
Ockner, R. K., and Manning, J. (1974). *J. Clin. Invest.* **54,** 326.
Ockner, R. K., Hughes, F. B., and Isselbacher, K. J. (1969). *J. Clin. Invest.* **48,** 2367.
Olson, J. A. (1961). *J. Biol. Chem.* **236,** 349.
Olson, N. C., and Zimmer, J. F. (1978). *J. Am. Vet. Med. Assoc.* **173,** 271.
Oltjen, R. R., Williams, E. E., Slyter, L. L., and Richardson, G. V. (1969). *J. Anim. Sci.* **29,** 816–822.
Palay, S. K., and Karlin, L. J. (1959). *J. Biophys. Biochem. Cytol.* **5,** 363.
Parsons, B. J., Smyth, D. H., and Taylor, C. B. (1958). *J. Physiol. (London)* **144,** 387.
Parsons, D. S., and Wingate, D. L. (1961). *Biochim. Biophys. Acta* **46,** 170.
Patterson, D. S. P. (1967). *Vet. Rec.* **80,** 260.
Patterson, D. S. P., and Berret, S. (1969). *J. Med. Microbiol.* **2,** 327.
Patterson, D. S. P., Allen, W. M., and Lloyd, M. K. (1967). *Vet. Rec.* **81,** 717.
Patterson, D. S. P., Allen, W. M., Berrett, S., Ivins, L. N., and Sweasey, D. (1968). *Res. Vet. Sci.* **9,** 117.
Payne, L. C., and Marsh, C. L. (1962a). *J. Nutr.* **76,** 151.
Payne, L. C., and Marsh, C. L. (1962b). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **21,** 909.
Pearse, A. G. E., Polak, J. M., and Bloom, S. R. (1977). *Gastroenterology* **72,** 746.
Penhale, W. J., Christie, G., McEwan, A. D., Selman, I. E., and Fisher, E. W. (1967). *Vet. Rec.* **81,** 416.
Penhale, W. J., Christie, G., McEwan, A. D., Fisher, E. W., and Selman, I. E. (1970). *Br. Vet. J.* **126,** 30.
Penhale, W. J., Logan, E. F., and Stenhouse, A. (1971). *Vet. Rec.* **89,** 623.
Peric-Golia, L., and Socic, H. (1968). *Am. J. Physiol.* **215,** 1284.
Peters, T. J. (1970). *Biochem. J.* **120,** 195.
Pfeiffer, N. E., and McGuire, T. C. (1977). *J. Am. Vet. Med. Assoc.* **170,** 809.
Phillips, R. W., and Knox, K. L. (1969). *J. Comp. Lab. Med.* **3,** 1.

Phillipson, A. T. (1977). *In* "Dukes' Physiology of Domestic Animals" (M. J. Swenson, ed.), p. 250. Cornell Univ. Press, Ithaca, New York.
Playoust, M. R., and Isselbacher, K. J. (1964). *J. Clin. Invest.* **43,** 878.
Playoust, M. R., Lack, L., and Weiner, I. M. (1965). *Am. J. Physiol.* **208,** 363.
Polak, J. M., Pearse, A. G. E., and Heath, C. M. (1975). *Gut* **16,** 225.
Portnoy, B. L., DuPont, H. L., Pruitt, D., Abdo, J. A., and Rodriguez, J. T. (1976). *J. Am. Vet. Med. Assoc.* **236,** 844–846.
Powell, D. W., Malawer, S. J., and Plotkin, G. R. (1968). *Am. J. Physiol.* **215,** 1226.
Powell, D. W., Tapper, E. J., and Morris, S. M. (1979). *Gastroenterology,* **76,** 1429–1437.
Prior, R. L., Hintz, H. F., Lowe, J. E., and Visek, W. J. (1974). *J. Anim. Sci.* **38,** 565.
Rao, G. A., and Johnston, J. M. (1966). *Biochim. Biophys. Acta* **125,** 465.
Redman, C. M., Siekevitz, P., and Palade, G. E. (1966). *J. Biol. Chem.* **241,** 1150.
Rhodes, J. B., Eichholz, A., and Crane, R. K. (1967). *Biochim. Biophys. Acta* **135,** 959.
Richards, K. L., and Douglas, S. D. (1978). *Microbiol. Rev.* **42,** 592–613.
Riklis, E., and Quastel, J. H. (1958). *Can. J. Biochem. Physiol.* **36,** 347.
Roberts, H. E., and Worden, A. N., and Rees Evans, E. T. (1954). *J. Comp. Pathol.* **64,** 283.
Roberts, M. C. (1974). *Equine Vet. J.* **6,** 28.
Roberts, M. C., and Hill, F. W. G. (1973). *Eq. Vet. J.* **5,** 171–173.
Roe, J. H., and Rice, E. W. (1948). *J. Biol. Chem.* **173,** 507.
Russell, S. W., Gomez, J. A., and Trowbridge, J. (1971). *Lab. Invest.* **25,** 509.
Ryle, A. P. (1965). *Biochem. J.* **96,** 6.
Ryle, A. P., and Hamilton, M. P. (1966). *Biochem. J.* **101,** 176.
Ryle, A. P., and Porter, R. R. (1959). *Biochem. J.* **73,** 75.
Sabesin, S. M., and Isselbacher, K. J. (1965). *Science* **147,** 1149.
Samloff, I. M. (1971). *Gastroenterology* **60,** 586.
Sander, C. H., and Langham, R. F. (1968). *Arch. Pathol.* **85,** 94.
Sandholm, M. (1974). *Res. Vet. Sci.* **17,** 32.
Sandholm, M. (1976). *Clin. Biochem.* **9,** 39.
Sandholm, J., and Kivisto, A. K. (1975). *J. Small Anim. Pract.* **16,** 201.
Sarda, L., and Desnuelle, P. (1958). *Biochim. Biophys. Acta* **30,** 513.
Sari, H., Entressangles, B., and Desnuelle, P. (1966). *Biochim. Biophys. Acta* **125,** 597.
Sasaki, H., Rubacalva, B., Baetes, D., Blazquez, E., Srikant, C. B., Orci, L., and Unger, R. H. (1975). *J. Clin. Invest.* **56,** 135.
Saunders, S. J., and Isselbacher, K. J. (1965). *Biochim. Biophys. Acta* **102,** 397.
Saunders, S. J., and Isselbacher, K. J. (1966). *Gastroenterology* **50,** 586.
Schachter, D., Finkelstein, J. D., and Kowarski, S. (1964). *J. Clin. Invest.* **43,** 787.
Schall, W. D. (1974). *In* "Current Veterinary Therapy" (R. W. Kirk, ed.), 5th ed., p. 742. Saunders, Philadelphia, Pennsylvania.
Schultz, S. G., and Curran, P. F. (1970). *Physiol. Rev.* **50,** 637.
Schultz, S. G., and Zalusky, R. (1964). *J. Gen. Physiol.* **47,** 1043.
Schultz, G., Schultz, K., and Hardman, J. G. (1975). *Metabolism* **24,** 429–437.
Schwartz, C. J., Kimberg, D. V., and Ware, P. (1975). *Gastroenterology,* **68,** 94–104.
Selman, I. E., McEwan, A. D., and Fisher, E. W. (1971). *Res. Vet. Sci.* **12,** 1.
Senior, J. R., and Isselbacher, K. J. (1962). *J. Biol. Chem.* **237,** 1454.
Serebro, H. A., Iber, F. L., Yardley, J. H., and Hendrix, T. R. (1969). *Gastroenterology* **56,** 506–511.
Shindel, N. M., Van Kruiningen, H. J., and Scott, F. W. (1978). *J. Am. Anim. Hosp. Assoc.* **14,** 738.
Skála, I., and Hrubá, F. (1964). *Am. J. Physiol.* **206,** 458.
Skou, J. C. (1965). *Physiol. Rev.* **45,** 596.
Smith, H. W. (1962). *J. Pathol. Bacteriol.* **84,** 147.
Smith, H. W., and Halls, S. (1967). *J. Pathol. Bacteriol.* **93,** 531.
Smith, H. W., O'Neil, J. A., and Simmons, E. J. (1967). *Vet. Rec.* **80,** 664.
Soll, A. H. (1977). *Gastroenterology* **73,** 899.
Soll, A. H., and Grossman, M. I. (1978). *Am. Rev. Med.* **29,** 495.
Stone, S. S., and Gitter, M. (1969). *Br. Vet. J.* **125,** 68.
Straus, E., Johnson, G. F., and Yalow, R. S. (1977). *Gastroenterology* **72,** 380.
Strauss, E. W. (1966). *J. Lipid Res.* **7,** 307.
Strombeck, D. R. (1978). *J. Am. Vet. Med. Assoc.* **173,** 1319.

Sutherland, E. W., and de Duve, C. (1948). *J. Biol. Chem.* **175,** 663.
Sutherland, E. W., and Rall, T. W. (1960). *Pharmacol. Rev.* **12,** 265.
Svendsen, P. (1969). *Nord. Veterinaermed.* **21,** Suppl. I.
Tapper, E. J., Powell, D. W., and Morris, S. M. (1978). *Am. J. Physiol.* **235,** E402-E409.
Tasker, J. B. (1967). *Cornell Vet.* **57,** 668.
Taylor, W. H. (1959a). *Biochem. J.* **71,** 73.
Taylor, W. H. (1959b). *Biochem. J.* **71,** 373.
Taylor, W. H. (1968). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiol. Soc., Sect. 6, Vol. V, p. 2567. Williams & Wilkins, Baltimore, Maryland.
Tennant, B., Harrold, D., and Reina-Guerra, M. (1968). *Cornell Vet.* **58,** 136.
Tennant, B., Harrold, D., Reina-Guerra, M., and Laben, R. C. (1969a). *Am. J. Vet. Res.* **30,** 345.
Tennant, B., Reina-Guerra, M., Harrold, D., and Goldman, M. (1969b). *J. Nutr.* **97,** 65.
Tennant, B., Harrold, D., and Reina-Guerra, M. (1972). *J. Am. Vet. Med. Assoc.* **161,** 993.
Tennant, B., Harrold, D., and Reina-Guerra, M. (1975). *Cornell Vet.* **65,** 457.
Tennant, B., Ward, D. E., Braun, R. K., Hunt, E. L., and Baldwin, B. H. (1978). *J. Am. Vet. Med. Assoc.* **173,** 654.
Tennant, B., Baldwin, B. H., Braun, R. K., Norcross, N. L., and Sandholm, M. (1979). *J. Am. Vet. Med. Assoc.* **174,** 848.
Theran, P., and Carpenter, J. (1968). *J. Am. Vet. Med. Assoc.* **152,** 65.
Thompson, G. A., and Meister, A. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1985.
Thompson, G. R., Ockner, R. K., and Isselbacher, K. J. (1969). *J. Clin. Invest.* **48,** 87.
Thompson, J. C. (1969). *Annu. Rev. Med.* **20,** 291.
Thompson, W. J., Chang, L. K., Rosenfeld, G. C., and Jacobson, E. D. (1977). *Gastroenterology* **72,** 251.
Thornton, J. R., Willoughby, R. A., and McSherry, B. J. (1972). *Can. J. Comp. Med.* **36,** 17.
Tracy, H. J., and Gregory, R. A. (1964). *Nature (London)* **204,** 935.
Turner, D. A. (1958). *Am. J. Dig. Dis.* **3,** 594.
Ugolev, A. M. (1965). *Physiol. Rev.* **45,** 555.
Ussing, H. H. (1947). *Nature (London)* **160,** 262.
Vahouny, G. V., and Treadwell, C. R. (1964). *Proc. Soc. Exp. Biol. Med.* **116,** 496.
Vahouny, G. V., Weersing, S., and Treadwell, C. R. (1965). *Biochim. Biophys. Acta* **98,** 607.
Vallee, B. L., Stein, E. A., Summerwell, W. N., and Fischer, E. H. (1959). *J. Biol. Chem.* **234,** 2901.
Valverde, I., Rigopoulou, D., Marco, J., Faloona, G. R., and Unger, R. H. (1970). *Diabetes* **19,** 614.
van de Kamer, J. H., ten Bokkel Huinink, H., and Weyers, H. A. (1949). *J. Biol. Chem.* **177,** 347.
van der Gagg, I., Happe, R. P., and Wolvehomp, W. T. C. (1976). *Vet. Pathol.* **13,** 172.
van der Gagg, I., Happe, R. P., and Lamers, C. B. H. W. (1978). *Vet. Pathol.* **15,** 573.
Van Kruiningen, H. J. (1968). *In* "Current Veterinary Therapy" (R. W. Kirk, ed.), 3rd ed., p. 521. Saunders, Philadelphia, Pennsylvania.
Van Kruiningen, H. J. (1971). *In* "Current Veterinary Therapy" (R. W. Kirk, ed.), 4th ed., p. 544. Saunders, Philadelphia, Pennsylvania.
Van Kruiningen, H. J. (1977). *Vet. Pathol.* **14,** 19.
Van Kruiningen, H. J., and Hayden, D. W. (1972). *Vet. Clin. North Am.* **2,** 29.
Van Kruiningen, H. J., Montali, R. J., Strandberg, J. D., and Kirk, R. W. (1965). *Pathol. Vet.* **2,** 521.
Vernon, D. F. (1962). *J. Am. Vet. Med. Assoc.* **140,** 1062.
Waldmann, T. A., Morell, A. G., Wochner, R. D., Strober, W., and Sternlieb, I. (1967). *J. Clin. Invest.* **46,** 10.
Waldmann, T. A., Wochner, R. D., and Strober, W. (1969). *Am. J. Med.* **46,** 275.
Walker, D. M. (1959). *J. Agric. Sci.* **52,** 357.
Walker, W. A., and Isselbacher, K. J. (1974). *Gastroenterology* **67,** 531.
Walsh, J. H., and Grossman, M. I. (1975). *N. Engl. J. Med.* **292,** 1324.
Wasserman, R. H., and Taylor, A. N. (1966). *Science* **152,** 791.
Wasserman, R. H., and Taylor, A. N. (1968). *J. Biol. Chem.* **243,** 3987.
Wasserman, R. H., Corradino, R. A., and Taylor, A. N. (1968). *J. Biol. Chem.* **243,** 3978.
Webb, D. W., Bartley, E. E., and Meyer, R. M. (1973). *J. Anim. Sci.* **35,** 1263.
Weiner, I. M., and Lack, L. (1962). *Am. J. Physiol.* **202,** 155.
Weiner, I. M., and Lack, L. (1968). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiol. Soc., Sect. 6, Vol. III, p. 1439. Williams & Wilkins, Baltimore, Maryland.
Wells, M. H., Shingleton, W. W., and Saunders, A. P. (1955). *Proc. Soc. Exp. Biol. Med.* **90,** 717.

Wetterfors, J. (1964). *Acta Med. Scand.* **176,** 787.
Wetterfors, J. (1965). *Acta Med. Scand.* **177,** 243.
Wetterfors, J., Liljedahl, S.-O., Plantin, L.-O., and Birke, G. (1965). *Acta Med. Scand.* **177,** 227.
Wheeler, H. O., and Mancusi-Ungaro, P. L. (1966). *Am. J. Physiol.* **210,** 1153.
Wiggans, D. S., and Johnston, J. M. (1959). *Biochim. Biophys. Acta* **32,** 69.
Wilkinson, G. T. (1969). *J. Small Anim. Pract.* **10,** 87.
Wilson, F., and Dietschy, J. (1971). *Gastroenterology* **61,** 912.
Wilson, T. H. (1962). "Intestinal Absorption." Saunders, Philadelphia, Pennsylvania.
Windmueller, H. G., and Levy, R. I. (1968). *J. Biol. Chem.* **243,** 4878.
Wiseman, G. (1968). *In* "Handbook of Physiology" (J. Field, ed.), Am. Physiol. Soc., Sect. 6, Vol. III, p. 1277. Williams & Wilkins, Baltimore, Maryland.
Wollenweber, J., Kottke, B. A., and Owen, C. A., Jr. (1965). *Clin. Res.* **13,** 410.
Wood, P. C. (1955). *J. Pathol. Bacteriol.* **70,** 179, 1955.
Word, J. D., Martin, L. C., Williams, D. L., Williams, E. I., Panciera, R. J., Nelson, T. E., and Tillman, A. D. (1969). *J. Anim. Sci.* **27,** 786–791.
Zachman, R. D., and Olson, J. A. (1963). *J. Biol. Chem.* **238,** 541.
Zilversmit, D. B. (1965). *J. Clin. Invest.* **44,** 1610.

9

Kidney Function

DELMAR R. FINCO

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

I. INTRODUCTION

The importance of the kidneys in life is dramatically apparent following bilateral nephrectomy or ureteral occlusion. Death occurs in most species in less than 1 week. Partial reduction of renal mass results in variable deviations from normal depending on the quantity of functional tissue remaining. Elevations of blood levels of nitrogenous wastes, such as urea and creatinine, may occur with severe impairment; this is called azotemia. A constellation of clinical signs, including lethargy, anorexia, vomiting, and altered urine output, occurs with severe impairment of renal function. These signs reflect consequences of severe renal insufficiency that occur regardless of cause of disease. The terms "renal failure," "uremia," and "uremic syndrome" are used to describe the clinical manifestations that may occur with renal dysfunctions.

The role of the kidneys in maintaining life is a composite of several functions. Water and electrolyte balance is controlled by the kidney by conservation in times of decreased intake or excess extrarenal loss, and by excretion in instances of excessive intake. Nutrients (glucose, amino acids, protein) are avidly conserved by the kidney so that they are nearly absent from normal urine. Hydrogen ions are excreted or conserved so that blood pH is kept within extremely narrow limits. End products of nitrogen metabolism, such as urea, creatinine, and allantoin, are removed from the body via the urine so that blood levels remain low and relatively constant. In addition to these regulatory duties, the kidney is an important endocrine organ. It produces renin and erythropoietin and performs a vital hydroxylation required for vitamin D activity. It responds to a variety of hormones, the most notable of which are antidiuretic hormone (ADH) and parathyroid hormone (PTH).

The evolution of this organ to fulfill the needs of mammals and birds is of interest. This story has been told eloquently by a pioneer in the study of the kidney, Homer Smith (Smith, 1959). Simple diffusion into primordial fluid with constant composition sufficed as a mechanism of excretion for single-celled and primitive organisms. As the complexity of organisms grew, extracellular fluid with a composition of the environment evolved. Simple excretory organs sufficed since extracellular water with wastes could be extruded and easily replaced by ingestion. As the seas increased in salinity, the threat of osmotic imbalance occurred. Some animals, such as the shark, solved this problem by retaining urea as a compound to balance the osmolality of the seas. Others depended on renal

excretion of excess salt via renal tubular systems. The animals that moved to fresh water from the ocean prior to its increase in salinity faced a different problem. The lack of osmotically active particles in the fresh water of their environment resulted in an influx of water into the tissues. Glomeruli evolved in order to facililate a high filtration system so that the water could be removed. The movement from fresh water to land was accompanied by the need to conserve water rather than excrete it. The high-filtration kidney was modified so that most of the water could be retained in the body while solute wastes were still excreted.

The evolution of the high-filtration, high-reabsorption kidney may help explain the apparent paradox of a continuous loss of water equivalent to 4–6% of cardiac output from the blood via glomerular filtration, only to have the tubules reclaim about 99.9% of this quantity. Certainly an engineer called upon to create an excretory organ for mammals would not choose this devious and energy-inefficient approach. Since our mission in clinical medicine is the treatment of existing organ disease rather than the design of natural new ones, we must deal with the model at hand. The evolution of the high-filtration, high-reabsorption kidney has clinical relevance. Although failure to excrete unwanted compounds (i.e., wastes) is a consistent problem with renal disease, the converse situation—unwanted excretion of desirable materials—is sometimes a problem associated with the high-filtration system. Thus, dehydration, hypokalemia, and hyponatremia are potential problems in some patients with generalized renal failure. More specific tubular defects (cystinuria, tubular acidosis, Fanconi syndrome) may result in loss of other compounds that are normally conserved.

II. NORMAL RENAL FUNCTIONS

A. The Nephron as a Functional Unit

Most renal functions are the sum of the activities of thousands of nephrons. Each nephron consists of several anatomical divisions. Bowman's capsule surrounds the glomerular capillaries and channels filtrate into the tubule. The convoluted portion of the proximal tubule assumes a meandering path within the cortex before a straight portion, the pars recta, descends toward the medulla. A marked thinning of the descending structure is identified as the beginning of the descending loop of Henle. This structure courses into the medulla, makes a hairpin turn, and ascends back into the cortex. At some point in its ascent the epithelium changes to a thicker character, which persists to the point of termination of the loop of Henle at the glomerulus from which the tubule originates. The afferent and efferent arterioles of the glomerulus are in confluence with the ascending loop of Henle. A thickening of tubular epithelial cells at this site is called the macula densa. Distal tubule is considered to be that segment of the nephron between the macula densa and the first union of two distal tubules into a collecting duct. Collecting ducts empty into the renal pelvis.

The number of nephrons varies among species, with larger species having more of them. For example, the cat has about 190,000 nephrons per kidney, the dogs has about 430,000, and the cow has about 4 million (Smith, 1951). In species such as the dog with large extremes in size among breeds, nephron size rather than nephron number varies with body size (Finco and Duncan, 1972). The overall function of the nephron may be summarized as perfusion of glomerular capillaries with blood, passage of some materials

(filtrate) through the capillary walls into Bowman's space, modification of this filtrate as it passes through tubular lumens, and eventual secretion as urine. Relevant aspects of these individual functions will be discussed subsequently.

B. Nephron Heterogeneity

Common clinical methods used to measure renal function do not distinguish among the contributions of various nephrons. However, both anatomical and physiological data indicate that all nephrons are not the same. Both inter- and intranephron heterogeneity have been described in a variety of species (Jamison, 1973; Lameire *et al.*, 1977; Valtin, 1977). Anatomically, differences exist between juxtamedullary (JM) and outer cortical (OC) nephrons. In most species JM nephrons have larger glomeruli and larger efferent arterioles than OC nephrons. The efferent arterioles have different perfusion patterns depending on their site of location in the kidney. Total proximal tubule length is greater for JM nephrons. Histochemical studies indicate that enzyme distribution is different between OC and JM nephrons. Renin is more prevalent in OC nephrons, while Na-K-ATPase content is higher in JM nephrons.

Functional studies on single nephrons and on nephron segments have revealed that single nephron glomerular filtration rate in OC nephrons is normally 80% that of JM nephrons. Such factors as perfusion pressure, changes in extracellular volume, and changes in sodium balance may alter the filtration rate of OC and JM glomeruli differently under certain conditions. Considerable attention has been given to shunting of blood from the OC to the JM areas of the kidney. Such shunting does not imply that blood is diverted through the kidney by nonglomerular channels. Although Trueta *et al.* (1948) popularized the nonglomerular shunting notion, it is generally acknowledged that aglomerular pathways would facilitate less than 5% of total renal blood flow. Shunting or distribution of blood flow between OC and JM glomeruli has been studied extensively. Numerous drugs have been tested and body states induced to establish whether shunting of blood to JM nephrons occurs. Under many conditions, normal blood flow is redistributed to the JM nephrons, and in most instances a decrease in renal resistance occurs in association with this redistribution (Lameire *et al.*, 1977).

It has been hypothesized that changes in renal function observed in abnormal states, such as sodium retention with heart failure, might be due to nephron heterogeneity and redistribution of blood from one functionally distinct group of nephrons to another group. However, because of conflicting or inadequate experimental data, shunting of blood has not been established as a cause of fluid imbalance in any condition of clinical interest (Lameire *et al.*, 1977).

Newer findings indicate that some of the conventional anatomical divisions of the nephron do not delineate areas of function adequately; greater heterogeneity exists within each nephron than formerly appreciated. For example, both anatomists and physiologists have defined the distal tubule as that part extending from the macula densa to the first junction with another nephron. Several lines of information point to functional differences within this segment. Three functionally distinct sections exist in OC nephrons, with the early segment similar to the ascending loops of Henle and the last segment similar to collecting ducts. Two functionally distinct portions exist in JM nephrons. They do not possess a segment with function similar to that of the collecting ducts as do OC nephrons.

C. Renal Perfusion

Although there are some variations, vascular patterns of renal perfusion are similar in the mammalian species that have been studied (Stein, 1976). The kidneys receive roughly 15–25% of cardiac output (Kaikara *et al.*, 1969). This rate of perfusion is highly disproportionate to the size of the kidneys when compared to other organs and is related to the evolution of the high-filtration system previously discussed. The blood flows through a series of arteries that divide and decrease progressively in size. The renal artery divides into several interlobar arteries of about equal size. Due to a lack of development of collateral circulation, each interlobar artery totally perfuses a distinct part of each kidney. Occlusion of these vessels or its branches results in ischemia of the supplied portion.

The arterial subdivisions eventually lead to the afferent glomerular arterioles which supply glomerular capillaries. Efferent arterioles drain the glomeruli and divide into another capillary network. The site perfused by this network is dependent on the anatomical location of the glomeruli from which the efferent arteriole originates (Beeuwkes, 1971). In the superficial cortex, efferent arterioles supply only the tubule of the parent glomerulus. Efferent arterioles in the midcortex supply either superficial or deep tubules of different glomerular origin. Juxtamedullary efferent arterioles provide capillary networks for the juxtamedullary and the medullary zones.

Clearance procedures discussed in Section VII and electromagnetic flowmeters provide reliable methods of measuring total renal blood flow (RBF) but give no information concerning intrarenal distribution of blood. Other methods (inert gas washout, microshpere injection) have been used for intrarenal blood flow comparisons, but none have been considered foolproof (Stein, 1976). The general consensus is that medullary blood flow rate is normally very small compared to that of the cortex.

Blood flow to the kidney as well as glomerular filtration rate remain fairly constant during variation in systemic arterial blood pressure from 80 to 180 mm Hg (Pitts, 1974). This phenomenon is known as autoregulation. It occurs in the isolated perfused kidney and thus is not dependent on renal innervation or extrarenal humoral agents. Several theories exist to explain autoregulation (Stein, 1976), but none have total acceptance. While renal perfusion is maintained by autoregulation under physiological conditions, it may not be maintained during diseases despite maintenance of normal systemic blood pressure. Renal disease or the application of neural or certain humoral stimuli to the kidney can decrease renal perfusion and filtration. Such phenomenon are commonplace in clinical medicine and will be discussed later.

As a basis for the subsequent discussion, the role of nerve stimulation in renal perfusion should be recognized. The kidney receives both sympathetic and parasympathetic fibers, but sympathetic fibers predominate (Davis and Freeman, 1976). Renal denervation causes little if any increase in renal blood flow (Berne, 1952), nor does α- or β-adrenergic blockade (Stein *et al.*, 1973). However, renal nerve stimulation of considerable magnitude and frequency will reduce total renal blood flow by 50% (Stein *et al.*, 1973). Neural stimulation that does not alter renal blood flow in the normal state may do so during disease. For example, total renal blood flow was normal during exercise of both normal dogs and renal denervated dogs with right atrioventricular valve insufficiency and pulmonic stenosis. In contrast, dogs with the same heart defects with renal nerves intact had renal blood flow reduced to 30% of normal with exercise (Millard *et al.*, 1972).

Severe pain, cold, severe exercise, and fright decrease renal blood flow in man and animals (Pitts, 1974), although species variation in susceptibility apparently has not been examined in any detail.

D. Glomerular Filtration

1. Nature of Filtrate

Material that passes from the capillary lumens into Bowman's space has been collected by micropuncture techniques and compared to the composition of blood. As predicted prior to the advent of such techniques, the composition of glomerular filtrate is nearly identical to that of blood from which the cells and protein have been removed. A small difference in ion concentration exists as a consequence of Gibbs–Donnan equilibrium secondary to protein restriction to the capillary lumen (Pitts, 1974). Another exception is that glomerular filtrate is not totally devoid of protein. Filtrate harvested from proximal tubules of rats by micropuncture techniques had albumin concentrations of 0.1–0.1 mg/dl (Oken and Flamenbaum, 1971). Lower molecular weight proteins are also present (Maack, 1975). The relevance of this protein is discussed in Section VI.

2. Anatomical Considerations for Filtration

In order for material to pass from the glomerular capillary lumen into Bowman's space, three potential anatomic barriers exist. These are the capillary endothelium, the basement membrane, and the capillary epithelium. The glomerular capillaries differ from other capillaries of the body in that the endothelial fenestrae are larger and occupy a greater percentage of total endothelial surface area, the basement membranes are thicker, adventitial connective tissue is absent, and the epithelial cells possess an elaborate arrangement of interdigitating foot processes (Farquhar, 1975). An intercapillary cell, the mesangial cell, appears to play no direct role in the filtration process but may act as a scavenger cell for particles lodged in the capillary wall. It is generally acknowledged that the endothelium can function to retain only the cellular elements of the blood since the fenestrae are 50–100 nm in diameter. Although the gaps between foot processes are very wide (25 nm), epithelial slit membranes which cover the slits could act as filters. The uninterrupted nature of the basement membrane suggests that it may also be a barrier. Controversy has existed over the relative contributions of the epithelium and basement membrane as the structures relevant to filter selectivity (Farquhar, 1975).

3. Physiological Factors in Filtration

Classic physiological concepts identify hydrostatic pressure of cardiac origin as the impetus for glomerular filtration. Additional factors include the colloidal osmotic force of plasma proteins in the blood and in the filtrate and hydrostatic pressure within Bowman's space. The permeability of the glomerulus is another factor that influences filtration (Pitts, 1974; Tucker and Blantz, 1977) and may be of great relevance in glomerular diseases. Estimates of the magnitude of some of these factors are given in Fig. 1. These values indicate that net force for filtration diminishes as blood flows through the glomerular capillaries because of the increase in colloid osmotic pressure associated with concentration of protein as fluid leaves the capillary lumens. More recent studies in rats utilizing more sophisticated methods of measurement have revealed that colloid osmotic pressure

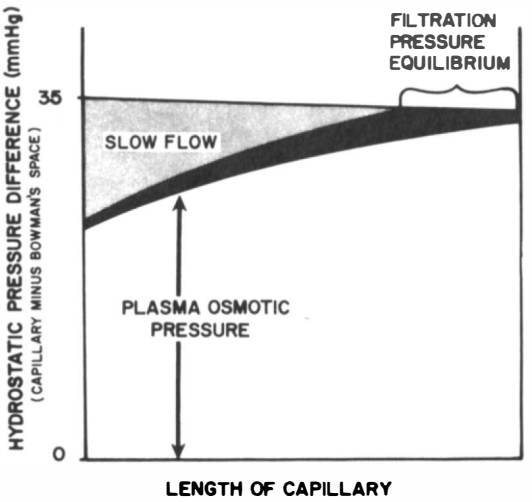


Fig. 1. Pressure factors relevant to glomerular filtration. Hydrostatic pressure difference represents the force responsible for filtration. Plasma colloid osmotic pressure impedes filtration. At slow plasma flow rates filtration fraction increases, and thus colloid osmotic pressure may rise sufficiently in some species so that filtration ceases before the end of the glomerular capillary bed (filtration pressure equilibrium).

balances hydrostatic pressure before the end of the glomerular capillary bed. This condition, termed filtration pressure equilibrium, indicates that the entire capillary bed is not being used for filtration. With these conditions an increase in plasma flow rate could be expected to increase filtration rate as follows. An increase in flow rate would decrease the proportion of plasma that became filtrate. Intraluminal colloid osmotic pressure would not rise as rapidly as at a slower flow rate, so a positive filtration pressure would exist over a greater span of the capillary loop. In the presence of filtration pressure equilibrium, plasma flow rate is a factor in determining glomerular filtration rate (GFR) (Tucker and Blantz, 1977). However, proof of the existence of filtration pressure equilibrium in domestic animals is not available at present, and some data suggest that it does not exist in the dog (Youngberg *et al.*, 1977).

4. Functional Nature of the Glomerular Filter

Certain characteristics of the glomerular filter are well known. Such factors as size and shape of molecules are significant in determining whether they pass through the filter (Pitts, 1974). More recently, the importance of electrical charge has been proved (Rennke and Venkatachalam, 1977; Brenner *et al.*, 1978). Negatively charged sialoproteins are components of all three layers of the capillary wall. These negative charges facilitate passage of cationic macromolecules and impede passage of anionic macromolecules through the filter. The relevance of this finding is that albumin has a size and shape which nearly allow it to pass through the glomerular filter under normal conditions. However, since both albumin and the sialoproteins of the glomerular structures are negatively charged, an electrical impediment to filtration normally exists. When the charge sialoprotein is altered, as may occur with some diseases, the impeding electrical force is removed and albuminuria may occur (Brenner *et al.*, 1978).

E. Tubular Modification of Filtrate

One method of renal excretion entails passage of a material through the glomerular filter without subsequent tubular reabsorption. The other mechanism is tubular excretion. Body homeostasis depends on tubular excretions, particularly for control of acid–base balance, potassium homeostasis, and secretion of some organic ions. Some of the secretory mechanisms occur by active processes, while others are passive because of electrical or concentration gradients.

Death would occur in less than 1 hour if all glomerular filtrate were lost from the body. The large volume of water is nearly all reabsorbed as it passes through the tubule. Many other components of filtrate are also nearly completely reabsorbed. As with excretion, the reabsorptive mechanisms include both active and passive transport.

The concept of tubular maxima (T_m) for reabsorption and excretion should be kept in mind. This refers to the point at which a particular active reabsorptive or secretory function is operating at maximal capacity. Further increases in substrate concentration at the site of tubular action will not increase the function. Many compounds handled by active transport by the renal tubules have T_m values. These values are expressed as milligrams transported per 100 ml of glomerular filtrate or milligrams transported per unit of time.

F. Conservation of Nutrients

The normal mammalian renal tubule has evolved as a highly efficient structure for reabsorption of nutrients from glomerular filtrate back into the body. At normal blood levels of glucose, reabsorption from filtrate occurs almost completely within the first 20% of the length of the proximal tubule (Von Baeyer *et al.*, 1973). Integral proteins of the brush border membranes actively transport glucose into the cells by mechanisms obeying Michaelis–Menten kinetics. The mechanism is stereospecific (D- versus L-glucose), sodium dependent, and inhibited by phlorizin (Silverman and Huang, 1976). It is shared by D-fructose and D-galactose, although the affinity of these sugars for the transport mechanism is much less than that of glucose. The glucose is transported from the cell to the renal interstitium by a different transport process that is phlorizin insensitive, indiscriminating of all D-hexoses, and independent of sodium transport. Although nearly all glucose is normally reabsorbed in the proximal convoluted tubule, distal sites also have this capability (Wen, 1976). Despite this system, urine from the normal animal still contains a small quantity of glucose that is undetectible by the urinalysis methods of assay.

The kidney has definite limits for glucose reabsorption. Blood and thus filtrate concentration as well as flow rate of fluid through the nephron are factors that determine whether glucosuria occurs (Pitts, 1974). The interpretation of glucosuria is discussed in Section VI on urinalysis.

Glomerular filtrate contains about 2.5–3.5 mmol of amino acids per liter (Pitts, 1974). Active reabsorption of these amino acids is conducted predominantly by proximal tubule cells. Much remains to be learned about amino acid transport. Studies indicate that renal tubular cells actively reabsorb amino acids by carrier mechanisms which exhibit stereospecificity (L- rather than D-amino acid preference), saturability, common transport mechanisms for some groups of amino acids with competitive inhibition within the

groups, and sodium dependence (Segal, 1976). Specifically, mutual competitive inhibition has been shown for the dibasic amino acids and for proline, hydroxyproline, and glycine. Glycine may be transported by three systems: one for glycine alone, one shared by alanine, and one with proline and hydroxyproline. Biochemical events in amino acid reabsorption are presently being uncovered; evidence exists for the participation of glutathione and γ-glutamyltransferase (GGT) (Meister, 1975).

The relevance of amino acid reabsorption is related not only to conservation of nutrients in the normal animal but also to diseases characterized by aminoaciduria. Unfortunately, simple tests to detect aminoaciduria do not exist as for glucosuria and so clinical detection is difficult. Aminoacidurias are often classified as generalized or specific, depending on whether one or all the transport systems are involved.

In disorders such as some hepatic diseases in which hyperaminoacidemia occurs, renal transport mechanisms may be saturated, and an overflow, generalized aminoaciduria occurs. Generalized aminoaciduria also exists as a component of the Fanconi syndrome. In this syndrome proximal tubular disease exists, which may result in glucosuria, phosphaturia, aminoaciduria, and bicarbonate wasting (Woolf, 1966). Some acquired causes of this syndrome that have been identified include heavy-metal toxicoses, ingestion of degraded tetracycline, and maleic acid administration to the dog (Silverman 1975; Schneider and Seegmiller, 1972). Aminoaciduria has been described as occurring occasionally in familial renal disease of Norwegian elkhound dogs and in basenji dogs (Finco, 1976; Bovee *et al.*, 1978a,b).

Defects in one amino acid transport mechanism lead to a specific aminoaciduria. Cystinuria has been described in at least 16 breeds of dogs and mongrel dogs and acts as a predisposing factor in the development of cystine calculi (Finco, 1971a). It is of interest that normal cats have 100–180 mg/dl of the amino acid felinine in their urine; the role of this compound in feline metabolism and its relevance in urine are apparently unknown (Westall, 1953; Tallen *et al.*, 1954; Trippett, 1957; Avizonis and Wriston, 1959; Greaves and Scott, 1960).

Metabolic intermediates may be present in the blood in low concentrations and could potentially be lost in the urine because of their small molecular weight. Tubular reabsorption of these compounds occurs, but some may appear in the urine (Pitts, 1974). Lactate has a T_m of 100 mg/dl for the dog, and the reabsorption mechanism is the same for both the D and L isomers. This is relevant since the lactate in parenteral fluids is a racemic mixture of D and L forms.

Very little information is available concerning the role of the normal kidney in lipid metabolism. Blood forms of lipid apparently do not pass the glomerular filter. The urine of many species normally is devoid of fats, as judged by urinalysis. Fat is a normal constituent of urine of dogs and cats.

G. Electrolyte Homeostasis

The narrow limits within which blood components are maintained is often the consequence of renal regulation. This is true for most electrolytes. An exception is the less significant role played by the kidney in calcium homeostasis in most domestic animals.

The prominent role of the kidney in electrolyte homeostasis is a reflection of the lack of discrimination by the intestinal tract. The feces of carnivores normally has a low electro-

lyte content regardless of quantities ingested. Oral intake of electrolytes usually exceeds body needs, and the kidney responds to sensing mechanisms by excreting the proper amount of electrolyte so that body balance is maintained.

1. Sodium Homeostasis

Of the large quantity of filtered sodium, most is automatically reabsorbed without regard for body homeostasis. According to available data, sodium in filtrate moves passively into proximal tubular cells by concentration and electrical gradients. It is actively extruded from the cells into interspaces between tubule cells and at the cell–interstitium border. It is then transferred from the interspaces into peritubular capillaries by hydrostatic and osmotic forces (Windhager and Giebisch, 1976). Fluid leaving the proximal tubule is isosmotic because proportionate quantities of particles and water have been reabsorbed. In the ascending loop of Henle, chloride reabsorption is active and sodium reabsorption is passive (Burg and Stoner, 1974). This is the site of action of furosemide and ethacrynic acid, and it thus appears that these diuretics act by inhibition of chloride reabsorption with secondary effects on sodium. The fine tuning of body sodium regulation is handled by the more distal portions of the tubule. In time of sodium deficiency, urinary excretion of sodium may be negligible. In time of excess intake, large quantities are secreted. Several factors have been studied for their effects on sodium homeostasis. Increasing the filtered load of sodium (GFR times serum sodium concentration) causes increased sodium excretion, particularly if due to hypernatremia (Schrier, 1976). Hemodilution (i.e., administration of isotonic saline) may cause increased sodium excretion through a decrease in peritubular osmotic pressure, which decreases renal uptake of reabsorbate from the interstitium (Schrier, 1976). Hemodynamic alterations may also influence sodium excretion, with increases in blood pressure and decreases in renal vascular resistance causing natriuresis. Shunting of blood from the OC to the JM nephrons has not been proved to be associated with changes in sodium excretion, however (Schrier, 1976). The effect of renal sympathetic nerve stimulation on sodium excretion is controversial (Schrier, 1976). Aldosterone secretion by the adrenal cortex is generally considered to be an important regulatory component. Its secretion may be initiated by several stimuli, including renin production by the kidney. Aldosterone increases tubular reabsorption throughout the nephron, but particularly in the latter portions. However, effects of aldosterone cannot explain many humoral renal regulatory responses for sodium homeostasis. The presence of another hormonal factor has been hypothesized, and considerable data to support the presence of this "natriuretic hormone" have been provided (Bricker *et al.*, 1975).

2. Potassium Homeostasis

Potassium is filtered through glomeruli and actively reabsorbed by the proximal tubules. Up to 70% of the potassium in filtrate is reabsorbed in the first two-thirds of the proximal tubule. It is likely that further reabsorption occurs in the loop of Henle secondary to active chloride reabsorption (Gabow, 1976). Potassium is excreted from tubular cells into the lumen in the distal tubule. This excretion occurs passively via a favorable electrical gradient, but the amount secreted is the major factor in regulation of extracellular potassium content. Several factors have been shown to influence potassium excretion. These include aldosterone, sodium delivery to the distal tubule, hydrogen ion secretion, the presence of unreabsorbable anion in the distal tubule, and urine flow rate (Gabow,

1976). Aldosterone affects potassium secretion independently of sodium reabsorption. The role of sodium delivery to the distal tubule in potassium homeostasis is not known, but increased delivery enhances potassium secretion. For both sodium and hydrogen. stoichiometric exchanges for potassium at tubular cell membranes are now known not to occur, contrary to statements in older texts. However, a "loose coupling" of the ions occurs inasmuch as states of metabolic alkalosis and hypokalemia coexist, with a cause–effect relationship between the two. The presence of unreabsorbable anions, such as sulfate or phosphate, enhances potassium secretion in the distal tubule, apparently by maintaining an electrical gradient for movement of potassium into the tubule lumen. Increased tubule fluid flow rate apparently increases potassium excretion by enhancing the concentration gradient for diffusion.

The normal kidney is able to undergo adaptation to an increased potassium load. The increased potassium excretion can be demonstrated in isolated perfused kidneys and thus is an intrinsic character of these organs (Silva *et al.*, 1977). The Na–K-ATPase in the basolateral membranes of renal tubular cells has a three- to fourfold increase in activity in association with the potassium adaptive process in normal animals. The enzyme activity is also increased in remnant kidneys and most likely plays a role in adaptation to potassium loads found during renal failure (Schon *et al.*, 1974). This enzyme increase is independent of aldosterone levels.

Cattle normally excrete a much larger percentage of the filtered load of potassium than do carnivores because of high dietary intake (Pickering, 1965). It is not known whether adaptive phenomena described for carnivores exist in bovine kidneys, or whether different mechanisms operate.

3. Phosphorus

The kidney is the major organ for control of serum phosphorus levels. Phosphorus is reabsorbed primarily in the proximal convoluted tubule by a sodium-dependent mechanism (Knox, 1977). Parathormone inhibits phosphorus reabsorption via an adenyl cylase–cyclic AMP system. The details of the operation of this system for inhibiting phosphorus absorption are unknown. Some data indicate that the site of action of PTH is in the straight portion of the proximal tubule (Knox, 1977). Phosphorus plays a role in the development of renal secondary hyperparathyroidism, which is discussed in Section V.

4. Chloride

Except for the ascending loop of Henle, chloride appears to be handled by passive mechanisms by the kidney in response to the need to satisfy the laws of electroneutrality.

5. Calcium

Serum calcium levels are not controlled by the kidney. Ionized and chelated calcium appear in glomerular filtrate in the same concentration as plasma, but protein-bound calcium is retained in the blood. The renal handling of calcium roughly parallels that of sodium until some divergence is observed in the distal tubules (Goldberg *et al.*, 1976). Hypercalcemia due to several causes may persist in animals with relatively normal renal function. This emphasizes the limited role played by the kidney in calcium homeostasis in most species. The horse may be an exception to this generality since large quantities of calcium are normally excreted by horse kidneys.

H. Acid–Base Balance

The lungs and the kidneys are the primary organs concerned with acid–base regulation. Although the lungs may cause sudden and dramatic changes in blood pH by altering pCO_2, the maneuver is not associated with loss of hydrogen ions (H^+) from the body. When pCO_2 is reestablished at normal levels, the H^+ reappears due to dissociation of H_2CO_3 to H^+ and HCO_3^- according to the law of mass action. The kidneys accomplish regulation of acid–base balance by excretion of H^+.

Maintenance of acid–base homeostasis requires the conservation of bicarbonate to act as a proton (H^+) acceptor. Bicarbonate is freely filtered through the glomerular membranes and may be lost from the body if tubular reabsorption is faulty. Most bicarbonate is reabsorbed by the proximal tubules. Prerequisites for the reabsorption are secretion of H^+ by the proximal tubule cells and the presence of carbonic anhydrase in the brush border of these cells. These are required since it is believed that bicarbonate cannot pass through the cell membranes; it must be converted to CO_2. Filtered bicarbonate is converted to CO_2 by the reaction $HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow H_2O + CO_2$. After CO_2 is formed, it diffuses into tubular cells and is reconverted to bicarbonate. Since H^+ ions are recycled in this process, no net secretion occurs in association with bicarbonate reabsorption. However, secreted H^+ may combined with other compounds that act as components of buffer pairs and be excreted in that way. Fluid leaving the proximal tubule has a pH only slightly lower than that of blood because the H^+-secreting mechanism in this section of the nephron cannot attain a high blood–tubular lumen gradient. The bicarbonate reabsorptive mechanism normally receives a "signal" to reabsorb sufficient amounts to keep blood levels normal. A T_m for bicarbonate has been found in intact animals. However, this T_m is apparently a regulatory maximum rather than one representing the maximal capability of the tubules for HCO_3^- reabsorption. The amount of reabsorption can be altered by several factors, including extracellular fluid volume and blood aldosterone, PTH, and calcium levels. Single-nephron perfusion studies do not demonstrate the T_m phenomenon that exists in the intact animal (Rector, 1976). Bicarbonate reabsorption also occurs at levels distal to the proximal tubule. In instances of bicarbonate deficit, the normal kidney reabsorbs essentially all bicarbonate filtered.

Hydrogen ion secretion can occur in the distal tubules and collecting ducts against an approximately 1000 : 1 concentration gradient. Various buffer bases, including NH_3, may accept protons, which minimizes the pH gradient and facilitates continued H^+ excretion. The NH_3 mechanism for facilitating H^+ secretion is particularly important in times of acidosis since the quantity of NH_3 produced by the kidney can be increased five- to tenfold under these conditions (Pitts, 1974). This mechanism enhances H^+ secretion because of the trapping of protons in the tubule lumen by NH_3 to form NH_4^+ according to the reaction $NH_3 + H^+ \rightarrow NH_4^+$. The p$K$ of this reaction is about 9.15 so at pH 7.15 there is a ratio of 100 NH_4^+ to 1 NH_3. However, at lower urine pH levels found in the distal tubule during acidosis (below 5), there are greater than 10,000 NH_4^+ to 1 NH_3. This situation favors diffusion of NH_3 from the cell to the tubular lumen with subsequent conversions to NH_4^+ and secretion as an ammonium salt (Klahr and Schoolwerth, 1977).

Ammonia is produced by cells throughout the entire length of the nephron. Because of the relevance of luminal pH in its conversion to NH_4^+, the distal tubules and collecting ducts are the most important sites of secretion. Some of the NH_3 eventually excreted as

NH_4^+ by the kidney originates from the blood, but most is produced in renal tubular cells from glutamine.

The functions of the kidney in acid–base balance may be simplistically defined as both conservation of bicarbonate and excretion of H^+ in times of acidosis, which is the opposite of the excretion of bicarbonate and retention of H^+ in times of alkalosis. However, the interrelationships between acid–base functions and homeostatic functions for other electrolytes do not allow the kidneys to fulfill all requirements under all conditions. It has been found that various degrees of acidosis are induced when identical milliequivalents of acid are given, depending on whether the acids are HCl, HNO_3, or H_2SO_4. The differences appear to depend on whether the anionic component of the acid is reabsorbed by the renal tubule and on the size of the load of sodium that is presented to the distal tubule (Schwartz and Cohen, 1978). The observation that other components of filtrate may influence renal excretion of H^+ helps explain paradoxes such as that observed clinically in vomiting dogs with alkalosis and acidic urine.

I. Maintenance of Water Homeostasis

All mammals and birds have various degrees of ability to concentrate urine above the osmolality of plasma. This provides a mechanism for conserving water when intake is sporadic. These animals also have the ability to excrete urine with an osmolality less than that of plasma so that excess body water can be excreted without solute depletion. The term "free water clearance" is used to describe this phenomenon, and quantitative values for it can be calculated.

The mechanisms by which urine concentration and dilution occur have been studied intensively in the last two decades. Many facets of the process are now understood, but many questions remain. Active transport of water has not been found in biological systems; its movement is secondary to hydrostatic and osmotic forces. In the proximal tubules, 60–80% of the glomerular filtrate is reabsorbed secondary to solute reabsorption and independent of body needs. While the proximal tubules play no primary role in water homeostasis, they may affect urine volume and concentration if reabsorptive defects prevent the usual quantities of water and solute from being absorbed. In these circumstances, the more distal parts of the nephron may be flooded beyond their capacity to reabsorb, and polyuria may ensue. Part of the action of osmotic diuretics (Gennari and Kassirer, 1974) and polyuria of the Fanconi syndrome have this pathogenesis. Fluid leaving the proximal tubule in the normal animal is isosmotic compared to plasma. Water reabsorption occurs from the descending loop of Henle but not from the ascending limb. These differences are relevant to the operation of the countercurrent system of urine concentration. The osmolality of urine increases in association with water removal in the descending loop of Henle but decreases in association with solute removal in the ascending limb. Fluid entering the distal tubule is hypo-osmotic compared to plasma. Within the distal tubule, reabsorption of water relative to solute varies. Urine remains hypo-osmotic in the dog, chinchilla, some strains of rats, and monkeys. It becomes iso-osmolar in the cat and in some strains of rats (Jamison, 1976; Suzuki, 1971). By the time the fluid reaches the collecting duct system, about 91% of the amount originally filtered has been reabsorbed, and essentially all of this has occurred without regard for body homeostasis. Modulation of the remaining 9% is normally performed by the collecting ducts under the

influence of ADH. However, this modulation results in production of a concentrated urine only as an event secondary to the presence of a hyperosmolar renal medullary interstitium. The maintenance of the medullary hyperosmolality depends upon the functioning of the renal countercurrent multiplier system. Details of this system are beyond the scope of this chapter. In fact, some of the concepts of its operation are the subject of considerable debate (Andreoli *et al.*, 1978) and are yet to be resolved. However, a working knowledge of its operation is important in understanding the possible mechanisms of polyuria in disease. Since water is not moved by active transport, conservation of filtrate depends on the establishment of an osmotic gradient for its reabsorption. The countercurrent system depends on both selective permeability of various tubule segments and active transport of solute to achieve the hyperosmolality of the renal medulla. Transport of NaCl by the ascending loop of Henle from the lumen into the interstitium provides at least part of the osmotic gradient. The loops of Henle and the collecting ducts provide a counterflow system which multiplies differences in the osmotic gradient at one point over the entire length of this system. The descending loop of Henle is permeable to water. Water moves from its lumen into the interstitium, and the intraluminal solute becomes concentrated. The lumen contains more sodium but less urea than the interstitium at the turn of the loop. The ascending loop of Henle is impermeable to water, but NaCl moves from the lumen to the interstitium. In the inner medulla it may leave passively due to a higher concentration in the lumen than in the interstitium. In the outer medulla it is actively pumped by cells of the thick ascending loop of Henle. Urea may diffuse into the ascending limb from the interstitium according to concentration differences. As previously mentioned, fluid changes in the distal tubule depend on the species involved. In the outer medullary collecting ducts, water may move from the lumen into the interstitium in the presence of ADH, but luminal urea is restrained. This water reabsorption concentrates the urea to very high levels. The inner medullary collecting ducts are permeable to urea, and thus both water and urea move into the interstitium. This explains how a high concentration of urea accumulates in the medulla, and how water conservation occurs (Fig. 2). Thus, active transport of NaCl, passive movement of urea secondary to NaCl transport, and selective permeability provide the medullary hyperosmolality. The role of urea in the urine-concentrating mechanism can be related to observations made by Jolliffe and Smith (1931) that the urine-concentrating ability of dogs fed a high-protein diet was superior to that of dogs on a low-protein diet.

The functions of the medullary vessels are important in the operation of this system. The vasa recta perfuse the medulla with a slow rate of blood flow. This functions to provide nutrients to the area and to provide a portal of exit for water and solute reabsorbed from the medullary tubular structures. The slow rate of blood flow through these vessels is important since it allows equilibrium between the tubules and interstitium without undue removal of solute from the medullary interstitium. As shown in Fig. 2, osmolality of vasa recta blood increases as they descend into the medulla but decreases as they ascend. Rapid blood flow through the medulla would not allow for the equilibration, and "washout" of the medulla would occur (Jamison, 1976).

Some studies indicate that urine in the renal pelvis influences urine-concentrating ability. Opening the pelvis during micropuncture studies results in less concentrated urine in rats (Chuang *et al.*, 1978), and such species as the desert rodent *Psammomys obesus* have an invaginating renal pelvis which appears to facilitate renal concentration (Kaibling *et al.*, 1975).

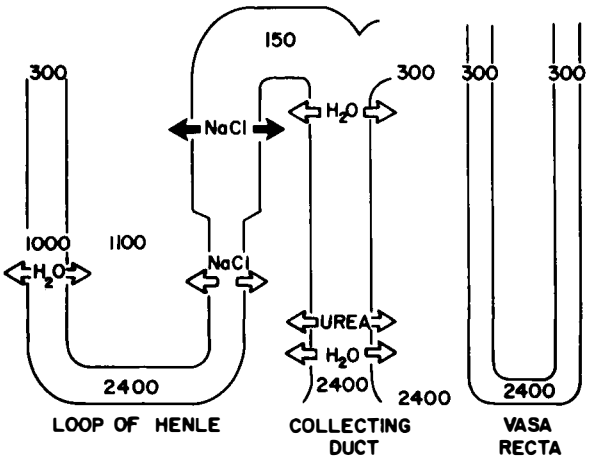


Fig. 2. Schematic representation of a popular theory of the urine-concentrating mechanism in the renal medulla. Numbers represent milliosmoles per liter. Solute in the descending loop of Henle is concentrated by passive movement of water into the hyperosmolar interstitium. The ascending limb is impermeable to water, but NaCl moves into the interstitium passively (open arrows) in the inner medulla and by active transport (opaque arrow) in the outer medulla, creating dilute urine in the distal tubule (150 mosmole/liter). Water may be reabsorbed in both the outer and inner medulla if collecting duct cells are rendered water permeable by ADH. Urea passively reabsorbed in the inner medulla in the presence of ADH contributes to interstitial hyperosmolarity. The vasa recta provide nutrition to the medulla and remove quantities of solute and solvent beyond that necessary to maintain normal interstitial volume and osmolality.

In summary, the countercurrent system operates maximally to conserve water and concentrate urine when (1) proximal tubule reabsorption does not allow overflow of the distal system, (2) NaCl is actively pumped from the ascending loop of Henle, (3) urea is available for contributing to medullary hyperosmolality, (4) ADH is present and operative on a cellular level, and (5) medullary blood flow rate is not excessive.

More recent advances in knowledge concerning the tubular action of ADH are germane to the discussion of renal concentrating ability. The ADH acts via attachment to receptor sites located at the basolateral surface of collecting duct cells. The attachment activates adenyl cyclase, which converts ATP to cyclic AMP. The effect of ADH on tubular cells to facilitate passage of water from the tubular lumen to the interstitium is dependent on this generation of cyclic AMP. Many factors, however, control the intracellular levels or action of cyclic AMP. These include prostaglandin E, calcium, magnesium, adrenal steroids, and adrenergic agents (Hays, 1976). These findings indicate that several intermediate steps exist between the appearance of ADH in the blood and the expression of its biological activity by production of a concentrated urine. These intermediate processes may be relevant in explaining the polyuria of many spontaneous diseases.

J. Renal Maturation and Senescence

The renal function of the neonatal animal has relevance in the practice of clinical medicine in all species. Unfortunately, our knowledge about this subject is fragmentary. Canine fetuses within 10 days of whelping were found to have urine specific gravity readings of 1.008–1.025 (Rahill and Subramanian, 1973). There is morphological evi-

dence of immaturity in the kidney of the newborn pup. Immature glomeruli are apparent in the subcapsular area until about 3 weeks of age. The glomerular filtration rate of individual superficial nephrons increased sevenfold during studies on pups between 2 and 77 days of age (Horster and Valtin, 1971). This increase was due not only to increased glomerular volume but also to increased glomerular permeability. Most growth of the kidney that occurs with maturation is due to lengthening of tubules. During maturation, tubular functions appear to lag behind glomerular function. Random urine samples at 2 days of age had an osmolality about twice that of plasma, and the ratio increased to seven times that of plasma at 77 days of age (Horster and Valtin, 1971). These studies suggest that the newborn pup has concentrating ability that improves with age, but, since the pups were not challenged to concentrate, maximal concentrating ability was not established. Studies on 2-day-old pups demonstrated that they could excrete an oral water load, but their ability to do so was inferior to that of adults (McCance and Widdowson, 1955). The pups had a lower urine flow rate per kilogram and a higher urine osmolality and excreted less of a 5% water load in 4 hours than adult dogs. Because of their indiscriminate water drinking, young calves may suffer from water intoxication as a consequence of overdrinking (Kirkbride and Frey, 1967). The ability of young calves to concentrate urine seems limited, since, with diarrhea, urine osmolality values were only 406 ± 27 (Thornton and English, 1976).

Few data are available for domestic animals concerning the effects of aging on renal function. In human beings, slight decreases in GFR and concentrating ability were observed with age (Dontas *et al.*, 1972; Slack and Wilson, 1976), but the effects were minor when compared to the total quantity of renal reserve. Morphological changes observed in association with aging in human beings include shrinkage of nephron size after the fourth decade, as determined by nephron dissection, and a gradual increase in thickness of both Bowman's capsules and the tubules (Darmady *et al.*, 1973). Differences in renal clearance were found among dogs of different ages by Asheim *et al.* (1961), but the range of ages studied was not reported. Cats from 2 months to 7.5 years of age had no difference in adenyl cyclase activity in their kidney tissues, but increases in lipid concentration of proximal tubules occurred with advancing age. Functional effects of aging were not examined (Kaler and Haensly, 1977).

Although morphological changes in the kidneys commonly are found in older domestic animals, it is unwarranted to conclude that these changes are associated with a major degree of renal dysfunction. The idea that old animals must have renal dysfunction of clinical relevance simply because they are old should be abandoned.

III. ALTERATIONS IN RENAL FUNCTION DUE TO EXTRARENAL FACTORS

The kidney performs many of its functions in response to and in synchrony with activities of other organs. Under certain circumstances the kidneys may remain anatomically intact but function in a manner detrimental to the well-being of the animal because of extrarenal factors. When azotemia occurs as a consequence of this situation, it is called extrarenal azotemia. Dietary factors and those which interfere with renal perfusion are termed prerenal; those which interfere with urine outflow are called postrenal. Some extrarenal factors affecting renal functions are discussed subsequently. It is important in

clinical medicine to be aware of such factors so that extrarenal causes of renal dysfunctions are not interpreted as renal disease.

A. Diet

Carnivores in particular may have some aspects of renal function influenced by diet. Its effect on concentrating ability has already been mentioned. In addition, feeding dogs meat or other high-protein diets may increase GFR up to 100% for several hours (Smith, 1951). A high-protein diet was also associated with GFR values in sheep that were higher than those found with a low-protein diet (Rabinowitz *et al.*, 1973). This effect on GFR could influenced results from more sensitive function tests. The effect of high-protein diets on the interpretation of tests for azotemia is discussed in Section VII.

B. Renal Perfusion

Since the kidney depends on hydrostatic force and plasma flow for filtration, it is not surprising that alterations in these factors influence renal function.

Perfusion defects (ischemia) can influence renal excretory functions without causing renal damage. This is because filtration may cease or be impaired while the parenchyma is still being perfused by capillaries originating from efferent arterioles. The degree and duration of impaired perfusion determine whether ischemic renal damage occurs. Several diseases and conditions exist in which renal parenchymal damage does not occur despite prolonged prerenal azotemia. In contrast to these naturally occurring conditions in which the kidney is spared, experimental complete normthermic renal artery occlusion for 4 hours results in ischemic necrosis of the kidneys that is irreversible (Hamilton *et al.*, 1948).

1. Dehydration

Dehydration can cause elevated blood urea nitrogen (BUN) and serum creatinine (SC) levels as a consequence of impaired renal perfusion. Dehydration azotemia was experimentally induced in dogs by controllable occlusion of the pylorus. The dehydration that occurred secondary to vomiting caused reduced cardiac output, decreased renal plasma flow, and markedly impaired GFR. The pronounced effect on filtration occurred as a consequence of increased colloid osmotic pressure of plasma proteins. The autoregulatory capacity of the kidney also appeared to be lost. The elevation of BUN was disproportionate to elevations in SC, and thus tissue catabolism also may have been occurring in this model (Balint *et al.*, 1975; Balint and Sturcz, 1959; Balint and Fekete, 1960; Balint and Forgacs, 1965; Balint and Visy, 1965). Dehydration in dogs may also cause changes in intrarenal patterns of perfusion. Outer cortical flow may be preserved longer during the period of dehydration than inner cortical flow. In addition, sudden and intermittent periods of ischemia of 1 to 60 minutes' duration occurred in localized areas of the cortex during dehydration (Kirkebo and Tyssebotn, 1977).

2. Decreased Cardiac Output

The effects of cardiac disease on renal function during exercise were mentioned in Section II. It appears that the specific cardiac lesion is immaterial; decreased cardiac output creates the potential for impaired renal function. Acute cardiac tamponade induced in dogs also caused a decrease in GFR (Mandin and Davidman, 1978).

3. *Hepatorenal Syndrome*

The term "hepatorenal syndrome" is used to describe a condition in human beings in which both organs are in a state of dysfunction. Hepatic failure occurs first and is followed by oliguria and renal dysfunction. Available data indicate that the renal dysfunction is extrarenal in origin. Renal lesions usually are absent, and kidneys transplanted from such patients have functioned normally in recipients (Conn, 1973; Kew, 1972; Papper, 1975). The pathogenesis of the renal failure remains speculative. Hyperbilirubinemia could be a contributing factor since, when induced in dogs by diversion of the bile duct into the postcava, it caused a decrease in GFR of 54% (Aylward *et al.*, 1973). The natural occurrence of hepatorenal syndrome has not been reported in domestic animals, however.

C. Specific Drug Effects on Renal Function

1. *Furosemide*

Furosemide has diuretic properties because it inhibits chloride transport in the ascending loop of Henle. However, some evidence exists that it has hemodynamic effects, which include action on the kidney. Renal blood flow and renal tissue oxygen tension transiently increase after furosemide administration to the normal dog (Nuutinen and Toononen, 1976). In dogs with induced unilateral pyelonephritis, GFR increased 31% in the diseased kidney following furosemide administration (Gutmann and Rieselbach, 1971). The mechanism by which furosemide causes these changes is not known; changes in intrarenal blood flow distribution (Birtch *et al.*, 1967) and suppression of individual nephron feedback inhibition (Schnermann, 1975; Wright, 1977) may be factors.

2. *Anesthetic Agents*

There is a paucity of information concerning the effects of anesthetic agents on renal function in domestic animals. Most of the classic renal function studies done on dogs were performed with dogs anesthetized with pentobarbital sodium. Studies comparing results in conscious and anesthetized dogs indicated that GFR and tubular secretion were usually not affected by the anesthetic for up to 6 hours unless deep levels of anesthesia were used (Corcoran and Page, 1943; Glauser and Selkurt, 1952). Halothane alone or in combination with N_2O was associated with a reduction of renal blood flow in the normal dog in several studies (Hill *et al.*, 1977; MacDonald, 1969) but actually caused an increase in blood flow in hypotensive dogs (MacDonald, 1969) in one study. Halothane anesthesia in the cat resulted in a marked decrease in blood flow during deep anesthesia (Westermark and Wahlin, 1969). It is possible that some of the effects of anesthesia are related to blood levels maintained (i.e., depth of anesthesia) as well as the anesthetic itself. The alterations reported here should be distinguished from nephrotoxic effects of some anesthetics that are mentioned in Section IV.

3. *Ionic Imbalances*

Alterations in renal function may occur during hypercalcemia. Long-term effects of hypercalcemia include parenchymal damage which may lead to renal failure. However, hypercalcemia has acute effects on the kidney, which include a decrease in GFR and RBF. These effects have been postulated to be due to direct effects of calcium on muscle of the renal vasculature (Chomdej *et al.*, 1977).

Potassium deficiency in dogs induced by dietary depletion over periods of up to 7 weeks resulted in a progressive decrease in GFR and RPF to about 77% of normal. Some decrease in urine-concentrating ability was also noted. All renal functions returned to normal after potassium repletion (Abbrecht, 1969).

4. Osmotic Diuretics

Osmotic diuretics are materials that are freely filtered by the glomeruli but are not reabsorbed significantly or at all by the tubules. A high intraluminal concentration is achieved as some water is reabsorbed. However, the osmotic activity in the lumen interferes with the normal passive reabsorption of water so that increased urine volume occurs. During hyperglycemia glucose acts as an osmotic diuretic because T_mG is exceeded. Mannitol, a nonmetabolizable sugar alcohol, acts as an osmotic diuretic since it is freely filtered but totally nonreabsorbable. Although the mechanisms are not known, osmotic diuretics increase RBF and GFR moderately. They also decrease the tubular reabsorption of sodium. This occurs as a consequence of a decrease in sodium concentration of proximal tubule fluid brought about by retention of water secondary to the osmotic agent. With the decrease in sodium concentration, there is less of a gradient for passive absorption into the cell of the tubule (Gennari and Kassirer, 1974). The greater flow of fluid to the more distal tubule prevents adequate reabsorption of sodium, urea, and other components of the fluid. As a consequence, urinary excretion of sodium, potassium, calcium, phosphorus, magnesium, and urea is increased during both acute and chronic osmotic diuresis (Gennari and Kassirer, 1974). Concentration of urine depends on passive movement of water secondary to osmotic gradients between the interstitium and the distal tubule or collecting duct. Since osmotically active particles remain in the tubular lumen, the presence of ADH and medullary hypertonicity have no effect on the rate of urine flow during osmotic diuresis. However, medullary blood flow rate increases and interstitial sodium and urea levels decrease during osmotic diuresis in dogs (Velasquez *et al.*, 1973). This means that ability to concentrate urine after the use of osmotic diuretics is dependent upon the reestablishment of medullary hypertonicity.

D. Renal Outflow Impairment

1. Acute Effects (Less Than 24 Hours)—Unilateral Obstruction

Complete ureteral occlusion results in a rapid onset of changes in renal function. Due to occlusion, ureteral pressure increases during the first 5 hours to about 60 mm Hg and is maintained at about 40 mm Hg at 12–24 hours. The RBF and GFR gradually decrease to about 40 % of normal at 12–24 hours. Some glomerular filtration continues despite lack of urine outflow because tubular reabsorption accommodates new filtrate. It is believed that GFR and RBF changes are at least in part due to constriction of afferent arterioles since backpressure countering filtration is decreased after 24 hours but decreased RBF persists. The vascular response appears to be a single-nephron phenomenon since blockage of individual tubules does not affect adjacent unblocked tubules (Wilson, 1977)

2. Acute Effects—Bilateral Obstruction

In contrast to unilateral obstruction, intratubular pressure remains high (30 mm Hg) in bilateral obstruction. Another difference is that a postobstruction diuresis occurs after release of acute bilateral obstruction but not after unilateral obstruction. The decrease in

RBF and GFR common to both types of obstruction persists for a variable time after release of bilateral obstruction, but there is a proportionately greater effect on GFR than on RBF (Wilson, 1977).

3. Physiological versus Pathological Dysfunction

Ischemia that affects GFR was differentiated from ischemia that resulted in renal cell injury when perfusion defects were discussed. The distinction between functional and morphological consequences of obstruction must also be considered. The two are not easily separated, but it does appear that most of the consequences of acute obstruction (i.e., less than 24 hours) are a result of physiological changes. Obstruction that leads to pathological changes can cause primary renal failure. The degree and duration of obstruction seem to be factors that influence the occurrence of renal damage.

In dogs with complete unilateral obstruction of 7 days, kidney function thereafter was 65% of control values (Kerr, 1954). In dogs with 14 days of complete unilateral obstruction, renal function was 46% of control values after 4 months and did not change thereafter (Vaughan *et al.*, 1973).

4. Functional Changes Related to Postobstruction Diuresis

Postobstruction diuresis may occur in animals with acute bilateral or chronic obstruction. Extrarenal factors, such as increased extracellular fluid volume, urea diuresis, and the presence of natriuretic compounds retained during obstruction, are believed to contribute to the polyuria. Renal damage as a cause of the diuresis becomes more likely the longer the period of obstruction. In dogs, a deficit of Na^+-K^+-ATPase has been associated with elevated solute excretion and diuresis following 3 and 7 days of ureteral obstruction (Williams *et al.*, 1976). The diuresis following ureteral obstruction is of clinical relevance since patients may develop water and electrolyte imbalances if losses are not balanced by intake.

IV. PRIMARY RENAL DYSFUNCTION—ACUTE FAILURE

A. Causes

1. Nephrotoxins

The kidney is particularly vulnerable to effects of noxious agents because of its high perfusion rate, its numerous enzyme systems, and its role as an excretory organ. Nephrotoxins encountered in domestic animals include heavy metals, oxalates or their precursors as glycols, a variety of antibiotics including the aminoglycoside group, acorn poisoning, and sulfonamides in cattle, and hemoglobin or myoglobin nephropathy. Fluoride-containing anesthetic agents may also cause nephrotoxicity (Kosek *et al.*, 1972; Churchill *et al.*, 1974).

Our knowledge concerning the mechanism by which nephrotoxins cause damage is limited but expanding. Some cause damage because they remain in the lumen and are concentrated as water is reabsorbed. Others are taken up by tubular cells and concentrated therein. They may react with and alter the activity of enzymes of specific organelles. Immunological reactions (immune complex nephritis) may be acute, and hypersensitivity reactions in the renal interstitium have been reported for several drugs (Muehrcke *et al.*, 1976). It has been found that some toxins are relatively inert until modified by host

enzymes. For example, ethylene glycol is converted to glycoaldehyde, glycolic acid, and oxalic acid, which are more toxic than the parent compound (Muehrcke *et al.*, 1976). In some instances the function of the cytoplasmic cytochrome system is important in the formation of nephrotoxic metabolites. This cytochrome system is normally involved in drug oxidations. Its action can be blocked or suppressed with cobalt chloride or piperonyl butoxide. Pretreatment of animals with these compounds markedly decreases the proximal tubular necrosis produced by cephaloridine because the oxidation of cephaloridine to a more nephrotoxic form is prevented (Mitchell *et al.*, 1977).

2. *Ischemia*

As previously indicated, impaired perfusion may alter renal function without apparent renal damage, but severe or prolonged ischemia produces acute renal failure. Species differences may exist concerning vulnerability to ischemic nephrosis. Experimental results suggest that the dog may not tolerate hypovolemia as well as the human being and thus may succumb to shock rather than eventually dying of anuria secondary to ischemic nephrosis (Phillips *et al.*, 1946). Objective data are not available concerning the vulnerability and incidence of ischemic failure in domestic animals.

3. *Infectious Agents*

Some infectious agents, such as *Leptospira* spp., may cause acute nephritis. When the infections cause massive lesions, death may occur acutely after onset of signs, and renal failure may be a contributing cause (Low *et al.*, 1956).

B. Pathogenesis of Acute Renal Failure

The common occurrence and high mortality of acute renal failure in human beings has resulted in considerable experimental study of laboratory models of this disease. Models of nephrotoxic acute failure have been produced by administering glycol, uranium salts, mercury, or hemoglobin, and models of ischemic failure have been produced by clamping the renal artery or injecting epinephrine. Unanimity of opinion does not exist on the events that take place in the development of acute failure (Harrington and Cohen, 1975; Oken, 1975; Levinsky, 1977; Stein *et al.*, 1978). One theory is that is is due to renal vasoconstriction. Another theory is that tubular blockage occurs as a consequence of intraluminal debris and interstitial edema. Yet another theory surmises that glomerular filtrate forms but is totally reabsorbed (passive backflow) because of tubular necrosis. Changes in the glomerular ultrafiltration coefficient have also been postulated. Many paradoxes and contradictions can be found when individual studies are compared, and it is apparent that definitive answers await further study. It is possible that different causes of acute renal failure have a different pathogenesis and that a unified concept is unrealistic.

A sudden decrease in nephron mass, such as that induced by unilateral nephrectomy, is associated with functional changes in the opposite kidney that precede morphological compensatory changes. The GFR and RPF increase within hours by mechanisms that are not understood.

C. Clinical and Biochemical Characteristics of Anuric Failure

Most human beings and many animals with acute renal failure have anuria or severe oliguria. However, nonoliguria or polyuric renal failure may also occur (Anderson *et al.*,

1977). Conversely, oliguria or anuria may occur as a late event in chronic progressive renal disease in the dog (Finco, 1979). Available data on the biochemical manifestations of naturally occurring acute renal failure in domestic animals are limited. Clinical impression suggests that biochemical alterations are more dependent on the presence or absence of oliguria than on the acuteness or chronicity of the renal insult. The subsequent discussion probably applies to anuric renal failure regardless of whether the renal disease is acute or chronic.

1. Urine Composition

Anuria is defined as the absence of urine, yet most patients described as anuric produce a small quantity of urine. For this reason the terms "anuric" and "oliguric failure" are often used loosely and interchangeably. Despite the small volume, urine specific gravity is usually low. In instances when small volume is associated with high specific gravity, first consideration should be given to prerenal factors (i.e., dehydration) that may be influencing urine output rather than primary acute renal failure. Although urine specific gravity or osmolality is low in acute failure, sodium concentration of the urine is higher than normal (Miller *et al.*, 1978; Stein *et al.*, 1978). This is attributed to impaired sodium reabsorption by the injured tubules. Specific data on domestic animals are sparse; in human beings normal urine or urine from patients with prerenal azotemia has less than 5 mEq of sodium per liter, while in patients with acute renal failure it is usually greater than 30 mEq. The apparent paradox of high specific gravity–low-sodium urine and low specific gravity–high-sodium urine is due to the minor contribution of sodium to urine osmolality, compared to urea and other components. Other urine findings with acute anuric failure are not unique; urinalysis is discussed in Section VI.

2. Blood Chemical Changes

Azotemia and an elevated serum phosphorus concentration are alterations observed with severe renal failure of any duration. There are no published data indicating that the degree of hyperphosphatemia or azotemia, or the relation of one to the other, is different in acute oliguric failure when compared to chronic failure. These blood components as well as others normally excreted by the kidney may increase progressively from day to day after the sudden onset of renal failure. In contrast, patients with chronic renal failure are often at a stable level of function; blood levels of retained elements remain relatively constant from day to day until further measurable functional changes occur.

Hyperkalemia is a common finding with anuric renal failure and has great clinical significance because of the role of this ion in neuromuscular transmission. Hyperkalemia occurs with anuria because of the release of intracellular potassium stores in association with normal cell injury and death. The preponderance of body potassium is intracellular; loss of but a small percentage to the extracellular compartment without rapid excretion leads to marked hyperkalemia. Intracellular to extracellular shifts in potassium that occur in association with metabolic acidosis may also contribute to the hyperkalemia. Dietary intake of potassium may complicate the situation if food intake is occurring. The cause of death in experimental anuria of dogs was identified as hyperkalemia in studies conducted several decades ago (Hoff *et al.*, 1941).

Anuric renal failure provides potential for positive water balance because of lack of urine outflow. However, this usually does not occur in domestic animals because of adipsia, normal water loss via the skin and lungs, and vomiting by carnivores. Iatrogenic

overhydration is a potential problem if parenteral fluids are administered to the anuric animal. Clinical evaluation as well as hematocrit and total serum protein levels may be used to evaluate the state of hydration.

Changes in blood components during acute anuric failure are a manifestation of the anuria. It is not surprising that extrarenal causes of anuria cause identical alterations. Urethral obstruction commonly occurs in the dog, cat, and ruminants. Rupture of the urinary bladder occurs quite commonly in dogs. The biochemical manifestations of these abnormalities have been studied (Burrows and Bovee, 1974, 1978, Finco and Cornelius, 1977). In obstructed cats, metabolic acidosis, mild hyponatremia, hyperkalemia, hypermagnesemia, hypocalcemia, hyperphosphatemia, hyperglycemia, and hyperproteinemia accompanied the azotemia. In dogs with bladder rupture, hyperkalemia, marked hyponatremia, hypochloremia, hyperphosphatemia, and compensated metabolic acidosis occurred.

Few data are available concerning the outcome of acute oliguric renal failure in domestic animals. In human beings it is associated with a fairly high mortality rate despite the use of hemodialysis. However, functional recovery may be up to 100% because of both regenerative capabilities of the kidney and compensatory mechanisms which the kidney possesses. In patients with acute renal failure, oliguria may be followed by a period of profound diuresis. Electrolyte and water depletion may occur during this phase unless losses are replenished.

V. PRIMARY RENAL DYSFUNCTION—CHRONIC FAILURE

A. Causes

Chronic renal failure is often the consequence of slow, insidious destruction of renal parenchyma. Because of the reserve capacity of the kidneys, clinical signs of disease may not develop for months or years following the initiation of disease. This long time interval has made it difficult to identify causes of chronic failure. Morphological examinations are of little value because renal responses to injury are limited in variety and tend to assume a common character regardless of cause as chronicity develops.

Bacterial pyelonephritis and causes of chronic glomerulonephritis have been identified as factors in specific cases of chronic failure but probably account for but a small percentage of all cases. Any cause of acute renal failure has the potential for leaving a patient with marginal renal function, but evidence for the progression of dysfunction once the initial insult has occurred is scant. In fact, direct evidence was obtained that experimental induction of acute leptospiral nephritis in dogs was not associated with chronic renal failure (Low *et al.*, 1967). Further studies will be required to identify the causes of chronic failure.

B. Pathophysiology of Chronic Renal Failure

In contrast to the abrupt sequence of events in acute anuric failure that reflect manifestations of sudden dysfunction, most changes take place gradually in chronic failure. These changes are initially undetectable both clinically and biochemically. It is only when over two-thirds of normal parenchyma has been damaged that clinical signs become apparent.

The nature and consequences of these changes have been studied extensively. Although unanimity of opinion does not exist, there is fair agreement concerning events that occur during chronic failure.

1. Morphological Adaptations

Both functional and morphological alterations occur after reduction of nephron mass. The functional responses may be detected almost immediately. For example, single-kidney GFR increased from 31.4 to 37.5 ml and RPF from 114 to 149 ml 24 hours after contralateral nephrectomy in dogs (Rous and Wakim, 1967). An increase in the RNA to DNA ratio was detected in the cells of the residual kidney 6 hours after unilateral nephrectomy of rats, and weight changes in the kidney were detectable by 12 hours (Van Urk *et al.*, 1978). Marked increases in weight are found in remaining dog kidneys 10 days after unilateral nephrectomy (Carriere and Gagnan-Brunette, 1977), but maximal increase in size does not occur until 8 weeks (Rous and Wakim, 1967). There is some evidence that decreases in renal mass prior to renal maturation may be associated with production of new nephrons (Bonvalet *et al.*, 1972; Imbert *et al.*, 1974). However, maturation is completed prenatally or during early postnatal life. The increase in renal size thereafter is brought about by changes in size of existing nephrons and the interstitium. Microdissections from dogs with chronic disease demonstrated that glomerular surface area and volume were nearly doubled and that tubule length and thickness were increased (Oliver *et al.*, 1941). The term "renal hypertrophy" has been used loosely to describe the increase in kidney size associated with unilateral nephrectomy. Some studies indicate that hypertrophy is the primary factor when one-half of renal mass is lost (i.e., unilateral nephrectomy) but that the degree of hyperplasia increases progressively as loss of renal mass increases (Kaufman *et al.*, 1976; Van Urk *et al.*, 1978). The ability to respond to renal insult by compensatory renal growth is better in the immature animal than in the adult (Galla *et al.*, 1974).

2. Functional Adaptations

In health the kidney responds to changes in intake by adjustments in excretion so that extracellular composition remains normal. The kidney with chronic disease may have severe morphological changes that could interfere with this regulatory function. Nevertheless, the diseased kidney operates to maintain homeostasis wtihin the limits of its capacity. It does so by orderly and meaningful adaptation to the conditions under which it must function. The intact nephron hypothesis was formulated in 1960 (Bricker *et al.*, 1960) and modified in 1969 (Bricker, 1969). In modified form, it can be stated as follows:

> In most forms of chronic renal disease associated with marked nephron destruction, the majority of nephrons that contribute to renal functions behave, in a regulatory context, as if they were normal. If glomerular or tubular functions are impaired in individual nephrons due to structural damage, there will be simultaneous and proportional changes in other functional systems in the same nephrons. Function in the composite group of surviving nephrons is characterized by relative homogeneity of glomerulo-tubular balance, appropriate adaptations of glomerular and tubular functions, and continuing responsiveness to the changing needs of the patient as the disease advances.

Several examples of adaptation and successful homeostasis by the diseased kidney are available. These examples amplify the fact that, under conditions of constant oral intake, fractional excretion (i.e., fraction of quantity filtered that must be excreted to maintain body balance) of a solute such as sodium must increase proportionately on an individual

nephron basis as the number of functional nephrons decreases. Thus, when the number of nephrons is 10% of normal, fractional excretion of sodium per residual nephron is ten times what it was in health. The same phenomenon occurs for phosphorus, as increased PTH levels enhance renal excretion of this ion. Unfortunately, the increased levels of PTH adversely affect bone; thus, the beneficial effects on renal phosphorus regulations are a tradeoff for other undesirable consequences. The concept that adaptive phenomena brought about by reduced renal function may have longer-term complications has been called the "tradeoff hypothesis" (Bricker, 1972).

3. Uremic Toxins as a Cause of Abnormalities in Renal Failure

An early concept was that the signs and effects of renal failure could be explained by retention during renal failure of a compound normally excreted by the kidneys. The success of hemodialysis and peritoneal dialysis in alleviating most signs of renal failure supported this concept. Among the presumed culprits that have been incriminated over the years are urea, ammonia, creatinine, uric acid, hippuric acid, leucine, tyrosine, sulfates, phosphates, chlorides, potassium, acidosis, organic acids, indicans, guanidine and derivatives, such as methylguanidine and guanidinosuccinic acid, magnesium, phenols, aromatic oxy acids, urinary alkaloids, and hyperosmolality, as enumerated in a publication in the 1960's (Schreiner and Maher, 1961). However, no irrefutable proof exists that any one of these compounds is the uremic toxin. Urea deserves special mention since it is used as an indicator of renal failure. Despite its value as such an indicator, urea itself has little toxicity. Only when blood levels are greater than 300 mg/dl are any adverse signs expected (Giovannetti and Barsotti, 1975). More recently, attention has been given to the "middle molecules" as uremic toxins. These are molecules with molecular weights of 350–2000 which were incriminated as causes of uremic neuropathy if not removed by dialysis procedures (Brentano *et al.*, 1975; Furst *et al.*, 1975). These "middle molecules" appear to be a mixture of different peptides, but their specific identity has not been established (Furst *et al.*, 1975). Myoinositol has also been incriminated as a uremic toxin on the basis of tissue culture studies (Liveson *et al.*, 1977), but neither spinal fluid nor plasma levels of this compound correlated with nerve conduction velocities or electroencephogram (EEG) changes (Blumberg *et al.*, 1978).

In summary, the quest for "uremic toxins" goes on unabated; the matter is in a state of relative mystery at present.

C. Consequences of Chronic Renal Failure

1. Polyuria

Increased urine production has long been associated with chronic renal failure. It was disputed whether polyuria was a consequence of renal damage or an adaptive response to damage. Studies in dogs with unilateral disease caused by producing pyelonephritis or aminonucleoside nephrosis indicated that the diseased kidney could concentrate urine as well as the normal contralateral kidney (Bricker *et al.*, 1959). Likewise, dogs with remnant kidneys and normal dogs had equal ability to concentrate when normal dogs received equivalent solute loads by injection of urea (Coburn *et al.*, 1965), and remnant kidneys had equal concentrating ability in a uremic and nonuremic environment (Morrin *et al.*, 1970). These results indicate that the polyuria is an adaptive consequence of increased solute load per remaining nephron. An additional factor that may be associated

with the polyuria of renal failure is a lack of medullary osmotic gradient (Eknoyan, 1977). This could be secondary to the diuresis or a consequence of faulty NaCl transport by the ascending loop of Henle.

Failure to dilute urine adequately as well as concentration defects have been found in dogs with reduced renal mass (Coburn *et al.*, 1965). Nevertheless, some dogs with chronic renal disease may have hyposthenuria, which is defined as urine osmolality below that of plasma (Finco, 1979).

The failure to regulate water excretion as effectively as normal predisposes the patient with polyuric chronic renal failure to water imbalance. Dehydration is frequently found in association with uremia because of this regulatory defect. Thus, a factor with potential for causing prerenal azotemia may be superimposed on the primary renal dysfunction.

2. Hematological Abnormalities

A nonregenerative normocytic normochromic anemia often is observed in association with chronic renal failure. Its onset in relationship to degree of nephron dysfunction has not been well established, but it is frequently present when chronic azotemia is discovered.

It is known that a substance produced by the kidneys is required for normal erythropoiesis. There is disagreement over the method of action of the renal compound. Some evidence indicates that it acts directly on the bone marrow. Other evidence suggests that the active material is a product of the action of the renal compound on a circulating globulin (Erslev, 1975). Because of this controversy the terms "erythropoietin" and "erythropoietin-stimulating factor" are both used to describe the product of the kidneys. The effects of erythropoietin on bone marrow are discussed in Chapter 4. The exact site of origin of the renal material has not been established.

Lack of erythropoietin or erythropoietin-stimulating factor may not be the only reason for anemia during renal failure. Uremic serum contains factors that are directly toxic to bone marrow cell cultures (Lamperi *et al.*, 1974) or inhibitory to erythropoietin (Wallner *et al.*, 1977).

It is established that shortened red cell survival occurs in uremic human beings (Naets, 1975), but the cause is unknown. There are no data concerning red blood cell (RBC) hemolysis in uremia of domestic animals.

The anemia of chronic renal failure may be slowly progressive but often seems to reach a plateau. In some species extrarenal sources of erythropoietin may serve as the source of stimulus under these circumstances (Fisher, 1972).

3. Renal Secondary Hyperparathyroidism, Phosphorus, and Calcium

The development of renal secondary hyperparathyroidism is related to alterations in calcium metabolism that occur in renal failure. The interrelationship that exists between calcium and phosphorus is important in the pathogenesis of this disorder. Although the specific mechanisms have not all been elucidated, early in the course of loss of renal funtion there is an analytically imperceptible hyperphosphatemia and hypocalcemia. The hyperphosphatemia is due to decreased GFR. The hypocalcemia is probably a direct physiochemical effect of hyperphosphatemia during mild dysfunction. Later in the progression of the disease calcium deficit occurs in association with impaired gut absorption secondary to abnormal hydroxylation of vitamin D, increased urinary excretion, and tissue deposition secondary to injury. Hypocalcemia stimulates PTH release by the parathyroid

glands; PTH in turn has several effects. It inhibits tubular reabsorption of phosphate to cause phosphaturia and a decrease in serum phosphorus levels. It also inhibits proximal tubular reabsorption of both sodium and bicarbonate (Aurbach and Heath, 1974; Schmidt *et al.*, 1976). The stimulus for PTH release occurs early in the course of chronic progressive nephron destruction, long before the presence of azotemia. With the release of PTH and its enhancement of phosphorus excretion, the biologically significant but analytically imperceptible hyperphosphatemia and hypocalcemia are corrected for the existing level of renal function. If more renal function is lost, the actions and reactions are accentuated so that normophosphatemia and normocalcemia prevail. The mechanisms are effective in controlling blood phosphorus levels until about the time that azotemia develops. At this time the decrease in filtered phosphorus is inadequate for maintenance of normal serum levels even with maximal inhibition of tubular reabsorption. The importance of phosphorus in the development and progression of renal secondary hyperparathyroidism has been proved in dogs with induced renal insufficiency by demonstrating that control of serum phosphorus levels by dietary restriction prevented increased PTH levels (Slatopolsky *et al.*, 1971).

To recapitulate, animals with chronic renal failure have normal serum levels of calcium and phosphorus despite the decrease in filtered load because of the action of PTH on the phosphate reabsorptive mechanism. In advanced stages of failure, PTH cannot overcome the effects of decreased GFR, so blood levels of phosphorus increase, Total serum calcium levels remain within normal limits as determined by laboratory methods of measurement until further progression, but they may eventually decrease. The decrease in serum calcium that occurs is usually moderate, and clinical signs of hypocalcemia are rarely observed. Acidosis that may accompany renal failure is protective against hypocalcemia since a greater portion of total serum calcium is in the biologically active ionized form when hydrogen ion concentration increases.

Exceptions to the generality of normocalcemia or hypocalcemia in renal failure are known to exist. Hypercalcemia has been observed in renal failure in the horse (Tennant *et al.*, 1974) and occasionally in the dog (Finco and Rowland, 1978). Since hypercalcemia can cause renal failure as well as occasionally be an effect of it, it is important to establish proper cause–effect relationships during clinical diagnosis. Causes of primary hypercalcemia include parathyroid neoplasia, pseudohyperparathyroidism, and vitamin D toxicosis. The pathogenesis of hypercalcemia secondary to chronic renal failure is not well established. In the horse it may be associated with high dietary calcium intake and a prominent role played by the kidney in calcium homeostasis in this species. In the dog, lack of feedback inhibition of the hyperplastic parathyroid gland may play a role (Finco and Rowland, 1978).

The effects of renal secondary hyperparathyroidism on the skeleton may be dramatic. The biochemical events which occur in bone during uremia are not well understood. During uremia in the growing animal, bone growth is impaired and calcification is faulty. Decalcification also occurs in the adult. "Rubber jaw syndrome" may occur in some dogs, while on rare occasions exostosis occurs (Norrdin, 1975).

4. Other Blood Electrolyte Values

As previously mentioned, renal compensatory mechanisms operate during chronic failure in an effort to maintain normal body composition. Increased fractional excretion of sodium and renal adaptive mechanisms for increasing potassium excretion have been

discussed. These mechanisms are highly successful in maintaining normal blood levels of both sodium and potassium during chronic renal failure so long as polyuria exists. The normokalemia is a striking contrast to the hyperkalemia of oliguric renal failure. Although blood levels of sodium are usually normal, negative body balance of sodium may be present in dehydrated animals. One report indicates that sodium wasting may occur in dogs with chronic renal failure (Gartner, 1962).

Hypermagnesemia usually occurs in chronic renal failure. The acid–base balance in chronic renal failure is in the direction of metabolic acidosis. With acidosis, bicarbonate levels are decreased and chloride levels may be normal or increased.

5. Neurological Disorders

Abnormal cerebral function manifested as depression is noted clinically in dogs and cats with uremia, and cattle often grind their teeth. Dogs with induced uremia had EEG alterations that correlated with increases in brain calcium levels after induction of uremia, and thus some relationship between brain calcium levels and neurological signs may exist (Arieff *et al.*, 1975).

Peripheral neuropathy also occurs with chronic uremia. Motor nerve conduction velocity is decreased in human beings in association with primary axonal degeneration and segmental demyelination (Raskin and Fishman, 1976). Such measurements in domestic animals with naturally occurring disease have not been reported.

6. Gastrointestinal Signs

Both acute and chronic renal failure may cause vomiting in carnivores. It may be partially inhibited by ablation of the chemoreceptor trigger zone in the medulla, suggesting a circulating factor as one cause of vomiting (Borison and Herbertson, 1959). Chronic renal failure may also be associated with oral and gastrointestinal ulcers. The ulcers have been attributed by some to ammonia released by bacterial action on urea (Black, 1970). Others suggest that pancreatic enzymes play a role (Bounous, 1970). The gastrointestinal lesions may contribute to vomiting by initiating afferent impulses to the vomiting center.

Diarrhea or constipation has also been associated with azotemia.

A typical "uremic breath" may be identified in patients with advanced renal failure. Studies in human beings indicate that dimethylamine and trimethylamine are responsible for this odor (Simerhoff *et al.*, 1977).

7. Metabolic Alterations Associated with Uremia

Several alterations have been noted in metabolism as a consequence of renal failure. Glucose intolerance was found in the dog (Swenson *et al.*, 1973) as well as in other experimental animals (Bagdade, 1975). Several factors have been considered instrumental in its pathogenesis. These include resistance of tissues to enhancement of glucose uptake in response to insulin (insulin resistance), impaired regulation of growth hormone secretion, elevated blood levels of urea, and potassium deficiency (Bagdade, 1975). The degree of glucose intolerance is mild compared to diabetes mellitus, and hyperglycemia of sufficient magnitude to cause glucosuria does not occur.

Alterations in serum lipids and lipid metabolism have been found in several species during renal failure. Hypercholesterolemia occurs in association with the nephrotic syndrome in several species. Increases in blood levels of very low density lipoproteins (VLDL), which are mainly triglycerides, have been found in nonnephrotic human beings and rats with chronic renal failure. The elevations are apparently due to increased liver

production and decreased peripheral tissue uptake of VLDL (Bagdade, 1975). A decrease in high-density lipoproteins in chronic uremia may also occur. The presence of alterations in lipid metabolism in domestic animals with naturally occurring renal failure have not been examined thoroughly. Interest in the abnormalities found in human beings is related to a high incidence of atherosclerosis in patients with chronic renal failure. The development of such alterations in animals has not been reported.

Protein metabolism in renal failure is altered, but difficulty exists in separating the causes of these alterations. Animals with chronic renal failure often suffer from a caloric deficit and weight loss because of anorexia. Alterations that occur in protein metabolism may be due either to malnutrition itself or to deleterious effects of uremia on metabolism. Unfortunately, few studies have been done on protein metabolism of domestic animals during azotemia. Histidine is required by azotemic human adults, but the amino acid is nonessential for normal adults (Bergstrom *et al.*, 1970). In addition, the metabolic conversion of phenylalanine to tryosine is impaired in azotemia (Richards, 1975). Protein synthesis as measured by ^{15}N uptake is stimulated by dialysis (Bergstrom *et al.*, 1975), implying that anabolism is impaired during azotemia. Such findings made it seem doubtful that conversion of the catabolic state to an anabolic one could be expected in the patient with chronic renal failure. However, giving amino acids or their hydroxy and keto acid precursors has resulted in reversal of catabolism and even positive nitrogen balance in human beings with renal failure (Close, 1974; Kopple and Swendseid, 1977; Walser, 1978). These studies support the concept that altered protein metabolism in renal failure is due to both malnutrition and and deleterious consequences of uremia but show that the latter can be modified by proper nutritional management.

The rationale of a low-protein diet, which has been advocated for use during renal failure, may seem to contradict the information presented in the preceding discussion. The objective of protein restriction during renal failure is to avoid protein catabolism that may occur as a consequence of administering amino acids in excess of the body's ability to utilize them for protein synthesis. This excess may be due to ingestion of too much of all amino acids or due to an improper balance of amino acids. The consequence of the ingestion of quantities beyond those usable for anabolism is deamination and increased production of nitrogenous wastes. The goal of nutritional management of renal failure is to provide precisely the protein diet required for protein anabolism without an excess for catabolism. Unfortunately, the makeup of this ideal diet has not been ultimately defined for either domestic animals or human beings.

8. Effects of Renal Failure on Drug Metabolism

Compromised renal function affects the excretion and blood levels of drugs that are normally excreted by the kidneys. Another effect of renal failure on drug metabolism is related to plasma binding of drugs. The free rather than the bound drugs are biologically active, and yet the usual methods of measuring drug levels include both forms (Reidenberg, 1977a). In uremia, anionic drugs have decreased plasma binding, while cationic drugs have normal or increased binding (Reidenberg, 1976, 1977a). In addition, biotransformation of drugs is altered during the uremic state (Reidenberg, 1977b). Drug oxidations are normal or accelerated by the microsomal oxidative system, while reductions are slowed. Acetylations may be normal or slowed, and many hydrolyses are slowed. The preceding observations have been made in experimental animals and thus may be applicable to domestic species. However, little data are available on metabolism of specific drugs in uremic domestic species.

9. Effect of Uremia on Immunity

Uremia causes suppression of the immune system in a variety of species. Both humoral and cellular factors are impaired (LoGrippo *et al.*, 1970; Souhami, 1973; Touraine *et al.*, 1975; Dobbelstein, 1976; Raskova and Morrison, 1978). Abnormalities are found in acute as well as chronic uremia but seem more severe in the latter. Several explanations have been offered for the impaired immune response, but mechanisms have not been elucidated. The degree of suppression of immunity seems to vary from patient to patient. The relative rather than absolute nature of the suppression must be emphasized; transplant rejection occurs in uremic human patients if histocompatibility does not exist. Clinically, the degree of suppression of immunological responses does not seem to warrant routine antimicrobial therapy in the uremic patient.

VI. URINALYSIS

Urinalysis usually is performed as a screening method for acquiring a large amount of information about several body systems with minimal expenditure of resources. It is also performed specifically for evaluation of the kidneys. Its value in studying the kidneys is related to the detection of abnormal components in the urine that may be of renal origin (casts, protein). Urinalysis is of less value for evaluation of renal function since of all parts of the urinalysis only the specific gravity may reflect level of function. In the subsequent discussion, emphasis is placed on the principles of each part of the analysis and on the interpretation of the results rather than on methodology.

The method of collection of urine is of utmost importance in interpreting a urinalysis. The urinalysis request slip should contain provisions for recording this information (Fig.

URINE

VOID ☐ CATH. ☐ CYSTO. ☐ EXPRESS ☐
ROUTINE ANALYSIS
COLOR _____
TRANSP. _____
pH _____
SPEC. GRAV _____
PROTEIN _____
KETONE BODIES _____
GLUCOSE _____
BILIRUBIN _____
Hb OCCULT BLOOD _____
MICROSCOPIC _____
RBC _____
WBC _____
EPITHELIUM _____
CASTS _____
CRYSTALS _____
BACT. _____
MISC. _____
UROBILINOGEN _____

Fig. 3. Form for reporting urinalysis results. Notice that provisions are included for recording the method of collection of urine. CATH. refers to catheterization, CYSTO. to cystocentesis, and EXPRESS to manual compression of the urinary bladder.

3). In that way the urinalysis results can be properly interpreted beyond the time period of mental recall of method of urine procurement. Spontaneously voided urine may contain bacteria and white blood cells (WBC) that are normal constituents of the genitalia. In the normal animal, samples obtained by cystocentesis are devoid of bacteria, contain but few WBC, but may contain abnormal numbers of RBC due to trauma of cystocentesis. Samples of urine obtained by catheterization are devoid of lower-tract contamination if collected properly but may have increased numbers of RBC due to iatrogenic trauma.

Urine samples should be obtained in clean containers, or sterile ones if microbial studies are desired. The sample should be refrigerated if the urinalysis cannot be performed within 1 hour. Refrigerated samples should be warmed to room temperature prior to urinalysis, particularly to avoid errors in specific gravity measurement with a urinometer.

A. Urine Color, Odor, and Turbidity

Urine color is normally yellow in all domestic species. The cause of the coloration is reported to be urochrome, which is a combination of urobilin and urobilinogen with a peptide (Anonymous, 1965). The intensity of the color usually varies with urine concentration; thus, concentrated urine is dark yellow, and dilute urine is pale yellow.

Abnormal constituents may alter the color of urine. Bilirubin or its degradation products may give urine a darker yellow color than normal. Hemoglobin or hematuria may impart a red or brown color, and myoglobin a reddish brown appearance. Some drugs or diagnostic reagents may alter the color of urine. Bromsulphalein (BSP) and phenolsulfonphthalein (PSP) are both red in alkaline urine, and PSP is dark yellow in acid urine. Neoprontosil causes red urine, and methylene blue results in greenish urine.

Urine odor varies somewhat from species to species, and the specific reasons for the differences have not been well studied. Sex differences in urine odor within a species may also exist. Infection with urea-splitting organisms may result in a highly ammoniacal urine odor.

Turbidity of urine varies among species and among samples in the same individual. Horse urine is normally markedly turbid because of calcium carbonate content and mucus originating from glands in the renal pelvis. Cow urine becomes turbid on standing because of calcium carbonate crystals. Dog and cat urine is often clear but may be turbid in the absence of abnormalities.

B. Specific Gravity

As previously discussed, the amount of water in urine is determined by passive movement of water from tubule to interstitium secondary to osmotic gradients. Osmotic forces are determined by numbers of particles per unit of solvent. Specific gravity is used to estimate the number of particles per unit of solvent. Making this estimate has clinical value since urine specific gravity may be "abnormal" under a variety of circumstances. However, caution must be exercised in interpreting urine specific gravity readings because of the homeostatic role played by the kidney in water balance. In times of ingestion of large amounts of water, urine specific gravity as low as 1.001 is normal because of the need to excrete water for homeostasis. In times of dehydration, a vigorous effort to conserve water is expected, and urine specific gravity would be elevated to maximal

capabilities for that animal. Thus, the range of specific gravity readings between maximal diluting and maximal concentrating capabilities encompasses all specific gravity readings that are considered abnormal.

In contrast to the normal range, it is appropriate to consider the usual range of specific gravity readings for a species under existing environmental conditions and husbandry practices. Since carnivores and most herbivores do not normally overdrink, some concentrations of urine is usually anticipated. Interpretation of urine specific gravity as normal or abnormal is facilitated by considering the state of water balance during the time the urine was excreted. A urine of low specific gravity produced a dehydrated animal indicates abnormal water conservation by the kidney. Several causes of low urine specific gravity have been identified in domestic animals. States in which dilute urine may be produced in the dog are given in Table I.

Some error exists in using specific gravity measurements made with a urinometer for estimating osmolality of urine. Specific gravity readings are a reflection not only of the number of particles but of their size and attraction for one another. Thus, protein may falsely elevate specific gravity by 0.001 per 400 mg/dl and glucose 0.001 per 270 mg/dl (Wolf, 1969). Refractometers are calibrated to give readings of specific gravity but actually measure refractive index of the fluid. Refractive index is superior to specific gravity readings obtained with a urinometer and compares well with information obtained by osmometry (Wolf, 1969).

Refractometry has the advantages of accuracy, simplicity, and requiring but a few drops of urine. Urine with specific gravity readings above 1.035 must be diluted (1:1) with distilled water to obtain readings. The actual specific gravity is obtained by doubling results to the right of the decimal point. Refractometry is the preferred method of measuring urine concentration since it is more accurate than urinometer readings and simple to perform compared to osmometry. Osmometry is considered the definitive method of measuring solute–solvent ratios in urine since osmometers measure one of the colligative properties of urine (freezing point depression, vapor pressure). While specific gravity readings are expressions of the relative density of urine compared to water, osmotic pressure readings are expressed as milliosmoles per kilogram of water. Normal serum

TABLE I

Causes of Dilute Urine in the Dog

Condition	Postulated or known pathogenesis of dilute urine
Overdrinking	Compensatory polyuria
Renal failure	Osmotic diuresis; defective NaCl pump in ascending loop of Henle
Hepatic failure	Decreased urea production; toxic effects of hyperammonemia on tubules
Hyperadrenocorticism	Interference with ADH activity
Diabetes insipidus	ADH deficiency
Nephrogenic diabetes insipidus	ADH—refractory tubules
Pyometra	Endotoxin effects on tubules
Hypercalcemia	Interference with ADH activity; later, renal failure
Pyelonephritis	Unknown
Postobstruction (urinary)	Osmotic diuresis; natriuretic hormone; renal damage
Potassium deficiency	Unknown
Hyperreninemia	Primary polydipsia, secondary polyuria

osmolality in domestic animals is about 280–300 mosmol/kg. Urine values are discussed in Section VII with urine concentration tests.

C. Urine Protein Determination

As previously discussed, a small amount of protein is present in the urine of all species. The amount found in normal urine is determined by the net result of normal leakage through glomerular capillaries, protein reabsorption by the proximal tubules, and addition of proteins to the filtrate as it passes through the tubules, pelvis, ureters, bladder, urethra, and genital tract. It is important to establish whether the degree of proteinuria is normal or abnormal. In the clinical laboratory an effort is made to distinguish normal levels of protein in urine from pathological proteinuria by using semiquantitative tests that detect only pathological proteinuria. A "negative" protein reaction is reported when amounts in urine are not large enough to be indicative of disease. A study of common methods used to detect proteinuria revealed that false-positive and false-negative results were obtained with all procedures (Thysell, 1969). This suggests that a degree of skepticism should exist in interpreting protein results.

Dipstick methods are in common use for the assessment of urine protein in domestic animals. The procedure is dependent on color changes in tetrabromphenol blue at various protein levels. However, pH may also cause color changes. To keep pH constant, the reagent square is impregnated with citrate buffer. In domestic animals, it appears that false-positive values occur frequently. This may be related to the pH dependence of the sticks and leaching of buffer from the strip by prolonged contact with alkaline urine. The sticks are also more sensitive to albumin than to globulins (Bowie *et al.*, 1977), and severe proteinuria due to globulins may give a false-negative reading. Difficulty in interpretation of the color change also appears to be a problem since, in one study, different readings were obtained by different technicians on the same urine (James *et al.*, 1978).

It is advisable to evaluate all urine samples by an alternate procedure if they are positive for protein with dipsticks. The sulfosalicylic acid test and the nitric acid ring test are two semiquantitative methods that may be used. The sulfosalicylic acid test may give false-positive values with X-ray contrast media and large doses of penicillin, however. The Coumassie blue method of protein determination requires the addition of urine to a single reagent and the determination of the optical density of the mixture (Bradford, 1976). It gives quantitative results that may be of value in cases of equivocal proteinuria or in cases in which a value for 24-hour protein excretion is desired. With this method, 95% of 157 dogs without urinary tract disease had urine protein values of less than 150 mg/dl. Ten beagle dogs had 24-hour urine protein output of 70 $\pm$ 49 mg measured by the Coumassie method (Barsanti and Finco, 1979). The specific gravity of urine should be considered when random samples are evaluated. Protein concentration (milligrams per deciliter) times volume per 24 hours gives 24-hour protein output. When 24-hour volume is not measured, urine specific gravity gives a rough idea about volume since the two are usually inversely related. Protein–creatinine ratios in urine may correct for variable volumes on the basis of the following presumptions. Creatinine excretion can be expected to be fairly constant between 24-hour periods except in animals with acute renal dysfunction or severe renal failure. The concentration of creatinine in urine depends on urine volume just as protein concentration does. With 24-hour creatinine excretion being constant, proteinuria can be related to creatinine concentration, and the urine volume factor cancels.

$$\text{24-Hour protein output} = \text{urine volume} \times \text{protein concentration} \quad (1)$$

$$\text{24-Hour creatinine output} = \text{urine volume} \times \text{creatinine concentration} \quad (2)$$

$$\frac{\text{Protein concentration}}{\text{Creatinine concentration}} = n, \text{ regardless of urine volume} \quad (3)$$

This manipulation allows more precise evaluation of protein, or any other urinary constituent, than do random sample determinations of concentration, but it is probably less desirable than 24-hour collections.

Once it is established that the magnitude of proteinuria is abnormal, its source and cause should be considered. Proteinuria in the absence of RBC or inflammatory cells is usually of renal origin. It may be due to a glomerular leak or to a lack of proximal tubular reabsorption, or both. Proteinuria in the presence of RBC and WBC in the urine is difficult to interpret. Hemorrhage is associated with direct leakage of RBC and plasma proteins; the presence of WBC implies inflammatory responses with potential leakage of plasma proteins. When RBC or WBC are present and the protein reaction is positive, the possibility of concomitant disease at separate sites or of differing causes exists. It may be necessary in such instances to eliminate the cause of the WBC or RBC and establish whether proteinuria disappears or persists.

In human beings, proteinuria is reported in such conditions as fever, passive renal congestion, and the biped postural stance in the absence of renal or urinary tract disease (Rubin and Balish, 1971). The effect of such factors in domestic animals has not been reported.

Proteinuria has been classified into types in human beings (Hardwicke, 1975), and the same types exist in domestic animals. Overflow proteinuria occurs as a consequence of increased levels of small molecular weight proteins in the blood. Such proteins pass through the normal glomerular filter and appear in the urine when tubular resorptive mechanisms are saturated. Newborn calves may have proteinuria as a consequence of absorption of proteins of colostrum from the gut into the blood. Small molecular weight components are subsequently lost in the urine. The proteinuria disappears when gut absorption ceases after the first few days of life (Pierce, 1959, 1961). The appearance of Bence Jones light-chain proteins in the urine of animals with multiple myeloma is another example of overflow proteinuria. These proteins may not be detectable by conventional urine protein methods but are detectable by urine elctrophoresis.

Glomerular proteinuria occurs as a consequence of injury to glomeruli. Amyloidosis and glomerulonephritis are two broad categories of disease in domestic animals which cause glomerular proteinuria. The defect in the filter may vary in magnitude among diseases or in the same animal at different stages of disease. Glomerular proteinurias are often relatively selective so that albumin is the main component of the urinary protein. Severe proteinuria in the absence of RBC and WBC is usually of glomerular origin. Tubular proteinuria occurs as a consequence of normal passage of small molecular weight proteins through the glomerular filter combined with defective tubular reabsorption. Proteins in the urine of these patients are predominantly small molecular weight globulins, and amino acids may also be present (Fanconi syndrome). Basenji dogs with glucosuria, aminoaciduria, and proteinuria probably have this type of abnormality. In general, tubular proteinurias result in mild to moderate protein loss (Rubin and Balish, 1971).

A high molecular weight mucoprotein (Tamm–Horsfall mucoprotein) has been isolated from the normal urine of several species, including dogs and cats (Lewis *et al.*, 1970;

Schenk *et al.*, 1971). This mucoprotein is apparently secreted by cells of the ascending loop of Henle and the macula densa portion of the distal tubule. It is believed to be a component of some urinary casts. Its concentration in urine is normally too low to be detected by semiquantitative screening methods.

D. Occult Blood Reactions

The dipstick method used for the detection of blood is highly sensitive and fairly specific. It detects both myoglobin and hemoglobin, however, and false-positive readings may be obtained with H_2O_2 or sodium hypochlorite. The principle of the positive reaction is the peroxidase activity of the porphyrin molecule. Hydrogen peroxide in the strip is converted to oxygen, which then oxidizes *o*-tolidine to cause the formation of a blue color. Microbial peroxidase or oxidizing contaminants may cause false-positive reactions.

Detection of positive occult blood in urine indicates leakage of free hemoglobin through glomeruli (hemoglobinuria), leakage of myoglobin through glomeruli (myoglobinuria), extravasation of RBC directly into the urogenital tract (hematuria), or contaminants previously listed. The size and shape of the hemoglobin molecule are such that a small percentage of hemoglobin in plasma that is unbound to haptoglobin passes the glomerular filter and appears in urine. This occurs in diseases in which intravascular hemolysis is occurring. Hemorrhage anywhere from glomerular capillaries to the tip of the genitalia may cause hematuria; thus, its presence should not be considered a localizing sign.

Differentiating hemoglobin from myoglobin in urine requires a special analysis since sequestered blood may have the same color as myoglobin (Glauser *et al.*, 1972). Differentiating hemoglobinuria from hematuria may be difficult, but the following observations are helpful. With hemoglobinuria, plasma may also be observed to have hemolysis. The urine may be clear red with hemoglobinuria but cloudy red with hematuria. The urine sediment may have no RBC or a disproportionately low number of RBC with hemoglobinuria. Care must be exercised to distinguish intravascular hemolysis from hemolysis of RBC once they are in urine. Red cells may hemolyze in urine of low osmolality (Vaughan and Wyker 1971). Hemolysis is reported to occur in urine with specific gravity readings below 1.005, but it may also occur in more concentrated specimens depending on the permeability of RBC to the urine solutes. In cases of hemolysis of RBC within urine, RBC "ghosts" (cell membrane) are often discernible in the urinary sediment when it is examined microscopically. Dipstick methods of detecting hemoglobin are more sensitive to free hemoglobin than to intact RBC. For this reason, the degree of hemolysis in the urine may influence the magnitude of the reading (Vaughan and Wyker, 1971).

E. Glucosuria

Dipstick procedures use the glucose oxidase method of glucose measurement. This enzyme converts glucose to gluconic acid. In the presence of hydrogen peroxide and peroxidase incorporated into the strip, an indicator in the strip (*o*-tolidine) is oxidized to cause a color change. While the method is specific for glucose compared to other sugars, contamination of urine with H_2O_2 or sodium hypochlorite can cause false-positive reactions. Conversely, ascorbic acid in urine may prevent positive samples from giving a positive reaction (Mayson *et al.*, 1972).

The strip is not sufficiently sensitive to detect the small quantity of glucose found in the

urine of normal animals but gives positive results when glucose concentration is 10 mg/dl or more (Mayson *et al.*, 1972).

Glucosuria occurs when the renal tubular maximum for reabsorption from filtrate is exceeded. This may occur with any cause of hyperglycemia or with decreased tubular glucose reabsorptive capacity. Generally, hyperglycemia greater than 180 mg/dl results in glucosuria, but flow rate of fluid through the tubules and nephron heterogeneity with regard to resorptive capacity are also factors. Common causes of hyperglycemia sufficient to cause glucosuria are parenteral therapy with dextrose and diabetes mellitus. Any cause of the Fanconi syndrome may result in glycosuria during normoglycemia since faulty renal tubular reabsorption is the offender in this syndrome.

F. Urine pH

The pH of urine is usually determined by renal regulation of blood bicarbonate and H^+ levels. Since pH is a measure of free ions, the actual amount of acid being excreted by the kidney depends not only on pH, but on the amount of undissociated hydrogen present in buffer salts. The titratable acidity of urine is defined as the amount of base required to titrate urine back to the pH of blood. This value gives an estimate of hydrogen ion excretion, while urine pH does not. Nevertheless, measurement of urine pH is useful since unusual results provide one with insight concerning many abnormalities. The urine pH of domestic animals usually is determined by diet. Animals on diets of high animal protein content usually have acidic urine, while those on cereal diets or most forages have neutral or slightly alkaline urine. The extremes of urine pH values for mammalian kidneys are 4.5–8.5.

Urine pH becomes more acidic when a large acid load is imposed upon the extracellular compartments. The extracellular fluid and blood pH may return to normal if urinary excretion of acid is adequate, but acidemia may exist if the acid load was greater than the ability of the kidney to excrete H^+. Conversely, a highly alkaline urine should be interpreted as an indication of an extracellular H^+ deficit which may or may not be associated with alkalemia. Alkaline urine may also be due to the production of ammonia by urea-splitting microbes that gained access to the urine either within or outside the body. Alkaline urine or less acidic urine also has been associated in several species with an "alkaline tide" or alkalemia which follows food ingestion and excretion of H^+ by the stomach.

The limits in deductions that can be made about systemic acid–base balance from urine pH measurements must be emphasized. Although urine pH values that are not extremely acid or alkaline are expected in normal domestic animals, the presence of such values does not provide assurance that body acid–base balance is normal. Impaired ability of the kidney to excrete H^+ can lead to acidosis, while values for urine pH are not unusual. With chronic generalized renal failure, urine pH is usually moderately to markedly acidic when acidosis is present.

Proximal renal tubular acidosis and distal renal tubular acidosis are two groups of diseases of human beings that probably exist in animals. In proximal tubular acidosis, renal reabsorption of bicarbonate is faulty. This may initially cause alkaline urine, but it soon becomes acidic as plasmic bicarbonate levels fall and as normal distal H^+ secretion persists. Urine pH in such patients is markedly acidic. With distal tubular acidosis, proximal bicarbonate reabsorption is normal, but distal acidification is faulty, so that urine pH is acidic but greater than 5.3 (Thier *et al.*, 1977).

Urine pH may be altered by bacterial contamination after it is procured. Acidification or alkalinization may occur depending on the end products of metabolism of the contaminating organism. Urine pH does not change in sterile urine stored at room temperature for several hours (Finco, 1979).

Aciduria during alkalemia occurs in the vomiting dog; this paradox may be associated with anion reabsorption, as previously mentioned.

G. Acetonuria

Ketone bodies refer to acetoacetate, β-OH-butyrate, and acetone. These compounds are products of fat degradation; the quantity produced is markedly increased under conditions in which oxidation of fats provides the bulk of the energy. Ketone bodies appear in filtrate and are reabsorbed by the tubules until the T_m is exceeded. Under normal conditions, very low plasma levels of ketone bodies exist, and they are absent from the urine. Species differences exist regarding circumstances under which ketone bodies appear in the urine. They are commonly found in the ruminant during starvation, but rarely in the dog and cat. Diabetes mellitus is the disease classically associated with ketonuria.

The urine ketone reaction is based on the reaction of acetoacetic acid or acetone with nitroprusside. No reaction occurs with β-OH-butyrate. False-positive reactions may occur with BSP, phenyl ketones, or L-dopa metabolites.

H. Bile Pigments in Urine

The interpretation of bilirubinuria or urobilinogenuria is discussed in Chapter 6.

I. Urine Sediment

1. General Considerations

The examination of urine sediment is the single most important part of urinalysis. Because it is slightly more time-consuming than the dipstick analysis, there may be a tendency among veterinarians to perform only dipstick tests and specific gravity determinations. This approach is inexcusable considering the value of sediment examination and the false sense of security that is left when urinalysis is reported to be done but sediment is not examined.

Quantitative overinterpretation of the sediment is another problem. Although the technician reports many microscopic findings numerically as cells, casts, etc., per low-power or high-power field, the numerous variables that influence these counts must be kept in mind. Early studies of urine sediment in human beings were made to determine 24-hour urinary content of various elements. Such studies have not been reported in domestic animals, and so our concept of normal versus abnormal is based on the examination of random samples of urine and the interpretation of normal on semiquantitative bases. Some of the variables that influence the concentration of elements in urinary sediment include the volume of urine used, how that volume relates to 24-hour urine production, the period of time between formation of urine and its examination (some components disintegrate), the speed and time of centrifugation, the volume used to resuspend the sediment for viewing, the volume of suspension used to prepare the slide, and the thickness of the slide preparation. Each laboratory must standardize its procedure and define the limits for normal with those procedures in order to obtain meaningful results. An acceptable proce-

dure is to centrifuge 10 ml or urine in a conical tube for 5 minutes at 700 g. Although the speed of centrifugation for urine sediment examination is often stated as revolutions per minute, this value is meaningless unless the length of the centrifuge arm is known. The supernatant fluid is removed except for 0.5 ml, which is used to gently resuspend the sediment. One drop is placed on a cleaned glas slide, and a 22-mm square coverslip placed on the drop. After 1 minute to allow for sedimentation, the slide is scanned under low power ($\times 100$) with reduced light. Final identifications are usually made at $\times 440$ under reduced light. At least ten fields should be counted and numbers of elements averaged (Duncan and Prasse, 1976). Several stains for urinary sediment are available commercially. The experienced technologist generally finds these stains unnecessary, but they may be helpful to the novice. New methylene blue stain may be used by mixing a drop directly with the sediment. However, cellular elements do not always take up the stain. Heat-drying of the slide with subsequent application of stain gives a better stain. Neither type of stained slide should be used for semiquantitative measurements since the concentration of components in the sediment has been altered by the manipulations.

2. *Epithelial Cells*

These cells are normal components of urine sediment. Their number may vary depending on the presence of inflammation or degeneration, neoplasia, and the method of urine collection. The concept that the site of origin of epithelial cells can be deduced from their morphological character appears to have been borrowed from human medicine. Small and cuboidal epithelial cells are from the bladder, and squamous cells from the lower urethra and genital organs (Osbaldiston, 1970). The validity of these observations has not been proved in domestic species.

Epithelial cells in sediment have little diagnostic significance in themselves. It is necessary to correlate their presence with other findings in the urinalysis or with other studies in the animal.

3. *Casts*

Casts are cylindrical structures that are formed from cells, cell fragments, and macromolecules that are present in the lumens of renal tubules. They conform to the shape of the lumen from which they originate. The total composition of various types of casts is still the object of study. When relatively intact RBC, WBC, or epithelial cells make up the bulk of the cast, they are identified by reference to these cells. Hyaline casts are clear, structureless, colorless objects barely discernible from the background. Immunofluorescence studies have revealed that hyaline casts from human patients are made up of Tamm–Horsfall mucoprotein but do not contain serum proteins (Orita *et al.*, 1977). Granular casts are homogeneous in appearance except for the presence of small (fine) to large (coarse) granules. Immunofluorescence studies have revealed that the granules are composed of fractions of various serum proteins (Rutecki *et al.*, 1971; Orita *et al.*, 1977). These findings contradict those of others, who concluded that granules in casts were remnants of tubular epithelial cells (Relman and Levinsky, 1971). Waxy casts are similar to hyaline casts except that they are distinctive because of a hard, refractile outline. They are believed to be degenerative stages of cellular casts.

The size of casts appears to be related to their site of formation. Small-diameter casts are probably of more proximal origin, while broad casts are likely to be of collecting duct origin.

Some of the conditions conducive to cast formation have been identified. Tamm–Horsfall mucoprotein is readily precipitated by highly acidic urine pH and by high electrolyte concentrations (McQueen and Engel, 1966). Thus, casts are often formed in acidic urine and may be dissolved *in vitro* by alkaline urine. Rate of urine flow also seems to be a factor, with slow flow in the distal segments of the nephron favoring cast formation.

The presence of casts in the urine is interpreted as an indication of abnormal concentrations of their precursors in the tubular lumens. Thus, proteinuria, pyuria, hematuria, and cell desquamation of renal origin may provide the material for cast formation. Urine flow rate and pH are factors which interact with these components.

A few casts per low-power field are considered normal. Data for domestic animals are not available, but in normal human beings 5000–10,000 casts are excreted per 24 hours (Relman and Levinsky, 1971). Most of the casts normally found are of the hyaline type.

4. Crystals

Several types of crystals may appear in the urine of domestic animals. These may be categorized as one group which are formed from normal components of urine and another

TABLE II

Components of Urinary Sediments

- Cells
  - Epithelial cells (normal, neoplastic)
  - RBC
  - WBC
- Crystals
  - Magnesium ammonium phosphate
  - Urate
  - Ammonium biurate
  - Oxalate
  - Cystine
  - Bilirubin
  - Tyrosine
  - Drugs (sulfonamides)
- Casts
  - Granular
  - Cellular (epithelial, RBC, WBC)
  - Hyaline
  - Fatty
  - Waxy
- Miscellaneous
  - Lipid droplets
  - Spermatozoa
  - Mucous threads
  - Amorphous material
- Infectious agents
  - Bacteria
  - Mycotic agents
  - Parasites, parasite ova
    - *Capillaria*
    - *Stephanurus*
    - *Dioctophyme*
    - *Dirofilaria microfilaria*
- Physical contaminants

group which originates from exogenous compounds, abnormal metabolism, or abnormal excretion.

The formation of crystals from compounds normally in the urine occurs as a consequence of physiochemical factors, such as changes in urine concentration or pH. Many components of urine are held in colloidal suspension; disruption of the colloid results in precipitation of its components. Phosphate crystals in dogs and cats and carbonate crystals of cattle and horses fall in this category. They reflect conditions of urine formation and should not be interpreted as abnormal. Since Dalmatian dogs normally excrete urates as well as allantoin as end products of purine metabolism, urate crystals are also normal for this species. Bilirubin crystals may also be normal in concentrated specimens.

Abnormal crystalluria usually is indicated by the presence of the remaining crystals listed in Table II. Ammonism biurate and tyrosine crystals are observed in canine urine in association with hepatic dysfunction, oxalate crystals may be observed in some cases of ethylene glycol toxicity, and sulfonamide crystals may be observed after therapy with these drugs.

5. *Miscellaneous Components*

Other constituents of urine sediment that are considered normal include lipid droplets, which are particularly common in dog and cat urine. Spermatozoa are normal in urine from the male and may be present in urine obtained from dogs by cystocentesis (Hubbert, 1972). Mucous threads and amorphous debris vary considerably from one urine sample to another within a species. No specific pathological significance seems to be attached to their presence.

6. *Infectious Agents*

Infectious agents may appear in the urine as a consequence of contamination of normal urine during collection, infection localized to the genitourinary tract, or occasionally polysystemic infection which includes the urinary tract.

The presence of bacteria in urinary sediment must be interpreted with caution because of possible contamination of urine during or after procurement. False-negative results from examination of urine sediment for bacteria may occur with cocci infections since Brownian movement may be confused with the bacteria. Mycotic agents in urine are usually contaminants or evidence of polysystemic infection.

Parasite ova in urine usually present no diagnostic problem. Occasionally, ova of enteric origin may contaminate voided urine and must be distinguishable from the parasites listed in Table II.

VII. TESTS OF RENAL FUNCTION

A. Tests for Azotemia

Historically, BUN, SC, and nonprotein nitrogen determinations have been used as indices of retention of nitrogenous wastes by the kidney. The latter has fallen in disfavor, and at present BUN and SC are used exclusively. An understanding of the genesis, metabolism, and excretion of urea and creatinine should aid in their assessment as renal function tests. The subsequent discussion summarizes information obtained in domestic animals and man.

1. Urea Metabolism

Small quantities of urea are ingested in animal foodstuffs because urea is present in all tissues of mammals. Urea also enters the gut in pancreatic secretions (Altman and Dittmer, 1971) and secondary to water movement into the intestines. Little or no urea is found in the stool of azotemic human beings (Brown *et al.*, 1971), so urea is either well absorbed or metabolized as it passes through the gastrointestinal tract.

Urea synthesis provides a mechanism for excretion of ammonia during catabolism of amino acids. The urea cycle incorporates two molecules of ammonia into each urea molecule. Formation of urea is an energy-requiring reaction, which occurs almost exclusively in the liver (White *et al.*, 1973). Although apparently rare in domestic animals, biochemical defects in the urea cycle were found in a dog with signs of hyperammonemia (Strombeck *et al.*, 1975).

The rate of urea formation depends on the rate of protein (i.e., amino acid) catabolism. An increase in BUN may reflect an accelerated rate of protein catabolism rather than decreased urinary excretion of urea. In a study involving 14 clinically normal dogs fed diets of known protein content for 3 days, BUN decreased from 16.0 mg/dl with a 8.5% protein diet to 13.7 mg/dl with a 5% protein diet. Preprandial and 4-hour postprandial studies indicated that BUN increased from 13.7 to 16.0 mg/dl with the 5% protein diet and from 16.0 to 26.8 mg/dl with the 8.5% protein diet (Anderson and Edney, 1969). In another study, BUN was elevated for 10–18 hours after food ingestion (Street *et al.*, 1968). The degree of elevation of BUN in these studies was probably not clinically important since the normal canine range (10–30 mg/dl) was not exceeded. However, higher-protein diets could feasibly cause clinically important increases, and thus an 18-hour fast has been recommended to eliminate the influence of diet on BUN (Street *et al.*, 1968). Consideration should be given to the likelihood that the patient with marginal renal function and impaired urea clearance is more likely to have an increase in BUN as a result of diet. For this reason, patients with an elevation of BUN that decreases into the normal range with fasting should be scrutinized for renal dysfunction with more sensitive tests.

Any process inducing protein catabolism can result in an increased BUN. In man, specific causes include (1) hemorrhage into the small bowel with digestion, absorption, and catabolism of amino acids; (2) fever; (3) burns; (4) corticosteroid administration; (5) starvation; (6) infection; and (7) tetracycline administration (Dossetor, 1966; Kopple and Coburn, 1974; Shils, 1963). The magnitude of increase in BUN associated with enteric hemorrhage is apparently related to the amount of hemorrhage. Hypovolemia associated with hemorrhage or dehydration may lead to concomitant prerenal retention of nitrogenous wastes, as previously discussed (Section III). Fever apparently results in increased BUN because of increased tissue catabolism. The magnitude of increase in BUN with burns is infrequently stated, but one human being with 50% third-degree burns had a BUN of 60–80 mg/dl coincident with a normal SC (Dossetor, 1966).

The effect of corticosteroids on BUN was determined in a study comparing azotemic human renal transplant recipients given prednisolone with chronic renal failure patients of comparable dysfunction who had not been given corticosteroids. The SC was from 2 to more than 5 mg/dl in both groups. Patients treated with an average of 70 mg prednisolone per day had BUN values from 1.5 to 2 times that of azotemic patients not given corticosteroids (Kopple and Coburn, 1974).

Starvation was reported in a man in which SC was 0.7 and BUN was 250 mg/dl. The BUN decreased to normal with an adequate diet (Kumar *et al.*, 1972).

Administration of tetracycline to azotemic human beings was followed by acidosis, anorexia, vomiting, and weight loss. The BUN and serum phosphorus concentration increased, but SC remained unchanged. Patients without renal compromise given tetracycline at the same dosage did not develop these symptoms. Because SC was not altered by tetracycline in the azotemic group, it was concluded that the increase in BUN was due to protein catabolism rather then nephrotoxicosis (Shils, 1963). Tetracycline (28 mg/kg BID) was administered for 5 days to a dog with a stable level of azotemia (BUN 80 mg/dl; SC 3.5 mg/dl). No change in degree of azotemia was detected in samples taken every other day for 2 weeks (Finco, 1979). Comprehensive studies are needed to evaluate the effect of tetracycline on domestic animals with azotemia.

Nonrenal factors reported to cause decreased BUN values are (1) anabolic steroids, (2) diminished protein intake, and (3) severe hepatic insufficiency. Anabolic steroids apparently prevent the increase in BUN associated with the use of tetracycline in man (Shils, 1963).

The ingestion of decreased quantities of high-quality protein results in decreased BUN because nearly all ingested protein is used for protein synthesis. However, inadequate caloric intake coincident with low protein intake may not result in decreased BUN because the protein ingested may be catabolized for energy. Conversely, intravenous administration of large quantities of amino acids and adequate amounts of nonprotein sources of energy to azotemic human beings and dogs results in anabolism and decreased BUN (Abel *et al.*, 1973; Van Buren *et al.*, 1972). Thus, the fate of amino acids (i.e., protein anabolism versus catabolism) as well as their quantity must be considered in relating diet to BUN.

Severe hepatic insufficiency causes decreased BUN, apparently because of impaired urea synthesis.

Acid–base status may influence the rate of BUN increase in acute renal failure. Experiments in rats made azotemic by ureteral ligation revealed that those in which acidosis was prevented (blood pH 7.32) by treatment with sodium bicarbonate had BUN values of 233 $\pm$ 8.3 mg/dl. Untreated rats and physiological saline-treated rats had blood pH values of 7.04 and BUN values of 299 $\pm$ 17 and 303 $\pm$ 18 mg/dl, respectively (Simon and Luke, 1971). The mechanism responsible for this significant difference was not investigated.

Vigorous prolonged exercise in human beings (cross-country ski racing) caused about a 60% increase in BUN. During racing, production rates of urea increased 60–80% over resting values, and urinary excretion of urea was reduced (Refsum and Stromme, 1974).

Once formed, urea is distributed via the vascular system and passively diffuses throughout total body water. Urea administered to nephrectomized dogs reached equilibrium with body fluids within 1½ hours (Schloerb, 1960). Brains of dogs with induced acute uremia had equal blood and brain concentrations of urea (Arieff *et al.*, 1975), indicating that urea passes the blood–brain barrier in the azotemic patient.

2. Urea Excretion

Renal excretion of urea is quantitatively the most important, but routes of excretion of lesser importance have been identified. In clinically normal human beings, studies with isotope-labeled urea indicated that at least 25% of the urea synthesized is subsequently degraded by enteric organisms (Walser and Bodenlos, 1959). Bacterial ureases catalyze the breakdown of urea to ammonia and carbon dioxide. When enteric bacteria were killed by oral administration of neomycin, nearly all ^{14}C-labeled urea given orally was recovered

in the urine. In azotemic human beings, the number of strains of anaerobic bacteria that produce urease increases as BUN increases, suggesting that enzyme induction occurs (Brown *et al.*, 1971). The hydrolysis of urea, and presumably hydrogen ion trapping by ammonia, results in a pH of the colon that is higher in acidotic, uremic human beings than in clinically normal human beings (Brown *et al.*, 1971). It seems likely that the same phenomena occur in other omnivores and carnivores.

Although these findings would imply that the gastrointestinal tract serves as a significant route of exit of urea from the body, such is not the case. Nearly all ammonia from bacterial degradation is used to resynthesize urea in the liver. Thus, a futile cycle occurs, and enteric loss of urea because of bacterial degradation is minimal (Mitch *et al.*, 1975).

Such a futile cycle does not occur during ruminant digestion. Ammonia in the ruminants derived from urea may be utilized to form amino acids from keto and hydroxy acid precursors that are normal products of digestion.

In man, small quantities of urea may be lost via perspiration (Altman and Dittmer, 1971). Prior to the use of hemodialysis, induction of profuse sweating was considered an adjuvant treatment of uremia (Sobel, 1964). Because of poorly developed sweat glands in the dog and cat, urea loss by perspiration is probably negligible.

Severe vomiting and diarrhea have been suggested as mechanisms by which urea may be lost from the body (Dossetor, 1966). Quantitative data on loss by these routes are not available.

The concept that low-protein diets decrease the work load on the kidney by decreasing the amount of urea to be excreted is incorrect, because urea excretion is a passive phenomenon. Urea appears in glomerular filtrate in the same concentration as in plasma as a result of simple filtration through the glomerular basement membrane (Smith, 1951). Energy for filtration is from hydrostatic pressure of cardiac origin rather than renal metabolism. As urea traverses the renal tubules, it diffuses passively according to concentration gradients. Because large amounts of water are reabsorbed as glomerular filtrate passes through the tubules, there is passive diffusion of urea with water from the tubule lumens back into the blood. Because of physicochemical factors, urea diffuses through biological membranes less readily than does water. Consequently, the amount of urea reabsorbed by renal tubules usually is proportionately less than the amount of water and is related to the rate of water movement through the tubules. More rapid passage of fluid allows less reabsorption of urea. At the highest urine flow rate attainable in the dog, about 40% of filtered urea is reabsorbed (Shannon, 1936). As urine flow rate decreases, more urea is absorbed. Urea that is reabsorbed diffuses into the interstitium and then into the general circulation via the renal vasculature. Its role in the interstitium as a urine-concentrating agent is discussed in Section II.

In summary, urea is freely filtered through the glomerulus, passively diffuses from the tubules back into the body at a rate dependent on flow rate through the tubules, and is excreted by mechanisms requiring no energy expenditure by the kidneys.

The term "blood urea nitrogen" is ingrained in the medical literature despite the fact that serum or plasma may be used for the analysis. Although "serum urea nitrogen" or "plasma urea nitrogen" would more precisely describe such analyses, urea has the same concentration in RBC as in serum or plasma. The difference in values for the three methods is small, represented only by the mass of hemoglobin and RBC membranes.

Caution should be exercised in interpreting British literature since values for blood urea rather than BUN may be used. Blood urea and BUN levels are related on the basis of

molecular weight (60 versus 28, respectively). The BUN values are only 0.47 of blood urea levels.

3. Creatinine Metabolism

Small quantities of creatinine are ingested by animals consuming diets containing animal tissues since creatinine is distributed throughout body water. Muscle tissue contains phosphocreatine, which undergoes spontaneous cyclization with loss of inorganic phosphate to form creatinine. Creatinine is apparently well absorbed from the intestinal tract (Goldman, 1954).

Most of the creatinine eventually excreted originates from endogenous creatine. The amino acids arginine and glycine combine to form guanidinoacetate in the pancreas, kidneys, and small intestine. In the liver methionine provides a methyl group for conversion of guanidinoacetate to creatine (Tyler, 1972). Creatine circulates in the plasma and is taken up by muscle, where it stores energy in the form of phosphocreatine. The serum concentration of creatine in man is about 0.45 mg/dl, but values for domestic animals are not available (Altman and Dittmer, 1971).

Creatine undergoes no catabolic reactions other than decomposition to creatinine (Bloch *et al.*, 1941). Creatine is excreted in small quantities in the urine of man (Bleiler and Schell, 1962; Bloch *et al.*, 1941; Hoberman *et al.*, 1948), but older literature indicates that large quantities of creatine are present in the urine of the dog (Hawk, 1910; Van Pilsum, 1959). Another study indicates that creatine clearance in the dog approaches zero at low plasma concentrations (Pitts, 1934). These conflicting data indicate that conclusions concerning urinary excretion of creatine by the dog cannot be made. This is important since creatine is the sole precursor of creatinine; an understanding of creatine excretion is needed to evaluate its effect on creatinine metabolism.

Creatine conversion to creatinine is a nonenzymatic, irreversible process that occurs at a rate of 1.6–2% daily (Bloch *et al.*, 1941; Hoberman *et al.*, 1948). Creatinine is distributed throughout body water, but it diffuses more slowly than urea and requires about 4 hours for equilibration in the dog (Schloerb, 1960). The quantity of creatinine formed each day depends on the total body content of creatine, which in turn depends on dietary intake, rate of synthesis of creatine, and muscle mass. Dietary intake of creatine may inhibit its endogenous synthesis (Walker, 1960, 1961). Sex differences in muscle mass are probably not significant in the dog and cat but could be relevant in other domestic species, such as cattle. Other factors, such as disease of muscle, tissue wasting, and character of muscle as influenced by physical training, may also affect the size of the creatine pool and thus the daily production of creatinine. As a consequence of the latter factor, a working animal is likely to excrete more creatinine in the urine than a sedentary one of comparable size.

Even in patients with normal renal function, a direct relationship seems to exist between daily creatinine production and SC. The SC level is 30% higher in clinically normal human males than in females (Doolan *et al.*, 1962). An SC concentration of 1.5 mg/dl may be normal in a muscular working animal but may indicate impairment of function in a sedentary one. Withdrawal of meat from the diet of clinically normal human beings for a period of 6 weeks resulted in a decrease in SC of about 25% (Bleiler and Schell, 1962). The decrease in SC associated with loss of muscle mass was termed "significant" (Doolan *et al.*, 1962; Newman, 1971), but specific values were not given.

Severe, prolonged exercise of man caused an increase in SC and BUN of about 60% (Refsum and Stromme, 1974). Because urine creatinine excretion during the exercise period was normal, it was concluded that the increased SC was due to increased creatinine production.

Some diurnal variation in SC occurs in human beings. In one study afternoon samples were about 10% higher than morning values (Doolan *et al.*, 1962).

4. Creatinine Excretion

The concentration of creatinine in sweat ranges from 0.1 to 1.3 mg/dl (Altman and Dittmer, 1971). Lowered ratios of BUN to SC in uremic human beings who are vomiting profusely (Dossetor, 1966) suggests proportionately less creatinine than urea in vomit, but quantitative studies have not been reported. Creatinine is absent from the feces of uremic human beings, probably because of intestinal reabsorption or bacterial metabolism or both. Bacterial metabolism of creatinine appears to be a major method of removal from the body. Colonic flora of the rat can be induced to catabolize creatinine (Jones and Burnett, 1972). Isotope studies in azotemic human beings revealed that 15.9–65.7% of the creatinine was metabolized or excreted via nonrenal routes (Jones and Burnett, 1974). Metabolic products identified were CO_2 and methylamine. Evidence for recycling of components of creatinine breakdown are lacking.

In all mammalian species, creatinine is freely filtered through the glomerulus and appears in glomerular filtrate in the same concentration as plasma (Smith, 1951). Species differences exist with regard to tubular action on creatinine. In man, creatinine is secreted by renal tubules. In the dog, an extremely weak proximal tubular secretory mechanism exists for creatinine (O'Connell *et al.*, 1962; Swanson and Hakim, 1962). The mechanism is slightly stronger in the male. In dogs with reduced renal mass, simultaneous determination of inulin and creatinine clearance revealed that the latter is a valid method for ascertaining GFR in the female but slightly overestimates GFR in the male (Robinson *et al.*, 1974).

Evaluation of the mechanism of renal excretion of creatinine in the cat gave equivocal results in one study, but it was concluded that creatinine was probably excreted only by glomerular filtration (Eggleton *et al.*, 1951).

Endogenous creatinine clearance is nearly the same as inulin clearance in horses, cows, and swine (Osbaldiston, 1970), but critical evaluations of the handling of creatinine have apparently not been made in those species.

5. Precision of BUN and SC Measurement

Two general techniques are used for urea analysis (Davidsohn and Henry, 1969). With enzymatic methods, urea is converted to ammonia by urease. The ammonia concentration is measured by a color reaction. These methods are specific because urease acts only on urea. Free blood ammonia concentration does not interfere since it is present in such low concentration compared to urea. The other general method of urea analysis is based on the formation of colored products from direct combination of urea with compounds such as diacetylmonoamine. This method is less specific, and dibasic amino acids may also cause a color reaction. With spectrophotometric methods of urea analysis, the two methods give comparable results when BUN is $\leqslant$100 mg/dl. Above 100 mg/dl, results with the diacetylmonoxamine method are 5–15% higher. A dipstick method of estimating blood

urea by the urease method is available, but the accuracy is poor. It has been recommended that dipsticks be used only for emergency analyses and that samples reading greater than 20 mg/dl be analyzed by more reliable methods (Bold *et al.*, 1970).

Clinical measurement of SC involves a reaction between alkaline picrate solution and creatinine. Several products of the creatinine–picric acid reaction have been proposed (Blass *et al.*, 1974). Unfortunately, the reaction is not specific; over 50 compounds interfere, including glucose, fructose, ascorbic acid, hippuric acid, protein, urea, ketones, histidine, arginine, glycocyamidine, and pyruvic acid. Interfering compounds are collectively called noncreatinine chromogens (Henry, 1964; Newman, 1971; Narayanan and Appleton, 1972; Blass *et al.*, 1974).

More specific methods of measuring SC have been devised. A clay (Lloyd's reagent) can be used to selectively absorb creatinine prior to reaction with alkaline picrate (Henry, 1964). Using Lloyd's reagent, studies on dog sera revealed that creatinine accounted for only 50% of the color developed with alkaline picrate (Balint and Visy, 1965). In another study, SC determined using Lloyd's reagent was 0.67 ± 0.16 mg/dl in 27 normal dogs (Finco, 1971b), compared to values of 1–2 mg/dl given as normal (Coles, 1967). Unfortunately, the procedure utilizing Lloyd's reagent is not adaptable to automated equipment. Although a cation-exchange method has been devised for removal of noncreatinine chromogens by automated equipment (Polar and Metcoff, 1965) it is not in common use. Consequently, nearly all SC data from commercial laboratories are obtained by the nonspecific alkaline picrate method.

A specific chemical method for creatinine determination has been reported (Van Pilsum, 1956). However, this method is too complicated for use in clinical laboratories.

Noncreatinine chromogens do not appear in the urine of the dog (Balint and Visy, 1965). For this reason, alkaline picrate methods apparently give accurate results for urine creatinine measurements (Narayanan and Appleton, 1972). The absence of urinary excretion of noncreatinine chromogens is important for two reasons. First, creatinine clearance in animals is erroneous if both urine and serum are analyzed by the alkaline picrate method. Urine creatinine concentration U_c is accurately measured, but plasma creatinine concentration P_c is erroneously high. Since the U_c/P_c ratio is calculated in making clearance calculations, results for clearance will be low. Second, it indicates that extrarenal mechanisms exist for the elimination of noncreatinine chromogens. This implies that noncreatinine chromogen concentration in blood does not increase in parallel with creatinine during the progression of renal dysfunction. Consequently, SC determined by the alkaline picrate method becomes a progressively more accurate estimate of actual SC concentration as the degree of renal dysfunction increases.

6. Interpretation of BUN and SC Values

Both BUN and SC are relatively insensitive in detecting renal dysfunction. About 75% of the nephrons must be nonfunctional before values outside the normal range are obtained (Bernstein, 1965). There are no data to indicate that either compound is more sensitive than the other in detecting renal dysfunction.

Normal values for each laboratory should be established in order to interpret results properly. Generally, normal BUN values are 10–30 mg/dl, and SC levels are 1–2 mg/dl (including noncreatinine chromogens) for domestic animals.

In most domestic species, serum concentrations of both urea and creatinine are crude estimates of glomerular filtration rate (Fig. 4). On the basis of the mechanism of renal

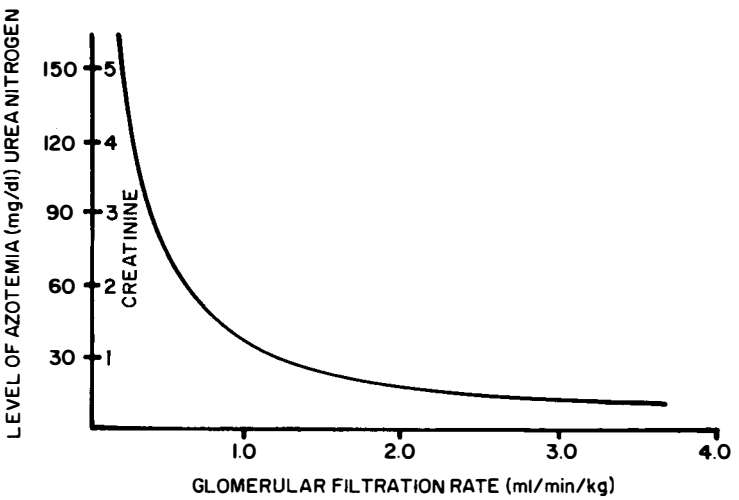


Fig. 4. Schematic representation of the relationship between azotemia and glomerular filtration rate. Notice that values for blood urea nitrogen and serum creatinine concentrations do not exceed the normal range until renal dysfunction is marked. Blood values theoretically double with each halving of function. The relationship between urea nitrogen and creatinine values in this figure should not be interpreted strictly since several factors independently affect blood levels of each (see text).

excretion of the two compounds, creatinine is a better indicator of GFR. However, studies in man indicate that SC does not increase in proportion to the decrease in creatinine clearance (Dooland *et al.*, 1962; Enger and Blegen, 1964; Goldman, 1954). This discrepancy may be attributed to the nonrenal losses of creatinine previously discussed and suggests that nonrenal losses increase progressively as the degree of azotemia increases. On a practical basis, these findings indicate that a patient with SC of 3 mg/dl probably has many more than twice as many functional nephrons as a patient with SC of 6 mg/dl. It may also mean that two patients of the same size with the same SC may have different creatinine clearance values. The same statements are valid for comparison of comparable BUN values and emphasize that measurement of serum levels alone is not a precise method of assessing existing renal function.

In man it is reported that BUN correlates more directly with symptoms of uremia than SC. Patients with a high BUN are more likely to have uremic symptoms than patients with a low BUN, even when the two groups have similar SC values (Kassirer, 1971).

Levels of BUN and SC should be interpreted while considering two types of errors. One error is concluding that renal disease does not exist when it actually does. Because of factors previously discussed it should be apparent that animals with renal dysfunction approaching, but less than, 75% may not have elevated BUN and SC measurements, and thus errors in interpretation would be made. Errors of misinterpretation associated with diagnosis of disease when it does not exist probably are also made. Studies in animals to evaluate the incidence of such errors have not been carried out. A study of human patients referred to a nephrology service was conducted to compare GFR with BUN and SC values. In this study, normal BUN and SC values were found in patients proved abnormal by GFR measurements in 26 and 22% of the evaluations, respectively. Abnormally high BUN and SC values were found in patients with normal renal function in 9 and 4% of the patients, respectively (Rickers *et al.*, 1978). While it cannot be stated with certainty that

the same percentages of error are encountered with the use of BUN and SC in domestic animals, it is likely that they do. The error of false-positive values occurs with readings that are slightly above the normal range. Other, more sensitive tests of renal function are required to resolve the issue in such cases.

Some effort has been made to use BUN-SC ratios to extract information on the pathogenesis of azotemia in human beings. Studies in the dog revealed that BUN-SC ratios were of no value in differentiating prerenal, renal, or postrenal failure or in differentiating acute renal failure from chronic renal failure (Finco and Duncan, 1976). Because ruminant microflora may metabolize a greater percentage of urea than do enteric flora in nonruminants, BUN values may not increase proportionately to SC levels in renal failure in these species until anorexia occurs. This phenomenon should be recognized in evaluating the level of dysfunction but does not appear to be of further significance.

B. Urine Concentration Tests

Patients with polydipsia and polyuria may have a renal defect in urine-concentrating ability. Nonrenal diseases may also affect the renal concentrating mechanism. In the case of diabetes insipidus, ADH deficiency is the problem. In other conditions, such as hyperadrenalcorticism, the exact nature of the tubular aberration is not known. The foregoing categories of disease (generalized renal versus primary nonrenal) are ones in which the polyuria is primary and the polydipsia is a compensatory mechanism. Primary polydipsia with compensatory polyuria may also occur in domestic animals. With this situation, polyuria ceases when water intake is restricted. In contrast, animals with primary polyuria have impaired ability to concentrate urine even when challenged to do so. For the nephrologist, it is unfortunate that failure to respond to stimuli for concentration of urine is not specific for renal failure but includes many other diseases as well (Table I).

1. Indications for Urine Concentration Tests

When polydipsia and polyuria exist, a urine concentration test may distinguish the primary factor from the compensatory one. For the diagnosis of generalized renal disease, the test is indicated when polyuria but not azotemia is present. As indicated in Section V, polyuria usually precedes azotemia during the progression of chronic renal failure. Urine concentration tests serve no purpose in animals with unequivocal azotemia since renal dysfunction has already been established.

2. Types of Urine Concentration Tests

Stimulus for the production of a concentrated urine may be elicited by inducing endogenous secretion of ADH by witholding water. This occurs in dogs with a body water deficit of 2–3% (Pitts, 1974). Both abrupt and gradual water deprivation tests have been described. The gradual deprivation test has been advocated because of concern that any cause of polyuria may secondarily affect the concentrating mechanism by causing medullary washout of solute. Under such circumstances, production of concentrated urine would be impossible until the medullary hypertonicity were reestablished.

The amount of time required for the normal kidney to reestablish medullary hypertonicity is not well defined in domestic animals. One study in dogs found that repletion following water diuresis had not occurred after 10 hours (Boylan *et al.*, 1962). In dogs, washout reported as a consequence of furosemide occurred within 5 minutes of intra-

venous administration of the drug. On the other hand, not all causes of polyuria are associated with medullary washout. Dogs with induced diabetes insipidus maintain a degree of medullary hyperosmolality (Appelbloom *et al.*, 1965), and dogs with spontaneous diabetes insipidus respond to Pitressin tannate within 24 hours (Finco, 1979). Various other diseases in domestic animals have not been examined for the status of the renal medullary gradients, nor have comparative trials of abrupt and gradual water deprivation tests been made. Because the cost of hospitalization makes the gradual deprivation test expensive to perform and because its superiority has not been documented, the abrupt deprivation test is a logical choice over the gradual test for initial study. If the animal fails to concentrate urine with the abrupt test, a gradual deprivation test may be considered.

An alternate form of urine concentration test is performed by supplying an exogenous source of ADH. Regardless of the animal's state of hydration, concentrated urine should be produced in the presence of ADH if the kidney is normal. Aqueous Pitressin, Pitressin tannate in oil, or synthetic preparations of ADH may be used for the test. Since the half-life of aqueous Pitressin is extremely short, constant infusion is required to maintain adequate blood levels for the test. This complicates the test compared to the use of Pitressin tannate in oil. The question of secondary washout of medullary gradient is more germane to a concentration test performed with aqueous Pitressin during minutes compared to conducting a Pitressin tannate test over a period of many hours. One synthetic ADH preparation (1-deamino-8-D-arginine-vasopressin) has been shown to have activity in the dog for about 12 hours after mucosal application (Greene *et al.*, 1979). This drug may have future application in urine concentration tests. However, on the basis of past experience, Pitressin tannate in oil is recommended for diagnostic use in animals at present.

The combined use of water deprivation and Pitressin in a single procedure has been advocated for the diagnosis of polyuria problems in both the human being and the dog (Mulnix *et al.*, 1976).

3. Methods of Conducting Urine Concentration Tests

The various tests for urine concentration probably all have merit if conducted carefully and interpreted properly. The tests subsequently described are those which the author has found satisfactory in dogs and cats.

a. Abrupt Water Deprivation Test. This test is conducted so that a definite stimulus for ADH release is elicited prior to the conclusion of the test. In the past, the procedure involved withholding water for a specified number of hours and then determining the degree of urine concentration. This technique has been found to be inaccurate because of variation among patients in rate of dehydration following water deprivation. With this technique some patients may be dehydrated to dangerous degrees and others may not be challenged. The preferable end point is a specific degree of dehydration rather than time of water deprivation.

Prior to conducting the test it is necessary to evaluate the animal's state of hydration. Performing a water deprivation test in a dehydrated animal is meaningless and dangerous. It is meaningless because clinically detectable dehydration is usually 5% or greater (Finco, 1972), and such an animal would already be secreting ADH and concentrating urine if capable. It is dangerous because imposing water deprivation on an animal that is already dehydrated may lead to hypovolemia and shock. Performing a water deprivation test in an

azotemic animal is also contraindicated. The test gives no added information in cases of primary renal azotemia, and dehydration aggravates the azotemia by superimposing a prerenal factor on the underlying disease. Patients with prerenal azotemia are usually producing a concentrated urine spontaneously.

The stepwise procedure for conducting the abrupt water deprivation test is the following:

1. While still providing water, have the patient fast for 4 hours.
2. After the fast, obtain a blood sample for measurement of packed cell volume (PCV), total serum solids, and serum osmolality if feasible. Obtain urine for refractometry measurements and osmolality if feasible. The osmolality samples should be stored and analyzed with the samples obtained at the conclusion of the test.
3. Weigh the animal with an accurate balance scale with the urinary bladder empty.
4. Remove all sources of drinking water. Fasting is preferable, but dry food may be given if the period of deprivation is prolonged.
5. Monitor the patient at appropriate intervals for evidence of dehydration. Body weight measurements are the primary guide. Serum total solids, PCV determinations, and clinical signs are less accurate and should be used only as secondary indices. Appropriate intervals for observation are highly variable among patient; they can be judged by weight evaluations during the early stages of the test. The first observation after initiating the test should be made at 4 hours. Body weight determinations should be made with the bladder empty, and consideration should be given to the weight changes that occur from eating, defecation, or other losses.
6. Terminate the test when 5–7% of body weight is lost or if undesirable signs develop during the test. Since urine produced during maximal dehydration should give the best indication of concentrating ability, it is desirable to empty the animal's bladder once sufficient weight loss has occurred and to wait for newly formed urine for analysis. Four-hour fasting serum samples and urine are obtained for analysis at the conclusion of the procedure. The interpretation of results of the test is discussed subsequently.

b. Pitressin Tannate Test. This test may be used in instances when it is important to avoid the risk of water deprivation to the patient. Since Pitressin has some vasomotor activity, it is contraindicated in some conditions in human beings. While the same contraindications may exist in animals, adverse effects from the drug are rare. The test is conducted as follows:

1. The contents of the Pitressin tannate vial are thoroughly mixed. The vial is placed in hot water to disperse the drug in the oil. Failure to take these precautions may result in ADH remaining in the bottom of the vial, where it settled during storage.
2. Preinjection measurements of urine concentration are made.
3. The drug is administered IM at a dose of 0.25 U/kg up to a maximal dosage of 5 U for dogs.
4. The animal is allowed water *ad libitum* during the study. Although not essential, comparison of water intake and urine volume for 24-hour periods before and after injection may aid in the interpretation of results.
5. Urine is collected at intervals during the 24 hours following administration of drug and analyzed for concentration. Time of optimal concentrating ability appears to vary from one animal to another. Generally, samples are obtained at 6, 12, and 24 hours after Pitressin injection.

4. Interpretation of Results of Urine Concentration Tests

The same results would be anticipated whether exogenous or endogenous ADH were utilized, but some studies indicate that urine-concentrating ability is better after water deprivation than after injection of Pitressin (West *et al.*, 1955). Although not confirmed by controlled studies, it is the author's impression that the water deprivation test is a more reliable test than the Pitressin tannate test.

In the past, a urine specific gravity of 1.025 has been considered to be an adequate indication of concentrating ability for the dog and cat (Osborne *et al.*, 1972). Unfortunately, this figure was transposed from studies of human beings, whose maximal concentrating ability is about 1.040. Studies on normal beagle dogs indicated that 16 hours or more of water deprivation resulted in specific gravity readings of 1.058 ± 0.008 (Balazs *et al.*, 1971). In another study of 20 dogs, mean concentrating ability was 1.062 ± 0.007 for specific gravity and 2289 ± 251 mosmole/hg for osmolality with 39.3 ± 16 hours of deprivation that resulted in 4–16% weight loss (Hardy and Osborne, 1979).

In studies in which normal cats were subjected to water deprivation until a 5–8% loss of body weight occurred, urine osmolality was 2196 ± 533 mosmole/kg and urine specific gravity was 1.064 ± 0.015 on 18 tests in 11 cats fed commercial dry food (Greenwood and Finco, 1979). However, whether values obtained from abrupt deprivation of normal animals would be the same as values from normal animals that had polyuria prior to deprivation has not been established. Another problem is the influence of diet on concentrating ability, as mentioned in Section II. Any guidelines to be used for differentiating normal from abnormal concentrating ability must be tentative until more information is collected. At present, for the dog and the cat with the use of the abrupt water deprivation test, urine specific gravity readings of 1.040 or above are considered evidence of normal concentrating ability. Readings between 1.030 and 1.040 are considered questionable and worthy of further investigation. Readings below 1.030 are considered abnormal. For the Pitressin tannate test, values sightly lower (0.005) may be used.

If osmolality readings are made, pre- and posttest serum samples may be compared for the water deprivation test in order to document the adequacy of water deprivation. Serum osmolality should be higher in the posttest sample. Food ingestion seems to markedly alter serum osmolality results, and thus at least a 4-hour fast is recommended prior to sampling. Posttest urine and serum osmolality ratios may also be examined to establish the effectiveness of the renal concentrating mechanism.

C. Phenolsulfonphthalein Excretion Test

Phenolsulfonphthalein is an organic anionic dye that is excreted primarily by the kidney (Poutsiaka *et al.*, 1962). Renal excretion is performed by proximal tubular secretion (Heetderks, 1964). A report of studies in rabbits indicated that at high blood levels tubular reabsorption as well as tubular secretion of PSP occurs (Gerdes *et al.*, 1978), but such blood levels are not achieved during the renal function tests subsequently described.

Although PSP is excreted by the proximal tubules, the tests for renal function are a better index of renal blood flow than tubular secretion. This is because the tubular maximum is not approached with the low blood levels attained, and therefore blood flow to the tubules is the determining factor in the amount of dye that is excreted (Relman and Levinsky, 1971).

Various intervals for collection of urine have been used after the single intravenous injection of PSP. Longer periods of collection make the test less sensitive, since the

half-life curves for drug excretion tend to blend together as they become asymptotic. Empirical trials suggest that the 20-minute PSP test is acceptable for detecting renal dysfunction. The following procedure is used to perform this test in the dog:

1. To avoid problems with complete emptying of the bladder at the end of the test, the urinary bladder should be empty or nearly empty at the beginning. This is achieved by allowing the animal to void spontaneously or by catheterizing it with a flexible catheter which can be fitted to a syringe. If catheterization is done, it is convenient to leave the catheter in place and immediately proceed with step 2.
2. Regardless of body size, inject 6 mg (0.1 ml) of PSP intravenously. It is imperative that the drug be given IV since SQ injection gives erroneous low values for renal function.
3. At about 10 minutes after injection of PSP, the animal should be catheterized if this has not already been done. Care should be taken to save all urine produced after the injection of PSP.
4. At about 15 minutes after injection, the bladder is completely emptied, and rinsing is begun by injecting and withdrawing sterile saline. All rinses are added to the urine collected after dye injection. Rinsing is completed at exactly 20 minutes after the injection of PSP. If difficulty is encountered in aspirating injected saline during the rinsing procedure, it may be helpful to inject a bolus of air into the bladder or to reposition the catheter.
5. The 20-minute urine specimen is submitted for laboratory analysis. Analysis is conducted by adding 10 ml of 2.5 *N* NaOH, diluting to 1.0 liter, mixing, and comparing absorbance at 550 nm to a standard curve.

The percentage of the total dose excreted by normal dogs is 43.8 ± 11.1 (Finco, 1971b). No correlation exists between body size and amount secreted. This implies that larger dogs with greater renal mass secrete an amount comparable to smaller dogs because of lower plasma concentration of dye in the larger dogs (Finco, 1971b). For purposes of clinical interpretation, any value of less than 30% excretion should be considered abnormal.

D. Plasma Sulfanilate Test

Studies suggest that sodium sulfanilate is excreted by glomerular filtration in the dog and that the kidney is the major route of excretion of the compound (Rosenfeld, 1955). One method of estimating renal function is to determine the rate of disappearance of such a material from the body (blood) once the material has mixed with body fluids.

This principle was applied to sulfanilate excretion to establish normal values for the dog (Rosenfeld, 1955). The procedure is conducted as follows (Carlson and Kaneko, 1971a):

1. Five percent sodium sulfanilate is injected IV at a rate of 0.1 ml/lb body weight.
2. At least 1.0 ml of heparinized blood is obtained at 30, 60, and 90 minutes after the injection, and the time accurately recorded.
3. The blood is analyzed for sodium sulfanilate as described elsewhere (Kaneko *et al.*, 1971).
4. The optical density readings are plotted on semilog paper, a line of best fit is drawn, and the time interval required for halving of blood concentration ($T_{\frac{1}{2}}$) is read from the graph (Fig. 5).

Values from normal dogs are 66 ± 11 minutes (Rosenfeld, 1955), and values of 50–80 minutes are considered normal. The test has been reported to be sufficiently sensitive to detect unilateral nephrectomy in normal dogs (Carlson and Kaneko, 1971a,b).

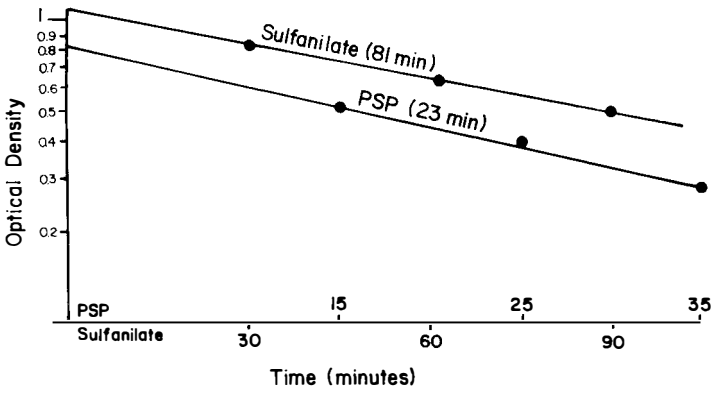


Fig. 5. Blood $T_{\frac{1}{2}}$ values for sulfanilate and PSP after unilateral nephrectomy of a dog with ectopic ureter. The $T_{\frac{1}{2}}$ value for sulfanilate is just beyond the normal range of 50–80 minutes, and the $T_{\frac{1}{2}}$ value for PSP is the upper limit of the normal range of 18–24 minutes. Values for $T_{\frac{1}{2}}$ were read from the graph by determining the amount of time required for halving of zero-time optical density.

Values for 22 determinations on 11 normal cats were 45.1 ± 7.0 minutes (Greenwood and Finco, 1979). Methods of excretion of sulfanilate by the cat have not been studied.

E. Plasma PSP Test

Tests have been devised to circumvent urine collection of PSP in dogs, cats, and cattle by measuring the disppearance of dye from the plasma (Brobst, 1967; Mixner and Anderson, 1958; Osbaldiston and Fuhrman, 1970). The principles on which these tests are based are identical to those described for sulfanilate. However, since PSP is predominantly protein bound, its distribution is restricted to the vascular space, and equilibrium is reached sooner than with sulfanilate. The procedure of Brobst has been modified slightly by the author for use on dogs and cats as follows:

1. Aqueous solution of sodium PSP (20 mg of PSP per ml H_2O) is prepared and sterilized by autoclaving.
2. A dose of 5.0 mg/kg is injected IV, and 3.0 ml of blood are withdrawn at 15, 25, and 35 minutes after injection and placed in sodium EDTA anticoagulant.
3. One milliliter of plasma from each sample is mixed in a centrifuge tube with 3.0 ml of acetone and 0.5 ml of 4.0 *N* NaOH. The tubes are stoppered and shaken vigorously for 1 minute. After centrifugation at 1000 *g* for 2 minutes, the supernatant fluid is transferred to 10-mm cuvettes.
4. Optical density is read against a blank prepared from 1.0 of water handled as plasma in step 3.
5. The $T_{\frac{1}{2}}$ is determined by plotting optical density readings on semilog paper (Fig. 5). Values for normal dogs are 18–24 minutes.

An attempt has been made to simplify the plasma PSP test in dogs by procuring but one blood sample at 60 minutes after injection of dye (Kaufman and Kirk, 1973). This procedure is based on the assumption that the volume of distribution of PSP is constant among dogs and that one point will accurately determine the plasma decay rate of the compound. On the basis of experience with three-point measurements of both PSP and

sulfanilate, the author is skeptical of the validity of this assumption and does not recommend the use of this test.

F. Other Plasma Tests

p-Aminohippurate and inulin have also been used for estimations of renal function in animals (Harvey and Hoe, 1966). The principles of these tests are the same as those described for sulfanilate and PSP.

G. Clearance Methods

The development of the clearance concept represented a major advancement in the measurement of renal function (Smith, 1951). Data have accumulated to indicate that inulin can be used to measure GFR, and *p*-aminohippuric acid at low blood levels can be used to measure RPF. The clearance formula is expressed as follows:

$$C = \frac{U_v U_c}{P_c}$$

where C is clearance; U_v, urine volume, U_c, urine concentration; and P_c, plasma concentration.

Physiology textbooks can be consulted for a more detailed description of the clearance concept. While the clearance formula and inulin and *p*-aminohippuric acid clearances are standards by which other measures of renal function are judged, they are too time-consuming for clinical use in veterinary medicine. Since both compounds are foreign to the body, it is necessary to induce and maintain constant blood levels by giving priming doses followed by constant infusion of solutions. In research dogs and cats, the determination of inulin and creatinine clearances requires 4–6 human hours of effort per animal.

Attempts to circumvent these problems have been made by using a single-injection technique. This gives a high blood level of compound initially, which decreases as the material is distributed and excreted. Rather than $T_{\frac{1}{2}}$ calculations, which previously were described for single-injection techniques, mathematical models have been derived to calculate the results as clearance values. The validity of the single-injection technique for accurate measurement of GFR and RPF is doubted by some (Smith, 1951) but accepted by others (Bryan *et al.*, 1972). A comparison between the constant-infusion and single-injection methods of assessing renal function in dogs was made using sodium [^{125}I] iodothalamate and sodium [^{131}I]iodohippurate (Phil, 1974). The two methods gave the same results for GFR but different results for RPF (iodohippurate clearance).

The single-injection method using isotopes may have application in referral centers or in drug evaluations (Powers *et al.*, 1977) but is not applicable under usual practice circumstances.

H. Endogenous Creatinine Clearance

In contrast to clearance methods previously mentioned, the endogenous creatinine clearance is feasible with clinic cases. Blood levels of creatinine are relatively constant over short intervals of time unless some factor acutely affects renal function. Thus, the clinician need only have the blood level of creatinine measured and make an accurately

timed collection of urine. Both 20-minute and 24-hour endogenous creatinine clearance values have been determined for the dog (Finco, 1971b; Bovee and Joyce, 1979). Twenty-minute clearance determinations performed on 27 normal dogs of both sexes weighing from 5.6 to 31.3 kg gave values of 2.8 ± 0.96 ml/minute per kilogram or 60.0 ± 21.9 ml/minute per square meter of body surface area (Finco, 1971b). Twenty-four hour clearance determinations performed on 36 normal female beagle dogs gave values of 3.7 ± 0.77 ml/minute per kilogram or 57.6 ± 9.3 ml/minute per square meter of body surface area (Bovee and Joyce, 1979). However, the results may not be directly comparable since nonspecific plasma chromogens that overestimate SC were measured in the latter study. With both studies, the ranges of values for the normal dogs were large (26–70 and 32–113 ml/minute per square meter, respectively).

The technique for performing this test is simple, but certain precautions are necessary. The test is dependent on total collection of urine during the time interval that is chosen. If urine is erroneously left in the bladder at the beginning of the test, creatinine will be measured that was not cleared from the blood during the test time chosen. Clearance values will be erroneously high. If all urine is not removed from the bladder at the termination of the test, not all creatinine cleared from the blood during the test period will be measured, and clearance values will be erroneously low. The precision required in emptying the bladder increases progressively as the time period for collection is decreased. When the 20-minute clearance test is performed, it is necessary to rinse the bladder several times with sterile saline both prior to initiating the test and at its conclusion. The rinses prior to the start are discarded; the rinses made prior to the conclusion of the test are added to the urine that is collected. The amount of creatinine excreted during the test period is the product of creatinine concentration of the rinse–urine mixture times its volume. Clearance is calculated by dividing urine creatinine excretion by plasma creatinine concentration.

I. Use of Radiochemicals

The need for specialized equipment eliminates isotope measurement methods from use in common clinical practice. However, some veterinary referral centers have the facilities for carrying out some of the procedures subsequently discussed, and thus a casual acquaintances with the technique seems warranted.

Isotope-labeled compounds ([^{14}C]inulin, [^{125}I]iodohippurate, [^{131}I]iodohippurate, and others) have been used for measurements of GFR and RPF by constant-infusion and single-injection methods, as previously discussed.

Kidney function also has been assessed by intravenous injection of isotopes and direct measurement of their accumulation in the kidney by monitoring devices placed over the kidneys. For substances, such as labeled hippurate, that are secreted by the proximal tubules, the time–concentration curve obtained by the detection device has three main components. An initial steep rise represents the arrival of the isotope to the kidney and lasts about 20 seconds. The activity increases during the second, or secretory, phase, during which the isotope is concentrated in the kidney. This phase lasts about 5 minutes. The third phase is the removal phase, which is affected mainly by the efficiency of drainage to the bladder (Joekes, 1972). Considerable information is obtained merely by visualizing the renal image obtained a few minutes after the injection of radioisotope. Such images do not provide quantitative information concerning function but allow com-

parisons to be made between the two kidneys. This may identify unilateral lesions that could be missed by methods outlined previously. ^{99m}Tc-Labeled dimercaptosuccinic acid appears to be a favorite for renal parenchymal imaging (Daly *et al.*, 1978), although [^{123}I]-orthoiodohippurate is also advocated (Zielinski *et al.*, 1977).

J. Urinary Enzymes

Increases in serum enzyme activity are commonly used to detect organ abnormalities. Such enzyme measurements are not true tests of function, but they are sensitive indicators of organ damage. Serum enzymes or isoenzymes unique to the kidney that may be used for detection of changes in this organ have not been found.

The appearance of enzymes in the urine may offer unique opportunities for the detection of renal injury. An enzyme with a large molecular weight should not pass from the blood through the glomerular filter, and its appearance in urine would indicate that it was of urogenital tract origin. Studies on the application of urine enzyme assay to the detection of renal injury have been reviewed (Mattenheimer, 1971, 1977; Raab, 1972; Dubach and Schmidt, 1978). Attention has been focused on enzymes present in the brush border of the proximal tubules and on enzymes in lysosomes. Complicating factors that have been encountered are the presence of enzyme inhibitors and accelerators in urine that often must be removed prior to analysis. Of the domestic species, some results have been reported from the dog (Conzelman *et al.*, 1970; Ellis *et al.*, 1973). However, data accumulated up to this time have not been encouraging for clinical application of this concept.

K. A Perspective of Renal Function Tests

In nephrology, the question of whether renal function is normal is of frequent concern. The question should be answered reasonably quickly and at the least cost to the owner of the animal. This implies that the evaluation will not be time-consuming for the veterinarian or veterinary assistants.

The only part of urinalysis that relates directly to function is urine specific gravity. A low specific gravity reading is not diagnostic of renal dysfunction, but merely creates the suspicion that renal or extrarenal disease exists. Other parts of the urinalysis (i.e., many casts per high-power field, glomerular proteinuria) may indicate that renal damage has occurred but do not give information about level of function. The relative simplicity of performing BUN and SC measurements makes them logical methods for assessing renal function. Normal values for these blood components and ability to concentrate urine are taken as evidence that renal dysfunction is not an imminent threat to life. Clinical investigation is often discontinued at this point unless there is a specific reason to question the normal state of the kidneys. Such findings as proteinuria or casts could lead to that question. In their absence, the clinician acknowledges that the normal BUN, SC, and concentrating ability are compatible with up to two-thirds loss of renal parenchyma. On the other hand, abnormal levels of BUN or SC indicate that prerenal, primary renal, or postrenal abnormalities exist. When azotemia is severe, little if any added information of clinical relevance will be obtained by performing more sensitive renal function tests. Additional tests to evaluate renal function are appropriate when BUN, SC, and urine specific gravity readings are equivocal or when such values are normal but it is of importance to know that considerable renal reserve exists.

Some general concepts concerning renal function are relevant to a discussion of more sensitive tests. As previously discussed, renal blood flow, glomerular filtration, and tubular function are three general categories of renal function. Tubular function may be simplistically divided into proximal and distal portions. The more sensitive renal function tests previously described each depend primarily on one component of these renal functions. Urine concentration depends upon distal tubule–collecting duct function, PSP excretion depends on renal blood flow, and both sulfanilate and creatinine excretion depend on glomerular filtration. While different renal functions form the basis for the various tests, the usefulness of the tests is not dictated by the anatomical site of the renal lesion. Although exceptions exist, most renal diseases are characterized by a proportionate loss of the various functions. Thus, renal blood flow, glomerular filtration, proximal tubular function, and distal tubular function are all affected even when an anatomical lesion seems localized to one structure. As a consequence, all the renal function tests have validity in most instances regardless of the type of disease. When exceptions to this generality exist, they usually occur early in the course of nephron destruction. Because of this generality, choices of renal function tests should not be made exclusively on the basis of the presumed site of anatomical defect in the kidney.

The renal function tests previously described have some differences in simplicity and feasibility. They may also have some differences in sensitivity, but few comparative studies have been made of these tests in each of the various domestic species.

The urine concentration tests have the advantages of requiring but a few relatively inexpensive pieces of equipment and only modest amounts of time to perform. Their sensitivity in detecting renal dysfunction is debatable because limits of concentrating ability at various levels of renal dysfunction have not been defined for domestic species. As a generality, however, it appears that concentrating ability is unaffected by loss of 50% of renal mass but adversely affected when 75% is absent. Thus, polyuria and impaired concentrating ability usually precede azotemia. Exceptions may exist in the dog with primary glomerular disease, in which concentrating ability may still exist when azotemia supervenes. The cat may retain considerable concentrating ability with azotemia. Cats with spontaneous disease and those with renal mass reduced by nephrectomy and arterial ligation both have considerable concentrating ability at the onset of azotemia (Greenwood and Finco, 1979).

One major disadvantage of the urine concentration tests is their lack of specificity. Lack of ability to concentrate normally does not differentiate renal disease from myriad non-renal diseases that affect urine concentrating ability secondarily.

The urine PSP test is relatively simple to perform but does require urethral catheterization and urine collection. The sensitivity of the 20-minute excretion test appears to be greater than that of the urine concentration tests and nearly the same as that of the plasma sulfanilate test (Finco, 1979). The analysis for PSP in urine is technically much simpler than that for plasma sulfanilate or plasma PSP.

The plasma tests (sulfanilate, PSP) have a decided advantage in terms of simplicity of patient manipulation since one IV injection and three withdrawals of blood are made. As previously indicated, the analysis of the plasma samples is more complex, and thus a tradeoff is made between ease with the animal and ease in the laboratory. The plasma sulfanilate test appears to be more sensitive for detecting renal dysfunction than the plasma PSP test in the cat (Greenwood and Finco, 1979).

Although the creatinine clearance test is theoretically the most sound because of the use

of the clearance concept, it also has limitations. The large variation in values from normal dogs indicates that broad ranges must be quoted for normal. The 24-hour test requires hospitalization of the animal, while the PSP tests and sulfanilate tests do not. A direct comparison of the sensitivity of the endogenous creatinine clearance tests with that of other tests described has not been reported, but the broad normal ranges make it doubtful that it is superior to others.

In summary, the choice of the renal function test to use if BUN, SC, and urine specific gravity tests are inadequate is not clear-cut. Further comparisons among tests are required to establish the superiority of any one over the others.

REFERENCES

Abbrecht, P. H. (1969). *J. Clin. Invest.* **48,** 432-442.

Abel, R. M., Beck, C. H., Abbott, W. M., Ryan, J. A., Barnett, G. O., and Fischer, J. F. (1973). *N. Engl. J. Med.* **288,** 696-699.

Altman, P. L., and Dittmer. D. S. (1961). Fed. Am. Soc. Exp. Biol., Washington, D.C.

Anderson, R. J., Linas, S. L., Berns, A. S., Henrich, W. L., Miller, T. R., Gabow, P. A., and Schrier, R. W. (1977). *N. Engl. J. Med.* **296,** 1134-1138.

Anderson, R. S., and Edney, A. T. (1969). *Vet. Rec.* **84,** 348-349.

Andreoli, T., Berliner, R. W., Kokko, J. P., and Marsh, D. J. (1978). *Am. J. Physiol.* **235,** F1-F11.

Anonymous (1965). *In* "Hawk's Physiological Chemistry" (B. L. Oser, ed.), pp. 1153-1205. McGraw-Hill, New York.

Appelbloom, J. W., Brodsky, W. A., and Scott, W. M. (1965). *Am. J. Physiol.* **208,** 38-45.

Arieff, A. E., Guisado, R., and Massry, S. G. (1975). *Kidney Int.* **7,** S194-S200.

Asheim, A., Person, F., and Person, S. (1961). *Acta Physiol. Scand.* **51,** 150-162.

Aurbach, G. D., and Heath, D. A. (1974). *Kidney Int.* **6,** 331- 345.

Avizonis, P. V., and Wriston, J. C. (1959). *Biochim. Biophys. Acta* **34,** 279-281.

Aylward, T. D., Schowengerdt, C. G., and Bove, K. (1973). *J. Surg. Res.* **15,** 1-3.

Bagdade, J. D. (1975). *Nephron* **14,** 153-162.

Balazs, T., Sekella, R., and Pauls, J. F. (1971). *Lab. Anim. Sci.* **21,** 546-548.

Balint, P., and Fekete, A. (1960). *Acta Physiol. Acad. Sci. Hung.* **17,** 277-286.

Balint, P., and Forgacs, I. (1965). *Acta Physiol. Acad. Sci. Hung.* **27,** 47-58.

Balint, P., and Sturcz, J. (1959). *Acta Med. Acad. Sci. Hung.* **13,** 9-17.

Balint, P., and Visy, M. (1965). *Acta Physiol. Acad. Sci. Hung.* **28,** 265-272.

Balint, P., Laszlo, K., Szocs, E., and Tarjan, E. (1975). *Acta Med. Acad. Sci. Hung.* **32,** 193-205.

Barsanti, J. A., and Finco, D. R. (1979). *Am. J. Vet. Res.* **40,** 1583-1589.

Beeuwkes, R. (1971). *Am. J. Physiol.* **221,** 1361-1374.

Bergstrom, J., Furst, P., Josephson, B., and Noree, L. O. (1970). *Life Sci.* **9,** 787.

Bergstrom, J., Furst, P., and Noree, L. O. (1975). *Clin. Nephrol.* **3,** 187-192.

Berne, R. M. (1952). *Am. J. Physiol.* **171,** 148-158.

Bernstein, L. M. (1965). "Renal Function and Renal Failure." Williams & Wilkins, Baltimore, Maryland.

Birtch, A. G., Zakheim, R. M., Jones, L. G., and Barger, A. C. (1967). *Circ. Res.* **21,** 869-878.

Black, D. A. (1970). *Arch. Intern. Med.* **126,** 906-909.

Blass, K. G., Thibert, R. J., and Lam, L. K. (1974). *Z. Klin. Chem. Klin. Biochem.* **12,** 336-343.

Bleiler, R. A., and Schell, H. P. (1962). *J. Lab. Clin. Med.* **59,** 945-955.

Bloch, K., Schoenheimer, P., and Rittenberg, D. (1941). *J. Biol. Chem.* **138,** 155-166.

Blumberg, A., Esslen, E., and Burgi, W. (1978). *Nephron* **21,** 186-191.

Bold, A. M., Menzies, I. S., and Walker, G. (1970). *J. Clin. Pathol.* **23,** 85-89.

Bonvalet, J., Champion, M., Wanstok, F., and Berjal, G. (1972). *Kidney Int.* **1,** 391-396.

Borison, H. L., and Herbertson, L. M. (1959). *Am. J. Physiol.* **197,** 850-852.

Bounous, G. (1970). *Am. J. Surg.* **119,** 264-268.

Bovee, K. C., and Joyce, T. (1979). *J. Am. Vet. Med. Assoc.* **174,** 488-491.

Bovee, K. C., Joyce, T., Reynolds, R., and Segal, S. (1978a). *Metab. Clin. Exp.* **27,** 45-52.

Bovee, K. C., Joyce, T., Reynolds, R., and Segal, S. (1978b). *Science* **201,** 1129-1131.

Bowie, L., Smith, S., and Gochman, N. (1977). *Clin. Chem.* **23,** 128–130.
Boylan, J. W. and Asshauer, E. (1962) *Pfluegers Arch.* **276,** 99–116.
Bradford, M. M. (1976). *Anal. Biochem.* **72,** 248–254.
Brenner, B. M., Hostetter, T. H., and Humes, H. D. (1978). *N. Engl. J. Med.* **298,** 826–833.
Brentano, J. L., Man, N. K., Sausse, A., Cuilte, G., Zinfraff, J., Drueke, T., Jungers, P., and Billon, J. P. (1975). *Kidney Int.* **7,** S352–S356.
Bricker, N. S. (1969). *Am. J. Med.* **46,** 1–11.
Bricker, N. S. (1972). *N. Engl. J. Med.* **286,** 1093–1099.
Bricker, N. S., Dewey, R. R., Lubowitz, H., Stokes, J., and Kirkensgaard, T. (1959). *J. Clin. Invest.* **38,** 516–523.
Bricker, N. S., Morrin, P. A. F., and Kime, S. W., Jr. (1960). *Am. J. Med.* **28,** 77–98.
Bricker, N. S., Schmidt, R. W., Favre, H., Fine, L., and Bourgoignie, J. J. (1975). *Yale J. Biol. Med.* **48,** 293–303.
Brobst, D. F., Carter, J. M., and Herron, M. (1967). *Gaines Vet. Symp., Proc. Gaines Res. Cent., White Plains, N.Y.* 15.
Brown, C. L., Hill, M. J., and Richards, P. (1971). *Lancet* **ii,** 406–408.
Bryan, C. W., Jarchow, R. C., and Maher, J. G. (1972). *J. Lab. Clin. Med.* **80,** 845–856.
Burg, M., and Stoner, L. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 31.
Burrows, C. F., and Bovee, K. C. (1974). *Am. J. Vet. Res.* **35,** 1083–1088.
Burrows, C. F., and Bovee, K. C. (1978). *J. Am. Vet. Med. Assoc.* **172,** 801–805.
Carlson, G. P., and Kaneko, J. J. (1971a). *J. Am. Vet. Med. Assoc.* **158,** 1229–1234.
Carlson, G. P., and Kaneko, J. J. (1971b). *J. Am. Vet. Med. Assoc.* **158,** 1235–1239.
Carriere, S., and Gagnan-Brunette, M. (1977). *Can. J. Physiol. Pharmacol.* **55,** 105–110.
Chomdej, B., Bell, P. D., and Navar, L. G. (1977). *Am. J. Physiol.* **232,** F490–F496.
Chuang, E. L., Reineck, H. J., Osgood, R. W., Kunan, R. T., and Stein, J. H. (1978). *J. Clin. Invest.* **61,** 633–639.
Churchill, D., Knaack, J., Chirito, E., Barre, P., Cole, C., Muehrcke, R., and Gault, M. H. (1974). *Am. J. Med.* **56,** 575–582.
Close, J. H. (1974). *N. Engl. J. Med.* **290,** 663–667.
Coburn, J. W., Gonick, H. C., Rubini, M. E., and Kleeman, C. R. (1965). *J. Clin. Invest.* **44,** 603–614.
Coles, E. H. (1967). "Veterinary Clinical Pathology." Saunders, Philadelphia, Pennsylvania.
Conn, H. O. (1973). *Gastroenterology* **65,** 321–340.
Conzelman, G. M., Flanders, L. E., Springer, K., and Crout, D. W. (1970). *Lab. Anim. Care* **20,** 192–197.
Corcoran, A. C., and Page, I. H. (1943). *Am. J. Physiol.* **140,** 234–239.
Daly, M. J., Milutinovic, J., Rudd, T. G., Phillips, L. A., and Fealkow, P. J. (1978). *Radiology* **128,** 701–704.
Darmady, E. M., Offer, J., and Woodhouse, M. A. (1973). *J. Pathol.* **109,** 195–207.
Davidsohn, I., and Henry, J. B. (1969). "Clinical Diagnosis by Laboratory Methods." Saunders, Philadelphia, Pennsylvania.
Davis, J. O., and Freeman, R. H. (1976). *Physiol. Rev.* **56,** 2–44.
Dobbelstein, H. (1976). *Nephron* **15,** 409–414.
Dontas, A. S., Marketos, S. G., and Papanayiotou, P. (1972). *Postgrad. Med. J.* **48,** 295–303.
Doolan, P. D., Olsen, E. L., and Meil, G. B. (1962). *Am. J. Med.* **32,** 65–79.
Dossetor, J. B. (1966). *Ann. Intern. Med.* **65,** 1287–1299.
Dubach, U. C., and Schmidt, U. (1978). *J. Clin. Chem. Clin. Biochem.* **16,** 197–211.
Duncan, J. R., and Prasse, K. W. (1976). *Vet. Clin. North Am.* **6,** 647–661.
Eggleton, M. G., and Habib, Y. A. (1951), **112,** 191–200.
Eknoyan, G. (1977). *In* "Pathophysiology of the Kidney" (N. A. Kurtzman and M. Martinez-Maldonado, eds.), pp. 842–881. Thomas, Springfield, Illinois.
Ellis, B. G., Price, R. G., and Topham, J. C. (1973). *Chem.-Biol. Interact.* **7,** 101–113.
Enger, E., and Blegen, E. M. (1964). *Scand. J. Clin. Lab. Invest.* **16,** 273–280.
Erslev, A. J. (1975). *Am. J. Med.* **58,** 25–30.
Farquhar, M. G. (1975). *Kidney Int.* **8,** 197–211.
Finco, D. R. (1971a). *J. Am. Vet. Med. Assoc.* **158,** 327–335.
Finco, D. R. (1971b). *J. Am. Vet. Med. Assoc.* **159,** 336–340.
Finco, D. R. (1972). *J. Am. Anim. Hosp. Assoc.* **8,** 155–165.
Finco, D. R. (1976). *Am. J. Vet. Res.* **37,** 87–91.

Finco, D. R. (1979). Unpublished observations.
Finco, D. R., and Cornelius, L. M. (1977). *Am. J. Vet. Res.* **38,** 823–829.
Finco, D. R., and Duncan, J. R. (1972). *Am. J. Vet. Res.* **33,** 2447–2450.
Finco, D. R., and Duncan, J. R. (1976). *J. Am. Vet. Med. Assoc.* **168,** 593–601.
Finco, D. R., and Rowland, G. N. (1978). *J. Am. Vet. Med. Assoc.* **173,** 990–994.
Fisher, J. W. (1972). *Pharmacol. Rev.* **24,** 459–508.
Furst, P., Bergstrom, J., Gordon, A., Johnsson, E., and Zimmerman, L. (1975). *Kidney Int.* **7,** S272–S275.
Gabow, P. (1976). *In* "Renal and Electrolyte Disorders" (R. W. Schrier, ed.), pp. 143–167. Little Brown, Boston, Massachusetts.
Galla, J. H., Klein-Robbenhaar, T., and Hayslett, J. P. (1974). *Yale J. Biol. Med.* **47,** 218–226.
Gartner, K. (1962). *Berl. Muench Tieraerztl. Wochenschr.* **75,** 109–112.
Gennari, F. J., and Kassirer, J. P. (1974). *N. Engl. J. Med.* **294,** 714–720.
Gerdes, U., Kristensen, J., Moller, J. V., and Sheikh, M. I. (1978). *J. Physiol. (London)* **277,** 115–129.
Giovannetti, S., and Barsotti, G. (1975). *Nephron* **14,** 123–133.
Glauser, K. F., and Selkurt, E. E. (1952). *Am. J. Physiol.* **168,** 469–477.
Glauser, S. C., Wagner, H., and Glauser, E. M. (1972). *Am. J. Med. Sci.* **264,** 135–139.
Goldberg, M., Agus, Z. S., and Goldfarb, S. (1976). *In* "Kidney and Urinary Tract Physiology II" (K. Thurau, ed.), pp. 211–256. Univ. Park Press, Baltimore, Maryland.
Goldman, R. (1954). *Proc. Soc. Exp. Biol. Med.* **85,** 446–448.
Greaves, J. P., and Scott, P. P. (1960). *Nature (London)* **187,** 242.
Greene, C. E., Wong, P., and Finco, D. R. (1979). *J. Am. Anim. Hosp. Assoc.* **15,** 371–377.
Greenwood, L., and Finco, D. R. (1979). Unpublished observations.
Gutmann, F. D., and Rieselbach, R. E. (1971). *J. Lab. Clin. Med.* **77,** 14–22.
Hamilton, P. B., Phillips, R. A., and Hiller, A. (1948). *Am. J. Physiol.* **152,** 517–522.
Hardwicke, J. (1975). *Clin. Nephrol.* **3,** 37–41.
Hardy, R. M., and Osborne, C. A. (1979). *J. Am. Vet. Med. Assoc.* **174,** 479–484.
Harrington, J. T., and Cohen, J. J. (1975). *N. Engl. J. Med.* **292,** 89–91.
Harvey, D. G., and Hoe, C. M. (1966). *J. Small. Anim. Pract.* **7,** 361–373.
Hawk, P. B. (1910). *J. Biol. Chem.* **8,** 465–477.
Hays, R. M. (1976). *Kidney Int.* **9,** 223–230.
Heetderks, D. R. (1964). *J. Urol.* **92,** 221–233.
Henry, R. J. (1964). "Clinical Chemistry." Harper, New York.
Hill, G. E., Lunn, J. K., Hodges, M. R., Stanley, T. H., Sentker, C. R., Johansen, R., and Hatt, J. (1977). *Anesth. Analg. (Cleveland)* **56,** 690–695.
Hoberman, H. D., Sims, E. A. H., and Peters, J. H. (1948). *J. Biol. Chem.* **172,** 45–58.
Hoff, H. E., Smith, P. K., and Winkler, A. W. (1941). *J. Clin. Invest.* **20,** 607–624.
Horster, M., and Valtin, H. (1971). *J. Clin. Invest.* **50,** 779–795.
Houck, C. R. (1948). *Am. J. Physiol.* **153,** 169–175.
Hubbert, W. T. (1972). *Cornell Vet.* **62,** 13–20.
Imbert, M. J., Berjal, G., Moss, N., deRaiffignac, C., and Bonvalet, J. P. (1974). *Pfluegers Arch.* **346,** 279–290.
James, G. P., Bee, D. E., and Fuller, J. B. (1978). *Clin. Chem.* **24,** 1934–1939.
Jamison, R. L. (1973). *Am. J. Med.* **54,** 281–289.
Jamison, R. L. (1976). *In* "The Kidney" (B. M. Brenner and F. C. Rector, eds.), pp. 391–441. Saunders, Philadelphia, Pennsylvania.
Joekes, A. M. (1972). *Br. Med. Bull.* **28,** 200–204.
Jolliffe, N., and Smith, H. W. (1931). *Am. J. Physiol.* **98,** 572–581.
Jones, J. D., and Burnett, P. C. (1972). *Clin. Chem.* **18,** 280–284.
Jones, J. D., and Burnett, P. C. (1974). *Clin. Chem.* **20,** 1204–1212.
Kaibling, B., de Rouffignac, C., Barrett, J. M., and Kriz, W. (1975). *Anat., Histol., Embryol.* **148,** 121–143.
Kaikara, S., Rutherford, R. B., Schwentker, E. P., and Wagner, H. N., Jr. (1969). *J. Appl. Physiol.* **27,** 218–222.
Kaler, L. W., and Haensly, W. E. (1977). *Am. J. Vet. Res.* **38,** 897–901.
Kassirer, J. P. (1971). *N. Engl. J. Med.* **285,** 385–389.
Kaufman, C. F., and Kirk, R. W. (1973). *J. Am. Anim. Hosp. Assoc.* **9,** 66–70.
Kaufman, J. M., Siegel, N., Lytton, B., and Hayslett, J. P. (1976). *Invest. Urol.* **13,** 441–444.

Kerr, W. S. (1954). *J. Appl. Physiol.* **6,** 762–772.
Kew, M. (1972). *Gut* **13,** 748–753.
Kirkbride, C. A., and Frey, R. A. (1967). *J. Am. Vet. Med. Assoc.* **151,** 742–746.
Kirkebo, A., and Tyssebotn, I. (1977). *Acta Physiol. Scand.* **101,** 257–263.
Klahr, S., and Schoolwerth, A. C. (1977). *In* "Pathophysiology of the Kidney" (N. A. Kurtzman and M. Martinez-Maldonado, eds.), pp. 296–335. Thomas, Springfield, Illinois.
Knox, F. G. (1977). *Physiologist* **20,** 25–31.
Kopple, J. D., and Coburn, J. W. (1974). *J. Am. Med. Assoc.* **227,** 41–44.
Kopple, J. D., and Swendseid, M. E. (1977). *Nephron* **18,** 1–12.
Kosek, J. C., Mazze, R. I., and Cousins, M. J. (1972). *Lab. Invest.* **27,** 575–580.
Kumar, R., Steen, P., and McGeown, M. G. (1972). *Lancet* **ii,** 1005.
Lameire, N. H., Lifschitz, M. D., and Stein, J. H. (1977). *Annu. Rev. Physiol.* **39,** 159–184.
Lamperi, S., Bandiani, G., Fiorio, P., Muttini, P., and Scaringi, G. (1974). *Nephron* **13,** 278–287.
Levinsky, N. G. (1977). *N. Engl. J. Med.* **296,** 1453–1458.
Lewis, R. A., Schwartz, R. H., and Schenk, E. A. (1970). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **29,** 574.
Liveson, J. A., Gardner, J., and Bornstein, B. (1977). *Kidney Int.* **12,** 131–136.
LoGrippo, G. A., Rupe, C. E., Hayaski, H., and LeSher, D. (1970). *Henry Ford Hosp. Med. J.* **18,** 83–90.
Low, D. G., Hiatt, C. W., Gleiser, C. A., and Bergman, E. N. (1956). *J. Infect. Dis.* **98,** 249–259.
Low, D. G., Mather, G. W., Finco, D. R., and Anderson, N. V. (1967). *Am. J. Vet. Res.* **28,** 731–739.
Maack, T. (1975). *Am. J. Med.* **58,** 57–64.
McCance, R. A., and Widdowson, E. M. (1955). *J. Physiol. (London)* **129,** 628–635.
MacDonald, A. G. (1969). *Br. J. Anaesth.* **41,** 644–654.
McQueen, E. G., and Engel, G. B. (1966). *J. Clin. Pathol.* **19,** 392–396.
Mandin, H., and Davidman, M. (1978). *Am. J. Physiol.* **234,** F117–F122.
Mattenheimer, H. (1971). *Med. Clin. North Am.* **55,** 1493–1508.
Mattenheimer, H. (1977). *Ann. Clin. Lab. Sci.* **7,** 422–432.
Mayson, J. S., Schumaker, O., and Nakamura, R. M. (1972). *Am. J. Clin. Pathol.* **52,** 297–299.
Meister, A. (1975). *Med. Clin. North Am.* **1,** 649–666.
Millard, R. W., Higgins, C. B., Franklin, D., and Vatner, S. F. (1972). *Circ. Res.* **31,** 881–888.
Miller, T. R., Anderson, R. J., Linas, S. L., Henrich, W. L., Berns, A. S., Gabow, P. A., and Schrier, R. W. (1978). *Ann. Intern. Med.* **89,** 47–50.
Mitch, W. E., Lietman, P. S., and Walser, M. (1975). *Proc. Am. Soc. Nephrol. Annu. Meet., 8th, Washington, D.C.* Abstr. 65.
Mitchell, J. R., McMurtry, R. J., Statham, C. N., and Nelson, S. D. (1977). *Am. J. Med.* **62,** 518–526.
Mixner, J. P., and Anderson, R. R. (1958). *J. Dairy Sci.* **41,** 306–313.
Morrin, P. A., Joint, M. S., Handa, S. P., and Frame, J. (1970). *Invest. Urol.* **8,** 273–283.
Muehrcke, R. C., Volini, F. I., Morris, A. M., Moles, J. B., and Lawrence, A. G. (1976). *Ann. Clin. Lab. Sci.* **6,** 477–513.
Mulnix, J. A., Rijnberk, A., and Hendriks, H. J. (1976). *J. Am. Vet. Med. Assoc.* **169,** 1327–1330.
Naets, J. P. (1975). *Nephron* **14,** 181–194.
Narayanan, S., and Appleton, H. D. (1972). *Clin. Chem.* **18,** 270–274.
Newman, G. H. (1971). *Postgrad. Med.* **50,** 236–239.
Norrdin, R. W. (1975). *Cornell Vet.* **65,** 173–186.
Nuutinen, L. S., and Toononen, S. (1976). *Ann. Chir. Gynaecol. Fenn.* **65,** 272–276.
O'Connell, J. B., Romeo, J. A., and Mudge, G. H. (1962). *Am. J. Physiol.* **203,** 985–990.
Oken, D. E. (1975). *Annu. Rev. Med.* **26,** 307–319.
Oken, D. E., and Flamenbaum, W. (1971). *J. Clin. Invest.* **50,** 1498–1505.
Oliver, J., Bloom, F., and MacDowell, M. (1941). *J. Exp. Med.* **73,** 141–159.
Orita, Y., Nobuko, I., Naohiko, U., Keiko, A., Keiko, S., Ando, A., Fujiwara, Y., Hirano, S., and Abe, H. (1977). *Nephron* **19,** 19–25.
Osbaldiston, G. W. (1970). *In* "Clinical Biochemistry of Domestic Animals" (J. J. Kaneko and C. E. Cornelius, eds.), 2nd ed., Vol. 1, pp. 1–60. Academic Press, New York.
Osbaldiston, G. W., and Fuhrman, W. (1970). *Can. J. Comp. Med.* **34,** 138–141.
Osborne, C. A., Low, D. G., and Finco, D. R. (1972). "Canine and Feline Urology." Saunders, Philadelphia, Pennsylvania.

Papper, S. (1975). *Clin. Nephrol.* **4,** 41–44.
Phillips, R. A., Dole, V. P., Hamilton, P. B., Kendall, E., Archibald, R. M. and Van Slyke, D. D. (1946). *Am. J. Physiol.* **145,** 314–336.
Pickering, E. C. (1965). *Proc. Nutr. Soc.* **34,** 73–80.
Pierce, A. E. (1959). *J. Physiol. (London)* **148,** 469–488.
Pierce, A. E. (1961). *J. Physiol. (London)* **156,** 136–149.
Pihl, B. (1974). *Scand. J. Urol. Nephrol.* **8,** 147–154.
Pitts, R. F. (1934). *Am. J. Physiol.* **109,** 532–541.
Pitts, R. F. (1974). "Physiology of the Kidney and Body Fluids." Yearbook Publ., Chicago, Illinois.
Polar, E., and Metcoff, J. (1965) *Clin. Chem.* **11,** 763–770.
Poutsiaka, J. W., Keysser, H., Thomas, B. G., and Linegar, C. R. (1962). *Toxicol. Appl. Pharmacol.* **4,** 55–69.
Powers, T. E., Powers, J. D., and Garg, R. C. (1977). *Am. J. Vet. Res.* **38,** 1933–1936.
Raab, W. P. (1972). *Clin. Chem.* **18,** 5–25.
Rabinowitz, L., Gunther, R. A., Shoji, E. S., Freedland, R. A., and Avery, E. H. (1973). *Kidney Int.* **4,** 188–207.
Rahill, W. J., and Subramanian, S. (1973). *Lab. Anim. Sci.* **23,** 92–96.
Raskin, N. H., and Fishman, R. A. (1976). *N. Engl. J. Med.* **294,** 204–210.
Raskova, J., and Morrison, A. B. (1978). *Lab. Invest.* **38,** 103–109.
Rector, F. C. (1976). *In* "The Kidney" (B. M. Brenner and F. C. Rector, eds.), pp. 318–343. Saunders, Philadelphia, Pennsylvania.
Refsum, H. E., and Stromme, S. B. (1974). *Scand. J. Lab. Invest.* **33,** 247–254.
Reidenberg, M. M. (1976). *Clin. Pharmacokinet.* **1,** 121–125.
Reidenberg, M. M. (1977a). *Am. J. Med.* **62,** 466–470.
Reidenberg, M. M. (1977b). *Am. J. Med.* **62,** 482–485.
Relman, A. S., and Levinsky, N. G. (1971). *In* "Diseases of the Kidney" (M. B. Strauss and L. G. Welt, eds.), pp. 87–139. Little, Brown, Boston, Massachusetts.
Rennke, H. G., and Venkatachalam, M. A. (1977). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36,** 2619–2626.
Richards, p. (1975). *Nephron* **14,** 134–152.
Rickers, H., Brochner-Mortensen, J., and Rodbro, P. (1978). *Scand. J. Urol. Nephrol.* **12,** 39–44.
Robinson, T., Harbison, M., and Bovee, K. C. (1974). *Am. J. Vet. Res.* **35,** 487–491.
Rosenfeld, S. (1955). M.S. Thesis, Univ. of California, Davis.
Rous, S. N., and Wakim, K. G. (1967). *J. Urol.* **98,** 30–35.
Rubin, M. I., and Balish, T. (1971). *Pediatr. Clin. North Am.* **18,** 245–264.
Rutecki, G. J., Goldsmith, C., and Schreiner, G. E. (1971). *N. Engl. J. Med.* **284,** 1049–1052.
Schenk, E. A., Schwartz, R. H., and Lewis, R. A. (1971). *Lab. Invest.* **25,** 92–95.
Schloerb, P. R. (1960). *Am. J. Physiol.* **199,** 661–665.
Schmidt, R. W., Bricker, N. S., and Gavellas, G. (1976). *Kidney Int.* **10,** 287–294.
Schneider, J. A., and Seegmiller, J. E. (1972). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stansbury, J. B. Wyngaarden, and D. S. Fredrickson, eds.), pp. 1581–1605. McGraw-Hill, New York.
Schnermann, J. (1975). *Clin. Nephrol.* **3,** 75–81.
Schon, D. A., Silva, P., and Hayslett, J. P. (1974). *Am. J. Physiol.* **227,** 1323–1330.
Schreiner, G. E., and Maher, J. F. (1961). "Uremia: Biochemistry, Pathogenesis, and Treatment." Thomas, Springfield, Illinois.
Schrier, R. (1976). *In* "Renal and Electrolyte Disorders" (R. W. Schriver, ed.), pp. 45–77. Little, Brown, Boston, Massachusetts.
Schwartz, W. B., and Cohen, J. J. (1978). *Am. J. Med.* **64,** 417–428.
Segal, S. (1976). *N. Engl. J. Med.* **294,** 1044–1051.
Shannon, J. A. (1936). *Am. J. Physiol.* **117,** 206–225.
Shils, M. E. (1963). *Ann. Intern. Med.* **58,** 389–408.
Silva, P., Brown, R. S., and Epstein, F. H. (1977). *Kidney Int.* **11,** 466–475.
Silverman, M. (1975). *Biochim. Biophys. Acta* **457,** 304–351.
Silverman, M., and Huang, L.)1976). *Am. J. Physiol.* **231,** 1024–1032.
Simenhoff, M. L., Burke, J. F., Saukkonen, J. J., Ordinario, A. T., and Doty, R. (1977). *N. Engl. J. Med.* **297,** 132–135.

Simon, D., and Luke, R. G. (1971). *Proc. Soc. Exp. Biol. Med.* **137,** 1073–1074.
Slack, T., and Wilson, D. M. (1976). *Mayo Clin. Proc.* **51,** 296–300.
Slatopolsky, E., Cagler, S., Pennell, J. P., Taggart, D. D., Canterbury, J. M., Reiss, E., and Bricker, N. S. (1971). *J. Clin. Invest.* **50,** 492–499.
Smith, H. W. (1951). "The Kidney Structure and Function in Health and Disease." Oxford Univ. Press, London and New York.
Smith, H. W. (1959). "From Fish to Philosopher. The Story of Our Internal Environment." Ciba, Summit, New Jersey.
Sobel, J. (1964). *Lancet* **ii,** 760–761.
Souhami, R. L. (1973). *Br. J. Exp. Pathol.* **54,** 380–387.
Stein, J. H. (1976). *In* "The Kidney" (B. Brenner and F. Rector, eds.), Chap. 5. Saunders, Philadelphia, Pennsylvania.
Stein, J. H., Boonjarern, S., Mauk, R. C., and Ferris, T. F. (1973). *J. Clin. Invest.* **52,** 39–47.
Stein, J. H., Lifschitz, M. D., and Barnes, L. D. (1978). *Am. J. Physiol.* **234,** F171–F181.
Street, A. E., Chesterman, H., Smith, G. K., and Quinton, R. M. (1968). *Toxicol. Appl. Pharmacol.* **13,** 363–371.
Strombeck, D. R., Meyer, D. J., and Freedland, R. A. (1975). *J. Am. Vet. Med. Assoc.* **166,** 1109–1111.
Suzuki, A. (1971). *Jpn. J. Pharmacol.* **21,** 75–85.
Swanson, R. E., and Hakim, A. A. (1962). *Am. J. Physiol.* **203,** 980–984.
Swenson, R. S., Peterson, D. T., Eshleman, M., and Reaven, G. M. (1973). *Kidney Int.* **4,** 267–272.
Tallan, H. H., Moore, S., and Stein, W. H. (1954). *J. Biol. Chem.* **211,** 927–938.
Tennant, B. C., Lowe, J. E., and Tasker, J. B. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 670.
Thier, S. O., McCurdy, D. K., and Rastegar, A. (1977). *In* "Pathophysiology of the Kidney" (N. A. Kurtzman and M. Martinez-Maldonado, eds.), pp. 335–355. Thomas, Springfield, Illinois.
Thornton, J. R., and English, P. B. (1976). *Aust. Vet. J.* **52,** 335–337.
Thysell, H. (1969). *Acta Med. Scand.* **185,** 401–407.
Touraine, J. L., Touraine, F., Revillard, J. P., Brochier, J., and Traeger, J. (1975). *Nephron* **14,** 195–208.
Tripett, S. (1957). *J. Chem. Soc.* 1929–1930.
Trueta, J., Barclay, A. E., Daniel, P. M., Franklin, K. J., and Prichard, M. M. L. (1948). "Studies on the Renal Circulation." Blackwell, Oxford.
Tucker, B. J., and Blantz, R. C. (1977). *Am. J. Physiol.* **232,** F477–F483.
Tyler, F. H. (1972). *In* "Metabolic Basis of Inherited Disease" (J. B. Stanbury, ed.), pp. 1204–1218. McGraw-Hill, New York.
Valtin, H. (1977). *Am. J. Physiol.* **233,** F491–F501.
Van Buren, C. T., Dudrick, S. J., and Baumbauer, E. (1972). *Surg. Forum* **23,** 83–84.
Van Pilsum, J. F. Kito, M. E., and Hess, J. (1976). *J. Biol. Chem.* **222,** 225–236.
Van Urk, H., Malamud, D., Soler-Montesinos, L., and Malt, R. A. (1978). *Lab. Invest.* **38,** 674–676.
Vaughan, E. D., Jr., Sweet, R. E., and Gillenwater, J. Y. (1973). *J. Urol.* **109,** 979–982.
Vaughan, E. D., Jr., and Wyker, A. W. (1971). *J. Urol.* **105,** 709–711.
Velasquez, M. T., Notargiacomo, A. V., and Cohn, J. N. (1973). *Am. J. Physiol.* **224,** 322–327.
Von Baeyer, H., Von Conta, C., Haeberle, D., and Deetjen, P. (1973). *Pfluegers Arch.* **343,** 273–286.
Walker, J. B. (1960). *J. Biol. Chem.* **235,** 2357–2361.
Walker, J. B. (1961). *J. Biol. Chem.* **236,** 493–498.
Wallner, S. F., Kurnick, J. E., Vautrin, R., and Ward, H. P. (1977). *Am. J. Hematol.* **3,** 45–55.
Walser, M. (1978). *Nephron* **21,** 57–74.
Walser, M., and Bodenlos, L. J. (1959). *J. Clin. Invest.* **38,** 1617–1626.
Wen, S. F. (1976). *Am. J. Physiol.* **231,** 468–475.
West, C. D., Traeger, J., and Kaplan, S. A. (1955). *J. Clin. Invest.* **34,** 887–898.
Westall, R. G. (1953). *Biochem. J.* **55,** 244–248.
Westermark, L., and Wahlin, A. (1969). *Acta Anaesthesiol. Scand.* **13,** 185–208.
White, A., Handler, P., and Smith, E. L. (1973). "Principles of Biochemistry." McGraw-Hill, New York.
Williams, R. D., Fanestil, D. D., and Blackard, C. E. (1976). *Invest. Urol.* **14,** 148–152.
Wilson, D. R. (1977). *Annu. Rev. Med.* **28,** 329–339.
Windhager, E. E., and Giebisch, G. (1976). *Kidney Int.* **9,** 121–133.
Wolf, A. V. (1969). *Am. J. Med.* **46,** 837–843.

Woolf, L. T. (1966). "Renal Tubular Dysfunction." Thomas, Springfield, Illinois.
Wright, F. (1977). *Am. J. Physiol.* **233,** F1-F7.
Youngberg, S. P., Marchand, G. R., Spillman, W. S., Osswald, H., and Knox, F. G.)1977). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36,** 2609-2613.
Zielinski, F. W., Holly, F. E., Robinson, G. D., and Bennett, L. R. (1977). *Radiology* **125,** 753-759.

10

Fluids, Electrolytes, and Acid-Base Balance

JOHN B. TASKER

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

I. INTRODUCTION

Water is the most abundant substance in the body—it represents approximately 60% of the body as a whole and more than 90% of the plasma. It is the medium in which vital chemical processes occur. It is the substance on which the volume of the body fluids depends, and thus it is necessary for circulation, by which nutrients and oxygen are distributed to the tissues, and for excretion, the elimination from the body of useless products of metabolism. While water determines the volume of the body fluids, the substances dissolved in that water determine the biological properties of the fluid. In the normal organism, the concentrations of body fluid constituents are held under strict control by a multitude of mechanisms which, in sum, maintain health. When these mechanisms fail, the composition of the body fluids deviates from that which is favorable to normal functioning of the organism. This jeopardizes the well-being of the animal and often results in death.

In the last 20 years, the clinical significance of body fluid derangements has become widely appreciated in veterinary medicine. The administration of corrective fluid therapy to animal patients is commonplace. In many instances, such therapy is but one part of a regimen of comprehensive supportive care. In other instances, however, appropriate fluid therapy is the sole factor which determines the survival of the patient. Rational management of such patients to a successful outcome requires a clear understanding of the anatomy and physiology of the body fluids, the pathological mechanisms by which the normal properties become deranged, the significance of such derangements, the means by which these disturbances can be identified accurately, and, finally, the procedures which can be used to correct such disturbances in a prompt, safe, and effective manner.

The purpose of this chapter is to provide information which will help the reader achieve better understanding of this complex but very important subject.

II. PHYSIOLOGY OF BODY FLUIDS

A. Units of Solute Measurement

1. Mass

In most instances in clinical chemistry, the quantity of a substance is expressed by reference to the mass, or weight, of the substance present. In the metric system, the gram is the basic unit of mass employed. Since most substances of biological importance are dissolved in the body fluids, it is customary to refer to the *concentration* of substance in a standard volume of solution. In some cases the standard volume is the milliliter, but more often it is a liter or the deciliter (100 ml). Thus, we find plasma protein usually reported as grams of protein per deciliter of serum or plasma, and glucose reported as milligrams of glucose per deciliter of plasma, serum, or whole blood, depending upon the specimen used for analysis.

In general chemistry, an arbitrary system has been developed to indicate the relative mass of the elements. This value is called the atomic mass. The basis for this system is the assignment of an atomic mass of 16 to oxygen and an atomic mass of 1 to hydrogen. The relative mass of all other elements is expressed by comparison with the reference values; i.e., that of sodium is 23 and that of potassium is 39.1. A mass in grams of any element which is equivalent to its atomic mass contains a specific number of atoms (6.02×10^{23}, Avogadro's number). This is the gram-atomic mass. Twenty-three grams of sodium and 39.1 gm of potassium are the gram-mass weights of these substances and contain equal numbers of atoms.

When two or more atoms are associated in molecules, the molecular mass is the sum of the atomic mass of the constituent elements. The moelcular mass of sodium chloride, NaCl, is 58.5 (sodium, 23, plus chloride, 35.5), and the molecular mass of calcium chloride, $CaCl_2$, is 111 (calcium, 40, plus 2 × chloride, 71; 40 + 71 = 111). The molecular mass (or atomic mass) of a substance, expressed in grams, is called a *mole* or a gram-molecular (atomic) mass. Tables of atomic and molecular mass can be found in many chemistry texts and reference manuals.

When 1 molecular mass, 1 mole, of a substance is dissolved in 1 kg of water, the concentration is described as a 1 *molal* solution. When the reference volume is 1 liter of the final solution, 1 mole of a substance per liter of final solution, it is described as a 1 *molar* solution. When 58.5 gm of sodium chloride are dissolved in 1 kg of water, the concentration of the solution is described as a 1.0 *molal* solution of NaCl. When 58.5 gm of sodium chloride are contained in a 1-liter volume of solution, it is a 1.0 *molar* solution. The difference between these two expressions is very slight; the terms are defined here merely to indicate correct terminology.

2. Combining Capacity (Equivalency)

Electrolytes are substances which in solution exist as positive- and negative-charged particles called ions but which in the solid state are associated in molecules which bear no charge. Sodium chloride is an electrolyte; when dissolved in water, it dissociates into sodium and chloride ions, which bear positive and negative charges, respectively. Ions

with a positive charge are cations; those with a negative charge are anions. The combination of cations and anions in electrolyte molecules is related to the charge on the particles, not to the mass of the particle. This is also true of combinations of acids and bases. In chemistry, an *equivalent* is the mass in grams of a substance which will combine with or replace 1 gm of hydrogen ion. The atomic mass of hydrogen is 1; thus, 1 gm of hydrogen ion, 1 equivalent, is also 1 mole (6.02×10^{23} atoms). One equivalent of chloride ion is 35.5 gm (1 mole, 6.02×10^{23} atoms). For univalent atoms, 1 mole equals 1 equivalent. Therefore, 1 mole (equivalent) of H^+, plus 1 mole (equivalent) of Cl^- equals 1 mole (equivalent) of HCl. However, for multivalent ions, 1 equivalent is equal to the molecular (atomic) mass divided by the charge on the particle. Thus, 1 mole of calcium ion, Ca^{2+} (atomic mass 40.08) is 40.08 gm, but 1 equivalent is 20.04 gm (atomic weight divided by charge, 40.08/2 = 20.04). In general chemistry, a solution which contains 1 equivalent of solute per liter is called a 1 normal solution. The term "normal" should not be used in medical terminology since it is not apparent whether "normal" refers to equivalents in solution or to similarities to "normal" animals (e.g., "normal saline solution").

3. *Osmotic Pressure*

Osmotic properties of solutions are not related to the mass of solute present nor to its charge. They are related only to the *number* of particles in solution. The unit of measurement is the osmole. One osmole of a nondissociable substance is equivalent to the atomic or molecular mass in grams. As indicated previously, this quantity of any substance contains 6.02×10^{23} atoms or molecules (particles). An osmole of a substance which dissociates in solution into two or more ions is the molecular weight in grams divided by the number of particles into which each molecule dissociates. For example, 180 gm of glucose, 1 mole, contains 6.02×10^{23} molecules (particles) and is thus 1 osmole, while 58.5 gm of sodium chloride, also 1 mole of that substance, represents 2 osmoles since each molecule, when in solution, dissociates into two ions (particles). The number of particles obtained from 1 mole of sodium chloride in solution is equal to 2 × Avogadro's number, i.e., $2 \times 6.02 \times 10^{23}$.

Osmolarity is the number of osmoles per liter of solution; osmolality is the number of osmoles per kilogram of water. Although these two values are very similar, osmolality is the more correct indication of osmotic properties and is the preferred expression for clinical use.

In biological fluids, the solute concentrations are relatively low. Concentrations of solute are usually less than 1 mole, 1 equivalent, or 1 osmole/liter. To minimize the use of decimals, it is conventional to express these small quantities of solute by the use of the metric prefix "milli" to indicate units which are one-thousandth part of the standard unit. Conventional terms, therefore, are millimoles per liter (mmole/liter) or milligrams per deciliter (mg/dl), milliequivalents per liter (mEq/liter), and milliosmoles per kilogram of water (mosmol/kg). Typical plasma electrolyte concentrations are presented in Table I. It can be seen that only when expressed in milliequivalents per liter do the concentrations of cations and anions appear to be equal.

An attempt has been made in recent years to standardize the units and terminology use in clinical chemistry. The resulting *Systeme Internationale d'Unites* (SI units) has not been accepted immediately, but changes in this direction may be anticipated. In the SI terminology, all solute concentrations are expressed in moles and millimoles per liter,

TABLE I

Plasma Electrolyte Concentrations Expressed in Different Units

Plasma electrolyte	Normal concentration			
	mg/dl	mmole/liter	mEq/liter	mosmole/kg
Cations				
Sodium (Na^+)	326.6	142.0	142.0	142.0
Potassium (K^+)	16.8	4.3	4.3	4.3
Calcium (Ca^{2+})[a]	5.0	1.25	2.5	1.25
Magnesium (Mg^{3+})[a]	1.3	0.55	1.1	0.55
Total cations	349.7	148.1	149.9	148.1
Anions				
Chloride (Cl^-)	369.2	104.0	104.0	104.0
Bicarbonate (HCO_3^-)	146.4	24.0	24.0	24.0
Phosphate[b] ($H_2PO_4^-$, HPO_4^{2-})	3.4	1.1	2.0	1.1
Proteins[b]	7000	2.5	14.0	2.5
Others (sulfate lactate, etc.)	—	5.5	5.9	5.5
Total anions	7519.0	135.5	149.9	137.0
Total milliosmoles from electrolytes				285.1

[a] Only ionized calcium and magnesium have been considered here.

[b] Phosphate concentration is that of inorganic phosphorus. Since a variable equilibrium exists between $H_2PO_4^-$ and HPO_4^{2-}, the actual valence and milliequivalents must be estimated. The same variability is true of protein anions as well.

blood gases are expressed in kilopascals, and osmolality is expressed in milliKelvins of freezing point depression (Lehmann, 1976) (see Appendix I).

B. Distribution and Composition of Body Fluids

1. Body Fluid Compartments

The total body water is divided into two major compartments: intracellular fluid (ICF) and extracellular fluid (ECF). The latter is subdivided into the intravascular fluid, which is the plasma, and the interstitial fluid, the fluid outside the vessels and between the cells of the body tissues. The ECF also includes the "transcelluar fluids," which are specialized fluids of the body formed by active transport processes rather than passive transudation from plasma as the interstitial fluid is. The transcellular fluids include the fluids of the salivary glands, pancreas, liver and biliary tree, thyroid gland, skin, gonads, mucous membranes of the respiratory and gastrointestinal tracts, and kidneys, as well as the cerebrospinal fluid, the fluids of the eye, and the contents of the gastrointestinal tract (Hays, 1972).

Numerous studies have been made of the volumes of the fluid spaces in various animals. The method used for such determinations is a tracer dilution technique in which a substance is injected into the body which is believed to be distributed more or less exclusively in a specific body fluid compartment. After a suitable time has elapsed for equilibration of the tracer substance in the body, its concentration in the plasma is determined. Corrections to a theoretical concentration at "zero" time can be made to account

TABLE II

Volume of Body Fluid Compartments[a,b]

Species	Subjects	Plasma	ECF	TBW	ICF	ISF	Reference
Horse	Standardbred, adults	6.3					Kohn *et al.* (1978)
	Draft horses, adults	4.9					Dalton and Fisher (1963)
Pony		4.3	38.8			34.5	Alexander (1974)
	10 months to 4 years	5.1		67.7			Deavers *et al.* (1973)
Cattle	Lactating			70.9			Aschbacher *et al.* (1965)
	Nonlactating			68.3			Aschbacher *et al.* (1965)
	Nonlactating—fat			56.3			Aschbacher *et al.* (1965)
	Mature			64.5			Seif *et al.* (1973)
	129 days			69.4			Hansard (1964)
	1175 days			50.2			Hansard (1964)
	2 months	5.4	37.1			31.7	Payne *et al.* (1967)
	3 years	3.7	26.3			22.6	Payne *et al.* (1967)
	2 years—winter	2.8					Howes *et al.* (1963)
	2 years—summer	3.2					Howes *et al.* (1963)
	1–4 weeks		50.2	76.5	26.6		Fayet (1971)
	0–2 weeks	7.7	56.4	86.1	29.7	48.7	Phillips *et al.* (1971)
	7–15 weeks	7.9					Haxton *et al.* (1974)
	Birth	5.3					Mollerberg *et al.* (1975)
	1 day	6.5					Mollerberg *et al.* (1975)
	90 days	4.9					Mollerberg *et al.* (1975)
Sheep	Adult	4.9	25.8	56.3	30.5	20.9	Huber (1971)
	8 months			66.6			Reid *et al.* (1963)
	20 months			58.1			Reid *et al.* (1963)
	2.5–14 years			54.6			Anand and Parker (1966)
	137 days			62.7			Hansard (1964)
	350 days			56.5			Hansard (1964)
	Adult ewes	5.1	15.5	53.7	38.2	10.4	English (1966)
	Adult wethers	4.0	14.9	43.0	28.1	10.9	English (1966)
	Lambs	5.6	19.7		14.1		English (1966)
Goats		4.2	21.5	69.0	47.5	17.3	Manston (1971)
Swine	Birth	6.4		81.7			Pownall and Dalton (1973)
	30 days	5.8		70.9			Pownall and Dalton (1973)
	1 day			80.1			Tollerz (1964)
	19 days			72.7			Tollerz (1964)
	60 days			65.1			Hansard (1964)
	300 days			46.2			Hansard (1964)
Dogs	Birth			84.0			Sheng and Huggins (1971)
	Adult—summer	3.6					Hannon and Durrer (1962)
	Adult—winter	4.1					Hannon and Durrer (1962)
	Adult	5.1					Lombardi (1972)
		5.9	28.5	56.2	27.6		Reece (1972)
Man	Young adult male	4.5	27.0	60.0	33.0	22.5	Hays (1972)

[a] Percentage of body weight.

[b] Abbreviations: ECF, extracellular fluid; TBW, total body water; ICF, intracellular fluid; ISF, interstitial fluid.

for loss of tracer by excretion or alteration during the equilibration period. By knowing the amount of tracer substance injected and its concentration in the body fluid space being measured, one can calculate the volume of the space in which dilution occurred. Dyes, chemicals, and radioactive materials have been used as tracers. The choice depends on ease of measurement, restriction of the substance to the fluid compartment being studied, and lack of toxic effects.

Table II lists some of the studies which have been made of the body fluid compartments. A large number of investigations have been made to determine body fluid volumes in various species; those presented are only a small fraction of the total, but they are sufficient to indicate some important factors related to this subject. A very wide range of data has been obtained. Much of this variation is due to different tracers being used which are distributed to various degrees in the space being measured. Further variation is the result of technical differences from study to study in the performance of the testing procedure. In spite of these influences, however, certain general observations can be made.

1. When expressed as a percentage of body weight, the relative distribution of fluid in the compartments is similar for all domestic animal species and man.

2. Total body water is a much greater proportion of body weight in young animals than in adults. Although this is evident in all species, it is especially noticeable in the data of Fayet (1971) and Phillips *et al.* (1971) for calves (Table II): Total body water is reported to exceed 75% of body weight and ECF to be greater than 50%, while for adult cattle total body water is reported to be near 60% (Aschbacher *et al.*, 1965) and ECF closer to 25% (Payne *et al.*, 1967).

3. Much of the difference in total body water data among animals of the same species is due to variations in body fat. Since fat is a water-poor tissue, large amounts of fat reduce the proportion of body weight which is water. This is apparent in the data from Aschbacher *et al.* (1965) on cows but would be expected in all species. This may explain much of the observed difference between young and old animals.

2. *Body Fluid Composition*

The composition of the body fluids is given in Table III. Although the data have been taken from studies on human beings, the information in this table is a general approximation of actual solute concentrations found in many animals. Careful studies show variation in these values from one part of the body to another, from one day to another, and from one individual to another. Significant differences exist among species, some of which will be apparent from a consideration of typical serum chemistry values for clinical laboratory tests (see Table IX).

One specific cell in which there is a remarkable variation in intracellular electrolyte concentration is the erythrocyte. In most mammals the erythrocyte has a much higher concentration of potassium, and a lower concentration of sodium, than plasma. In the dog and cat, however, the sodium and potassium concentrations of normal erythrocytes are very similar to those of ECF. Among sheep, there are certain genetically determined high red cell potassium sheep and other low red cell potassium sheep. Some of these differences are shown in Table IV.

In general, the interstitial fluid and intravascular fluid are in equilibrium and have similar solute concentrations. The differences which exist between these fluids are due to

TABLE III

Electrolyte Composition of the Body Fluids[a]

Constituent	Serum (mEq/liter)	Interstitial fluid (mEq/liter)	Intracellular fluid (muscle) (mEq/liter)
Cations			
Sodium	142.0	145.1	12.0
Potassium	4.3	4.4	150.0
Calcium	5.5	2.4	4.0
Magnesium	1.1	1.1	34.0
Total cations	149.9	153.0	200.0
Anions			
Chloride	104.0	117.4	4.0
Bicarbonate	24.0	27.1	12.0
Phosphate	2.0	2.3	40.0
Protein	14.0	0	54.0
Other	5.9	6.2	90.0
Total anions	149.9	153.0	200.0

[a] From Rose (1977).

the protein being confined to the intravascular space. This results in a Gibbs-Donnan equilibrium being established, in which unequal distribution of both cations and anions results from nondiffusibility of the protein anions (Hays, 1972).

The principal ions of the ECF are sodium, chloride, and bicarbonate. In contrast to this, the principal ions of the ICF are potassium, magnesium, phosphate, and protein. Although the cell is permeable to both sodium and potassium, the marked difference in concentration of these cations found in most cells is due to the sodium-potassium pump, which eliminates sodium from cells and concentrates potassium within cells.

TABLE IV

Sodium and Potassium Content of Mammalian Erythrocytes[a,b]

Species	Sodium	Potassium
Dog	107	9
Cat	104	6
Horse	—	88
Ox	79	22
Sheep[c]	16	64
	84	18
Swine	11	100

[a] From Prankerd (1961).

[b] Milliequivalents per liter cells.

[c] There is a marked variation among sheep in the intracellular cation concentration.

C. Osmotic Forces

The volume of fluid in the body as a whole and the distribution of that fluid within the body fluid compartments are determined largely by osmotic forces. Since the structural barriers which demarcate these different spaces are, in almost all instances, freely permeable to water, water distribution is determined by solute concentrations in each space. Total body water is a reflection of total solute in the body which "holds" water. Water absorption from the gastrointestinal tract and from the renal tubules is a passive process resulting from the existence of osmotic gradients.

The relative and absolute quantities of fluid in the ICF and in the ECF are determined by the solute in each space which functions osmotically to retain greater or lesser quantities of water in each space. In the normal animal, extracellular sodium and intracellular potassium are the chief determinants of extracellular and intracellular fluid volume, respectively. Sodium, in addition, is the chief determinant of total body water volume.

Partition of water between the intravascular space and the interstitial space is also influenced by osmotic forces. The confinement of plasma protein to the intravascular space results in a small osmotic (oncotic) force, which pulls water into the vessels. This is balanced by the hydrostatic pressure in the vessels, which tends to drive water in the opposite direction.

Fluid volume disorders of major clinical importance may result from abnormalities in solute concentration, which, in turn, result in maldistribution of water in the body.

D. Essential Parameters

The well-being of the organism depends upon cellular biochemical processes continuing at an optimal rate. These biochemical reactions require suitable substrate availability and the removal of the products of the reactions. The speed of the reactions is influenced greatly by the concentrations of the reactants and especially by the hydrogen ion concentration in the reaction medium.

The availability of nutrients in the cells affects individual reactions that require these nutrients. The water, electrolyte, and acid-base status of the body, on the other hand, determines the cellular environment in which *all* such reactions occur. Disturbances in these parameters thus have a universal effect on cellular function. Since these disturbances can occur suddenly, and since relatively minor absolute changes can cause dramatic alterations in cell function, the problems described in this chapter are of constant concern to the clinician and are also fascinating to the physiologist.

III. PATHOPHYSIOLOGY OF BODY WATER

A. Water Balance

Water is available to the animal from three sources: pure water, i.e., drinking water; water contained in foodstuffs (the water content of dry hay is 10–12%, and the water content of canned dog food is approximately 70%); and water produced *de novo* by oxidative metabolism in the body. (Oxidation of 1 gm of fat, carbohydrate, or protein results in synthesis of 1.07, 0.60, or 0.41 gm of water, respectively.)

Water derived from these sources is balanced by losses of water in urine and feces, in expired air from the respiratory tract, and by evaporation from the skin. Although the gains and losses of water vary greatly, they are carefully balanced in the normal animal so that the amount of water in the body fluctuates very little. Since there is little control over fecal water loss and "insensible" losses from skin and lungs, the principal regulation of water loss from the body is by the kidney. Since there is little control over water intake in foodstuffs and that derived from metabolism, the principal regulation of water input is thirst, i.e., control over consumption of drinking water.

Useful figures to describe actual water balance in various species are not available. Studies have been made, but the results are useful only under the specific conditions of the individual study. Metabolic water production can be determined only by knowledge of the substrates used for energy production in the animal. Insensible losses from skin and lungs are very difficult to measure directly and vary greatly with ambient temperature and exercise. Fecal water losses vary with the species of animal and with the type of diet an animal is consuming. Water drunk and renal water loss reflect all of the above variables plus the salt and water content of the diet. Drinking water intake in adult cats, for example, was reported to average 5 ml/day when the cats were eating canned cat food but increased to almost 150 ml/day when fed an expanded dry cat food and to over 200 ml/day when fed a nonexpanded dry cat food (Jackson and Tovey, 1977). In another experiment, the addition of 4% sodium chloride to a cat food increased water consumption from 99 ml/day to 169 ml/day (Hamar *et al.*, 1976). These figures illustrate the great variation in water balance data due to the specific conditions under which any study is done.

Water in the body passes freely across most tissues and membranes in accordance with osmotic forces, and the fluid compartments, with few exceptions, are thus in osmotic equilibrium. That is, they are of the same osmolality. The volume of fluid in each space, therefore, is determined by the solute in each space. What determines the volume of fluid in the body as a whole? The central determinant appears to be what has been called *effective circulating volume,* "that part of the ECF that is effectively perfusing the tissues" (Rose, 1977). Diminution in this parameter leads to diminished blood pressure, stimulation of arterial baroreceptors, and increase in sympathetic tone. The latter causes a series of cardiovascular changes which *compensate* to some degree for the diminished circulating volume. More importantly, these physiological responses lead to increased renin secretion, which, in addition to causing vascular changes, results in increased secretion of aldosterone, the salt-retaining hormone. Increased aldosterone results in increased renal tubular resorption of sodium, increased renal water conservation, and expanded ECF volume. The increased sympathetic tone also stimulates hypothalamic thirst centers and causes increased water consumption.

Thus, water deficit in the body, resulting in diminished effective circulating volume and altered sympathetic tone, is corrected, in part, by increased water resorption in the kidney and increased water consumption. When overhydration leads to excessive circulating fluid volume, the body water is reduced by a reversal of these mechanisms.

In addition to these pressure-induced changes in water balance, important osmotic influences are also effective in altering water balance. Changes in osmolality of the ECF alter both the thirst centers and the closely related hypothalamic centers which regulate production and release of antidiuretic hormone (ADH). Increased ECF osmolality results in stimulation of the thirst centers and increased release of ADH, additional mechanisms

which result in increased water intake and diminished renal water loss and thus tend to correct hypovolemia when this is associated with the hyperosmotic state. Overhydration associated with the hypoosmotic state is corrected by the reverse of these mechanisms.

B. Dehydration

Water balance in the normal animal results from adequate supplies of drinking water, normal renal function, normal central nervous system function, and nominal losses of water from the digestive tract, skin, and lungs. When one or more of these controlling factors are abnormal, a serious threat to water balance occurs. Compensations can and do occur, but, when these fail, dehydration results.

When access to drinking water is denied, or the central nervous system is depressed so the animal does not drink, or the inability to drink or swallow prevents successful intake of water, the threat of serious dehydration is present. The actual development of significant negative water balance is prevented or delayed by several compensating factors. With normal renal function, urinary water loss is greatly diminished; with normal intestinal function, fecal water loss is greatly diminished. In certain desert rodents this response is so successful that metabolic water and the small amount of water in food is sufficient to maintain water balance without any drinking water. Among domestic animals, the herbivores have capacious digestive tracts containing large volumes of fluid. This represents a reservoir of water which sustains normal volumes of ICF and ECF for many days after water intake is curtailed. Eventually, of course, dehydration results from diminished water intake in most animals.

A far more serious cause of dehydration is uncontrollable loss of water from the body. Vomiting and diarrhea, polyuria, severe sweating, and even polypnea may result in large water losses. When physiological mechanisms cause increased water conservation as well as increased thirst and augmented water intake, water balance can be maintained in spite of greatly abnormal losses. In many instances, however, compensatory mechanisms are also impaired; e.g., central nervous depression prevents increased water consumption, or poor management denies the necessary increased water intake. At these times, life-threatening dehydration occurs.

Patients with diabetes insipidus lack the capacity for renal conservation of water. There is greatly increased water loss but no dehydration (in most patients) because thirst mechanisms have caused polydipsia and a new steady state of water balance has been achieved. If water deprivation occurs, either by accident or by intention, marked negative water balance will develop quickly, resulting in dehydration, hypovolemia, shock, and death.

The clinical syndrome of shock is not necessarily associated with dehydration and negative water balance. It is characterized by inadequate effective circulating volume. This may be due to dehydration, but it may also be due to cardiovascular alterations or maldistribution of ECF. It is important to distinguish among the meanings of dehydration, hypovolemia, and shock. Dehydration is a pathological state resulting from a clinically significant reduction in total body water. Hypovolemia is an abnormal reduction in blood volume. Shock is a clinical syndrome resulting from inadequate capillary perfusion. While the three conditions may be coexistent, they are not synonymous.

C. Overhydration

Although dehydration is the most common water balance disorder encountered in spontaneous diseases of domestic animals, overhydration, when it occurs, is also a body fluid disorder with life-threatening significance. This condition is almost always an iatrogenic disturbance. Physiological thirst mechanisms and normal renal function prevent overhydration in the normal animal. Even disease processes which result in excessive drinking (psychogenic polydipsia) or diminished renal excretion of water (''renal shutdown'') do not result in fluid balance problems if but one problem is present. It is extremely rare for an animal to have both disturbances at the same time.

The usual cause of overhydration is excessive administration of therapeutic solutions. This may develop gradually over a period ranging from a few hours to a few days when voluminous fluid therapy is administered to anuric patients, regardless of the actual rate of fluid administration. It may also develop acutely in any patient, even those with normal renal function, when an excessive rate of fluid administration is employed. In either event, overhydration may result in hypervolemia and lead to cardiovascular stress, pulmonary edema, and neurological disturbances.

IV. PATHOPHYSIOLOGY OF OSMOTIC PRESSURE

A. Control of Osmolality

The total volume of body water and the distribution of water among the body fluid compartments are determined by the amount of solute present. Confinement of specific solutes to each body fluid compartment creates an osmotic force which draws available water into the space until osmotic equilibrium is established. A regulatory system is present which responds to fluctuations in ECF osmolality and regulates solute concentration.

The osmolar regulating center is located in the supraoptic nuclei of the hypothalamus. The nerve cell bodies respond to changes in the effective osmolality of the blood by causing changes in the secretion of ADH. This is probably mediated by changes in the ICF volume; i.e., when plasma osmolality in high, water is drawn out of the receptor cells, intracellular volume is reduced, intracellular osmolality is increased, and ADH is released. The reverse occurs when osmolality is reduced. Since ADH regulates the renal excretion of water, a beneficial sequence of events occurs. Abnormally high plasma osmolality stimulates ADH release, which causes renal retention of water. Water retention causes solute dilution in the ECF and reduction in plasma osmolality.

As noted previously, hypothalamic centers also regulate thirst. These centers are stimulated by osmolal fluctuations also. Thus, increased plasma osmolality results in increased thirst. The resulting water intake dilutes the ECF and reduces plasma osmolality. There appears to be an inherent ''setting of the osmostat'' which maintains plasma osmolality at approximately 280–300 mosmol/kg. There is slight species variation in this value, with large animals (horses and cattle) being on the lower side, 270–300 mosmol/kg, and small animals (dogs and cats) being on the high side, 280–310 mosmol/kg.

It should be noted that osmotically sensitive nerve cells respond only to ''effective osmoles.'' This expression relates to the diffusibility of a solute. Sodium is an ''effective osmole'' because it is largely excluded from the ICF. Its accumulation in the ECF creates

an imbalance between intra- and extracellular osmolality, and water moves out of the cell to reestablish osmotic equilibrium. Urea, on the other hand, is an ineffective osmole, since it is freely diffusible into cells. When urea concentration is increased, plasma osmolality is increased, but it is of no significance. It is due to ineffective osmoles, the urea conentration is equilibrated between intra- and extracellular fluid, and osmotic disequilibrium is not present; no water shift between body fluid compartments occurs. Hyperosmolality due to azotemia is therefore of little clinical significance.

B. Role of Sodium

The major influence on ECF osmolality is sodium. This element and its related anions account for virtually all of the osmotically effective solute in the ECF. Sodium thus plays a central role in water and osmotic regulation in the body. Sodium retention in the body results in water retention, and sodium depletion usually results in water depletion as a result of the regulatory mechanisms described above. It is therefore important to review the essential aspects of sodium balance in the animal body.

The normal human diet contains ample quantitites of sodium because of its content in foods of animal origin, which are relatively high in sodium. The ubiquitous saltshaker insures that additional amounts of this inexpensive substance are always available. The scientist working with herbivores realizes, however, that the diet of herbivores is very low in sodium and that supplementation with a source of sodium is necessary for optimal production. This is especially true of high-producing dairy cows and working horses, in which appreciable losses of sodium occur in the milk and sweat, respectively. Interesting hypotheses concerning salt appetite in animals have been presented by Michell (1974).

When dietary salt is adequate, the principal regulation of body sodium is by the kidney. This is mediated through the effects of aldosterone on the renal tubule. When this renal regulation of sodium has been compromised, serious body fluid disorders result. In adrenal insufficiency, the lack of mineralocorticoid hormones results in inability to reabsorb sodium and associated inability to excrete potassium. The resulting hyponatremia and hyperkalemia play an important role in the clinical syndrome presented and are useful in diagnosis. In chronic renal failure, the lack of tubular sodium conservation results in sodium depletion with concomitant water depletion. In congestive heart failure, there is abnormal aldosterone-mediated sodium retention with concomitant water retention.

C. Hypernatremia

Hypernatremia is an uncommon clinical abnormality which is always associated with hyperosmolality of the ECF. This results in a shift of water from the intracellular space into the hyperosmotic extracellular space until osmotic equilibrium has been reached. The loss of intracellular volume is associated with marked alterations in cell function, which are especially significant in the central nervous system.

Hypernatremia does not occur when there is a normal thirst mechanism, adequate supply of pure water, and ability to drink. When it is encountered as a clinical problem, one of these factors has been involved. Thus, in salt poisoning of pigs or other animals, there is a high sodium intake without free access to pure water. Hypernatremia has also been observed in dogs in which pure water intake has been denied, because of anesthesia or vomiting or because some unusual liquid has been substituted for ordinary drinking

water. These peculiar conditions are more likely to result in hypernatremia if parenteral salt-containing fluids are administered simultaneously. Although uncommon, these factors have produced bizarre clinical problems of a serious nature.

D. Hyperosmotic States Unrelated to Hypernatremia

In addition to hypernatremia, accumulation of significant quantities of other osmotically active solutes may also cause hyperosmotic abnormalities. Hyperglycemia is the most common cause of hyperosmotic abnormalities other than those due to hypernatremia. Each increase in plasma glucose of 100 mg/dl is associated with an increase of 5.5 mosmol/kg in plasma osmolality. The effect of a modest degree of hyperglycemia (ca. 200 mg/dl) is negligible, but when hyperglycemia is severe (>500 mg/dl) a significant osmotic problem exists in the body. A clinical syndrome of hyperosmotic coma has been described in some diabetic patients. Alcohol and other exogenous intoxicants are additional causes of hyperosmotic states.

E. Hyponatremia

Hyponatremia is a frequent clinical finding, especially in animals with large losses of intestinal fluid. Horses with gastrointestinal disease almost always have marginal to severe hyponatremia. It is also characteristic of adrenal insufficiency. Hyponatremia can be caused by, or aggravated by, the administration of large amounts of sodium-free fluids, such as pure water orally or electrolyte-free, carbohydrate (e.g., glucose)-containing solutions parenterally. When hyponatremia is not associated with the accumulation of other osmotically active solute, hypoosmolality is present. This results in movement of water from ECF to the intracellular space and cellular overhydration, which leads to neurological signs due to alterations in nerve cell function. These signs are severe when the osmotic changes have developed abruptly but are less prominent when the hypoosmotic state has occurred slowly. There seem to be compensatory intracellular responses which, with time, reduce the effective intracellular osmoles and minimize the cellular overhydration (Rose, 1977).

The hyperglycemia of diabetes mellitus creates extracellular hyperosmolality. This draws water out of the cells, expands the ECF, and reduces extracellular solute concentrations. In this situation, hyponatremia is not an indication of hypoosmolality but a consequence of hyperosmolality. Attempts to correct this hyponatremia directly would be contraindicated.

F. Sodium Concentration as a Predictor of Osmolality

Since sodium concentration is the principal determinant of ECF osmolality, increased sodium concentration is *always* associated with *hyperosmolality*. Similarily, *hypoosmolality* cannot develop when sodium concentration is normal. Note, however, that hyperosmolality can exist when the sodium concentration is normal and that hypoosmolality is not always present when hyponatremia occurs. In these instances, other unusual solute accumulations are responsible for creating osmotic effects in excess of that predicted from the sodium concentration. The sodium concentration indicates that the osmolality is at least comparable with the sodium level, but it may be much more.

V. SALT AND WATER INTERRELATIONSHIPS

It can be seen from the foregoing discussion that there is an inextricable relationship between salt (sodium) and water in the maintenance of body fluid volume and osmolality. *Volume* (water) *abnormalities* are corrected physiologically by modulation of sodium balance via altered sodium appetite and renal sodium excretion (aldosterone). The resulting changes in body sodium cause more or less "holding" of water in the body and tend to correct the original disturbances. *Abnormalities in osmolality* of the body fluids result in modulation in water balance via alterations in thirst and renal water excretion (ADH).

In most instances, these homeostatic mechanisms are beneficial. Occasionally, however, they are harmful. In congestive heart failure, for example, the effective circulating volume is diminished, and sodium retention occurs as a result. This causes increased plasma osmolality, increased water intake, and increased ECF volume. In this condition, however, there is no dehydration, and the increased water intake aggravates the edematous state of the patient. It is necessary to restrict the amount of sodium intake by the patient in order to interrupt this untoward response.

In the hypoosmotic state, ADH secretion is inhibited and water excretion is enhanced. If the primary abnormality is excessive water intake, the response is beneficial. When hypoosmolality is associated with dehydration, as is often the case in equine diarrhea, inhibition of ADH release is counterproductive.

In some clinical syndromes a significant sodium abnormality exists but may be inapparent due to the close relationship between salt and water. In the salt retention abnormality of heart failure mentioned above, hypernatremia is not seen owing to the coexisting water retention. In the sodium depletion problem in chronic renal failure, hyponatremia is often not seen owing to the concurrent water loss. In both instances significant sodium disturbances are present which are camouflaged by the water changes which followed; i.e., ECF sodium *concentrations* are usually normal.

VI. PATHOPHYSIOLOGY OF ACID-BASE BALANCE

Normal body chemistry is influenced greatly by intracellular enzymes. These enzymatic reactions occur optimally only within a very narrow range of pH. For this reason, the maintenance of normal pH in the body fluid is of fundamental importance. Since water and electrolyte balance are closely linked with acid-base balance (Nuttall, 1965) and diseases that alter one of these often alter the others, acid-base balance is customarily included in discussions of fluid balance.

A. Definition of pH

The pH of a solution is a measure of its acidity and is equal to the negative logarithm of the hydrogen ion concentration. It is thus inversely related to hydrogen ion concentration, $[H^+]$. In aqueous solution the product of $[H^+]$ and hydroxyl ion concentration, $[OH^-]$, is constant: 1×10^{-14} Eq/liter. When the two species are present in equal concentration (1×10^{-7}), the reaction is neutral, and the pH is 7. When $[H^+]$ is greater than 1×10^{-7}, the solution is acidic, and the pH is less than 7. When $[OH^-]$ is greater than 1×10^{-7}, $[H^+]$ is less than 1×10^{-7}, the solution is alkaline, and the pH is greater than 7.

B. Buffers

The fluids of the body contain several buffer systems which tend to prevent sudden changes in pH as acids or bases enter the body from without or are produced as a result of normal physiological processes. An understanding of buffers is thus of importance in understanding acid-base control.

A buffer system is a mixture of a weakly dissociated acid and a salt of that acid. For example, a mixture of acetic acid and sodium acetate forms a buffer system. This mixture tends to prevent changes in the pH of a solution in the following manner. If a large amount of strong acid (H^+) were added to water, the quantity of free hydrogen ion in the water would be large and the pH of the solution would fall sharply. However, if the acid were added to the buffer described above the following reaction would occur:

$$H^+ + C_2H_3O_2^- \rightarrow HC_2H_3O_2 \tag{1}$$

Because the added acid resulted in formation of acetic acid, much of which was undissociated, the increase in free H^+ in the solution was minimized and the change in pH was therefore reduced.

Similarly, if a strong base (OH^-) were added to water, the pH would rise sharply as the OH^- converted some of the hydrogen ions to water:

$$OH^- + H^+ \rightarrow H_2O \tag{2}$$

However, if OH^- were added to the buffer solution described above, the change in pH would be minimized by reactions (3) and (4).

$$OH^- + H^+ \rightarrow H_2O \tag{3}$$

$$HC_2H_3O_2 \rightarrow H^+ + C_2H_3O_2^- \tag{4}$$

As the hydroxyl ion converted hydrogen ions to water, some acetic acid molecules would dissociate to restore the hydrogen ion concentration.

The tendency of a weak acid to dissociate is indicated by its dissociation constant K. The hydrogen ion concentration of a buffer solution such as that described above can be determined by the following formula:

$$[H^+] = K\frac{[\text{acid}]}{[\text{salt}]} \tag{5}$$

When this relationship is expressed in terms of pH, it becomes

$$\text{pH} = \text{p}K + \log\frac{[\text{salt}]}{[\text{acid}]} \tag{6}$$

This is the Henderson–Hasselbalch equation, which is basic to the chemistry of pH regulation in the body. It should be recognized in passing that the pH changes only when the *ratio* between the components of the buffer changes. Changes in the absolute concentrations of the buffer pair do not result in changes in pH if the ratio between them is not changed.

C. Blood Buffers

The blood is a complex liquid. It contains many buffer pairs which help to control blood pH. The total buffer capacity of the blood is the cumulative effect of these individual

buffer pairs. The significant buffer pairs in the plasma are bicarbonate–carbonic acid (HCO_3^-/H_2CO_3), monohydrogen phosphate–dihydrogen phosphate ($HPO^{2-}_4/H_2PO_4^-$), and proteinate–protein (protein$^-$/H-protein). In erythrocytes, organic phosphate and hemoglobin are additional important buffers. It should be recognized that the addition of acid or base to a complex mixture of buffers such as plasma results in alterations in the equilibrium of all buffer pairs simultaneously. Thus, these buffers *share* the burden of preventing marked changes in blood pH.

In spite of the fact that the buffers function together in the plasma, the bicarbonate–carbonic acid buffer pair is the most important to the clinician. This is because it is the most abundant buffer in the plasma, it is the easiest to measure, and it is the buffer system over which the body has the most control. Since changes in pH affect all plasma buffers, clinical evaluation of the bicarbonate–carbonic acid buffer pair makes possible inferences about the total plasma buffer.

The Henderson–Hasselbalch equation applied to this important buffer pair becomes

$$\text{pH} = 6.1 + \log \frac{[HCO_3^-]}{[H_2CO_3]} \tag{7}$$

($6.1 = pK$ for the HCO_3^-/H_2CO_3 buffer pair).

The pH of the plasma is dependent upon the concentrations of bicarbonate and of carbonic acid and, of more importance, on the *ratio* between these two constituents.

D. Genesis of Carbonic Acid and Bicarbonate

Carbon dioxide is generated by various decarboxylation reactions in the cells of the body. It diffuses out of the cells, across the interstitial fluid, and into the plasma. Most of the carbon dioxide is in the molecular form; very little is hydrated to form carbonic acid. When the CO_2 enters the erythrocytes, hydration is catalyzed by the enzyme carbonic anhydrase, and H_2CO_3 is formed very rapidly. A large proportion of the H_2CO_3 dissociates to hydrogen and bicarbonate ions since, at the pH of the blood, this satisfies the requirements of the Henderson–Hasselbalch equation.

E. Regulation of Carbonic Acid Concentration

An equilibrium exists between the partial pressure of CO_2 in alveolar air, partial pressure of gaseous CO_2 dissolved in the blood, and the carbonic acid of the blood. Because of this relationship, changes in the carbonic acid concentration in the blood are directly related to pulmonary function.

The conventional method of evaluating the carbonic acid or carbon dioxide in the blood is the determination of the partial pressure of carbon dioxide (pCO_2). The pCO_2 is usually expressed in millimeters of mercury. The carbonic acid concentration in milliequivalents per liter can be determined by multiplying pCO_2 by a factor of 0.03. Because of this constant relationship between pCO_2 and carbonic acid concentration, these terms can be used interchangeably in discussions of mechanisms in the body.

The respiratory center in the medulla oblongata is sensitive to changes in pCO_2 in the blood. When pCO_2 rises sigificantly above the normal value of approximately 40 mm Hg, the respiratory center is stimulated and respiration is increased. Increased respiration decreases the pCO_2 of the alveolar air, and this in turn decreases the pCO_2 of the blood.

The respiratory center is also sensitive to changes in blood pH. If the blood pH falls significantly below the normal value of 7.4, respiration is stimulated (even when the pCO_2 is normal), and the pCO_2 is reduced.

The opposite stimuli, i.e., decreased pCO_2 and increased pH, cause decreased respiration and an increase in pCO_2 in the blood.

F. Regulation of Bicarbonate Concentration

The principal organ regulating bicarbonate concentrations is the kidney. At normal plasma bicarbonate concentrations the kidney tends to reabsorb most of the bicarbonate in the tubular fluid. When bicarbonate concentration rises, however, this ion is not usually reabsorbed but is excreted in the urine.

G. Excretion of Acid

Although the blood buffers can prevent a sudden change in pH of the body fluid when excess acid or alkali enters the ECF, a mechanism is necessary for the excretion of hydrogen ions. This is accomplished in the kidney by excretion of dihydrogen phosphate and ammonium ion. To the extent that these ions are excreted in the urine, hydrogen ion is removed from the body.

H. Abnormalities of Acid–Base Balance

Disturbances of acid–base balance have been described as respiratory or nonrespiratory (metabolic). Respiratory disturbances are the result of abnormalities in pulmonary gaseous exchange and are always characterized by alterations in the pCO_2 of the blood. The remaining disturbances of acid–base balance have many different causes, e.g., renal failure, vomiting, diarrhea, and shock. These have been referred to collectively as metabolic disturbances. However, a more accurate and informative description would be nonrespiratory disturbances. These disturbances are always characterized by an alteration in the bicarbonate concentration in the blood.

There are four general classifications of acid–base disturbance: respiratory acidosis, respiratory alkalosis, nonrespiratory acidosis, and nonrespiratory alkalosis. In each of these four types of disturbance the process may be uncompensated or compensated. Compensation is the result of various homeostatic mechanisms in the body, inducing a second acid–base disturbance which tends to correct the abnormality of hydrogen ion concentration caused by the primary disturbance. The compensatory mechanism is opposite the primary disturbance in two respects. If the primary disturbance is respiratory, the compensatory mechanism is nonrespiratory; if the primary disturbance is nonrespiratory, the compensatory mechanism is respiratory. Furthermore, if the primary disturbance causes acidosis, the compensatory mechanism is one that tends to cause alkalosis; if the primary disturbance causes alkalosis, the compensatory mechanism is one that tends to cause acidosis. Thus, the compensatory mechanism tends to neutralize the effects of the primary disturbance.

When a disturbance of acid–base balance has been recognized, it is important to try to classify the disturbance with respect to the primary mechanism responsible for it. This not only permits localization of the abnormality but is essential for the selection of rational

therapy. In most cases of acid-base disturbance, identification of the abnormal mechanism involved is possible if the pH, pCO_2, and bicarbonate concentration are known. It should be remembered that direct measurement of two of these will permit evaluation of the third by use of the Henderson–Hasselbalch equation.

1. Respiratory Acidosis

This condition is the result of hypoventilation. Carbon dioxide is not adequately eliminated, and the pCO_2 (H_2CO_3) of blood rises. If there is no compensatory reaction, the blood pH falls as the carbonic acid concentration increases.

Compensation for this disturbance occurs in the kidney. Increased quantities of H^+ are excreted, and increased bicarbonate ion is reabsorbed. Chloride ion is excreted instead of bicarbonate.

It will be recalled that the Henderson–Hasselbalch equation shows that pH is not dependent upon the absolute concentration of carbonic acid or bicarbonate but on the *ratio* between them. The typical findings in a normal individual, a patient with uncompensated respiratory acidosis, and one with partially compensated respiratory acidosis are shown in Table V.

In the uncompensated patient the increase in $pCO_2(H_2CO_3)$ results in a decrease in the bicarbonate/carbonic acid ration, and pH falls. As the bicarbonate increases due to renal compensation, the ratio is changed toward normal, and the pH becomes less abnormal. Evaluation of pH, pCO_2, and bicarbonate concentration indicates unequivocally that the problem is an acidosis and that it is of respiratory origin; it also indicates the degree to which compensation has occurred.

The most common clinical situation in veterinary medicine in which respiratory acidosis is recognized is anesthesia with volatile anesthetics and a closed-system apparatus. Under these conditions ventilation may be seriously reduced without hypoxia developing. The high oxygen content of the gas mixture supplied to the patient maintains high pO_2 in the blood and prevents an hypoxic stimulation of respiration. The well-oxygenated blood falsely indicates to the surgeon that ventilation is adequate. However, anesthesia of the respiratory center results in decreased respiratory movements and inadequate CO_2 elimination. Although CO_2 accumulates (hypercapnia, hypercarbia), the respiratory center does not respond by initiating increased respiration, and serious respiratory acidosis may develop, *in spite of adequate oxygenation of the blood.*

Other causes of respiratory acidosis are widespread pulmonary disease, intrathoracic lesions that prevent normal ventilation, and lesions or drugs affecting the central nervous

TABLE V

Clinicopathological Change in Respiratory Acidosis

Condition	pCO_2	H_2CO_3	HCO_3^-	HCO_3^-/H_2CO_3	pH
Normal	40	1.2	24	20 : 1	7.40
Uncompensated respiratory acidosis	90	2.7	24[a]	8.8 : 1	7.20
Partially compensated respiratory acidosis	90	2.7	38	14 : 1	7.32

[a] Although the bicarbonate concentration in uncompensated respiratory acidosis is shown unchanged to indicate no compensation, there is actually a slight increase in bicarbonate at this time due to equilibrium with the increased carbonic acid.

system which interfere with its regulation of normal respiration. In contrast to respiratory acidosis resulting from closed-system anesthesia, these other causes of hypoventilation are frequently accompanied by cyanosis because of coexisting hypoxia.

2. *Respiratory Alkalosis*

This condition is the result of hyperventilation and is characterized by decreased pCO_2 in the alveolar air and blood (Table VI). If there is no compensatory reaction, the ratio of bicarbonate to carbonic acid increases, and the pH is increased.

Compensation for this disturbance is by increased renal excretion of bicarbonate instead of chloride. This results in an increase in plasma chloride and a decrease in plasma bicarbonate. As plasma bicarbonate decreases, the bicarbonate–carbonic acid ratio decreases, and the blood pH changes toward normal.

Respiratory alkalosis may be seen in animals in pain or under psychological stress, when increased respiration occurs. It may also occur in animals (especially dogs) which hyperventilate to prevent overheating when the ambient temperature is elevated. It is not of frequent occurrence in veterinary medicine.

3. *Nonrespiratory Acidosis*

"Metabolic" acidosis is by far the most common type of acid-base disorder encountered in veterinary practice. It results from the loss of bicarbonate from the ECF. This reduces the bicarbonate–carbonic acid ratio and the pH (Table VII).

Compensation for nonrespiratory acidosis occurs by hyperventilation. Thus, respiratory alkalosis is superimposed on the primary nonrespiratory acidosis, and the disturbance in pH is ameliorated as the bicarbonate–carbonic acid ratio is returned toward normal.

The most common cause of metabolic acidosis in veterinary practice is diarrhea. The secretions of the intestinal tract are alkaline and contain a high concentration of bicarbo-

TABLE VI

Clinicopathological Change in Respiratory Alkalosis

Condition	pCO_2	H_2CO_3	HCO_3^-	HCO_3^-/H_2CO_3	pH
Normal	40	1.2	24	20:1	7.4
Uncompensated respiratory alkalosis	20	0.6	24[a]	40:1	7.55
Partially compensated respiratory alkalosis	20	0.6	20	33.3:1	7.50

[a] Although the bicarbonate concentration in uncompensated respiratory alkalosis is shown unchanged to indicate no compensation, there is actually a slight decrease in bicarbonate at this time due to equilibrium with the decreased carbonic acid.

TABLE VII

Clinicopathological Change in Metabolic Acidosis

Condition	pCO_2	H_2CO_3	HCO_3^-	HCO_3^-/H_2CO_3	pH
Normal	40	1.2	24	20:1	7.40
Uncompensated metabolic acidosis	40	1.2	15	12.5:1	7.20
Partially compensated metabolic acidosis	32	0.96	15	15.6:1	7.30

nate. When the alimentary canal is functioning normally these secretions are largely reabsorbed in the lower digestive tract, but in diarrhea the secretions are lost from the body. There is a serious depletion of bicarbonate as a result.

In certain other abnormalities of the digestive tract, e.g., obstruction or ileus, intestinal secretions may accumulate in the digestive tract and, being sequestered in this location, represent a significant loss of bicarbonate from the plasma and interstitial fluid. In certain acute intestinal disorders, especially in the horse, there may be a large accumulation of fluid in the intestine before diarrhea is recognized. These animals may also have a serious metabolic acidosis.

The accumulation in the body of organic acids is another common cause of metabolic acidosis. In these instances the accumulating organic acids are buffered by bicarbonate, and the bicarbonate concentration is decreased. This can be seen in Eq. (8), where the organic anion is indicated by the symbol A.

$$\begin{array}{l} HA + HCO_3^- \rightarrow H_2CO_3 + A^- \\ \qquad\qquad\quad \hookrightarrow H_2O + CO_2 \end{array} \tag{8}$$

The hydrogen ions provided by the organic acid have been removed by the conversion of bicarbonate to carbonic acid, but bicarbonate has been depleted in the process and has been replaced by the organic anion.

Since ketone bodies are mostly organic acids, this may be seen in the clinical ketosis of cattle and sheep as well as in diabetes mellitus. Shock and cellular hypoxia from other causes are characterized by a severe metabolic acidosis. This is because the failure of normal aerobic cellular metabolism results in the accumulation of lactic and pyruvic acids. Renal failure also is characterized by metabolic acidosis. This may be due in part to the retention of phosphate, sulfate, and other acids by the failing kidneys but is also related to the inability of the kidney to excrete H^+ and to reabsorb bicarbonate.

4. Nonrespiratory Alkalosis

Metabolic alkalosis is characterized by the accumulation in the ECF of abnormally large amounts of bicarbonate ion (Table VIII). This increases the bicarbonate–carbonic acid ratio and the pH. Compensation for this abnormality is hypoventilation. This increases the pCO_2 of the blood and tends to decrease the bicarbonate–carbonic acid ratio and the pH toward normal.

In compensating for metabolic alkalosis, the kidney may excrete increased quantities of bicarbonate as an additional compensatory measure. If the bicarbonate concentration were reduced while the pCO_2 were increasing, the change in bicarbonate–carbonic acid ratio would be greater and the compensation would be more complete.

Metabolic alkalosis is most commonly seen in digestive tract disorders in the ruminant.

TABLE VIII

Clinicopathological Change in Metabolic Alkalosis

Condition	pCO_2	H_2CO_3	HCO_3^-	HCO_3^-/H_2CO_3	pH
Normal	40	1.2	24	20:1	7.40
Uncompensated metabolic alkalosis	40	1.2	38	31.5:1	7.60
Partially compensated metabolic alkalosis	50	1.5	38	25.3:1	7.50

Any abnormality that results in the sequestration of abomasal juice in the abomasum or forestomachs may be a cause of metabolic alkalosis. This is seen in high intestinal obstruction and in atony, torsion, or displacement of the abomasum. The large amount of hydrochloric acid in the gastric juice is secreted by the mucosal cells by the following overall process (White *et al.*, 1964):

$$NaCl + H_2CO_3 \rightarrow NaHCO_3 \text{ (plasma)} + HCl \text{ (secreted)} \quad (9)$$

The HCl which results is secreted in the gastric juice and the sodium bicarbonate moves into the plasma. If the gastric juice is subsequently reabsorbed in the lower digestive tract, no acid–base disorder occurs. However, if abnormalities of the upper digestive tract prevent gastric juice from moving into the intestine for reabsorption, serious metabolic alkalosis will occur as hydrogen ions are continuously removed and bicarbonate is continuously contributed to the ECF.

Profuse vomiting in monogastric animals may cause metabolic alkalosis by the same mechanism, although this is not as commonly encountered as might be expected.

Severe potassium depletion may be a cause of metabolic alkalosis since hydrogen ions tend to move into the cells to replace the lost potassium. This by itself tends to cause alkalosis. In addition, there may be increased hydrogen ion secretion in the urine as a result of increased hydrogen ion concentration in the tubule cells. Whenever hydrogen ion is lost from the ECF, alkalosis occurs, which is characterized by increased bicarbonate, as shown in Eq. (10).

$$H_2CO_3 \rightarrow H^+ + HCO_3^- \quad (10)$$

The removal of H^+ favors the dissociation of H_2CO_3 to restore the hydrogen ion concentration and produce abnormally high concentrations of bicarbonate.

5. *Mixed Disturbances*

Abnormalities of acid–base balance are usually the result of either a primary disturbance of respiratory control of carbon dioxide or nonrespiratory factors which alter bicarbonate concentration. Each of these may be associated with an opposite, compensatory change. In some patients, however, a mixed disturbance exists which, at first inspection, may be confusing.

For example, if a patient had a pCO_2 of 80 mm Hg and a plasma bicarbonate concentration of 10 mEq/liter, it would not be comparable with any of the simple disturbances, with or without compensation, described above. One would have to conclude that the patient had both a primary respiratory acidosis and a primary metabolic acidosis, a mixed disturbance. This type of abnormality could be seen in a patient with intestinal obstruction (metabolic acidosis) that had been anesthetized without adequate ventilation (respiratory acidosis).

VII. PATHOPHYSIOLOGY OF POTASSIUM METABOLISM

Potassium serves an important role in the maintenance of intracellular osmotic pressure. In addition, this element is essential to many physical and chemical processes in the body. Potassium concentration in the ECF influences the development of membrane potentials

and in this way significantly influences nerve transmission and muscle function. Potassium is also important in carbohydrate metabolism and electron transport since a number of the enzymes in these metabolic pathways are potassium dependent.

A. Intake of Potassium

Potassium is the most abundant cation in the ICF of both plants and animals. For this reason, this element is present in high concentration in almost all normal animal foods. The diet of herbivores contains especially large quantities of potassium. For example, a high-quality alfalfa–timothy hay mixture which was analyzed contained 393 mEq potassium per kilogram. Ten kilograms of this hay daily was adequate to maintain the body weight of several adult horses weighing approximately 450 kg (Tasker, 1967a). The daily potassium intake of these horses was equal to that in 283 gm of potassium chloride! In contrast, the sodium contained in 10 kg of this hay was equal to that of less than 20 gm of sodium chloride.

Although the proportions of sodium and potassium in the diet of carnivores are different from those in the diet of herbivores, there is seldom, if ever, a deficiency of potassium in the normal diet of any animal.

B. Potassium Excretion

Because of the large quantities of potassium consumed by normal animals, large amounts must be excreted to prevent potassium intoxication of the organism. Most of the potassium is excreted in the urine, but an additional quantity is lost in sweat and digestive tract fluids. Animals with voluminous feces of high water content lose a significant quantity of electrolytes from the digestive tract, but in other animals the loss by this route is negligible.

The kidney is the principal organ for potassium excretion. It does this by glomerular filtration as well as by tubular secretion. Ordinarily, there is little need to conserve potassium in the body, and the principal renal effect is to prevent the accumulation of toxic quantities. However, when potassium intake is interrupted, renal conservation of this element is necessary to prevent potassium depletion. The kidney is much less able to conserve potassium during these periods than sodium. Although sodium restriction is immediately followed by resorption of virtually all sodium from the urine, potassium restriction does not result in a similar response. Under these conditions, potassium excretion continues for several days in spite of body depletion.

Tubular resorption of potassium occurs continually, as does that of sodium. However, when aldosterone causes increased resorption of sodium, it does so by promoting the exchange of sodium in the tubular fluid for potassium in the renal tubular cell. Thus, aldosterone promotes excretion of potassium. When sodium is scarce in the tubular fluid, potassium excretion by this mechanism is curtailed.

Renal control of potassium excretion has been studied in the dog by Lemieux *et al.* (1964) and in the ox by Pickering (1965). Suki (1976) has reviewed control of potassium metabolism in man, and Hintz (1976) has studied potassium metabolism in ponies. Ward (1966a) and Keynes and Harrison (1967) have reviewed potassium metabolism in ruminants.

C. Potassium Distribution in the Body

The potassium concentration in the ECF is very low, approximately one-thirtieth of the sodium concentration. The reverse is true of most cells of the body: The potassium concentration is many times greater than the sodium concentration. This is presumably the result of the active extrusion of sodium from the cell by the so-called sodium pump.

Several abnormal situations can influence the degree to which potassium is confined to the cell, but in the normal individual more than 90% of the body potassium is in the ICF. Because of the preponderance of muscle tissue in the body, most of the body potassium is in muscle.

The concentration of potassium in the fluids of the body is shown in Table III.

D. Disturbances of Potassium Metabolism

While sodium concentration in the plasma is a reliable index of the status of the body with respect to sodium, plasma potassium concentration is not always a meaningful indication of the status of the body with respect to potassium. This is because most of the potassium is intracellular, and there is no consistently reliable relationship between intra- and extracellular potassium concentrations.

The normal cell, bathed by normal ECF, maintains a disequilibrium between ECF potassium and ICF potassium. This is the result of the active extrusion of sodium from the cell, the sodium pump, which is a process requiring energy. When intracellular carbohydrate metabolism is defective, as in diabetes mellitus, the sodium pump does not work optimally, and intracellular potassium is lost in the ECF. Subsequently, after insulin administration, intracellular carbohydrate metabolism is accelerated, and potassium is quickly redistributed from the ECF to the ICF. In neither of these conditions does the ECF potassium concentration reflect the nature of intracellular stores.

When cells of the body, especially muscle cells, become necrotic, large amounts of potassium move into the ECF. Once again, the elevated ECF potassium does not reflect general intracellular concentrations.

The most common clinical problem in which serum potassium values are seriously misleading is acidosis. When the extracellular concentration of hydrogen ions is increased (acidosis), the cells tend to take up hydrogen ions and release potassium ions into the ECF. Thus, a marked *elevation* of serum potassium may occur when the cells are *depleted.* In alkalosis, low serum potassium values may be found as hydrogen ions leave the cells and plasma potassium enters. Such findings do not always indicate potassium depletion.

1. Hypokalemia

Decreased concentration of potassium in the serum is called hypokalemia or hypopotassemia. This may be the result of decreased intake of potassium. It will be remembered that the kidney excretes the large quantities of potassium which are consumed daily but does not curtail potassium excretion promptly when intake ceases. Potassium depletion occurs even if the serum concentration is not recognizably altered. When anorectic animals continue to drink water or when they are kept hydrated by parenteral administration of potassium-free fluids for many days, frank hypokalemia may be recognized.

Potassium depletion and hypokalemia are also seen when the patient has lost large quantities of fluids by vomiting or diarrhea. This is especially apt to occur when fluid losses are replaced with potassium-free fluids.

Prolonged administration of adrenal steroids that have some mineralocorticoid activity may promote potassium excretion and result in hypokalemia. Certain diuretics also may cause potassium depletion (see Section XI, A, 13).

Alkalosis, regardless of cause, results in increased exchange of potassium ions for hydrogen ions in the renal tubular fluid. This also may cause marked hypokalemia.

Human patients with potassium deficiencies show neurological, muscular, and cardiac abnormalities. These do not usually appear until the serum potassium concentration has dropped below 2.5 mEq/liter. The neurological signs are drowsiness, irritability, mental confusion, and even coma. Severe muscular weakness is a common complaint. Electrocardiographic abnormalities are also seen in hypokalemia. The characteristic changes are depression of the T waves and prolongation of the Q–T interval (Clark and McCrady, 1966). Electrocardiographic changes seen in potassium deficiency in cattle have been described by Cox *et al.* (1966).

Necrosis of the myocardium has been reported in patients with chronic potassium depletion. Renal abnormalities also occur in potassium deficiency. Inability to concentrate the urine has resulted in a syndrome similar to diabetes insipidus. Characteristic histological lesions have been reported in the renal tubules of potassium-deficient patients (Epstein, 1962). Abbrecht (1969) studied the effect of potassium deficiency on renal function in the dog. A review of clinical aspects of hypokalemia in man has been published (Lindeman, 1976).

2. Hyperkalemia

Increased potassium within the cell is apparently not harmful; the toxic effects of excess potassium in the body are the direct result of increased levels in the ECF.

Hyperkalemia is most frequently the result of acidosis causing a redistribution of body potassium, i.e., the movement of intracellular potassium into the ECF. It may also occur in renal failure, although the significance of this in chronic renal failure is often overemphasized. Hyperkalemia resulting from renal failure is most likely to occur if renal failure is acute, if intake of potassium is continued in large amounts, if extensive cellular necrosis occurs simultaneously, or if serious acidosis exists.

The principal danger in hyperkalemia is cardiac arrest. Electrocardiographic abnormalities may be observed when the serum potassium is 7 mEq/liter and are usually severe when the serum potassium is greater than 8 mEq/liter. Death usually occurs when the serum potassium level is 10–12 mEq/liter. The characteristic electrocardiographic abnormalities are high, peaked T waves, prolongation of the P–R interval, disappearance of P waves, and prolongation of the QRS complex (Epstein, 1962; Clark and McCrady, 1966). Electrocardiographic changes due to hyperkalemia have been described by Gentile and Venturoli (1960) in the horse and by Greenspan *et al.* (1965) in the dog. Ward (1966b) has reported the death of a cow following oral administration of potassium chloride. Clinical aspects of hyperkalemia in man have been reviewed by Whang (1976).

VIII. ROLE OF ANIONS

The principal of electroneutrality requires that the total charges of cations and anions must balance. The cations in feedstuffs, principally potassium, sodium, calcium, and magnesium, are balanced, for the most part, by organic anions, phosphates, and chloride.

Catabolism of organic anions results in formation of sulfate ion. Bicarbonate ion is formed endogenously by the hydration of carbon dioxide. Sulfate is the source of sulfur and, in one form or the other, is an essential constituent of heparin, mucopolysaccharides, amino acids, and other important substances in the body. Phosphate is an important constituent of bones and teeth and of many metabolically important compounds.

Both sulfate and phosphate are of importance in nutrition and in metabolism, but neither is of special importance in the study of electrolyte pathophysiology in the body fluids. Concentrations of each are relatively low in the ECF (Table III). Chloride and bicarbonate, on the other hand, are intimately related to one another, and both are related to sodium. Both are present in relatively large quantities in the ECF (Table III). These anions, therefore, are of central importance in the understanding of both electrolyte and acid–base balance in the body.

Chloride is the only quantitatively important anion which is reabsorbable in the renal tubule. As such, it has profound effects on tubular electrolyte conservation. In the presence of abundant chloride ion, sodium reabsorption is accompanied by passive movement of chloride with no alteration in electroneutrality. However, when chloride is absent from the tubulur fluid, sodium reabsorption can occur only by exchange with hydrogen and potassium ions since electroneutrality must be preserved. The absence of chloride from the filtrate, therefore, may cause significant alterations in potassium and/or acid–base balance. A common example of this is the ''paradoxical aciduria'' seen in patients with a severe loss of gastric juice. In veterinary medicine, this occurs most frequently in abomasal sequestration of gastric secretions in ruminants or in vomiting by monogastric animals. The loss of hydrochloric acid results in hypochloremia and metabolic aklalosis. If the patient is sodium-depleted, as is often the case, a strong stimulus to renal sodium reabsorbtion occurs. Since chloride is reduced in the tubular fluid, sodium is reabsorbed in exchange for hydrogen and potassium ions. Hydrogen ion secretion by the tubule is accompanied by the production of bicarbonate which passes into the blood. The net effect of this sequence of events is aggravation of alkalosis (since bicarbonate production is required) and inappropriate secretion of potassium and hydrogen ion (paradoxical aciduria). The therapeutic provision of chloride ion results in sparing of potassium and hydrogen losses and amelioration not only of the hypochloremia, but of the potassium depletion and metabolic alkalosis as well.

IX. CLINICAL EVALUATION OF PATIENTS

An accurate appraisal of the nature of fluid balance disorders is a prerequisite for rational therapy and valid prognosis. Furthermore, identification of specific electrolyte and acid–base disorders may yield strong evidence about the nature of the primary disease process in the patient. For example, in the hyperventilating patient, confirmation of primary metabolic acidosis strongly indicates that the syndromes associated with this derangement, such as renal failure and diabetes mellitus, be considered. In another hyperventilating patient, however, confirmation of respiratory alkalosis would suggest a primary disorder of ventilation and the possibility of a central nervous disorder.

The most objective and specific information about water, electrolyte, and acid–base disorders is obtained from laboratory tests. Much can be learned, however, from historical and physical evaluation of the patient. Regardless of the source of information it must be

kept clearly in mind that the purpose of such evaluation is to answer the following essential questions: (1) Is there a fluid *volume* abnormality in the patient? (2) Is there an *acid-base* abnormality in the patient? (3) Is there an *osmolar* abnormality in the patient? (4) Is there a *specific electrolyte* abnormality in the patient?

A. History

The history of a patient's illness tells us much about alterations in water balance. Information can usually be obtained that indicates volume of water intake. When intake is decreased, either from deprivation or from adipsia, negative water balance may have developed or may be imminent, depending on the extent of compensating reductions in water loss. When polydipsia has been reported, excessive and uncontrollable losses of water have often occurred.

A history of diarrhea, polyuria, emesis, sialosis, or copious wound drainage suggests a significant loss of a peculiar body fluid with attendant depletion from the body of special constituents of the fluid. The consequence of copius vomition, for example, is often metabolic alkalosis and hypochloremia since gastric juice has an unusually high concentration of hydrochloric acid.

Short-term (day-to-day or hour-to-hour) fluctuations in body weight can be used as an indication of body water changes. Over a longer time the usefulness of this indicator is compromised by confusion with weight changes due to body tissue catabolism in anorectic patients. In water deprivation studies of renal concentrating ability and during fluid therapy of anuric patients, frequent determination of body weight is a valuable indicator of significant changes in total body water.

B. Physical Examination

Further understanding of the biochemical disorder(s) in a patient can be obtained from a careful physical examination. For example, alterations in respiration may reflect acid-base disorders. Special caution should be exercised in the interpretation of such respiratory disorders, however, since it is extremely difficult to determine from clinical observation of respiratory movements whether alveolar ventilation is increased or decreased. Thus, in polypnea (panting), ventilation may be reduced, and, conversely, in some instances of hyperventilation the rate of respiration may be quite slow. Furthermore, even when hyperventilation can be confirmed, a primary respiratory disturbance must be differentiated from a compensatory reaction. This is not always obvious to the examiner.

The physical examination is particularly useful in the recognition of dehydration. The usual procedure for detecting such changes is evaluation of skin turgor. The normal elasticity of the skin is diminished markedly when dehydration is severe. Measuring the time required for an elevated fold of skin to return to its normal position is the clinical procedure used for this determination. Considerable experience is required for reliable information to be obtained. It is especially important that the same body area be used on all animals examined since the observations vary to a large extent depending on the natural loosèness or tautness of the skin in different parts of the body. Dryness of the mucous membranes and sinking of the eyes are additional physical signs that may be used as indicators of dehydration. It must be recognized that sinking of the eyes may be due to the loss of retroorbital fat in cachexia as well as to dehydration.

Although rules of thumb which relate physical signs of dehydration to specific body water volume deficits are largely empirical and are, at best, rough approximations, it is generally agreed that dehydration is undetectable by physical signs until the water volume loss is equal to 3–5% of body weight and that extreme signs of dehydration are associated with volume deficits of 12–15% of body weight. Thus, barely detectable changes in skin turgor in a 500-kg horse are assumed to reflect a water deficit of approximately 15–20 kg (liters), and marked abnormality in skin turgor in an 20-kg dog may reflect a water deficit of 2.5–3.0 liters.

When the clinical examination reveals a specific syndrome or definite abnormality, additional assumptions may be made about the biochemical disorder(s) in the patient. Recognition by the clinician of signs of shock suggests the probability of the hypovolemia and lactic acidosis which are typical of shock. Detection of anuria raises concern for hyperkalemia, metabolic acidosis, and overhydration. Bradycardia may be indicative of potassium disturbances. Abomasal displacements may cause concern for the biochemical changes which result from sequestration of abomasal fluids.

A compilation of the fluid derangements frequently associated with specific clinical syndromes or diseases is provided in Section XI. It must be emphasized that these "typical" abnormalities are not constantly present. Many vomiting dogs do not have the "typical" triad of hypochloremia, hypokalemia, and metabolic alkalosis; many cows with displaced abomasum do not have the same "typical" triad. Occasionally, when patients are evaluated thoroughly, it is found that the actual disturbance present is diametrically opposite the one described as "typical" for the disease or syndrome present. Such shocking discoveries make it clear that the more objective and specific information available from laboratory test results is needed for the most accurate evaluation of clinical patients.

C. Electrocardiography

The electrocardiogram (ECG) is often an indicator of serious potassium abnormalities. It is most reliable in hyperkalemia. This abnormality causes tall, peaked T waves and flattened P waves. The P–R interval and QRS complex may be prolonged, and bradycardia is usually present.

When hypokalemia is present, the ECG may also indicate bradycardia, the Q–T interval may be prolonged, and small biphasic T waves may be present.

A more detailed description of potassium-induced changes in the ECG has been provided by Bolton (1975). Cotton *et al.* (1971) have emphasized the greater reliability of the ECG findings in hyperkalemia and the unreliability of this means of detecting hypokalemia. Additional studies of electrocardiograph–electrolyte relationships have been published by Coulter and Engen (1972) and by Feldman and Ettinger (1977).

X. LABORATORY EVALUATION OF PATIENTS

A. Routine Hematology

The routine hemogram is useful in the identification of patients with significant fluid balance disturbance through the peripheral erythroid values—packed cell volume, hemoglobin, total erythrocyte count—and, when it is included in the hemogram, the refractomet-

ric evaluation of plasma total solids. These laboratory results are influenced greatly by hemoconcentration and hemodilution. They are inexpensive, easily obtained indicators of hydration status of the patient.

It is not necessary to determine more than one of the three erythroid values for this purpose since changes in hydration status affect all three to the same degree. Since other conditions may alter the erythroid values (anemia, polycythemia, epinephrine release) or the plasma total solids (hypoproteinemia, hyperglobulinemia), abnormalities in these laboratory values may not be an indication of a fluid balance change. However, by the routine simultaneous determination of both an erythroid value and total solids concentration, the correct interpretation is more obvious. When these two different measurements are abnormal to the same degree and in the same direction, the probability is much greater that the disorder is one of hemoconcentration or hemodilution resulting from a fluid balance abnormality. If only one of these values is abnormal, a specific disorder involving red cells or plasma solids is more likely. For this reason, it is strongly recommended that routine hemograms include both measurements.

There is no predictable effect of hemoconcentration or hemodilution on the leukocytes. Leukocyte kinetics are more complex and more volatile than erythrocyte kinetics or plasma solids regulation. It is seldom possible to attribute leukocytosis or leukopenia to fluid volume abnormalities.

B. Routine Urinalysis

The urine specific gravity and urine pH are influenced by abnormalities in the fluid balance and acid-base status, respectively. When renal tubular function and the ADH system are normal, urine specific gravity is usually a reflection of water balance: Dilute urine is a reflection of excess fluid intake (voluntary drinking or fluid therapy), and highly concentrated urine is a reflection of limited fluid intake. However, many normal animals, especially cats, regularly produce urine of high specific gravity when fluid balance is normal. Furthermore, in the presence of renal disease or defective ADH function, the urine specific gravity may be quite misleading as an indicator of hydration status; i.e., the urine specific gravity may be very low in spite of severe dehydration of the patient.

The urine pH may be a useful reflection of systemic acid-base status. In most metabolic disorders, an unusually low urine pH is associated with systemic acidosis, and an unusually high pH is associated with alkalosis. In respiratory conditions, however, the correlation may not exist (Simmons, 1962). The paradoxical aciduria of hypochloremic metabolic alkalosis is another instance in which the expected correlation is not present. Moreover, herbivores, the urine of which is typically alkaline in the normal state, develop an acidic urine upon fasting irrespective of the systemic acid-base status.

C. Serum Electrolyte Determinations

Laboratory determination of serum or urine sodium and potassium concentration requires the use of a flame photometer. No quick office procedures are available for these tests. Both electrolytes are usually determined simultaneously. Plasma or serum can be used but calcium-chelating anticoagulants must be avoided since they usually contribute either sodium or potassium to the specimen. Heparin is satisfactory (the cation contribution from the sodium salt of heparin is negligible). Plasma or serum must be separated

from the red cells promptly in those species that have high red cell potassium levels to avoid leakage from cells to plasma or serum.

Since the principal location of sodium in the body is the ECF, and since the plasma or serum is a component of that fluid, determination of serum or plasma sodium is a reliable indicator of its *concentration* in the body. It must be recognized that determination of sodium *concentration* does not always indicate with accuracy the total amount of sodium in the body. Sodium depletion or excess may be present when the sodium concentration is normal if water volume has changed to preserve isosmotic conditions.

The principal location of potassium in the body is the ICF. Determination of potassium in ECF, i.e., plasma or serum—the fluid conveniently available for laboratory analysis—does not reveal directly the intracellular concentration or the whole-body state of potassium. In the most commonly misleading clinical example, the patient with diarrhea is usually depleted of potassium, but the coexisting metabolic acidosis causes extracellular translocation of potassium to produce normal or even elevated potassium levels in the ECF. Thus, the calf with neonatal diarrhea, although potassium-depleted, often is presented with hyperkalemia, and the horse with acute diarrhea, also with potassium depletion, frequently is found to have normal serum or plasma potassium concentration at the time of presentation. Since abnormally elevated extracellular potassium concentration is toxic, regardless of the intracellular concentration or whole-body state, this finding warrants direct corrective action. The finding of normal extracellular potassium concentration in a potassium-depleted subject is often misleading, however, and may cause the clinician to give inadequate attention to correction of the serious potassium deficit extant. The interpretation of laboratory determinations of potassium in serum or plasma must include an evaluation of acid–base balance as well as an estimation of potassium intake and output from the body.

Serum or plasma concentration of chloride can be determined electrometrically, photometrically, or by titration. The results usually reflect changes in sodium concentration, with which the chloride concentration varies directly, or changes in bicarbonate, with which the chloride varies inversely. When chloride concentration is studied in relation to sodium and bicarbonate, certain electrolyte interrelationships become apparent. For this reason, chloride is usually included in laboratory electrolyte profiles. For example, hypochloremia without equivalent hyponatremia indicates loss of hydrogen chloride (HCl in gastric secretions) from the body rather than a loss of sodium chloride.

Spurious electrolyte results can occur as a result of severe hyperlipidemia. Since the alteration is most conspicuous in the sodium value, this problem is referred to as factitious hyponatremia (Harrington and Cohen, 1973). In severe hyperlipidemia, as, for example, in diabetes, the plasma or serum has an appreciable lipid phase in addition to the usual aqueous phase. Any volume of the specimen taken for analysis will include both phases even though the substances to be determined, e.g., electrolytes, are confined to the aqueous phase. The quantity of substance present, expressed in terms of concentration in the volume of specimen analyzed, will be smaller than the true concentration in the aqueous phase of the sample. Since it is the latter concentration which is physiologically significant, the reported result is misleading.

D. Blood pH and pCO_2

The procedures of choice for evaluating acid–base balance are the determination of blood pH and pCO_2. From these two results bicarbonate concentration can be calculated

from the Henderson–Hasselbalch equation or from convenient nomograms which express the mathematical relationships of that equation. The instrument required for these determinations is a sensitive voltmeter with specially adapted blood pH and pCO_2 electrodes. The specimen must be analyzed under conditions which simulate the *in vivo* state of the blood. Whole blood is used which is drawn in heparin to prevent clotting. Calcium-binding anticoagulants should not be used since they alter blood buffers. The specimen must be collected and analyzed without exposure to air since equilibration of blood gases (CO_2) with air will alter the acid–base status of the sample. The specimen must be analyzed at body temperature since hydrogen ion activity is altered by changes in temperature. Metabolic activity in the specimen (blood cell metabolism) must be minimized if the specimen is to reflect *in vivo* conditions. This can be accomplished by immediate laboratory evaluation or by chilling the sample immediately and rewarming when analysis is to be done. The holding period, even when the sample is chilled, should not exceed 3 hours. The most reliable blood pH and pCO_2 values are obtained from arterial blood specimens. However, in most clinical situations an accurate evaluation can be made with properly collected venous blood specimens. Such results differ from arterial blood values only very slightly when sample collection is done carefully. (Note: When cardiopulmonary function is to be evaluated and/or when blood pO_2 is to be determined, arterial specimens must be obtained.)

Brobst (1975) and Haskins (1977) have reviewed the clinical evaluation of acid–base status in veterinary patients.

E. Bicarbonate and Total CO_2

When a comprehensive evaluation of acid–base status is to be made, two of the three unknowns in the Henderson–Hasselbalch equation must be determined. From these two, the third can be calculated. Blood pH and pCO_2 are usually the two parameters evaluated, although either of these plus bicarbonate concentration would be equally informative. Whenever respiratory acid–base disturbances can be eliminated from consideration, the metabolic acid–base status of patients can be evaluated by determination of the bicarbonate concentration alone. Such evaluations cannot reveal the extent of respiratory compensation for the metabolic disorder or the extent of pH derangement, and, since the possibility of primary respiratory disturbances cannot be excluded with certainty without pCO_2 evaluation, patient evaluation by this means is not completely reliable or satisfactory. However, the instrumentation required for the estimation of bicarbonate concentration is more widely available, and the sample collection is less exacting. For these reasons, patient evaluation by the estimation of bicarbonate concentration may be possible when more comprehensive evaluation is not convenient.

The concentration of bicarbonate in plasma or serum can be estimated by titration with standard acid and the use of pH indicators. More often, however, it is evaluated by the determination of "CO_2 content" or "total CO_2" of the plasma or serum specimen. These terms are unfortunate and potentially misleading since they imply the determination of carbon dioxide in the specimen which might be thought to be related to pCO_2 and thus reflect the respiratory component of acid–base regulation. "Total CO_2" or "CO_2 content" procedures are indicators of bicarbonate (metabolic) disturbances, however, for the following reason. The techniques involve the measurement of the carbon dioxide gas liberated from the specimen by the addition of acid. This is shown by:

$$H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow H_2O + CO_2\uparrow \qquad (11)$$

The addition of excess acid to the sample forces this reaction to the right, with the evolution of CO_2 gas, which is measured. The "total CO_2" obtained from the specimen is actually the sum of CO_2 derived from bicarbonate and that obtained from carbamino CO_2, H_2CO_3, and dissolved CO_2 in the specimen. It is thus not a direct determination of bicarbonate concentration. However, bicarbonate is always the predominant source of this "total CO_2" and is usually approximately 95% of the total. Conspicuous increases or decreases in "total CO_2" thus are a reflection of changes in bicarbonate concentration.

When interpreting changes in bicarbonate concentration or "total CO_2" without knowledge of blood pH and pCO_2 values, one must appreciate that increases are expected in metabolic alkalosis *and* compensated respiratory acidosis and that decreases occur in metabolic acidosis *and* compensated respiratory alkalosis. Thus, for each abnormality, two diametrically opposite primary conditions may be present. Careful clinical evaluation of the patient is necessary if misinterpreation of the laboratory results is to be avoided.

F. Buffer Base, Base Excess, and Standard Bicarbonate

These values are occasionally provided by laboratories as part of blood gas–acid/base studies. They are usually derived mathematically from the direct measurements of pH and pCO_2.

The *buffer base* is an indication of the sum of all buffer anions in the blood or plasma under standarized conditions. Its value in normal human beings is approximately 40 mEq/liter. The *base excess* indicates deviations from normal in the buffer base; it is approximately 0 ± 3 mEq/liter in normal human beings. The *standard bicarbonate* is the plasma bicarbonate concentration under specific conditions which eliminate respiratory influences on the value obtained.

These three derived values all reflect changes in the nonrespiratory (metabolic) aspect of acid–base balance. In each case, increased values reflect increases in buffer anions and suggest metabolic alkalosis; decreased values reflect decreased buffer anions and suggest metabolic acidosis. The metabolic changes indicated by these results are not necessarily primary disturbances. Metabolic changes which are compensating for primary respiratory disorders also are reflected by alterations in these values.

G. Plasma and Urine Osmolality

Serum (or plasma) osmolality is measured to identify alterations in the "colligative properties" of the ECF. These properties are related to the number of particles in solution, rather than the kind or mass of substance present. Such properties are the freezing point, boiling point, vapor pressure, and osmotic pressure of the specimen. Although the osmotic pressure is the property of clinical interest, techniques for evaluating osmolality of clinical specimens are based on the measurement of either the freezing point or the vapor pressure of the specimen.

One osmole of solute dissolved in 1 kg of water depresses the freezing point by 1.86°C. Instruments have been designed which measure very accurately the freezing point of a specimen. This value can be compared with that of a sodium chloride standard solution of known osmolality to provide the osmolality of the specimen. More recently, instruments have been designed to measure the vapor pressure of a specimen. These instruments have the advantage of requiring a very small sample volume. Instruments which measure osmolality are called osmometers.

The measured osmolality of a solution of known composition is usually slightly less than that calculated from the solute concentration(s). This is due to incomplete dissociation of electrolytes as well as certain interionic forces which prevent individualization of the constituent particles. Although this is of theoretical interest in physical chemistry, it is of little importance in clinical measurements.

Specimens used for evaluating ECF osmolality may be either serum or heparinized plasma; the use of calcium-binding anticoagulant salts should be avoided.

Urine osmolality is determined in the same manner as serum osmolality. Because of variation in urine concentration, the measured range of osmolality may be extremely broad, i.e., from 50 to >2000 mosmol/kg H_2O. Since renal concentration of urine is accomplished by water moving across *osmotic* gradients, osmolality of urine is the most direct measurement of this function. Urine specific gravity and urine refractive index are used more frequently as an indication of urine concentration only because they are more practicable in the office laboratory. These measures of urine concentration are not measures of colligative properties of the specimen and are therefore only indirect indications of variations in osmotic pressure of the sample.

H. Urine Electrolyte Concentrations

The measurement of sodium, potassium, and chloride concentration in urine is occasionally of value in the evaluation of water and electrolyte disorders. Such measurements have many limitations, however, which must be appreciated. In the normal animal and in many clinical patients, urine electrolyte concentrations are a direct reflection of dietary intake and can be predicted from that information. In addition, measurements on random urine samples reflect only the *concentration* of electrolytes at the time the specimen was collected and may be quite misleading in regard to electrolyte balance in the patient or to renal electrolyte control. Reliable information concerning these functions is best obtained from 24-hour urine collections, from which one can determine the total excretion of electrolytes on a daily basis. Administration of parenteral fluids or diuretics to patients may alter urine electrolyte concentrations markedly so that results on such samples may be misleading. The indications, limitations, and interpretation of urine electrolyte data have been reviewed by Harrington and Cohen (1975).

I. Other Serum Chemistry

The blood urea nitrogen (BUN) concentration is a useful piece of information in the evaluation of clinical patients with fluid balance disorders. It draws attention to the possibility of renal disease as an explanation for severe water, electrolyte, or acid-base disturbances. Moreover, when renal and postrenal diseases can be eliminated, the BUN is an indication of "prerenal azotemia," which is usually due to dehydration or shock. Thus, in the horse with an acute intestinal syndrome, the magnitude of azotemia is a useful indicator of the severity of dehydration present.

J. Anion Gap

The sum of measured serum cation concentrations (sodium and potassium) exceeds the sum of measured serum anion concentrations (chloride and bicarbonate) when these quantities are expressed in milliequivalents per liter. This is because a greater quantity of

anions is not measured in the usual procedures (phosphate, sulfate, organic acids, negatively charged protein ions). The difference in their concentrations is called the anion gap. Some calculations of the anion gap may ignore the concentration of potassium since it is quite consistent and quite small. In such instances the anion gap is about 5 mEq less than if potassium were included in the calculation. When potassium is included, the formula for calculating the anion gap is as follows:

$$\text{Anion gap} = ([Na^+] + [K^+]) - ([Cl^-] + [HCO_3^-]) \quad (12)$$

The anion gap in domestic animals normally falls between 10 and 25 mEq/liter when calculated by the above formula.

The anion gap concept was originally used, in a somewhat rearranged form, to estimate serum sodium concentration when only chloride and bicarbonate concentration had been measured (Benjamin, 1961). Later, it was used to confirm the accuracy of laboratory results when all four electrolyte concentrations had been determined; i.e., anion gaps outside the expected range focused attention on peculiar patient abnormalities or laboratory errors in the measurement of one or more of the electrolytes. More recently, the usefulness of the anion gap in the understanding of clinical problems has been emphasized (Oh and Carroll, 1977; Smithline and Gardner, 1976).

If the laboratory determinations are accurate, increases in the anion gap usually reflect accumulation of unmeasured anions; decreases in the anion gap reflect either increases in unmeasured cations or decreases in unmeasured anions. Increased anion gap is seen, therefore, when ketone anions accumulate in diabetes, lactate accumulates in shock, or phosphate accumulates in uremia. Decreased anion gap is less often seen but may result from hypercalcemia or hypoalbuminemia. Computerized preparation of laboratory reports has made it convenient to report anion gaps routinely whenever electrolytes are measured.

K. Osmolar Gap

The osmolar gap is another calculation which can be made when appropriate laboratory data are available (Smithline and Gardner, 1976; Tasker, 1975). It is the difference between measured osmolality (by osmometer) and osmolality predicted from determinations of the concentrations of major osmotically-active serum constituents: osmolar gap equals osmolality (measured) − osmolality (calculated). Several different equations have been suggested for determining the calculated serum osmolality. Most of these have been reviewed by Green (1975). The most detailed is Eq. (13).

$$\text{Osmolarity (calculated)} = \frac{1.86([Na^+] + [K^+]) + [\text{glucose}]/18 + [\text{urea N}]/2.8}{\%\ \text{serum water}} \quad (13)$$

When calculated by this formula, the osmolar gap is approximately zero (±15) in most species. Deviations from the expected results may reflect laboratory errors or special disease conditions. Decreases in the osmolar gap always reflect laboratory errors. Increases in the osmolar gap may reflect laboratory errors but also may indicate increases in unmeasured osmotically active solute or decreases in serum water content. The formula presented above [Eq. (13)] for calculation of serum osmolality includes serum water content, glucose, and urea nitrogen components. When these have been measured accurately, large osmolar gaps reflect increased concentration of osmotically active solute other than glucose and urea. Such substances, to influence the serum osmolality significantly,

must be substances of low molecular weight which can accumulate in appreciable quantity in the serum before death occurs. A number of specific toxic substances cause abnormal osmolar gaps—ethanol, mannitol, ethylene glycol, and salicylic acid are a few of the most notorious. In other instances, abnormal osmolar gaps have been identified in patients in shock and in moribund patients, neither of which have had exposure to toxic chemicals or drugs (Boyd *et al.*, 1971). In such patients, it is assumed that endogenous substances have accumulated. Lactic acid certainly is a possible contributor to the osmolar gap in such circumstances, but it does not explain the entire amount. This type of abnormal osmolar gap has been observed occasionally in moribund horses. When routinely calculated, the osmolar gap may provide useful information about laboratory accuracy and about the diagnosis and prognosis of the patient.

L. Normal Values

Reference values for the clinical chemistry procedures mentioned above are given in Table IX for common domestic animals. Such values depend greatly on the methods employed in the laboratory as well as on the sampling procedures used and on possible differences in age, sex, breed, geographical location, and other variable factors. They are provided only as a guide for use when more appropriate reference ranges are not available.

XI. CLINICAL SYNDROMES ASSOCIATED WITH DISTURBANCES OF WATER, ELECTROLYTE, AND ACID-BASE BALANCE

Veterinary patients with a wide variety of clinical syndromes of water, electrolyte, and acid-base disturbance have been described in the literature. Such descriptions are often helpful in anticipating special problems requiring specific therapy. It must be emphasized, however, that the metabolic consequences of a given syndrome can never be predicted with certainty. The variable factors which influence the final patient state are so diverse and numerous that no two patients will be alike in their *status praesens* or in their response to treatment. Descriptions of "classical" or "typical" patterns of derangement fail to

TABLE IX

Reference Values for Blood Gas and Electrolyte Determinations

Electrolyte or gas	Ox	Horse	Dog	Cat
Sodium (mEq/liter)	132-152	132-146	140-155	147-156
Potassium (mEq/liter)	3.9-5.8	2.6-5.0	3.7-5.8	4.0-5.3
Chloride (mEq/liter)	97-111	99-109	105-120	115-123
pH	7.35-7.50	7.32-7.44	7.31-7.42	7.24-7.40
pCO_2 (mm Hg)	35-44	38-46	29-42	29-42
Bicarbonate (mEq/liter)	20-30	24-30	17-24	17-24
Osmolality (mosmol/kg H_2O)	270-300	270-300	280-305	280-305

describe the many patients with the same disease entity which have quite different fluid balance abnormalities. Such descriptions are especially misleading in respect to occasional patients in which the abnormalities are exactly the opposite of the classical pattern, e.g., metabolic acidosis when metabolic alkosis is expected and hypokalemia when hyperkalemia is expected. For this reason, therapy based on the presumption of a classical disturbance may be contraindicated in the individual patient. Specific laboratory tests are necessary to identify, with certainty, problems in each patient.

In spite of these caveats, it is often helpful to know the patterns of fluid disturbances which have been associated with specific disease syndromes in animals.

A. Dogs

1. Vomiting

The classical fluid balance changes due to vomiting in man are metabolic alkalosis with hypochloremia and hypokalemia. These are the result of loss of hydrochloric acid and potassium from the gastric secretions. Secretion of each hydrogen ion into the gastric juice is accompanied by release of one bicarbonate ion into the ECF, as shown by the following reactions which occur in the gastric parietal cells.

$$CO_2 \text{ (from plasma)} + H_2O \rightarrow H_2CO_3 \tag{14}$$

$$H_2CO_3 \rightarrow H^+ \text{ (gastric juice)} + HCO_3^- \text{ (plasma)} \tag{15}$$

Thus, bicarbonate accumulates as hydrogen ions are lost, and metabolic alkalosis develops.

This "classical" pattern of "gastric alkalosis" is occasionally seen in vomiting dogs and may be quite severe. However, the individual patient is subject to a variety of modifications of this pattern, e.g. variations in volume and composition of vomitus, variations in proportions of gastric and intestinal secretions lost, and variations in duration of vomiting. For this reason, the pattern of fluid disturbance in a specific patient cannot be predicted with certainty.

2. Diarrhea

When diarrhea is voluminous and prolonged, large volumes of bicarbonate- and electrolyte-rich fluids are lost. The typical pattern of disturbance associated with diarrhea is thus dehydration, metabolic acidosis, and depletion of sodium, potassium, and other electrolytes. Decreased *concentrations* of these electrolytes in the body fluids may or may not occur, depending on the relative proportions of water and solute lost. When water intake has been increased by drinking or by administration of electrolyte-free solutions, subnormal electrolyte concentrations in the serum are especially likely.

3. Intestinal Obstruction

Intestinal obstruction prevents normal gastrointestinal functions from continuing. Luminal accumulations of fluid cranial to the obstruction often occur. This sequestration of electrolyte-rich fluid occurs at the expense of extracellular and intracellular fluid. Vomiting may aggravate fluid deficits. Hypovolemia may lead to shock. The resulting disturbance in the patient is complex and unpredictable, but dehydration, electrolyte depletion, and acid–base derangement (usually metabolic acidosis) may be severe.

4. Acute Renal Failure

The sudden cessation of renal function may lead to metabolic acidosis and hyperkalemia. If fluid intake is continued, overhydration may develop.

5. Chronic Renal Failure

Gradual loss of renal function leads to sodium depletion and dehydration, but, if these occur *pari passu,* hyponatremia will not be evident. Metabolic acidosis and potassium depletion are often significant findings.

6. Diabetes Mellitus

Hyperglycemia and glucosuria cause a number of severe fluid balance disturbances (Cotton *et al.,* 1971; Ling *et al.,* 1977). The solute diuresis causes depletion of sodium and potassium and may lead to dehydration. The defect in carbohydrate metabolism leads to ketosis, which, in turn, results in metabolic acidosis. Severe ketoacidosis may cause extracellular translocation of potassium, resulting in hyperkalemia even if the patient is depleted of potassium. This life-threatening hyperkalemia may be followed by equally life-threatening hypokalemia after correction of the ketoacidosis. Sudden changes in extracellular concentrations of glucose, potassium, and bicarbonate due to excessively rapid corrective measures may lead to serious central nervous system complications.

A syndrome of hyperosmolar (nonketotic) coma has occasionally been recognized in dogs (Schaer *et al.,* 1974) and cats (Schaer, 1975) with severe hyperglycemia. The neurological abnormalities are apparently due to marked elevation in ECF osmolality resulting from hyperglycemia, since correction of the osmolar disturbance results in marked improvement.

It should be recognized that the hyponatremia which is often observed in diabetic animals is not an indication of hypoosmolality of the ECF. In fact, misguided attempts to correct this abnormality may aggravate an existing hyperosmolar state.

7. Diabetes Insipidus

This syndrome is characteristically associated with uncontrollable loss of water from the body. Neither dehydration nor electrolyte/acid-base disturbances are usually evident if drinking water is freely available. This syndrome has been reviewed by Madewell *et al.* (1975) and Bovee (1977).

The determination of plasma osmolality may provide useful information for differentiating diabetes insipidus from psychogenic polydipsia. In the former, polydipsia is secondary to excessive water loss from the ECF. This may lead to slight negative water balance and serum osmolality slightly in excess of normal (or at least in the high normal range). In psychogenic polydipsia, on the other hand, polydipsia is the primary disturbance, and polyuria is the mechanism by which excess water is eliminated. This may lead to slight positive water balance and serum osmolality which is in the low normal range (Harrington and Cohen, 1973).

8. Congestive Heart Failure

In this disease active reabsorption of sodium from the kidneys is increased, and passive reabsorption of water occurs as a result. This leads to marked increase in ECF volume (up to 50% of body weight) and edema. Plasma protein may be decreased due to hemodilu-

tion, but serum sodium concentration is usually normal since water and sodium have been retained to an equal degree (Hamlin, 1972).

9. Adrenal Insufficiency

The hallmark of adrenal insufficiency is decreased mineralocorticoid activity, which leads to hyponatremia and hyperkalemia. The sodium diuresis which occurs results in negative water balance, hypovolemia, and shock or chronic dehydration. These, in turn, may lead to azotemia.

It has been suggested that the ratio of sodium to potassium in the serum is a more obvious indication of mineralocorticoid deficiency than the actual concentration of each and that ratios below normal are indicative of adrenal insufficiency. This has led others to assume that subnormal Na/K ratios are virtually pathognomonic of this disease. Although such findings are most often the result of adrenal insufficiency, other conditions, especially chronic diarrhea, may also be associated with subnormal Na/K ratios.

10. Shock

The characteristic finding in shock is hypovolemia with lactic acidosis due to anaerobic metabolism in the tissues. The increased lactate is buffered by bicarbonate. This leads to reduction of bicarbonate and blood pH. Hypovolemia is present, and hyperkalemia may be seen.

11. Gastric Dilatation–Volvulus

It might be assumed that this gastric disease would be characterized by the "gastric alkalosis" described in the section on vomiting due to the sequestration of gastric juice. However, it has been shown that metabolic acidosis is more likely to occur (Muir and Lipowitz, 1978; Wingfield *et al.*, 1974, 1976). This is probably due to the development of shock in affected animals. A marked rise in serum potassium has also been observed following the relief of dilatation and torsion in experimental animals. The source of the potassium influx has not been determined. It has been emphasized, however, that it may have a role in the sudden death that may occur after surgical correction of spontaneous gastric torsion.

12. Heat Stroke

Studies of simulated heat stroke in dogs have demonstrated increased blood pH and decreased pCO_2, i.e., respiratory alkalosis (Hanneman *et al.*, 1977). Panting, tachycardia, and hyperthermia occurred during the exposure to increased ambient temperature.

13. Diuretic Therapy

Furosemide and the thiazides are commonly used diuretics which inhibit sodium and chloride reabsorption in the nephron. In addition to promoting the loss of these ions and water from the body, these drugs can cause potassium depletion and significant hypokalemia (Ross, 1975).

B. Cats

1. Urethral Obstruction

Cats with urethral obstruction of 24–48 hours' duration were found to have severe metabolic acidosis and hyperkalemia. Following treatment with fluid therapy and sodium

bicarbonate, hypokalemia occasionally developed (Burrows and Bovee, 1978; Finco and Cornelius, 1977; Schaer, 1977).

2. *Feline Panleukopenia*

This disease is a notorious cause of dehydration in cats. Experimental and clinical investigations have been concerned with virology, serology, and hematology. There is little in the literature regarding the special characteristics of the water, electrolyte, and acid–base derangements which occur.

C. Ruminants

1. *Abomasal Disorders*

Left and right abomasal displacements and torsion of the abomasum are characterized by a triad of metabolic abnormalities similar to those seen after vomiting in monogastric animals, i.e., hypochloremia, hypokalemia, and metabolic alkalosis (Schotman, 1971; Poulsen, 1973, 1974). The severity of these changes varies greatly from patient to patient and depends also on the nature of the abdominal displacement. Torsion of the abomasum and right-sided displacements are generally associated with more severe abnormalities than left-sided displacements. In some patients, postsurgical recovery is seriously impeded until correction of the metabolic abnormalities has been accomplished.

Paradoxical aciduria is seen in some cows with abomasal disorders (Gingerich and Murdick, 1975). A significant correlation has been reported between postsurgical outcome and serum chloride concentration in cows with right-sided torsions (Smith, 1978).

2. *Intestinal Obstruction*

Although the triad of hypokalemia, hypochloremia, and metabolic alkalosis is often considered pathognomonic for primary disorders of the abomasum, it is often seen in cows with cecal volvulus and small intestinal obstruction (intussusception) as well (Whitlock *et al.*, 1976; Hammond *et al.*, 1964). This must be kept in mind in differential diagnosis. (It is also possible that this triad develops due to ileus without any physical abnormalities of the gastrointestinal tract.)

3. *Neonatal Diarrhea*

Neonatal diarrhea in calves is characterized by dehydration and metabolic acidosis (Tennant *et al.*, 1972). Although depletion of sodium, chloride, and potassium (Lewis and Phillips, 1972) occurs, decreased serum concentrations of these electrolytes are seldom seen in the untreated calf. In fact, hyperkalemia is a typical presenting sign due to the severe acidosis and decreased renal function in affected animals. Hyperkalemia may be the cause of death in some affected animals.

4. *Salt Deficiency*

Polyuria, pica, and decreased milk production have been reported in dairy cattle in which inadequate dietary salt was identified. Serum sodium, chloride, and osmolality were decreased. Urine and saliva concentrations of sodium and potassium and the sodium/potassium rations in these fluids were considered most reliable indicators of sodium deficiency (Whitlock *et al.* 1975).

5. *"Downer" Cows*

Much has been written about the role of potassium in paretic cows and the rationale for potassium therapy in treatment. Although potassium depletion is occasionally encountered as a cause of this syndrome, and some cows respond favorably to replacement therapy, many cows have normal potassium intake and normal serum levels and do not respond favorably to such treatment. The "downer" cow syndrome has many diverse causes (Curtis *et al.*, 1970).

6. *Water Intoxication*

A syndrome of water intoxication is occasionally observed in calves which consume large volumes of water in a short period of time (Gibson *et al.*, 1976). The most frequent sign of this problem is intravascular hemolysis and hemoglobinuria. In severe cases, however, marked abnormalities of the central nervous system result from the development of cerebral edema.

7. *Grain Overload*

This syndrome in cattle has been studied extensively (Blood and Henderson, 1974; Vestweber *et al.*, 1974). Fermentation of excessive quantities of carbohydrates in the rumen results in rapid production of lactic acid, which leads to systemic metabolic acidosis. Increased osmolality of the rumen contents leads to rapid influx of water from the ECF, hypovolemia, anuria, and shock.

D. Horses

1. *Acute Diarrhea in Adult Horses*

Acute diarrheal disease in adult horses is a serious medical problem (Tasker, 1966, 1967b). Severe dehydration often occurs. Hyponatremia and hypochloremia are common clinical findings. Metabolic acidosis is marked. Affected animals are usually depleted of potassium. However, the serum potassium is usually normal owing to the effects of acidosis. Treatment of such animals with alkalinizing solutions which do not contain supplemental potassium may lead to severe hypokalemia.

2. *Acute Diarrhea of Foals*

Severe diarrhea in foals, as with adult horses, tends to cause metabolic acidosis and dehydration as well as depletion of sodium, chloride, and potassium. Foals tend to develop hyperkalemia, however, as calves do, due to the severity of acidosis and concomitant oliguria; hyperkalemia in adult horses with diarrhea is extremely rare.

3. *Chronic Diarrhea*

Water, electrolyte, and acid–base abnormalities in horses with chronic diarrhea are less severe than those in acute illness. Hyponatremia, hypokalemia, hypochloremia, and metabolic acidosis may be present to some degree, but the extent of these abnormalities is unpredictable due to variations in compensatory factors in individual patients.

4. *Intestinal Obstruction*

The horse with acute intestinal obstruction develops dehydration, shock, metabolic acidosis, and electrolyte depletion (Datt and Usenik, 1975; Donawick and Hiza, 1973;

Tennant, 1975). These findings are very similar to those in horses with acute diarrheal illness. Hyponatremia and hypochloremia may be seen. Although there is potassium depletion, the serum concentration is often normal due to coexisting acidosis.

5. Anhidrosis (Dry-Coat Syndrome)

Veterinarians working with race horses in the tropics have reported a fluid and electrolyte abnormality called the anhidrosis syndrome (Correa and Calderin, 1966; Currie and Seager, 1976). This problem is seen primarily in thoroughbred horses imported from temperate climates. It is characterized by fever, dyspnea, and the absence of sweating following exercise. Severe dehydration, hyponatremia, and hypochloremia have been reported. Although several theories have been advanced to explain this problem, complete elucidation of the mechanism has not been achieved.

6. Overfeeding

Experiments intended to produce laminitis by overfeeding a high-starch ration to ponies resulted in metabolic acidosis and hypokalemia (Harkema *et al.*, 1978). Some animals died of shock.

7. Endurance Competitions

Horses have been studied during the course of endurance rides (Carlson and Mansmann, 1974; Carlson *et al.*, 1976). Horses judged to be "normal" during the event were found to have metabolic alkalosis, dehydration, hyponatremia, hypochloremia, and hypokalemia. Other horses were identified as "exhausted." The latter group was more severely dehydrated, and acid–base and electrolyte values were more variable than in the "normal" horses.

8. Short-Term Strenuous Exercise (Racing)

Studies of horses under racing conditions to evaluate fluid balance alterations have emphasized the fluctuations in acid–base parameters which occur (Krzywanek *et al.*, 1976; Milne, 1974). The principal change observed was metabolic acidosis associated with an increase in blood lactic acid.

9. Endotoxic Shock

Studies of ponies following the administration of endotoxin revealed the development of severe metabolic acidosis associated with increased blood lactic acid concentration (Beadle and Huber, 1977). Hemoconcentration and hyponatremia were also observed. Serum osmolality increased as serum sodium declined. This suggests that azotemia, hyperglycemia, or accumulation of other unknown solute occurred during the period of study.

XII. FLUID THERAPY

A thorough discussion of fluid therapy is beyond the scope of this chapter. It should be apparent from the foregoing presentation, however, that derangements of water, electrolyte, and acid–base balance occur commonly in clinical illnesses of domestic animals.

Rational therapy is based on accurate diagnosis and on the principals of therapeutics. In the context of fluid therapy for the correction of water, electrolyte, and acid–base disor-

ders, the prerequisite accurate diagnosis is not necessarily a diagnosis of the primary disease entity in the patient, but an accurate characterization of the fluid balance disorder present. Sections IX and X were devoted to a discussion of how to obtain four basic pieces of information; i.e., whether a volume, acid–base, osmolar, or specific electrolyte abnormality exists in the patient. Once this information has been acquired, it is possible to plan rational corrective therapy.

Since fluid therapy may be used to correct a variety of disorders of volume, osmolality, acid–base balance, and specific electrolyte concentrations, different kinds of fluids must be available. Table X includes the most commonly used parenteral solutions and additives.

The basic aim of fluid therapy is to restore body fluids to normal with respect to the four important characteristics described above. Water and electrolyte solutions are selected to accomplish this purpose. Although the rate and route of administration are important considerations in fluid therapy, providing an appropriate *volume* of the right *kind* of fluid is the most challenging task.

TABLE X

Composition of Typical Fluid Therapy Products

Product	Electrolyte concentration (mEq/liter)							
	Cations					Anions		
	Na^+	K^+	Ca^{2+}	Mg^{2+}	NH_4^+	Cl^-	HCO_3^-	$Lactate^-$
Plasma, normal	140	4	5	2		103	25	5
Commonly used parenteral solutions								
5% dextrose	0	0	0	0	0	0	0	0
0.9% sodium chloride	154	0	0	0	0	154	0	0
Lactated Ringer's solution	130	4	3	0	0	109	0	28
2.5% dextrose in half-strength lactated Ringer's solution	65	2	1.5	0	0	54.5	0	14
Special solutions								
Ringer's solution	145	4	6	0	0	155	0	0
3% sodium chloride solution	513	0	0	0	0	513	0	0
Gastric replacement (human)	63	17	0	0	70	150	0	0
Duodenal replacement (human)	80	36	5	3	0	63	0	60
Ammonium–potassium chloride solution (bovine)	0	75	0	0	75	150	0	0
Alkalinizing solutions								
1.3% sodium bicarbonate	155	0	0	0	0	0	155	0
1/6 *M* sodium lactate	167	0	0	0	0	0	0	167
Acidifying solution								
1/6 *M* ammonium chloride	0	0	0	0	168	168	0	0
Additives								
5% sodium bicarbonate	600	0	0	0	0	0	600	0
Potassium chloride (1 mEq/ml)								
Sodium bicarbonate (1 mEq/ml)								
Potassium chloride powder (1 gm = 14 mEq)								
Sodium bicarbonate powder (1 gm = 12 mEq)								

A rule of thumb for estimating volume requirements is often used. This rule is that severe clinical signs of dehydration are associated with volume deficits of at least 10% of body weight. Since kilograms and liters are approximately equivalent, such a 10% deficit is equal to 1 liter per 10 kg body weight. A 22-lb (10-kg) dog with severe signs of dehydration has an estimated fluid deficit of 1 liter; a 1000-lb (450-kg) horse with similar signs has an estimated fluid deficit of 45 liters. This rule of thumb is merely a rough approximation which is used as a guideline for clinical purposes.

Selection of the appropriate kind of fluid for each patient is the most important aspect of fluid therapy. The formulation of each parenteral solution is designed for the correction of a different clinical problem. It should be obvious that the administration of an acidifying solution to an acidotic patient is contraindicated. Rational fluid therapy should follow this sequence:

1. Evaluate the patient, using appropriate laboratory tests if possible.
2. Select fluids, the composition of which complements the ECF of the patient, i.e., alkalinizing fluids for the patient in metabolic acidosis, high-potassium fluids for the patient with potassium depletion.
3. Administer fluids to the patient according to estimated volume requirements.
4. Reevaluate the patient, and repeat treatment as indicated.

It is very difficult to estimate specific patient requirements for bicarbonate in metabolic acidosis, but the following rule of thumb has been suggested:

$$\text{Body weight (kg)} \times 30\% \times \text{plasma bicarbonate deficit (mEq/liter)} = \text{bicarbonate required (mEq)}$$

In using this rule, the plasma bicarbonate deficit is the difference between the patient value and an arbitrary figure taken as normal, usually 24 mEq/liter. The 30% times body weight is a generous estimate of ECF volume. Since plasma bicarbonate concentration is an indication of its concentration in all ECF, the formula indicates the total deficit in the ECF. Some have felt that this equation underestimates total body requirements for correction of such problems as metabolic acidosis and have used a factor of 60% instead. In either case, the result obtained is but a crude approximation of patient requirements, and reevaluation of patients after treatment is always indicated.

Lactated Ringer's solution is the classic balanced electrolyte solution. It provides major ECF electrolytes in approximately the same concentration found in plasma. For this reason, rehydration of patients with this solution does not usually create iatrogenic electrolyte disturbances, and this fluid is therefore usually recommended for routine clinical purposes. It should be noted that lactate or other organic anions (e.g., acetate) are often used in parenteral solutions in place of bicarbonate since bicarbonate-containing solutions cannot be sterilized by heat. If metabolism of the organic anion proceeds in the normal manner, bicarbonate is produced in equivalent quantities. Thus, when lactated Ringer's solution is administered to a patient with the ability to metabolize lactate, it has the same effect as if the solution contained 28 mEq/liter of bicarbonate ion.

It can be seen from Table X that some fluids commonly used in fluid therapy are quite different in composition from normal plasma. Isotonic (5%) dextrose in water has no electrolvtes at all. Infusion of large volumes of this solution to severly dehydrated patients would tend to create electrolyte disturbances. Although 0.9% sodium chloride solution is often called "physiological saline," it contains no bicarbonate or bicarbonate precursor

and no potassium. Infusion of large volumes of this fluid would tend to aggrevate metabolic acidosis or hypokalemia.

A common error in the clinical use of fluids is to use the expression "Ringer's solution" as an abbreviated form of "lactated Ringer's solution." Table X contains a description of each of these solutions. It is obvious that they are quite different! Ringer's solution is seldom used in contemporary clinical medicine. It is not a balanced electrolyte solution since it contains no bicarbonate or bicarbonate precursor and, like "physiological saline," contains half again more chloride than normal.

As shown in Table X, special solutions have been designed for special purposes. When the needs of the patient have been determined with certainty through the use of appropriate laboratory tests, a special solution can be selected which specifically complements the patient's ECF. Thus, a "gastric replacement solution" is designed for the patient with the classic disorder of hypochloremia, hypokalemia, and metabolic alkalosis due to loss of gastric fluid, and a "duodenal replacement fluid" may be most appropriate for the patient with loss of intestinal fluids. The ammonium-potassium chloride solution listed in Table X has been formulated for treatment of cattle with especially severe disorders due to upper gastrointestinal disease (Whitlock *et al.*, 1976). Since these special solutions contain unphysiological concentrations of certain electrolytes, they should be used only when known to be specifically indicated. Random use without accurate laboratory evaluation of the patient may be harmful.

Table X also contains a description of additives which are available for preparing solutions especially needed for certain patients. The use of such solutions is especially recommended in certain common diseases of animals (Tasker, 1966).

Recognition and treatment of the primary disease process in a patient are obvious goals of the clinician. Of equal, and sometimes of greater, importance to the eventual recovery of the patient, however, are the recognition and correction of secondary effects of the illness. The fluid balance problems described in this chapter may impede or prevent recovery of the patient in some instances even when the specific treatment of the primary disease has been adequate. A comprehensive approach to the patient which includes evaluation and correction of water, electrolyte, and acid-base derangements will greatly improve the quality of patient care.

REFERENCES

Abbrecht, P. H. (1969) *J. Clin. Invest.* **48,** 432-442.
Alexander, F. (1974). *Br. Vet. J.* **130,** 17-22.
Anand, R. S., and Parker, H. R. (1966). *Am. J. Vet. Res.* **27,** 899-902.
Aschbacher, P. W., Kamal, T. H., and Cragle, R. G. (1965). *J. Anim. Sci.* **24,** 430-433.
Beadle, R. E., and Huber, T. L. (1977). *J. Equine Med. Surg.* **1,** 371-375.
Benjamin, M. M. (1961). "Outline of Veterinary Clinical Pathology," 2nd ed. Iowa State Univ. Press, Ames.
Blood, D. C., and Henderson, J. A. (1974). "Veterinary Medicine," 4th ed. Williams & Wilkins, Baltimore, Maryland.
Bolton, G. R. (1975). "Handbook of Canine Electrocardiography." Saunders, Philadelphia, Pennsylvania.
Bovee, K. C. (1977). *Vet. Clin. North Am.* **7,** 603-611.
Boyd, D. R., Addis, H. M., Chilimindris, C., Lowe, R. J., Folk, F. A., and Baker, R. J. (1971). *Arch. Surg.* **102,** 363-372.
Brobst, D. (1975). *J. Am. Vet. Med. Assoc.* **166,** 359-364.
Burrows, C. F., and Bovee, K. C. (1978). *J. Am. Vet. Med. Assoc.* **172,** 801-806.
Carlson, G. P., and Mansmann, R. A. (1974). *J. Am. Vet. Med. Assoc.* **165,** 262-264.

Carlson, G. P., Ocen, P. O., and Harrold, D. (1976). *Theriogenology* **6,** 93-104.
Clark, D. R., and McCrady, J. D. (1966). *Vet. Med. Small Anim. Clin.* **61,** 751-760.
Correa, J. E., and Calderin, G. G. (1966). *J. Am. Vet. Med. Assoc.* **149,** 1556-1560.
Cotton, R. B., Cornelius, L. M., and Theran, P. (1971). *J. Am. Vet. Med. Assoc.* **159,** 863-870.
Coutler, D. B., and Engen, R. L. (1972). *J. Am. Vet. Med. Assoc.* **160,** 1419-1422.
Cox, J. L., Becker, D. E., and Jensen, A. H. (1966). *J. Anim. Sci.* **25,** 203-206.
Currie, A. K., and Seager, S. W. J. (1976). *Proc. Annu. Meet. Am. Assoc. Equine Pract., 22nd, Dallas,* pp. 249-251.
Curtis, R. A., Cote, J. F., and Willoughby, R. A. (1970). *Mod. Vet. Pract.* **51,**(7), 25-28.
Dalton, R. G., and Fisher, E. W. (1963). *Br. Vet. J.* **119,** 384-386.
Datt, S. C., and Usenik, E. A. (1975). *Cornell Vet.* **65,** 152-172.
Deavers, S., Rosborough, J. P., Garner, H. E., Huggins, R. A., and Amend, J. F. (1973). *J. Appl. Physiol.* **34,** 341-343.
Donawick, W. J., and Hiza, M. A. (1973). *Tijdschr. Diergeneeskd.* **98,** 980-982.
English, P. B. (1966). *Res. Vet. Sci.* **7,** 258-275.
Epstein, F. H. (1962). *In* "Clinical Disorders of Fluid and Electrolyte Metabolism" (M. H. Maxwell and C. R. Kleeman, eds.), pp. 38-70. McGraw-Hill, New York.
Fayet, J. C. (1971). *Br. Vet. J.* **127,** 37-44.
Feldman, E. C., and Ettinger, S. J. (1977). *Vet. Clin. North Am.* **7,** 487-496.
Finco, D. R., and Cornelius, L. M. (1977). *Am. J. Vet. Res.* **38,** 823-830.
Gentile, G., and Venturoli, M. (1960). *Nuova Vet.* **36,** 167-174.
Gibson, E. A., Counter, D. E., and Barnes, E. G. (1976). *Vet. Rec.* **98,** 486.
Gingerich, D. A., and Murdick, P. W. (1975). *J. Am. Vet. Assoc.* **166,** 227-230.
Green, R. A. (1975). *Bull. Am. Soc. Vet. Clin. Pathol.* **4**(3/4), 9-25.
Greenspan, K., Wunsch, C., and Fisch, C. (1965). *Am. J. Physiol.* **208,** 954-958.
Hamar, D., Chow, F. C. H., Dysart, M. I., and Rich, L. J. (1976). *J. Am. Anim. Hosp. Assoc.* **12,** 514-517.
Hamlin, R. L. (1972). *J. Am. Anim. Hosp. Assoc.* **8,** 224-228.
Hammond, P. B., Dzuik, H. E., Usenik, E. A., and Stevens, C. E. (1964). *J. Comp. Pathol.* **74,** 210-222.
Hanneman, G. D., Higgins, E. A., Price, G. T., Funkhouser, G. E., Grape, P. M., and Snyder, L. (1977). *Am. J. Vet. Res.* **38,** 955-958.
Hannon, J. P., and Durrer, J. L. (1963). *Am. J. Physiol.* **204** 517-519.
Hansard, S. L. (1964). *Am. J. Physiol.* **206,** 1369-1372.
Harkema, J. R., Robinson, N. E., and Scott, J. B. (1978). *Am. J. Vet. Res.* **39,** 741-744.
Harrington, J. T., and Cohen, J. J. (1973). *Arch. Intern. Med.* **131,** 810-825.
Harrington, J. T., and Cohen, J. J. (1975). *N. Engl. J. Med.* **24,** 1241-1243.
Haskins, S. C. (1977). *J. Am. Vet. Med. Assoc.* **170,** 429-434.
Haxton, J. A., Schneider, M. D., and Kaye, M. P. (1974). *Am. J. Vet. Res.* **35,** 835-837.
Hays, R. M. (1972). *In* "Clinical Disorders of Fluid Electrolyte Metabolism" (M. H. Maxwell and C. R. Kleeman, eds.), 2nd ed, pp. 1-43. McGraw-Hill, New York.
Hintz, H. F., and Schryrer, H. F. *J. Anim. Sci.* **42,** 637-643.
Howes, J. R., Hentges, J. F., Jr., and Feaster, J. P. (1963). *J. Anim. Sci.* **22,** 183-187.
Huber, T. L. (1971). *Am. J. Vet. Res.* **32,** 887-890.
Jackson, O. F., and Tovey, J. D. (1977). *Feline Pract.* **7**(4), 30-34.
Keynes, R. D., and Harrison, F. A. (1967). *Vet. Rec.* **81,** 244-250.
Kohn, C. W., Muir, W. W., and Sams, R. (1978). *Am. J. Vet. Res.* **39,** 871-874.
Krzywanek, H., Milne, D. W., Gabel, A. A., and Smith, L. G. (1976). *Am. J. Vet. Res.* **37,** 291-294.
Lehmann, H. P. (1976). *Am. J. Clin. Pathol.* **65,** 2-18.
Lemieux, G., Warren, Y., and Gervais, M. (1964). *Am. J. Physiol.* **206,** 743-749.
Lewis, L. D., and Phillips, R. W. (1972). *Cornell Vet.* **62,** 596-607.
Lindeman, R. D. (1976). *Am. J. Med. Sci.* **272,** 5-17.
Ling, G. V., Lowenstine, L. J., Pulley, L. T., and Kaneko, J. J. (1977). *J. Am. Vet. Med. Assoc.* **170,** 521-530.
Lombardi, M. H. (1972). *Am. J. Vet. Res.* **33,** 1825-1834.
Madewell, B. R., Osborne, C. A., Norrdin, R. A., Stevens, J. R., and Hardy, R. M. (1975). *J. Am. Anim. Hosp. Assoc.* **11,** 497-506.
Manston, R. (1971). *Res. Vet. Sci.* **12,** 168-169.

Michell, A. R. (1974). *Br. Vet. J.* **130,** vi.
Milne, D. W. (1974). *J. S. Afr. Vet. Assoc.* **45,** 345–354.
Mollerberg, L., Ekman, L., and Jacobsson, S. (1975). *Acta Vet. Scand.* **16,** 178–185.
Muir, W. W., and Lipowitz, A. J. (1978). *J. Am. Vet. Med. Assoc.* **172,** 683–690.
Nuttall, F. Q. (1965). *Arch. Intern. Med.* **116,** 670–680.
Oh, M., and Carroll, H. (1977). *N. Engl. J. Med.* **297,** 814–817.
Payne, E., Ryley, J. W., and Gartner, R. J. W. (1967). *Res. Vet. Sci.* **8,** 20–26.
Phillips, R. W., Lewis, L. D., and Knox, K. L. (1971). *Ann. N.Y. Acad. Sci.* **176,** 231–243.
Pickering, E. (1965). *Proc. Nutr. Soc.* **24,** 73–80.
Poulsen, J. S. D. (1973). Ph.D. Thesis, R. Vet. Coll., Stockholm.
Poulsen, J. S. D. (1974). *Nord. Veterinaermed.* **26,** 1–26.
Pownall, R., and Dalton, R. G. (1973). *Br. Vet. J.* **129,** 583–588.
Prankerd, T. A. J. (1961). "The Red Cell." Thomas, Springfield, Illinois.
Reece, W. O. (1972). *Am. J. Vet. Res.* **33,** 1494–1498.
Reid, J. T., Bensadoun, A., Paladines, O. L., and Van Niekerk, B. D. H. (1963). *Ann. N.Y. Acad. Sci.* **110,** 327–342.
Rose, B. D. (1977). "Clinical Physiology of Acid-Base and Electrolyte Disorders." McGraw-Hill, New York.
Ross, J. N. (1975). *In* "Textbook of Veterinary Internal Medicine" (S. Ettinger, ed.). Vol. 2, pp. 825–864. Saunders, Philadelphia, Pennsylvania.
Schaer, M. (1975). *J. Am. Anim. Hosp. Assoc.* **11,** 42–46.
Schaer, M. (1977). *Vet. Clin. North Am.* **7,** 407–414.
Schaer, M., Scott, R., Wilkins, R., Kay, W., Calnert, C., and Wolland, M. (1974). *J. Am. Ahim. Hosp. Assoc.* **10,** 357–361.
Schotman, A. J. H. (1971). *Neth. J. Vet. Sci.* **4,** 5–23.
Seif, S. M., Johnson, H. D., and Hahn, L. (1973). *J. Dairy Sci.* **56,** 581–586.
Sheng, H., and Huggins, R. A. (1971). *Proc. Soc. Exp. Biol. Med.* **137,** 1093–1099.
Simmons, D. (1962). *In* "Clinical Disorders of Fluid and Electrolyte Metabolism" (M. Maxwell and C. Kleeman, eds.), pp. 71–114. McGraw-Hill, New York.
Smith, D. (1978). *J. Am. Vet. Med. Assoc.* **173,** 108–111.
Smithline, N., and Gardner, K. (1976). *J. Am. Med. Assoc.* **236,** 1594–1597.
Suki, W. N. (1976). *Am. J. Med. Sci.* **272,** 31–41.
Tasker, J. B. (1966). *Vet. Med. Small Anim. Clin.* **61,** 765–776.
Tasker, J. B. (1967a). *Cornell Vet.* **57,** 649–657.
Tasker, J. B. (1967b). *Cornell Vet.* **57,** 668–67.
Tasker, J. B. (1975). *Bull. Am. Soc. Vet. Clin. Pathol.* **4**(1), 3–6.
Tennant, B. C. (1975). *Proc. Annu. Meet. Am. Assoc. Equine Pract., 21st, Boston* pp. 426–439.
Tennant, B. C., Harrold, D., and Reina-Guerra, M. (1972). *J. Am. Vet. Med. Assoc.* **161,** 993–1007.
Tollerz, G. (1964). *Acta Vet. Scand.* **5,** 24–34.
Vestweber, G. E., Leopold, H. W., and Smith, J. E. (1974). *Am. J. Vet. Res.* **35,** 1537.
Ward, G. M. (1966a). *J. Dairy Sci.* **49,** 268–276.
Ward, G. M. (1966b). *J. Am. Vet. Med. Assoc.* **148,** 543–544.
Whang, R. (1976). *Am. J. Med. Sci.* **272,** 19–29.
White, A., Handler, P., and Smith, E. L. (1964). "Principles of Biochemistry." McGraw-Hill, New York.
Whitlock, R. H., Kessler, M. J., and Tasker, J. B. (1975). *Cornell Vet.* **65,** 512–526.
Whitlock, R. H., Tennant, B. C., and Tasker, J. B. (1976). *Proc. Int. Conf. Prod. Dis. Farm Anim., 3rd, Wageningen, Neth.* pp. 67–69.
Wingfield, W. E., Cornelius, L. M., and Deyoung, D. W. (1974). *J. Small Anim. Pract.* **15,** 41–53.
Wingfield, W. E., Betts, C. W., and Rawlings, C. A. (1976). *J. Am. Anim. Hosp. Assoc.* **12,** 136–141.

11

Pituitary and Adrenal Function

JOHN S. WILKINSON

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

I. INTRODUCTION

In the second edition of this volume it was noted that it was not possible to deal comprehensively with the pituitary and adrenal glands. Since then there has been a very considerable increase in the literature, and only a selection of this material can be included. Exclusion does not indicate necessarily poor quality but rather the difficulty of covering the entire field. For further information consult reviews by Blackwell and Guillemin (1973), Vale *et al.* (1977), and Cox (1975).

The characteristics and actions of the pineal hormones are still being unraveled, and the prostaglandins are now much better understood but appear to have little clinical importance in the syndromes being discussed.

A. Hypothalamic Hormones

There are many more hypothalamic hormones than we can consider in this chapter. They are all releasing factors or releasing factor inhibitors. We can deal only with corticotropin (ACTH) releasing factor (CRF), growth hormone (GH) (somatotropin) releasing factor (SRF), somatostatin, which blocks SRF, and antidiuretic hormone (ADH) [for further details, see Peterson and Guillemin (1973)]. For the sake of convenience those aspects of the hypothalamic hormones germane to our discussion are considered in the section dealing with control of the adenohypophyseal hormones (Section II, B).

B. Pituitary and Adrenal Glands

1. Hormones

a. Adenohypophysis. The anterior pituitary produces tropic hormones which are involved in apparently all metabolic processes. Adrenocorticotropin and GH are but two of some eight hormones, and more are being discovered, for example, the possible natriuretic hormone described by Szalay *et al.* (1975).

b. Neurohypophysis. The pars nervosa produces two hormones: ADH (vasopressin) and oxytocin.

c. Adrenal Cortex. The hormones of this gland are too numerous to mention individually, and additions are constantly being made to those known. The most important are cortisol (hydrocortisone), corticosterone, aldosterone, and androgens and estrogens.

d. Adrenal Medulla. Two hormones are produced: epinephrine and norepinephrine.

2. Structure of Pituitary and Adrenal Hormones

The hormones being discussed are either lipid-soluble steroids (adrenal cortex) or water-soluble polypeptides and glycoproteins (pituitary) and catecholamines (adrenal medulla). The pituitary and medullary hormones are ineffective therapeutically when

given orally because they are digested. The steroids are absorbed unchanged and can therefore be administered by mouth. The anterior pituitary hormones show slight species variations in structure. These differences are sufficient to render them mildly antigenic when used in heterologous species.

a. Adenohypophysis. The hormones of the adenohypophysis are glycoproteins or peptides (ACTH and GH) of low relative molecular mass (20,000–24,000 Mr). Species specificity in amino acid content (Wilhelmi, 1968) may account for the variations in response achieved by different workers. Adrenocorticotropin has been synthesized and is available commercially.

b. Neurohypophysis. The hormones of the neurohypophysis are polypeptides of about 1100 Mr. Their structure is known, and species differences occur. In the pig and hippopotamus, lysine replaces the arginine in position 8.

c. Adrenal Cortex. The steroid hormones of the adrenal cortex and gonads are of the same basic structure, the cyclopentanophenanthrene ring. Most biologically active steroids are Δ^4,3-one; that is, they have a double bond between positions 4 and 5 and an oxo (keto) group at position 3. Most androgens and estrogens have an oxo group in the 17 position (17-keto- or 17-oxosteroids, usually abbreviated as 17-KS), although hydroxyl groups may replace the oxo groups in either of these positions. A longer chain at the 17 position, and sometimes a hydroxyl group also, imparts gluco- or mineralocorticoid activity. Cortisol and corticosterone have a hydroxyl group in the 11 position and are sometimes called 11-hydroxycorticosteroids (11-OHCS).

d. Adrenal Medulla. The medullary hormones (catecholamines) differ from each other only in that epinephrine has a methyl group which norepinephrine lacks.

3. Mechanisms of Hormone Actions

The mechanisms of many hormonal actions are still unknown or uncertain. It has been held that they activate enzyme systems; for instance, it was thought that epinephrine and glucagon activated hepatic phosphorylase. This activation has now been shown to be indirect, being mediated through cyclic 3′,5′-adenosine monophosphate (cAMP) by activation of the enzyme adenyl cyclase.

It has been suggested that some hormones may combine with a cell constituent to actually form an enzyme. Karlson and Sekeris (1966) postulated that hormones may function as gene activators by stimulating synthesis of messenger ribonucleic acid (mRNA) "coded" as specific enzymes. This would account for the well-known effect of cortisol as an enzyme inducer.

C. Classification of Endocrine Disease

Disease processes in the endocrine system can be classified as hyperfunctional, hypofunctional, nonfunctional, dysfunctional, and ectopic. Examples of all these entities can be found in variations of pituitary adrenal function, although dysfunction and ectopic endocrinopathy of the pituitary adrenal aims have not yet been documented in veterinary medicine.

1. Hyperfunction

a. Adrenal Cortex. Adrenocortical hyperfunction is the most common syndrome of excessive endocrine activity. It may be primary, due to adrenocortical neoplasia, or secondary, due to excessive ACTH production.

b. Adrenal Medulla. Medullary hyperfunction due to neoplasia has been suspected but not undeniably established.

2. Hypofunction

a. Adenohypophysis. Pituitary cachexia may be the end result of almost all pituitary or hypothalamic diseases, whether it is due to neoplasia, ischemia, or inflammation.

In man, individual anterior pituitary hormone deficiencies have been described (Odell, 1966). It is reasonable to assume that similar deficiencies may cause hypothyroidism and adrenal failure in animals. Clarification of this point awaits estimation of peripheral levels of individual tropic hormones.

b. Adrenal Cortex. There may be a number of causes of adrenocortical failure. The gland may be destroyed by nonfunctional neoplastic tissue, inflammation, or infarction. "The existence of autoimmune disturbances of the thyroid, adrenal and testis is no longer questioned" (Solomon and Blizzard, 1963), and Goudie *et al.* (1968) demonstrated that, in the human being, adrenal antigens are associated with the microsomal fraction of the cell. Autoimmunity has to be considered as a possible cause of primary adrenocortical atrophy in the dog also.

3. Nonfunction

Lesions may be present in endocrine tissue without producing any alteration in function; e.g., some adrenal neoplasms, adrenal calcification, and pituitary cysts are without apparent endocrine effect.

4. Dysfunction

In the adrenal in man there is a condition that is due to failure of an enzyme reaction, which blocks the progression from the basic androgen and results in the accumulation of the androgen substrate and its leakage into the blood. In cattle and sheep faults in the metabolism of the thyroid gland result in synthesis of an abnormal thyroglobulin and iodoproteins, respectively.

5. Ectopic Endocrinopathy

This is the result of the production of hormones by nonendocrine tissue, usually neoplastic. In man various neoplasms have been shown to cause hypercalcemia due to parathyroid hormone production, hypoglycemia due possibly to the release of an insulin-like substance or an insulin potentiator, polycythemia due to excessive erythropoietin, hyponatremia due to excessive ADH release, and hyperadrenocorticoidism due to excessive amounts of an ACTH-like substance. The mechanics of these aberrations are not yet known, but it is becoming clear that tumors may produce almost any hormone. All cells have the same genetic components and therefore the potential to produce any peptide. Specialization of function is achieved by control of some of these processes by "re-

pressors.'' Malignant cells that produce hormones have possibly lost or escaped from this control. In dogs hypercalcemia as a sequel to some cases of lymphosarcoma and hypoglycemia due to an embryonal nephroma have been described.

D. Species Prevalence

Clinical biochemical changes associated with alterations in endocrine function have been studied most widely in the dog, and, unless specifically stated otherwise, the discussion following in this chapter refers to dogs.

II. HORMONES OF THE ADENOHYPOPHYSIS

A. Site of Production

There has been considerable speculation as to the cells responsible for the synthesis of the adenohypophyseal hormones. It is becoming increasingly likely that individual hormones are synthesized by individual cells, and with sufficiently specific techniques it may be possible to demonstrate as many cell types as there are hormones.

The chromophobe cells, because they had no distinctively staining granules, were at first thought to be nonfunctional, either precursor, resting, or exhausted forms of the ''functional'' cells. Cushing described in man a disease in which a basophil tumor was associated with adrenocortical hyperplasia. Early accounts of a similar condition in dogs also ascribed the condition to basophil neoplasia (Coffin and Munson, 1953). ''Functionally active'' chromophobe neoplasms are now commonly reported in man and animals. This more recent histological evaluation has been confirmed by the demonstration of ACTH activity associated with particulate material derived from chromophobe cells (Knutson, 1966).

It has been suggested that specific hormones may originate from specific zones within the adenohypophysis (Section II,B).

B. Control

Regulation of adenohypophyseal secretion is mediated neurally through the cerebral cortex and midbrain, chemically through circulating levels of cortisol, thyroxine, glucose, and possibly tropic hormones, and through specific releasing factors. The blood supply to the adenohypophysis is rather unusual and plays a very important role in the regulation of secretion.

1. Blood Supply

In many mammals there are two distinct portal systems supplied from the hypothalamic artery. One has its primary plexus in the hypothalamus (median eminence) and drains by the long portal veins, which lie mostly on the surface of the pituitary stalk, to the secondary plexus in the anterior lobe. The other system has its primary plexus in the region of the lower pituitary stalk and neural lobe of the neurohypophysis and drains via the short portal veins to the sinusoids of the anterior lobe.

These portal systems are the only blood supply to the pituitary in a number of species,

there being no arterial supply. The distribution of the individual secondary plexuses within the adenohypophysis is quite discrete. In fact, "the affluent blood of a single portal vessel seems to enter the sinusoids of a rather restricted zone of the pars distalis" (McCann *et al.*, 1968). It is speculated that a given tropic hormone is secreted preferentially by cells receiving blood from a specific portal vessel. If this could be proved, it would support the argument that specific zones within the adenohypophysis are responsible for secreting specific hormones and possibly account for the occurrence of individual tropic hormone deficiencies. Perfusion of the adenohypophysis following severe hemorrhage is maintained by dilation of the hypothalamic hypophyseal vascular bed (Kopaniky and Gann, 1975).

2. *Feedback Mechanisms*

Production of tropic hormones is suppressed by elevated levels of target organ hormones and possibly tropic hormone. This may be a direct suppression within the pituitary cell or may be effected at a hypothalamic level through the releasing factors.

3. *Releasing Factors*

Extracts of different portions of the hypothalamus specifically stimulate the secretion of individual tropic hormones. These hypothalamic areas are able to "sense" any need for alterations in pituitary secretion. When an increase is necessary, the releasing factor is synthesized in the neurones and passes down the axons to be liberated into one of the pituitary portal systems and carried to the adenohypophysis. Here it promotes either liberation, synthesis, or both, of the corresponding tropic hormone.

Psychic stimuli enhance hypothalamic liberation of releasing factors by pathways from the cerebral cortex. Factors inhibiting release are also present. Landau (1970) suggests that there is a peripherally produced ACTH inhibitor, and Relkin (1969) describes a similar activity, possibly pineal in origin.

4. *Adrenocorticotropin Production and Release*

Adrenocorticotropin is produced in the pituitary, from which it is liberated at need in response to CRF. The CRF may control the synthesis of mRNA. Antidiuretic hormone has a CRF-like effect, and it may increase CRF production. The production of CRF is controlled by pathways from the cerebral cortex and perhaps by circulating levels of cortisol and ACTH. Cortisol was originally thought to concentrate in the area in the midbrain where CRF originates but more recently has been shown to be more or less evenly distributed throughout the brainstem. Emotional stress is mediated through the cerebral cortex. Plasma cortisol levels remain above normal in a proportion of calves for up to 3 weeks after transport (Shaw and Nichols, 1964). A rapid decrease in plasma glucose concentration enhances ACTH liberation (James *et al.*, 1970). Infusion of cortisol into the adenohypophysis reduces ACTH release. This may be physiologically important. Hypothalamic implantation of thyroxine stimulates ACTH production in rats, but it is doubtful whether a physiological role for thyroxine can be deduced from this experimental observation. The production of ACTH is enhanced as much by frustration as by thermal stress. Jones *et al.* (1975) found 6 out of 13 fetal sheep with high ACTH levels. All 13 had high plasma corticosteroid levels, and it is possible the low ACTH levels in 7 of the 13 resulted from the high corticosteroid level.

Adrenocortical response to ACTH drops sharply after a single dose of *o,p'*-DDD and

then gradually recovers, partially at least. "Normal" output can be maintained with one dose weekly.

There is no basic change in shock, but, when hypovolemia reduces adrenal perfusion, output in response to ACTH is very much increased by even small increments in the plasma volume following fluid therapy (Mack and Egdahl, 1970). Prolonged stimulation eventually destroys the gland, therefore rendering it insensitive (Panaretto and Ferguson, 1969). In the fetus sensitivity of the zona reticularis and zona fasciculata to ACTH is minimal until just before parturition, although the zona glomerulosa is responsive and aldosterone output is increased by ACTH. In a series of papers Alvarez-Buylla and Alvarez-Buylla (1964, 1967, 1968a,b) proposed that the pituitary is merely a storage organ the function of which can be adequately taken over by a portion of salivary or adrenal gland. They also demonstrated that a conditioned reflex can be evoked in this system, and they postulated that the vagus is involved. These results await clarification and confirmation. Araujo *et al.*, (1975) suggested that the transplanted gland provides a milieu in which remnants of the pituitary will grow.

Kraicer *et al.* (1969) likened the *in vitro* release of ACTH to the action of muscle; high K^+ concentration in the incubation medium increased ACTH release but only in the presence of Ca^{2+}. Corticosterone in the incubation medium prevented the release of ACTH, confirming that the feedback works at the pituitary level.

Organophosphate insecticides reduce endogenous corticosterone synthesis and block steroidogenesis in response to ACTH (Civen and Brown, 1974). In dogs norepinephrine inhibits ACTH output in response to stress, while ACTH indirectly influences catecholamine output since this is dependent upon steroid levels, although pharmacological doses of steroids in fact suppress catecholamine secretion. Cold shock in sheep is due to the continued stimulation of the cortex by ACTH with its eventual collapse. Laychock and Rubin (1975) showed that ACTH increases synthesis of prostaglandins and suggested that these are a link in ACTH-induced steroidogenesis; calcium appears to be necessary also. Most polypeptide hormones can be found in more than one form in plasma. "Big" ACTH is found in plasma of patients with ectopic production of ACTH. In normal people with increased pituitary output the secretion is "little" (1–39) ACTH. The "intermediate" form is found in animals in which the major adrenal secretion is corticosterone, while "little" ACTH is found in those that secrete mainly cortisol (Coslovsky and Yalow, 1974).

5. Growth Hormone Production and Release

A GH-releasing factor of hypothalamic origin has been demonstrated. There is apparently a feedback mechanism, since peripheral GH-secreting tumors cause a decrease in pituitary eosinophils. MacLeod and Abad (1968) emphasized that the feedback inhibition is on the synthesis of GH rather than on the releasing factor.

The most potent stimulus to GH secretion is hypoglycemia. This is probably due to a lack of carbohydrate substrate for metabolism within the GH-regulating center (Glick, 1968) in the hypothalamus. However, Trenkle and Burroughs (1967) were unable to demonstrate an increase in plasma GH in cattle in response to insulin-induced hypoglycemia. Roth *et al.* (1964) demonstrated that absolute hypoglycemia was not essential but that a rapid fall in plasma glucose levels would stimulate GH release. Both reduction in brain norepinephrine, which probably interferes with neurohumoral transmission, and reduction in cortisol reduce GH release or reduce somatomedin activity. Fasting does not cause a rise in GH in sheep.

C. Actions

1. Adrenocorticotropin

a. Adrenal Cortex. The adrenal cortex responds to ACTH within 2–3 minutes by increasing its output of steroid hormones. This is possibly achieved by enhanced trapping of plasma cholesterol, by specific stimulation of 20-hydroxylation of cholesterol (Hall and Young, 1968) or conversion to Δ^5-pregnenolone (Ney *et al.*, 1967), or possibly by influencing cholesterol synthesis *de novo.* An increase in adrenal free fatty acids (FFA) parallels the steroid response to ACTH, and there is a similar and perhaps causal increase in lipase and decrease in triglycerides.

Ion flux through the cell and across cell membranes appears to be an important factor in steroidogenesis, and it has been suggested that ACTH may exert an important influence here, either directly or through increased levels of cAMP. The enzyme adenyl cyclase, the function of which is to produce cAMP from ATP, has been shown *in vivo* to increase very rapidly following ACTH stimulation. Complicated stimulatory and inhibitory factors have been identified in steroid synthesis, and ACTH has been said to act by increasing synthesis of a protein which blocks the inhibitory action of another protein.

Many steps in intermediary metabolism are dependent on sufficient levels of the electron donor nicotinamide adenine dinucleotide (NADH), and it appears that steroid synthesis is no exception. It has been shown that ACTH activates the enzyme glucose-6-phosphate dehydrogenase, the activity of which determines the levels of available NADH.

The cortical hyperplasia which follows chronic stimulation by ACTH involves frequent mitosis. This requires prolonged high levels of synthesis of protein and mRNA. These increases, in response to ACTH, take several hours to reach a peak but are prolonged. The relative insensitivity of the suppressed adrenal cortex to ACTH may be due to the need first to synthesize protein and nucleic acid. Conversely, the gland which has previously been stimulated and then stressed shows an immediate increase in synthesis. However, the hormonal products of this synthesis are not all released immediately; some are temporarily stored and slowly released. This results in a much more prolonged rise in plasma cortisol level, although the levels do not reach the height that would be achieved were all liberated at once (Jones and Stockham, 1966). In addition, ACTH increases the half-life of corticosteroids.

The role of ACTH in the regulation of the zona glomerulosa of the adrenal cortex was for a long time uncertain, but it has now been established that under certain conditions ACTH causes an increase in aldosterone secretion. While the sodium pool is normal or enlarged, or sodium intake is high, the response of the zona glomerulosa to ACTH is minimal. However, when sodium is depleted, cortical sensitivity is much enhanced, and ACTH then causes an increase in aldosterone secretion. In the fetal lamb, the inner adrenal cortex is insensitive until just prior to birth; however, the zona glomerulosa responds earlier (Venning *et al.*, 1962). Lee *et al.* (1968), however, suggested that in the rat this pituitary glomerulotropic effect is not due to ACTH but to some unidentified substance which stimulates the adrenal cortex indirectly. During acute stress in sheep, ACTH appears to be a more important determinant of aldosterone secretion than angiotensin (Lun *et al.*, 1975).

b. Extraadrenal Systems. Adrenocorticotropin also acts outside the adrenal, affecting skin pigmentation, peripheral lipolysis, and leukocyte formation.

The main physiological stimulus to the melanocyte is the specific melanocyte-stimulating hormone, but ACTH has a minor effect which may lead to increased pigmentation.

The lipolytic effect of ACTH on the adrenal cortex has been mentioned (Section II,C,1,a). It has been shown that this lipolysis is not confined to the adrenal cortex but is also found in the peripheral fat depots, where adenyl cyclase concentration is increased. The rather specific redistribution of fat which is seen in hyperadrenocorticism in the human being may be the result of this lipolysis. It may also contribute to the extremely fatty liver found in the dog.

Adrenocorticotropin has a direct leukocytosis-inducing effect (Massimo *et al.*, 1966), which is distinct from the effect of cortisol upon the bone marrow. It also reduces capillary permeability in inflammation and affects ovulation in the sow. Prolonged exposure to ACTH and or other pituitary hormones induces the porcine stress syndrome and sudden death with pale, soft, exudative-like myopathy (Kraeling and Rampacek, 1977).

2. Growth Hormone

It has been known for some time that GH requires a complementary agent in order to work. The recent demonstration of somatomedins brings GH into line with other hormones in that it has one or more end organs upon which to act.

Growth hormone stimulates mainly the liver to produce somatomedins, through which it has its effect on growth and metabolism in general. Somatomedins are also found in plasma, kidney, muscle, and lymph, and it has been suggested that perhaps each tissue has its own somatomedin. Hypophysectomy reduces somatomedin concentrations by as much as one-half.

Somatostatin is a hypothalmic SRF-blocking factor. It also blocks thyrotropin-releasing factor (TRF) and inhibits glucagon and to a lesser extent insulin.

a. Protein Metabolism. This has been reviewed by Daughaday *et al.* (1975), who has emphasized once more the interrelated roles of insulin and GH. In the absence of a pituitary gland, growth is retarded or even absent. This can be partially corrected by administration of GH, which causes retention of nitrogen, but the full expression of growth occurs only if adequate insulin is also present. It is thought that uptake of amino acids is enhanced by these two hormones in concert, but this would not account for all the increase in nitrogen retention, and it is possible that they stimulate other stages of protein synthesis. These may include synthesis of mRNA or enzymatic activity of the ribosomes.

Weber *et al.* (1966) showed that rats carrying transplantable GH-secreting tumors had increased levels of liver glycogen (300%), nitrogen (200%), RNA (200%), and gluconeogenic enzymes. In sheep, GH administration for 4 weeks depressed wool growth, but after treatment was stopped wool growth rose above control values for the following 20 weeks. Possibly the large amount of liver nitrogen that resulted from GH administration was "switched" to wool production (Wheatley *et al.*, 1966).

b. Carbohydrate Metabolism. Plasma glucose levels are sometimes elevated by GH. The design of the experiment, the species of test animal, and the type of GH used all influence the result of its administration and may account for the differences recorded by different authors.

Sirek *et al.* (1964) showed that GH causes a rise in plasma glucose levels in normal and

hypophysectomized but not in Houssay (hypophysectomized–pancreatectomized) dogs, indicating that insulin as well as GH is necessary to produce the rise.

Wallace and Bassett (1966) showed that administration of ovine GH to sheep for 4 weeks caused a rise in plasma insulin levels. They noted also that GH caused an increase in immunoreactive insulin in dogs. Campbell and Rastogi (1968) stated that GH causes a much greater hyperinsulinemia than do glucosteroids. Growth hormone may divert insulin from carbohydrate to protein metabolism. There is some evidence of a hypothalamic hypophyseal tropic hormone responsible for stimulating the β cells to produce insulin (Chieri *et al.*, 1976).

c. Lipid Metabolism. Gans and Butz (1964) demonstrated in dogs that GH was one of the many factors which elevated plasma cholesterol levels. It also increased the plasma–liver cholesterol pool. Cholesterol synthesis per unit weight of liver tissue was not increased, but there was an increase in liver size, which could have accounted for the increased cholesterol production.

Most workers have found that GH mobilizes FFA from peripheral fat depots, and Randle *et al.* (1963) indicated that the level of plasma FFA may be related to plasma glucose levels. Levine and Luft (1964), however, suggested that there may be a second and heretofore unseparated pituitary hormone which is responsible for the "diabetogenic" effects of GH, including FFA mobilization. Sirek *et al.* (1967), on the other hand, found that bovine GH lowered plasma FFA levels in normal, hypophysectomized, and Houssay dogs within 30 minutes of injection without any appreciable change in plasma glucose. Bassett and Wallace (1966) also noticed a decline in FFA immediately after administration of ovine GH to sheep, but this was followed later by an increase in plasma FFA. Plasma glucose levels also fell in the first hour but rose to hyperglycemic levels during the next 7 hours, and blood ketones tended to follow the same pattern.

D. Excretion

Excretory pathways for the anterior pituitary hormones have not been fully elucidated. The gonadotropins are excreted in the urine. It is probable that the other pituitary hormones are also excreted, but investigations have been contradictory. Girard and Greenwood (1968) were unable to find human GH in urine, although the kidney has been suggested as the main site of degradation of the hormone.

E. Normal Values

Differences in methods of estimation of the adenohypophyseal hormones can cause considerable variations in values. Radioimmunoassay is the method of choice for most pituitary hormones but requires specialized equipment. Bioassays are less specific and less reliable but are the only methods available for some hormones in some species. Specific values are not included since methods and therefore results are still very variable.

F. Disease

Hypo-, hyper-, and nonfunctional pituitary disease states have been recorded. Hypofunctional states commonly arise from pressure changes due to tumors which initially may have produced hyperfunction. Degenerative changes with hemorrhage and

necrosis are common (Jubb and Kennedy, 1970). In contrast to man, in whom infarction is a common cause, pituitary cachexia in domestic animals does not usually follow hypophyseal necrosis. Hyperfunctional states are frequently due to neoplasms but hyperplasia may be the source of pathologically high output. Prolonged high output due to physiological stress as in cold shock in sheep will cause acute adrenocortical failure and death.

Pituitary dwarfism, described by Siegel (1977), Scott *et al.* (1978), Allan *et al.* (1978), and Willeberg *et al.* (1975) in dogs, is often due to cystic changes, but cysts are common and not usually associated with altered pituitary function. The condition is a simple autosomal recessive in German shepherds and Carelian bear dogs, possibly due to the same progenitor. It does occur in other breeds and has been described in a cat (Hartigan and McGilligan, 1976).

1. Peripheral Levels of Tropic Hormones

Dev and Lasley (1969) found no difference in plasma GH concentration between normal and dwarfsnorter cattle. Rijnberk *et al.* (1968a) described the presence of exophthalmus-producing factor in sera and one pituitary from cases of adrenal hyperfunction.

It is postulated that in pituitary-induced adrenal hyperfunction (Cushing's Syndrome, cold shock) there would be elevated levels of ACTH. With the failure of the feedback mechanism in primary adrenocortical failure, high values of ACTH would also be anticipated, but low values would be found in cases with a primary deficit of adenohypophyseal ACTH. In man, increased ACTH-like activity has been demonstrated in cases of Cushing's syndrome associated with bronchial carcinoma. Similar assumptions may be made concerning elevation of plasma GH levels in acromegaly and lowered levels in panhypopituitarism. Allan *et al.* (1978) found plasma GH levels to be low in all of 5 cases of pituitary dwarfism in which it was assayed.

2. Alterations in Peripheral Blood Chemistry

Biochemical alterations in impaired adenohypophyseal function are mainly the result of changes in end organ function. Relatively few changes are primarily due to the alterations in tropic hormone secretion. Some of the diseases of the anterior pituitary are so rare that no biochemical investigations have been made.

Adrenocorticotropin has a number of extraadrenal effects, but it may not be possible to distinguish between these and the results of excessive corticosteroid secretion induced by ACTH. The lipolysis which ACTH is known to cause is probably the origin of the fatty liver, but glucosteroids are also involved in fat metabolism and may be responsible. Both steroids and ACTH also induce a neutrophilia.

Panhypopituitarism and pituitary dwarfism are associated with biochemical changes, which are the results of end organ failure. Plasma somatomedin concentrations were consistently less than normal in Willeberg's (1975) series but Allan *et al.* (1978) found two out of five cases in which concentrations were normal. They also found reduced adrenocortical and thyroid responses to ACTH and TSH, respectively. Acromegaly may exist more frequently than it is diagnosed, but we know nothing of the chemical changes that occur. One case of acromegaly and diabetes mellitus has been described in a dog, but separation of the causes of the biochemical changes is impossible (Groen *et al.*, 1964). Foley (1956) suggested that variations in pituitary–adrenal function, as indicated by increased insulin sensitivity, occurred in dwarf cattle.

III. HORMONES OF THE NEUROHYPOPHYSIS

Antidiuretic hormone but not oxytocin has been associated with biochemical changes in disease and the following discussion concerns ADH alone.

A. Site of Production

Antidiuretic hormone is produced in the supraoptic and possibly the paraventricular centers of the hypothalamus. It is probable that individual neurones are specific in their secretion of ADH or oxytocin.

B. Control

The neurohypophysis differs from the adenohypophysis in some of its control mechanisms. There is still neural control via the hypothalamus, although the influence of the cerebral cortex probably is less important. The mechanics of release of ADH are at present uncertain; it appears to be not simply due to change in osmolality nor in the speed of diffusion of material across the blood–brain barrier. It is probable that it is sodium concentration in cerebrospinal fluid (CSF) which is the determining factor. There is no evidence that a feedback mechanism is involved, nor has a releasing factor been demonstrated.

1. Blood Supply

Transmission of the neurohumor to the end organ is not dependent on the pituitary portal systems. The hormones are liberated directly from the axons of the hypothalamic neurones into capillaries of the posterior pituitary gland.

2. Antidiuretic Hormone Production and Release

The paraventricular and supraoptic nuclei in the hypothalamus are the source of ADH. In this area there are organelles which are responsive to fluctuations of as little as 2% in plasma crystalloid osmotic pressure. The ADH is elaborated in the neurones in these centers in response to need. Synthesis is enhanced by chronic dehydration and depressed by overhydration. The initial neurohumor may not be functional; activation may occur farther down the axon. The neurosecretory material passes down the axon bound to a protein, neurophysin, and is stored in bulbous expansions of the axon close to the basement membrane of the capillaries of the posterior pituitary. This stored material is liberated in response to an acute stimulus such as acute hemorrhage, but after an initial large and rapid response the rate of secretion soon declines. The mechanism of the dissociation of ADH from the carrier protein and its release into the capillaries is not yet clear. In the plasma the hormone is not protein bound.

Stimuli for release include alterations in blood volume and increases in intrathoracic pressure, which probably reach the hypothalamus through the baroceptors in the atrium and carotid arteries; pain and emotion also cause an increase in ADH output. Bradykinin, a polypeptide produced by autolytic or bacterial breakdown of tissue, causes an antidiuresis, possibly by stimulating release of ADH (Silva and Malnic, 1964). Pain itself may also result from the action of a similar polypeptide pain-producing factor. Angiotensin has a central effect in increasing ADH secretion (Uhlich *et al.*, 1975). There is also a postprandial fall in urine flow in sheep with an increase in ADH excretion (Brook *et al.*, 1968).

C. Actions

1. Vasopressor Action

It is suggested that some of the effect of ADH upon the kidneys is due to its direct vasopressor action upon arterioles although Johnson *et al.* (1977) demonstrated an increase in the fractional blood flow and possibly absolute flow also. However, at the usual plasma levels there appears to be no effect on the general vasculature. It requires the stimulus of severe, acute hemorrhage to elevate plasma ADH levels sufficiently to cause peripheral vasoconstriction.

2. Water Turnover

Antidiuretic hormone achieves its effect on the renal tubules by influencing the synthesis of cAMP, which has replaced ADH in some experiments. The increase in cAMP causes an increase in permeability of the tubule to water and perhaps to urea also (Lee *et al.*, 1967). Both calcium and potassium ion concentrations have been said to influence the sensitivity of the tubule to ADH, a finding which is not universally supported. Ullman and co-workers (1965) showed that tubular sensitivity to ADH was increased during an "acid tide," whereas the need to retain hydrogen ions was associated with reduced sensitivity.

Other factors influencing water transport have been suggested. Fourman and Kennedy (1966), for example, postulated that the vasopressor action of ADH alters the hemodynamics of the renal medulla, thus influencing the countercurrent mechanism. Levels of ADH too low to influence peripheral resistance have been shown to increase renal blood flow. The increase in glomerular filtration rate seen in response to ADH may result from an increase in the number of functional nephrons. Antidiuretic hormone may suppress drinking through the thirst center in the hypothalamus. The reduction in intake in response to ADH injection in some cases of diabetes insipidus is too rapid to have been achieved by other homeostatic mechanisms.

3. Electrolyte Metabolism

The influence of ADH on sodium, potassium, and chloride excretion appears to be a function of the state of hydration and alimentation of the experimental animal and of the specific response to the type of ADH used. Directly conflicting views have been obtained. For example, Brook and colleagues (1968) found that arginine vasopressin had no effect upon kaliuresis in sheep, whereas Kuhn (1967) found that it enhanced it. Kuhn (1967) also found that lysine vasopressin was not kaliuretic in sheep but was in dogs. These results, however, may not be directly comparable since there were differences in experimental design. In the normally hydrated or dehydrated dog ADH has no effect on electrolyte excretion, whereas in the water-loaded dog it causes an increase in sodium and in potassium and chloride loss. In sheep Gans (1964) found that saliuresis depended on the dose of arginine vasopressin used.

4. Steroid Secretion

The increase in secretion of adrenal steroids in response to ADH stimulation is probably not of physiological significance since it appears only at pharmacological levels of the hormone.

D. Excretion

Antidiuretic hormone is in part excreted unchanged by both kidneys and liver and in part enzymatically degraded by both these organs.

E. Disease

Syndromes of over- and underproduction of ADH have been reported in man and animals. The syndrome of inappropriate secretion of ADH is mentioned later (Section III,E,2,a). It has been associated with a wide variety of diseases, both thoracic and extrathoracic.

Diabetes insipidus is due to reduced ADH secretion in the face of chronic dehydration and hyperosmolarity of plasma. The disease may arise from the following:

1. A primary derangement of the sensing elements in the hypothalamus (not associated with obvious lesions).
2. Destruction of the hypothalamus or neurohypophysis by degenerative processes or pressure atrophy. Koestner and Capen (1967) suggested that the disease in dogs is due to diminished synthesis of the transport protein due to gradually increasing pressure on the supraoptic nucleus by the growing tumor. Sudden and total loss of ADH production is rare; the rate at which the syndrome progresses reflects the rate of growth of the underlying neoplasm. Rogers *et al.* (1977) described a case in a cat in which trauma had damaged the hypothalamic–hypophyseal tracks, but some residual function remained. Calcium ions inhibit the activity of ADH; hypercalcemia is often associated with polyuria. Steroids are said to have a similar effect by suppressing phosphodiesterase, which destroys AMP (Hays and Levine, 1974).
3. Insensitivity of the renal tubule to ADH. Nephrogenic diabetes insipidus has been described by Lage (1973). Diabetes insipidus arises most commonly from pressure changes due to pituitary tumors or degenerative changes in the hypothalamus. It frequently accompanies adrenocortical hyperplasia of pituitary origin and the adiposogenital syndrome.

1. Peripheral Level of ADH

There are no records, of which the author is aware, of levels of ADH in disease. One may anticipate high levels with intrathoracic neoplasms and following surgery and reduced levels in pituitary or hypothalamic disease.

2. Alterations in Peripheral Blood and Urine Chemistry

a. Inappropriate Secretion of ADH. There is hyponatremia and hypoosmality of plasma in the presence of hypertonic urine and normal adrenal steroid secretion (Breitschwerdt and Root, 1979).

b. Diabetes Insipidus. The cardinal biochemical sign in this disease is a persistently low urinary osmolality or specific gravity. Serum osmolality is elevated, but no other serum changes occur. It should be emphasized here that in some cases of pituitary failure the lack of ADH is masked by the deficiency of cortical steroids secondary to lack of ACTH. In the absence of cortisol, blood volume is so reduced that glomerular filtration reaches levels so low that water reabsorption can occur even in the absence of ADH. The glucosteroids also tend to antagonize the action of ADH, and in the absence of this

antagonism minimal levels of ADH maintain water balance. Alexander *et al.* (1975) demonstrated that the dehydration associated with diabetes insipidus causes hemolysis, possibly due to "osmotic stress."

IV. HORMONES OF THE ADRENAL CORTEX

A. Site of Production

The adrenal cortex is divided into three distinct zones: the outer zona glomerulosa, the middle zona fasciculata, and the inner zona reticularis. Aldosterone is secreted by the zona glomerulosa. The other two zones may function as a single entity producing glucosteroids and sex hormones throughout their substance (Cameron and Grant, 1967). If they have separate functions, then the fasciculata produces the glucosteroids and the reticularis the sex hormones, although the glomerulosa does produce low concentrations of glucosteroids while the fasciculata can produce aldosterone. There is also a fourth, fetal, zone, which is of no importance in the normal adult but which may be involved in adrenal hyperplasia (Craig, 1964).

B. Control

Although for convenience the glucosteroids and aldosterone are considered separately, their control is not entirely independent. Adrenocorticotropin stimulates both glucosteroid and, under special conditions, aldosterone secretion. Riegle *et al.* (1968) noted a decline in cortical sensitivity to ACTH associated with aging in goats, but Breznock and McQueen (1970) found no such deterioration in dogs.

1. Glucosteroids

a. Adrenocorticotropin. This, the major controlling factor in steroid synthesis, has been discussed earlier (Section II,C).

b. Corticosteroid Feedback. Morrow *et al.* (1967) demonstrated *in vitro* that some adrenal steroids suppress adrenal protein synthesis. This finding suggests the possibility of a negative feedback control. Louis *et al.* (1974) found that the injection of prostaglandin F2a also increases output of cortisol; the pathway is unknown.

2. Aldosterone

a. Renin–Angiotensin System. In each nephron there is a group of cells close to the afferent glomerular arteriole. This is the juxtamedullary apparatus, which is thought to be sensitive to changes in pressure and pO_2 that are due to alterations in renal perfusion. Hypovolemia and cardiac insufficiency both reduce renal perfusion and stimulate the juxtamedullary apparatus to produce renin. The hormone activates a precusor, angiotensinogen, liberating angiotensin I; this is converted to angiotensin II, which has two main functions. First, it is a most potent vasoconstrictor, and, second, it stimulates the adrenal to secrete more aldosterone. The importance of this system, at least in the sheep, was established by Blairwest *et al.* (1968), who demonstrated that a kidney was essential for sustained hypersecretion of aldosterone in hypophysectomized, sodium-depleted sheep. It

is interesting that this system is not important in the cat. Fredlund *et al.* (1977) demonstrated that the sensitivity of the glomerulosa cell *in vitro* to angiotensin was influenced by the concentration of K^+ in the substrate. Production of aldosterone was low when K^+ concentration was also low.

Low plasma sodium levels initiate aldosterone secretion in the absence of the renin–angiotensin system but only if the pituitary remains intact. This is not due to increased ACTH production. Angiotensin is a more effective stimulus to the zona glomerulosa in the sodium-depleted animal.

b. Plasma Electrolyte Levels. The zona glomerulosa responds to a decrease in plasma sodium and an increase in plasma potassium by increasing aldosterone output. This may be achieved by increasing the conversion either of cholesterol to pregnenolone or of corticosterone to aldosterone.

c. Adrenocorticotropin. The zona glomerulosa, normally unresponsive, is rendered sensitive to ACTH by sodium depletion (Section II,C).

C. Actions

The division of adrenal hormones into glucosteroids, which influence carbohydrate metabolism, and mineralocorticoids, which effect electrolyte metabolism, is artificial and erroneous. All steroids have effects on both metabolic systems which quantitatively are considerably different but qualitatively are similar. Cortisol is a typical glucosteroid, but it has significant effect on salt and water metabolism. The term "glucosteroid" is itself misleading, for both fat and protein metabolism are also subject to steroid influences. It is, however, convenient to separate the different but interrelated metabolic processes of these hormones. For further details, see Gorski and Gannon (1976).

1. Carbohydrate Metabolism

Fundamentally, the glucosteroids are insulin antagonists, although they may increase insulin secretion (Campbell and Rastogi, 1968). There are species variations in the degree of antagonism and in the manner in which it is produced. Impaired peripheral utilization and increased hepatic gluconeogenesis are two of the more important ways in which carbohydrate metabolism is altered.

Hypophysectomy in dogs causes a reduction in body glucose pool, diminished flux of glucose from the liver into the blood, and reduced uptake of glucose by peripheral tissues. Steroid administration increases the glucose pool and hepatic glucose output in these animals. However, glucosteroid excess in both hypophysectomized and normal dogs inhibits peripheral utilization, perhaps by blocking the action of insulin. This inhibition may be immediate in onset; Lecocq *et al.* (1964) found utilization to be reduced by 38% within 30 minutes of the start of a cortisol infusion in normal dogs or to be delayed, requiring some time to develop, in man (Mills, 1964).

Azuma and Eisenstein (1964) consider that this impairment in peripheral glucose utilization is more important than increased hepatic gluconeogenesis in the development of hyperglycemia in the dog. Basset *et al.* (1966) demonstrated that daily injections of cortisol caused hyperglycemia but no comparable depression of the rate of utilization once higher levels had been reached. Cortisol acts only to reduce utilization at low plasma

glucose levels. Pugh (1968) found that the synthetic steroid prednisolone acetate reduced peripheral utilization and raised plasma glucose levels in sheep.

Kitabchi *et al.* (1968) demonstrated in man that physiological levels of glucosteroids increased blood insulin levels in response to an oral glucose load, but they were unable to say whether this was a direct effect or was due to an increase in rate of intestinal absorption of glucose.

Hockaday (1965) demonstrated that an increase in blood pyruvate followed glucose and insulin infusions in adrenalectomized but not in entire cats. This can be interpreted as suggesting either that peripheral utilization is impaired after adrenalectomy or that pyruvate production from fatty acid or amino acid metabolism is increased.

The importance of impaired peripheral utilization has been stressed, but hepatic gluconeogenesis from amino acids is an important factor in the development of steroid-induced hyperglycemia.

2. Fat Metabolism

Efficient fat absorption requires adrenocortical steroids, but whether this is a direct effect on the gut mucosa is not certain.

Glucosteroids are essential for the mobilization of FFA from peripheral tissues by norepinephrine, but the mechanism is uncertain. Possibly as a result of this action, the production of ketone bodies is reduced by steroid lack. Brown fat function is maintained by glucosteroids.

3. Protein Metabolism

Glucosteroids are closely involved in protein metabolism. The are both antianabolic, i.e., they reduce synthesis, and catabolic, increasing breakdown. The ability of the liver to "trap" amino acids is increased; this reduces the amount available to the peripheral tissues. The liver channels amino acids into urea, glucose, and hepatic protein. The alimentary and urogenital tracts also accumulate nitrogen in this way, whereas the peripheral tissues lose it.

Serum proteins may also reflect the changes in metabolism. Degradation of albumin is increased and may exceed synthesis, which is also elevated. Immune globulin concentrations are initially increased due to lysis of lymphocytes but slowly fall due to suppression of lymphocyte mitosis.

There is a marked increase in hepatic RNA and in some enzyme systems, which may be increased up to 1000-fold, whereas other systems are reduced in concentration. Perhaps herein lies the main antagonism between insulin and the glucosteroids. Although insulin tends to reduce the levels of hepatic glucose-6-phosphatase, fructose-1,6-diphosphatase, and other glycogenolytic enzymes, steroids enhance their activity.

4. Inflammation and Immune Response

The prime therapeutic importance of glucosteroids is the suppression of the inflammatory and immune responses in which their action is enhanced by estrogens.

a. Capillary Permeability. Loss of fluid from the capillary is reduced by steroids.

b. Vasodilation. Glucosteroids prevent vasodilation by reducing histamine liberation, by increasing the vasoconstrictor effect of norepinephrine, and by reducing the

liberation of the potent vasodilator kinins (Cline and Helman, 1966). These are polypeptides produced from inactive kininogens during the inflammatory process.

c. Phagocytosis. Activity of the reticuloendothelial system is increased by small doses of glucocorticoids, but in excess they suppress phagocytosis (Nichol *et al.* 1965). Leukocyte diapedesis is reduced, and this may be more important than reduction of capillary permeability in combating inflammation.

d. Collagen Formation. This is an important part of the repair process which is suppressed by glucosteroids; they may also cause dissolution of formed fibrous tissue. This may be valuable under some circumstances, but it may lead to extension of local infection and prevent wound and fracture repair. Ehrlich and Hunt (1968) state that this suppression can be prevented by vitamin A.

e. Antibody Reaction. The production of antibody depends on cell division. Mitosis in lymphoid tissue is decreased and immune globulin production is reduced by excessive glucosteroids. There is a more immediate suppression of the cellular response to an antigen–antibody reaction.

5. Calcium and Phosphorus Metabolism

Calcium metabolism shows some quite marked changes in response to glucosteroids. There is a marked depression of uptake from the gut, the result of steroid inhibition of vitamin D. There is evidence also for an inhibition of parathyroid hormone (PTH) action on bone, although this may be offset by the increased production of PTH that steroids induce. This requires further study. There is also some evidence that cortisol may interfere with the hypocalcemic effect of calcitonin on bone (Thompson *et al.*, 1968). Talmage *et al.* (1970) suggest that neither calcitonin nor PTH is involved in steroid-induced bone turnover. It is claimed that some, at least, of the bone thinning associated with hypercorticoidism arises from interference with protein metabolism, that is, bone matrix production. Cortisol suppresses renal reabsorption of phosphorus, and urinary loss increases. Calcium loss is also increased.

6. Salt and Water Metabolism

Aldosterone is the hormone with the greatest effect on sodium metabolism. It stimulates the distal renal tubule to reabsorb Na^+ and to excrete K^+, H^+, NH_4^+, and Mg^{2+}. These results probably originate from an increase in synthesis of RNA and in tubular enzyme function. Cortisol and aldosterone are closely linked in electrolyte balance; for example, cortisol may enhance the kaliuresis produced by aldosterone. It may do this by causing a release of intracellular K^+. Cortisol appears to be responsible for keeping in the exchangeable pool the sodium that is retained by aldosterone activity.

The fluid retention of cardiac failure, cirrhosis, and nephrosis is usually due to secondary hyperaldosteronism together with some other, unidentified factor. It differs from the results of aldosterone administration to subjects not suffering from these conditions, in whom aldosterone has only limited effects. In "normal" subjects given aldosterone, sodium retention occurs to a limited extent only, and a natriuresis develops in the face of continued aldosterone administration. This has been called the "escape" phenomenon; its mechanics are uncertain. Hyperaldosteronism is not present in all cases of fluid retention

of cardiac, hepatic, or renal origin, and a number of other factors have to be elucidated before the full picture is understood.

However, despite their potency, the mineralocorticoids are not always effective in correcting the alteration in the distribution of water within the body that results from overall steroid deficiency. Swingle and Swingle (1967a) showed that, in adrenalectomized dogs maintained on a high-salt diet and mineralocorticoids over long periods, plasma volume is reduced to as little as 55% of the initial volume. They ascribed this change to alterations in vasoconstrictive activity of the peripheral circulation. These alterations can be corrected with glucosteroids. This hypovolemia reduces the glomerular filtration rate. Both are increased very rapidly by cortisol, too rapidly to have been brought about by salt and water retention. Water is mobilized from the intracellular to the intravascular compartment by a route that is still conjectural. Cortisol in pharmacological doses can cause so much loss of potassium in the urine that an alkalosis develops.

Glucosteroids also affect water metabolism. They increase free water clearance, that is, loss of water not associated with electrolyte excretion. They are necessary for maximal tubular impermeability to water in the absence of ADH. The increase in free water clearance has been attributed to an inhibition of ADH activity, but this theory does not receive universal support.

7. Lysosomes

At least some of the influence of glucosteroids on cellular metabolism may be stabilization of lysosomal membrane, although Cline and Helman (1966) maintain that this is pharmacological rather than physiological. The lysosomes are intracellular sacs of autolytic enzymes that escape when the cell is damaged by such conditions as endotoxin shock and ischemia.

8. Vitamin B

An interesting if somewhat unexpected interrelationship exists between nicotinamide and glucocosteroids. Greengard and colleagues (1966) demonstrated that glucosteroids would prevent the development of signs and cause remission of developed signs in dogs maintained on nicotinamide-deficient diets. This may be due to the influence of steroids on NAD in tissues.

9. Catecholamines

The interrelationship between the adrenal cortex and medulla is important. There is convincing evidence that the metabolism of catecholamines is dependent on glucosteroids. Coupland (1968) found that corticosterone, in tissue culture, increased methylation of norepinephrine, and Wurtman *et al.* (1968) suggested that this may be achieved by induction of the enzyme which transfers the methyl group. Steroids appear to be necessary for the action of catecholamines as well as for their release.

10. Bone Marrow and Blood Picture

Clinically, one of the more important effects of the glucosteroids is that on the peripheral blood picture.

a. Leukocytosis. There is an immediate increase in the total white cell count, which is more than accounted for by an increase in the neutrophil count. In man this occurs

within 5 hours of prednisolone administration (Cream, 1968). This leukocytosis is at least partly due to a reduction in diapedesis from the capillaries. Whether this accounts for all or whether some derives from mobilization from the storage pool by some as yet uncertain route is not clear.

b. Eosinopenia. The other cell most markedly affected is the eosinophil. There is a reduction in numbers, the mechanism of which is again not fully elucidated. Archer (1963) demonstrated that in the horse there was a very close relationship between the plasma histamine level and the blood eosinophil count and argued that this was cause and effect. However, other workers have been unable to demonstrate such a simple relationship in other species. Blenkinsopp and Blenkinsopp (1967) demonstrated that dexamethasone caused a migration of eosinophils into the reticuloendothelial system in cats, which accounted for the immediate decline in numbers. Long-term administration caused a suppression of production. Whatever its origin, the change in the eosinophil count tends to reflect, inversely, the circulating cortisol concentration (see Section VI,A).

c. Lymphopenia. Immediate lysis of lymphocytes follows an increase in plasma cortisol. This short-term action is further emphasized by the subsequent long-term effect of lymphoid suppression (Section IV,A,3).

d. Monocytosis and Basophilia. Monocytes and basophils do not present such a consistent pattern of change. A monocytosis is the result of high levels of circulating steroids according to Jasper and Jain (1965), while Schalm (1965) describes a basophilia accompanying hypercorticoidism.

e. Red Cell Count. A single injection of a glucosteroid causes a temporary increase in erythropoiesis with an increase in red cell count and a subsequent fall to below initial levels. Repeated injections cause a persistent elevation of red cell count. Donati and Gallagher (1968) recorded that a similar effect of ACTH is mediated through the adrenal cortex. The origin of the increase is uncertain. Donati and Gallagher (1968) suggested that this may be due to alterations in metabolism and increased oxygen requirement caused by steroids. If this is the case the increased red cell count may be mediated through erythropoietin. Adrenocortical failure in man causes anemia, but hemoconcentration due to contraction of plasma volume in the dog masks any similar change.

11. Parturition

A functional fetal adenohypophyseal system is essential for normal parturition. Interference with the function of or failure of normal development of this system results in prolonged gestation. This may result from *Veratrum* alkaloid poisoning in sheep on day 14 of pregnancy, which causes cyclopian monsters with inadequate adenohypophyseal development. Similar prolongation of pregnancy is seen in the hereditary failure of the system to develop, as is seen in Friesian and Guernsey cattle. Conversely, fetal adrenal hyperplasia is a cause of abortion in Angora goats. Multiple pregnancies are usually terminated earlier than normal, a reflection of the higher steroid levels which result from normal fetal adrenal secretion but from more than the single fetus.

As term approaches, the fetal hypothalamus receives a signal, perhaps from fetal thermoreceptors, that stimulates the anterior pituitary to release ACTH, which causes an

increase in fetal plasma cortisol. This precedes the increase in maternal plasma estrogen and is thought to induce the enzyme which synthesizes the estrogen in the cotyledons. Fairclough *et al.* (1975) showed an eight- to ten-fold increase in bovine fetal plasma levels of corticosteroids over the last 10 days in uterus, but there was no apparent change in the dam's plasma. Challis and Thorburn (1975) explained the increase in fetal cortisol to be at least partly due to an increase in cortisol-binding globulin. For further details concerning the effects of hormones upon parturition, see Nathanielz (1978).

12. Central Nervous System

Intravenous glucocorticoids in therapeutic doses decrease CSF secretion by 50%. There is no change in chemical composition. Clinically in man and in various experimental animals the effects of adrenal steroids upon the central nervous system have been described. Until we know more of the changes in psyche in dogs, it will not be possible to assess their effects in this species (Relkin, 1969). However, a recent case was described in which wool chewing was associated with adrenal hypercorticoidism (Chastain, 1978), and a gallimaufry of signs indicating neurotoxicity was recorded in a dog which ate 250 mg prednisolone followed several days later by 150 ml of a corticosteroid paste (Knecht *et al.* 1978). An intolerance of other dogs which developed during long-term therapy, abated when steroids were withdrawn (Dickerson, 1978). For further details of the effects of steroid hormones, see Leung and Munck (1975).

D. Excretion

Glucosteroids and aldosterone, together with their inactive metabolites, are excreted by both the liver and kidneys. In the plasma the active steroids are transported bound to the carrier proteins transcortin and albumin. In the liver a proportion of them is inactivated by conversion to tetrahydro derivatives and then to 17-KS, cortol, or cortolones. Both active and inactive steroids are enzymatically conjugated with glucuronic or sulfuric acid. Most of the conjugates are then excreted into the bile, but some reflux into the blood to be excreted in the urine. There is some enterohepatic recirculation of the steroids excreted in the bile.

The relative importance of each pathway varies among species and depends on the relative efficiency of hepatic excretion. This influences the amount of conjugate which refluxes into the plasma, and so determines the relative amounts of protein-bound (unconjugated) and free (conjugated) steroids in the plasma.

Renal clearance is controlled by the degree of protein binding, protein-bound steroids being retained by the glomerulus. The conjugated steroids pass readily into the glomerular filtrate. Most is excreted in the urine, but a proportion of the filtered load returns to the circulation, probably by simple backdiffusion in the distal tubule (Beisel *et al.*, 1964). Deck and Siegenthaler (1967) suggested that aldosterone glucuronide is actively excreted by the proximal kidney tubule.

E. Normal Values

It is perhaps in this field that the most important recent advances have been made. With new techniques more accurate quantitative and qualitative examinations have been made of the steroid constituents of adrenal effluent blood, peripheral blood, and urine. These

investigations have also been extended in their scope to include animals which may prove of value in research but which have been neglected until the present time (Tables I–IV).

1. Glucosteroids

Unfortunately, it is not possible to compare directly results from different groups working on the same species since different methods give different results. There is no universal agreement on the way in which the results should be presented. However, as the techniques become more specific, there should be fewer of these variations, and eventually they will be eliminated.

Cortisol and corticosterone are the steroids secreted in the highest concentration in most species. There are specific variations in the cortisol/corticosterone ratio, and there may be, as there is in the dog, very marked variations among and in individuals. A circadian rhythm has been well established in some species.

In the plasma much cortisol is normally bound to an α-globulin, transcortin, for transport, and some may be bound to albumin (Paterson and Harrison, 1968). The remainder is "free," having been conjugated in the liver with glucuronide or sulfate. Both cortisol and aldosterone are adsorbed onto the red cell membrane. The proportion so adsorbed is a function of the hematocrit, transcortin level, and steroid concentration. It is decreased in pregnancy (Bartter and Slater, 1966).

Circadian rhythm, the regular fluctuation of activity of glands over a 24-hour period, has been well established in some individuals and species, but the evidence is less clear in others. Rijnberk *et al.* (1968a) demonstrated an unequivocal rhythm in steroid secretion in six out of eight dogs, as did Campbell and Watts (1973), with a maximum at 10.00 a.m. and a minimum at 10.00 p.m. However, in some individuals this pattern was reversed, both in normal dogs and in dogs with adrenocortical hyperfunction. Halberg (1975) noted that the hemogram altered in response to biorhythms. This is, then, another factor to be considered in the interpretation of the hemogram. Holley *et al.* (1975) found circadian rhythm in sheep, and Zolovick *et al.* (1966), Hoffsis *et al.* (1970), and James *et al.* (1970) in horses.

a. Cattle. Cupps (1967) was able to recover 32% of radioactivity following intramuscular injection of labeled cortisol into adrenalectomized bulls. Of this, 86.3% was in the feces and 14.7% in the urine. Urinary radioactivity reached highest levels within 3–4 hours and disappeared by 28 hours after injection. Activity appeared in the feces at about 8 hours after injection and reached a maximum by 12–14 hours. It remained steady for about 16–18 hours and then disappeared over the next 40 hours. Unger *et al.* (1961) emphasized the problems of interfering chromogens in cattle. Plasma corticoid levels are very high in the neonate, falling rapidly over the first 24 hours and then more slowly, reaching baseline levels within 2 weeks. (See Table I.)

b. Dog The proportion of cortisol bound to protein varies with the concentration: Below 2 μg/dl, 88% is bound; at 10 μg/dl, only 56% is bound (Plager *et al.*, 1963). Gold (1961) showed that cortisol is excreted as cortone, β-cortolone, tetrahydrocortisol, and tetrahydrocortol in the ratio 1.4 : 2.0 : 2.2 : 1.2. Corticosterone is excreted in urine and feces in the ratio 5 : 2 (Taylor and Scratcherd, 1963). Urquhart and Li (1968) examined meticulously the influence of ACTH on adrenocortical secretion. (See Table II.)

TABLE I

Normal Plasma Levels of Adrenal Steroids in Cattle[a]

Adrenal steroid (μg/dl)	Reference
Total corticoids	Willet and Erb (1972)
10.5–71.5 (30.0 ± 4)	
Corticosterone	
3.0–4.9 (4.0 ± 0.3)	
Cortisol	
5.9–14.7 (9.6 ± 1.1)	
At birth, 12.1	
At 12 hours, 4.9	
Twelfth day, 1.1	
Dam at birth, 5.0	
Lactating, 0.5–2.7 (mean 1.0)	
At birth, 11.6	Hudson *et al.* (1976)
At 24 hours, 2.5	
Corticosteroids	
Mature, dry, barren, 5.84 ± 3.13	Heitzman *et al.* (1970)
50–15 days before parturition, 7.06 ± 3.67	
15–0 days before parturition, 8.01 ± 3.41	
0–15 days after parturition, 8.31 ± 5.2	
15–50 days after parturition, 5.02 ± 1.71	
90 days after parturition, 6.4 ± 2.52	
Lactating milked, 9.4 ± 0.09	Wagner and Oxenreider (1972)
Lactating suckled, 6.8 ± 0.05	
Dry 4.5 ± 0.04	
0200–1000 hours, 7.34 ± 0.53	
1000–1800 hours, 7.51 ± 0.68	
1800–0200 hours, 5.31 ± 0.40	

[a] In micrograms per deciliter; mean ±SD by the competitive protein binding technique.

c. Cat. Cortisol and corticosterone have both been identified in plasma (Taylor and Scratcherd, 1963). Rivas and Borrell (1971) found the resting level of cortisol in cats to be 1.64 μg/dl. After three daily doses of ACTH this rose to 20.52 μg/dl; after 6 days it was 26.03 μg/dl, but after 14 days the level was down to 12.34 μg/dl. (See Table III.)

Concentrations of steroid metabolites are too low in cat urine to measure by the usual techniques. Those Porter–Silber chromogens present are probably not corticosteroids (Borrell, 1963a,b). At least 50% of corticosterone metabolites are excreted by the liver within 4–5 hours; only 1% can be found in the urine (Taylor and Scratcherd, 1963). About 80% of cortisone is excreted in bile, and about 4% in urine (Taylor and Scratcherd, 1967).

d. Horse. Zolovick *et al.* (1966) have shown circadian rhythm in the horse; plasma corticoids were highest at 1000 hours, but the highest level of cortisone was found at 0200 hours. The lowest level for cortisol and corticosterone corresponded with the maximum for cortisone (0200 hours), while cortisone was at low ebb at about 1500 hours. When total combined levels were considered, maximal values were found at 1000 hours and minimum levels at 2000 hours. Moss and Rylance (1967) did not find free cortisol in horse urine, though James *et al.* (1970) did. (See Table IV.)

TABLE II

Plasma Cortisol Concentration in Normal Dogs

Time (hours)	Concentration (μg/dl)	Mean	Method[a]	References
0800	1.3–9.7	4.0	F	Rijnberk *et al.* (1968a)
1700	0.4–7.0	3.6		Rijnberk *et al.* (1968a)
0800	2.8			McManus *et al.* (1970)
1700	1.5			McManus *et al.* (1970)
	2–15	6.8	F	Halliwell *et al.* (1971)
	0.2–2.5	1.3	CPB	Halliwell *et al.* (1971)
	2.8–9.2	5.7	F	Campbell and Watts (1973)
	0.9–12.4	3.8	CPB	Schechter *et al.* (1973)
	0.3–1.3	0.7	CPB	Opitz (1974)
	2.2		F	Richkind and Edqvist (1973)
	0.16–4.2[b]		CPB	Schwartz-Porsche *et al.* (1976)
	0.5–1.7[c]			Schwartz-Porsche *et al.* (1976)
	0.9–3.9	2.3	RIA	Becker *et al.* (1976)
0900–1000	0.6–2.85		RIA	Chen *et al.* (1978)
	3.8–13.5	8.3	F	Feldman and Tyrell (1977)
	2.5–10.3		F	Lubberink (1977)

[a] Abbreviations: CPB, competitive protein binding; F, fluorimetry; RIA, radioimmunoassay.
[b] Random sampling over 24 hours.
[c] Morning samples only.

TABLE III

Plasma Corticosteroid Concentration in Normal Cats

Concentration (μg/dl)[a]	Method	Reference
1.66 ± 0.12	Fluorimetric	Rivas and Borrell (1971)
26.03 ± 9.65[b]	Fluorimetric	Rivas and Borrell (1971)

[a] Mean ±SD.
[b] After 7 daily injections of ACTH.

TABLE IV

Plasma Cortisol Concentration in Normal Horses

Time (hours)	Concentration (μg/dl)[a]	Method[b]	Reference
1000	2.70 ± 0.5	CPB	Bottoms *et al.* (1972)
2200	1.37 ± 0.4		
Morning	7.0 ± 0.5[c]	F	James *et al.* (1970)
	5.12 ± 1.67[d]	CPB	Hoffsis *et al.* (1970)

[a] Mean ±SD.
[b] CPB, competitive protein binding; F, fluorimetry.
[c] Corticosterone.
[d] Cortisol and corticosterone.

TABLE V

Plasma Cortisol Concentration in Normal Pigs

Concentration (μg/dl)[a]	Method[b]	Reference
1.4 ± 0.22[c]	CPB	Bottoms *et al.* (1972)
2.3 ± 0.74 (corticosterone)	CPB	Stith and Bottoms (1972)
15.0 ± 6.2 (piglets)	RIA	Brenner and Gurtler (1977)
9.7 ± 3.7 (dams)		
1-2	CPB	Liptrap and Raeside (1978)

[a] Mean ± SD.
[b] CPB, competitive protein binding; RIA, radioimmunoassay.
[c] Measured at 1000 hours.

e. Pig. The very high levels of plasma 17-OHCS found in newborn piglets gradually drop to "adult" levels within 12 months (Dvorak, 1967). Cortisol, cortisone, and possibly tetrahydrocorticosterone, tetrahydrocortisol, and aldosterone are present in pig urine (Tegeler and Schulke, 1967). Clark *et al.* (1965) found that urinary levels of 17-OHCS were too low to measure in the resting animal, but concentrations rose to 59 mg/24 hours after ACTH administration. (See Table V.)

f. Sheep. See Table VI.

2. Oxosteroids (Ketosteroids)

In man the excretion of 17-oxosteroids is used as a measure of androgen secretion since testosterone is excreted in this form. In the dog some small portion of testosterone may be excreted in this form, but the majority is not. There is no sex difference. Rijnberk *et al.* (1968a) reported values ranging from 0.02 to 0.24 (mean 0.06) mg/kg in 24 hours, and Adlin and Channick (1966) found 0.8–1.2 (mean 1.0) mg/24 hours. Clark *et al.* (1965) demonstrated marked qualitative and quantitative variations in 17-oxosteroid excretion in entire male and female pigs. Orchidectomized animals of both sexes have similar 17-oxosteroid excretion.

In addition to the major steroids there are numerous others which have been demon-

TABLE VI

Plasma Cortisol Concentration in Normal Sheep[a]

Animal	Concentration (μg/dl)[b]
Ewes and rams	2.0
During estrus	26.0
Spayed ewes	4.9
Castrated males	20.9
Hypophysectomized males	0.5

[a] Data from Basset *et al.* (1970); measured by the competitive protein binding method.
[b] Mean.

strated in trace concentrations in either peripheral or adrenal vein blood (Oertel and Eik-Ness, 1962). At the moment and with no evidence to the contrary, it is assumed that these traces are escaped precursors of the physiologically important end products. This is only an assumption, and it may yet be shown that these apparently insignificant compounds have some specific function of their own.

3. Mineralocorticoids

Aldosterone is probably the most potent of all the adrenal steroids. It is present in adrenal effluent blood in all species which have been investigated, but there are few data about its concentration.

a. Sheep. In the resting, sodium-replete state, sheep secrete aldosterone at 0.71 ± 0.41 μg/minute. When sheep are sodium-depleted, the rate rises to 1.7 ± 0.41 μg/minute (Coghlan *et al.*, 1966).

b. Cattle. Normal calves secrete 0.08 ± 0.03 μg/min per gland. After 14 days of negative sodium balance the rate rises to 1.06 ± 0.90 μg/min per gland (Whipp *et al.*, 1967). In calves Lopez and Phillips (1976) found neonatal plasma aldosterone concentration to be 533.6 ± 159.2 pg/ml, falling to 246.0 ± 56.9 by the eighth day.

c. Dogs. Following laparotomy to induce Goldblatt hypertension, dogs excreted 29.1 ± 7.0 μg/24 hours. One week later this figure had declined to 8.6 ± 1.1 μg/24 hours. After hypertension was established, excretion was 14.5 ± 2.7 μg/24 hours. Hechter *et al.* (1955) demonstrated a very wide range (0–236 μg/hour) in "electrocortin" secretion; adrenal venous blood concentrations ranged from 10 to 65 μg/dl.

F. Disease

Aldosterone has not yet been shown to play a major role in disease in domestic animals. In man primary and secondary aldosteronism both exist. The former, due to an aldosterone-secreting tumor, is characterized by fluid retention, hypokalemia, and an increase in exchangeable body sodium. Serum sodium levels may not be altered. Secondary aldosteronism contributes to the fluid retention of congestive heart disease, cirrhosis, and nephrosis. Primary aldosteronism has not been reported in animals. All the syndromes which in man are associated with secondary aldosteronism occur in domestic animals, but aldosterone secretion in them has not yet been evaluated. Severe sodium deficiency may develop in cattle grazing in areas of low pasture sodium. This has been demonstrated to occur on the high plains of Victoria, Australia. Similar but less marked deficiencies may be seen on pastures irrigated with low-sodium water (river water). Under these conditions urinary salt loss is minimal and the salivary Na/K ratio drops; both are indications of increased aldosterone secretion. Hypertrophy of the zona glomerulosa has been associated with salt-deficient diets, and atrophy is seen in adrenal hypofunction.

Aldosterone levels are high in scouring calves; in calves which die the level is higher immediately before death than it is in those which live (Lopez *et al.*, 1975).

Evidence of biochemical changes in endocrine disease is almost entirely confined to the dog, and unless specifically stated otherwise all the following comments concern alterations in glucosteroid metabolism in this species.

1. Plasma Cortisol Levels

The wide variations in normal resting values have made it hard to say what plasma cortisol concentrations constitute abnormal levels. However, the development of more specific tests for steroid analyses has resulted in a much narrower range of normal values. Most samples are still assayed by fluorimetry, but the isotopic methods of competitive protein binding and radioimmunoassay (RIA) are better. Problems do arise, however. Competitive protein binding measures cortisol alone. If individual animals secrete cortisol and corticosterone constantly in the same proportions (the ratio varies from 40 parts corticosterone to 1 of cortisol to 1 : 30), then both methods will give directly comparable results. If the ratio fluctuates, then the more specific RIA is probably the better choice (Tables IV–VII).

Resting plasma values are not a satisfactory diagnostic aid since many cases of Cushing's syndrome have figures within the normal range. However, stimulation of the adrenal cortex with ACTH results in plasma cortisol values well above those seen in normal dogs. Neoplasms are usually nonresponsive to homeostatic mechanisms and may not react to ACTH, in which case one or more of the other diagnostic tests can be used (See Section VI,A).

Schwartz-Porsche *et al.* (1976) were unable to detect cortisol in cases of Addison's disease (<0.05 μg/dl), and ACTH stimulation did not increase output to measurable levels. Glucosteroid levels are altered in a number of situations for which there appears to be little explanation. In cattle, starvation for 4 days is not associated with changes in cortisol levels, but the first 24 hours of refeeding are (Athanasiou and Phillips, 1978). A postnatal decline in plasma cortisol occurs in calves, but the values drop less in animals that go on to develop scours later (Hudson *et al.*, 1976). Silage feeding to cows results in a reduction of adrenocortical sensitivity, and stress-susceptible pigs have a much higher metabolic clearance rate of corticosteroid than do normal pigs.

TABLE VII

Plasma Cortisol Concentration in Dogs with Adrenal Disease

Concentration (μg/dl)	Mean	Method[a]	Time of sampling (hours)	Reference
Adrenal hyperfunction				
4–22	14 ± 5	F		Campbell and Watts (1973)
7.3–52.4	16.9	F		Halliwell *et al.* (1971)
1.9–4.8	3.5	CPB		Halliwell *et al.* (1971)
5.0–36.6		F		Lubberink (1977)
1.8		CPB	1100	McManus *et al.* (1970)
2.8			0800	McManus *et al.* (1970)
1.1–13.2	5.7	CPB		Opitz (1977)
4.5–22.2	12.3	F	0800	Rijnberk *et al.* (1968b)
4.3–24.3	11.9		1700	Rijnberk *et al.* (1968b)
2.6–12.5	5.7	CPB		Schechter *et al.* (1973)
Adrenal hypofunction				
0.32–1.48		RIA		Chen *et al.* (1978)
0.05		CPB		Schwartz-Porsche *et al.* (1976)

[a] F, fluorimetry; CPB, competitive protein binding; RIA, radioimmunoassay.

Copulation, presence of receptive sows, or aggressive boars elevates plasma corticoid levels in boars. They are elevated in bovine ketosis. Altman *et al.* (1969) reported a very high incidence of adrenal neoplasms in castrate male goats, while entire males developed none.

2. *Urinary Steroids*

Now that plasma cortisol can be measured relatively simply, the estimation of urinary steroids for diagnostic purposes, relying as it does on the collection of 24-hour samples, is obsolete except in the mepyrapone test (Section VI,B).

3. *Salt and Water Metabolism*

It is not always possible to differentiate between the effects of the pituitary and the adrenal on water metabolism. A pituitary tumor may interfere with ADH secretion as well as with control of cortisol secretion. This may be demonstrable by the ADH (vasopressin) test (Section VI,D). The possibility that the adenohypophysis produces a natriuretic hormone further complicates the picture.

In primary adrenal hyperfunction the response to exogenous ADH is much less marked. Regardless of origin, however, cases of hypercorticoidism produce urine of low specific gravity because of the increase in free water clearance which the glucosteroids induce. Some samples may be at the low end of the normal range (<1.018), whereas others are well into the diabetes insipidus range (<1.005). In adrenocortical insufficiency there is a negative water balance with dehydration, hemoconcentration, and a concentrated urine.

Alterations in plasma Na^+ and K^+ levels are of little diagnostic significance in hyperfunctional states, although, terminally, marked alterations may occur (Wilkinson *et al.*, 1965; Rijnberk *et al.*, 1968b). It is worth commenting here that plasma Na^+ levels show a very wide normal range in cattle and are therefore of little diagnostic value. Adrenalectomy causes an immediate saliuresis and rise in plasma K^+ concentration, which plateaus for several days until rising again just before death (Anderson and Clark, 1975). The hyponatremia is partly due to a shift from the extravascular pool into the tendons, cartilage, and bones. Hiatt *et al.* (1975) demonstrated a specific effect of adrenalectomy upon tissue reactions to infusions of potassium. Electrocardiogram changes developed when plasma K^+ concentration was only 2.0 mEq/liter above normal, while in control dogs these changes did not develop until the plasma K^+ level was increased by 5.6 mEq/liter.

In adrenal hypofunction plasma Na^+ and K^+ values may not be outside the normal range, but the normal Na:K ratio of 30 : 1 falls terminally to as low as 12 : 1 or less. Plasma Na^+ levels as low as 100 mEq/liter and plasma K^+ levels up to 8.0 mEq/liter have, however, been found. This level of plasma K^+ is toxic to the myocardium and produces diagnostic alterations in the electrocardiogram. Seiber (1964) described a case of pituitary hypofunction in which a high plasma K^+ level reflected the lack of ACTH, but the plasma Na^+ level was also high. Kirk (1974) described briefly 11 cases of a syndrome in horses diagnosed as adrenocortical failure secondary to prolonged treatment with steroids. None showed the elevated packed cell volume and blood urea nitrogen (BUN) seen in the dog. However, his values for plasma sodium were, with one exception, within the reference range given by Tasker (1971). Potassium values were higher, and one was given as 19.9 mEq/liter, a concentration inconsistent with life in dogs. The Na:K ratio in horses is given as 40 : 1. Low plasma bicarbonate and chloride values are also found since both these anions are lost in urine with sodium.

4. *Calcium and Magnesium Metabolism*

Alterations in calcium metabolism are said to occur in adrenal hyperfunction. The occurrence of calcinosis cutis in dogs with adrenal hyperplasia would support this, although the lesion may be a manifestation of calciphylaxis and not an alteration in overall calcium metabolism. Hypercalcemia is found regularly in experimentally adrenalectomized dogs and cats, although it is not very prominent in the spontaneous disease. Wilkinson *et al.* (1965) found 2 of 15 samples from 8 cases of adrenal hyperfunction to have plasma Ca^{2+} levels above, and 4 below, the normal range. In a cow with suspected adrenal hypofunction secondary to pituitary failure Howard *et al.* (1968) reported fluctuating plasma Ca^{2+} levels. On 2 days these reached the extraordinarily high levels of 20.8 and 15.6 mg/dl. There was also a severe hypoproteinemia at one stage of the disease. Unfortunately, no estimations of plasma cortisol, Na^+, or K^+ level were made.

Hypomagnesemia was also found in experimentally adrenalectomized dogs (Walser *et al.*, 1963), but the reverse was found in all of 12 samples from 8 cases of hyperfunction (Wilkinson *et al.*, 1965).

Osteoporosis is a common finding in Cushing's syndrome in man, but it is only rarely recorded in the dog. Its origin has not been fully elucidated, but it may be due to disturbances of calcium balance or alterations in protein metabolism.

5. *Carbohydrate Metabolism*

With few exceptions hyperglycemia is found in all cases of corticoid excess; it may progress to overt diabetes. In the dog primary adrenocortical insufficiency leads to increased insulin sensitivity, but hypoglycemia does not always follow. Hypophysectomized dogs succumb to hypoglycemia if not treated (Houssay, 1965); however, in untreated bilaterally adrenalectomized dogs the blood glucose level falls, but it does not reach hypoglycemic levels (Swingle and Swingle, 1967b). This suggests that GH is more important than glucosteroids in glucose homeostasis.

The hypoglycemia of newborn piglets cannot be explained in terms of steroid lack since high plasma steroid levels are found immediately after birth. However, these levels may well contribute to the problem by reducing peripheral utilization of glucose and thus exacerbating any existing tissue deficit. Inhibition of glucose metabolism is thought to be at least partly responsible for the dermatopathy of Cushing's syndrome.

6. *Fat Metabolism*

Accumulation of fat in the liver is a frequent if not invariable autopsy finding in untreated adrenal hyperfunction. This may be due to excess ACTH causing an excessive peripheral lipolysis with resynthesis in the liver, or it may be due to excessive glucosteroid depression of lipoprotein synthesis.

Plasma cholesterol values are altered in many endocrine diseases. Adrenal hyperfunction is, with rare exceptions, accompanied by raised plasma levels. This may reflect reduced secretion of thyroid-stimulating hormone in a gland compressed by neoplastic tissue.

7. *Protein Metabolism*

With the exception of some enzymes there is little biochemical evidence of altered protein metabolism in adrenal hyperfunction, although osteoporosis and muscular weak-

ness are attributed to the marked protein antianabolic or catabolic effect of glucosteroids. In those cases in which they have been assayed serum proteins have shown no significant change.

a. Serum Alkaline Phosphatase (SAP). This enzyme is elevated to quite remarkable levels in adrenal hyperfunction. This has been shown to be a "new" isoenzyme of both different thermolability and electrophoretic mobility from those present in normal dogs (Dorner *et al.*, 1974; Rogers, 1976; Hoffman, 1977). Occasional cases are seen in which this elevation does not occur.

b. Serum Glutamic Pyruvic Transaminase (SGPT) (Alanine Aminotransferase). Enzyme induction of glutamic pyruvic transaminase in liver tissue has been shown to result from steroid administration. The raised serum values not infrequently seen in adrenal hyperfunction or steroid administration may arise from this or from liver damage due to a specific hepatopathy (Hoe, 1956; Rogers and Ruebner, 1977).

c. Blood Urea Nitrogen. Blood urea nitrogen levels terminally exceed 100 mg/dl in most cases of adrenocortical failure; values in other conditions are not altered. This high value is due to decreased renal blood flow resulting from the decrease in plasma volume, which, experimentally, may fall to 50% of the normal value.

Duncan *et al.* (1977) described myotonia as part of the syndrome of adrenal hyperfunction in dogs. They offered no explanation, but high concentrations of plasma cortisol are known to cause extensive myopathy in skeletal but not in cardiac muscle, alter amino acid transport, and reduce the ability to convert amino acids to muscle protein. This is seen very clearly in cold shock in sheep where very high plasma concentrations of amino acids and muscle enzymes occur.

8. Peripheral Blood Picture

The effect of steroids on the blood picture is mentioned in Section IV,C,10. The "hemogram of stress" is quite distinctive. There is a well-developed leukocytosis, and the differential count reveals a neutrophilia, a lymphopenia, and usually an eosinopenia. The monocytosis and basophilia noted by Jasper and Jain (1965) and Schalm (1965), respectively, are not commonly reported by other authors. Changes in the red cell picture do not appear to be of any great significance. The decrease in plasma volume in adrenal failure is due to loss of fluid only; the hematocrit and hemoglobin level are therefore increased. A true polycythemia may or may not occur in adrenal hyperfunction.

9. Reproductive Function

Van Rensburg (1965), in reviewing adrenal function and fertility, mentioned that an enhanced plasma cortisol response to ACTH had been described in habitually aborting Angora goats, and the fetuses showed adrenal hyperplasia. He also commented that in cattle cystic ovaries had been associated with adrenal hyperplasia.

An association between the zona glomerulosa and fertility was remarked upon (Cupps *et al.*, 1964) in cattle; testicular degeneration and zona glomerulosa hypertrophy were found in 16 bulls.

Holm *et al.* (1961) investigated the prolonged gestation syndrome in Friesian cattle and demonstrated adrenal insufficiency in the fetus, and Frerking and Benten (1977) described

adrenal neoplasia and pituitary hypoplasia in 5 of 11 calves associated with prolonged gestation.

Androgen output from the adrenals in hyperfunction is variable; excessive secretion with the development of comedones is not uncommon, although less common is enlargement of the clitoris. Altman *et al.* (1969) reported a very high incidence of adrenal neoplasia in castrate male goats, while entire males developed none.

10. Immune Complex Disorders

Plechner and Shannon (1977) described a congenital disease in Doberman pinschers under this heading. It is associated with low (when compared with the reference range for all dogs) plasma cortisol levels. Normals for Dobermans were not given. The interrelationship between the endocrine system and immune status is discussed by Plechner (1979).

G. Effects of Prolonged Glucosteroid Administration

Glucosteroids are being used therapeutically more frequently and in higher doses. The peripheral blood levels produced are pharmacological and are much higher than any physiological levels. This may have severe effects upon the control of endogenous ACTH and glucosteroids. The production of CRF is depressed, and protein synthesis both in the adenohypophysis and adrenal cortex is suppressed. Higher doses and longer therapy increase the degree of suppression. This has been well established in man and is also of importance in domestic animals. It is possible to produce an easily recognizable, fully developed adrenocortical hypercorticoidism (Cushing's syndrome) by sufficiently prolonged steroid therapy. An equally serious complication is the likelihood of adrenocortical collapse occurring during or after any "stress" such as surgery in any patient who has received more than minimal steroid therapy (Foord, 1967; Kirk, 1974).

V. HORMONES OF THE ADRENAL MEDULLA

A. Site of Production

The adrenal medulla is the main site of production of the catecholamines, although, in man at least, there are small extramedullary glands which also produce epinephrine and norepinephrine. It is probable that the two hormones are produced by different cells. In cattle the medulla can be divided into outer epinephrine-rich and inner norepinephrine-rich zones (Zamora *et al.* 1967).

B. Control

1. Tropic Hormone

There appears to be no tropic hormone involved.

2. Neural Control

Control of the adrenal medulla is primarily neural. Like the cortex, the medulla is influenced by the hypothalamus, which receives stimuli from the cerebral cortex and ascending tracts in the spinal cord. Innervation to the medulla is from spinal nerves

T_{10}–T_{13}. Acetylcholine from the preganglionic neurones stimulates the cells to liberate epinephrine or norepinephrine. The manner in which one hormone rather than the other is released has not been demonstrated. *In vivo,* pain, rage, fear, and cold all enhance liberation of catecholamines.

3. Feedback Mechanisms

Norepinephrine synthesis is self-limiting in splenic nerve preparations (Stjarne *et al.* 1967). This may apply to the adrenal medulla *in vivo*. The sulfonylureas inhibit the release of catecholamines from the adrenal medulla, an effect which may be, in part, the origin of the hypoglycemia caused by this group of drugs (Hsu *et al.,* 1975). Both norepinephrine secretion and epinephrine secretion increase after hemorrhage of greater than 10% of blood volume, but the increase in epinephrine secretion is far greater than that of norepinephrine (Zileli *et al.,* 1974).

C. Actions

The actions of the hormones of the adrenal medulla, like those of the cortex, are aimed at increasing survival at times of stress. They enhance both skeletal and myocardial muscular activity, enabling the animal either to fight or to flee. They also mobilize both glucose and fatty acids, making both readily available for the production of energy. Basal metabolism is enhanced in a manner which simulates the thyroid hormone and is in fact dependent upon the presence of thyroid hormone (Therminarias *et al.,* 1975). There is an increase in hepatic heat production, which is due to the increased metabolism of lactate following glycogenolysis.

Their functions differ only quantitatively. Norepinephrine has a much greater vasoconstricting effect than epinephrine and thus elevates blood pressure to a greater extent. Increase in cardiac output, elevation of basal metabolic rate, and bronchial dilation are greater following the administration of epinephrine.

1. Glycogenolysis

Epinephrine causes hyperglycemia by rapid mobilization of glycogen. One of the enzymes necessary for this process is a phosphorylase which may be present in the inactive form. Epinephrine activates this through adenyl cyclase. Muscle phosphorylase is also activated, but muscle glycogenolysis does not contribute directly to the hyperglycemia. The enzyme necessary for the final step in glucose formation is absent, and glucose 6-phosphate is metabolized to lactate. This enters the circulation and may be converted to glucose in the liver or used by the heart for energy. Neural as well as humoral factors may be involved in hepatic glycogenolysis (see Chapter 1).

2. Lipolysis

Epinephrine and norepinephrine have roughly equivalent lipolytic actions. They cause a considerable increase in plasma FFA. This lipolysis is mediated through increased adenyl cyclase levels and therefore elevated cAMP.

3. Electrolytes

Epinephrine causes an increase in plasma Mg^{2+} (Lavor, 1968) but not plasma Ca^{2+} levels (Shim *et al.,* 1968), although plasma phosphorus levels are decreased.

4. Peripheral Blood Picture

a. Red Cells. Epinephrine causes an immediate increase in red cell count, hematocrit, and hemoglobin concentration. It has been said that this is due to expulsion of the cells from the spleen, but since the plasma protein concentration increases to the same extent as the other factors it is probably due to a shift in water from the intra- to the extravascular compartment.

b. White Cells. Epinephrine causes a neutropenia which is due to a fall in eosinophils. This eosinopenia does not occur in the absence of glucosteroids.

5. Water Metabolism

Catecholamines affect water loss by the kidney, but for maximal affect vasopressin has to be present (Schrier *et al.*, 1975).

D. Excretion

In man, epinephrine and norepinephrine are degraded to metanephrine and normetanephrine. These may be converted to vanillylmandelic acid, which is then excreted in the urine or conjugated in the liver and excreted in the bile and urine.

E. Normal Values

There is very little information about circulating levels of the medullary hormones in domestic animals or of their excretory products in urine.

F. Disease

There is no information concerning blood catecholamines or urinary excretion in cases of disease. Excessive production of catecholamines may be due to a pheochromocytoma, a neoplasm which has only recently been diagnosed antemortem (Twedt *et al.*, 1975). Roth *et al.* (1968) suggested that there is a failure in the feedback mechanism in functional tumors of the adrenal medulla. Retrospective evidence of functional pheochromocytomas was adduced by Howard and Nielsen (1965) in the dog and by Wright and Conner (1968) in cattle. The signs to be expected are intermittent hyperglycemia and glycosuria with neutropenia and an increase in plasma FFA, but Twedt *et al.* (1975) reported elevated levels of serum alkaline phosphatase (SAP), SGPT, sodium, and potassium. The significance of these changes is uncertain.

White and Cheyne (1977) described a case in which the primary pheochromocytoma had metastasized to the femur, but there were no clinical signs of a metabolic disturbance. The hyperglycemia of hemorrhage, endotoxin shock, and similar conditions are due at least in part to epinephrine release, although neural factors are also partly responsible, and the secretion of glucagon is enhanced (Jarhult *et al.*, 1975).

VI. TESTS OF FUNCTION

A. Thorn Test

The Thorn test, as originally described, measured adrenocortical response to ACTH by

the alteration in eosinophil count. Now the more usual and more accurate test involves the measurement of plasma levels of cortisol before and after ACTH stimulation.

1. Blood Eosinophil Response

Administration of ACTH by all routes has been advocated; eosinophil counts are made prior to and at 4 and 7 hours after the dose. The test has been evaluated in dogs, cattle (Pehrson and Wallin, 1966; Forenbacher, 1963), horses (Forenbacher, 1963), and pigs (Forenbacher, 1963). A decline in eosinophils of about 70% indicates normal function; a fall of 20% or less is diagnostic of adrenocortical failure. Tests giving intermediate values should be repeated. Occasionally, intramuscular ACTH is destroyed, and no response is obtained; this can be eliminated by using an intravenous preparation.

The use of eosinopenic response has two drawbacks: (1) An eosinophilia does not invariably accompany adrenocortical failure, and the test may have too low a baseline for a significant fall to be achieved. (2) The eosinophil count is not related to the plasma cortisol level when this is low.

Osbaldiston and Greve (1978) have refined the test in dogs and use the neutrophil/lymphocyte (N/L) ratio rather than the eosinophil response. A sample is taken prior to an injection of 10 units of an aqueous solution of ACTH by deep intramuscular injection. After 4 hours a second sample is taken and the N/L ratio determined by examination of the films (this may be done on as few as 40 cells). An adequate response will produce at least a 30% increase in the ratio. Only normal values were reported, but by extrapolation it is assumed that hypofunction will cause little or no change and hyperfunction will produce higher values.

a. Cattle. Pehrson and Wallin (1966) concluded that an intravenous infusion of 200 IU of ACTH would produce in normal cattle (1) a 50% decrease in circulating eosinophils, (2) a 100% increase in neutrophils, (3) an increase of at least 10% in total white cell count, and (4) an increase in blood glucose level of at least 5 mg/dl.

They based the preinjection values on the mean of two samples. They bled two or three times over 6–10 hours after the injection. If two criteria are not fulfilled, the first should be repeated. If three are missing, adrenocortical failure is likely, although failure of all four is, of course, a better indication. The test may be used to assess the adrenal component of sterility. Sybesma and Van der Veen (1962) used 80 units of a depot preparation and took a 60% decrease in eosinophils to indicate normal function.

b. Dog

i. Eosinophil response. Two samples are taken at a 30-minute interval to assess resting eosinophil levels. Either 0.1–0.5 IU ACTH per kilogram or a standard dose of 20 USP united (Siegel and Belshaw, 1968) intramuscularly is then given and blood samples collected for total eosinophil counts at 4 and 7 hours later.

ii. Plasma cortisol response. While this method gives occasionally false-negative responses in hyperfunctional states, a failure of the cortex to respond is a very accurate confirmation of adrenal hypofunction. The response may be measured by either urinary steroid excretion or plasma cortisol levels. Unfortunately, there is little uniformity in the

way the test has been performed. There has been no standardization of preparation of ACTH used, route of administration, or methods by which plasma and urinary corticosteroids are assayed. (Tables VIII and IX.)

Submaximal stimulation of adrenocortical function causes a minor response in normal dogs and an excessive response in most cases of hyperfunction. Halliwell *et al.* (1971) noted that the percent increase before and after ACTH administration depends upon the method of assay, values of >25.0 μg/dl by fluorimetry and 15 μg/dl by competitive protein binding being the minimal values expected 5 hours after ACTH stimulation in hyperfunction.

In hyperfunctional conditions the test is used to distinguish between adrenal tumor and pituitary-dependent adrenal hyperplasia. Adrenal cells which have been maximally stimulated for some time are likely to respond excessively, while most tumors are ACTH independent and the increase in response is likely to be less (but see Chastain *et al.*, 1978).

The test may be used to differentiate between primary and secondary failure. When the disease is adrenal in origin there is unlikely to be any response to exogenous ACTH, but when the condition arises from a lack of ACTH the gland will respond to exogenous ACTH. In the dog, however, where adrenocortical atrophy has been induced by prolonged medication with steroids, cortical response to ACTH may be markedly reduced (Wilson *et al.*, 1967), and in man some cases of panhypopituitarism show a similar refractoriness to ACTH stimulation (Chakmakjian *et al.*, 1968).

The availability of the synthetic β^{1-24}-corticotropin makes it possible to standardize the test. Feldman and Tyrell (1977) advocated 0.25 mg intramuscularly and bled at 60 and 90 minutes (peak at 60). While Campbell and Watts (1973) advocated the same dose but

TABLE VIII

Plasma Cortisol Response to ACTH in Normal Dogs[a]

Response (μg/dl)[b]	Method	Time	Dose and preparation of ACTH	Reference
8.0–33	F	45 minutes	0.25 mg Synacthen	Campbell and Watts (1973)
18.5–33.7 (26.2)	F	60 minutes	0.25 mg Cortrosyn	Feldman and Tyrrel (1977)
3.0–14.9 (9.7)	F	5 hours	20 units CG	Halliwell *et al.* (1971)
0.3–7.3 (2.6)	CPB			Halliwell *et al.* (1971)
13.3–26.0	F	5 hours	60–80 units CG	Halliwell *et al.* (1971)
3.3–13.7	CPB			Halliwell *et al.* (1971)
11.3–24.1		60 minutes		Lubberink (1977)
10.6–22.5	F	90 minutes	0.25 mg Cortrosyn IV	Lubberink (1977)
8.7–20.6		120 minutes		Lubberink (1977)
40.4	CPB	10 hours	20 units	McManus *et al.* (1970)
3.0		24 hours		McManus *et al.* (1970)
7.6–17.6	CPB		20–250 units Synacthen	Opitz (1977)
20.69	CPB	45 minutes	2 units/kg CG IM	Richkind and Edqvist (1973)
9.5–22.2	CPB		1 unit/0.45 kg CG IM	Schechter *et al.* (1973)
7.9–21.0 (13.2)	CPB	2 hours		Schwartz-Porsche *et al.* (1976)

[a] Abbreviations: F, fluorimetry; CPB, competitive protein binding; CG, corticotropin gel.

[b] Numbers in parentheses indicate mean.

TABLE IX

Plasma Cortisol Response to ACTH in Adrenal Disease[a]

Response (μg/dl)	Method	Time	Dose and preparation of ACTH	Reference
33.0	F	45 minutes	25 mg Synacthen	Campbell and Watts (1973)
28.4–92.0	F	5 hours	10–20 units CG	Halliwell *et al.* (1971)
17.5–30.0	CPB			Halliwell *et al.* (1971)
24.0, 30.0, 85.0	F		40 units CG	Lorenz *et al.* (1973)
25.4–46.5	F	90 minutes	0.25 mg Cortrosyn	Lubberink (1977)
40.4	CPB	10 hours	20 units	McManus *et al.* (1970)
13.3–56.0	CPB		20–25 units Synacthen	Opitz (1977)
10.0–44.0	CPB	2 hours	1 unit/0.45 kg CG	Schechter *et al.* (1973)

[a] Abbreviations: F, fluorimetry; CPB, competitive protein binding; CG, corticotropin gel.

found the peak at 45 minutes, Lubberink (1977) found that the majority of Cushing's patients peaked at 90 minutes. Its use in Addison's disease was described by Baarschers *et al.* (1975) and in normal dogs by Schwartz-Porsche *et al.* (1976).

Epinephrine is necessary in the dog for the test to function, and, although medullary tissue is usually present in the common form of adrenocortical failure, it may also have been destroyed. This presents yet another complicating factor in interpreting the results (Henry *et al.*, 1953).

B. Mepyrapone Test

The mepyrapone test is used to assess anterior pituitary function and depends on normal adrenocortical function. An intrinsic step in the production of glucosteroids is the 11-hydroxylation of cortisol precursors, more specifically 11-deoxycortisol. Mepyrapone selectively blocks this process, and the precursors accumulate and are liberated into the circulation. They do not inhibit ACTH production as cortisol does, and this leads to increased production of ACTH. This stimulates more active steroidogenesis up to the stage of 11-hydroxylation with increased excretion of androgens, which may be measured as 17-ketosteroids.

One or two 24-hour samples of urine are collected for 24–48 hours prior to the administration of 50 mg mepyrapone per pound orally, and 24-hour samples of urine are collected for 2 days afterward. This usually produces an increase over baseline values of 17-ketogenic steroids (17-KGS) of about 1.6 mg in the first and about 2.3 mg in the second 24-hour period (Siegle and Belshaw, 1968). A poor response, that is, little or no increase, usually indicates an inability of the pituitary to produce more ACTH, that is, hypopituitarism. A poor response is also given by some adrenocortical neoplasms which have escaped from ACTH control (Siegel and Belshaw, 1968).

As with many investigations, there are indications that all is not as simple as it seems. Adlin and Channick (1966) investigated the result of prolonged mepyrapone administration in dogs and showed that when there was an increase in urinary steroid excretion it was of the order of 100% (from 1.0 to 2.0 mg/24 hours) but that a number of animals showed no response. It must be emphasized, however, that they were using 17-KS estimation, not a 17-KGS method.

C. Dexamethasone Suppression Test

Output of ACTH is suppressed by dexamethasone, which does not interfere with estimation of plasma cortisol levels. In man a low dose regime (0.5 mg/kg, every 6 hours) will differentiate between normal (in which the plasma cortisol level falls) and patients with hyperfunction. A high dose regime (2.0 mg/kg, every 6 hours) will differentiate between those cases which are due to neoplasia of the pituitary or adrenal cortex (in which plasma cortisol does not fall) and those in which the high output of cortisol is due to increased adrenocortical sensitivity to ACTH or loss of sensitivity to cortisol in the hypothalamus, thus causing failure of the feedback mechanism.

The test has been refined for use in dogs (Halliwell *et al.*, 1971). Samples of blood are taken at 9.00 A.M. and 9.00 P.M., and the dog is given dexamethasone orally (0.1 mg/kg). Another sample is taken at 9.00 A.M. the following morning, and cortisol levels are assayed. Normal dogs showed a drop in levels to 5 μg/dl (fluorimetric) or less. While dogs with hyperfunction show a less marked decline, only one fell to less than 5 μg/dl. Using a different dose rate, Rijnberk *et al.* (1968b) found an occasional case due to hyperplasia which gave anomalous results, but neoplastic lesions showed no such suppression. Richkind and Edqvist (1973) showed that 0.01 mg/kg dexamethasone given intravenously caused a drop of 80% of pretreatment concentration in normal dogs.

The effects of ACTH, exercise, surgery, and hypoglycemia on plasma cortisol levels in the horse were described by James *et al.* (1970), those of ACTH and dexamethasone in cats by Rivas and Borrell (1971), mepyrapone in pigs by Stith and Bottoms (1972), ACTH and dexamethasone in pigs by Sebranek *et al.* (1973), dexamethasone and ACTH in horses by Hoffsis *et al.* (1970), hypoglycemia, vasopressin, and mepyrapone in pigs by Donald *et al.* (1968).

D. Tests of Water Turnover or Posterior Pituitary Function

1. Water Deprivation Test

This test is designed to measure the degree of urinary concentration achieved in response to dehydration and may be carried out in two ways.

a. Set Time of Deprivation. In this test the animal is allowed water and food *ad libitum* for 24 hours. The bladder is then emptied by catheterization if necessary, and the specific gravity of the urine noted. All water is withdrawn for the period of the test, usually 12–24 hours. At the end of this period the bladder is again emptied, and finally 1 hour later the patient is once more catheterized. The specific gravity of this sample is compared with that of the first. A significant increase in concentration suggests the presence of endogenous ADH and the ability of the tubule to react to it; a failure to concentrate argues for the absence of ADH or failure of the tubules to react.

b. Controlled Weight Loss. The previous test takes no account of variations in severity of disease and may subject some patients to little, but others extreme, distress. A test which depends on the loss of a fixed amount of fluid uses as its end point a factor which is physiologically related to the disease process.

Barlow and de Wardener (1959) used a loss of 3% of the body weight as the end point in human beings, and a similar end point is advised for use in veterinary medicine. A

disadvantage of this test is that it requires more frequent catheterization and weighing (usually at hourly intervals, although this depends on the rapidity with which weight is being lost). Failure to concentrate significantly when this amount of dehydration has occurred is interpreted in the same way as in the set time test. An increase in specific gravity of 0.005 or less should be considered an inadequate response.

2. *ADH (Vasopressin) Test*

The corollary to the water deprivation test is the ADH test. Failure to concentrate under the stress of water deprivation is due to a lack of endogenous ADH or to an inability of the tubules to respond. In any subject who fails to concentrate, the next step is to administer ADH. This is available in three forms: as an aqueous or oily solution and as a snuff. Either injectable form may be used in this test (dose 1.0 unit/kg body weight). If the aqueous solution is used, urine samples must be taken at hourly intervals for 4 hours after inoculation; if the oily solution is used in this test, samples should be taken at 8, 12, 16, and 24 hours. As vasopressin is a labile drug, if one dose fails to show satisfactory concentration the test should be repeated using a fresh solution or a larger dose. If there is concentration of more than 0.005, diabetes insipidus has been proven. Nephrogenic diabetes insipidus, insensitivity of the kidney to ADH, has been described (Lage, 1973).

3. *Water Loading Test*

Mulnix *et al.* (1976) modified these tests for measuring renal concentrating ability. Urinary concentration is measured by osmometry. When concentrating ability is maximal, Pitressin is injected and osmolality measured at 1 and 2 hours after the injection. Distinction between severe diabetes insipidus, on the one hand, and partial diabetes insipidus and hyperadrenocorticism, on the other, can be made with this procedure. The patient is put in a metabolism cage, and baseline values for urine production per 24 hours are determined. On the third day food is withheld but water is given *ad libitum* for 12 hours before the onset of the test. Water deprivation is started, the bladder emptied hourly, the dog weighed, and urine volume and osmolality measured. When the increase in osmolality is less than 5% between two readings, ADH is given (3 units Pitressin tannate in oil), and samples are taken 1 and 2 hours later. The response is considered to be normal when urine osmolality is greater than plasma osmolality after dehydration and urine concentration does not increase by more than 10% 1 hour after ADH administration. In dogs with hyperadrenocorticism urine osmolality was greater than plasma osmolality after dehydration and increased some 20% following ADH administration. Dogs with partial diabetes insipidus showed comparable results, but in those with severe diabetes insipidus urine concentration rose by some threefold.

Musselman (1975) described the use of a water loading test as a diagnostic aid. The bladder is emptied, and 10 ml of water per pound body weight is given by stomach tube. Dogs with normal adrenocortical function will excrete at least two-thirds of this within the next 5 hours. When function is impaired, much less is excreted.

REFERENCES

Adlin, E. V., and Channick, B. J. (1966). *Endocrinology* **78,** 511–521.
Alexander, C. S., Swaim, W. R., and Garcia, M. C. (1975). *Proc. Soc. Exp. Biol. Med.* **150,** 295–298.
Altman, N. H., Spencer Streett, C., and Tarner, J. Y. (1969). *Am. J. Vet. Res.* **30,** 583–589.

Alvarez-Buylla, R., and Alvarez-Buylla, E. R. (1964). *Acta Physiol. Lat. Am.* **14,** 245–250.
Alvarez-Buylla, R., and Alvarez-Buylla, E. R. (1967). *Acta Physiol. Lat. Am.* **17,** 253–257.
Alvarez-Buylla, R., and Alvarez-Buylla, E. R. (1968a). *Acta. Physiol. Lat. Am.* **18,** 7–12.
Alvarez-Buylla, R., and Alvarez-Buylla, E. R. (1968b). *Acta Physiol. Lat. Am.* **18,** 13–18.
Anderson, J. G., and Clark, D. R. (1975). *Am. J. Vet. Res.* **36,** 1037–1040.
Araujo, R. L., Queiroz, E. A., Saraiva, B. M., and Junqueira-Alvarenga, R. (1975). *Acta Physiol. Lat. Am.* **25,** 12–22.
Archer, R. K. (1963). "The Eosinophil Leucocyte," pp. 71–80. Blackwell, Oxford.
Athanasiou, V. N., and Phillips, R. W. (1978). *Am. J. Vet. Res.* **39,** 953–957.
Azuma, T., and Eisenstein, A. B. (1964). *Endocrinology* **75,** 521–526.
Baarschers, J. J., Hommes, U. E., and Poll, P. H. A. (1975). *Tijdschr. Diergeneeskd.* **100,** 894–896.
Barlow, E. D., and de Wardener, H. E. (1959). *Q. J. Med.* **28,** 235–258.
Bartter, F. C., and Slater, J. D. H. (1966). *J. Physiol. (London)* **184,** 29P.
Basset, J. M., and Wallace, A. L. C. (1966). *Metab., Clin. Exp.* **15,** 933–938.
Basset J. M., Mills, S. L., and Reid, R. L. (1966). *Metab., Clin. Exp.* **15,** 922–932.
Basset, J. M., Thorburn, G. D., and Wallace, A. L. C. (1970). *J. Endocrinol.* **48,** 251–263.
Becker, M. J., Helland, D., and Becker, D. N. (1976). *Am. J. Vet. Res.* **37,** 1101–1102.
Beisel, W. R., Cos, J. J., Horton, R., Chao, P. Y., and Forsham, P. H. (1964). *J. Clin. Endocrinol. Metab.* **24,** 887–893.
Blackwell, R. E., and Guillemin, R. (1973). *Annu. Rev. Physiol.* **35,** 357–390.
Blairwest, J. R., Coghlan, J. P., Denton, D. A., Goding, J. R., Wintour, M., and Wright, R. D. (1968). *Aust. J. Exp. Biol. Med. Sci.* **46,** 295–318.
Blenkinsopp, E. C., and Blenkinsopp, W. K. (1967). *J. Endocrinol.* **37,** 463–469.
Borrell, S. (1963a). *Biochem. J.* **89,** 51–53.
Borrell, S. (1963b). *Biochem. J.* **89,** 54–57.
Bottoms, G. D., Roesel, O. F., Rausch, F. D., and Akins, E. L. (1972). *Am. J. Vet. Res.* **33,** 785–790.
Breitschwerdt, E. B., and Root, C. R. (1979). *J. Am. Vet. Med. Assoc.*, **175,** 181–186.
Brenner, K. V., and Gurtler, H. (1977). *Arch. Exp. Vet. Med.* **31,** 741–747.
Breznock, E. M., and McQueen, R. D. (1970). *Am. J. Vet. Res.* **31,** 1269–1273.
Brook, A. H., Radford, H. M., and Stacy, B. D. (1968). *J. Physiol. (London)* **197,** 723–734.
Cameron, E. H. D., and Grant, J. K. (1967). *J. Endocrinol.* **37,** 413–420.
Campbell, J., and Rastogi, K. S. (1968). *Can. J. Physiol. Pharmacol.* **46,** 421–429.
Campbell, J. R., and Watts, C. (1973). *Br. Vet. J.* **129,** 134–145.
Chakmakjian, Z. H., Nelson, H., and Bethune, J. E. (1968). *J. Clin. Endocrinol. Metab.* **28,** 259–265.
Challis, J. R., and Thorburn, G. D. (1975). *Br. Med. Bull.* **31,** 57–61.
Chastain, C. B. (1978). *J. Am. Vet. Med. Assoc.* **172,** 573–574.
Chastain, C. B., Mitten, R. W., and Kluge, J. P. (1978). *J. Am. Vet. Med. Assoc.* **172,** 586–588.
Chen, C. L., Kumar, M. S. A., Williard, M. D., and Liao, T. F. (1978). *Am. J. Vet. Res.* **39,** 179–181.
Chieri, R. A., Basabe, J. C., and Farina, J. M. (1976). *Horm. Metab. Res.* **8,** 329–332.
Civen, M., and Brown, C. B. (1974). *Pestic. Biochem. Physiol.* **4,** 254–258.
Clark, A. F., Raeside, J. I., and Solomon, S. (1965). *Endocrinology* **76,** 427–433.
Cline, J. J., and Helman, K. L. (1966). *Science* **153,** 1135–1137.
Coffin, D. L., and Munson, T. D. (1953). *J. Am. Vet. Med. Assoc.* **123,** 402–408.
Coghlan, J. P., Wintour, M., and Scoggins, B. A. (1966). *Aust. J. Exp. Biol. Med. Sci.* **44,** 639–664.
Coslovsky, R., and Yalow, R. S. (1974). *Biochem. Biophys. Res. Commun.* **60,** 1351–1356.
Coupland, R. E. (1968). *J. Endocrinol.* **41,** 487–490.
Cox, R. I. (1975). *Adv. Vet. Sci. Comp. Med.* **19,** 287–305.
Craig, P. (1964). Personal communication.
Cream, J. J. (1968). *Br. J. Haematol.* **15,** 259–269.
Cupps, P. T. (1967). *J. Dairy Sci.* **50,** 992.
Cupps, P. T., Laben, R. C., Fowler, M. E., and Briggs, J. R. (1964). *J. Dairy Sci.* **4,** 433–436.
Daughaday, W. H., Herington, A. C., and Philips, L. S. (1975). *Annu. Rev. Physiol.* **37,** 211–244.
Deck, K. A., and Siegenthaler, W. E. (1967). *Acta Endocrinol. (Copenhagen)* **55,** 648–655.
Dev. V. G., and Lasley, J. F. (1969). *J. Anim. Sci.* **29,** 384–388.
Dickerson, G. W. (1978). *J. Am. Vet. Med. Assoc.* **173,** 1290.
Donald, R. A., Murphy, S. S., and Nabarro, J. D. N. (1968). *J. Endocrinol.* **41,** 509–518.

Donati, R. M., and Gallagher, N. I. (1968). *Med. Clin. North Am.* **52,** 231–241.
Dorner, J. L., Hoffmann, W. E., and Long, G. B. (1974). *Am. J. Vet. Res.* **35,** 1457–1458.
Duncan, I. D., Griffiths, I. K., and Nash, A. S. (1977). *Vet. Rec.* **100,** 30–31.
Dvorak, M. (1967). *Vet. Med. (Prague)* **12,** 43–48.
Ehrlich, H. P., and Hunt, T. K. (1968). *Ann. Surg.* **167,** 324–328.
Fairclough, R. J., Hunter, J. T., Welsh, R. A. S., and Payne, E. (1975). *J. Endocrinol.* **65,** 135–140.
Feldman, E. C., and Tyrell, J. B. (1977). *J. Am. Anim. Hosp. Assoc.* **13,** 494–499.
Foley, C. W. (1956). *J. Anim. Sci.* **15,** 1217.
Foord, H. E. (1967). *Vet. Rec.* **81,** 242.
Forenbacher, S. (1963). *Proc. World Vet. Congr., 17th, Hanover* **2,** 1209.
Fourman, J., and Kennedy, G. C. (1966). *J. Endocrinol.* **35,** 173–176.
Frerking, H., and Benten, K. V. (1977). *Dtsch. Tieraerztl. Wochenschr.* **84,** 239–240.
Fredlund, P., Saltman, S., Kondo, T., Douglas, J., and Catt, K. J. (1977). *Endocrinology* **100,** 481–488.
Gans, J. H. (1964). *Am. J. Vet. Res.* **25,** 918–922.
Gans, J. H., and Butz, R. L. (1964). *Am. J. Vet. Res.* **25,** 1695–1701.
Girard, J., and Greenwood, F. C. (1968). *J. Endocrinol.* **40,** 493–503.
Glick, S. M. (1968). *Ann. N.Y. Acad. Sci.* **148,** 471–487.
Gold, N. I. (1961). *J. Biol. Chem.* **236,** 1924–1929.
Gorski, J., and Gannon, F. (1976). *Annu. Rev. Physiol.* **38,** 425–450.
Goudie, R. B., McDonald, E., Anderson, J. R., and Gray, K. (1968). *Clin. Exp. Immunol.* **3,** 119–131.
Greengard, P., Sigg, E. B., Fratta, I., and Zak, S. B. (1966). *J. Pharmacol. Exp. Ther.* **154,** 624–631.
Groen, J. J., Frenkel, H. S., and Offerhaus, L. (1964). *Diabetes* **13,** 492–499.
Halberg, F. (1975). *Adv. Exp. Med. Biol.* **54,** 1–47.
Hall, P. F., and Young, D. G. (1968). *Endocrinology* **82,** 559–568.
Halliwell, R. E. W., Schwartzman, R. M., Hopkins, L., and McEvoy, D. (1971). *J. Small Anim. Pract.* **12,** 453–462.
Hartigan, P. J., and McGilligan, C. A. (1976). *Ir. Vet. J.* **30,** 188–189.
Hays, R. M., and Levine, S. D. (1974). *Kidney Int.* **6,** 307–322.
Hechter, O., Macchi, I. A., Korman, H., Frank, E. D., and Frank, H. A. (1955). *Am. J. Physiol.* **182,** 29–34.
Heitzman, R. J., Adams, T. C., and Hunter, G. T. (1970). *J. Endocrinol.* **47,** 133–134.
Henry, W. L., Oliver, L., and Rainey, E. R. (1953). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **12,** 66.
Hiatt, N., Katayanagi, T., and Miller, A. (1975). *Proc. Soc. Exp. Biol. Med.* **149,** 168–171.
Hockaday, T. D. R. (1965). *J. Endocrinol.* **33,** 163.
Hoe, C. M. (1956). Personal communication.
Hoffman, W. E. (1977). *J. Am. Anim. Hosp. Assoc.* **13,** 237–241.
Hoffsis, G. R., Murdock, P. W., Tharp, V. L., and Ault, K. (1970). *Am. J. Vet. Res.* **31,** 1379.
Holley, D. C., Beckman, D. A., and Evans, J. W. (1975). *J. Endocrinol.* **65,** 147–148.
Holm, L. W., Parker, H. R., and Galligan, S. J. (1961). *Am. J. Obstet. Gynecol.* **81,** 1000–1008.
Houssay, B. A. (1965). Personal communication.
Howard, E. B., and Nielsen, S. W. (1965). *J. Am. Vet. Med. Assoc.* **147,** 245–252.
Howard, J. R., Adams, W., and Sloss, M. W. (1968). *J. Am. Vet. Med. Assoc.* **152,** 17–24.
Hsu, C. Y., Brooker, G., Peach, M. J., and Westfall, T. C. (1975). *Science* **187,** 1086–1087.
Hudson, S., Mullord, M., Whittleston, W. G., and Payne, E. (1976). *Br. Vet. J.* **132,** 551–556.
James, V. H. T., Horner, M. W., Moss, M. S., and Rippon, A. E. (1970). *J. Endocrinol.* **48,** 319–335.
Jarhult, J., Hillman, J., and Mellander, S. (1975). *Acta Physiol. Scand.* **93,** 129–134.
Jasper, D. E., and Jain, N. C. (1965). *Am. J. Vet. Res.* **26,** 844–850.
Johnson, M. D., Park, C. S., and Malvin, R. L. (1977). *Am. J. Physiol.* **232,** F111–1116.
Jones, C. T., Boddy, K., Robinson, J. S., and Ratcliffe, J. G. (1975). *J. Endocrinol.* **67,** 26P.
Jones, J. T., and Stockham, M. A. (1966). *J. Physiol. (London)* **184,** 741–756.
Jubb, K. V. F., and Kennedy, P. C. (1970). "Pathology of Domestic Animals," 2nd ed., Vols. 1 and 2. Academic Press, New York.
Karlson, P., and Sekeris, C. E. (1966). *Acta Endocrinol. (Copenhagen)* **53,** 505–518.
Kirk, M. D. (1974). *Vet. Med. Small Anim. Clin.* **69,** 1383–1386.
Kitabchi, A. E., Buchanan, K. D., Vance, J. E., and Williams, R. H. (1968). *J. Clin. Endocrinol. Metab.* **27,** 1479–1486.
Knecht, C. D., Henderson, R., and Richardson, R. C. (1978). *J. Am. Vet. Med. Assoc.* **173,** 91–92.

Knutson, F. (1966). *Acta Endocrinol. (Copenhagen)* **52,** 305-318.
Koestner, A., and Capen, C. C. (1967). *Pathol. Vet.* **4,** 513-536.
Kopaniky, D. E., and Gann, D. S. (1975). *Endocrinology* **97,** 630-635.
Kraeling, R. R., and Rampacek, G. B. (1977). *J. Anim. Sci.* **45,** 71-80.
Kraicer, J., Milligan, J. N., Gosbee, J. L., Conrad, R. G., and Branson, C. M. (1969). *Endocrinology* **85,** 1144-1153.
Kuhn, E. (1967). *Arch. Int. Pharmacodyn. Ther.* **168,** 417-424.
Lage, A. (1973). *J. Am. Vet. Med. Assoc.* **163,** 251-253.
Landau, B. (1970). *J. Endocrinol.* **47,** 389-390.
Lavor, P. (1968). *Ann. Biol. Anim., Biochim., Biophys.* **8,** 461-464.
Laychock, S. G., and Rubin, R. P. (1975). *Prostaglandins* **10,** 529-540.
Lecocq, F. R., Mebane, D., and Madison, L. L. (1964). *J. Clin. Invest.* **43,** 237-240.
Lee, E., Cross, R. B., and Thornton, W. (1967). *Aust. J. Exp. Biol. Med. Sci.* **45,** P. 6.
Lee, T. C., van der Wal, B., and De Wied, D. (1968). *J. Endocrinol.* **43,** 465-475.
Leung, K., and Munck, A. (1975). *Annu. Rev. Physiol.* **37,** 245-272.
Levine, R., and Luft, R. (1964). *Diabetes* **13,** 651-655.
Liptrap, R. M., and Raeside, J. I. (1978). *J. Endocrinol.* **76,** 75-85.
Lopez, G. A., and Phillips, R. W. (1976). *Proc. Soc. Exp. Biol. Med.* **151,** 415-417.
Lopez, G. A., Phillips, R. W., and Lewis, L. D. (1975). *Am. J. Vet. Res.* **36,** 1245-1247.
Lorenz, M. D., Scott, D. W., and Pulley, L. T. (1973). *Cornell Vet.* **63,** 646-665.
Louis, T. M., Stellflug, J. N., Tucker, H. A., and Hafs, H. D. (1974). *Proc. Soc. Exp. Biol. Med.* **147,** 128-133.
Lubberink, A. A. M. E. (1977). "Diagnosis and Treatment of Canine Cushing's Syndrome." Rijksuniversiteit, Utrecht.
Lun, S., Espiner, E. A., and Hart, D. S. (1975). *N.Z. Med. J.* **82,** 238.
McCann, S. M., Dharival, A. P. S., and Porter, J. C. (1968). *Annu. Rev. Physiol.* **30,** 589-640.
Mack, E., and Egdahl, R. H. (1970). *Surg., Gynecol. Obstet.* **131,** 65-71.
MacLeod, R. M., and Abad, A. (1968). *Endocrinology* **83,** 799-806.
McManus, J. L., Nimmons, G. B., and Buchta, W. (1970). *Can. Vet. J.* **11,** 78-80.
Massimo, L., Gaiero, G., and Braito, A. (1966). *Minerva Med.* **6,** 22-24.
Mills, I. H. (1964). "Clinical Aspects of Adrenal Function," p. 49. Blackwell, Oxford.
Morrow, L. B., Burrow, G. N., and Mulrow, P. J. (1967). *Endocrinology* **80,** 883-888.
Moss, M. S., and Rylance, H. J. (1967). *J. Endocrinol.* **37,** 129-137.
Mulnix, J. A., Rijnberk, A., and Hendricks, H. J. (1976). *J. Am. Vet. Med. Assoc.* **169,** 1327-1330.
Musselman, E. E. (1975). *Vet. Med. Small Anim. Clin.* **70,** 1433-1437.
Nathanielz, P. W. (1978). *Annu. Rev. Physiol.* **40,** 411-445.
Ney, R. L., Dexter, R. L., Davis, W. W., and Garren, L. D. (1967). *J. Clin. Invest.* **46,** 1916-1924.
Nichol, T., Vernon-Roberts, B., and Quantock, D. C. (1965). *J. Endocrinol.* **33,** 365-383.
Odell, W. D. (1966). *J. Am. Med. Assoc.* **197,** 1006-1016.
Oertel, G. W., and Eik-Ness, K. B. (1962). *Endocrinology* **70,** 39-42.
Opitz, M. (1977). *Proc. Cong., World Small Anim. Vet. Assoc., 6th, Amsterdam* pp. 93-94.
Osbaldiston, G. W., and Greve, T. (1978). *Cornell Vet.* **68,** 308-318.
Panaretto, B. A., and Ferguson, K. A. (1969). *Aust. J. Agric. Res.* **70,** 115-124.
Paterson, J. Y. F., and Harrison, F. A. (1968). *J. Endocrinol.* **40,** 37-47.
Pehrson, B., and Wallin, O. (1966). *Acta Vet. Scand.* **7,** 35-46.
Peterson, R. E., and Guillemin, R. (1974). *Am. J. Med.* **57,** 591-600.
Plager, J. E., Knopp, R., Slaunwhite, R., and Sandberg, A. A. (1963). *Endocrinology* **73,** 353-358.
Plechner, A. J. (1979). *Calif. Vet.* **33,** 12-15.
Plechner, A. J., and Shannon, M. S. (1977). *Mod. Vet. Pract.* **58,** 341.
Pugh, D. M. (1968). *Br. Vet. J.* **124,** 259-265.
Randle, P. T., Garland, P. B., Hales, C. N., and Newsholme, E. A. (1963). *Lancet* **1,** 785-789.
Relkin, R. (1969). *N.Y. State J. Med.* **69,** 2133-2145.
Richkind, M., and Edqvist, L. E. (1973). *Acta Vet. Scand.* **14,** 745-757.
Riegle, G., Przekop, F., and Nellor, J. E. (1968). *J. Gerontol.* **23,** 187-190.
Rijnberk, A., der Kinderen, P. J., and Thijssen, J. H. H. (1968a). *J. Endocrinol.* **41,** 387-395.
Rijnberk, A., der Kinderen P. J., and Thijssen, J. H. H. (1968b). *J. Endocrinol.* **41,** 397-406.

Rivas, C., and Borrell, S. (1971). *J. Endocrinol.* **51,** 283–290.
Rogers, W. A. (1976). *J. Am. Vet. Med. Assoc.* **168,** 934–937.
Rogers, W. A., and Ruebner, B. H. (1977). *J. Am. Vet. Med. Assoc.* **170,** 603–606.
Rogers, W. A., Valdez, H., Anderson, B. C., and Comella, C. (1977). *J. Am. Vet. Med. Assoc.* **170,** 545–547.
Roth, J., Glick, S. M., Yalow, R. S., and Berson, S. A. (1964). *Diabetes* **13,** 355–361.
Roth, R. H., Stjarne, L., Levine, R. J., and Giarman, N. J. (1968). *J. Lab. Clin. Med.* **72,** 397–403.
Schalm, O. (1965). "Veterinary Hematology," 2nd ed., p. 440. Lea & Febiger, Philadelphia, Pennsylvania.
Schechter, R. D., Stabenfeldt, G. H., Gribble, D. G., and Ling, G. V. (1973). *J. Am. Vet. Med. Assoc.* **162,** 629–639.
Schrier, R. W., Berl, T., Harbottle, J. A., and McDonald, K. M. (1975). *Nephron* **15,** 186–196.
Schwartz-Porsche, D., Weiss, J., and Hollihn, U. (1976). *Zentralbl. Veterinaermed., Reihe A* **23,** 754–774.
Scott, D. W., Kirk, R. W., Hampshire, J., and Altzuler, N. (1978). *J. Am. Anim. Hosp. Assoc.* **14,** 183–191.
Sebranek, J. G., Marple, D. N., Cassens, R. G., Briskey, E. J., and Kastenschmidt, L. L. (1973). *J. Anim. Sci.* **36,** 41–44.
Seiber, S. E. (1964). Personal communication.
Shaw, K. G., and Nichols, R. E. (1964). *Am. J. Vet. Res.* **25,** 252–253.
Shim, S. S., Copp, D. H., and Patterson, F. P. (1968). *Can. J. Physiol. Pharmacol.* **46,** 43–45.
Siegel, E. T. (1977). "Endocrine Diseases of the Dog." Lea & Febiger, Philadelphia, Pennsylvania.
Siegel, E. T., and Belshaw, B. E. (1968). *Curr. Vet. Ther.* **3,** 545–551.
Silva, M., and Malnic, G. (1964). *J. Pharmacol. Exp. Ther.* **146,** 23–26.
Sirek, A., Schoeffling, K., Webster, M., and Sirek, O. V. (1964). *Can. J. Physiol. Pharmacol.* **42,** 299–301.
Sirek, O., Sirek, A., Przybylska, K., Doolan, H., and Niki, A. (1967). *Endocrinology* **81,** 395–397.
Solomon, I. L., and Blizzard, R. M. (1963). *J. Pediatr.* **63,** 1021–1033.
Stith, R. D., and Bottoms, G. D. (1972). *Am. J. Vet. Res.* **33,** 963–966.
Stjarne, L., Lishajko, F., and Roth, R. H. (1967). *Nature (London)* **215,** 770–772.
Swingle, W. W., and Swingle, A. J. (1967a). *Proc. Soc. Exp. Biol. Med.* **125,** 811–814.
Swingle, W. W., and Swingle, A. J. (1967b). *Endocrinology* **81,** 406–408.
Sybesma, W., and Van der Veen, H. E. (1962). *Tijdschr. Diergeneeskd.* **87,** 691–698.
Szalay, L., Bencsath, P., Takacs, L., Ponec, J., and Lichardus, B. (1975). *Experientia* **31,** 1298–1299.
Talmage, R. V., Park, H. Z., and Jee, W. (1970). *Endocrinology* **86,** 1080–1084.
Tasker, J. B. (1971). *In* "Clinical Biochemistry of Domestic Animals" (J. J. Kaneko and C. E. Cornelius, eds.), 2nd ed., Vol. 2, p. 95. Academic Press, New York.
Taylor, W., and Scratcherd, T. (1963). *Biochem. J.* **86,** 114.
Taylor, W., and Scratcherd, T. (1967). *Acta Endocrinol. (Copenhagen), Suppl.* **119,** 139.
Tegeler, G., and Schulke, B. (1967). *Arch. Exp. Vet. Med.* **21,** 777–788.
Therminarias, A., Chirpaz, M. F., and Tanche, M. (1975). *Rev. Can. Biol.* **34,** 101–109.
Thompson, J. S., Palmer, G. M. A., Eliel, L. P., and Cutler, G. A. (1968). *Endocrinology* **83,** 470–474.
Trenkle, A., and Burroughs, W. (1967). *J. Anim. Sci.* **26,** 1497.
Twedt, D. C., Tilley, L. P., Ryan, W. W., Liu, S. K., Wilkins, R. J., and Johnson, G. F. (1975). *J. Am. Anim. Hosp. Assoc.* **11,** 491–496.
Uhlich, E., Weber, P., Eigler, J., and Groschel-Stewart, U. (1975). *Klin. Wochenschr.* **53,** 177–180.
Ullman, T. D., Czazkcs, W. J., and Menczel, J. (1965). *J. Clin. Invest.* **44,** 754–764.
Unger, F., Rosenfeld, G., and Dorfman, R. I. (1961). *Am. J. Vet. Res.* **22,** 313–318.
Urquhart, J., and Li, C. C. (1968). *Am. J. Physiol.* **214,** 73–85.
Vale, W., Rivier, C., and Brown, M. (1977). *Annu. Rev. Physiol.* **39,** 473–527.
Van Rensburg, S. J. (1965). *J. S. Afr. Vet. Med. Assoc.* **36,** 491–505.
Venning, E. H., Dyrenfurth, T., Dosseter, J. B., and Beck, J. C. (1962). *Metab., Clin. Exp.* **11,** 254–264.
Wagner, W. C., and Oxenreider, S. L. (1972). *J. Anim. Sci.* **34,** 630–635.
Wallace, A. L. C., and Bassett, J. M. (1966). *Metab., Clin. Exp.* **15,** 95–97.
Walser, M., Robins, B. H., and Buckett, J. W. (1963). *J. Clin. Invest.* **42,** 456–465.
Weber, G., Singhal, R. L., Hird, H. I., and Furth, J. (1966). *Endocrinology* **79,** 865–870.
Wheatley, I. S., Wallace, A. L. C., and Bassett, J. M. (1966). *J. Endocrinol.* **35,** 341–353.
Whipp, S. C., Beary, M. E., Usenik, E. A., Weber, A. F., and Good, A. L. (1967). *Am. J. Vet. Res.* **28,** 1343–1349.
White, R. A. S., and Cheyne, I. A. (1977). *J. Small Anim. Pract.* **18,** 579–589.
Wilhelmi, A. E. (1968). *Yale J. Biol. Med.* **41,** 199–207.

Wilkinson, J. S., Medway, W., Siegel, E. T., Schwartzman, R., and Craig, P. (1965). *J. Am. Vet. Med. Assoc.* **147,** 1650.
Willeberg, P., Kastrup, K. W., and Andresen, E. (1975). *Nord. Veterinaermed.* **27,** 448–454.
Willet, L. B., and Erb, R. E. (1972). *J. Anim. Sci.* **34,** 103–111.
Wilson, R. B., Kline, K. J., Clark, T. J., Hendricks, E. C., and Grossman, M. S. (1967). *Am. J. Vet. Res.* **28,** 313–322.
Wright, B. J., and Conner, G. H. (1968). *Cancer Res.* **28,** 251–263.
Wurtman, R. J., Casper, A., Pohorecky, L. A., and Bartter, F. C. (1968). *Proc. Natl. Acad. Sci. U.S.A.* **61,** 522–528.
Zamora, C. S., Weber, A. F., and Whipp, S. C. (1967). *Am. J. Vet. Res.* **28,** 1351–1361.
Zileli, M. S., Gedik, O., Adalar, N., and Caglar, S. (1974). *Endocrinology* **95,** 1477–1481.
Zolovick, A., Upson, D. W., and Eleftheriou, B. E. (1966). *J. Endocrinol.* **35,** 249–253.

12

Thyroid Function

JIRO J. KANEKO

I. INTRODUCTION

Disorders of the thyroid gland are the most common endocrine disorders in man, and an extensive historical and scientific literature is available. In domestic animals, however, thyroid function and its disorders are less well known, but the literature is expanding at a rapid pace. Thyroid function and its diseases have become progressively more important

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

as the life span of some domestic species has increased. This is already true concerning thyroid function in the dog.

Recent advances in the understanding of thyroid physiology and in the development and refinement of methods of testing thyroid function have added impetus to the study of thyroid disease. In this chapter, the anatomy, physiology, and diseases of the thyroid will be briefly reviewed as a corollary to the understanding of thyroid function. Emphasis is placed upon an understanding of the physiological bases of a variety of thyroid function tests, most of which are now readily available to the veterinary clinician.

II. ANATOMICAL CONSIDERATIONS

The thyroid gland of animals is a bilobed structure which overlays the trachea and is located just below the larynx. Anatomical variation of the gland is quite marked among species and to a lesser extent within a given species. The isthmus connecting the two lobes of the thyroid is the region which varies most markedly among species. Man and the pig have a large, discrete isthmus which forms a pyramidal lobe connecting the two lateral lobes. The cow has a fairly wide band of glandular tissue which forms the connecting isthmus. In the horse, sheep, goat, cat, and dog, the isthmus is a narrow remnant and may even be nonexistent. The size of the lobes is increased, and the isthmus may be present in these animals under conditions of increased thyroid stimulation.

The thyroid gland is a highly vascularized tissue with a large blood flow. The glandular tissue of the thyroid is composed of follicles the lumens of which contain thick, clear fluid, the colloid. The size of the follicles and their cells varies according to the functional state of the gland. The cells can vary from the less active squamous type to the highly active tall columnar cells. Interspersed among the follicles are the thyroid "C" cells, the source of calcitonin, the hormone associated with calcium metabolism.

III. PHYSIOLOGY

The thyroid gland is unique among the endocrine glands in that an integral part of its hormone, L-thyroxine (T_4), is a micronutrient, iodine, which is available to the animal in only limited amounts. The minimal daily iodine requirement of the adult beagle dog is 140 μg (Belshaw *et al.*, 1975). This is compensated for by the presence of a very efficient trapping mechanism. Also, while most endocrine glands store only a small amount of their hormones, the thyroid manages to store quantities of its hormone that are sufficient for from one to several weeks (biological half-time) depending upon the species. The thyroid gland is also one of the larger of the endocrine glands and contains about 20% of the total body iodine. Its iodine content and size vary with iodine intake and the state of thyroid function, but it usually contains 10–40 mg iodine per 100 gm tissue.

A. Iodine Metabolism

1. Absorption and Excretion

Iodine may be absorbed into the body in a wide variety of soluble chemical forms but usually as iodides (I^-), as iodates (IO_4^-), and as the hormonal forms. Iodide may be

absorbed from any moist body surface, but its chief route of entry into the general circulation is by absorption through the small intestine. The I^- in the circulation is trapped principally by the thyroid gland, with some trapping by the salivary gland and a minimal amount by the gastric mucosa, placenta, and mammary gland. In ruminants, 70–80% of an oral dose is absorbed in the rumen, and 10% in the omasum (Barua *et al.*, 1964).

The main route of excretion of I^- is by the kidneys, through which almost all the I^- that was not trapped by the thyroid is lost in the urine. A small but significant amount is lost in the saliva, and minimal amounts are lost in the feces, sweat, and milk. In ruminants, a significant amount may be lost in the feces as well (Bustad *et al.*, 1957, 1962).

2. *Functions of the Gland*

The role of the thyroid in the synthesis and release of thyroid hormones is shown in Fig. 1, and the structures of the important thyroid hormones are shown in Fig. 2. The T_4 molecule contains 65.3% iodine. Initially, the I^- that is absorbed into the general circulation is taken up by the thyroid follicular cells by a highly efficient trapping and concentrating mechanism. It does this against a concentration gradient which can be from 20- to 500-fold across the thyroid cell membrane. It is an active transport process, thought to be enzymatic in nature, requires oxygen, and can be assumed to utilize high-energy phosphate bonds in the form of ATP (Freinkel and Ingbar, 1955; Slingerland, 1955). While I^- may enter the thyroid cell by diffusion or by deiodination of preexisting iodinated compounds within the gland, active transport is the most important concentrating mechanism. This trapping of I^- is also the basis for the use of uptake of radioactive iodine (^{131}I) as a test of thyroid function (Section IV, L). The uptake of I^- is stimulated by thyroid-stimulating hormone (TSH) and blocked by thyrotoxic agents, such as thiocyanate (SCN^-) and perchlorate (ClO_4^-), and by large amounts of I^- itself. The sites of these and other blocks in the thyroxine biosynthetic pathway are also shown in Fig. 1.

As shown in Fig. 1, the first step after trapping involves an oxidation of iodide (I^-) to

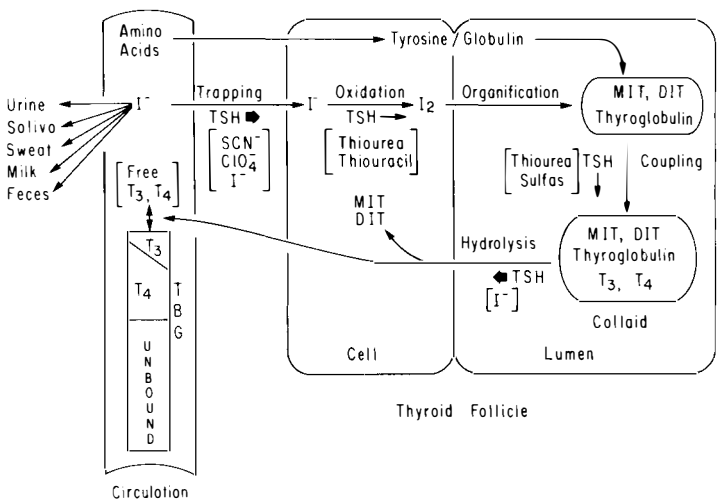


Fig. 1. Pathways of iodine metabolism and thyroid hormone synthesis. Sites, direction, and relative degree of thyroid-stimulating hormone action are shown by arrows. Goitrogenic blocking agents and their sites are shown in brackets.

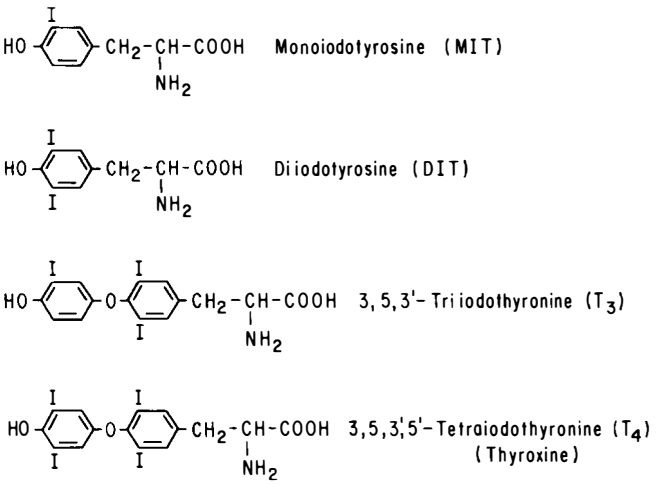


Fig. 2. Chemical structures of the major iodinated compounds of the thyroid gland.

iodine (I_2) by a peroxidase. This reaction is inhibited by thyrotoxic agents, such as thiouracil and thiourea. The I_2 next conjugates with the tyrosine moiety of thyroglobulin to form monoiodotyrosine (MIT) diiodotyrosine (DIT) (Fig. 2). This reaction is sensitive to blocking by sulfa drugs and paraaminobenzoic acid. The iodinated tyrosines are next coupled to form either L-3,5,3′-triiodothyronine (T_3) or L-3,5,3′,5′-tetraiodothyronine (T_4), the principal thyroid hormones. The latter reactions occur in the follicular lumen, where thyroglobulin acts as the acceptor protein upon which T_3 and T_4 are synthesized. Thyroglobulin is a glycoprotein which under normal conditions is found only in the thyroid follicle. The thyroid hormones bound to thyroglobulin are stored in the follicular lumen as colloid.

Another major action of TSH is to stimulate the release of thyroid hormones from the gland. It appears to activate the enzymatic hydrolysis of colloid to release its T_4, T_3, MIT, DIT, and other iodinated compounds. Both T_4 and T_3 are released into the circulation, while MIT and DIT are metabolized and their iodine is recycled within the gland. About 90% of the hormone released into the circulation is T_4, and 10% is T_3. The oxidation and coupling steps are two additional sites of TSH action (Fig. 1).

Thyroxine is transported in the plasma mainly bound to a specific plasma protein, thyroxine-binding globulin (TBG). A small amount is bound to thyroxine-binding prealbumin and to albumin. On the other hand, T_3 is bound only to TBG (Klebanoff, 1965) and less tightly than T_4. About 99.95% of the T_4 in plasma is bound to the protein, and only a small fraction, 0.05%, is present in the free or unbound state. These fractions are diagramatically shown in Fig. 1. This free L-thyroxine, T_4f, is thought to function metabolically as an active form paralleling the action of T_3, the active hormone. Since T_4 functions in cell metabolism and in TSH feedback control, measurement of T_4f would be among the definitive tests of thyroid function.

There are two principal factors of importance in the regulation of the amount of T_4f. These are the total amount of T_4 in the plasma and the amount of the binding protein, TBG. The amount of T_4f in the plasma is usually maintained by a balance of these two

factors and, in general, when T_4 increases, T_4f increases and, when TBG increases, T_4f decreases:

$$T_4f = k\frac{[T_4]}{TBG}$$

Only about one-third of the binding sites for T_4 on the TBG molecule are normally occupied, but this ratio is usually constant enough so that T_4 can be considered proportional to TBG. Thus, T_4 is also, in most instances, directly proportional to the amount of T_4f, and therefore T_4 or protein-bound iodine (PBI) also becomes a direct measure of thyroidal activity.

B. Mechanism of Thyroid Hormone Action

After the administration of T_4, its first effects are noted in 24–28 hours, and its maximal effects are noted in 7–10 days (Table I). The requirements for T_4 for these activities vary, and increased amounts are required for growth, pregnancy, and lactation. Triiodothyronine takes a shorter time and is more active, perhaps due to its low affinity for TBG. It is now thought to be the principal active form of the thyroid hormone, and T_4f acts in a manner similar to T_3. The mechanism of thyroid hormone action at the cellular level has recently been reviewed, and a multifaceted concept of its action has been suggested (Sterling, 1979).

For many years, the mitochondrion, the site of cellular energy metabolism, has been the focus of attention as the site of thyroid hormone action. Uncoupling of oxidative phosphorylation (ox-phos) has been an attractive hypothesis and has remained prevalent for many years since the first reports in the early 1950's. Under normal conditions, 3 moles of ATP (P) are synthesized per atom of oxygen ($\frac{1}{2}O_2$ = O) utilized in the cytochrome oxidase system; hence, P/O ratio = 3. If less than 3 moles of ATP are formed per unit O in a system under the influence of a compound such as the thyroid hormone, the system is said to be uncoupled; i.e., P/O ratio < 3. Thyroxine has been repeatedly shown to uncouple ox-phos in *in vitro* systems. These findings have been extended to the whole animal to explain that, in the presence of T_3 or T_4, ox-phos is uncoupled and the animal must consume increased amounts of O_2 in order to generate the equivalent amount of ATP

TABLE I

Effects of Thyroid Hormone

Category	Effect
Clinical	Tremors, nervousness, exophthalmos, hyperactivity, rapid weight loss (adult)
Physiological	Temperature increase, heart function increase
Calorigenic	Increase in basal metabolic rate (O_2 consumption)
Carbohydrate metabolism	Increased glucose turnover, absorption
Protein metabolism	Anabolic, positive N balance
Lipid metabolism	Decrease in blood cholesterol
Development	Stimulation of growth and maturation
Reproductive	Fertility, pregnancy, ovulation
Hematological	Tendency for polycythemia, anemia in absence

needed. The large amount of oxidative energy not incorporated into ATP is dissipated as heat. Thus, the increased oxidative effects and O_2 consumption promoted by thyroid hormone are theorized to be the result of the uncoupling of ox-phos.

Another well-known effect of thyroid hormone is the stimulation of cellular protein synthesis, which was originally put forth by Tata *et al.* (1963) and Tapley (1964), and this occurs during the latent period when the calorigenic effect of thyroid hormone occurs. Triiodothyronine is now known to stimulate messenger RNA (mRNA) synthesis in addition to protein synthesis. In fact, increased transcription to form mRNA is now thought to be one of the major types of action of thyroid hormone. In this way, with increased translational synthesis of protein, the anabolic effects of thyroid hormone would be the result of a generalized stimulation of protein synthesis.

Another function of thyroid hormone is the stimulation of the "sodium pump" (Na-K-ATPase) of the cell membrane with resulting increase in oxygen consumption (Edelman, 1974). Oubain, an inhibitor or Na-K-ATPase, also inhibits the increases in O_2 consumption induced by T_4 or T_3. Thus, the sodium pump appears to be another significant way in which thyroid hormones stimulate increased energy expenditure.

A direct effect of thyroid hormones on cell membrane transport has also been suggested from studies on 2-deoxyglucose uptake (Segal *et al.*, 1977). It has been proposed by Pliam and Goldfine (1977) that T_3 binds to receptor sites on the cell membrane and is transported into the cell.

A number of groups have proposed a combination of the above mechanisms to explain the mechanism of action of thyroid hormone. Sterling (1979) suggested a model patterned after the generalized steroid hormone model, in which (1) T_3 is bound to a receptor site on the plasma membrane, (2) T_3 is transported across the membrane into the cell, (3) T_3 in the cell is rapidly and reversibly bound to a carrier protein, (4) T_3 rapidly exchanges between receptor sites on the mitochondrion or on nuclear chromatin, and (5) T_3 interacts in metabolic processes.

C. Regulation of the Thyroid Gland

1. Thyroid-Stimulating Hormone

The major control of the thyroid gland is exercised by TSH secreted by the anterior pituitary gland, which in turn is mediated through the hypothalamus. The secretion of TSH is regulated by a system of feedback control based on the product of the target gland, thyroxine. The iodine levels in the blood and thyroid also affect the control mechanism. The secretion of TSH by the pituitary is dependent upon the levels of unbound or "free" thyroxine, T_4f, in the blood. By negative feedback inhibition, high levels of T_4f depress TSH, and vice versa. Although the iodine-containing thyroid hormones may act directly on the thyroid gland, their principal regulatory effect is on the pituitary and certain regions of the hypothalamus. Both structures respond to increased thyroxine levels by depressing TSH release, but the pituitary response may be the faster acting. A transmitting agent from the hypothalamus, thyrotropin-releasing factor (TRF), stimulates the pituitary to release TSH when levels of T_4f are low.

The pituitary TSH has a number of effects on the thyroid gland. The gland increases in size, the height of the follicular cell increases, and there is a loss of colloid. The response of the thyroid gland to TSH is also affected by the level of stable iodine intake. When the

level of stable iodine intake is high, the action of TSH is suppressed and the size and activity of the follicular cells are decreased. When the level of stable iodine intake is low, there is an increase in the number and size of cells and in uptake and release of iodine, which is most likely the result of an increase in TSH secretion.

2. Long-Acting Thyroid Stimulator

A second thyroid stimulatory factor has been discovered in serum which appears to be closely involved in the mechanism of thyrotoxicosis (Adams, 1958; McKenzie, 1958). It differs from TSH in that it is cleared from the blood more slowly and produces its thryoid-stimulating effects many hours (8–24) after those observed for TSH. It is therefore referred to as long-acting thyroid stimulator (LATS). Studies of LATS have been confined largely to man, in whom it has been closely correlated with hyperthyroidism (Lipman, 1967). Present knowledge of this factor with regard to its origin, nature, and role in thyroid function is meager.

IV. THYROID FUNCTION TESTS

The diagnosis of diseases of the thyroid gland in animals is very often obscured by the nonspecific nature and variety of clinical signs. Routine, rapid laboratory aids to diagnosis such as hematology or urinalysis are of limited value as adjuncts to the physical examination. Thus, the specific laboratory tests of thyroid function are of great importance in the diagnosis of thyroid disease. In recent years, improvements and refinements of standard tests and the rapid development of new tests have provided the veterinary clinician with a variety of tests. A select few are likely to become the most definitive measures of circulating thyroid hormone levels or thyroid function, and these are readily available as part of the diagnostic armamentaria for thyroid disease in animals.

A. Hematology

A moderate normocytic normochromic anemia is sometimes associated with clinical hypothyroidism in the dog. This anemia has also been observed in man and in experimental animals and is known to be of a depression type (Cline and Berlin, 1963). The stained blood smear characteristically shows little or no evidence of active erythrogenesis, such as anisocytosis, polychromasia, or nucleated red cells. Leptocytosis may be especially prominent. The hemogram is characteristic of the depression type of anemia associated with a variety of chronic diseases, such as neoplasia and chronic infection. This anemia is therefore not diagnostic of hypothyroidism, but, conversely, in cases of unexplained hypoplastic or depression anemia, hypothyroidism should be considered.

B. Basal Metabolic Rate

This test measures the O_2 consumption of the animal under rigidly standardized conditions and is the classical test of thyroid function in man. It has found little application in veterinary medicine due to the difficulty of maintaining animals under basal conditions. Even under anesthesia, basal conditions are not maintained.

C. Serum Cholesterol

The concentration of plasma or serum cholesterol is affected by the status of thyroid activity, and in general it varies inversely with the degree of activity. Thyroid hormone increases both the rate of cholesterol synthesis and the rate of its catabolism by the liver (Koppers and Palumbo, 1972). In hypothyroid states, lipid and cholesterol catabolism is decreased to a lesser extent than the synthesis of cholesterol. The effect of these changes is a relative net increase in cholesterol synthesis, resulting in an increase in serum cholesterol.

The major cholesterol carrier in human plasma is low-density lipoprotein. In the dog, low-density lipoprotein and a second lipoprotein, high-density lipoprotein, both function as cholesterol carriers (Mahley and Weisgraber, 1974).

Previously, considerable reliance was placed upon the use of serum cholesterol as an index of thyroid function because hypothyroidism is generally associated with an elevation in serum cholesterol. However, serum cholesterol elevations are frequently seen in a variety of conditions unrelated to thyroid activity. These include the nature of the diet, nephrotic syndrome, hepatic function, biliary obstruction, and diabetes mellitus. This variety of factors influencing cholesterol level limits its usefulness as a test of thyroid function. The diagnostic accuracy of serum cholesterol level for hypothyroidism in the dog is about 60%. However, when the concentrations are very high, >500 mg/dl, and diabetes mellitus is eliminated, its diagnostic accuracy is greatly increased, and this test can be of great value. To be of greatest diagnostic significance, this test should be employed in combination with other tests of thyroid function.

Conversely, it would seem reasonable that lowered serum cholesterol concentrations might be similarly employed as a test for hyperthyroid states, but the inconsistencies are such that this is not possible. On the other hand, cholesterol concentration decreases quickly and consistently in response to thyroid therapy and can be employed as a guide to proper dosage. In thyroidectomized horses with clinical evidence of hypothyroidism, Lowe *et al.*, (1974) demonstrated a 50% decrease in serum cholesterol shortly after feeding thyroprotein.

A detailed discussion of cholesterol metabolism and its association with various diseases is given in Chapter 2. Table II gives normal values of cholesterol concentration for a number of domestic animals. For the dog, 135–270 mg/dl serum or plasma is considered normal.

D. Serum Protein-Bound Iodine

A direct approach to testing thyroid function is to measure the amount of thyroid hormone in the blood. As already described, almost all of the T_4 in plasma is bound to plasma proteins, mainly TBG. Also, about 90% of the iodine bound to plasma protein is that of T_4, with the remainder being from T_3 and other iodine derivatives. Since this percentage is usually quite constant, a measure of the amount of iodine bound to protein in plasma is a direct reflection of the amount of circulating thyroid hormone. The amount of PBI varies directly with the degree of thyroid activity and is low in the hypothyroid and high in the hyperthyroid animal. Prior to modern clinical chemistry, it was the standard and most useful index of thyroid function. A more accurate measure of T_4 is a modification of PBI known as butanol-extractable iodine. This is the organic iodine which is

TABLE II

Plasma Cholesterol Concentration in Various Species[a]

Species	Total (mg %)	Free (mg %)	Ester (mg %)	Reference
Cat	95–130			Kritchevsky (1958)
	93 ± 24	30 ± 10	63 ± 23	Boyd (1942)
	98 ± 7.3			Morris and Courtice (1955)
Cow	80–120			Kritchevsky (1958)
	110 ± 32	37 ± 15	73 ± 15	Boyd (1942)
Pregnant, nonlactating	241.9			Lennon and Mixner (1957)
Lactating, nonpregnant	96.1			Lennon and Mixner (1957)
Heifers, 15–18 months	105.6			Lennon and Mixner (1957)
Calves	123			Lennon and Mixner (1957)
Dog	125–250			Kaneko (1963)
	110–135			Kritchevsky (1958)
	110 ± 28	51 ± 20	59 ± 19	Boyd (1944)
	194 ± 35			Morris and Courtice (1955)
Low-fat diet	140			Boyd and Oliver (1958)
High-fat diet	280			Boyd and Oliver (1958)
Fowl (chicken, ducks, geese)	100–200			Kritchevsky (1958)
Cockerel	100 ± 23	34 ± 9	66 ± 19	Boyd (1942)
Nonlaying hens	116 ± 2–152 ± 2			Leveille *et al.* (1957)
Laying hens	208 ± 15–285 ± 24			Leveille *et al.* (1957)
Goat	80–130			Kritchevsky (1958)
Horse	83–140			Kritchevsky (1958)
	96.8 ± 2.8	15.7	81.1	Norcia and Furman (1959)
	128 ± 12			Morris and Courtice (1955)
Rabbit	30–70			Kritchevsky (1958)
	45 ± 18	22 ± 13	23 ± 12	Boyd (1942)
Sheep	70			Boyd and Oliver (1958)
	64 ± 12			Morris and Courtice (1955)
Swine (low-fat diet)	35.6–53.7	5.7–10.9	28–48	Reiser *et al.* (1959)

[a] Modified from Carroll (1963).

soluble in butanol, is predominantly T_4, and has also been found to be a reliable index of thyroid function in man (Schultz *et al.*, 1954).

Methods for the determination of PBI are beyond the scope of this section even though the procedure has been greatly simplified. The automated chemical analysis of PBI made this a fairly common test and increased its accuracy, and it continues to be used (Bush, 1972). The determination of PBI is one of the most rigorous of quantitative chemical determinations, yet the results, particularly in animals, are often equivocal. In each step of the procedure, from the initial venipuncture to the final determination, precautions must be taken to avoid contamination with any iodine containing compounds and resultant errors. Contamination can be checked by measuring total serum iodine (SI), and, if it is greater than 25 μg/dl, the PBI should be considered unsatisfactory. A normal range for SI of 14–52 μg/dl (Taurog and Chaikoff, 1946) and 5–20 μg/dl (Siegel and Belshaw, 1968) has been reported. Prior to sampling, the history of previous medication should be ascertained so that errors from this source may be avoided. Automated chemical analyzers have largely reduced the human error in the laboratory but cannot reduce prior contamination. Iodine-containing compounds such as sodium or potassium iodide increase the PBI,

TABLE III

Some Drugs Which Affect Thyroid Function Tests

Compound	Effect on ^{131}I uptake	Effect on PBI	Effect on T_3-uptake	Duration of effect (average period)
Iodides				
Lugol's, cough syrup, vitamin preparations	Decrease	Increase	None	10–30 days
Iodine antiseptics	Decrease	Increase	None	10–30 days
Iodine-containing drugs	Decrease	Increase	None	10–30 days
X-ray contrast media				
Iodoalphionic acid (pheniodol)	Decrease	Increase	None	3–12 months
Iodopyracet (Diodrast)	Decrease	Increase	None	2–7 days
Iodized oil (*Oleum iodatum*)	Decrease	Increase	None	½–3 years
Most iv contrast media	Decrease	Increase	None	2–6 weeks
Most gallbladder media	Decrease	Increase	None	3 weeks to 3 months
Hormones				
Thyroid extract	Decrease	Increase	Increase	4–6 weeks
Triiodothyronine	Decrease	Decrease	Decrease	2–4 weeks
ACTH	Decrease	None	Increase	2 weeks
Estrogens	None	Increase	None	—
Oral contraceptives	None	Increase	None	—
Androgens	None	None	Increase	—
Thiocarbamide compounds				
Thiouracil	Decrease	Decrease	None	5–7 days
Propylthiouracil	Decrease	Decrease	None	5–7 days
Thiocyanate	Decrease	Decrease	None	14–21 days
Phenylbutazone	Decrease	None	Increase	14 days
Bromides	Decrease	None	—	10–30 days
Antihistamines	Decrease	None	—	7 days
Diphenylhydantoin (Dilantin)	None	Decrease	Increase	7–10 days
Salicylates	None	None	Increase[a]	—

[a] Effect depends on the method.

require about 10–14 days for disappearance from the blood, and are the chief sources of error in PBI determinations. The iodinated dye, Diodrast, used to visualize the urinary tract, persists for 10–20 hours, while dessicated thyroid or thyroxine given as medication requires 1–2 weeks to disappear. Some of these compounds are listed in Table III. Many cough syrups and some vitamin preparations contain iodine and also cause an increase in PBI. Care should be taken that the area selected for venipuncture is not cleansed with an iodine-containing antiseptic. All glassware with which the blood sample will come in contact should be specially cleaned and used only for PBI sampling. Disposable syringes and needles are excellent for this purpose and may be used without further cleaning. In routine practice, if iodine compounds or thyroid medication have been given, the patient should be off the medication at least 1 and preferably 2 weeks before sampling.

The normal range for man is 4.0–8.0 μg/dl serum, as compared to 1.8–4.5 μg/dl for the dog. The range of PBI in all animals (Table IV) is considerably lower than that in man. The apparent decreased dependence of the dog upon thyroid hormone has been observed by a number of authors (Danowski *et al.*, 1946; Mayer, 1947; Glock, 1949). The usefulness of PBI determination in dogs is somewhat obscured by these low normal values and the normal values which are often found in patients in which other parameters of thyroid function (including biopsy) indicated a definite hypofunction of the thyroid (Kaneko *et al.*, 1959). Extrathyroidal synthesis of iodine-containing compounds by the dog is a possible explanation for this phenomenon. The determination of PBI was of considerable value at one time but has now been supplanted by newer methods of measuring thyroid hormone (Table V).

TABLE IV

Concentration of Serum Protein-Bound Iodine in Domestic Animals

Species	PBI (μg/dl serum)[a]	Reference
Dog	2.3 (1.5–3.5)	Siegel and Belshaw (1968)
	3.4 ± 1.0 (1.5–5.1)	Mallo and Harris (1967)
	2.3 ± 0.8 (1.1–4.3)	Quinlan and Michaelson (1967)
	1.99 ± 0.24	Theran and Thornton (1966)
	2.6 ± 0.18	O'Neal and Heinbecker (1953)
	2.7 (1.8–4.5)	Kaneko (1970)
Cat	3.5 (2.5–6.0)	Kaneko (1970)
Horse, thoroughbred	1.86 ± 0.29 (1.2–2.5)	Irvine (1967b)
	2.2 ± 0.6 (1.5–3.5)	Kaneko (1964)
	(1.6–2.7)	Trum and Wasserman (1956)
Pig		
Landrace	2.7 ± 0.1	Sorenson (1962)
Large white	4.4 ± 0.2	Sorenson (1962)
Dairy cattle	(2.73–4.11)	Long *et al.* (1951)
Lactating	3.7 ± 0.3	Sorenson (1962)
Pregnant heifer	5.0 ± 0.7	Sorenson (1962)
Nonpregnant heifer	3.3 ± 0.1	Sorenson (1962)
Beef cattle	2.19	Long *et al.* (1951)
Steer	2.5 ± 0.3	Sorenson (1962)
Sheep	3.8 ± 1.0 (3.6–4.0)	Hackett *et al.* (1957)
Goat, miniature	4 (2–5)	Ragan *et al.* (1966)

[a] Values are means with standard deviations, if available. The ranges are given in parentheses.

TABLE V

Serum Thyroxine and Triiodothyronine Concentrations in Animals[a]

Species	T_4-CPB (μg/dl)	T_4-RIA (μg/dl)	T_3-RIA (ng/dl)	Reference
Dog	0.3–2.3 (1.3 ± 0.5)			Kaneko (1979)
		0.6–3.6 (2.3 ± 0.8)		Kaneko *et al.* (1978)
		0.74–4.1 (2.48 ± 0.52)	45–175 (94 ± 24)	Belshaw and Rijnberk (1979)
			82–138 (107 ± 18)	Kaneko (1979)
	1.13–4.71 (3.1 ± 1.1)			Kallfelz (1973)
Cat	0.1–2.5 (1.0 ± 0.5)			Kaneko (1979)
	Male 1.0–3.6 (1.7 ± 0.5)			Bigler (1976)
	Female 0.8–3.7 (2.1 ± 0.6)			Bigler (1976)
Horse	0.9–2.8 (1.9)			Kaneko (1979)
	1.46–3.38 (2.57 ± 0.71)			Kallfelz and Lowe (1970)
			20–130[b]	Blackmore (1979)
		(1.74)[b]	(78)	
	(2.70)			Irvine and Evans (1975)
Cow	4.2–8.6 (6.3 ± 1.0)			McCrady *et al.* (1973)
	(5.10 ± 1.30)			Kallfelz and Erali (1973)
Pig	(2.10 ± 0.42)			Kallfelz and Erali (1973)
Goat	(5.25 ± 2.08)			Kallfelz and Erali (1973)
Sheep	(6.05 ± 1.64)			Kallfelz and Erali (1973)
Monkey (*Macaca mulatta*)		(4.1 ± 0.6)	(160 ± 34)	Belchetz *et al.* (1978)

[a] Observed ranges; means ±SD in parentheses.
[b] Calculated from the author's data.

E. Thyroxine Determination by Column Chromatography

A more specific approach to the determination of total circulating T_4 is the column chromatographic procedure for extracting T_4 from the serum (Pilleggi *et al.*, 1961; Fisher *et al.*, 1965). This procedure uses an anion-exchange resin to remove T_4, T_3, other iodoamino acids, and iodides from the serum. These compounds are then eluted off the resin. Only the fractions containing T_4 and T_3 are collected, and iodine is measured in these fractions. This test is superior to the PBI test because of very much less interference by inorganic iodine or iodine-containing compounds. However, iodine-containing radiographic contrast media will interfere with the test (Kaihara *et al.*, 1969).

F. Thyroxine Determination by Competitive Protein Binding

The competitive protein binding (CPB) method for T_4 does not depend on the colorimetric determination of iodine (Murphy and Pattee, 1964). It is based on the T_4-binding properties of TBG. The procedure involves the extraction of T_4 from patient serum and the incubation of T_4 with human TBG which has been saturated with ^{125}I-T_4. The ^{125}I-T_4 on the TBG is displaced from the TBG by the patient T_4 in an amount proportional to the amount of patient T_4. The displaced TBG is counted for radioactivity, and the amount of T_4 is read directly in micrograms per deciliter from a standard curve.

The advantage of thyroxine determination by CPB (T_4-CPB) is that iodine compounds or iodides do not interfere. Interference is caused only by compounds which compete with T_4 for the binding sites. Only diphenylhydantoin and salicylates compete and interfere when given in large amounts, but in small amounts their interference is negligible (Sparagana *et al.*, 1969). These and other comparative studies (Lucis *et al.*, 1969) indicate that T_4-CPB is a reliable method for serum T_4 determination. Statistical comparisons of T_4-CPB with thyroxine determination by radioimmunoassay (T_4-RIA) indicated that there was no significant difference (Kaneko *et al.*, 1975). Also, in horses, values obtained by T_4-CPB decreased dramatically to almost zero at 2 days after thyroidectomy (Lowe *et al.*, 1974). Feeding of thyroprotein to these horses resulted in a rapid rise of T_4-CPB values to above normal levels (Table V).

G. Thyroxine Determination by Radioimmunoassay

An improved assay for T_4 by RIA is now recommended and has essentially supplanted all other methods for determination of serum T_4. The principle of RIA is described in Chapter 13. The reagents for RIA are now readily available in kit form, and the methodology is improved and simplified in comparison to the earlier cumbersome assays. Interference by iodine-contained drugs is minimal, as with T_4-CPB. Belshaw and Rijnberk (1979) reported that, other than in hypothyroidism, only diphenylhydantoin, corticosteroids, or Cushing's disease result in very low T_4-RIA or T_3-RIA values. The high degree of specificity, sensitivity, and higher accuracy within a more extended range are among the greatest advantages of T_4-RIA. In principle, T_4-RIA is similar to the CPB assay except that the binding protein is an anti-T_4 antibody. When this method is used, the mean normal T_4-RIA value in dogs is 2.3 ± 0.8 μg/dl, with an observed range of 0.6–3.6 μg/dl (Kaneko *et al.*, 1978), which is comparable to the 1.5–4.0 μg/dl cited as normal by Sims *et al.* (1977). These findings are also in keeping with the well-known observation that T_4 concentrations in dogs and all domestic animals are considerably lower than the normal 8–15 μg/dl in human beings. This observation emphasizes the need for utmost care by the clinical laboratory which is called upon to assay T_4 in animals (Table V).

H. Thyroxine Determination by Enzyme-Labeled Immunoassay

An important recent advance in thyroid testing is the development of an immunoassay test comparable in every way to radioimmunoassay except that T_4 is bound to an enzyme rather than being labeled with radioiodine. This method (T_4-EIA) has some obvious advantages: (1) There is no need for radioactive reagents and their special requirements; (2) the method is useful in any laboratory capable of assaying enzymes; and (3) it has

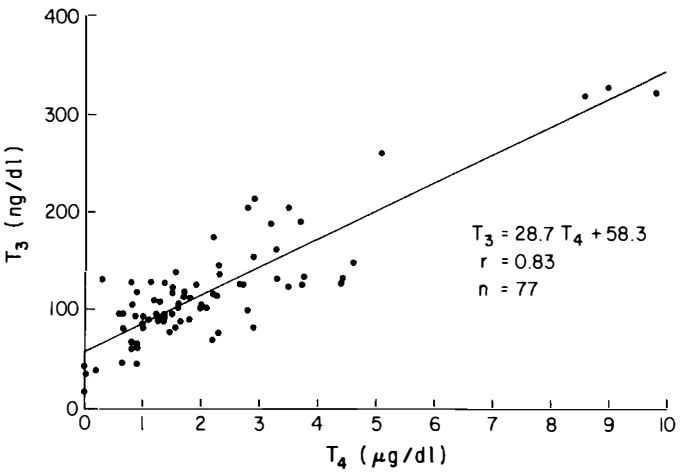


Fig. 3. Relationship between T_3-RIA and T_4-RIA values in the dog.

potential for automation. A presently available system couples malate dehydrogenase to T_4 which competitively binds to an antibody.* The activity of malate dehydrogenase is measured by standard enzyme methods.

The potential for this principle is enormous and may eventually make all RIA systems obsolete. There have been no reports of T_4-EIA assays in clinical disease of dogs, but the principle has been applied to assays for immunoglobulins using horseradish peroxidase as the enzyme label. The procedure is popularly known as the enzyme-labeled immunoadsorbent assay or the acronym ELISA.

I. Triiodothyronine Determination by Radioimmunoassay

The improvements in RIA procedures have been extended to similar assays for triiodothyronine (T_3-RIA). In the dog, the mean normal T_3-RIA value is 107 ± 18 ng/dl, with an observed range of 82–138 ng/dl (Kaneko, 1979). It has been found to parallel closely values obtained by T_4-RIA in dog sera (Fig. 3), T_3-RIA and T_4-RIA should be carried out simultaneously to improve upon the diagnostic accuracy of either one alone. Free T_3 and T_4 are thought to be the metabolically active forms of thyroid hormone at the cellular level, and the concentrations of the hormones provide the most reliable single tests of thyrometabolic status.

J. "Free" Thyroxine

"Free" thyroxine is thought to be the metabolically active fraction of the total circulating T_4. As previously described (Section III,A,2), the amount of T_4f is governed by the equilibrium between T_4f, TBG, and T_4.

The concentration of T_4f is potentially one of the most accurate parameters of thyrometabolic status. The concentration of T_4f, however, is extremely low and difficult to determine, and its determination is based, in part, upon analysis of T_4. Therefore,

*Syva Co., Palo Alto, California.

interferences can occur. The T_4f concentration for the beagle dog is 3.53 ± 0.34 ng/dl serum (Michaelson, 1969).

K. [^{131}I]Triiodothyronine Uptake

The uptake of [^{131}I]triiodothyronine (T_3-Uptake) by red blood cells (RBC) was observed and proposed by Hamolsky *et al.* (1957, 1959) as an *in vitro* test of thyroid function. This test measures the *in vitro* partition of ^{131}I-labeled T_3 between TBG of serum and a secondary binding agent, in this case, RBC. Serum TBG is the primary binding agent, and it binds T_4 more firmly than it does T_3. Therefore, when ^{131}I-T_3 is added, it is bound to any unbound TBG, and the excess is bound to RBC or other secondary binding agents such as resins. A radioactive count of the RBC (or resin) will thus be inversely proportional to the amount of unbound TBG. The uptake is therefore low in hypo- and high in hyperthyroidism. The T_3-Uptake should not be confused with the T_3-RIA described above.

There is a large group of modifications to the basic test which utilize different binding techniques or agents, and many are available in kit form. Pain and Oldfield (1969) surveyed six T_3-Uptake methods, including red cell uptake, resin uptake, resin sponge uptake, Sephadex, T_3-charcoal–hemoglobin, and thyroid binding index. They concluded that the original red cell T_3-Uptake gave overall satisfactory results, but only two of the newer methods, Sephadex and T_3-charcoal–hemoglobin, gave satisfactory results for both hyper- and hypothyroidism. On the basis of ease of performance, T_3-charcoal–hemoglobin (Irvine and Standeven, 1968) is the test of choice.

The T_3-Uptake tests have found widespread use as thyroid function tests in human beings because of their simplicity and performance *in vitro*. They are useful with contaminated sera, during pregnancy, and as an adjunct to other tests. The ranges, however, usually overlap, and the results are so poorly correlated with thyroid activity in animals as to be virtually useless. Using the T_3-Uptake by resin sponge, Kallfelz (1969) reported an uptake of 40–56% (49.75 ± 5.51%) for dogs, which is very close to the 50.6–57.9% (54.3 ± 2.0%) reported by Bush (1972). Wilson *et al.* (1961) reported T_3-Uptakes by the red cell to be about 10–30% for dogs. The T_3-Uptake by resin sponge was 46–57% (51.5 ± 2.9%) in nonpregnant mares but was not evaluated for thyroid function (Kuhns *et al.*, 1969). The thyroid binding index in horses was 0.80–1.20 (1.03 ± 0.14) (Kaneko, 1964). Thus far, there have been no reports which firmly support the value of T_3-Uptake as a valid test for thyroid disease in animals (Kallfelz and Erali, 1973).

L. Thyroidal Radioiodine Uptake

The uptake of radioiodine (^{131}I or ^{125}I) as a tracer for thyroid function is based upon the principle that the metabolic behavior of ^{131}I is inseparable from the behavior of the nonradioactive isotope of iodine, ^{127}I. As such, injected or ingested tracer amounts of ^{131}I follow the same metabolic pathway in the animal as stable iodine, as shown in Fig. 1. The radioiodine uptake test has been used for more than 3 decades (Hamilton and Soley, 1939) and remains one of the most definitive tests for thyroid function. With the development of accurate assays for T_4, ^{131}I uptake tests now are largely relegated to research studies on iodine metabolism.

The use of tracer doses of ^{131}I adds no significant amount of iodine to the system (1 μCi

$^{131}I = 8 \times 10-^{6}$ μg iodine) and results in only minimal exposure of the subject to radiation. The amount of ^{131}I used as tracer depends on a variety of factors, such as size and age of patient, thyroid gland size, amount of dietary iodine, and the instrumentation used for detection. Metz (1969) developed a mathematical formulation for calculation of the minimal dose of ^{131}I, which can be as little as 1 μCi for man. Since high doses can cause functional impairment to the thyroid gland, it is important that only a minimal dose be used. For practical clinical purposes, this minimum should be established, and about 10 μCi is used for the dog, although some systems require as little as 5 μCi. The dose is given intravenously, although the oral route also suffices.

The high-energy γ radiation of ^{131}I also permits ease of counting radioactivity *in vitro* and *in vivo,* and, by appropriate procedures, various parameters of thyroid function can be assessed. A number of procedures have been used to study thyroid physiology in goats and cattle (Pipes and Turner, 1956), sheep (Bustad *et al.,* 1958), swine (Seigneur *et al.,* 1959), and horses (Trum and Wasserman, 1956).

A standard technique is to monitor the amount of ^{131}I taken up by the gland using an external scintillation detector placed over the thyroid region of the neck and to express the uptake as a percentage of the injected dose. Ideally, the uptake should be measured frequently after injection, and a time–uptake curve should be constructed for 3–4 days. For clinical purposes in the dog, a single measurement at 72 hours is usually satisfactory for the hypothyroid, but the uptake curve is required for the differential diagnoses of hyperthyroidism. The 72-hour time interval is the time during which the normal dog accumulates a maximal amount of administered isotope.

The procedure in the dog has been to measure the thyroidal uptake of ^{131}I 72 hours after injection. The percent uptake is calculated from the following:

$$\frac{\text{Neck count} - (\text{stifle count} \times 1.1)}{\text{Standard count}} \times 100 = \% \text{ uptake}$$

The standard count represents the injected dose of ^{131}I as counted in a thyroid neck mock-up or phantom. The factor 1.1 is used to correct the stifle count to obtain the body background. The body background can also be obtained by shielding the thyroid region of the neck and counting. When this method is used, an uptake of 10–40% at 72 hours is considered to be within the normal (euthyroid) range for the dog. Values below 10% are considered indicative of hypofunction of the thyroid, and those above 40% as indicative of hyperfunction. A slightly lower euthyroid range (7–37%) was reported by Lombardi *et al.* (1962). The normal uptake at 24 hours is 7–35%, and that at 48 hours is 8–38%. The correlation of the 72-hour thyroidal ^{131}I uptake with the degree of thyroid function and its use as a reliable diagnostic test of thyroid disease in dogs has been shown (Kaneko *et al.,* 1959; Lombardi *et al.,* 1962).

It is well known that a large number of iodine-containing compounds, in addition to exogenous thyroid hormone, may interfere with thyroid function tests (Table III), and most of these compounds depress the ^{131}I uptake. These include various forms of iodine compounds used in diagnostic radiology, expectorants, vitamin preparations, and topical tinctures of iodine. The length of time during which uptake studies may be affected varies from a few hours to as long as several years depending on the compounds. In general, it is well to defer the uptake test for at least 3 weeks if iodine compounds have been administered.

M. Protein-Bound Radioiodine and the Conversion Ratio

These tests may be carried in conjunction with the ^{131}I uptake test. Following the accumulation of ^{131}I by the thyroid gland, ^{131}I-labeled thyroid hormone is synthesized and released into the circulation. The amount of ^{131}I bound to plasma protein ($PB^{131}I$) thus reflects the status of thyroid activity in the same manner as the amount of nonradioactive iodine (^{127}I) bound to plasma protein, i.e., the standard PBI. The PB ^{131}I and PBI differ, however, in the techniques employed for their measurement and expression of the results. Injected ^{131}I is taken up by the thyroid and released into the circulation of [^{131}I]thyroxine, which is then bound to plasma protein. The $PB^{131}I$ is conveniently separated from the ^{131}I iodide ion of the circulation by resin techniques (Scott and Reilly, 1954; Zieve *et al.*, 1956). This test usually requires a higher dose (50 μCi) of ^{131}I than used in the standard uptake test in order to obtain satisfactory count rates of the serum. An accurate measure of the injected dose is also required because the PB ^{131}I is expressed as the percentage of the injected dose per liter of serum. A modification of the $PB^{131}I$ test is to measure the radioactivity of the isolated plasma proteins and that of the whole plasma; the result is expressed as the conversion ratio (CR):

$$\frac{\text{Plasma protein radioactivity}}{\text{Total plasma radioactivity}} \times 100 = \frac{PB^{131}I}{PB^{131}I + {}^{131}I} \times 100 = CR\ (\%)$$

The CR describes the percentage of the total ^{131}I in the plasma which is accounted for by ^{131}I bound to protein. As such, it reflects the amount of labeled thyroid hormone formed by the gland and is a measure of thyroid function. A CR of 2–6% has been reported (Lombardi *et al.*, 1962) for normal dogs. This test is useful in the diagnosis of hyperthyroidism in man, but its diagnostic value in thyroid disease of animals awaits further confirmation.

N. Thyroxine Secretion Rate

The output of thyroxine by the thyroid gland is also a direct indicator of thyroid function and can be determined by several methods. Most data on thyroxine secretion rates (TSR) in domestic animals have been obtained by a technique based on the amount of exogenous L-thyroxine necessary to inhibit the release of ^{131}I by the thyroid, i.e., by thyroxine substitution. Another method has been to calculate the TSR from the fractional turnover rate of injected [^{131}I]thyroxine and the T_4 or PBI. The TSR has been determined in most domestic animals, including the cow (Pipes *et al.*, 1963), sheep (Henneman *et al.*, 1955), goat (Flamboe and Reineke, 1959), pig (Sorenson, 1962), horse (Irvine, 1967a), and dog (Kallfelz, 1973). There is a fairly wide variation in the reported values, which is probably the result of the differences in technique and conditions of the studies. The TSR is also affected by age, lactation, diet, season, and training in the case of horses. In animals, the TSR appears to vary from a low of about 0.108 mg/100 kg per day in the horse (Irvine, 1967a) to a high of about 0.46 mg/100 kg per day in the cow (Sorenson, 1962) or 0.49 mg/100 kg per day for the dog (Kallfelz, 1973).

O. Response to Thyroid-Stimulating Hormone

The response of an animal to TSH injection is also a means of differentiating primary hypothyroidism from a hypothyroid state which is secondary to pituitary hypofunction.

The response is best followed by T_4-RIA, T_3-RIA, or both. In pituitary hypofunction, the absence of a stimulus (TSH) for normal thyroid hormone metabolism accounts for the manifestations of hypothyroidism. If the thyroid gland has remained functional, the TSH response test will indicate significant increases in values obtained by T_4-RIA or T_3-RIA. This technique has been applied in the dog using an IM injection of 10 IU of TSH prior to performance of the standard 72-hour ^{131}I uptake test (Kaneko *et al.*, 1959). It has been extended (Siegel and Belshaw, 1968) so that an ^{131}I uptake curve is first determined for 72 hours, 3 IU of TSH are injected twice a day for 3 days, and then 10 IU of TSH are injected just before a second 72-hour ^{131}I uptake curve is obtained.

The PBI determination has also been used as an index of TSH response (Siegel and Belshaw, 1968). A control serum sample for PBI is obtained, 10 IU of TSH is injected IM, and, after 24 hours, a second serum sample for PBI is obtained. The normal dog responds with a mean increase of 3 μg/dl. In secondary hypothyroidism with functional thyroid tissue, an increase of at least 1 μg/dl is seen while, in primary hypothyroidism, there is less than 0.5 μg/dl increase in the PBI.

When this method is used, the mean peak response of T_4 concentration as determined by RIA to 10 IU of TSH is observed at 16 hours postinjection and is about fourfold greater than the value observed at zero time (Kaneko *et al.*, 1978). This is comparable to similar studies using T_4-CPB in which peak responses were observed at 12 hours (Kallfelz, 1973; Hoge *et al.*, 1974). These observations suggest that a practical approach to standardizing the TSH response test for clinical purposes would be to take the zero-time sample at about 5:00 p.m., inject 10 IU TSH IM, and then take the second sample on the following morning at 9:00 a.m. A doubling of the T_4-RIA value in response to TSH is the minimal normal response. A failure to respond minimally can be regarded as definitive evidence of thyroid gland dysfunction. On the other hand, unequivocally low T_4-RIA or T_3-RIA values at zero time followed by a significant response to TSH is suggestive of pituitary dysfunction. Confirmation of pituitary dysfunction would require a TSH assay, which is not currently available for animals.

P. Thyroid-Stimulating Hormone

The principles of radioimmunoassay have been successfully applied for the assay of TSH in human beings. When the same method* is used for the dog, the tentative normal value is 5.9 ± 4.1 μU/ml (Kaneko *et al.*, 1975). Since the antibody is antihuman TSH, considerable question is raised regarding the validity of the assay. Until an assay using anticanine TSH is developed, this question will remain. The human TSH method used in monkeys (*Mucaca mulatta*) resulted in 0.2–2.6 μU/ml (1.53 μU/ml) (Belchetz *et al.*, 1978).

Q. Response to Thyrotropin-Releasing Factor

The response to thyrotropin-releasing factor has been used as a test for the diagnosis of pituitary hypothyroidism in human beings, and the test has been evaluated in the dog (Kraft and Gerbig, 1977). No response to TRF was observed with T_4-RIA, T_3-RIA, or T_3-Uptake. The TSH concentration was not measured, but presumably the human TRF

*Nuclear Medical System, Long Beach, California.

preparation did not stimulate TSH release since the thyroid hormone levels did not increase. At present, the TRF response test appears to be inappropriate for use in dogs.

R. Summary of Tests of Thyroid Function

The myriad available tests and the rapid advancements in methodology currently permit only a recommendation of certain tests for use with animals, with the expectation that advances will occur rapidly. Initial screening by routine hematological examination and measurement of serum cholesterol should be performed followed by both T_4-RIA and T_3-RIA. If the results are still equivocal, the TSH response test should then be used.

V. DISORDERS OF IODINE METABOLISM

Thyroid disease has been most extensively studied in the dog, and several recent reviews are available (Bush, 1969; Capen *et al.*, 1975). Rijnberk (1971) has published an extensive study of iodine metabolism and thyroid disease in the dog. Lowe *et al.* (1974) have described the clinical effects of experimental thyroidectomy in mares and stallions.

Goiter may be defined as any enlargement of the thyroid gland which is not due to inflammation or malignancy. There are two general types of goiters: (1) nontoxic goiters, which produce normal amounts of hormone (simple goiter) or less than normal amounts of hormone (hypothyroid), and (2) toxic goiters, which are characterized by excessive production of hormone (hyperthyroid). The complex steps in the synthesis of thyroxine have been described in Sections III,A,2. Almost all steps involve either hormonal or enzymatic action, and it is apparent that a defect or a deficiency at any step can result in thyroid disease.

The simple form of nontoxic goiter is said to be "compensated" because there has been a compensatory increase in thyroid activity and mass (hyperplasia and hypertrophy) such that the tissue secretes as much T_4 as does a normal gland. When the secretion of T_4 is less than normal, hypothyroidism occurs.

Hypothyroidism may be the result of a variety of factors. Iodine deficiency (endemic goiter) is a well-known cause of goiter. Goitrogenic materials, either natural substances or drugs, induce goiters by their blocking effects on steps in the iodine metabolic pathways (Section III,A,2). Thyroiditis with similarities to Hashimoto's thyroiditis in man has also been reported in about 12% of beagle dogs (Beierwaltes and Nishiyama, 1968). Antithyroglobulin antibodies were reported in these dogs. In the adult dog, follicular atrophy is probably the most common cause of hypothyroidism (Clark and Meier, 1958; Siegel and Belshaw, 1968). There are also a number of rare types of familial goiters associated with defects in hormone synthesis (dyshormonogenesis) in man (Stanbury, 1966) which may find their counterparts in sheep (Rac *et al.*, 1968) and cattle (Van Zyl *et al.*, 1965). Finally, hypothyroidism may be secondary to a pituitary insufficiency, as described earlier.

Hypothyroidism, particularly in the dog, is an important differential diagnosis. The hypothyroid dog is typically obese and lethargic with a dry skin, myxedema, and a sparse hair coat. It is therefore an important consideration in the differential diagnoses of the dermatoses. The requirement of T_4 for normal reproduction, growth, and development is well known and also makes thyroid disease an important consideration in reproduction and

its disorders. Experimentally thyroidectomized mares and stallions also failed to grow, were lethargic, and had decreased rectal temperatures and increased serum cholesterol (Lowe *et al.*, 1974). Their hair coats were also dull and coarse.

Hyperthyroidism or toxic goiter is characterized by increased T_4. It has been reported to occur in dogs but is a rare condition (Meier and Clark, 1958). These dogs usually exhibit symptoms of hypermetabolism as a result of the excess T_4, such as hyperexcitability and hyperactivity. The most common form of hyperthyroidism is a toxic diffuse hyperplasia of the thyroid (Graves' disease in man). Less commonly a multinodular form or a single adenoma occurs, and in animals they may all be collectively described as adenomas (Smith and Jones, 1966). In recent years, there has been evidence that the thyroid in Graves' disease is responding to a substance other than TSH. This substance is long-acting thyroid stimulator, which is present only in serum of Graves' disease patients and has already been discussed (Section III,C).

Tumors of the thyroid, on the other hand, appear to be of relatively frequent occurrence (Lucke, 1964). Almost all, however, are benign, and their significance as a clinical disease lies in their cosmetic effect since Brodey and Kelly (1968) found no evidence of clinical thyroid disease in their study of thyroid tumors.

REFERENCES

Adams, D. D. (1958). *J. Clin. Endrocrinol. Metab.* **18,** 699.

Barua, J., Cragle, R. G., and Miller, J. K. (1964). *J. Dairy Sci.* **47,** 539.

Beierwaltes, W. H., and Nishiyama, R. H. (1968). *Endocrinology* **83,** 501.

Belchetz, P. E., Gredley, G., and Himsworth, R. (1978). *J. Endocrinol.* **76,** 427.

Belshaw, B. E., and Rijnberk, A. (1979). *J. Am. Anim. Hosp. Assoc.* **15,** 17.

Belshaw, B. E., Cooper, T. B., and Beeker, D. V. (1975). *Endocrinology* **96,** 1280.

Bigler, B. (1976). *Schweiz. Arch. Tierheilkd.* **118,** 417.

Blackmore, D. L. (1979). Personal communication.

Boyd, E. M. (1942). *J. Biol. Chem.* **143,** 131.

Boyd, E. M. (1944). *Can. J. Res., Sect. E* **22,** 39.

Boyd, G. S., and Oliver, M. F. (1958). *In* "Cholesterol" (R. P. Cook, ed.), p. 187. Academic Press, New York.

Brodey, R. S., and Kelly, D. (1968). *Cancer (Philadelphia)* **22,** 406.

Bush, B. M. (1969). *J. Small Anim. Pract.* **10,** Part I, 95; Part 2, 185.

Bush, B. M. (1972). *Res. Vet. Sci.* **13,** 177.

Bustad, L. K., George, L. A., Jr., Marks, S., Warner, D. E., Barnes, C. M., Herde, K. E., and Kornberg, H. A. (1957). *Radiat. Res.* **6,** 380.

Bustad, L. K., Warner, D. E., and Kornberg, H. A. (1958). *Am. J. Vet. Res.* **18,** 893.

Bustad, L. K., Barnes, C. M., George, L. A., Jr., Herde, K. E., Horstman, V. G., Kornberg, H. A., McKenney, J. R., Persing, R. L., Marks, S., Seigneur, L. J., and Warner, D. E. (1962). *In* "Use of Radioisotopes in Animal Biology and the Medical Sciences," Vol. I, p. 401. Academic Press, New York.

Capen, C. C., Belshaw, B. E., and Martin, S. L. (1975). *In* "Textbook of Veterinary Internal Medicine. Vol. 2: Endocrine Disorders" (S. E. Ettinger, ed.), p. 1378. Saunders, Philadelphia, Pennsylvania.

Carroll, E. J. (1963). *In* "Clinical Biochemistry of Domestic Animals" (C. E. Cornelius and J. J. Kaneko, eds.), p. 58. Academic Press, New York.

Clark, S. T., and Meier, H. (1958). *Zentralbl. Veterinaermed.* **5,** 17.

Cline, M. J., and Berlin, N. I. (1963). *Am. J. Physiol.* **204,** 415.

Danowski, T. S., Man, E. B., and Winkler, A. W. (1946). *Endocrinology* **38,** 230.

Edelman, I. S. (1974). *N. Engl. J. Med.* **290,** 1303.

Fisher, D. A., Oddie, T. H., and Epperson, J. (1965). *J. Clin. Endocrinol. Metab.* **25,** 1580.

Flamboe, E. E., and Reineke, E. P. (1959). *J. Anim. Sci.* **18,** 1135.

Freinkel, N., and Ingbar, S. H. (1955). *J. Clin. Endocrinol. Metab.* **15,** 598.
Glock, G. E. (1949). *J. Endocrinol.* **6,** 6.
Hackett, P. L., Gaylor, D. W., and Bustad, L. K. (1957). *Am. J. Vet. Res.* **18,** 338.
Hamilton, J. G., and Soley, M. H. (1939). *Am. J. Physiol.* **127,** 557.
Hamolsky, M. W., Stein, M., and Freedberg, A. S. (1957). *J. Clin. Endocrinol. Metab.* **17,** 33.
Hamolsky, M. W., Golodetz, A., and Freedberg, A. S. (1959). *J. Clin. Endocrinol. Metab.* **19,** 103.
Henneman, H. A., Reineke, E. P., and Griffin, S. A. (1955). *J. Anim. Sci.* **14,** 419.
Hoge, W. R., Lund, J. E., and Blakemore, J. C. (1974). *J. Am. Anim. Hosp. Assoc.* **10,** 167.
Irvine, C. H. G. (1967a). *J. Endocrinol.* **39,** 313.
Irvine, C. H. G. (1967b). *Am. J. Vet. Res.* **28,** 1687.
Irvine, C. H. G., and Evans, M. J. (1975). *J. Reprod. Fertil., Suppl.* **23,** 709.
Irvine, W. J., and Standeven, R. M. (1968). *J. Endocrinol.* **41,** 31.
Kaihara, S., Carullit, N., and Wagner, H. N., Jr. (1969). *J. Nucl. Med.* **10,** 281.
Kallfelz, F. A. (1969). *J. Am. Vet. Med. Assoc.* **152,** 1647.
Kallfelz, F. A. (1973). *Am. J. Vet. Res.* **34,** 535.
Kallfelz, F. A., and Erali, R. P. (1973). *Am. J. Vet. Res.* **34,** 1449.
Kallfelz, F. A., and Lowe, J. E. (1970). *J. Am. Vet. Med. Assoc.* **156,** 1888.
Kaneko, J. J. (1963). *In* "Clinical Biochemistry of Domestic Animals" (C. E. Cornelius and J. J. Kaneko, eds.), p. 310. Academic Press, New York.
Kaneko, J. J. (1964). *Proc. Annu. Conv. Am. Assoc. Equine Practitioners, 10th, Denver,* p. 125.
Kaneko, J. J. (1970). *In* "Thyroid Function" (J. J. Kaneko and C. E. Cornelius, eds.), 2nd ed., p. 293. Academic Press, New York.
Kaneko, J. J. (1979). Unpublished observations.
Kaneko, J. J., Tyler, W. S., Wind, A. P., and Cornelius, C. E. (1959). *J. Am. Vet. Med. Assoc.* **135,** 10.
Kaneko, J. J., Baker, B., and Mills, R. (1975). *Bull. Am. Soc. Vet. Clin. Pathol.* **4,** 30.
Kaneko, J. J., Comer, K. M., and Ling, G. V. (1978). *Calif. Vet.* **32,** 9.
Klebanoff, S. J. (1965). *In* "Physiology and Biophysics" (T. C. Ruch and H. D. Patton, eds.), 19th ed., p. 1147. Saunders, Philadelphia, Pennsylvania.
Koppers, L. E., and Palumbo, P. J. (1972). *Med. Clin. North Am.* **56,** 1013.
Kraft, W., and Gerbig, T. (1977). *Dtsch. Tierarztl. Wochenschr.* **84,** 185.
Kritchevsky, D. (1958). "Cholesterol," p. 279. Wiley, New York.
Kuhns, L. J., Tallman, D. F., and Sippel, W. L. (1969). *J. Comp. Lab. Med.* **3,** 27.
Lennon, H. D., and Mixner, J. P. (1957). *J. Dairy Sci.* **40,** 1424.
Leveille, G., Fisher, H., and Weiss, H. S. (1957). *Proc. Soc. Exp. Biol. Med.* **94,** 383.
Lipman, L. M. (1967). *Am. J. Med.* **43,** 486.
Lombardi, M. H., Comar, C. L., and Kirk, R. W. (1962). *Am. J. Vet. Res.* **23,** 412.
Long, J. F., Gilmore, L. O., Curtis, G. M., and Rife, D. C. (1951). *J. Anim. Sci.* **10,** 1027.
Lowe, J. E., Baldwin, B. H., Foote, R. H., Hillman, R. B., and Kallfelz, F. A. (1974). *Cornell Vet.* **64,** 276.
Lucis, O. M., Cummings, G. T., Matthews, S., and Burry, C. (1969). *J. Nucl. Med.* **10,** 160.
Lucke, V. M. (1964). *J. Small Anim. Pract.* **5,** 351.
McCrady, J. D., Hightower, D., Williams, G. D., Kyzar, J. R., and Bridges, C. H. (1973). *Cardiovasc. Res. Centr. Bull. (Houston)* **12,** 21.
McKenzie, J. M. (1958). *Endocrinology* **63,** 372.
Mahley, R. W., and Weisgraber, K. H. (1974). *Circ. Res.* **35,** 713.
Mallo, G. L., and Harris, A. L. (1967). *Vet. Med. Small Anim. Clin.* **62,** 533.
Mayer, E. (1947). *Endocrinology* **40,** 165.
Meier, H., and Clark, S. T. (1958). *Zentralbl. Veterinaermed.* **5,** 120.
Metz, C. E. (1969). *J. Nucl. Med.* **10,** 475.
Michaelson, S. M. (1969). *Mod. Vet. Pract.* **50,** 43.
Morris, B., and Courtice, F. C. (1955). *Q. J. Exp. Physiol. Cog. Med. Sci.* **40,** 127.
Murphy, B. E. P., and Pattee, C. J. (1964). *J. Clin. Endocrinol. Metab.* **24,** 187.
Norcia, L. M., and Furman, R. H. (1959). *Proc. Soc. Exp. Biol. Med.* **100,** 759.
O'Neal, L. W., and Heinbecker, P. (1953). *Endocrinology* **53,** 60.
Pain, R. W., and Oldfield, R. K. (1969). *Tech. Bull. Regist. Med. Technol.* **39,** 139.
Pilleggi, V. J., Lee, N. D., Golub, O. J., and Henry, R. J. (1961). *J. Clin. Endrocinol. Metab.* **21,** 1272.
Pipes, G. W., and Turner, C. W. (1956). *Univ. Mo. Agric. Exp. Stn., Res. Bull.* No. 617, p. 1.

Pipes, G. W., Bauman, T. R., Brooks, J. R., Comfort, J. E., and Turner, C. W. (1963). *J. Anim. Sci.* **22,** 476.
Pliam, N. B., and Goldfine, I. D. (1977). *Biochem. Biophys. Res. Commun.* **79,** 166.
Quinlan, W., and Michaelson, S. M. (1967). *Am. J. Vet. Res.* **28,** 179.
Rac, R., Hill, G. N., and Pain, R. W. (1968). *Res. Vet. Sci.* **9,** 209.
Ragan, H. A., Horstman, V. G., McClellan, R. D., and Bustad, L. K. (1966). *Am. J. Vet. Res.* **116,** 161.
Reiser, R., Sorrels, M. F., and Williams, M. C. (1959). *Circ. Res.* **7,** 833.
Rijnberg, A. (1971). "Iodine Metabolism and Thyroid Disease in the Dog," State Univ. of Utrecht, Utrecht.
Schultz, A. L., Sandhaus, S., Demorest, H. L., and Zieve, L. (1954). *J. Clin. Endocrinol. Metab.* **14,** 1062.
Scott, K. G., and Reilly, W. A. (1954). Metab. Clin. Exp. **3,** 506.
Segal, J., Schwartz, H., and Gordon, A. (1977). *Endocrinology* **101,** 143.
Seigneur, L. J., Test, L. D., and Bustad, L. K. (1959). *Am. J. Vet. Res.* **20,** 14.
Siegel, E. T., and Belshaw, B. E. (1968). *In* "Current Veterinary Therapy" (R. W. Kirk, ed.), 3rd ed., p. 545. Saunders, Philadelphia, Pennsylvania.
Sims, M. H., Redding, R. W., and Nachreiner, R. F. (1977). *J. Am. Vet. Med. Assoc.* **171,** 178.
Slingerland, D. W. (1955). *J. Clin. Endocrinol Metab.* **15,** 131.
Smith, H. A., and Jones, T. C. (1966). "Veterinary Pathology," p. 1073. Lea & Febiger, Philadelphia, Pennsylvania.
Sorenson, P. H. (1962). *In* "Use of Radioisotopes in Animal Biology and the Medical Sciences," Vol. I, p. 455. Academic Press, New York.
Sparagana, M., Phillips, G., and Kucera, L. (1969). *J. Clin. Endocrinol. Metab.* **29,** 191.
Stanbury, J. B. (1966). *In* "The Metabolic Basis of Inherited Disease" (J. B. Stanbury, J. B. Wyngaarden, and O. S. Fredrickson, eds.), p. 215. McGraw-Hill, New York.
Sterling, K. (1979). *N. Engl. J. Med.* **300,** 117.
Tapley, D. F. (1964). *Mayo Clinic Proc.* **39,** 626.
Tata, J. R., Ernster, L., Lindberg, O., Arrhenius, E., Pederson, S., and Hedman, R. (1963). *Biochem. J.* **86,** 408.
Taurog, A., and Chaikoff, I. L. (1946). *J. Biol. Chem.* **163,** 313.
Theran, P., and Thornton, G. W. (1966). *J. Am. Vet. Med. Assoc.* **148,** 562.
Trum, B. F., and Wasserman, R. H. (1956). *Am. J. Vet. Res.* **17,** 271.
Van Zyl, A., Schulz, K., Wilson, B., and Pansegrouw, D. (1965). *Endocrinology* **76,** 353.
Wilson, R. B., Dickson, W. M., and Dost, F. N. (1961). *Vet. Med. (Kansas City, Mo.)* **46,** 285.
Zieve, L., Vogel, W. C., and Schultz, A. L. (1956). *J. Lab. Clin. Med.* **47,** 663.

13

Reproductive Hormones

LARS-ERIC EDQVIST AND
GEORGE H. STABENFELDT

I. INTRODUCTION

Clinical reproductive endocrinology includes the study of diseases of the endocrine glands involved in reproduction and their secretory products, the reproductive hormones. In order to obtain a satisfactory understanding of the complex endocrinological events that occur during normal and abnormal reproductive function, quantification of the specific hormones involved is necessary. Previously, biological and chemical methods were used. Biological assay systems measured the effect of a hormone on its target tissue and the resulting hormone-induced effect in the tissue, e.g., weight gain or histological change

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

was used as the measure of hormone concentration. These assays were relatively imprecise, time-consuming, and, as a result, expensive. Later these techniques were replaced for steroid hormones by chemical determinations. These assay systems usually required large volumes of starting material (e.g., blood), which made serial sampling impossible. Some steroid hormones were studied in 24-hour urine aliquots. Collection of such urine aliquots from domestic species was difficult, time-consuming, and not practical under field conditions.

More recently, major progress has occurred in hormone analytical techniques because of the development of radioimmunoassay techniques (Berson and Yalow, 1959) and competitive protein binding techniques (Murphy, 1964). These techniques have added new dimensions to the study of reproductive mechanisms. The assay systems are sensitive, specific, relatively inexpensive, and require small amounts of assay material. They have been of special value for studying endocrinological reproductive functions in domestic animals in that they have made possible the study of dynamic endocrine changes through the assay of serial blood samples from the same animal. Radioimmunoassay and competitive protein binding techniques are suitable not only for physiological research, but also as diagnostic aids for the identification and elucidation of clinical reproductive problems. In clinical practice, these methods are becoming important from both a diagnostic and therapy-monitoring point of view.

Because this book deals with clinical biochemistry in domestic animals, the main emphasis of the chapter is on the determination of hormones and the use of the data as diagnostic aids. General reproductive endocrinology in domestic species is broadly covered. Readers specifically interested in this subject are referred to specialized books dealing with this matter.

A. Definition of Hormones

Hormones are chemical substances synthesized and secreted by ductless endocrine glands directly into the bloodstream, by which they are carried to other parts of the body, where they regulate (decrease or increase) the rates of specific biochemical processes. The endocrine glands include the pituitary (the adenohypophysis and neurohypophysis), thyroid, parathyroid, adrenal cortex and medulla, islets of Langerhans of the pancreas, ovary, testis, placenta, and pineal gland.

B. Chemical Classes of Reproductive Hormones

1. Protein Hormones

a. Releasing Hormones. Several types of hormones are involved in the regulation of reproduction. Releasing hormones are peptide hormones which are produced within the hypothalamus and transferred via the hypothalamohypophyseal portal veins to the adenohypophysis, where they regulate the synthesis and/or release of adenohypophyseal hormones. The gonadotropin-releasing hormone (Gn-RH), a decapeptide with the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, regulates the release of two important reproductive hormones, namely, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Thyrotropin-releasing hormone, a tripeptide (pGlu-His-Pro-NH_2) which releases thyroid-stimulating hormone (TSH), also causes the release of

prolactin in several species. Furthermore, both a prolactin-inhibiting hormone and a prolactin-releasing hormone have been demonstrated in hypothalamic tissues.

b. Hypophyseal Hormones. Luteinizing hormone and FSH are glycoproteins containing 13–25% carbohydrate. The molecular weight of LH in domestic animals (bovine, ovine, porcine, equine) is about 30,000. Ovine FSH and equine FSH have a molecular weight of about 32,000. Prolactin is a protein with a molecular weight of approximately 23,000 (bovine, ovine, porcine).

The cells of the adenohypophysis can be divided according to their staining characteristics. Cells which can be stained specifically are called chromophils. This group of cells can be further divided into basophils (affinity for basic stains) and acidophils (affinity for acid stains). Luteinizing hormone and FSH are produced within basophilic cells; it has even been demonstrated that LH and FSH can be present within the same cell (Phifer *et al.*, 1973). Prolactin, on the other hand, has been localized in acidophilic cells. As pointed out previously, the releasing hormones are important regulators of the synthesis and/or release of these hormones from the adenohypophysis.

The posterior pituitary is responsible for storage and release of oxytocin and antidiuretic hormone (vasopressin). These two hormones are synthesized primarily in the regions of the paraventricular nucleus and supraoptic nucleus of the hypothalamus. The hormones are transported to the posterior pituitary by axoplasmic fluid. Release of these hormones occurs as a result of stimulation of the nerve cell bodies in the nuclei. Oxytocin, an important reproductive hormone, is an octapeptide with a molecular weight of 1000.

c. Placental Gonadotropins. During early pregnancy in the mare, fetal trophoblastic cells (embedded within the uterus as endometrial cups) secrete large amounts of a gonadotropin, pregnant mare serum gonadotropin (PMSG). The molecular weight of PMSG, a glycoprotein, is uncertain; it was reported to be 28,000 (Bourrillon and Got, 1959), although later investigations indicated a higher molecular weight (Gospodarowicz, 1972). No specific placental gonadotropins have been demonstrated in other domestic species.

d. Subunits. Luteinizing hormone and FSH, as well as TSH, consist of two nonidentical subunits designated α and β. Chemical and biological studies have indicated that the α subunit is identical for the different hormones, while the β subunit is unique for each hormone and thus determines its biological activity. Subunits, by themselves, possess little or no biological activity. Individual subunits are probably not released into the circulatory system under normal circumstances.

2. Steroid Hormones

This group of hormones is derived from a common precursor molecule, cholesterol, via the metabolic pathway schematically outlined in Fig. 1. More than 1500 biologically active steroids have been isolated from biological material or have been produced synthetically. The molecular weight of steroid hormones is low, usually below 500 (Table I). Examples of steroids which play an important role in reproductive processes are estrogens, androgens, progestagens, and corticosteroids, with the main source being the gonads and the adrenal cortex. The structures of the most important sex steroids are given in Fig. 2. The most common steroid hormones are usually designated by a trivial name,

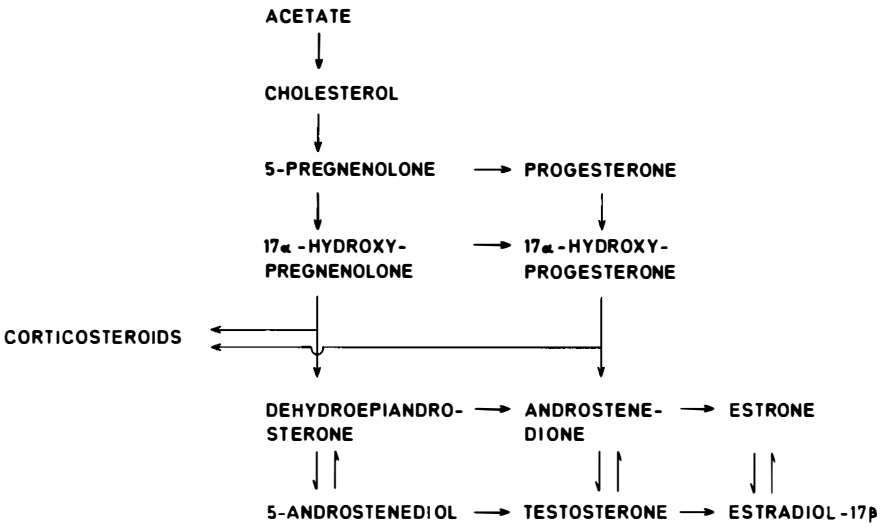


Fig. 1. Pathway for the synthesis of biologically active steroids from acetate. The steroids secreted from the gonads and the adrenal are formed from acetate and cholesterol.

e.g., estradiol, progesterone, or testosterone. The International Union of Pure and Applied Chemistry (IUPAC)* has recommended systemic names for steroid hormones. These systemic names describe the chemical and stereoisometric characteristics of the particular steroid hormone (Table I).

3. Prostaglandins

Prostaglandins constitute a group of 20-carbon unsaturated fatty acids with molecular weights usually between 300 and 400 (Table I). Prostaglandins are not hormones in the strictest sense, and the expressions "parahormones" or "local hormones" have been used. This is because prostaglandins are not secreted from any particular gland and the biological half-life of prostaglandins is usually extremely short, allowing, in most cases, only a local action. In actuality, several different prostaglandins are found in a number of types of mammalian tissues. One prostaglandin released from the uterus, prostaglandin $F_{2\alpha}$, plays an important role in regulating reproductive cycles in domestic species through the control of luteal activity in nonpregnant animals. The structures of prostaglandin $F_{2\alpha}$ and its main metabolite, 15-keto-13,14-dihydroprostaglandin $F_{2\alpha}$, are given in Fig. 2.

II. ASSAY METHODS

For the foreseeable future, competitive protein binding and especially radioimmunoassay techniques will likely be the laboratory methods of choice in reproductive diagnostic endocrinology of domestic species. The radioimmunoassay technique was originally introduced for the measurement of plasma insulin (Berson and Yalow, 1959) and the competitive protein binding technique for the determination of plasma cortisol concen-

*Revised Tentative Rules for Nomenclature of Steroids. *Biochim. Biophys. Acta* **164,** 453 (1968).

TABLE I

Nomenclature and Molecular Weight of Some Biologically Important Steroids and Prostaglandins

Trivial name	Systemic name	Molecular weight
Androstenedione	4-Androstene-3,17-dione	286
Cortisol	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione	363
Estradiol-17β	1,3,5,(10)-Estratriene-3,17β-diol	272
Estrone	3-Hydroxy-1,3,5(10)-estratrien-17-one	270
17α-Hydroxyprogesterone	17α-Hydroxy-4-pregnene-3,20-dione	331
Pregnenolone	3β-Hydroxy-5-pregnen-20-one	317
Progesterone	4-Pregnene-3,20-dione	315
Testosterone	17β-Hydroxy-4-androsten-3-one	288
$PGF_{2\alpha}$	9α,11α,15-Trihydroxyprosta-5,13-dienoic acid	354
15-Keto-13,14-dihydro-$PGF_{2\alpha}$	9α,11α-Dihydroxy-15-ketoprost-5-enoic acid	354

trations (Murphy, 1964). Both these techniques utilize the same basic principle, which is based on the ability of nonradioactive hormone to compete with a fixed amount of radioactive hormone for the binding sites on a fixed amount of protein. The nonradioactive hormone reduces the number of free binding sites on the protein, thus decreasing the availability of the binding sites to the labeled hormone. At equilibrium, the free hormone is separated from the protein-bound hormone, and the reaction is quantitated by the determination of antibody-bound and/or free-labeled hormone (Fig. 3). The degree

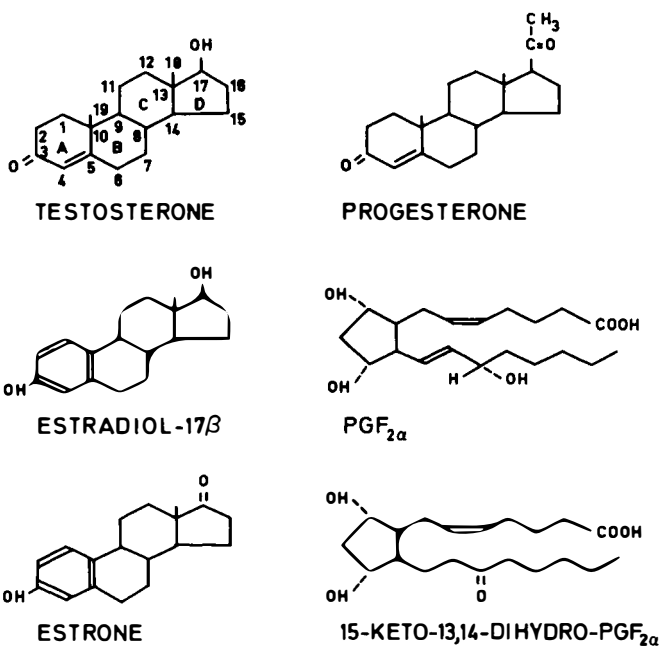


Fig. 2. The number sequence for the carbon atoms of the steroid skeleton and lettering sequence for the four rings are shown for testosterone. The structures of three other important sex steroid hormones---estrone-17β, and progesterone—as well as the structures of prostaglandin $F_{2\alpha}$ and its blood plasma metabolite 15-keto-13,14-dihydroprostaglandin $F_{2\alpha}$ are also depicted.

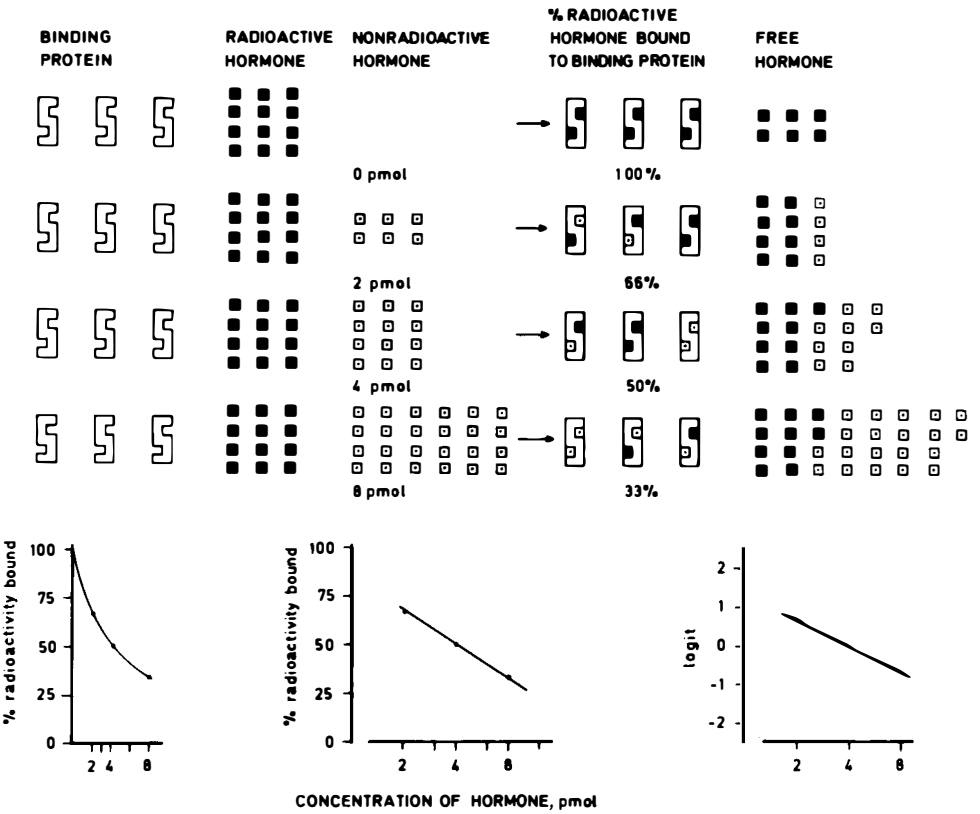


Fig. 3. The principle of competitive protein binding and radioimmunoassay techniques is based upon the ability of the binding protein to bind the radioactive hormone. Excess radioactive hormone is added to ensure saturation of the hormone-binding sites on the binding protein. The addition of increasing amounts of nonradioactive hormone (2, 4, and 8 pmol) results in a proportional decrease in the quantity of radioactive hormone bound to the binding protein. Separation of radioactive hormone bound to the binding protein from free hormone must be achieved before quantification can be done. In the lower half of the figure this reaction is depicted in three different ways. In the panel to the left and in the middle, percent radioactive hormone bound to the binding protein is given on the ordinate, and on the abscissa is given the amount of hormone in an arithmetic scale (left) and on a log scale (middle). In the panel to the right, the logit of the response variable is given on the ordinate and the amount of hormone plotted on a log scale. The method depicted in the middle and to the right can be used for determination of parallelism. The logit/log transformation (right) is frequently used when radioimmunoassay or competitive protein binding data are submitted to computer analysis.

of inhibition of binding of the radioactive hormone to the binding protein is a function of the concentration of nonradioactive hormone present in the solution. As a basis for the quantification, a standard curve is developed with fixed amounts of labeled hormone and binding protein incubated together in the presence of a known and graded concentration of unlabeled hormone (Fig. 3).

A. Radioimmunoassay

1. Production of Antibodies

Radioimmunoassay techniques utilize antibodies to the hormone to be measured as binding protein. Hormones such as LH and FSH, which are glycoproteins with molecular

weights of around 30,000, are antigenic because of their size and chemical composition. The smallest peptide hormone that has been shown to be immunogenic is vasopressin with a molecular weight of 1080 (Roth *et al,,* 1966).

At present, there are only a few polypeptide reproductive hormones available with a purity suitable for both immunization and preparation of the radioactive tracer. If an assay for bovine LH utilizes an antibody to bovine LH, radiolabeled bovine LH as tracer, and bovine LH as the standard, the assay system is completely species specific and is said to be of the homologous type. Such a system represents the ideal radioimmunoassay system for measuring a polypeptide hormone. Due to the limited availability and sometimes lack of suitable purity of polypeptide hormone preparations, heterologous assay systems have been developed. In these cases, an antiserum to a polypeptide hormone of one species has been used for the determination of the same polypeptide hormone in another species. The standard hormone used for quantification of the assay should, however, originate from the same species for which the measurements are performed. There are polypeptide hormone antisera available which show a high degree of cross-reactivity. One such antiserum of special interest in the field of reproductive hormones in domestic species is an LH antiserum raised against ovine LH. This antiserum reacts specifically with LH from other species and has been used for the determination of LH in approximately 45 species including the cow, sheep, pig, cat, and dog (Niswender *et al.,* 1968).

Steroid hormones and prostaglandins have considerably lower molecular weights and are thus not immunogenic per se. However, these structures can be rendered immunogenic if covalently linked to large carrier molecules such as bovine serum albumin, and specific antibodies can be elicited in this fashion (Landsteiner and Van der Scherr, 1936). In order for such a hormone–protein conjugate to be immunogenic, approximately 10–20 hormone molecules should be present per molecule of protein. In the case of bovine serum albumin, about 30% of the sites available for conjugation should be occupied.

Most naturally occurring steroid hormones or prostaglandins contain hydroxyl or ketone groups, which are used to prepare derivatives containing active groups such as carboxyl or amino groups. These groups are then activated so that they react with amino or carboxyl groups of the protein molecule. The specificity of the antisera obtained by immunization with a steroid–protein conjugate is dependent on the site used for conjugating the steroid to the protein. More specific antisera are obtained if the hapten (steroid) is attached to the protein at a site remote from the characteristic functional groups of the hormone (Lindner *et al.,* 1970; Niswender and Midgley, 1970; Exley *et al.,* 1971; Abraham, 1974).

The most frequent species used for immunization purposes are sheep and rabbits. Numerous reports are available on different schedules of immunization. One of the most popular and efficient schedules involves multiple injections of the antigen emulsified in complete Freund's adjuvant in the back and neck of the animal (Vaitakaitus *et al.,* 1971; Abraham, 1974). During immunization, the developing antibody titer is monitored, and bleedings are performed when a suitable titer has been achieved. Antisera seem to be quite stable when stored at −20°C, although the usual preferred temperature is −70°C. A few milliliters of a high-titer antiserum are usually sufficient for millions of radioimmunoassay determinations.

2. Radioactive Hormone

In radioimmunoassay techniques for polypeptide hormones, the antigen (hormone) is most commonly used for preparing the radioactive tracer. Usually, radioactive iodine, ^{125}I, is used for radioiodination of the antigen. The two most frequently used techniques

for iodination are the chloramine-T procedure (Hunter and Greenwood, 1962; Hunter, 1970) and the lactoperoxidase procedure (Thorell and Johansson, 1971). Peptide hormones containing tyrosyl or histidyl residues can usually also be iodinated using these techniques.

Most radioimmunoassay systems for steroid hormones and prostaglandins utilize tritiated forms of these molecules which are available commercially. Since tritium has a considerably longer half-life than iodine, the tritium tracers can be used in many cases over several years, while the iodinated tracers often have to be prepared monthly. There are, however, certain advantages in using iodinated tracers for steroid hormones and prostaglandins. A simpler and cheaper counting system can be used, i.e., γ counting as opposed to liquid scintillation counting. Another advantage of radioiodine over tritium is its higher activity per molecule of radiolabeled hormone, which increases the sensitivity of the assay (Midgley and Niswender, 1970). Direct incorporation of iodine in the skeleton of steroid hormones usually results in a loss of the immunoreactivity. The approach taken for radioiodination of steroid hormones has been to link a tyrosyl or histidyl molecule to the steroid molecule, making direct radioiodination possible (Niswender, 1973; Cameron *et al.*, 1975), or to iodinate a compound such as tyramine and conjugate the iodinated compound to the steroid molecule (Lindberg and Edqvist, 1974).

3. Separation of Antibody-Bound and Free Hormone

An essential part of any radioimmunoassay system is an efficient procedure for the separation of antibody-bound and free hormone (Fig 4i). Several different approaches have been taken in order to achieve a rapid and efficient separation. Currently, the most frequent separation procedures in use are based on (1) antibodies coupled to an insoluble polymer, (2) precipitation of antibody-bound hormone, or (3) adsorption of free hormone.

Antibodies coupled to an insoluble polymer have been used for separating antibody-bound and free hormone in radioimmunoassay procedures for both protein hormones (Catt

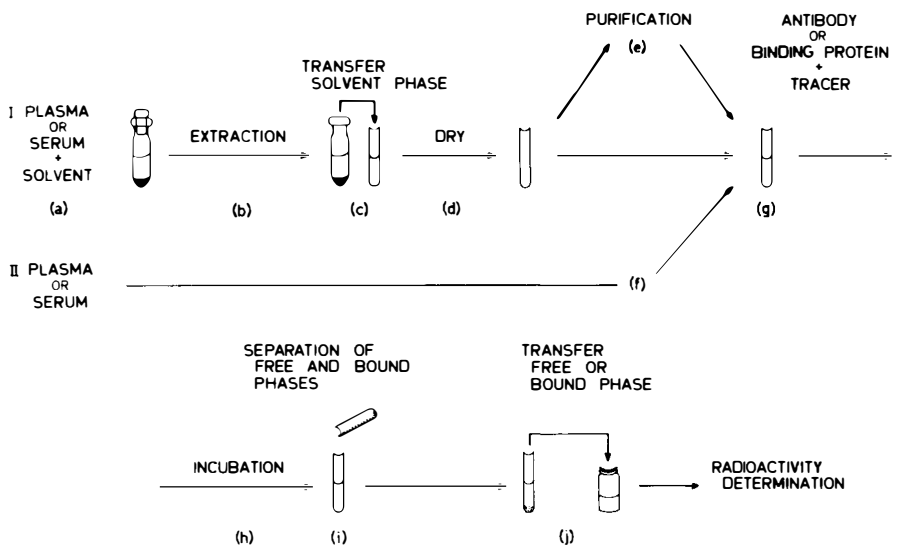


Fig. 4. General flow schematic for radioimmunoassay or competitive protein binding techniques.

et al., 1966; Wide and Porath, 1966) and steroid hormones (Abraham, 1969). One procedure involves the adsorption of the antibody to the interior surface of polystyrene test tubes. The standard or sample and the radioactive hormone are then added to the tube and allowed to react with the antibody. The reaction is terminated by decanting the tube, after which the antibody-bound or free radioactivity is determined. Another common procedure utilizes antibodies covalently coupled to an insoluble polymer (Wide and Porath, 1966). In this case, free and antibody-bound hormone are separated through centrifugation with the antibody coupled to the polymer present in the bottom of the tube. After removal of the supernatant containing the free hormone, the antibody-bound radioactivity can be determined.

Precipitation of antibody-bound hormone has been achieved through the addition of ammonium sulfate (Mayes and Nugent, 1970) or polyethylene glycol (Desbuqois and Aurbach, 1971), leaving the free hormones in the solution. The latter precipitation procedure has been found advantageous for the precipitation of prostaglandin–antibody complexes (Van Orden and Farley, 1973; Granström and Kindahl, 1976).

Precipitation of the antibody-bound hormone complexes can also be achieved through the addition of a second antibody prepared against the first antibody. Thus, in a radioimmunoassay technique for bovine LH which utilizes an antiserum to bovine LH raised in a rabbit (first antibody), the second antibody will be an antibody prepared against rabbit γ-globulin. The addition of the second antibody will result in a precipitate containing the antibody-bound LH, which can be separated from the supernatant by centrifugation. Systems using a second antibody which have been coupled to insoluble particles have also been described (Dericks-Tan and Taubert, 1975).

For steroid hormones, one of the most popular separation procedures is the adsorption of the free hormone to dextran-coated charcoal. After the addition of the charcoal, the separation of free and antibody-bound steroid is achieved through centrifugation. This method is rapid and efficient in separating free and bound steroid hormones. However, the charcoal can also adsorb some of the antibody-bound steroid, which is called "stripping." In order to control this, timing of the reaction is important as well as the temperature. Carrying out the reaction at 4°C has been found to limit this dissociation (Abraham, 1974).

4. Reliability Criteria

The reliability of a radioimmunoassay analysis depends on its specificity, sensitivity, accuracy, and precision.

a. Specificity. The specificity of a radioimmunoassay, or freedom from interference by substances other than the one to be measured, is dependent on several different factors, the most important being the specificity of the antiserum used. Demonstration of specificity for radioimmunoassay of large protein hormones such as LH and FSH is relatively difficult and relies more or less upon indirect criteria. Since it is not possible to synthesize these hormones, they have to be isolated and purified from biological material. The purity of such preparations is variable, and the most common cause of nonspecificity for these hormones is impurity. A relatively common finding is the cross-reaction of TSH with antibodies to LH, and vice versa. Antibody specificity is usually demonstrated by testing the binding of other hormones to the antibody. If, for example, bovine TSH inhibits the binding of bovine LH to an antibody to bovine LH, this indicates that the antiserum used is nonspecific or that the TSH preparation contains LH. If the inhibition curves are

parallel, the latter explanation is likely because the parallelism indicates the same binding kinetics. However, parallelism in itself is not adequate proof of specificity. Both LH and TSH are composed of two subunits. The α subunit is identical for the two hormones, while the β subunit is unique for each hormone. It is possible that the antiserum contains binding sites which will react only with the α subunit. In such a case, the dose–response curve of LH and TSH utilizing such an antisera will be parallel, and the assay system will not be hormone specific and thus will be invalid for the measurement of LH (Niswender and Nett, 1977). Further purification of a hormone interfering in an assay will usually reveal the reason for its interference.

In the case of radioimmunoassay techniques for steroid hormones and prostaglandins, the same proof of specificity has to be undertaken. Here the situation is simpler, since small molecular weight hormones can easily be purified and, in most cases, produced synthetically. Furthermore, comparison of radioimmunoassay results with results obtained by mass fragmentography gives very valuable information, since the latter technique can be considered as an absolute method of proof of structure and thus specificity.

Some idea as to the specificity of an antiserum to a steroid hormone can be gained from the position of the steroid molecule that is used as the anchoring point to the protein (Fig. 2). If an antiserum to estradiol-17β is produced through the use of an antigen conjugated via the hydroxyl group at carbon 17 of the steroid, the resulting antiserum will react almost equally well with estrone and thus would have a relatively poor specificity. This is because the only structural difference between the estradiol-17β and estrone steroid skeletons is the configuration at position 17 (Fig. 2). When this position is used for conjugation, the resulting antiserum will recognize the remaining steroid structure which is identical for both estrone and estradiol-17β. In general, steroid antibodies are more specific for the portion of the steroid molecule which protrudes from the carrier protein and less specific for the portion of the steroid used for linkage to the protein. Thus, in the case of estradiol-17β, highly specific antibodies have been developed after immunization with conjugates when carbon 6 of the B ring has been used as the site of attachment to the protein (Exley *et al.*, 1971; Boilert *et al.*, 1973; Kohen *et al.*, 1975; Niswender *et al.*, 1975).

By use of the same fundamental principle concerning the selection of appropriate sites for conjugation, specific antibodies have been produced against progesterone conjugated to the protein through carbon 11 (Niswender and Midgley, 1970; Thorneycroft and Stone, 1972; Kohen *et al.*, 1975) and to testosterone through carbon 1 and 3 (Furuyama *et al.*, 1970; Niswender and Midgley, 1970; Kohen *et al.*, 1975).

Protein hormone determinations by radioimmunoassay are often performed in blood serum or blood plasma. The influence of serum or plasma on the binding of the tracer to the antibody must be investigated. The assay of different amounts of serum or plasma should result in curves parallel to those obtained with protein hormone standard.

In most steroid hormone radioimmunoassays, the hormone is extracted by an organic solvent from a blood plasma sample (Fig. 4a–d). Most radioimmunoassay procedures designed to measure progesterone utilize antisera developed against a progesterone-11-protein conjugate. Most such antisera described show a minor cross-section with corticosteroids (DeVilla *et al.*, 1972; Spieler *et al.*, 1972; Thorneycroft and Stone, 1972). By using a nonpolar solvent such as petroleum ether for the extraction of progesterone from serum or plasma samples, about 80–90% of progesterone is extracted, leaving the more polar corticosteroids in the plasma (Johansson, 1969). The use of such a selective extrac-

tion system increases the overall assay specificity. Certain radioimmunoassay systems require a further purification of the plasma extract (Fig. 4e) in order to achieve an acceptable specificity. Such purification steps might include thin-layer chromatography, column chromatography, etc.

In the case of prostaglandin radioimmunoassay systems, demonstrating specificity is usually no problem. The main problem in radioimmunoassay of primary prostaglandins is that they are formed in large amounts from various sources, e.g., platelets, when the blood sample is taken (Samuelsson *et al.*, 1975; Granström and Kindahl, 1976). The concentrations of prostaglandin $F_{2\alpha}$ reported in blood serum or plasma appear, in most cases, to be 100–1000 times higher than the actual values (Granström and Kindahl, 1976). The primary prostaglandins have a very short half-life in the circulation (Hamberg and Samuelsson, 1971; Granström, 1972) and are rapidly converted to their corresponding 15-keto-13,14-dihydro derivatives. The latter have a considerably longer half-life and occur in higher concentrations than the parent compounds (Béguin *et al.*, 1972). Radioimmunoassay systems utilizing antibodies to 9α,11α-dihydroxy-15-ketoprost-5-enoic acid and 5α,7α-dihydroxy-11-ketotetranorprosta-1,16-dioic acid have been developed (Granström and Samuelsson, 1972; Granström and Kindahl, 1976). Most problems involved in the determination of the primary prostaglandins are avoided if their main metabolites, the 15-keto-13,14-dihydro compounds, are measured.

b. Sensitivity. The sensitivity of a radioimmunoassay is defined as the smallest quantity of hormone which the assay can reliably distinguish from a zero amount of the hormone. Usually, two kinds of sensitivity are evaluated. The sensitivity of the standard curve is defined as the smallest amount of hormone which is significantly different from zero at the 95% confidence limit. However, the most meaningful sensitivity to establish is the smallest amount of hormone that can be measured in the aliquot of biological fluid.

c. Accuracy. The accuracy of an assay is defined as the extent to which a measurement of a hormone agrees with the exact amount of the hormone. Accuracy is often determined by comparing radioimmunoassay data with values determined by other procedures, such as gravimetry, gas–liquid chromatography, and mass spectrometry. For steroid hormones and prostaglandins, accuracy is also often tested by recovery experiments. Different amounts of hormones are added to a biological fluid, e.g., blood plasma, which contains low concentrations of the hormone, and the amount of hormone measured in the assay is then compared with the amount of hormone added.

d. Precision. Two types of precision are usually evaluated. The within-assay precision is determined from duplicate measurements of the same sample within the same assay. The between-assay precision is evaluated from replicate determinations of the same sample in different assays. Usually, the between-assay variance is greater than the within-assay variance. Between-assay variance should be checked with each assay of a certain hormone through the use of a pool plasma containing a set amount of the hormone. A further refinement is to use two different plasma sets, one containing a lower and another a higher hormone concentration. Within-and between-assay variations in radioimmunoassay procedures are usually greater than for ordinary routine procedures used in clinical chemistry.

B. Competitive Protein Binding Assay

The basic principle of the competitive protein binding assay is similar to that of radioimmunoassay (Fig. 3). However, the competitive protein binding assay utilizes naturally occurring binding proteins instead of an antibody to the hormone. The criteria discussed for assay reliability of radioimmunoassay techniques are also applicable for techniques utilizing binding proteins.

Corticosteroid-binding globulin, or transcortin, is an α-globulin present in the blood of several species including human beings, dogs, domestic fowl, and horses. Transcortin has a relatively high affinity for progesterone, 17α-hydroxyprogesterone, cortisol, and corticosterone and, as a result, has been used for the determination of these hormones (Murphy, 1964; Neill *et al.*, 1967). The binding properties of transcortin from different species, and the steroid complexes formed, have been described (Seal and Doe, 1966; Murphy, 1967).

Androgens and estrogens are bound to another protein present in the blood plasma of some species. This protein is referred to as sex hormone steroid-binding globulin and has been used for the determination of androgens (Vermeulen and Verdonck, 1968) and estrogens (Korenman *et al.*, 1969).

Receptor assay systems for the determination of gonadotropic hormones have been described. However, the affinity of the receptor for the hormone is relatively low, which limits the sensitivity of these assay systems (Catt and Dufau, 1973).

Antibodies to hormones possess a higher affinity and specificity than do transcortin and sex hormone steroid-binding globulin. The general trend in hormone analytical procedures is that techniques utilizing binding proteins are gradually being replaced by radioimmunoassay techniques. However, the use of naturally occurring binding proteins is interesting in itself and has the possibility of furnishing information on the role of binding proteins and receptor proteins. Finally, it should be recognized that there is considerable variation in the occurrence of binding proteins in the blood of domestic species of animals.

C. Summary

The use of radioimmunoassay and competitive protein binding techniques has dramatically increased our knowledge and understanding of reproductive endocrine mechanisms in domestic species. These procedures are relatively simple to perform, inexpensive, sensitive, and specific. The equipment needed for carrying out these assays includes detectors for γ and tritium radiation and a high-capacity refrigerated centrifuge. Data gained from these techniques have resulted in characterization of endocrine changes throughout the estrous cycle and pregnancy in most domestic animals.

III. PHYSIOLOGY OF REPRODUCTIVE HORMONES IN THE FEMALE

This presentation is relatively brief as to its coverage of endocrinological events during the reproductive cycle. Readers interested in a more complete presentation of hormonal events involved in reproduction are referred to texts dealing specifically with the subject. Also, some hormones, such as those of the thyroid and adrenal glands, are not covered in this presentation.

A. Puberty

The onset of puberty in animals in which reproductive activity is related to a seasonally changing factor(s), such as photoperiodicity, is usually influenced more by the time between birth and onset of breeding season than by age. Among the domestic species, the horse, sheep, and cat are seasonal breeders. Most cats that are born during the spring and summer reach puberty during the next breeding season at ages ranging from 11 to 6 months of age (Kehrer and Starke, 1975). In sheep, there is considerable variation as to age and, at times within breed, as to the onset of puberty. A range of 6–17.5 months has been reported for different breeds (Van Tonder, 1972). The onset of puberty in the mare usually occurs during the breeding season following birth at an age of between 12 and 17 months (Mitchell and Allen, 1975).

In cattle, the onset of puberty varies among breeds and occurs usually between 10 and 15 months of age. However, cyclic ovarian activity is usually present before estrus is manifested (Gonzalez-Padilla *et al.*, 1975; Bane *et al.*, 1978). In gilts, the first estrus usually occurs at 4–9 months of age. In the bitch, the corresponding figure is 6–12 months of age. Once breeding activity commences, it often continues for the life of the animal, provided that a good health status is maintained. The counterpart of primate menopause is not observed per se in domestic animals.

B. Estrous Cycle

The major endocrine events which precede ovulation have been well documented in the cow (Glencross *et al.*, 1973; Akbar *et al.*, 1974; Chenault *et al.*, 1975; Lemon *et al.*, 1975), ewe (Bjersing *et al.*, 1972; Nett *et al.*, 1974; Baird and Scaramuzzi, 1976), sow (Guthrie *et al.*, 1972; Shearer *et al.*, 1972), mare (Evans and Irvine, 1975; Geschwind *et al.*, 1975; Noden *et al.*, 1975; Stabenfeldt *et al.*, 1975b), dog (Jones *et al.*, 1973; Smith and McDonald, 1974; Concannon *et al.*, 1975; Hadley, 1975a; Holst and Phemister, 1975; Nett *et al.*, 1975), and cat (Shille *et al.*, 1979b). Some follicle growth occurs during the luteal phase in spite of the inhibitory nature of progesterone, the main secretory product from the corpus luteum. While follicles are not ovulated during the luteal phase in most species, the mare occasionally ovulates during the luteal phase (Stabenfeldt *et al.*, 1972b). With regression of the corpus luteum (or corpora lutea), follicles grow rapidly prior to ovulation due to gonadotropin stimulation. During development the follicles secrete increasing amounts of estrogen, which is important for onset of sexual receptivity, as well as for the initiation of the surge release of gonadotropins that is essential for the ovulatory process (Fig. 5). Estrogens initiate the release of LH and FSH through the release of Gn-RH. In most species the preovulatory surge of gonadotropins begins approximately 24 hours before ovulation and is usually of short duration, for example, 8–10 hours in the cow. The mare is an exception in that large amounts of LH are released during an 8- to 9-day period, with ovulation occurring on the third day (Geschwind *et al.*, 1975).

Following ovulation a corpus luteum is formed under the influence of pituitary gonadotropins. In most species, both LH and prolactin are considered to be luteotropins, although the role of prolactin is less certain. If pregnancy does not ensue the corpus luteum regresses, permitting follicular growth, and estrogen secretion from the growing follicle initiates the surge release of gonadotropins, resulting in ovulation. This well-timed sequence occurs repetitively at set intervals if not interrupted by pregnancy. Figure 6

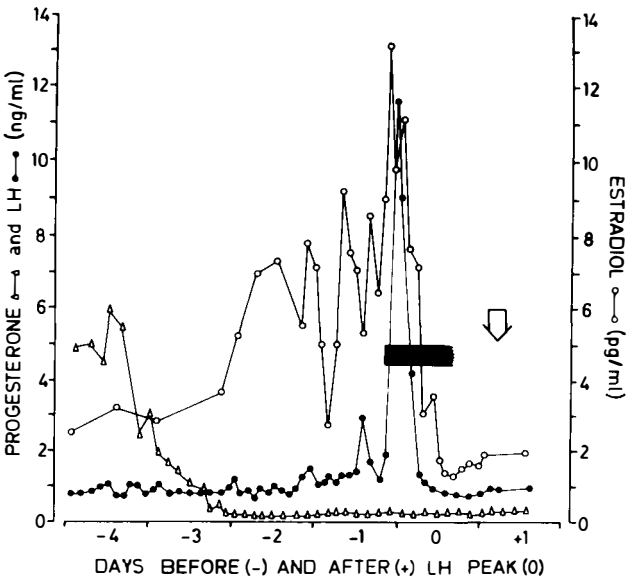


Fig. 5. Preovulatory events in the cow. The horizontal bar indicates the time of sexual receptivity, and the arrow indicates the approximate time of ovulation. [Progesterone data from Kindahl *et al.* (1976a) and estrogen, luteinizing hormone, estrus, and ovulation data from Chenault *et al.* (1975).]

presents a summary of estrous cycle activity in common domestic animal species. Seasonal breeders, such as the mare, ewe, and queen, undergo cyclic ovarian activity only during the breeding season, while the cow, sow, and bitch are affected little, if any, by photoperiod and can have cyclic ovarian activity the entire year. Estrous cycle length is approximately 3 weeks in the cow, mare, and sow and 2.5 weeks in the ewe. The bitch has a much longer estrous cycle, often being greater than 80 days from the beginning of follicle growth through complete regression of the corpus luteum. The interval between cyclic ovarian activity in the bitch is extended even further by the occurrence of a 4- to 5-month anestrum period following regression of the corpora lutea. The cat is an induced ovulator requiring coital stimulation for ovulation and thus for corpus luteum formation. In the absence of coitus, follicles develop every 15–20 days, with follicular growth and regression occupying approximately half, or 8 days, of each period. An ovulatory, nonfertile mating results in the formation of corpora lutea that persist for approximately 35 days.

C. Control of the Corpus Luteum

The regression of the corpus luteum (luteolysis) is the key event responsible for the well-timed estrus cyclicity seen in most domestic species. The importance of the uterus in the control of corpus luteum life span has been documented through hysterectomy in the cow, ewe, sow, and mare (du Mesnil du Buisson and Dauzier, 1959; Ginther and First, 1971; Stabenfeldt *et al.*, 1974a; Wiltbank and Casida, 1956). Removal of the uterus from these species during the luteal phase results in a pronounced prolongation of the luteal activity. It is now well established that the uterus in these species synthesizes and releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which causes the corpus luteum to regress (Pharris and Wyngarden, 1969; Harrison *et al.*, 1972; McCracken *et al.*, 1972). The temporal release

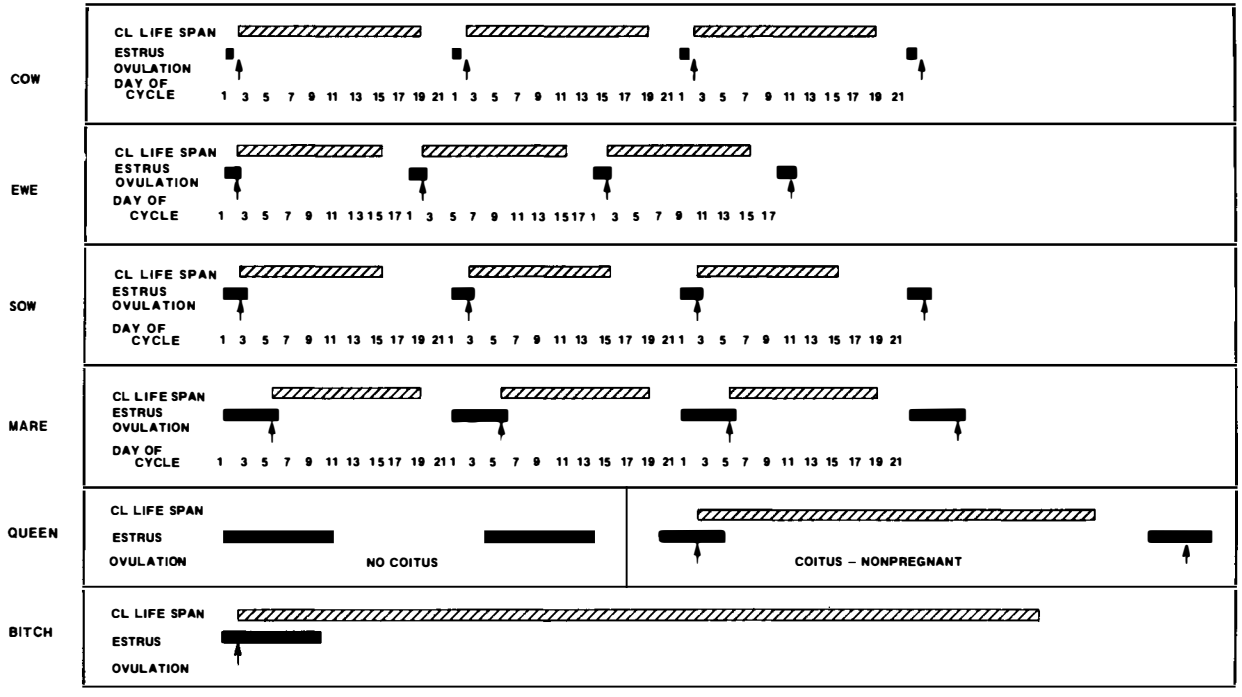


Fig. 6. Comparative data on the duration of estrus, time of ovulation, and duration of corpus luteum (CL) function in the cow, ewe, sow, mare, queen, and bitch. (From Stabenfeldt, 1974.)

patterns of $PGF_{2\alpha}$, usually in spurts lasting a few hours, have been described in the ewe (Baird *et al.*, 1976; Barcikowski *et al.*, 1974; Bland *et al.*, 1971; Harrison *et al.*, 1972; Roberts *et al.*, 1975), sow (Gleeson *et al.*, 1974; Moeljono *et al.*, 1977) and cow (Nancarrow *et al.*, 1973; Shemesh and Hansel, 1975). Some of the problems involved in determining $PGF_{2\alpha}$ (short half-life, formation by platelets at collection) can be avoided if the main blood plasma metabolite is determined. Data are available on the pattern of release and the concentration of the metabolite during luteolysis in the cow (Peterson *et al.*, 1975; Kindahl *et al.*, 1976a,b), ewe (Kindahl *et al.*, 1976c; Peterson *et al.*, 1976), mare (Kindahl *et al.*, 1976c; Neely *et al.*, 1979), and sow (Shille *et al.*, 1979a) (Fig. 7). Regression of corpora lutea is usually accomplished within 48 hours following the onset of the prostaglandin release. Factors that control the initiation of $PGF_{2\alpha}$ synthesis and release are not yet understood. In the dog and cat, $PGF_{2\alpha}$ does not appear to be involved in luteolysis although its precise role is still uncertain.

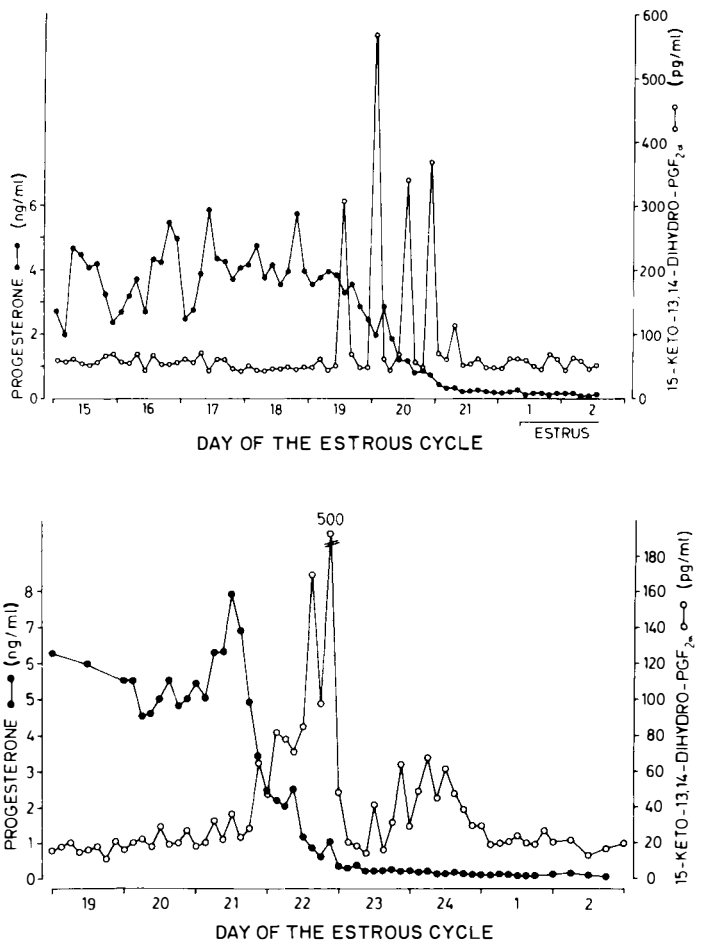


Fig. 7. The release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at the time of luteolysis, as indicated by changes in the main metabolite of $PGF_{2\alpha}$ [15-keto-13,14-dihydro-$PGF_{2\alpha}$ (○———○)] and progesterone (●———●) during the estrous cycle of the cow (upper) and the mare (lower). (Data from Kindahl *et al.*, 1976c.)

D. Early Pregnancy

Suppression of prostaglandin release is essential for the establishment of pregnancy in the species (cow, ewe, mare, and sow) having this compound as the luteolysin (Barcikowski *et al.*, 1974; Kindahl *et al.*, 1976a; Nett *et al.*, 1976; Peterson *et al.*, 1976; Moeljono *et al.*, 1977; Shille *et al.*, 1979a). The rapid elongation of fetal membranes, which precedes the critical time of the initiation of luteal regression by about 3 days in the nonpregnant animal, appears to be important for the prevention of prostaglandin release. In the sow it has been suggested that estrogens synthesized by the early preimplantation embryo may be the messenger for maternal recognition of pregnancy (Perry *et al.*, 1976; Bazer and Thatcher, 1977). Bazer and Thatcher (1977) suggested that the establishment of pregnancy involves redirection of PGF_2 secretion from the blood vascular system (endocrine) to the uterine lumen (exocrine). Significant increases in intraluminal PGF_2 have been observed in pigs (Frank *et al.*, 1978), cattle (Thatcher *et al.*, 1979), but not horses (Zavy, 1979) beginning about two weeks post-conception. Possible uterine antiluteolytic factors include PGE_1 (Hoyer *et al.*, 1978) and PGE_2 (Magness *et al.*, 1978). Increased production of PGE_2 by endometrial tissue has been demonstrated in ewes during early pregnancy (Ellinwood *et al.*, 1979). The net result is that luteal function is maintained in the cow, ewe, mare, and sow during early pregnancy. Suppression of $PGF_{2\alpha}$ release appears not to be important for the establishment of pregnancy in the dog and cat.

E. Pregnancy and Parturition

In the cow, the presence of a corpus luteum is necessary for the maintenance of pregnancy in a vast majority of animals (Edqvist *et al.*, 1973b; Fairclough *et al.*, 1975). In the ewe, the presence of corpora lutea is required for the first 50–60 days of gestation. After this time period, the fetoplacental unit secretes significant amounts of progesterone. It has been suggested that this progesterone synthesis is caused by a luteotropin of placental origin called ovine placental lactogen. The pig, like the cow, requires luteal support throughout gestation.

The necessity of the secondary corpora lutea formed in the mare between days 40 and 60 of gestation has been discussed over the years. The secondary corpora lutea may be a result of PMSG secretion by the endometrial cups which are formed from a circular band of cells of placental origin (chorionic girdle cells) that invade the endometrium and form isolated endocrine organs of temporary function (Allen, 1969). The secondary corpora lutea are formed either through the luteinization or rupture (ovulation) of follicles (Squires *et al.*, 1974). Recently, it has been demonstrated that PMSG has a close immunological relationship with equine LH (Farmer and Papkoff, 1979); further, a luteotropic effect of PMSG has been shown by incubation studies of CL (Squires *et al.*, 1979). The clinical importance of this resides in the fact that if fetal loss occurs after the formation of endometrial cups, the continuing PMSG production will support luteal activity, even to the point of making lysis of these CL difficult in conjunction with pharmacological administration of prostaglandin F_2. Progesterone support of pregnancy in the mare begins to be taken over by the placenta as early as day 50 of gestation but is not complete for all mares until approximately 140 days, a time that coincides with regression of both the primary and secondary corpora lutea (Holtan *et al.*, 1979).

The dog requires the presence of corpora lutea for practically all of gestation. Sokolowski (1971) found that ovariectomy as late as day 56 postbreeding resulted in

premature delivery. The cat appears to be less dependent upon the presence of corpora lutea for the maintenance of pregnancy in that ovariectomy at day 49 of gestation did not result in abortion; ovariectomy at day 46 did cause premature delivery.

One of the first important endocrine changes which occurs prior to parturition in the cow, ewe, and sow involves an increase in the synthesis and release of estrogens by the fetoplacental unit (Fig. 8). This increased estrogen synthesis is reflected in elevated plasma estrone concentrations in the pregnant cow beginning between 30 and 20 days prepartum (Henricks *et al.*, 1972a; Edqvist *et al.*, 1973a; Fairclough *et al.*, 1975). In the ewe, the increase in estrogen occurs abruptly about 2 days before parturition (Challis *et al.*, 1971; Liggins *et al.*, 1972; Thorburn *et al.*, 1974). The pig shows two distinct peaks of estrogen during gestation, one at around 25–30 days and the other beginning 1 week before delivery (Robertson and King, 1974) (Fig. 9).

A different estrogen pattern is observed in the mare in that significant estrogen production begins early in gestation (around 90 days), with high values obtained from day 150 to parturition (Nett *et al.*, 1973; Lovell *et al.*, 1975). The mare produces two estrogens during pregnancy that are unique to equids, equilin and equilenin, both of which have unsaturated B rings (Girard *et al.*, 1932). Little evidence of estrogen production during pregnancy has been reported for the dog (Edqvist *et al.*, 1975; Hadley, 1975b). Estrogen

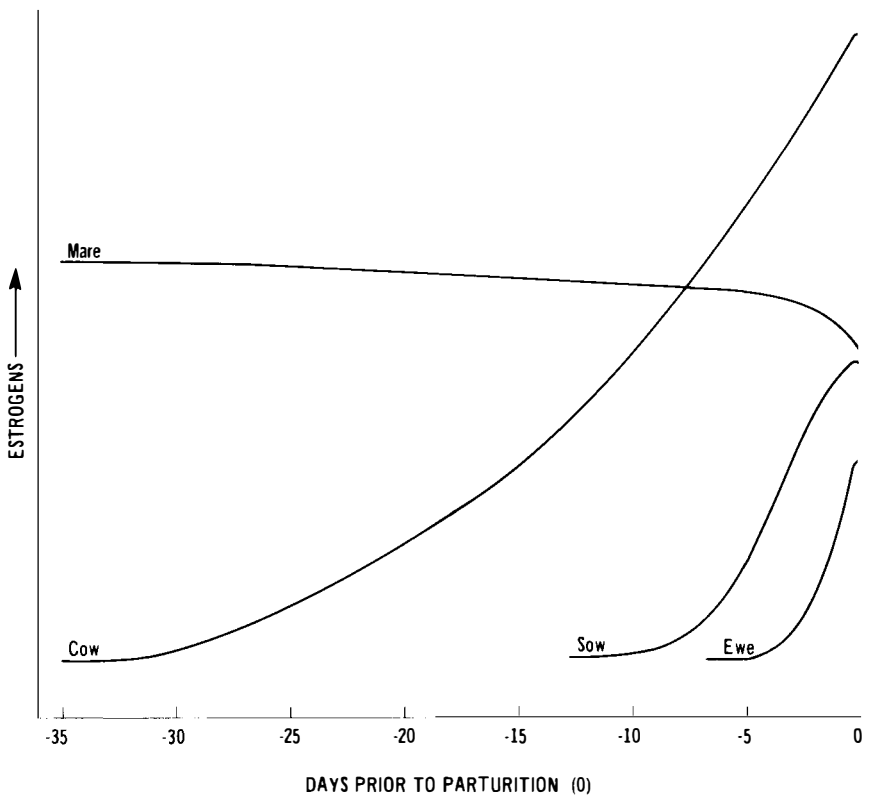


Fig. 8. Prepartum plasma estrogen patterns in the mare, cow, sow, and ewe [as presented by Nett *et al.* (1973), Edqvist *et al.* (1973a), Baldwin and Stabenfeldt (1975), and Thorburn *et al.* (1974), respectively].

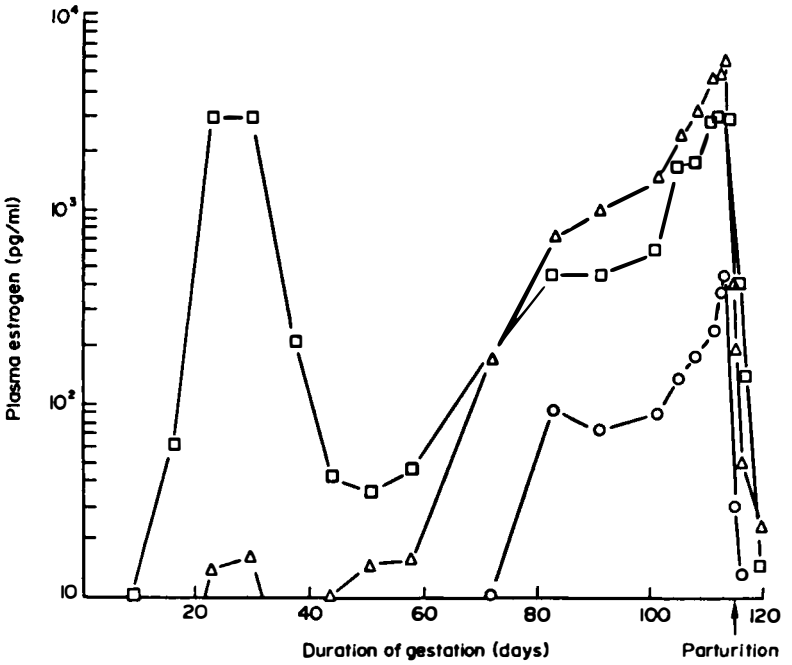


Fig. 9. The concentration of unconjugated estrone (△), estradiol-17β (○), and estrone sulfate (□) in the plasma of a gilt at implantation throughout gestation and at parturition. (Reproduced from Robertson and King, 1974.)

production by the fetoplacental unit in the cat also appears to be minimal during gestation (Verhage *et al.*, 1976).

In the cow and bitch, parturition is preceded by an abrupt fall in the peripheral blood plasma concentrations of progesterone between 48 and 24 hours prior to delivery (Stabenfeldt *et al.*, 1970; Edqvist *et al.*, 1973a; Smith and McDonald, 1974). In the ewe, mare, queen, and sow, partial withdrawal of progesterone occurs prior to delivery (Stabenfeldt *et al.*, 1972a; Baldwin and Stabenfeldt, 1975; Verhage *et al.*, 1976; Noden *et al.*, 1978). In the cow (Fairclough *et al.*, 1975; Edqvist *et al.*, 1976, 1978) and ewe (Liggins *et al.*, 1972; Thorburn *et al.*, 1974), it has been demonstrated that the release of prostaglandin initiates the regression of the corpus luteum and thus is responsible for the decline in progesterone. High estrogen and prostaglandin concentrations combined with low progesterone concentrations increase the contractile state of the uterus. Prostaglandins may also initiate cellular changes within the cervix which result in softening and dilation. Cervical stimulation, a result of the initial entry of the fetus into the pelvic canal, results in the reflex release of oxytocin from the posterior pituitary. This increases the intensity of the uterine contractions and thus aids the final steps of the delivery process.

IV. CLINICAL ASPECTS OF REPRODUCTIVE ENDOCRINOLOGY

The development of radioimmunoassay and competitive protein binding techniques for hormone determinations in domestic species has created laboratory procedures that are

relatively simple to perform, inexpensive, specific, and sensitive and that have potential usefulness as diagnostic aids in clinical work. For some reproductive hormones, relatively well defined indications for their clinical use are known at present and more are likely to be identified in the future.

For hormone analyses to be useful as a diagnostic tool, certain criteria have to be fulfilled. The concentration of the hormone at the site of sampling (usually a peripheral vein) should closely correlate with the release of the hormone from the endocrine gland. It is preferable that the release pattern of the hormone be steady, not of a pronounced pulsative character with large short-lasting fluctuations, which allows valid information to be obtained on the secretory status of the endocrine gland from one sample. Several reproductive hormones do not fulfill these criteria, and thus their determination is not as useful from a routine diagnostic view. For example, the duration of the LH peak observed in conjunction with ovulation is short in most domestic species except the horse. In the cow, the preovulatory LH peak has a duration of 8–10 hours, requiring samples to be obtained every 4 hours in order to detect the peak (Karg *et al.*, 1976). In the mare, the duration of the peak is considerably longer, 8–9 days, which allows a less frequent sampling interval (Geschwind *et al.*, 1975). However, the long duration of LH peak in the mare prevents the determination from being useful in predicting ovulation.

One reproductive hormone, progesterone, has been found to be of significant clinical value in most domestic species. Other hormones with established or potential clinical use will be discussed further for each species. In most cases the determination of hormones has been utilized as pregnancy diagnostic tests.

It is worth keeping in mind that the analysis of hormones as a diagnostic aid in solving clinical problems will not replace, but should only supplement, the information that can be gained by a careful clinical examination, including rectal palpation in the case of reproductive disorders.

A. Cattle

1. Progesterone

Several reports are available on the progesterone concentration in blood during early pregnancy in cattle (Pope *et al.*, 1969; Henricks *et al.*, 1970; Stabenfeldt *et al.*, 1970, 1976; Glencross *et al.*, 1973). Another finding of importance is that progesterone can be measured in the milk of lactating cows. The concentration of progesterone in milk has been shown to reflect with a high degree of accuracy the concurrent blood plasma concentration (Laing and Heap, 1971; Heap *et al.*, 1973; Hoffmann and Hamburger, 1973; Ginther *et al.*, 1974). Basically, the same type of assay system can be used for both blood plasma and milk. In general, three different approaches have been taken for the determination of progesterone in milk. These include the assay of progesterone in whole milk, milk fat, and fat-free milk (see Vol. 132, *British Veterinary Journal,* for further information).

The difference which exists in both blood plasma and milk progesterone concentrations 19–24 days after a fertile breeding as compared to a nonfertile breeding have been used as an early pregnancy diagnosis test (Shemesh *et al.*, 1968; Robertson and Sarda, 1971; Wishart *et al.*, 1975) (Fig. 10). The plasma progesterone concentration in pregnant cows at 21 days postbreeding is almost always 2 ng/ml (6.4 nmole/liter) and usually 6–8 ng/ml (19.1–25.5 nmole/liter), as compared to 0.5 ng/ml (1.6 nmole/liter) or less in the nonpregnant animal at the same time. The finding of elevated progesterone concen-

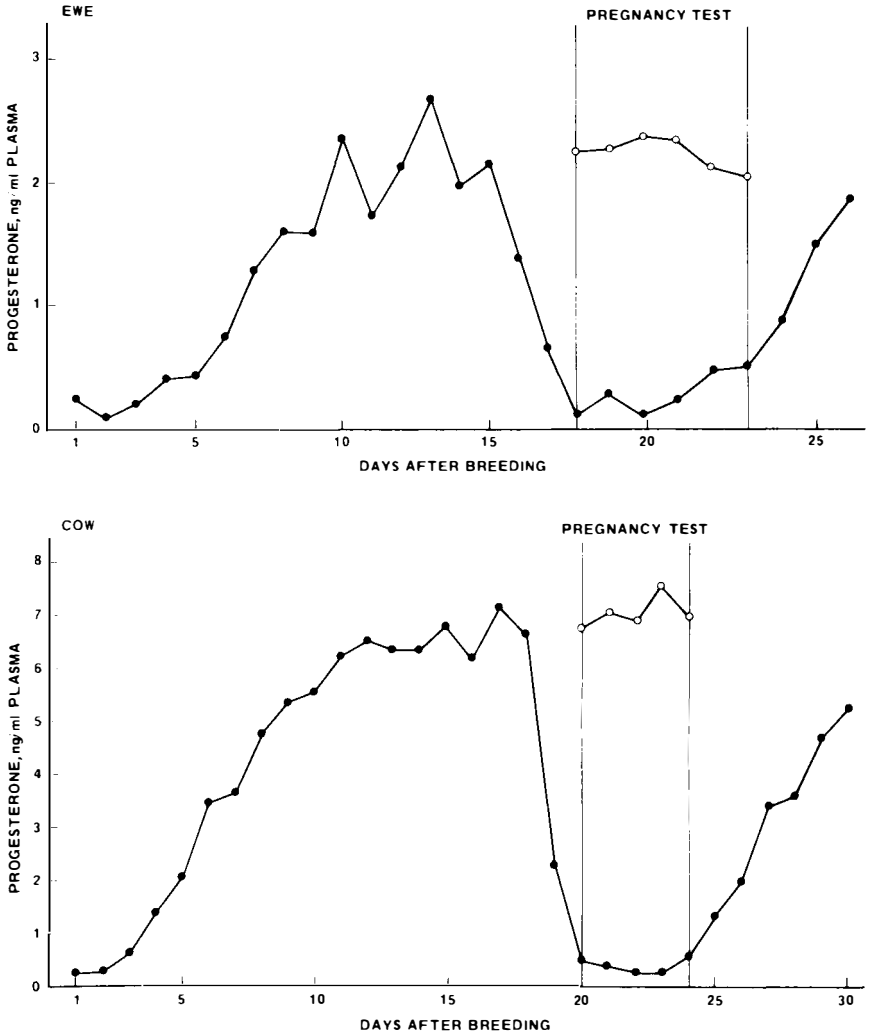


Fig. 10. Time after breeding for utilizing progesterone analysis as a means of pregnancy diagnosis. Progesterone content in nonpregnant (●———●) ewes (upper) and cows (lower) compared with progesterone content of pregnant animals (○———○). [Ewe and cow data from Stabenfeldt *et al.* (1969c; 1972a) and Stabenfeldt *et al.* (1969b; 1970), respectively.]

trations, however, is not necessarily indicative of the presence of a fetus *in utero*. If a cow has a prolonged estrous cycle after breeding, for example, a 27-day cycle instead of a 21-day cycle, this animal could have a corpus luteum on day 21, and thus the analytical progesterone result would be positive and the animal would be falsely considered pregnant. The accuracy of the forecast for pregnancy (positive forecast) thus can be relatively low, in most cases ranging between 75 and 90%. The negative forecast, however, will be more accurate since cows having low progesterone levels in milk or blood 21 days postbreeding will probably not be pregnant. The accuracy of the forecast will be dependent to some extent on the borderline value used for separating progesterone concentrations in pregnant cows from those in nonpregnant animals at an early stage to facilitate another

breeding as soon as possible. The accuracy of the positive forecast might be increased if progesterone analyses are also carried out on samples obtained at the time of insemination. Cows inseminated during the luteal phase will be in the luteal phase 21 days later, and, if a sample is analyzed at this time, the animal will be considered pregnant even though it is nonpregnant. The examination of cows at 40 days postbreeding to confirm the present of a fetus in animals previously designated pregnant will minimize the problem of false-positive diagnoses.

The use of progesterone determinations in cattle enable one to monitor the secretory status of the corpus luteum. If done at approximately 5-day intervals for the duration of an estrous cycle, ovarian cyclicity can be established in cases in which estrus is poorly expressed or missed. Progesterone determinations have also been used for the retrospective confirmation of the absence of an active progesterone-secreting corpus luteum at the time of insemination in cattle. If high progesterone levels are recorded at this time, this indicates that the insemination has been performed in the presence of a corpus luteum, e.g., at the wrong time. Since inadequate detection of estrus is the most common cause of low fertility in herds utilizing artificial insemination, the determination of progesterone levels at the time of insemination might be a useful tool when herds with fertility problems are encountered. The use of milk progesterone in these cases will allow the farmer to obtain the sample. Karg *et al.* (1976) indicated that improper timing of insemination, as judged from milk progesterone determinations, was performed in 15% of the cases in a controlled field test, while under practical field conditions the figure rose to 26%. In a limited field trial carried out in the Netherlands, corresponding figures were from 0 to 3.1% (Van de Wiel *et al.*, 1978), while in Sweden a figure of 4% was found (R. Oltner and L.-E. Edqvist, unpublished observations). The use of progesterone determinations in milk obtained at the time of insemination in cows with questionable heat signs and inconclusive genital tract findings can serve as a valuable tool for educating the staff responsible for this work.

Another area in which progesterone determinations are useful is the confirmation of a clinical diagnosis, e.g. anestrus, failure to manifest estrus, cystic ovaries, pyometra. They are also useful for following the effect of an applied treatment. In the case of ovarian cysts in cattle, treatment is usually done with human chorionic gonadotropin (HCG) or Gn-RH. However, when the cysts are partly luteinized, a better therapeutic effect can be obtained using $PGF_{2\alpha}$. Diagnosis of the type of cyst present by rectal examination sometimes produces inconclusive results. The determination of progesterone in such cows will aid in the establishment of a correct clinical diagnosis. Progesterone analysis is also useful for assessing the effect of a particular treatment. If milk progesterone analyses are utilized, the farmer can collect samples following the treatment, and the clinical response of the animal can be evaluated from subsequent progesterone analysis in milk samples. In cases of follicular cysts, the original progesterone level should be low, and, following successful treatment (HCG or Gn-RH), a progressive luteinization of the cysts should occur, resulting in increased progesterone concentrations in subsequent milk samples. In the case of a luteinized cyst, the original progesterone value will be higher than for the follicular cyst, and after treatment (prostaglandin) the progesterone level should decrease, reflecting the lysis of the luteal cyst (Hoffmann *et al.*, 1976). The same principle can also be applied in cases of pyometra and mummification of the fetus, wherein a persistence of luteal activity is a common finding.

Progesterone determinations in blood have been used to assess the success of superovu-

lation in cattle in conjunction with ova transfer (Henricks *et al.*, 1972b; Lemon and Saumande, 1972; Spilman *et al.*, 1973; Booth *et al.*, 1975). Agthe and co-workers (1976) found a positive correlation between progesterone concentrations and the number of corpora lutea formed following superovulation, although others did not find such a clear relationship (Lamond and Gaddy, 1972; Rajamahendran *et al.*, 1976; Solti *et al.*, 1978).

2. *Other Hormones*

Prolonged gestation in both Holstein-Fresian and Guernsey cows has been described in conjunction with fetuses having malfunctioning pituitaries and adrenal glands. It has been shown that cows carrying such fetuses do not exhibit increased peripheral blood plasma concentrations of estrone around the expected due day (Stabenfeldt *et al.*, 1975a). The determination of blood plasma concentrations of estrone could aid in the diagnosis of the condition.

Placental lactogen has been demonstrated in pregnant cows, and in the future it is possible that the determination of placental lactogen could serve as a more accurate pregnancy diagnostic test than progesterone analysis (Buttle and Forsyth, 1976).

B. Sheep

1. *Progesterone*

The same basic principle as employed in cattle of using progesterone determination as an early test for pregnancy has also been used in sheep (Robertson and Sarda, 1971) (Fig. 10). In the ewe, the progesterone determination has to be carried out on blood samples since most breeds of sheep are not lactating at the time of breeding. Maximal luteal phase progesterone levels in the ewe are approximately 2–4 ng/ml (6–13 nmole/liter), while the concentration at estrus is from 0.15 to 0.8 ng/ml (0.5–2.5 nmole/liter). A relatively marked increase in the progesterone concentration from 2–4 ng/ml (6–13 nmole/liter) to 12–20 ng/ml (38–63 nmole/liter) occurs between days 60 and 125 of pregnancy. This rise is due to an increased progesterone production from the placenta/conceptus. The contribution of the corpus luteum to the progesterone level at this time is relatively low.

The accuracy of the forecast and the pitfalls associated with using progesterone determinations as a pregnancy test in the ewe are similar to those previously outlined for cattle. However, in the ewe, the accuracy of the forecast of such a test will be increased if samples are obtained relatively late in the gestation period. The progesterone concentration at this stage is considerably higher than during the luteal phase, allowing differentiation between nonpregnant and pregnant ewes. Furthermore, due to the limited reproductive season in some breeds, ewes not bred at this time enter anestrum and, as a result, have low progesterone concentrations. In a trial which determined plasma progesterone concentrations in 46 ewes on the eighteenth and seventyth day after mating, correct pregnancy diagnoses were made for 80.4% of ewes using data obtained on the eighteenth day and 93.5% using data obtained on the seventyth day (Weigl *et al.*, 1975).

Since the elevated progesterone concentration seen in blood during the later part of pregnancy in the ewe is due to an increased production by the placenta/conceptus, several attempts have been made to use progesterone determinations to differentiate between single and twin pregnancies, with the object being the adjustment of the feeding regimen of the dam. The use of progesterone determinations to predict litter size has, in most cases, been poor (Stabenfeldt *et al.*, 1972a; Sarda *et al.*, 1973; Emady *et al.*, 1974;

McNatty and Thurley, 1974), although some investigators have established a good correlation (Gadsby *et al.*, 1972; Van de Wiel *et al.*, 1976). Van de Wiel and co-workers (1976) predicted litter size in 34 Texel ewes using the plasma progesterone concentration at 70–110 days after mating. Using the classes 0–1, 2–3, and $\geq$ 4 lambs per ewe, fluorimetric and radioimmunoassay techniques for progesterone were 82.9 and 80.0% correct, respectively. When 194 ewes of different breeds were used, the fluorimetric technique produced correct classifications in 65% of the cases. In general, the larger the number of fetuses, the more accurate the prediction.

2. *Other Hormones*

Ovine placental lactogen (oPL) has been demonstrated in the peripheral blood of pregnant ewes (Kelly *et al.*, 1974). The increase in maternal oPL concentrations parallels the rise in progesterone which occurs during days 60–125 of pregnancy. The determination of oPL concentrations might be used as a basis for a specific pregnancy test in the ewe.

In the ewe, an abrupt increase of unconjugated estrogens (estrone and estradiol-17β) occurs about 2 days prior to parturition. It was demonstrated that a conjugated estrogen, namely, estrone sulfate, could be detected at around 100 days of gestation (Tsang, 1978). This steroid is very likely secreted from the fetoplacental unit. Whether determinations of estrone sulfate can be useful as a pregnancy test in the ewe remains to be elucidated.

C. Pig

1. *Progesterone*

Luteal phase progesterone concentrations in the pig are considerably higher than in cattle and sheep, namely, 20–50 ng/ml (65–160 nmole/liter), while concentrations at estrus are approximately 0.5 ng/ml (1.6 nmole/liter) (Stabenfeldt *et al.*, 1969a), similar to that observed in cattle and sheep. The difference in progesterone concentration between nonpregnant and pregnant animals 19–24 days after service has been used as an early pregnancy test (Robertson and Sarda, 1971; Edqvist *et al.*, 1972; Ellendorff *et al.*, 1976). In the pig, progesterone determination has to be performed on blood. Due to the sensitivity of the assay systems, the analyses can be performed on a small volume of blood (about ten drops), allowing the sample to be obtained through a small incision in an ear vein. The limitations associated with using progesterone determinations as a pregnancy test in the pig are similar to those previously discussed for cattle.

Progesterone analyses have also been used to determine ovarian activity in clinically anestrus gilts as well as to establish the stage of the estrous cycle in gilts (Larsson *et al.*, 1975). Larsson *et al.* (1975) found that correct dating of the estrous cycle could be done, in most cases, from two progesterone determinations obtained 1 week apart.

2. *Estrone Sulfate*

Previous measurements of estrogen concentrations in urine revealed a marked increase in estrogen around day 30 of pregnancy (Velle, 1958; Lunaas, 1962). Studies of blood concentrations of estrone sulfate in pigs during pregnancy showed patterns similar to those determined in urine (Robertson and King, 1974). In early pregnancy in the pig, it has been hypothesized that estrogen synthesized by the early preimplantation embryo may be the messenger for maternal recognition of pregnancy (Perry *et al.*, 1976) and that the elevated estrone sulfate concentrations in the maternal circulation during early pregnancy reflect

fetal synthesis. Determination of estrone sulfate in early pregnancy in the pig is thus a specific pregnancy test. The index of discrimination between estrone sulfate concentrations of pregnant versus nonpregnant pigs around 30 days after breeding is very high (Fig. 9).

In order to determine estrone sulfate concentrations, hydrolysis of the steroid has to be performed; then the estrone concentration can be determined by usual radioimmunoassay procedures. Most procedures developed for the determination of estrone sulfate are rather complicated and include several chromatographic steps (Loriaux *et al.*, 1971; Hawkins and Oakey, 1974). Recently, however, a relatively simple nonchromatographic radioimmunoassay method of estrone sulfate has been described (Carlström and Sköldefors, 1977).

During the latter part of pregnancy, the maternal blood concentrations of estrone sulfate, estrone, and estradiol-17β are very high and can thus be used to confirm pregnancy at this stage of the gestation period (Fig. 9).

D. Horse

1. PMSG

While pregnancy diagnosis has been done in mares through the measurement of PMSG, there are several drawbacks to its use. First, PMSG cannot be detected until about day 40 of gestation and thus is not useful for early pregnancy diagnosis. Second, the presence of PMSG does not guarantee the presence of a fetus. It has been shown that endometrial cups have some autonomy and can continue to secrete PMSG for a period of time in spite of loss of the fetus (Allen, 1969; Mitchell and Betteridge, 1973).

2. Progesterone

Progesterone analysis has been done to support the diagnosis of persistent corpus luteum (Stabenfeldt *et al.*, 1974b) (Fig. 11). The progesterone values of mares with persistent luteal activity (2–4 ng/ml; 6–13 nmole/liter) are often somewhat diagnostic of the clinical syndrome in that luteal activity is often dampened 14 days postovulation due to $PGF_{2\alpha}$

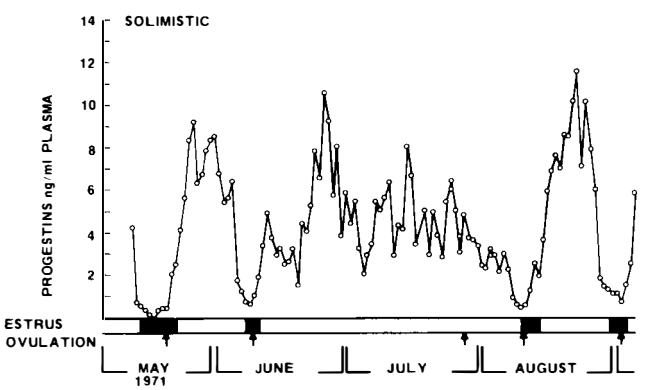


Fig. 11. Progestin concentrations in the blood of a nonpregnant mare with a persistent corpus luteum (luteal phase from about June 10 to August 10). Notice the regular estrous cycles before and after the period of the persistent corpus luteum. Also notice the ovulation that occurred late in July during the persistent luteal phase. Ovulation ↑; estrus, black horizontal bar. (Data from Stabenfeldt *et al.*, 1974b.)

release, albeit insufficient to cause complete luteolysis (Neely *et al.*, 1979). Progesterone analysis can also be useful in mares that fail to manifest sexual receptivity, yet have cyclic ovarian activity (Hughes *et al.*, 1973). Progesterone analysis at 5-day intervals over 20 days (approximately one estrous cycle length) can verify the presence or absence of cyclic ovarian activity. The time of ovulation can also be predicted within a 2- to 3-day interval with these results which can be helpful to the veterinary practitioner anticipating the next time of ovulation. Breeding may have to occur by artificial means in these situations.

3. Testosterone

Testosterone determinations have proven to be useful in verifying the diagnosis of granulosa-theca cell tumors in the mare (Stabenfeldt *et al.*, 1979). It was found that a large percentage of mares with ovarian tumors have elevated testosterone values. Leydig-like cells in the theca appear to be the source of testosterone. While testosterone concentrations are often 50–100% higher in affected animals (30–40 pg/ml; 104–139 pmole/liter) than in normal animals (20 pg/ml; 52 pmole/liter), the variation is relatively large, and several determinations may be advisable. Mares with granulosa-theca cell tumors that show male behavioral characteristics usually have testosterone concentrations >100 pg/ml (>347 pmole/liter) plasma.

Testosterone determinations in the male horse have been used as an aid in the diagnosis of cryptorchidism. Cox (1975) reported that horses with <40 pg/ml (<139 pmole/liter) plasma should be considered castrate, whereas animals with concentrations >100 pg/ml (>347 pmole/liter) should be considered as having testicular tissue present. Cox (1975) suggested that HCG administration is useful for resolving those animals which fall in the overlapping zone of 40–100 pg/ml (139–347 pmole/liter). While some cryptorchid animals have testosterone concentrations <100 pg/ml (<347 pmole/liter), most have values that range from 200 to 1000 pg/ml (694–3472 pmole/liter). Testosterone concentrations in intact males usually range from 1000 to 2000 pg/ml (3472–6944 pmole/liter) (Cox *et al.*, 1973; Berndtson *et al.*, 1974; Ganjam and Kenney, 1975). Ganjam and Kenney (1975) have suggested the use of estrogen analysis as an aid in detecting cryptorchidism, particularly in cases of bilateral involvement.

The experience of the authors indicates that the presence of aggressive type of behavior in suspected cases of cryptorchidism is not necessarily dependent upon elevated testosterone concentrations in that many patients referred because of behavioral problems are, in fact, castrate.

E. Dog

1. Progesterone

At present, there are few clinical reproductive situations in the female dog in which analysis of hormones affecting the reproductive system is useful. Occasionally, the authors have used progesterone analysis to confirm the occurrence of ovulation; progesterone concentrations usually begin to increase 24 hours following ovulation. This approach may be used in a retrospective analysis of a breeding cycle in an attempt to correlate the time of ovulation with other criteria such as vaginal cytological changes, or it may be used in conjunction with the breeding of an animal. In conjunction with the latter situation, bitches usually ovulate at the beginning of the period of sexual receptivity, remain sexually receptive during the first few days of luteal activity, and, in fact, are

fertile during this period of time (Holst and Phemister, 1974). Progesterone analysis can also be useful in cases of short estrous cycle intervals to determine if ovulatory failure is the basic cause. If so, progesterone concentrations would tend to remain low.

2. *Testosterone*

The most common endocrine test done in male dogs is for testosterone to check the secretory status of the Leydig cells. This is done in conjunction with fertility examinations, often times in stud animals that are presented as infertility cases following a prolonged show tour. Most of these animals have testosterone concentrations that are compatible with spermatogenesis, although values tend to be in the lower normal range. The range of testosterone values in dogs is considerable, varying from 1 ng/ml (3.5 nmole/liter) to 10 ng/ml (35.0 nmole/liter). The authors have observed a feminization syndrome in a dog in which concentrations of testosterone (25 pg/ml; 87 pmole/liter) compatible with the castrate state were found in the presence of small, hard testes; estrogen concentrations were only slightly elevated. Thus, it may be that the occurrence of feminizing syndromes requires only a small increase in estrogen, but in the virtual absence of testosterone.

F. Cat

1. *Estrogen*

There are currently no reproductive endocrine tests that are being used in the queen. Estrogen analysis may become useful in situations in which sexual receptivity is not manifested in conjunction with cyclic ovarian activity. Shille *et al.* (1979b) reported that some females failed to exhibit sexual receptivity even though normal follicular growth patterns were occurring as determined by estrogen analysis. The optimal breeding time for the cat is from 3 to 5 days after the beginning of follicular growth (first day of growth = day 1).

2. *Testosterone*

Testosterone analyses can be used to evaluate the endocrine portion of the testis of the male cat. The range of values is usually between 1 and 10 ng/ml (3.5–35 nmole/liter).

G. General Comments

In the foregoing presentation, concentrations of hormones have not been emphasized. This is because there is still some variability as to the values reported by various laboratories. It is important that clinical endocrinology laboratories understand and have experience with their assay systems in relation to particular clinical syndromes. For example, the actual concentration of progesterone during the follicular phase of the estrous cycle of a domestic animal species is approximately 100 pg/ml (318 pmole/liter) plasma. Some laboratories, however, report basal values of 1–2 ng/ml (3.2–6.4 nmole/liter) for progesterone. The authors have used 1 ng/ml (3.2 nmole/liter) as the lowest concentration of progesterone that is compatible with an actively secreting corpus luteum (especially for the cow, ewe, mare, and sow) and pay particular attention to values in this range, especially as to accuracy. It is possible to produce useful information with higher basal concentrations; however, the mark that divides active and inactive corpus luteum

function must be precisely known. The reference values developed by laboratories are dependent upon the type of assay used as well as the specificity of the binding protein, purification steps, and purity of reagents. There is a general tendency for values obtained from competitive protein-binding assays to be somewhat higher than those obtained by radioimmunoassay.

It is possible to have a wide range of values for a particular hormone and still have normal physiological conditions. Luteal phase progesterone concentrations in the cow, for example, range between 2 and 12 ng/ml (6–40 nmole/liter) with no adverse effect on the preparation of the animal for the nurture of a potential zygote(s). Hormone values can also depend upon the type of material used in the assay. In fat-free milk, luteal phase progesterone values are approximately 4 ng/ml (12 nmole/liter), in whole milk the corresponding value varies between 5 and 35 ng/ml (16–110 nmole/liter), and if milk fat is used the progesterone value is approximately 250 ng/ml (795 nmole/liter). Another factor influencing hormone values in blood is the time interval elapsed from bleeding to separation of serum or plasma. Storage of unseparated blood samples from cattle for 6 or 24 hours resulted in a significant lowering of the assayable progesterone concentration (Vahdat *et al.*, 1979). Sample handling procedures from blood collection to the freezing of serum or plasma should be standardized for each species and hormone determined.

REFERENCES

Abraham, G. E. (1969). *J. Clin. Endocrinol. Metab.* **29,** 866.

Abraham, G. E. (1974). *Acta Endocrinol. (Copenhagen), Suppl.* **183.**

Agthe, O., Luthman, F., and Holm, H. P. (1976). *Berl. Muench. Tieraerztl. Wochenschr.* **89,** 47.

Akbar, A. M., Reichert, L. E., Dunn, T. G., Kaltenbach, C. C., and Niswender, G. D. (1974). *J. Anim. Sci.* **39,** 360.

Allen, W. R. (1969). *J. Endocrinol.* **43,** 593.

Baird, D. T., and Scaramuzzi, R. J. (1976). *J. Endocrinol.* **70,** 237.

Baird, D. T., Land, R. B., Scaramuzzi, R. J., and Wheeler, A. G. (1976). *J. Endocrinol.* **69,** 276.

Baldwin, D. M., and Stabenfeldt, G. H. (1975). *Biol. Reprod.* **12,** 508.

Bane, A., Jansson, L., Edqvist, L.-E., and Swenson, C. (1978). *Comm. Cattle Prod., EAAP Meet., Stockholm.* Available on request from Department of Animal Breeding, 75007 Uppsala, Sweden.

Barcikowski, B., Carlson, J. C.. Wilson, L., and McCracken, J. A. (1974). *Endocrinology* **95,** 1340.

Bazer, F. W., and Thatcher, W. W. (1977). *Prostaglandins* **14,** 397.

Béguin, F., Bygdeman, M., Greén, K., Samuelsson, B., Toppozada, M., and Wiqvist, N. (1972). *Acta Physiol. Scand.* **86,** 430.

Berndtson, W. E., Pickett, B. W., and Nett, T. M. (1974). *J. Reprod. Fertil.* **39,** 115.

Berson, S. A., and Yalow, R. S. (1959). *Nature (London)* **184,** 1648.

Bjersing, L., Hay, M. F., Kann, G., Moor, R. M., Naftolin, F., Scaramuzzi, R. J., Short, R. V., and Younglai, E. V. (1972). *J. Endocrinol.* **52,** 465.

Bland, K. P., Horton, E. W., and Poyser, N. L. (1971). *Life Sci.* **10,** 509.

Boilert, B., Edqvist, L.-E., Johansson, E. D. B., Lindberg, P., and Martinsson, K. (1973). *Steroids* **22,** 891.

Booth, W. D., Newcomb, R., Strange, H., Rowson, L. E. A., and Sacher, H. B. (1975). *Vet. Rec.* **97,** 366.

Bourrillon, R., and Got, R. (1959). *Acta Endocrinol. (Copenhagen)* **31,** 559.

Buttle, H. L., and Forsyth, I. A. (1976). *J. Endocrinol.* **68,** 141.

Cameron, E. H. D., Scarisbrick, J. J., Morris, S. E., and Read, G. (1975). *In* "Steroid Immunoassay" (E. H. D. Cameron, S. G. Hillier, and K. Griffiths, eds.), p. 153. Alpha-Omega Publ., Cardiff.

Carlström, K., and Sköldefors, H. (1977). *J. Steroid Biochem.* **8,** 1127.

Catt, K. J., and Dufau, M. L. (1973). *In* "Receptors for Reproductive Hormones" (B. W. O'Malley and A. R. Means, eds.), p. 379. Plenum, New York.

Catt, K. J., Niall, H. D., and Tregear, G. W. (1966). *J. Lab. Clin. Med.* **100,** 316.

Challis, J. R. G., Harrison, F. A., and Heap, R. B. (1971). *J. Reprod. Fertil.* **25,** 306.
Chenault, J. R., Thatcher, W. W., Kalra, P. S., Abrams, R. M., and Wilcox, C. J. (1975). *J. Dairy Sci.* **58,** 709.
Christie, D. W., Bell, E. T., Horth, C. E., and Palmer, R. F. (1971). *Acta Endocrinol. (Copenhagen)* **68,** 543.
Concannon, P. W., Hansel, W., and Visek, W. J. (1975). *Biol. Reprod.* **13,** 112.
Cox, J. E. (1975). *Equine Vet. J.* **7,** 179.
Cox, J. E. Williams, J. H., Rowe, P. H., and Smith, J. A. (1973). *Equine Vet. J.* **5,** 85.
Dericks-Tan, J. S. E., and Taubert, H. D. (1975). *Acta Endocrinol. (Copenhagen)* **78,** 451.
Desbuquois, B., and Aurbach, G. D. (1971). *J. Clin. Endocrinol. Metab.* **33,** 732.
DeVilla, G. O., Roberts, K., Wiest, W. G., Mikhail, G., and Flickinger, G. (1972). *J. Clin. Endocrinol. Metab.* **35,** 458.
du Mesnil du Buisson, F., and Dauzier, L. (1959). *Ann. Zootech.* **8,** Suppl., p. 147.
Edqvist, L.-E., Einarsson, S., Ekman, L., Gustafsson, B., and Lamm, A. M. (1972). *Proc. Int. Congr. Anim. Reprod., 7th, Munich* **1,** 635.
Edqvist, L.-E., Ekman, L., Gustafsson, B., and Johansson, E. D. B. (1973a). *Acta Endocrinol. (Copenhagen)* **72,** 81.
Edqvist, L.-E., Ekman, L., Gustafsson, B., and Lindell, J.-O. (1973b). *Acta Endocrinol. (Copenhagen), Suppl.* **177,** 43.
Edqvist, L.-E., Johansson, E. D. B., Kasström, H., Olsson, S.-E., and Richkind, M. (1975). *Acta Endocrinol. (Copenhagen)* **78,** 554.
Edqvist, L.-E., Kindahl, H., and Stabenfeldt, G. H. (1976). *Proc. Int. Congr. Anim. Reprod., 8th, Cracow* **3,** 357.
Edqvist, L.-E., Kindahl, H., and Stabenfeldt, G. H. (1978). *Prostaglandins* **16,** 111.
Ellendorff, F., Meyer, J. N., and Elsaesser, F. (1976). *Br. Vet. J.* **132,** 543.
Ellinwood, W. E., Nett, T. M., and Niswender, G. D. (1979). *Biol. Reprod.* **21,** 845.
Emady, M., Hadley, J. C., Noakes, D. E., and Arthur, G. H. (1974). *Vet. Rec.* **95,** 168.
Evans, M., and Irvine, C. (1975). *J. Reprod. Fertil., Suppl.* **23,** 195.
Exley, D., Johnson, M. W., and Dean, P. D. G. (1971). *Steroids* **18,** 605.
Fairclough, R. J., Hunter, J. T., and Welch, R. A. S. (1975). *Prostaglandins* **9,** 901.
Farmer, S. W., and Papkoff, H. (1979). *Biol. Reprod.* **21,** 425.
Frank, M., Bazer, F. W., Thatcher, W. W., and Wilcox, C. J. (1978). *Prostaglandins* **15,** 151.
Furuyama, S., Mayes, D. M., and Nugent, C. A. (1970). *Steroids* **16,** 415.
Gadsby, J. E., Heap, R. B., Powell, D. G., and Walters, D. E. (1972). *Vet. Rec.* **90,** 339.
Ganjam, V. K., and Kenney, R. M. (1975). *J. Reprod. Fertil., Suppl.* **23,** 67.
Geschwind, I. I., Dewey, R., Hughes, J. P., Evans, J. W., and Stabenfeldt, G. H. (1975). *J. Reprod. Fertil., Suppl.* **23,** 207.
Ginther, O. J., and First, N. L. (1971). *Am. J. Vet. Res.* **32,** 1687.
Ginther, O. J., Nuti, L., Wentworth, B. C., and Tyler, W. J. (1974). *Proc. Soc. Exp. Biol. Med.* **146,** 354.
Girard, A., Sandulesco, G., Fridenson, A., and Rutgers, J. T. (1932). *C. R. Acad. Sci.* **194,** 909.
Gleeson, A. R., Thorburn, G. D., and Cox, R. I. (1974). *Prostaglandins* **5,** 521.
Glencross, R. G., Munro, I. B., Senior, B. E., and Pope, G. S. (1973). *Acta Endocrinol. (Copenhagen)* **73,** 374.
Gonzalez-Padilla, E., Wiltbank, J. N., and Niswender, G. D. (1975). *J. Anim. Sci.* **40,** 1091.
Gospodarowicz, D. (1972). *Endocrinology* **91,** 101.
Granström, E. (1972). *Eur. J. Biochem.* **27,** 462.
Granström, E., and Kindahl, H. (1976). *In* "Advances in Prostaglandin and Thromboxane Research" (B. Samuelsson, and R. Paoletti, eds.), Vol. 1, p. 81. Raven, New York.
Granström, E., and Samuelsson, B. (1972). *FEBS Lett.* **26,** 211.
Guthrie, H. D., Henricks, D. M., and Handlin, D. L. (1972). *Endocrinology* **91,** 675.
Hadley, J. C. (1975a). *J. Reprod. Fertil.* **44,** 445.
Hadley, J. C. (1975b). *J. Reprod. Fertil.* **44,** 453.
Hamberg, M., and Samuelsson, B. (1971). *J. Biol. Chem.* **246,** 6713.
Harrison, F. A., Heap, R. B., Horton, E. W., and Poyser, N. L. (1972). *J. Endocrinol.* **53,** 215.
Hawkins, R. A., and Oakey, R. E. (1974). *J. Endocrinol.* **60,** 3.
Heap, R. B., Gwyn, M., Laing, J. A., and Walters, D. E. (1973). *J. Agric. Sci.* **81,** 151.
Henricks, D. M., Dickey, J. F., and Niswender, G. D. (1970). *Biol. Reprod.* **2,** 346.

Henricks, D. M., Dickey, J. F., Hill, J. R., and Johnston, W. E. (1972a). *Endocrinology* **90,** 1336.
Henricks, D. M., Hill, J. R., Dickey, J. F., and Lamond, D. R. (1972b). *Nature (London)* **235,** 222.
Hoffmann, B., and Hamburger, R. (1973). *Zuchthygiene* **8,** 154.
Hoffmann, B., Günzler, O., Hamburger, R., and Schmidt, W. (1976). *Br. Vet. J.* **132,** 469.
Holst, P. A., and Phemister, R. D. (1974). *Am. J. Vet. Res.* **35,** 401.
Holst, P. A., and Phemister, R. D. (1975). *Am. J. Vet. Res.* **36,** 705.
Holtan, D. W., Squires, E. L., Lapin, D. R., and Ginther, O. J. (1979). *J. Reprod. Fertil., Suppl.* **27,** 457.
Hoyer, G. L., Colcord, M. L., and Weems, C. W. (1978). *Proc. Annu. Meet. Am. Soc. Anim. Sci.* Abstr. 367.
Hughes, J. P., Stabenfeldt, G. H., and Evans, J. W. (1973). *Proc. Annu. Meet. Am. Assoc. Equine Practitioners, 18th, 1972, San Francisco* p. 119.
Hunter, W. M. (1970). *Acta Endocrinol. (Copenhagen), Suppl.* **142,** 134.
Hunter, W. M., and Greenwood, F. C. (1962). *Nature (London)* **194,** 495.
Johansson, E. D. B. (1969). *Acta Endocrinol. (Copenhagen)* **61,** 592.
Jones, G. E., Boyns, A. R., Cameron, E. H. D., Bell, E. T., Christie, D. W., and Parkes, M. F. (1973). *J. Endocrinol.* **57,** 331.
Karg, H., Claus, R., Hoffmann, B., Schallenberg, E., and Schams, D. (1976). *In* "Nuclear Techniques in Animal Production and Health," p. 487. IAEA, Vienna.
Kehrer, A., and Starke, P. (1975). *Berl. Muench. Tieraerztl. Wochenschr.* **88,** 101.
Kelly, P. A., Robertson, H. A., and Friesen, H. G. (1974). *Nature (London)* **248,** 435.
Kindahl, H., Edqvist, L.-E., Bane, A., and Granström, E. (1976a). *Acta Endocrinol. (Copenhagen)* **82,** 134.
Kindahl, H., Edqvist, L.-E., Granström, E., and Bane, A. (1976b). *Prostaglandins* **11,** 871.
Kindahl, H., Granström E., Edqvist, L.-E., Neely, D. P., Hughes, J., and Stabenfeldt, G. H. (1976c). *Proc. Int. Congr. Anim. Reprod., 8th, Cracow* **3,** 145.
Kohen, F., Bauminger, S., and Lindner, H. R. (1975). *In* "Steroid Immunoassay" (E. H. D. Cameron, S. G. Hillier, and K. Griffiths, eds.), p. 11. Alpha-Omega Publ., Cardiff.
Korenman, S. G., Perrin, L. E., and McCallum, T. P. (1969). *J. Clin. Endocrinol. Metab.* **29,** 879.
Laing, J. A., and Heap, R. B. (1971). *Br. Vet. J.* **127,** XIX.
Lamond. D. R., and Gaddy, R. G. (1972). *J. Reprod. Fertil.* **29,** 307.
Landsteiner, K., and Van der Scherr, J. (1936). *J. Exp. Med.* **63,** 325.
Larsson, K., Edqvist, L.-E., Einarsson, S., Häggström, A., and Linde, C. (1975). *Nord. Veterinaermed.* **27,** 167.
Lemon, M., and Saumande, J. (1972). *J. Reprod. Fertil.* **31,** 501.
Lemon, M., Pelletier, J., Saumande, J., and Signoret, J. P. (1975). *J. Reprod. Fertil.* **42,** 137.
Liggins, G. C., Grieves, S. A., Kendall, J. Z., and Knox, B. S. (1972). *J. Reprod. Fertil., Suppl.* **16,** 85.
Lindberg, P., and Edqvist, L.-E. (1974). *Clin. Chim. Acta* **53,** 169.
Lindner, H. R., Perel, E., and Friedlander, A. (1970). *In* "Research on Steroids IV" (M. Finkelstein, C. Conti, A. Klopper, and Cassano, C., eds.), p. 197. North-Holland Publ., Amsterdam, 1970.
Loriaux, D. L., Ruder, H. J., and Lipsett, M. B. (1971). *Steroids* **18,** 463.
Lovell, J. D., Stabenfeldt, G. H., Hughes, J. P., and Evans, J. W. (1975). *J. Reprod. Fertil., Suppl.* **23,** 449.
Lunaas, T. (1962). *J. Reprod. Fertil.* **4,** 13.
Magness, R. R., Huie, J. M., and Weems, C. W. (1978). *J. Anim. Sci.* **46** Suppl 1, 376, Abstr. 401.
McCracken, J. A., Carlson, J. C., Glew, M. E., Goding, J. R., Baird, D. T., Gréen, K., and Samuelsson, B. (1972). *Nature (London)* **238,** 129.
McNatty, K. P., and Thurley, D. C. (1974). *N.Z. J. Agric. Res.* **17,** 309.
Mayes, D., and Nugent, C. A. (1970). *Steroids* **15,** 389.
Midgley, A. R., and Niswender, G. D. (1970). *Acta Endocrinol. (Copenhagen), Suppl.* **147,** 320.
Mitchell, D., and Allen, W. R. (1975). *J. Reprod. Fertil., Suppl.* **23,** 531.
Mitchell, D., and Betteridge. (1973). *Proc. Int. Congr. Anim. Reprod. 7th, Munich, 1972* **1,** 567.
Moeljono, M. P. E., Thatcher, W. W., Bazer, F. W., Frank, M., Owens, L. J., and Wilcox, C. J. (1977). *Prostaglandins* **14,** 543.
Murphy, B. E. P. (1964). *Nature (London)* **201,** 679.
Murphy, B. E. P. (1967). *J. Clin. Endocrinol. Metab.* **27,** 973.
Nancarrow, C. W., Buckmaster, J., Chamley, W., Cox, R. I., Cumming, I. A., Cummins, L., Drinan, J. P., Findlay, J. K., Goding, J. R., Restall, B. J., Schneider, B. J., and Thorburn, G. D. (1973). *J. Reprod. Fertil.* **32,** 320.
Neely, D. P., Kindahl, H., Stabenfeldt, G. H., Edqvist, L.-E., and Hughes, J. P. (1979). *J. Reprod. Fertil., Suppl.* **27,** 181.

Neill, J. D., Johansson, E. D. B., Datta, J. K., and Knobil, E. (1967). *J. Clin. Endocrinol. Metab.* **27,** 1167.
Nett, T. M., Holtan, D. W., and Estergreen, V. L. (1973). *J. Anim. Sci.* **37,** 962.
Nett, T. M. Akbar, A. M., and Niswender, G. D. (1974). *Endocrinology* **94,** 713.
Nett, T. M., Akbar, A. M., Phemister, R. D., Holst, P. A., Reichert, L. E., Jr., and Niswender, G. D. (1975). *Proc. Soc. Exp. Biol. Med.* **148,** 134.
Nett, T. M., Staigmiller, R. B., Akbar, A. M., Diekman, M. A., Ellinwood, W. E., and Niswender, G. D. (1976). *J. Anim. Sci.* **42,** 876.
Niswender, G. D. (1973). *Steroids* **22,** 413.
Niswender, G. D., and Midgley, A. R. (1970). *In* "Immunologic Methods in Steroid Determination" (F. G. Peron and B. V. Caldwell, eds.), p. 149. Appleton, New York.
Niswender, G. D., and Nett, T. M. (1977). *In* "Reproduction in Domestic Animals" (H. H. Cole, and P. T. Cupps, eds.), p. 119. Academic Press, New York.
Niswender, G. D., Midgley, A. R., and Reichert, L. E. (1968). *In* "Gonadotrophins" (E. Rosemberg, ed.), p. 199. Geron-X, Los Altos, California.
Niswender, G. D., Nett, T. M., Myer, D. L., and Hagerman, D. D. (1975). *In* "Steroid Immunoassay" (E. H. D. Cammeron, S. G. Hillier and K. Griffiths, eds.), p. 61. Alpha-Omega Publ., Cardiff.
Noden, P.A., Hafs, H. D., and Oxender, W. D. (1975). *J. Reprod. Fertil., Suppl.* **23,** 189.
Noden, P. A., Oxender, W. D., and Hafs, H. D. (1978). *Am. J. Vet. Res.* **39,** 1965.
Perry, J. S., Heap, R. B, Burton, R. D., and Gadsby, J. E. (1976). *J. Reprod. Fertil., Suppl.* **25,** 85.
Peterson, A. J., Fairclough, R. J., Payne, E., and Smith, J. F. (1975). *Prostaglandins* **10,** 675.
Peterson, A. J., Tervit, H. R., Fairclough, R. J., Havik, P. G., and Smith, J. F. (1976). *Prostaglandins* **12,** 551.
Pharris, B. B., and Wyngarden, L. J. (1969). *Proc. Soc. Exp. Biol. Med.* **130,** 92.
Phifer, R. F., Midgley, A. R., and Spicer, S. S. (1973). *J. Clin. Endocrinol. Metab.* **36,** 125.
Pope, G. S., Gupta, S. K., and Munro, I. B. (1969). *J. Reprod. Fertil.* **20,** 369.
Rajamahendran, R., Laguë, P. C., and Baker, R. D. (1976). *Can. J. Anim. Sci.* **56,** 37.
Roberts, J. S., Barcikowski, B., Wilson, L., Skarnes, R. C., and McCracken, J. A. (1975). *J. Steroid Biochem.* **6,** 1091.
Robertson, H. A., and King, G. J. (1974). *J. Reprod. Fertil.* **40,** 133.
Robertson, H. A., and Sarda, I. R. (1971). *J. Endocrinol.* **49,** 407.
Roth, J., Glick, S. M., Klein, L. A., and Petersen, M. J. (1966). *J. Clin. Endocrinol. Metab.* **26,** 671.
Samuelsson, B., Granström, E., Greén, K., Hamberg, M., and Hammarström, S. (1975). *Annu. Rev. Biochem.* **44,** 669.
Sarda, I. R., Robertson, H. A., and Smeaton, T. C. (1973). *Can. J. Anim. Sci.* **53,** 25.
Seal, U. S., and Doe, R. P. (1966). *In* "Steroid Dynamics" (G. Pincus, T. Nakao, and J. F. Tait, eds.), p. 63. Academic Press, New York.
Shearer, I. J., Purvis, K., Jenkin, G., and Haynes, N. B. (1972). *J. Reprod. Fertil.* **30,** 347.
Shemesh, M., and Hansel, W. (1975). *Proc. Soc. Exp. Biol. Med.* **148,** 123.
Shemesh, M., Ayalon, N., and Lindner, H. R. (1968). *J. Reprod. Fertil.* **15,** 161.
Shille, V., Karlbom, I., Einarsson, S., Larsson, K., Kindahl, H., and Edqvist, L.-E. (1979a). *Zentralbl. Veterinaermed., Reihe A.***26,** 169.
Shille, V. M., Lundström, K. E., and Stabenfeldt, G. H. (1979b). *Biol. Reprod.* **21,** 953.
Smith, M. S., and McDonald, L. E. (1974). *Endocrinology* **94,** 404.
Sokolowski, J. H. (1971). *Lab. Anim. Sci.* **21,** 696.
Solti, L., Greve, T., and Koefoed-Johnsen, H. H. (1978). *Acta Vet. Scand.* **19,** 298.
Spieler, J. M., Webb, R. L., Saldarini, R. J., and Coppola, J. A. (1972). *Steroids* **19,** 751.
Spilman, C. H., Seidel, G. E., Larson, L. L., Vukman, G. R., and Foote, R. H. (1973). *Biol. Reprod.* **9,** 116.
Squires, E. L., Douglas, R. H., Steffenhagen, W. P., and Ginther, O. J. (1974). *J. Anim. Sci.* **38,** 330.
Squires, E. L., Stevens, W. B., Pickett, B. W., and Nett, T. M. (1979). *Am. J. Vet. Res.* **40,** 889.
Stabenfeldt, G. H. (1974). *J. Am. Vet. Med. Assoc.* **164,** 311.
Stabenfeldt, G. H., Akins, E., Ewing, L. L., and Morrisette, M. C. (1969a). *J. Reprod. Fertil.* **20,** 443.
Stabenfeldt, G. H., Ewing, L. L., and McDonald, L. E. (1969b). *J. Reprod. Fertil.* **19,** 433.
Stabenfeldt, G. H., Holt, J. A., and Ewing, L. L. (1969c). *Endocrinology* **85,** 11.
Stabenfeldt, G. H., Osburn, B. I., and Ewing, L. L. (1970). *Am. J. Physiol.* **218,** 571.
Stabenfeldt, G. H., Drost, M., and Franti, C. E. (1972a). *Endocrinology* **90,** 144.
Stabenfeldt, G. H., Hughes, J. P., and Evans, J. W. (1972b). *Endocrinology* **90,** 1379.

Stabenfeldt, G. H., Hughes, J. P., Wheat, J. D., Evans, J. W., Kennedy, P. C., and Cupps, P. T. (1974a). *J. Reprod. Fertil.* **37,** 343.

Stabenfeldt, G. H., Hughes, J. P., Evans, J. W., and Neely, D. P. (1974b). *Equine Vet. J.* **6,** 158.

Stabenfeldt, G. H., Edqvist, L.-E., and Drost, M. (1975a). *Acta Endocrinol. (Copenhagen), Suppl.* **199,** 385.

Stabenfeldt, G. H., Hughes, J. P., Evans, J. W., and Geschwind, I. I. (1975b). *J. Reprod. Fertil.* **23,** 155.

Stabenfeldt, G. H., Kindahl, H., and Edqvist, L.-E. (1976). *In* "Nuclear Techniques in Animal Production and Health," p. 525. IAEA, Vienna.

Stabenfeldt, G. H., Hughes, J. P., Kennedy, P. C., Meagher, D. M., and Neely, D. P. (1979). *J. Reprod. Fertil., Suppl.* **27,** 277.

Thatcher, W. W., Wilcox, C. J., Bazer, F. W., Collier, R. J., Eley, R. M., Stover, Diann G., and Bartol, F. F. (1979). *Proc. Beltsville Agric. Res. Center, Symp. Anim. Reprod.* **3,** 259.

Thorburn, G. D., Nicol, D. H., Bassett, J. M., Shutt, D. A., and Cox, R. I. (1974). *J. Reprod. Fertil., Suppl.* **16,** 61.

Thorell, J. I., and Johansson, B. G. (1971). *Biochim. Biophys. Acta* **251,** 363.

Thorneycroft, I. H., and Stone, S. C. (1972). *Contraception* **5,** 129.

Tsang, C. P. W. (1978). *Theriogenology* **10,** 97.

Vahdat, F., Hurtgen, J. P., Whitmore, H. L., Johnston, S. D., and Ketelson, C. L. (1979). *Theriogenology* **12,** 371.

Vaitakaitus, J., Robbins, J. B., Nieschlag, E., and Ross, G. T. (1971). *J. Clin. Endocrinol. Metab.* **33,** 988.

Van de Wiel, D. F. M., Visscher, A. H., and Dekker, T. P. (1976). *In* "Nuclear Techniques in Animal Production and Health," p. 547. IAEA, Vienna.

Van de Wiel, D. F. M., Van Eldik, J., Koops, W., Postma, A., and Oldenbroek, J. K. (1978). *Tijdschr. Diergeneeskd.* **103,** 91.

Van Orden, D. E., and Farley, D. B. (1973). *Prostaglandins* **4,** 215.

Van Tonder, E. M. (1972). *J. S. Afr. Vet. Assoc.* **43,** 329.

Velle, W. (1958). *Am. J. Vet. Res.* **19,** 405.

Verhage, H. G., Beamer, N. B., and Brenner, R. M. (1976). *Biol. Reprod.* **14,** 579.

Vermeulen, A., and Verdonck, L. (1968). *Steroids* **11,** 609.

Weigl, R. M., Tilton, J. E., Haugse, C. N., Light, M. R., and Buchanan, M. L. (1975). *N.D. Farm Res.* **33,** 11.

Wide, L., and Porath, J. (1966). *Biochim. Biophys, Acta* **130,** 257.

Wiltbank, J. N., and Casida, L. E. (1956). *J. Anim. Sci.* **15,** 134.

Wishart, D. F., Head, V. A., and Horth, C. E. (1975). *Vet. Rec.* **96,** 34.

Zavy, M. T. (1979). PhD thesis, University of Florida, Gainesville, Florida, USA.

14

Skeletal Muscle Function

GEORGE H. CARDINET III
AND JULIA STEPHENS-ORVIS

I. INTRODUCTION

The skeletal muscle mass comprises 30–50% of the body weight; hence, skeletal muscle cells (myofibers) constitute the largest mass of cells in the body which have similar morphological and physiological properties. The protoplasmic properties of contractility and conductility which characterize myofibers are an expression of the functional properties of their sarcoplasmic organelles. Studies of muscles have provided excellent examples of the integration of form and function whereby molecular and organelle structure and function can be directly related to cellular morphology and function, which in turn can be related to the morphology and function of organs and organisms.

Owing to the contractile property of myofibers, the function of skeletal muscle is

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

generally described to be contraction. This definition of skeletal muscle function is, of course, correct, but it represents an oversimplification and unfortunately has resulted too often in the view that all muscles of the body are a homogeneous mass of contractile cells. Skeletal muscles of the body might be more appropriately considered as organs. The resultant act of contraction differs among muscles of the body, and the precise manner in which different muscles contract also differs. In addition, skeletal muscles differ in their vascular supply, nerve supply, and constituent fiber populations, all of which confer differences in their morphology and function. Singly, or in concert with other muscles, skeletal muscles function in virtually all systems of the body.

An attempt is made in this chapter to outline the great diversity that exists in skeletal muscle. By so doing, it is hoped that the knowledge and techniques developed, especially during recent years, will find their rightful introduction and application to increase our recognition and understanding of neuromuscular disorders in clinical veterinary medicine.

There are, no doubt, numerous neuromuscular disorders in domestic animals that are still unrecognized, and the observations made almost 30 years ago by Innes (1951) are worthy of citing here.

> I have drawn attention before to the fact that no diseases are recognized until they are found by looking with great care. One need only scan the veterinary literature of the last twenty years for this to be confirmed, and a long list of conditions could be compiled which might have been discovered years previously if more meticulous methods of pathological examinations had been used. Veterinary pathology has still a long road to travel before it achieves the profundity of human pathology, and this is not said in any deprecatory sense of past achievements. There are many reasons why this is so, but lack of volume of material studied and inattention to the lessons to be learnt from medical work have played a part. In veterinary work, unfortunately, also the question of economics always intrudes, and in the end we are constantly influenced by a consideration of what is, or is not, important.
>
> In conducting autopsy examinations of animals there is usually an immense amount of bias in selection of tissues for histological work; in the case of skeletal muscles (from my own experience) no doubt they are mostly ignored unless macroscopical changes are very obvious.

Since 1951, some advances have been made. Most notable have been the comparative biomedical investigations (Harris, 1979) which resulted from the original recognition and descriptions of hereditary muscular dystrophies in the mouse (Michelson *et al.*, 1955), chicken (Asmundson and Julian, 1956), and hamster (Homburger *et al.*, 1962). More recently, hereditary muscular dystrophies have been reported in mink (Hegreberg *et al.*, 1974; Hamilton *et al.*, 1974), sheep (McGavin and Baynes, 1969), and turkeys (Harper and Parker, 1964, 1967). However, in domestic animals which are more commonly considered of clinical importance, there is a need for vast improvements in our knowledge and understanding of neuromuscular diseases.

II. SARCOPLASMIC ORGANELLES AND MUSCULAR CONTRACTION

A. Excitation–Conduction

The morphology of skeletal myofibers is not extensively reviewed here, and the reader is referred to any current histology text for details (e.g., Bloom and Fawcett, 1975). The cell or plasma membranes of skeletal myofibers (sarcolemma) are specialized for the protoplasmic properties of excitation and conduction at the neuromuscular junction, the site of contact between a motoneuron axon terminal and a skeletal myofiber (Fig. 1). At

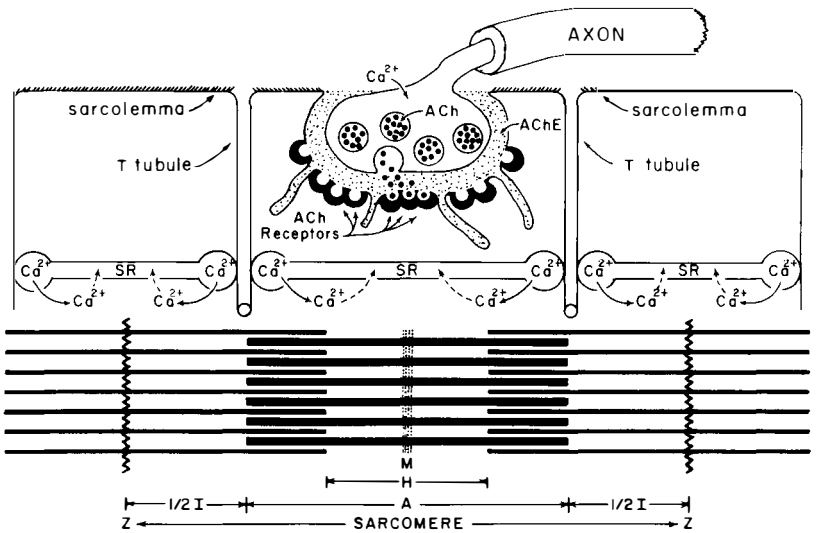


Fig. 1. Excitation of myofibers involves neuromuscular transmission in which (1) there is a calcium-dependent release of the neurotransmitter acetylcholine (ACh) at the axon terminal, (2) diffusion of ACh across the synaptic cleft, (3) interaction of ACh with ACh receptors on the postsynaptic membrane (end plate), and (4) the formation of an end plate potential caused by increased conductance of sodium and potassium at the end plate. The developed end plate potential generates a muscle action potential which spreads away from the neuromuscular junction in all directions over the surface of the myofiber and into its depths via the transverse (T) tubules. Within the depths of the myofibers, excitation is coupled to contraction through the release of calcium ions from terminal cisternae of the sarcoplasmic reticulum (SR). With increased sarcoplasmic calcium concentrations, the contractile events associated with the interaction of actin and myosin filaments occur. The Ach is hydrolyzed by AchE (acetylcholinesterase) present in the synaptic cleft.

the neuromuscular junction, the sarcolemma (end plate) is thrown into primary and secondary folds. The axon terminal is situated within the primary fold, and it contains numerous vesicles (quanta) of the neurotransmitter, acetylcholine. Acetylcholine is released at the axon terminal by a process of exocytosis in which the vesicles fuse with the axolemma. Each vesicle or quantum of acetylcholine contains approximately 10,000 molecules. The acetylcholine which is released eventually becomes hydrolyzed by acetylcholinesterase, which is located among the primary and secondary folds between the presynaptic axolemma and postsynaptic sarcolemma.

Excitation of the myofiber is initiated by the interaction of acetylcholine with acetylcholine receptors, which are located on the postsynaptic sarcolemma (end plate). The receptors are most numerous on the crests of the secondary folds. Combination of acetylcholine with receptors results in a transient and localized increased conductance (permeability) of the sarcolemma to sodium and potassium, which depolarizes the myofiber's membrane (end plate) potential. The amplitude of the depolarization is increased with increased numbers of acetylcholine–receptor combinations. The acetylcholine–receptor interaction is transient and abolished by hydrolysis of acetylcholine by acetylcholinesterase and the diffusion of acetylcholine away from the receptors.

Quantal release of acetylcholine occurs spontaneously and in association with the arrival of a nerve impulse at the axon terminal. The quantal release of acetylcholine is initiated by calcium uptake at the axon terminal, and a reduction of calcium ions at the axon terminal reduces the quantal release of acetylcholine.

At rest, individual quanta of acetylcholine are spontaneously released at a slow rate and cause transient, low-amplitude depolarizations at the end plate. These are referred to as miniature end plate potentials. When a nerve impulse (nerve action potential) is conducted along an axon and arrives at the axon terminal, there is a release of 100–200 quanta of acetylcholine. With the increased number of acetylcholine-receptor combinations, there is a greater conductance of sodium and potassium, which forms a large amplitude depolarization, referred to as an end plate potential.

The end plate potential produced by the acetylcholine–receptor interaction generates conduction of a wave of depolarization along the sarcolemma (muscle action potential). The muscle action potential spreads out from the end plate in all directions and is propagated into the depths of the myofiber along the transverse (T) tubules. The transverse tubules, invaginations of the sarcolemma, enter the depths of the myofiber at a plane which is transverse to its long axis, and their lumina openly communicate with the intercellular fluid space. Excitation and conduction are coupled to contraction within the depths of the myofibers at the triads where the T tubules come into intimate contact with the terminal cisternae of the sarcoplasmic reticulum, an intrafiber smooth membrane organelle. The sarcoplasmic reticulum functions in the uptake and storage of calcium within its lumina as well as the release of calcium ions into the aqueous sarcoplasm bathing the myofilaments and other organelles.

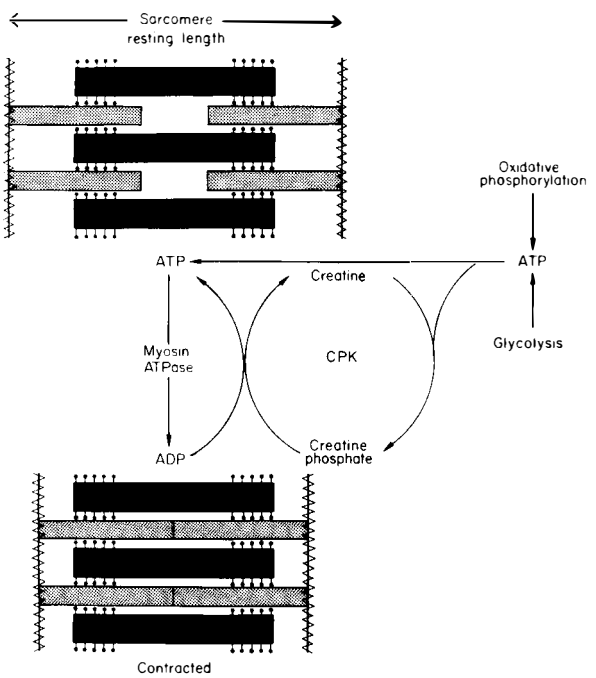


Fig. 2. Muscular contraction involves the shortening of sarcomeres by sliding of the overlapping arrays of thick (myosin) and thin (actin) myofilaments. The energy for contraction is derived from the hydrolysis of ATP in the presence of an ATPase associated with the cross-bridges of the myosin filaments. The ATP is generated by the energy metabolism of the myofiber, principally by anaerobic glycolysis or oxidative phosphorylation. The utilization of ATP may be direct from those sources or indirect from the phosphorylation of ADP from creatine phosphate by creatine phosphokinase (CPK).

B. Contraction

Muscular contraction results in the transformation of chemical energy into mechanical energy or shortening. The energy for contraction is derived from the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate (Fig. 2). That hydrolysis is catalyzed by adenosine triphosphatase (ATPase). Chemically, the tranformation of energy is associated with the cyclical association and disassociation of the contractile proteins actin and myosin, while mechanically the transformation is associated with shortening of sarcomeres, which is achieved by sliding of the overlapping arrays of thick (myosin) and thin (actin) myofilaments (Hanson and Huxley, 1953; Huxley and Hanson, 1954; Huxley and Niedergerke, 1954; H. E. Huxley, 1969; A. F. Huxley, 1974).

The ATP required for contraction is produced in mitochondria by oxidative phosphorylation and in the sarcoplasm by glycogenolysis and glycolysis. Muscle may use ATP directly from those sources or indirectly from phosphorylation of ADP by the dephosphorylation of creatine phosphate in the presence of creatine phosphokinase (Davies, 1965) (Fig. 2).

1. Contractile Proteins

The contractile proteins are myosin, the principal component of thick myofilaments, and actin, which is the principal component of thin myofilaments. Lateral projections, or cross-bridges, of the thick myofilaments are the constituents of myosin which form the reactive sites of cyclical association and disassociation between myosin and actin during contraction. The force generated for sliding of the filaments is believed to result from changes in the angle of cross-bridge attachments (Fig. 3).

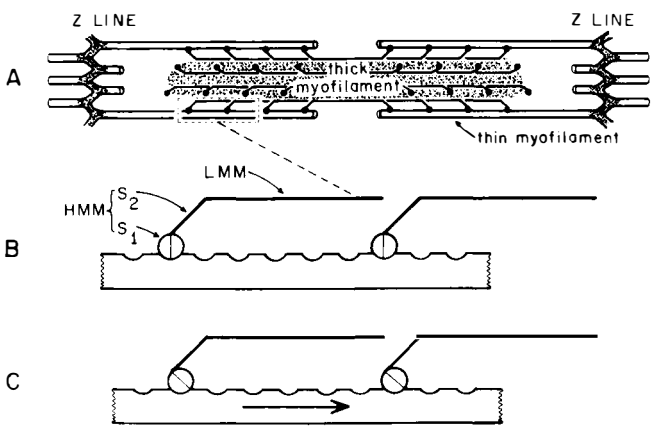


Fig. 3. Schematic presentation of myosin cross-bridges on thick myofilaments. (A) Portions of the myosin molecules project from the thick myofilaments and make contact with the thin myofilaments. (B) The light meromyosin portions of the myosin molecules (LMM) form the major structural component of the thick myofilament, while the heavy meromyosin component (HMM) forms the cross-bridge between the thick and thin myofilaments. The cross-bridges of myosin are composed of two fractions: (1) the S_1 fraction, a globular protein fraction which binds to actin and possesses the ATPase activity of myosin, and (2) the S_2 fraction, a fibrous protein fraction which forms a flexible linkage between the S_1 fraction and the light meromyosin portion of myosin. (C) The force for sliding of the myofilaments is believed to result from changes in the angle of attachment between the S_1 globular head and actin filament.

To gain an understanding of the physiochemical changes which occur at the cross-bridges, the composition and properties of myosin need to be considered. Myosin is a structural and enzymatic protein composed of two polypeptide chains (heavy chains), each with an approximate molecular mass of 200,000 (Gershman *et al.*, 1969; Gazith *et al.*, (1970), and four polypeptide chains (light chains), the molecular masses of which range from 16,000 to 25,000 (Lowey and Risby, 1971; Sarkar *et al.*, 1971; Weeds and Lowey, 1971). The heavy chains are arranged into a double helix to form a long fibrous portion, and at one end each heavy chain is folded into a globular portion. The four light chains are contained within the globular portion and consist of two classes: (1) two identical DTNB light chains, which disassociate from the globular portions with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and (2) two related but different species of "alkali light chains," which disassociate at pH 11 (Weeds, 1969; Gazith *et al.*, 1970; Weeds and Lowey, 1971). The principal biochemical properties of myosin are its actin-binding capacity and ATPase activity. Disassociation of the alkali light chains from the globular regions results in a loss of those properties, while disassociation of the DTNB light chains does not. Furthermore, the alkali light-chain composition appears to influence the relative actin-activated ATPase activity of different myosins (Wagner and Weeds, 1977).

Proteolytic digestion of myosin with trypsin results in the formation of two fragments: (1) heavy meromyosin, which is composed of the globular portion of myosin and a short initial segment of the fibrous portion, and (2) light meromyosin, which is composed of the remaining long fibrous portion of the molecule. The heavy meromyosin fragments correspond structurally to the cross-bridges, while the light meromyosin fragments comprise the bulk of the thick myofilaments (Huxley, 1963) (Fig. 3). Papain digestion results in two subfragments of heavy meromyosin: subfragment 1 (S_1), the globular head region, and subfragment 2 (S_2), the short fibrous portion. The actin-binding capacity, ATP-binding capacity, and ATPase activity which characterize myosin reside in the S_1 fragment, while the S_2 fragment comprises the flexible linkage between the globular head of the cross-bridge and the light meromyosin fragment. Thus, the portion of the myosin molecule that possesses the characteristic chemical properties of myosin (S_1) is structurally located on the terminal portion of the cross-bridges at the site of interaction with the actin of the thin myofilaments.

The thin filaments are composed of two F-actin strands arranged in a double helical configuration (Hanson and Lowy, 1963). The F-actin strands are polymers of the globular protein G-actin, and each G-actin monomer possesses a complementary binding site for the myosin S_1 fragment. Upon combining with myosin, actin activates the ATPase activity of the myosin S_1 fragment.

Contraction is a physiochemical cycle in which the cyclic interaction of actin and myosin (association, change in angulation of cross-bridge attachments, and disassociation) and hydrolysis of ATP are interdependent and complex (Lymn and Taylor, 1971; Eisenberg and Kielley, 1974) (Fig. 4). In the noncontracting state, actin and myosin are combined at the cross-bridges (Step1, Fig. 4), and the angle of attachment between the cross-bridge heads and the actin filaments is 45°. With the addition of ATP, ATP is bound to the globular head and there is a rapid disassociation of actin and myosin (Step 2, Fig. 4). The ATP is cleaved to form a myosin–products complex, and the globular head moves to a new position (Step 3, Fig. 4), which permits the angle of attachment to become 90° when the myosin–products complex recombines with the actin filament (Step 4,

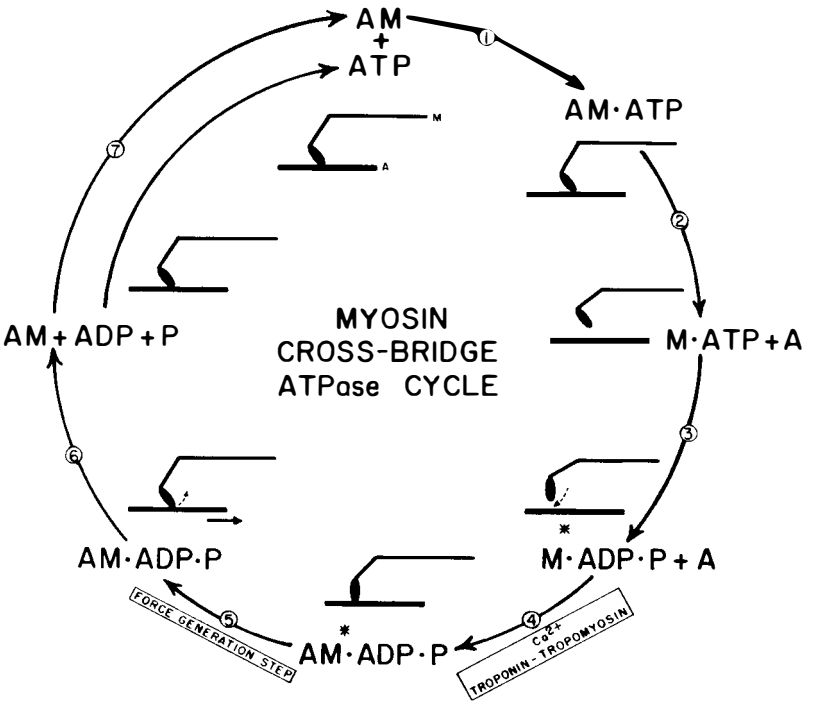


Fig. 4. Muscular contraction results from the cyclical association and dissociation of actin (A) and myosin (M) in which conformational changes occur in the cross-bridge linkages between the thick and thin myofilaments, associated with the hydrolysis of ATP.

Fig. 4). This recombination step between the myosin-products complex and actin is controlled by the regulatory proteins troponin and tropomyosin in response to calcium ion concentrations. The force for contraction is generated by movement of the cross-bridge head to a 45° angle of attachment (Step 5, Fig. 4), and the cycle is completed with the detachment of the hydrolytic products of ATP from the head (Step 6, Fig. 4). With the formation of ATP through rephosphorylation (Step 7, Fig. 4), the cycle may be repeated. Measurements indicate that each cycle (stroke) shortens a sarcomere by 12 nm (Barden and Mason, 1978).

2. Regulatory Proteins

Two proteins, in concert with calcium, regulate the interaction of actin and myosin and hence contraction (Ebashi *et al.*, 1969). One is the fibrous protein tropomyosin, which is present on the thin actin filaments arranged along their length. The other protein is troponin, a globular protein which combines with tropomyosin at intervals along the thin myofilament. At low sarcoplasmic calcium concentrations, tropomyosin molecules block the myosin-binding sites on actin, which prevents the interaction of actin and myosin. At higher concentrations, calcium ions combine with troponin, a calcium-receptive protein, and through some conformational change this results in the movement of tropomyosin to free the myosin-binding sites on actin. That permits actin and myosin to combine and initiate the cyclical changes associated with that interaction (Fig. 4).

C. Excitation–Conduction–Contraction Coupling

Excitation–conduction–contraction coupling occurs at the junction of the sarcolemma (transverse tubule) and the intrafiber sarcoplasmic reticulum. With the propagation of a muscle action potential over the surface of a myofiber and into its depths along the tubules, calcium ions are released by the terminal cisternae of the sarcoplasmic reticulum. The increased concentration and diffusion of calcium ions in the aqueous sarcoplasm result in the uptake and binding of calcium by troponin. In turn, calcium uptake by troponin results in the movement of tropomyosin to free the myosin-binding sites on actin and thus allow actin and myosin to combine. When calcium ion concentrations are decreased through uptake of calcium by the sarcoplasmic reticulum, the process is reversed, and actin and myosin combination is inhibited.

III. HETEROGENEITY OF SKELETAL MUSCLE

A. Gross Muscle Coloration

Differences in gross coloration of muscles have been recognized for a long time, and early observations were reviewed by Needham (1926). A variation in color is noted not only among species of animals but also among individual muscles within the same individual. The color of some muscles ranges from deep red to pale, whereas other muscles exhibit intermediate shades of coloration. Early workers believed that the differences in coloration were due to differences in the content of blood within the muscles; however, it was later shown that the red coloration was associated with the presence of myoglobin. The terms "red" and "white" were introduced to distinguish muscles on the basis of their gross coloration. Subsequently, numerous biochemical, histochemical, and physiological studies have been conducted with selected muscles from a variety of species. As a result, the terms "red" and "white" have come to imply a more specific meaning, relating to physiological properties and populations of myofiber types within a muscle.

B. Physiological Properties

Muscle coloration was observed to be associated with significant differences in the physiological properties of muscular contraction (Ranvier, 1873, 1874, 1880). It was demonstrated that the speed of contraction of red muscles was slower than that of white muscles, and a number of other investigations tended to support Ranvier's observations in a variety of animals (Kronecker and Stirling, 1878; Lee *et al.*, 1916; Denny-Brown, 1929). In addition, redness of a muscle was associated with the development of tetanus at lower frequencies of stimulation, the development of smaller twitch tensions, and a greater resistance to fatigue. Conversely, white muscles required greater frequencies of stimulation for the development of tetanus, developed larger twitch tensions, and tended to fatigue quickly. Hence, muscles were found to differ in their physiological properties of contraction, and the terminology of slow-contracting and fast-contracting muscles evolved. Moreover, since speed of contraction was closely associated with gross muscle coloration, the terms "red" and "white" came to be used interchangeably with "slow" and "fast," respectively. There are numerous exceptions, however, to this association of

gross coloration with physiological properties of contraction. Therefore, direct associations should not be assumed.

The morphological and functional unit of skeletal muscles is the motor unit. The motor unit consists of (1) the motoneuron, the cell body of which lies in the anterior or ventral horn of the spinal cord and the axon of which extends along the anterior or ventral root and peripheral nerve, (2) the neuromuscular junctions, and (3) the myofibers innervated by the neuron. There are different types of motoneurons based on their rates of discharge: (1) phasic motoneurons with a fast discharge rate and (2) tonic motoneurons with a slow rate (Granit *et al.*, 1957). In addition, the phasic motoneurons are characterized by shorter after-hyperpolarization potentials, faster conduction velocities, and larger axons than the tonic motoneurons (Eccles *et al.*, 1958). Investigations of these parameters in motoneurons of slow-contracting and fast-contracting muscles indicate that tonic motoneurons which discharge at rates of 10–20 per second innervate slow-contracting muscles, while phasic motoneurons which discharge at rates of 30–60 per second innervate fast-contracting muscle (Eccles *et al.*, 1958). Thus, there are at least two types of motor units which differ in their physiological properties and type of motoneuron innervation. Physiological measurements of isolated motor units in the cat have revealed two types of fast-contracting units and one type of slow-contracting unit (Burke *et al.*, 1973). One type of fast-contracting unit (FR) and the slow-contracting unit (S) were resistant to fatigue, while the other type of fast-contracting unit (FF) fatigued rapidly.

C. Quantitative Biochemistry

1. Metabolic Pathways Which Generate Energy for Contraction

Quantitative differences in enzyme activities and various substrate concentrations have been reported between red and white muscles. Those biochemical differences between red and white muscles reflect differences in their principal metabolic pathways active in the generation of energy (ATP) for muscular contraction (Fig. 5). In general, white muscles are biochemically suited to derive energy for contraction by substrate phosphorylation via anaerobic glycolysis. White muscles tend to have higher concentrations of glycogen and creatine phosphate as well as higher activities for enzymes associated with glycogenolysis and glycolysis, with the exception of hexokinase. Red muscles, on the other hand, generally have higher concentrations of triglycerides and myoglobin and are better suited to derive their energy by oxidative phosphorylation via the electron transport system following the oxidation of fatty acids and glucose via the Krebs cycle (Green, 1951; Lawrie, 1952, 1953; Ogata, 1960; Domonkos, 1961; Domonkos and Latzkovits, 1961a,b; George and Talesara, 1961; Beatty *et al.*, 1963, 1966b, 1967; Blanchaer, 1964; Dawson and Romanul, 1964; George and Bokdawala, 1964; George and Iype, 1964; Dawson and Kaplan, 1965; Cosmos, 1966; Cosmos and Butler, 1967; Beecher *et al.*, 1968; Gutmann, 1968; Bass *et al.*, 1969; Kubĭsta *et al.*, 1971; Crabtree and Newsholme, 1972; Staudte and Pette, 1972; Baldwin *et al.*, 1973b; Pette, 1975).

Whereas anaerobic glycolysis and glycogenolysis are greater in white muscles, red muscles have (1) greater glycogen synthetase activities (Stubbs and Blanchaer, 1965; Bocek and Beatty, 1966; Beatty and Bocek, 1970), (2) higher glycogenesis (Bocek *et al.*, 1966a,b), and (3) higher hexokinase activities (Peter *et al.*, 1968; Bass *et al.*, 1969; Cardinet *et al.*, 1972; Crabtree and Newsholme, 1972; Pette, 1975). Explanations for

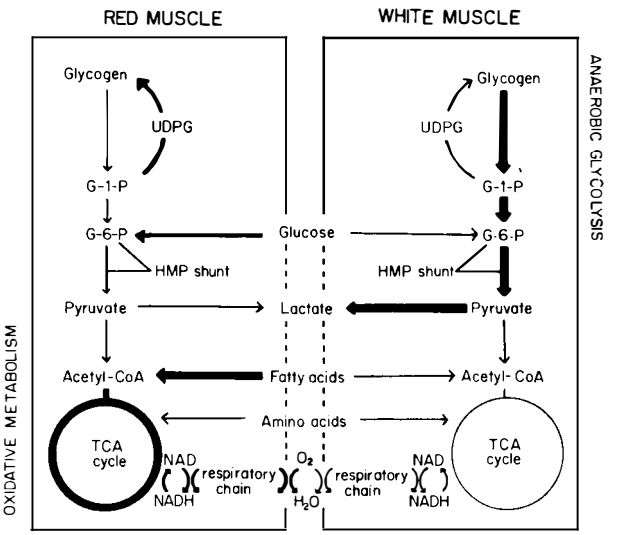


Fig. 5. Schematic representation of some differences in energy-yielding metabolic pathways of red and white muscles. In red muscles, energy for contraction is derived primarily by oxidative phosphorylation resulting from the oxidation of fatty acids, carbohydrates, and perhaps amino acids via the tricarboxylic acid cycle (TCA). White muscles derive their energy primarily via anaerobic glycogenolysis and glycolysis through the degradation of glycogen and glucose to lactate. Aerobic glycolysis [hexose monophosphate (HMP) shunt] is a minor pathway in both types of muscle.

those differences are not self-evident since similar relative activities for glycogenesis and glycogenolysis as well as hexokinase and glycolysis might be anticipated. Rather than having a relationship with other glycolytic enzymes, hexokinase activities demonstrate a constant relationship with mitochondrial citrate synthase activity.

Aerobic glycolysis via the pentose cycle does not appear to be a principal metabolic pathway in skeletal muscle (Glock and McLean, 1954; Green and Landau, 1965; Beatty *et al.*, 1966a,c; Beatty and Bocek, 1970).

The role of amino acids in the energy metabolism of muscle is uncertain. Transaminase activities are higher in red muscle of the chicken (Cardinet *et al.*, 1972). Also, amino acid uptake, incorporation, and turnover in skeletal muscle protein are greater in red muscle (Goldberg, 1967; Gutmann, 1968). Studies with ^{3}H-labeled amino acids have shown that, with the exception of alanine, all amino acids tested are more rapidly incorporated into red muscle protein (Citoler *et al.*, 1967). Alanine produced in muscle may function in the transport of amino groups to the liver, particularly during exercise (Felig and Wahren, 1971; Felig, 1977); however, that role is disputed by some. In the liver, alanine is reconverted to form glucose for transport back to muscle as part of the glucose-alanine cycle between muscle and liver.

Oxidation of fatty acids is an important energy source for muscle and is dependent on several factors, including carbohydrate availability, state of physical conditioning, and the intensity and duration of exercise (Issekutz *et al.*, 1965, 1966; Paul and Issekutz, 1967; Molé *et al.*, 1971; Holloszy *et al.*, 1971; Pande and Blanchaer, 1971; Therriault *et al.*, 1973; Issekutz *et al.*, 1975). Red muscle has been shown to contain larger quantities of lipid than white muscle (Beatty and Bocek, 1970). The activities of palmitate-activating enzyme and carnitine acyltranferases were twice as high in red as in white muscle

mitochondria. Studies indicate that red muscle is potentially capable of deriving more usable energy from free fatty acids than is white muscle (Pande and Blanchaer, 1971).

2. *Myosin ATPase: Metabolic Pathway Utilizing Energy for Contraction*

As previously discussed, the actin-activated myosin ATPase activity catalyzes the cyclical physiochemical interactions of actin and myosin during contraction (Fig. 4). Furthermore, the intrinsic speed of contraction (sarcomere shortening) has been demonstrated to be proprotional to the activity of actin-activated myosin ATPase (Barany, 1967; Mommaerts, 1969), and differences exist between the myosin ATPase activities of fast-contracting and slow-contracting muscles (Close, 1972). From those observations it is postulated that the rate-limiting step of sarcomere shortening during contraction is the hydrolysis of ATP. Concomitant with the different actin-activated ATPase activities of myosins from fast-contracting and slow-contracting muscles, their alkali light-chain compositions also differ (Lowey and Risby, 1971; Sarkar *et al.*, 1971). Slow- and fast-contracting muscles contain similar amounts of myosin; however, the ATPase activities of slow-contracting muscles are lower than in fast-contracting muscles, and their pH dependency and lability in acid and alkaline conditions differ (Barany *et al.*, 1965; Seidel, 1967).

D. Histology and Histochemistry

Different types of myofibers were recognized by early microscopists. By examination of unstained sections or in combination with various lipid stains, two basic types of myofibers were described. One type of myofiber was dark or opaque and contained numerous granules and lipid droplets between the myofibrils. The other type of myofiber was light or translucent and contained few granules and lipid droplets. These myofibers were referred to as dark and light myofibers, respectively. In addition, myofibers intermediate to dark and light myofibers were described (Bullard, 1912–1913). The most common finding was that mammalian muscles contained variable percentages of dark and light myofibers and therefore were mixed with respect to their myofiber-type composition. However, in certain instances, such as in the rabbit, guinea pig, and chicken, some muscles were found to contain predominantly dark myofibers and their gross coloration was red, while other muscles were found to contain predominantly light myofibers and their gross coloration was white. Although there was no unanimous agreement by early investigators, the tendency for myofibers of red muscles to be darker than the myofibers of white muscles led to the designation of dark and light myofibers as red and white myofibers, respectively. Histochemical and biochemical studies of red and white muscles have further tended to support the concept that gross muscle coloration is associated with the myofiber-type composition of a muscle (Nachmias and Padykula, 1958; Dubowitz and Pearse, 1960; Stein and Padykula, 1962; Romanul, 1964; Beatty *et al.*, 1966b; Bocek and Beatty, 1966; Cosmos and Butler, 1967; Gauthier, 1969).

With the development and introduction of histochemical techniques to studies of muscle, the heterogeneity of myofibers became more obvious. Since these techniques localized enzyme systems at the cellular level, their application involved implications of biochemical and functional heterogeneity of myofibers which had been suggested by the early microscopists. Histochemical studies of succinate dehydrogenase (SucDH), reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), and reduced

nicotinamide adenine dinucleotide phosphate-tetrazolium reductase demonstrated that the red or dark granular myofibers had higher activities for these enzymes than the white or less granular light myofibers (Padykula, 1952; Wachstein and Meisel, 1955; Nachmias and Padykula, 1958; Dubowitz and Pearse, 1960). In conjunction with electron microscopic observations, the activities of these enzymes were localized to mitochondria. The granules and higher activities of these enzymes in the dark or red myofibers correspond to greater numbers of mitochondria. Studies of red and white myofibers of the rat diaphragm and semitendinosus have revealed that the mitochondria of red myofibers are large with abundant cristae, while the white myofibers have fewer mitochondria which are smaller and have fewer cristae. Associated with the large mitochondria of the red myofibers are lipid inclusions. Therefore, the granules and lipid droplets described by early microscopists correspond to mitochondria and lipid inclusions, respectively (Padykula and Gauthier, 1963, 1967; Gauthier and Padykula, 1966; Gauthier, 1969).

A reciprocal histochemical profile for red and white myofibers was observed between mitochondrial enzymes and phosphorylase, an indicator of anaerobic glycolysis (Dubowitz and Pearse, 1960). Red myofibers with high mitochondrial enzyme activities had low phosphorylase activities, while the reverse was true of white myofibers, and a classification of myofibers was introduced in which red and white myofibers were designated as type I and type II myofibers, respectively (Dubowitz and Pearse, 1960). Owing to the fact that various intermediate histochemical reactions of myofibers exist between the extreme or classical characteristics described for red or type I myofibers and white or type II myofibers, other classifications have been proposed. Stein and Padykula (1962) proposed a classification of A, B, and C myofibers based on their SucDH reactions; that classification roughly corresponds with the classification of white, intermediate, and red muscle myofibers, respectively (Padykula and Gauthier, 1967). As many as eight myofiber types have been described in rat muscle, where esterase activity was found to be more precisely reciprocal with phosphorylase activity, suggesting a reciprocity between anaerobic glycolysis and lipid metabolism (Romanul, 1964).

In addition to employing histochemical stains for enzymes of ATP-generating metabolic pathways, myofibers have been differentiated histochemically into type I and type II myofibers based on their staining reaction for myofibrillar ATPase (Engel, 1962, 1965). Furthermore, type II myofibers, classified by the myofibrillar ATPase staining reaction, have been subdivided into type IIA, type IIB, and type IIC myofibers on the basis of their acid lability (Brooke and Kaiser, 1970). Of all the histochemical techniques applied, the myofibrillar ATPase reaction best differentiates myofiber types. If the actin-activated myosin ATPase activity is rate limiting in the speed of contraction, the histochemical method for myofibrillar ATPase would be anticipated to be the most specific method for the differentiation and association of myofiber types with contraction speed.

Histochemical localization of glycogen synthetase has varied. In human muscle its activity has been usually parallel with that of phosphorylase in type II or white fibers (Engel, 1962) but reciprocal and higher in type I or red fibers of rat and monkey muscle (Hess and Pearse, 1961; Bocek and Beatty, 1966) which is in agreement with quantitative results in the rat and monkey.

Since gross muscle coloration is often associated with the myofiber-type composition of a muscle and its contraction speed, myofiber types have been equated with speed of contraction. Therefore, red or type I myofibers have been referred to as slow or tonic, and white or type II myofibers as fast or phasic. That concept was based primarily on indirect evidence, and, while most evidence tended to support that concept, there were many

exceptions. Some red muscles which were slow contracting contained myofibers with intermediate staining intensities for mitochondrial enzymes, while some fast-contracting muscles contained significant numbers of red myofibers. It was suggested that fast-twitch myofibers were both red and white, while slow-twitch myofibers were intermediate (Barnard *et al.*, 1971). Also, studies of diaphragm muscles in small mammals (Gauthier and Padykula, 1966) and thyroarytenoid muscles in rabbits (Hall-Craggs, 1968) showed that some red muscles were fast contracting rather than slow. Since those muscles contained myofibers with many characteristics of red myofibers based on mitochondrial enzyme histochemistry, it was concluded that not all red myofiber types are slow contracting.

More direct evidence that supports and clarifies the interrelationships of physiological properties and histochemical properties of myofibers has come from combined studies of single motor units (Edstrom and Kugelberg, 1968; Burke *et al.*, 1973). In the rat, fatiguability of motor units was correlated with the histochemical properties of the myofibers comprising the units (Edstrom and Kugelberg, 1968). Fatiguable motor units were composed of type A myofibers, while fatigue-resistant motor units were composed of type C myofibers according to the myofiber typing classification of Stein and Padykula (1962). In the cat, three physiological types of motor units were classified in the medial gastrocnemius muscle on the basis of their contraction times and fatiguability. Each type of motor unit was found to have a unique histochemical profile for its myofibers (Burke *et al.*, 1973). Two types of motor units were fast contracting and stained dark with myofibrillar ATPase but differed in their sensitivity to fatigue. One type (FR) was resistant to fatigue and contained myofibers which were darkly stained for mitochondrial enzymes, while the other type (FF) fatigued rapidly and contained myofibers which were lightly stained for mitochondrial enzymes. The third type of motor unit was slow contracting, and its myofibers stained lightly for myofibrillar ATPase and darkly for mitochondrial enzymes. The physiological and histochemical properties and classifications of myofibers are summarized and illustrated in Table I and Fig. 6. Assessment of the histochemical profiles of myofibers in each physiological type of unit revealed that each motor unit was homogeneous with respect to its myofiber-type composition (Fig. 7).

IV. NEURAL TROPIC INFLUENCES ON MUSCLE

Tropic influences of nerve on muscle may be defined as those functions of the nerve that affect or regulate the metabolism of the muscle (Guth, 1968). This definition should encompass structural and physiological properties as well, since the structure of the myofiber and its organelles, physiological properties, and biochemical properties are all interdependent.

The implication of neuronal influences on muscle integrity have long been recognized by studies of denervation whereby numerous morphological, physiological, and biochemical changes result when a myofiber is separated from its axon (Gutmann, 1962). More precise implications concerning the influence of the motoneuron on speed of contraction and myofiber types have been demonstrated in a series of experiments in which nerves to fast and slow muscles have been cross-united (Buller *et al.*, 1960; Buller and Lewis, 1965; Close, 1965; Romanul and Van Der Meulen, 1966, 1967; Dubowitz, 1967; Robbins *et al.*, 1969; Barany and Close, 1971; Weeds *et al.*, 1974; Sreter *et al.*, 1975). In this experimental design, motoneurons which normally innervate slow muscles come to innervate muscles which are normally fast, while motoneurons which normally innervate fast

TABLE I

Physiological and Histochemical Properties and Classification of Myofiber Types

	Myofiber types		
Physiological properties			
Twitch contraction	Slow twitch	Fast twitch	Fast twitch
Fatiguability	Resistant	Resistant	Fatiguable
Histochemical properties			
Myofibrillar ATPase stain (pH 9.4)	Light	Dark	Dark
Preincubation at pH 4.5	Dark	Light	Dark
Preincubation at pH 4.2	Dark	Light	Light
NADH-TR stain	Intermediate to dark	Dark	Light
Phosphorylase stain	Light	Dark	Dark
Classifications			
Engel (1962, 1965)	I	II	II
Stein and Padykula (1962)	B	C	A
Padykula and Gauthier (1967)	Intermediate	Red	White
Brooke and Kaiser (1970)	I	IIA	IIB
Ashmore and Doerr (1971)	β-Red	α-Red	α-White
Peter *et al.* (1972)	Slow-twitch-oxidative	Fast-twitch-oxidative-glycolytic	Fast-twitch-glycolytic
Burke *et al.* (1973)	S	FR	FF
	(Slow-twitch-fatigue-resistant)	(Fast-twitch-fatigue-resistant)	(Fast-twitch-fatiguable)

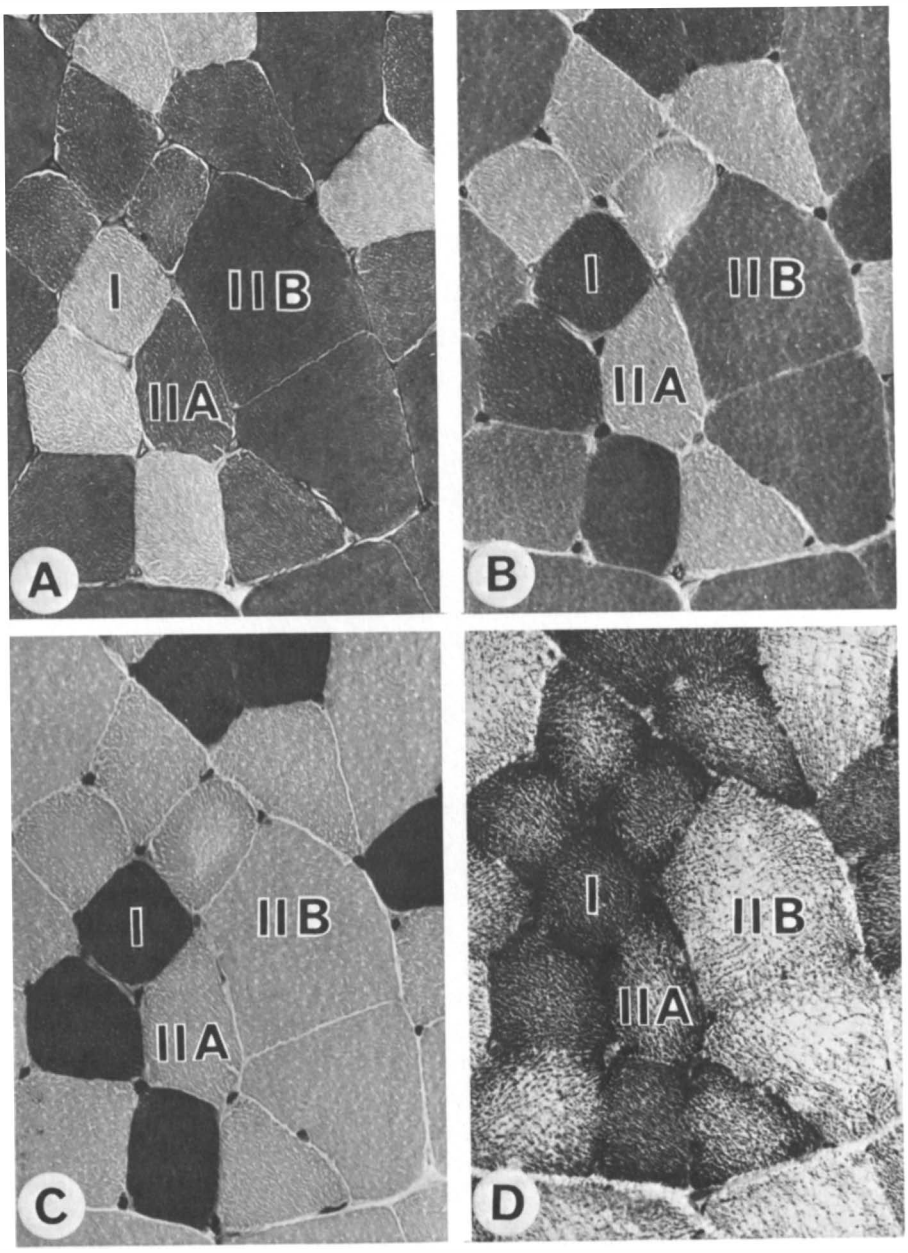


Fig. 6. Serial sections of cat medial gastrocnemius muscle incubated for the histochemical demonstration of (A) myofibrillar ATPase, incubated at pH 9.4; (B) myofibrillar ATPase, preincubated at pH 4.5; (C) myofibrillar ATPase, preincubated at pH 4.2; and (D) NADH-tetrazolium reductase. Type I myofibers comprise slow-twitch motor units that are resistant to fatigue. Type IIA myofibers comprise fast-twitch motor units that are resistant to fatigue, while type IIB myofibers comprise fast-twitch motor units that fatigue rapidly.

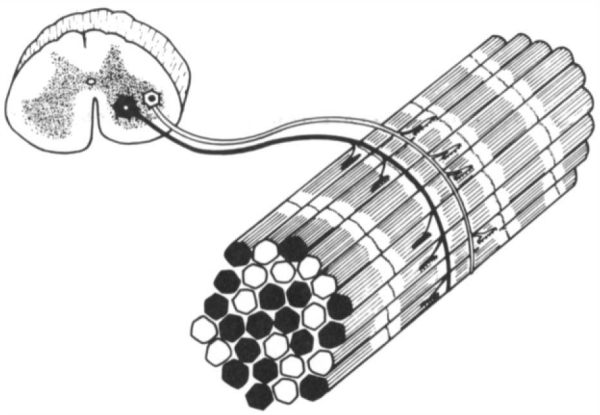


Fig. 7. Schematic representation of the homogeneity of skeletal muscle motor units. Motor units have a homogeneous myofiber-type composition whereby slow-contracting motor units are composed of only type I myofibers (lightly staining fibers), while fast-contracting motor units are composed of only type IIA or only type IIB myofibers (dark staining myofibers). Only one type of fast-contracting unit is illustrated.

muscles come to innervate muscles which are normally slow. These experiments have resulted in a reversal of contractile properties; i.e., fast-contracting muscles become slow, and slow-contracting muscles become fast. Accompanying those changes in the speed of contraction is a corresponding change in the enzyme histochemical profiles of the myofibers. Hence, slow muscles which have myofiber populations tending to have histochemical characteristics of high oxidative, low glycolytic, and low myofibrillar ATPase activities are changed to myofiber populations which tend to have low oxidative, high glycolytic, and high myofibrillar ATPase activities when innervated by a nerve which normally innervates a fast-contracting muscle. Converse changes occur by cross-union of a fast-contracting muscle with the nerve of a slow-contracting muscle (Romanul and Van Der Meulen, 1966, 1967; Dubowitz, 1967; Robbins *et al.*, 1969). Therefore, the motoneuron influences (1) the type of energy metabolism employed by a myofiber and all the structural changes in fiber organelles that this implies, and (2) the myofiber's physiological properties of contraction. The changes in contraction speed brought about by neuronal influences are presumably due to a direct effect on the type of myosin ATPase produced within a myofiber (Barany and Close, 1971; Weeds *et al.*, 1974; Sreter *et al.*, 1975).

Precisely how motoneurons exert their influence on myofibers is unknown. Two general postulates exist: (1) The influence of motoneurons is due to the frequency of their impulse discharge (Vrbova, 1963; Salmons and Vrbova, 1969; Pette *et al.*, 1973; Sreter *et al.*, 1973; Lomo *et al.*, 1974); or (2) the influence of motoneurons is mediated by specific chemical substances liberated by the motoneuron (Buller *et al.*, 1960). However mediated, the evidence suggests that the motoneuron directs the expression of the genetic complement in myofibers and that the myofiber maintains the potential for differentiation and redifferentiation at the direction of the nervous system.

V. ADAPTATIONS TO EXERCISE

Exercise induces major biochemical adaptations in skeletal muscle. The nutritional state, intensity and duration of exercise, and degree of physical fitness are all factors that

qualitatively and quantitatively affect the metabolic pathways used in the generation of energy for muscular contraction. The main fuels for muscular contraction are fatty acids and glucose, which are supplied by intramuscular and extramuscular depots during exercise. Intramuscular depots are the triglyceride and glycogen inclusions of the sarcoplasm, while the extramuscular depots are adipose tissues and the liver (Issekutz *et al.*, 1966; Paul and Issekutz, 1967; Holloszy *et al.*, 1971; Paul, 1971; Terjung *et al.*, 1972; Baldwin *et al.*, 1973a; Therriault *et al.*, 1973; Lindholm *et al.*, 1974a). The rate-limiting factor in the extramuscular supply of glucose to working muscle is glucose uptake by the myofiber. The rate-limiting factor in the plasma free fatty acid (FFA) supply appears to be the rate of FFA release from adipose tissues (Issekutz *et al.*, 1966).

At rest, oxidation of FFA and stored triglycerides contributes the bulk of the fuel used for maintaining muscle tone, whereas the oxidation of glucose accounts for only 10–20% of the CO_2 produced (Havel, 1971). At the onset of exercise, energy is initially derived from creatine phosphate and anaerobic glycogenolysis, which is accompanied by an increased production of lactate. However, as the duration of exercise is increased and blood flow increases, there is a shift to aerobic metabolism in which glucose, FFA, and triglycerides are oxidized and lactate production decreases (Havel, 1971; Rowell, 1971; Wahren *et al.*, 1971; Baldwin *et al.*, 1973b). At low to moderate exercise intensities, the oxidation of fatty acids provides the major source of energy. At moderate to high exercise intensities, the oxidation of fatty acids decreases and carbohydrates account for 50% or more of the amount of substrate utilized (Hagenfeld and Wahren, 1971; Saltin and Karlsson, 1971; Therriault *et al.*, 1973; Wahren, 1977). At moderately high exercise intensities, muscle glycogen and triglyceride depots decrease and muscle glycogen accounts for approximately 70% of the glucose oxidized, while 70% of the fatty acids oxidized are derived from muscle triglyceride depots. At maximal work loads, glycogen utilization is high and the major source of fuel (Hultman and Nilsson, 1971; Fröberg *et al.*, 1971).

Evidence exists that not all fibers are activated at once but that motoneurons are recruited with respect to their oxidative capacities (Baldwin *et al.*, 1973b). Measurements of rates of glycogen and triglyceride depletion in rats indicate that, in prolonged work of moderate intensity, the red and intermediate fibers are the first to become activated. Once the work load exceeds the capacity of the red and intermediate fibers, the white motor units are then activated by the central nervous system (Baldwin *et al.*, 1973a,b).

With training, muscle glycogen is increased (Gollnick *et al.*, 1972). The rate of lactate production is closely related to the intensity of exercise (Hermansen, 1971; Wahren, 1977), and lactate production is lower in trained than untrained subjects performing the same exercise. The increased plasma lactate concentrations are directly correlated with decreases in plasma FFA due to a reduction in FFA release from adipose tissues (Edwards *et al.*, 1971; Hermansen, 1971; Jorfeldt, 1971; Karlsson, 1971; Saltin and Essén, 1971; Saltin and Karlsson, 1971, Lindholm and Saltin, 1974; Issekutz *et al.*, 1975; Snow and Mackenzie, 1977). Glycogenolytic and glycolytic enzyme activities are unchanged or only slightly decreased in both red and white muscles of trained rats, with the exception of hexokinase. Hexokinase activities are markedly increased in trained rats and guinea pigs (Holloszy *et al.*, 1971; Baldwin *et al.*, 1973b; Peter *et al.*, 1968; Lamb *et al.*, 1969). While the increase occurred in both red and white muscles, the percent increase was greatest in red muscles (Baldwin *et al.*, 1973b).

The capacity for aerobic metabolism is increased by training. Oxygen consumption is

similar in trained and untrained subjects for a given work load, but there is a lower production of CO_2 and hence a lower respiratory quotient (RQ) in trained subjects (Holloszy *et al.*, 1971, 1977; Saltin and Karlsson, 1971). While several factors may affect the RQ, a major factor would be the source of energy for contraction, and fat utilization yields lower RQ values than carbohydrate utilization (Paul, 1971). The capacity for oxidation of palmitate, oleate, linoleate, palmityl-CoA, and palmitylcarnitine, as well as the activities for carnitine palmityltransferase, palmityl-CoA dehydrogenase, and mitochondrial ATP-dependent palmityl-CoA synthetase, were doubled in rats subjected to a training program (Molé *et al.*, 1971). Also, the turnover of FFA is significantly greater in trained than untrained dogs (Issekutz *et al.*, 1966).

Myoglobin content is greater in trained rats (Holloszy *et al.*, 1971) and pigs (Jørgensen and Hyldgaard-Jensen, 1975), in which the myoglobin functions to facilitate O_2 utilization by enhancing the intrafiber O_2 transport between the sarcolemma and mitochondria. The mitochondrial protein content increases approximately 60% (Holloszy, 1967; Molé *et al.*, 1971), which is associated with increased size and numbers of mitochondria in trained rats (Gollnick and King, 1969). An increase in mitochondrial size, but not number, has been reported in trained men (Morgan *et al.*, 1971). Cristae double their density within muscle mitochondria of trained rats (Holloszy *et al.*, 1971). Mitochondrial coupling factor I, mitochondrial ATPase, and cytochrome *c* are increased in trained subjects, whereas mitochondrial creatine phosphokinase and adenylate kinase activities were not increased (Oscai and Holloszy, 1971). Increased SucDH activities following training have been reported in man (Gollnick *et al.*, 1972; Saltin *et al.*, 1977), rats (Holloszy, 1967), and pigs (Jørgensen and Hyldgaard-Jensen, 1975), as have levels of NADH–cytochrome *c* reductase, succinate oxidase, cytochrome oxidase, citrate synthase, and NADH in trained rats (Holloszy, 1967; Kowalski *et al.*, 1969; Holloszy *et al.*, 1971) and guinea pigs (Barnard *et al.*, 1970).

In summary, trained subjects have increased capacities for aerobic metabolism. At work loads below maximal O_2 utilization, aerobic pathways are the principal sources of energy through the oxidation of fatty acids and to some degree glucose. This correlates with the evidence that red and intermediate fibers are activated first and their metabolic orientation is toward aerobic pathways. With work loads approaching maximal O_2 utilization, the sources of energy are derived principally from anaerobic pathways through glycogenolysis and glycolysis. White fibers are recruited only with increased work loads, and their metabolic energy derivation is mainly by anaerobic pathways. All of the complexities of energy derivation and fiber-type activities are not totally understood for various work load intensities and durations, but one can begin to conceive a general scheme of alterations in enzyme activities correlating to specific myofiber types in response to the type of exercise and needs for energy derivation.

VI. SERUM ENZYME DETERMINATIONS IN THE DIAGNOSIS OF NEUROMUSCULAR DISORDERS

A. General Principles

A valuable adjunct to the clinical diagnosis of neuromuscular diseases is the utilization of serum enzyme determinations. This involves the detection of enzymes in serum or

plasma. The activities or concentrations of the enzymes are usually low in serum or plasma because they are normally located within myofibers. Necrosis of myofibers is an example of a process by which serum activities of intracellular enzymes are elevated. Elevations in serum enzyme activities may also occur in association with increased cell permeability (leakage), increased enzyme production by the parenchymal cells, obstructions to normal enzyme excretory routes, increased amount of enzyme-forming tissue, delayed removal or inactivation of enzyme (Cornelius, 1967), and perhaps cell secretion.

B. Serum Creatine Phosphokinase

The most widely used serum enzyme determination in neuromuscular diseases of domestic animals is that for creatine phosphokinase (CPK). In muscle, this enzyme functions in making ATP available for contraction by the phosphorylation of ADP from creatine phosphate (Fig. 2).

$$\text{Creatine phosphate} + \text{ADP} \overset{\text{CPK}}{\rightleftarrows} \text{creatine} + \text{ATP}$$

The use of determinations of serum CPK (SCPK) offers greater promise of organ specificity in diseases of muscle than do other enzyme determinations employed to date. Analysis of tissues from human beings indicates that significant activities of CPK are present in skeletal muscle, myocardium, and brain, with lesser amounts in the gastrointestinal tract, uterus, urinary bladder, kidney, and thyroid (Dawson and Fine, 1967). The diversity of organs tested in other animals is not as broad, but those tested correlate well with these findings. The liver has negligible amounts of CPK (Oliver, 1955; Tanzar and Gilvarg, 1959; Colombo *et al.*, 1962; Eppenberger *et al.*, 1962; Cardinet *et al.*, 1967; Dawson and Fine, 1967).

Some normal values for SCPK activity have been determined in domestic animals (Table II); these vary with physical activity (Cardinet, 1969; Anderson, 1975; Blackmore and Elton, 1975; Rose *et al.*, 1977), age, and sex (Cardinet, 1969; Heffron *et al.*, 1976). Intramuscular injections may also increase SCPK activities due to local areas of muscle necrosis (Nevins *et al.*, 1973; Steiness *et al.*, 1978). The amount of CPK liberated following intramuscular injection of a drug depends on the properties of the injected solution and on such muscle factors as species differences in muscle CPK activity, local blood flow, susceptibility of the muscles, and local muscle binding of the drug (Steiness *et al.*, 1978). Therefore, an accurate history is important in evaluating SCPK activities.

There are three isoenzymatic forms of CPK. Creatine phosphokinase has a dimeric structure consisting of M (muscle) subunits and B (brain) subunits, which combine to form the three heterogeneous MM, MB, and BB isoenzymes (Dawson *et al.*, 1965, 1967). The isoenzymes can be separated by three different methods: (1) electrophoresis, (2) immunological techniques, and (3) ion-exchange chromatography (Fiolet *et al.*, 1977). The pattern of isoenzyme distribution varies among the organs of different species. Thus, identification of the isoenzymes present can be used to help determine the tissue source of elevations in SCPK (Eppenberger *et al.*, 1964a; Dawson *et al.*, 1965; Dawson and Fine, 1967; Van der Veen and Willebrands, 1966; Sherwin *et al.*, 1969; Klein *et al.*, 1973). Ontogenic studies in the rat revealed that all organs investigated contained only BB in early stages of fetal development. In skeletal muscle, BB forms slowly disappear and are initially replaced by MB forms followed by MM forms. Mixtures of isoenzymes occur during the transition. In the adult pattern, MB forms have been variously reported to be

TABLE II
Normal Values of Serum Creatine Phosphokinase Activity in Some Domestic Animals

Species	Age	Sex	SCPK activity (IU/liter) Mean	Standard deviation	Range	Reference
Canine[a]	0–4 months	Male	4.7	±0.3	1.2–8.2	Cardinet (1969)
		Female	3.0	±0.2	1.2–5.5	
	4–6 months	Male	2.4	±0.2	0.1–6.6	
		Female	1.8	±0.2	0.4–6.2	
	6–12 months	Male	1.8	±0.2	0.4–5.6	
		Female	0.8	±0.2	0.0–7.5	
	>12 months	Male	1.2	±0.3	0.2–2.6	
		Female	0.8	±0.1	0.0–1.8	
Canine[b]	>12 months	Both sexes	22.7	±7.3	9.9–28.8	Heffron *et al.* (1976)
Caprine[a]		Female	0.9	±0.9	0.0–2.5	Cardinet (1969)
Equine[a,c]	3–12 years		1.3	±0.9	0.0–3.6	Cardinet *et al.* (1967)
Feline[a]		Male	1.7	±1.1	0.4–3.4	Cardinet (1969)
		Female	1.9	±1.3	0.0–4.5	
Ovine[a]		Male	0.8	±0.6	0.0–2.9	Cardinet (1969)
		Female	0.5	±0.3	0.0–0.9	
Gallus domesticus[d]	27–134 days		38.1–59.1	—	—	Holliday *et al.* (1965)
	1 year	Male	109	—	—	
		Female	50	—	—	

[a] Enzyme activities determined without reducing agents.
[b] Enzyme activities determined in the presence of glutathione.
[c] Unexercised; not in training.
[d] Enzyme activities determined in the presence of cysteine.

present and absent. That inconsistency in noting the presence of MB forms in skeletal muscle of animals may be due to the source of skeletal muscle sampled, and all skeletal muscles may not contain the MB isoenzyme (Sherwin *et al.*, 1967; Thorstensson *et al.*, 1976). However, other studies indicate that, although there is more MM in white muscle of the rat than in red, there is no MB fraction in either (Dawson and Fine, 1967). Reasons for the discrepancies in the detection of MB forms in mammalian skeletal muscles are not evident at this time. The adult isoenzyme pattern in muscles of the rat appears at 90 days after birth, while in cardiac muscle the shift occurs earlier and the adult pattern has both MB and MM forms. In the brain, BB is the major isoenzyme throughout life (Eppenberger *et al.*, 1964a).

Determination of serum isoenzyme patterns has found clinical application in human medicine. The determination of MB forms is generally advocated as the best biochemical diagnostic tool for acute mycardial infarction (Fiolet *et al.*, 1977; Anonymous, 1978). Also, changes in serum isoenzyme patterns have been observed in various neuromuscular disorders and Duchenne muscular dystrophy (Goto *et al.*, 1969; Somer *et al.*, 1976). While the CPK isoenzyme patterns have not been employed in veterinary medicine, their application to clinical medicine needs to be investigated in cardiomyopathies and neuromuscular disorders of animals.

Elevations in total SCPK activities have been reported in the hereditary muscular dystrophies of chickens (Holliday *et al.*, 1965; Wagner *et al.*, 1971) and Syrian hamsters

(Eppenberger *et al.*, 1964b); in selenium-vitamin E deficiencies of cattle (Allen *et al.*, 1975; Van Vleet *et al.*, 1977), sheep (Whanger *et al.*, 1970), and swine (Ruth and Van Vleet, 1974); in myodegeneration due to ingestion of toxic plants in cattle (Henson *et al.*, 1965); in the arthrogryposis-hydranencephaly syndrome in calves (Hamada, 1974); in capture myopathies involving a moose (Haigh *et al.*, 1977) and pronghorns (Chalmers and Barrett, 1977); and in paralytic myoglobinuria involving horses (Gerber, 1964; Cardinet *et al.*, 1967; Lindholm *et al.*, 1974b) and a double-muscled heifer (Holmes *et al.*, 1972). Elevations in SCPK activities have also been reported in polioencephalomalacia and focal symmetric encephalomalacia of sheep, and it has been suggested that SCPK determinations may be of value in diagnosing diseases of the central nervous system (Smith and Healy, 1968). However, in diseases of the central nervous system which involve motor function, elevations of SCPK may be from skeletal muscle rather than from the central nervous system. This appears to be the case in central nervous system disorders in man (Cao *et al.*, 1969).

Diseases of muscle are classified, whenever possible, as to the origin or site of the primary lesion. Myopathies are those diseases in which the primary defect or disease process is considered to be limited to the myofibers, while neuropathies are those diseases of muscle which are secondary changes due to defects or diseases of the neuron (e.g., denervation). While SCPK determinations may be specific for diseases of muscle, they do not provide information relative to the origin of the disease process; however, elevations of SCPK are generally higher in myopathies than neuropathies because myonecrosis is a more prevalent pathological change in myopathies. More precise information regarding the origin of muscle diseases can be obtained by the use of histological and histochemical examination of muscle biopsies.

In a comparative study, the behavior of SCPK was found to be distinctly different from that of serum glutamic oxaloacetic transaminase (SGOT) during the course of paralytic myoglobinuria in horses (Fig. 8). The latter enzyme is discussed in the next section of this

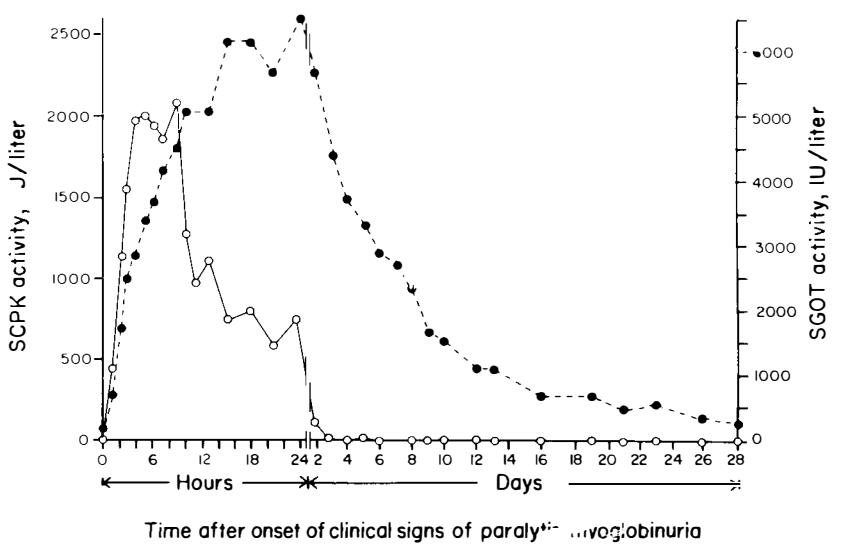


Fig. 8. The difference in the time course of elevations in SGOT and SCPK activities due to muscle necrosis (equine paralytic myoglobinuria). The SGOT activity remained elevated for much longer periods than SCPK activity. Key: (○) SCPK: (●) SGOT. (From Cardinet *et al.*, 1967.)

chapter. Elevations in SGOT activities were present for weeks after the onset of clinical disease, while SCPK activities remained elevated for only a few days. The course of elevations of these enzymes in this disease can be directly attributed to different disappearance rates of their activity in the plasma (Fig. 9).

While SCPK is more specific for myonecrosis than SGOT, the simultaneous determinations of SGOT and SCPK in the horse are potentially valuable diagnostic and prognostic aids owing to the different disappearance rates of their serum or plasma activities. (1) Elevated SCPK activities indicate that myonecrosis is active or has recently occurred; (2) persistent elevations of SCPK indicate that myonecrosis continues to be active; and (3) elevated SGOT due to myonecrosis accompanied by decreasing or normal SCPK activities indicates that myonecrosis is no longer active. It has not been established whether there are similar differences in the disappearance rates of SGOT and SCPK activities in the plasma of other animal species. Therefore, it is not known whether the same assessment of myonecrosis through the simultaneous determination of SGOT and SCPK activities can be applied to species other than the horse.

The normal SCPK values presented in Table II were determined by the method of Tanzer and Gilvarg (1959). Storage of serum at 0°–5°C results in a loss of activity; however, the addition of reducing agents such as cysteine or glutathione to the incubating medium results in higher values of serum activities, and the losses due to storage are minimized (Okinaka *et al.*, 1964; Weismann *et al.*, 1966; Hess *et al.*, 1968). The cysteine or glutathione reactivates the SCPK activity lost during storage, therefore, the addition of those reducing agents to the incubating medium appears to be the method of choice and necessitates the establishment of normal values when they are added to the incubating medium.

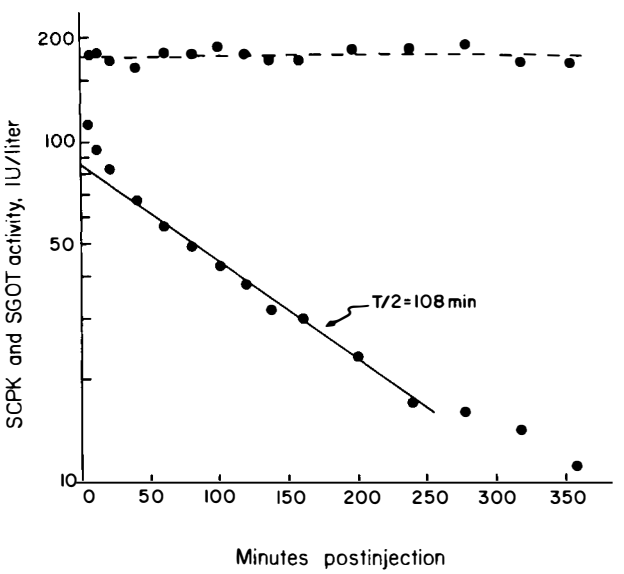


Fig. 9. Disappearance of SGOT and SCPK activities in the serum of a horse following the intravenous injection of GOT and CPK. The differences in the course of serum elevations of these two enzymes due to necrosis in the horse are the result of differences in their disappearance rates in the serum. Key: (———) SCPK; (---) SGOT. (From Cardinet *et al.*, 1967.)

C. Serum Glutamic Oxaloacetic Transaminase

Another enzyme which has been used as a diagnostic aid in neuromuscular disorders of domestic animals is serum glutamic oxaloacetic transaminase. Some normal values of SGOT activity reported for domestic animals are summarized in Table III. Normal values do not appear to differ greatly between sexes, although reported values for cows (Cornelius *et al.*, 1959a) are somewhat higher than values for bulls (Roussel and Stallcup, 1966). Differences associated with age have been reported in sheep (Lagace *et al.*, 1961), and there are seasonal differences in bulls (Roussel and Stallcup, 1966). Also, physical activity is associated with higher values in horses (Cornelius *et al.*, 1963; Cardinet *et al.*, 1963, 1967; Blackmore and Elton, 1975).

TABLE III

Normal Values of Serum Glutamic Oxaloacetic Transaminase Activity in Some Animals

Species	Comment	SGOT activity (Sigma-Frankel units/ml) Mean	Standard deviation	Range	Reference
Bovine	Bull, 1-97 weeks	23.7	±17.3	—	Roussel and Stallcup (1966)
	Cows, 2-10 years	43.8	±5.7	—	Cornelius *et al.* (1959a)
	Calves, 7-27 days	23.6	±3.7	—	Cornelius *et al.* (1959a)
Canine	>9 months	22.7	±5.4	—	Cornelius *et al.* (1959a)
	<2 years	26.6	±1.6	—	Hibbs and Coles (1965)
	4-12 months	22.1	±5.5	12-28	Crawley and Swenson (1965)
	1-5 years	25.8	±7.8	13-53	Crawley and Swenson (1965)
	>5 years	22.4	±5.2	14-30	Crawley and Swenson (1965)
Equine	Unexercised, not in training	165	±33.8	—	Cornelius *et al.* (1959a)
	>1 year	186	±52	120-336	Cornelius *et al.* (1963)
		151	±18.0	110-190	Cardinet *et al.* (1963)
		178	±52	99-354	Cardinet *et al.* (1967)
	Exercised, in training >1 year	346	±157	100-950	Cornelius *et al.* (1963)
		250	±31	185-320	Cardinet *et al.* (1963)
Feline	1 month	19.0	±4.8	12-27	Cornelius and Kaneko (1960)
Ovine	Lambs, 7-35 days	56	±31	—	Blincoe and Marble (1960)
	0-4 weeks	—	—	43-53	Lagace *et al.* (1961)
	5-8 weeks	—	—	<126	Lagace *et al.* (1961)
	>10 weeks	—	—	<81	Lagace *et al.* (1961)
Wild bighorn sheep	At capture, day 1	137	±15	—	Franzmann and Thorne (1970)
	After handling (day 3)	508	±101	—	Franzmann and Thorne (1970)
	After captivity (day 15)	136	±110	—	Franzmann and Thorne (1970)
	Grand mean	260	±196	—	Franzmann and Thorne (1970)
Domestic sheep		128	—	—	Franzmann and Thorne (1970)
Caprine adult[a]		222.9	±48.1	—	Lewis (1976)
Porcine	1-3 years	3.1	±14.1	—	Cornelius *et al.* (1959a)
Gallus domesticus	6 months	370	±186	—	Cornelius (1960)

[a] These values were converted from international units to Sigma-Frankel units.

Elevations of SGOT activities have been reported in white muscle disease of lambs, cows, and swine (Blincoe and Dye, 1958; Kuttler and Marble, 1958; Swingle *et al.*, 1959; Blincoe and Marble, 1960; Whanger *et al.*, 1970; Ruth and Van Vleet, 1974), tying up and paralytic myoglobinuria in horses (Cornelius *et al.*, 1963; Cardinet *et al.*, 1963, 1967; Lindholm *et al.*, 1974b), azoturia in a double-muscled heifer (Holmes *et al.*, 1972), hereditary muscular dystrophy in chickens (Cornelius *et al.*, 1959b), myodegeneration due to ingestion of toxic plants in cattle (Henson *et al.*, 1965), and capture myopathy in a moose (Haigh *et al.*, 1977) and pronghorns (Chalmers and Barrett, 1977). Although the use of SGOT determinations has proven valuable as a diagnostic aid, the enzyme lacks organ specificity since in addition to high concentrations in skeletal and cardiac muscle, GOT activities are also high in the liver as well as other organs (Cornelius *et al.*, 1959a; Nagode *et al.*, 1966; Cardinet *et al.*, 1967).

D. Serum Lactate Dehydrogenase

Lactate dehydrogenase (LDH) activities are high in various tissues of the body. Therefore, measurements of SLDH are not organ specific (Cornelius, 1971). Molecules of LDH are tetrameric, made up of four subunits of the two parent molecules, M (muscle) and H (heart). Various combinations of those subunits result in five isoenzymes of LDH, which can be separated by electrophoresis. The M molecule is found in purest form in skeletal muscle as the isoenzyme M_4, while the H molecule is found predominantly in the heart muscle as the isoenzyme H_4. The other three forms are molecular hybrids forming the isoenzymes M_3H, M_2H_2, and MH_3, and they are found in various amounts in different organs. The H_4 isoenzyme is maximally active at low concentrations of pyruvate and strongly inhibited by excess pyruvate (Dawson *et al.*, 1964), which favors the oxidation of lactate (Dawson *et al.*, 1964; Sund, 1969). The M form, on the other hand, maintains activity at relatively high pyruvate concentrations (Dawson *et al.*, 1964), which favors anaerobic reduction of pyruvate (Dawson *et al.*, 1964; Sund, 1969). Thus, tissues with essentially aerobic metabolism, such as heart muscle, contain mostly heart-specific isoenzymes, while tissues with more variable or flexible metabolic properties, such as skeletal muscle, contain predominantly the muscle-specific isoenzyme (Dawson *et al.*, 1964).

The LDH isoenzyme pattern within skeletal muscle seems to be under genetic control (Dawson *et al.*, 1964) but is influenced by environmental factors (Sjödin, 1976). Total LDH activity and muscle-specific LDH activity are higher in fast-twitch myofibers than in slow-twitch myofibers. Positive correlations have been found between both the individual percentage of fast-twitch myofibers and muscle lactate concentration, and between lactate concentration and total LDH activity and muscle-specific LDH activity, respectively (Tesch *et al.*, 1978). Since the fast-twitch myofibers derive more of their energy for contraction via anaerobic metabolism than do slow-twitch myofibers, it seems reasonable that fast-twitch myofibers should contain more of the muscle-specific LDH isoenzymes. Skeletal muscle LDH activity was found to increase with training in men (Suominen and Heikkinen, 1975; Sjödin *et al.*, 1976) and swine (Jørgensen and Hyldgaard-Jensen, 1975), whereas it has been reported to decrease with training in horses (Guy and Snow, 1977). The reasons for that difference are not obvious.

Elevated SLDH activities have been reported in selenium–vitamin E deficiency of cattle (Allen *et al.*, 1975), sheep (Whanger *et al.*, 1970), and swine (Ruth and Van Vleet, 1974) and in myoglobinuria in a double-muscled heifer (Holmes *et al.*, 1972) and in horses

(Rose *et al.*, 1977). Elevations in SLDH activity have also been reported in a variety of hepatic disorders (Cornelius, 1971). Therefore, unless isoenzyme analysis is utilized, the measurements of SLDH elevations are not organ specific.

VII. MUSCLE BIOPSY AND HISTOCHEMISTRY IN THE DIAGNOSIS OF NEUROMUSCULAR DISORDERS

The use of muscle biopsies in the evaluation of neuromuscular disorders (motor unit diseases) allows examination of myofibers, neuromuscular junctions, intramuscular nerve branches, connective tissues, and blood vessels. As discussed previously, histochemical examination of skeletal muscle provides information relative to the morphological, biochemical, and presumed physiological properties of myofibers. Therefore, the application of histochemical techniques in conjunction with routine light and electron microscopic examination of muscle biopsies offers the potential to evaluate and integrate the pathoanatomical, biochemical, and physiological manifestations of neuromuscular disorders. The application of histochemical techniques has become an essential diagnostic procedure in the evaluation of neuromuscular disorders in human beings (Engel, 1962, 1965, 1967, 1970; Dubowitz, 1966, 1968; Dubowitz and Brooke, 1973). Their application has been most helpful in determining which portion of the motor unit (neuron, myofiber, or both) is involved in the disease process. Their use has been particularly successful in distinguishing between neuropathies and myopathies and/or providing profiles specific for selected neuromuscular disorders (Engel, 1970; Dubowitz and Brooke, 1973). Routine histochemical procedures have been less informative in the evaluation of junctionopathies (disorders of neuromuscular transmission). However, immunoperoxidase methods are finding application in the evaluation of immune-mediated diseases of the neuromuscular junction in which deposits of immune complexes are localized along the end plate in myasthenia gravis (Engel *et al.*, 1977).

Histochemical techniques have been employed in the evaluation of neuromuscular disorders in animals of comparative biomedical research interest, such as in the hereditary muscular dystrophies of animals. However, only in recent years have such techniques begun to be applied to the evaluation of muscle biopsies in clinical cases of neuromuscular diseases in animals. A detailed consideration of muscle biopsy techniques in the evaluation of neuromuscular diseases is beyond the scope of this chapter. For details, the reader is referred to Dubowitz and Brooke (1973). A broader spectrum of neuromuscular disorders occurs in animals than is currently recognized. Neuromuscular disorders in animals are associated with endocrine, immune-mediated, infectious, metabolic, and neoplastic diseases, and continued application of those procedures will undoubtedly uncover many heretofore unrecognized neuromuscular disorders in animals (Cardinet and Holliday, 1979). While they have not been fully explored or precisely categorized, defects of carbohydrate, lipid, and electrolyte metabolism in skeletal muscle do exist in domestic animals common to clinical veterinary medicine.

REFERENCES

Allen, W. M., Bradley, R., Berrett, S., Parr, W. H., Swannack, K., Barton, C. R. Q., and Macphee, A. (1975). *Br. Vet. J.* **131,** 292-308.

Anderson, M. (1975). *Equine Vet. J.* **7,** 160–165.
Anonymous, (1978). *Lancet*—313–314.
Ashmore, C. R.. and Doerr, L. (1971). *Exp. Neurol.* **31,** 408–418.
Asmundson, V. S., and Julian, L. M. (1956). *J. Hered.* **47,** 248–252.
Baldwin, K. M., Reitman, J., Terjung, R., Winder, W., and Holloszy, J. (1973a). *Am. J. Physiol.* **225,** 1045–1050.
Baldwin, K. M., Winder, W. W., Terjung, R. L., and Holloszy, J. O. (1973b). *Am. J. Physiol.* **225,** 962–966.
Barany, M. (1967). *J. Gen. Physiol.* **50,** 197–218.
Barany, M., and Close, R. I. (1971). *J. Physiol. (London)* **213,** 455–474.
Barany, M., Barany, K., Reckard, T., and Volpe, A. (1965). *Arch. Biochem. Biophys.* **109,** 185–191.
Barden, J. A., and Mason, P. (1978). *Science* **199,** 1212–1213.
Barnard, R. J., Edgerton, V. R., and Peter, J. B. (1970). *J. Appl. Physiol.* **28,** 762–766.
Barnard, R. J., Edgerton, V. R., and Gillespie, C. A. (1971). *Arch. Biochem. Biophys.* **144,** 304–307.
Bass, A., Brdiczka, D., Eyer, P., Hofer, S., and Pette, D. (1969). *Eur. J. Biochem.* **10,** 198–206.
Beatty, C. H., and Bocek, R. M. (1970). *In* "Physiology and Biochemistry of Muscle as a Food" (E. J. Briskey, R. G. Cassens, and B. B. Marsh, eds.), Vol. 2, pp. 155–191. Univ. of Wisconsin Press, Madison.
Beatty, C. H., Peterson, R. D., and Bocek, R. M. (1963). *Am. J. Physiol.* **204,** 939–942.
Beatty, C. H., Basinger, G. M., and Bocek, R. M. (1966a). *Arch. Biochem. Biophys.* **117,** 275–281.
Beatty, C. H., Basinger, G. M., Dully, C. C., and Bocek, R. M. (1966b). *J. Histochem. Cytochem.* **14,** 590–600.
Beatty, C. H., Peterson, R. D., Basinger, G. M., and Bocek, R. M. (1966c). *Am. J. Physiol.* **210,** 404–410.
Beatty, C. H., Basinger, G. M., and Bocek, R. M. (1967). *J. Histochem. Cytochem.* **15,** 93–103.
Beecher, G. R., Kastenschmidt, L. L., Cassens, R. G., Hoekstra, W. G., and Briskey, E. J. (1968). *J. Food Sci.* **33,** 84–88.
Blackmore, D. J., and Elton, D. (1975). *Equine Vet. J.* **7,** 34–39.
Blanchaer, M. C. (1964). *Am. J. Physiol.* **206,** 1015–1020.
Blincoe, C., and Dye, W. B. (1958). *J. Anim. Sci.* **17,** 224–226.
Blincoe, C., and Marble, D. W. (1960). *Am. J. Vet. Res.* **21,** 866–869.
Bloom, W., and Fawcett, D. W. (1975). "A Textbook of Histology," 2nd ed. Saunders, Philadelphia, Pennsylvania.
Bocek, R. M., and Beatty, C. H. (1966). *J. Histochem. Cytochem.* **14,** 549–559.
Bocek, R. M., Basinger, G. M., and Beatty, C. H. (1966a). *Am. J. Physiol.* **210,** 1108–1111.
Bocek, R. M., Peterson, R. D., and Beatty, C. H. (1966b). *Am. J. Physiol.* **210,** 1101–1107.
Brooke, M. H., and Kaiser, K. K. (1970). *Arch. Neurol.* **23,** 369–379.
Bullard, H. H. (1912–1913). *Am. J. Anat.* **14,** 1–46.
Buller, A. J., and Lewis, D. M. (1965). *J. Physiol. (London)* **178,** 343–358.
Buller, A. J., Eccles, J. C., and Eccles, R. M. (1960). *J. Physiol. (London)* **150,** 417–439.
Burke, R. E., Levine, D. N., Tsairis, P., and Zajac, F. E. (1973). *J. Physiol. (London)* **234,** 723–748.
Cao, A., De Vigiliis, S., Lippi, C., and Trabalza, N. (1969). *Clin. Chim. Acta* **23,** 475–478.
Cardinet, G. H., III (1969). Unpublished observations.
Cardinet, G. H., III, and Holliday, T. A. (1979). *Ann. N.Y. Acad. Sci.* **317,** 290–313.
Cardinet, G. H., III, Fowler, M. E., and Tyler, W. S. (1963). *Am. J. Vet. Res.* **24,** 980–984.
Cardinet, G. H., III, Littrell, J. F., and Freedland, R. A. (1967). *Res. Vet. Sci.* **8,** 219–226.
Cardinet, G. H., III, Freedland, R. A., Tyler, W. S., and Julian, L. M. (1972). *Am. J. Vet. Res.* **33,** 1671–1684.
Chalmers, G. A., and Barrett, M. W. (1977). *J. Am. Vet. Med. Assoc.* **171,** 918–923.
Citoler, P., Benítez, L., and Maurer, W. (1967). *Exp. Cell Res.* **45,** 195.
Close, R. (1965). *Nature (London)* **206,** 831–832.
Close, R. I. (1972). *Physiol. Rev.* **52,** 129–197.
Colombo, J. P., Richterich, R., and Rossi, E. (1962). *Klin. Wochenschr.* **40,** 37–44.
Cornelius, C. E. (1960). *Calif. Vet.* **13,** 22–23.
Cornelius, C. E. (1967). *Proc. Am. Anim. Hosp. Assoc. Annu. Meet.*, pp. 82–87.
Cornelius, C. (1971). *In* "Clinical Biochemistry of Domestic Animals" (J. J. Kaneko and C. E. Cornelius, eds.), 2nd ed., Vol. 2, pp. 161–232. Academic Press, New York.
Cornelius, C. E., and Kaneko, J. J. (1960). *J. Am. Vet. Med. Assoc.* **137,** 62–66.

Cornelius, C. E., Bishop, J., Switzer, J., and Rhode, E. A. (1959a). *Cornell Vet.* **49,** 116–126.
Cornelius, C. E., Law, G. R., Julian, L. M., and Asmundson, V. S. (1959b). *Proc. Soc. Exp. Biol. Med.* **101,** 41–44.
Cornelius, C. E., Burnham, L. G., and Hill, H. E. (1963). *J. Am. Vet. Med. Assoc.* **142,** 639–642.
Cosmos, E. (1966). *Dev. Biol.* **13,** 163–181.
Cosmos, E., and Butler, J. (1967). *In* "Exploratory Concepts in Muscular Dystrophy and Related Disorders" (A. T. Milhorat, ed.), pp. 197–204. Excerpta Med. Found., Amsterdam.
Crabtree, B., and Newsholme, E. A. (1972). *Biochem. J.* **126,** 49–58.
Crawley, G. J., and Swenson, M. J. (1965). *Am. J. Vet. Res.* **26,** 1468–1471.
Davies, R. E. (1965). *In* "Muscle" (W. M. Paul, E. E. Daniel, C. M. Kay, and G. Monckton, eds.), pp. 49–69. Pergamon, Oxford.
Dawson, D. M., and Fine, I. (1967). *Arch. Neurol.* **16,** 175–180.
Dawson, D. M., and Kaplan, N. O. (1965). *J. Biol. Chem.* **240,** 3215–3221.
Dawson, D. M., and Romanul, F. C. A. (1964). *Arch. Neurol.* **11,** 369–378.
Dawson, D. M., Goodfriend, T. L., and Kaplan, N. O. (1964). *Science* **143,** 929–933.
Dawson, D. M., Eppenberger, H. M., and Kaplan, N. O. (1965). *Biochem. Biophys. Res. Commun.* **21,** 346–353.
Dawson, D. M., Eppenberger, H. M., and Kaplan, N. O. (1967). *J. Biol. Chem.* **242,** 210–217.
Denny-Brown, D. E. (1929). *Proc. R. Soc. London, Ser. B* **104,** 371.
Domonkos, J. (1961). *Arch. Biochem. Biophys.* **95,** 138–143.
Domonkos, J., and Latzkovits, L. (1961a). *Arch. Biochem. Biophys.* **95,** 144–146.
Domonkos, J., and Latzkovits, L. (1961b). *Arch. Biochem. Biophys.* **95,** 147–150.
Dubowitz, V. (1966). *J. Neurol., Neurosurg. Psychiatry* **29,** 23–28.
Dubowitz, V. (1967). *J. Physiol. (London)* **193,** 481–496.
Dubowitz, V. (1968). "Developing and Diseased Muscle." Spastics Int. Med. Publ., London.
Dubowitz, V., and Brooke, M. H. (1973). "Muscle Biopsy: A Modern Approach." Saunders, Philadelphia, Pennsylvania.
Dubowitz, V., and Pearse, A. G. E. (1960). *Histochemie* **2,** 105–117.
Ebashi, S., Endo, M., and Ohtsuki, I. (1969). *Q. Rev. Biophys.* **2,** 351–384.
Eccles, J. C., Eccles, R. M., and Lundberg, A. (1958). *J. Physiol. (London)* **142,** 275–291.
Edstrom, L., and Kugelberg, E. (1968). *J. Neurol., Neurosurg. Psychiatry* **31,** 424–433.
Edwards, R. H. T., Melcher, A., Hesser, C. M., and Wigertz, O. (1971). *Adv. Exp. Med. Biol.* **11,** 425–428.
Eisenberg, E., and Kielley, W. W. (1974). *J. Biol. Chem.* **249,** 4742–4748.
Engel, A. G., Lambert, E., and Howard, F. (1977). *Mayo Clin. Proc.* **52,** 267–280.
Engel, W. K. (1962). *Neurology* **12,** 778–794.
Engel, W. K. (1965). *In* "Neurohistochemistry" (C. W. M. Adams, ed.), pp. 622–672. Elsevier, Amsterdam.
Engel, W. K. (1967). *Pediat. Clin. North Am.* **14,** 963–995.
Engel, W. K. (1970). *Arch. Neurol.* **22,** 97–117.
Eppenberger, H. M., von Fellenberg, R., Richterich, R., and Aebi, H. (1962). *Enzymol. Biol. Clin.* **2,** 139–174.
Eppenberger, H. M., Eppenberger, M., Richterich, R., and Aebi, H. (1964a). *Dev. Biol.* **10,** 1–16.
Eppenberger, M., Nixon, C. W., Baker, J. R., and Homburger, F. (1964b). *Proc. Soc. Exp. Biol. Med.* **117,** 465–468.
Felig, P. (1977). *Ann. N.Y. Acad. Sci.* **301,** 57–72.
Felig, P., and Wahren, J. (1971). *Adv. Exp. Med. Biol.* **11,** 205–214.
Fiolet, J. W. T., Willebrands, A. F., Lie, K. I., and Ter Welle, H. F. (1977). *Clin. Chim. Acta* **80,** 23–35.
Franzmann, A. W., and Thorne, E. T. (1970). *J. Am. Vet. Med. Assoc.* **157,** 647–650.
Fröberg, S. O., Carlson, L. A., and Ekelund, L.-G. (1971). *Adv. Exp. Med. Biol.* **11,** 307–313.
Gauthier, G. F. (1969). *Z. Zellforsch. Mikrosk. Anat.* **95,** 462–482.
Gauthier, G. F., and Padykula, H. A. (1966). *J. Cell Biol.* **28,** 333–354.
Gazith, J., Himmelfarb, S., and Harrington, W. F. (1970). *J. Biol. Chem.* **245,** 15–22.
George, J. C., and Bokdawala, F. D. (1964). *J. Anim. Morphol. Physiol.* **11,** 124–132.
George, J. C., and Iype, P. T. (1964). *Pavo* **2,** 84–87.
George, J. C., and Talesara, C. L. (1961). *J. Cell. Comp. Physiol.* **58,** 253–260.
Gerber, H. (1964). *Zentralbl. Veterinaermed.* **1,** 135–150.
Gershman, L. C., Stratcher, A., and Dreizen, P. (1969). *J. Biol. Chem.* **224,** 2726–2736.

Glock, G. E., and McLean, P. (1954). *Biochem. J.* **56,** 171–175.
Goldberg, A. L. (1967). *Nature (London)* **216,** 1219–1220.
Gollnick, P. D., and King, D. W. (1969). *Am. J. Physiol.* **216,** 1502–1509.
Gollnick, P. D., Armstrong, R. B., Saubert, C. W., Piehl, K., and Saltin, B. (1972). *J. Appl. Physiol.* **33,** 312–319.
Goto, I., Nagamine, M., and Katsuki, S. (1969). *Arch. Neurol.* **20,** 422–429.
Granit, R., Phillips, C. G., Skoglund, S., and Steg, G. (1957). *J. Neurophysiol.* **20,** 470–481.
Green, D. E. (1951). *In* "Enzymes and Enzyme Systems" (J. T. Edsall, ed.), pp. 15–46. Harvard Univ. Press, Cambridge, Massachusetts.
Green, M. R., and Landau, B. R. (1965). *Arch. Biochem. Biophys.* **111,** 569–575.
Guth, L. (1968). *Physiol. Rev.* **48,** 645–687.
Gutmann, E. (1962). "The Denervated Muscle." Publ. House Czech. Acad. Sci., Prague.
Gutmann, E. (1968). *Symp. Biol. Hung.* **8,** 56.
Guy, P. S., and Snow, D. H. (1977). *Biochem. Biophys. Res. Commun.* **75,** 863–869.
Hagenfeldt, L., and Wahren, J. (1971). *Adv. Exp. Med. Biol.* **11,** 153–163.
Haigh, J. C., Stewart, R. R., Wobeser, G., and MacWilliams, P. S. (1977). *J.Am. Vet. Med. Assoc.* **171,** 924–926.
Hall-Craggs, E. C. B. (1968). *J. Anat.* **102,** 241–255.
Hamada, T. (1974). *Vet. Rec.* **95,** 441.
Hamilton, M. J., Hegreberg, G. A., and Gorham, J. R. (1974). *Am. J. Vet. Res.* **35,** 1321–1324.
Hanson, J., and Huxley, H. E. (1953). *Nature (London)* **172,** 530–532.
Hanson, J., and Lowy, J. (1963). *J. Mol. Biol.* **6,** 46–60.
Harper, J. A., and Parker, J. E. (1964). *Poult. Sci.* **43,** 1326–1327.
Harper, J. A., and Parker, J. E. (1967). *J. Hered.* **58,** 189–193.
Harris, J. B., ed. (1979). *Ann. N.Y. Acad. Sci.* **317.**
Havel, R. J. (1971). *Adv. Exp. Med. Biol.* **11,** 315–325.
Heffron, J. J. A., Bomzor, L., and Pattinson, R. A. (1976). *Vet. Rec.* **98,** 338–340.
Hegreberg, G. A., Camacho, Z., and Gorham, J. R. (1974). *Arch. Pathol.* **97,** 225–229.
Henson, J. B., Dollahite, J. W., Bridges, C. H., and Rao, R. R. (1965). *J. Am. Vet. Med. Assoc.* **147,** 142–145.
Hermansen, L. (1971). *Adv. Exp. Med. Biol.* **11,** 401–417.
Hess, J. W., Murdock, K. L., and Natho, G. J. W. (1968). *Am. J. Clin. Pathol.* **50,** 89–97.
Hess, R., and Pearse, A. G. E. (1961). *Proc. Soc. Exp. Biol. Med.* **107,** 569–571.
Hibbs, C. M., and Coles, E. H. (1965). *Proc. Soc. Exp. Biol. Med.* **118,** 1059–1062.
Holliday, T. A., Asmundson, V. S., and Julian, L. M. (1965). *Enzymol. Biol. Clin.* **5,** 209–216.
Holloszy, J. O. (1967). *J. Biol. Chem.* **242,** 2278–2282.
Holloszy, J. O., Oscai, L. B., Molé, P. A., and Don, I. J. (1971). *Adv. Exp. Med. Biol.* **11,** 51–61.
Holloszy, J. O., Rennie, M. J., Hickson, R. C., Conlee, R. K., and Hagberg, J. M. (1977). *Ann. N.Y. Acad. Sci.* **301,** 440–450.
Holmes, H. G., Ashmore, C. R., Robinson, D. W., Finn, J. P., and O'Dell, J. (1972). *Vet. Rec.* **90,** 625–630.
Homburger, F., Baker, J. R., Nixon, G. W., and Wilgram, G. (1962). *Arch. Intern. Med.* **110,** 660–662.
Hultman, E., and Nilsson, L. H. (1971). *Adv. Exp. Med. Biol.* **11,** 143–151.
Huxley, A. F. (1974). *J. Physiol. (London)* **243,** 1–43.
Huxley, A. F., and Niedergerke, R. (1954). *Nature (London)* **173,** 971–973.
Huxley, H. E. (1963). *J. Cell Biol.* **19,** 369–390.
Huxley, H. E. (1969). *Science* **164,** 1356–1366.
Huxley, H. E., and Hanson, J. (1954). *Nature (London)* **173,** 973–976.
Innes, J. R. (1951). *Br. Vet. J.* **107,** 131–143.
Issekutz, B., Miller, H. I., Paul, P., and Rodahl, K. (1965). *J. Appl. Physiol.* **20,** 293–296.
Issekutz, B., Miller, H. I., and Rodahl, K. (1966). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **25,** 1415–1420.
Issekutz, B., Shaw, W. A. S., and Issekutz, T. B. (1975). *J. Appl. Physiol.* **39,** 349–353.
Jørgensen, P. F., and Hyldgaard-Jensen, J. F. (1975). *Acta Vet. Scand.* **16,** 368–378.
Jorfeldt, L. (1971). *Adv. Exp. Med. Biol.* **11,** 409–417.
Karlsson, J. (1971). *Adv. Exp. Med. Biol.* **11,** 383–393.
Klein, M. S., Shell, W. E., and Sobel, B. E. (1973). *Cardiovasc. Res.* **7,** 412–418.
Kowalski, K., Gordon, E. E., Martinez, A., and Adamek, J. (1969). *J. Histochem. Cytochem.* **17,** 601–607.

Kronecker, H., and Stirling, W. (1878). *Arch. Anat. Physiol., Physiol. Abt.* **2,** 1.
Kubišta, V., Kubištová, J., and Pette, D. (1971). *Eur. J. Biochem.* **18,** 553-560.
Kuttler, K. L., and Marble, D. W. (1958). *Am. J. Vet. Res.* **19,** 632-636.
Lagace, A., Bell, D. S., Moxon, A. L., and Pounden, W. B. (1961). *Am. J. Vet. Res.* **22,** 686-688.
Lamb, D. R., Peter, J. B., Jeffress, R. N., and Wallace, H. A. (1969). *Am. J. Physiol.* **217,** 1628-1632.
Lawrie, R. A. (1952). *Nature (London)* **170,** 122-123.
Lawrie, R. A. (1953). *Biochem. J.* **55,** 298-305.
Lee, F. S., Guenther, A. E., and Meleney, H. E. (1916). *Am. J. Physiol.* **40,** 446-473.
Lewis, J. H. (1976). *Am. J. Vet. Res.* **37,** 601-605.
Lindholm, A., and Saltin, B. (1974). *Acta Vet. Scand.* **15,** 310-324.
Lindholm, A., Bjerneld, H., and Saltin, B. (1974a). *Acta Physiol. Scand.* **90,** 475-484.
Lindholm, A., Johansson, H., and Kjaersgaard, P. (1974b). *Acta Vet. Scand.* **15,** 325-339.
Lomo, T., Westgaard, R. H., and Dahl, N. A. (1974). *Proc. R. Soc. London, Ser. B* **187,** 99-103.
Lowey, S., and Risby, D. (1971). *Nature (London)* **234,** 81-85.
Lymn, R. W., and Taylor, E. W. (1971). *Biochemistry* **10,** 4617-4624.
McGavin, M. D., and Baynes, I. D. (1969). *Pathol. Vet.* **6,** 513-524.
Michelson, A. M., Russell, E. S., and Harman, P. J. (1955). *Proc. Natl. Acad. Sci. U.S.A.* **41,** 1079-1084.
Molé, P. A., Oscai, L. B., and Holloszy, J. R. (1971). *J. Clin. Invest.* **50,** 2323-2330.
Mommaerts, W. F. H. M. (1969). *Physiol. Rev.* **49,** 427-508.
Morgan, T. E., Cobb, L. A., Short, F. A., Ross, R., and Gunn, D. R. (1971). *Adv. Exp. Med. Biol.* **11,** 87-95.
Nachmias, V. T., and Padykula, H. A. (1958). *J. Biophys. Biochem. Cytol.* **1,** 47-59.
Nagode, L. A., Frajola, W. J., and Loeb, W. F. (1966). *Am. J. Vet. Res.* **27,** 1385-1393.
Needham, D. M. (1926). *Physiol. Rev.* **6,** 1-27.
Nevins, M. A., Saran, M., Bright, M., and Lyon, L. J. (1973). *J. Am. Med. Assoc.* **224,** 1382-1387.
Ogata, T. (1960). *J. Biochem. (Tokyo)* **47,** 726-732.
Okinaka, S., Sugita, H., Momoi, H., Toyokura, Y., Watanabe, I., Ebashi, F., and Ebashi, S. (1964). *J. Lab. Clin. Med.* **64,** 299-305.
Oliver, I. T. (1955). *Biochem. J.* **61,** 116-122.
Oscai, L. B., and Holloszy, J. O. (1971). *J. Biol. Chem.* **246,** 6968-6972.
Padykula, H. A. (1952). *Am. J. Anat.* **91,** 107-145.
Padykula, H. A., and Gauthier, G. F. (1963). *J. Cell Biol.* **18,** 87-107.
Padykula, H. A., and Gauthier, G. F. (1967). *In* "Exploratory Concepts in Muscular Dystrophy and Related Disorders" (A. T. Milhorat, ed.), pp. 117-127. Excerpta Med. Found., Amsterdam.
Pande, S. V., and Blanchaer, M. C. (1971). *Am. J. Physiol.* **220,** 549-553.
Paul, P. (1971). *Adv. Exp. Med. Biol.* **11,** 225-247.
Paul, P., and Issekutz, B. (1967). *J. Appl. Physiol.* **22,** 615-622.
Peter, J. B., Jeffress, R. N., and Lamb, D. R. (1968). *Science* **160,** 200-201.
Peter, J. B., Barnard, R. J., Edgerton, V. R., Gillespie, C. A., and Stempel, K. E. (1972). *Biochemistry* **11,** 2627-2633.
Pette, D. (1975). *Acta Histochem., Suppl.* **14,** 47-68.
Pette, D., Smith, M. E., Staudte, H. W., and Vrbova, G. (1973). *Pfluegers Arch.* **338,** 257-272.
Ranvier, L. (1873). *C. R. Acad. Sci.* **77,** 1030-1043.
Ranvier, L. (1874). *Arch. Physiol. Norm. Pathol.* **1,** 5-15.
Ranvier, L. (1880). "Lecons d'anatomie generale sur les systemes musculaires." De La Haye, Paris.
Robbins, N., Karpati, G., and Engel, W. K. (1969). *Arch. Neurol.* **20,** 318-329.
Romanul, F. C. A. (1964). *Arch. Neurol.* **11,** 355-368.
Romanul, F. C. A., and Van Der Meulen, J. P. (1966). *Nature (London)* **212,** 1369-1370.
Romanul, F. C. A., and Van Der Meulen, J. P. (1967). *Arch. Neurol.* **17,** 387-402.
Rose, R. J., Purdue, R. A., and Hensley, W. (1977). *Equine Vet. J.* **9,** 122-126.
Roussel, J. D., and Stallcup, O. T. (1966). *Am. J. Vet. Res.* **27,** 1527-1530.
Rowell, L. B. (1971). *Adv. Exp. Med. Biol.* **11,** 127-141.
Ruth, G. R., and Van Vleet, J. F. (1974). *Am. J. Vet. Res.* **35,** 237-244.
Salmons, S., and Vrbova, G. (1969). *J. Physiol. (London)* **201,** 535-549.
Saltin, B., and Essén, B. (1971). *Adv. Exp. Med. Biol.* **11,** 419-424.
Saltin, B., and Karlsson, J. (1971). *Adv. Exp. Med. Biol.* **11,** 289-299.
Saltin, B., Henriksson, J., Nygaard, E., and Andersen, P. (1977). *Ann. N.Y. Acad. Sci.* **301,** 3-29.

Sarkar, S., Sreter, F. A., and Gergely, J. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 946–950.
Seidel, J. C. (1967). *J. Biol. Chem.* **242,** 5623–5629.
Sherwin, A. L., Siber, G. R., and Elhilali, M. M. (1967). *Clin. Chim. Acta* **17,** 245–249.
Sherwin, A. L., Norris, J. W., and Bulcke, J. A. (1969). *Neurology* **19,** 993–999.
Sjödin, B. (1976). *Acta Physiol. Scand., Suppl.* **436,** 1–32.
Sjödin, B., Thorstensson, A., Frith, K., and Karlsson, J. (1976). *Acta Physiol. Scand.* **97,** 150–157.
Smith, J. B., and Healy, P. L. (1968). *Clin. Chim. Acta* **21,** 295.
Snow, D. H., and Mackenzie, G. (1977). *Equine Vet. J.* **9,** 134–140.
Somer, H., Dubowitz, V., and Donner, M. (1976). *J. Neurol. Sci.* **29,** 129–136.
Sreter, F. A., Gergely, J., Salmons, S., and Romanul, F. (1973). *Nature (London), New Biol.* **241,** 17–19.
Sreter, F. A., Luff, A. R., and Gergely, J. (1975). *J. Gen. Physiol.* **66,** 811–821.
Staudte, H. W., and Pette, D. (1972). *Comp. Biochem. Physiol. B* **41,** 553–540.
Stein, J. M., and Padykula, H. A. (1962). *Am. J. Anat.* **110,** 103–124.
Steiness, E., Rasmussen, F., Svendsen, O., and Nielsen, P. (1978). *Acta Pharmacol. Toxicol.* **42,** 357–364.
Stubbs, S. G., and Blanchaer, M. C. (1965). *Can. J. Biochem.* **43,** 463–468.
Sund, H. (1969). *In* "Biological Oxidations" (T. P. Singer, ed.), pp. 603–632. Wiley (Interscience), New York.
Suominen, H., and Heikkinen, E. (1975). *Eur. J. Appl. Physiol.* **34,** 249–254.
Swingle, K. F., Young, S., and Dang, H. C. (1959). *Am. J. Vet. Res.* **20,** 75–77.
Tanzer, M. L., and Gilvarg, C. (1959). *J. Biol. Chem.* **234,** 3201–3204.
Terjung, R. L., Baldwin, K. M., Molé, P. A., Klinkerfuss, G. H., and Holloszy, J. O. (1972). *Am. J. Physiol.* **223,** 549–554.
Tesch, P., Sjödin, B., and Karlsson, J. (1978). *Acta Physiol. Scand.* **103,** 40–46.
Therriault, D. G., Beller, G. A., Smoake, J. A., and Hartley, L. H. (1973). *J. Lipid Res.* **14,** 54–60.
Thorstensson, A., Elwin, K., Sjödin, B., and Karlsson, J. (1976). *Scand. J. Clin. Lab. Invest.* **36,** 821–826.
Van der Veen, K. J., and Willebrands, A. F. (1966). *Clin. Chim. Acta* **13,** 312–316.
Van Vleet, J. F., Crawley, R. R., and Amstutz, H. E. (1977). *J. Am. Vet. Med. Assoc.* **171,** 443–445.
Vrbova, G. (1963). *J. Physiol. (London)* **169,** 513–526.
Wachstein, M., and Meisel, E. (1955). *J. Biophys. Biochem. Cytol.* **1,** 483–487.
Wagner, P. D., and Weeds, A. G. (1977). *J. Mol. Biol.* **109,** 455–473.
Wagner, W. D., Peterson, R. A., and Anido, V. (1971). *Am. J. Vet. Res.* **32,** 2091–2093.
Wahren, J. (1977). *Ann. N.Y. Acad. Sci.* **301,** 45–55.
Wahren, J., Ahlborg, G., Felig, P., and Jorfeldt, L. (1971). *Adv. Exp. Med. Biol.* **11,** 189–203.
Weeds, A. G. (1969). *Nature (London)* **223,** 1362–1364.
Weeds, A. G., Lowey, S. (1971). *J. Mol. Biol.* **61,** 701–725.
Weeds, A. G., Trentham, D. R., Kean, C. J. C., and Buller, A. J. (1974). *Nature (London)* **247,** 135–139.
Weismann, U., Colombo, J. P., Adam, A., and Richterich, R. (1966). *Enzymol. Biol. Clin.* **7,** 266–284.
Whanger, P. D., Weswig, P. H., Muth, O. H., and Oldfield, J. E. (1970). *Am. J. Vet. Res.* **31,** 965–972.

15

Calcium, Phosphorus, and Magnesium Metabolism

MOGENS G. SIMESEN

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

I. CALCIUM AND INORGANIC PHOSPHORUS METABOLISM

Over 70% of the ash of the body consists of calcium and phosphorus. More than 99% of the total Ca and 80–85% of the P are contained in the skeleton and the teeth The minor portions present in the body fluids, although negligible in amount, play an extremely important role in the maintenance of normal body functions. The Ca in the extracellular fluid is critical for (1) normal neuromuscular exitability, (2) capillary and cell membrane permeability, (3) normal muscle contraction, (4) normal transmission of nerve impulses, and (5) normal blood coagulation.

Phosphorus outside the skeleton plays an even more fascinating role. It is involved in vital cellular structures and serves in the degradation and synthesis of numerous carbon compounds. High-energy phosphate bonds play a fundamental role in storage, liberation, and transfer of energy. Finally, the ability of P to be excreted either as $H_2PO_4^-$ or HPO_4^{2-} gives a broad margin for the acid-base metabolism in the body.

A. Calcium and Phosphorus in Composition and Formation of Bone

Normal adult bone is composed of approximately 45% water, 25% ash, 20% protein, and 10% fat. In mammals the ash is made up of 36% Ca, 17% P, and 0.8% Mg.

The major constituents of bone are collagen, which comprises the bulk of the inorganic matrix, and calcium phosphate, largely in the form of small crystals of hydroxyapatite. Other components include glucoproteins, acid mucopolysaccharides, lipids, calcium phosphate salts other than hydroxyapatite, and ions, such as Mg, CO_3^{2-}, Na, and F, which are associated with the mineral phase (Raisz, 1977).

Bone mineral is different from pure hydroxyapatite, which is a macrocrystalline mineral of the composition $Ca_{10}—(PO_4)_6—(OH)_2$. Calcium and phosphorus may be deposited initially not as hydroxyapatite but as amorphous calcium phosphate salts that are gradually transformed into hydroxyapatite crystals (Posner, 1973). These bone crystals are small and impure, and many ions other than the Ca, phosphate, and hydroxyl of pure synthetic hydroxyapatite are incorporated or adsorbed to their surfaces. There are substantial quantities of CO_3^{2-}, Na, K, and Mg in bone mineral, mainly in the hydration shell. The small apatite crystals, with their hydration shells, provide an enormous surface for exchange, which can rapidly take up limited quantities of a variety of bone-seeking elements. The amount of amorphous calcium phosphate, imperfect crystals, and incompletely mineralized bone tends to decrease with age. With maturation, the exchange of various ions between bone and extracellular fluid decreases (Vaughan, 1973).

Calcium and phosphorus always occur in an approximately 2 : 1 ratio, and this is not altered even in conditions of partial demineralization of the bones. Therefore, without ash percentage, the percentage of Ca and P is a poor indication of an animal's mineral reserve (Duncan, 1958). Cancellous bone is more readily resorbed than compact bone, and accordingly changes are best demonstrated in vertebrae or ribs.

Bone mineral is readily mobilized to maintain the level of serum Ca but less readily to maintain that of P, so that a low serum inorganic phosphate (P_i) level is the first sign of deficiency of P.

All bone formation involves (1) the production of a suitable matrix of organic material consisting of collagenous fibrils and a binding substance of mucoproteins and (2) the

precipitation of bone salts in the binding substance. Osteoblasts bring about both processes.

The precipitation of insoluble Ca salts at the site of bone formation is favored by secretion of a phosphatase, which in alkaline solution converts organic esters of phosphoric acid to P_i. Alkaline phosphatase (AP) is formed by osteoblasts (Robison, 1923). Some AP reaches the bloodstream and is found in the circulating blood.

The possibility and extent of deposition depend on the concentration of Ca and P in the serum and interstitial fluid. If Ca and/or P concentrations are too low, even the serum AP activity may not be effective enough to induce calcification. In rickets, the undersaturation of body fluids with Ca and P retards new bone formation in the epiphysis and also causes excessive resorption of bone from the diaphysis.

It is in cancellous bone with its extensive surface exposed to interstitial fluid that most of the bone turnover of Ca and P takes place. Mobilization and deposition of Ca are controlled by the output of parathyroid hormone and thyrocalcitonin.

Although the action of vitamin D is predominantly an enhancement of Ca absorption from the intestine, it is also essential for the normal growth of bones, exerting a specific effect at the site of calcification. Defects in this mechanism result in rickets.

B. Calcium and Phosphorus Absorption

The entry and exit of Ca and P from the body are subject to tight hormonal regulation, and the amounts of Ca and P in the extracellular fluid, cells, and bone are generally dependent on the amounts absorbed in the intestine and excreted in the kidney. When dietary intake of Ca is low, serum Ca levels can be maintained by bone resorption. When dietary intake of phosphate is low or deficient, serum P_i levels fall. The ability of the bone to provide needed phosphate is limited. When Ca and P supplies are adequate, the intestine controls Ca entry by selective absorption. In animals in Ca balance excretion is equal to the amount absorbed. When diets are rich in Ca balance, a transient hypercalcemia is often observed (Franklin *et al.*, 1948; Saarinen, 1950). When P intake is excessive, the kidney is able to excrete the load quite rapidly, but prolonged P loading can lead to secondary hyperparathyroidism (see Section IV, B, 4).

The efficiency of Ca absorption increases in conditions causing increased demands (Morrissey and Wasserman, 1971). Young sheep with high Ca requirements absorb Ca at a higher rate and with a greater efficiency than mature animals with lower requirements (Braithwaite and Riazuddin, 1971). During pregnancy and lactation or after periods of Ca deficiency, both the amount of Ca absorbed and the efficiency of absorption are increased. Phosphorus metabolism has been found to be closely linked to Ca metabolism. Results have indicated that P homeostasis is brought about largely by a control of urinary P excretion.

The pig, cow, and horse can adapt themselves to restrictions and increases in demand (Manston, 1967; Ramberg *et al.*, 1970; Schryver *et al.*, 1974; Van'T Klooster, 1976; Fox and Care, 1978). Calcium absorption decreases with increasing age.

The formation of insoluble Ca compounds in the intestine may reduce Ca absorption. It has been shown that oxalate reduces Ca absorption in rats due to formation of insoluble calcium oxalate, which is lost in the feces (Talapatra *et al.*, 1948). In ruminants, microbial decomposition of oxalates in the rumen has been demonstrated (Oslage *et al.*, 1960).

The degradation of oxalates may result, however, in alkalosis, thus upsetting mineral equilibrium and affecting Ca metabolism indirectly (Watts, 1959). Large quantities of oxalates (fresh leaves of sugar beets contain as much as 7% potassium oxalate), however, may overload the ruminal capacity of metabolizing oxalate, immobilize the Ca in the alimentary tract, and thus contribute significantly to the development of hypocalcemia.

Phytates, present in high levels in cereals, are of considerable importance in Ca and P metabolism in monogastric animals (Hoff-Jørgensen, 1946; Mellanby, 1949). Phosphorus bound in this manner is not utilized by monogastric animals unless it is decomposed (hydrolyzed) by the enzyme phytase. If this does not occur, the body is deprived, not only of P, but of Ca as well. This is due to the fact that this substance, inositolhexaphosphoric acid, at a pH of 5–7, forms a very insoluble Ca salt in the intestine. In ruminants, phytin is completely hydrolyzed in the rumen (Reid *et al.*, 1947). In ponies, sodium phytate has been found to be utilized as efficiently as monosodium phosphate (Schryver *et al.*, 1971).

It has been shown (Storry, 1961a,b) that abomasal digesta of sheep may contain materials which bind Ca above pH 5. Nucleic acids which are formed in the rumen and pass largely unchanged to the duodenum are potent Ca-binding agents (Chang and Carr, 1968) and are presumably in part responsible for this binding (Smith *et al.*, 1968). The chelating agent ethylenediaminetetraacetic acid (EDTA) has been shown to inhibit the absorption of Ca from the small intestine of sheep (Van'T Klooster and Care, 1966).

Calcium is absorbed primarily from the anterior portion of the small intestine, and most evidence indicates that increased acidity of the gastrointestinal fluid favors Ca absorption (Granström, 1908; Hart *et al.*, 1931; Lomba *et al.*, 1978). Oral administration of acid increases intestinal absorption, at the same time leading to increased urinary excretion of Ca.

The Ca/P ratio of the diet greatly influences the absorption of Ca and P. The dietary level of Ca or P tends to limit the absorption of the remaining element. As the Ca/P ratio is increased, the requirement for vitamin D increases (see Section IV). In nonruminants, a Ca/P ratio above 3 : 1 results in undesirable effects, whereas a Ca/P ratio below 1 : 1 is tolerated. Ruminants, on the other hand, tolerate a Ca/P ratio of 7 : 1 without undesirable effects, but with a Ca/P ratio lower than 1 : 1 decreased performance is observed (Wise *et al.*, 1963; Young *et al.*, 1966a,b; Ricketts *et al.*, 1970).

The principal site of Ca absorption is the small intestine (Phillipson and Storry, 1965; Schryver *et al.*, 1974; Van'T Klooster, 1976). Calcium is absorbed by passive or facilitated diffusion and by active transport (Wasserman, 1968). The active metabolite, 1,25-dihydroxycholecalciferol, of vitamin D_3 stimulates intestinal absorption of Ca and initiates the synthesis of a calcium-binding protein (CaBP) in the intestinal mucosa. In the steady state, there is a correlation between the concentration of intestinal CaBP and Ca transport, indicating a role for CaBP during the process of Ca absorption (Wasserman *et al.*, 1977a).

There is less information available on the intestinal absorption of P than on that of Ca. Wasserman and Taylor (1973) could find no evidence for a P binder analogous to CaBP in the chick intestinal mucosa. The absorption of P has been shown not to depend on the simultaneous transfer of Ca. Therefore, P is probably not absorbed merely as a co-ion to Ca. It is, however, known that the efficiency of intestinal phosphate absorption, like that of Ca, increases in response to dietary restriction of Ca or P (Fox and Care, 1978).

The small intestine is the major site of P absorption in ruminants, swine, and dogs, whereas the major sites of P absorption from the intestine of the horse are the dorsal large

colon and the small colon (Schryver *et al.*, 1972). Available evidence suggests that the rumen epithelium is relatively impermeable to the P ion as it is to Ca and Mg (Phillipson and Storry, 1965).

C. Vitamin D

Vitamin D is the major hormone controlling the intestinal absorption of Ca and probably also P_i (Wasserman and Taylor, 1973). Many features of vitamin D action on Ca transport have been identified. The development of this area depended upon the synthesis of radioactively labeled vitamin D so that its alterations and localization in various tissues and organs could be followed. Vitamin D_3, i.e., cholecalciferol, after absorption from the ingesta or having been produced in the skin in response to ultraviolet radiation, is transported to the liver. In the liver it is transformed to a biologically significant intermediate, 25-hydroxycholecalciferol (25-OH-D_3). The 25-OH-D_3 is then transported to the kidney, where two additional derivatives, 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] and 24,25-dihydroxycholecalciferol [24,25-$(OH)_2D_3$], are formed. Under conditions of Ca need or Ca deprivation, the form predominantly produced by the kidney is 1,25-$(OH)_2D_3$; otherwise, 24,25-$(OH)_2D_3$ is the major product. In concert with other calciotropic principles, such as parathyroid hormone (PTH) and thyrocalcitonin, the 1,25-$(OH)_2D_3$ hormone mediates Ca and P metabolism at target tissues, including intestine, bone, and kidney. In the intestine, receptors for 1,25-$(OH)_2D_3$ induce the synthesis of CaBP. In the absence of vitamin D, both active transfer and passive transfer of calcium across the intestinal mucosa are impaired. Although 1,25-$(OH)_2D_3$ is preferred by the intestinal system, it still responds to vitamin D_3 and 25-OH-D_3.

Bone is another target tissue for vitamin D action. The stimulation of bone resorption by vitamin D also implies the formation of a vitamin D-induced protein, which is required for its action at this site.

Kidney is a third well-recognized target tissue for vitamin D action. The vitamin D-induced CaBP, found initially in the intestine, has also been localized in the kidney (Taylor and Wasserman, 1972). As a direct effect of administration of vitamin D_3 metabolite on the reabsorption of P_i from the kidney tubule, an elevation of serum P_i is observed.

Factors controlling the conversion of 25-(OH)-D_3 to 1,25-$(OH)_2D_3$, either directly or indirectly, include the serum concentration of Ca, P, and PTH (Fox and Care, 1978).

In herbivorous animals, vitamin D and vitamin D metabolites characteristically cause a hyperphosphatemia and, to a lesser degree, a hypercalcemia. In omnivorous animals, hypercalcemia is the dominating picture (Dämmrich, 1963).

The development of assays for vitamin D metabolites and the following measurements of these have led to increased comprehension of diseases with a defect in vitamin D metabolism (Haussler and McCain, 1977).

D. Calcium and Phosphorus Excretion

I. Fecal Excretion

Fecal Ca, mainly dietary in origin, may be partitioned into exogenous and endogenous Ca. Exogenous fecal Ca is undigested Ca from the food. Endogenous fecal Ca is the fraction of fecal Ca derived from secretions of Ca into the digestive tract, mainly in the small intestine. Calcium-45 studies have indicated that the small intestine is by far the most important.

Knowledge of the amount of endogenous fecal Ca and P is important for determination of the "true" digestibility of food Ca and P. The apparent digestibility,

$$(\text{Food Ca} - \text{fecal Ca})/\text{food Ca}$$

is a valid basis for estimating the "true" digestibility only when the endogenous Ca or P is a small part of the total fecal Ca or P.

In cattle the amount of endogenous Ca has been estimated to be 4–7 gm/day (Visek *et al.*, 1953; Comar *et al.*, 1953), and it has been shown that the amount of endogenous fecal Ca is directly proportional to body weight and is little influenced by short-term dietary changes. Hansard *et al.* (1954) showed that the level of endogenous Ca in feces changes appreciably with age, becoming progressively greater in old animals. At the onset of lactation either a decrease in secretion of Ca into the gut or a more efficient reabsorption of endogenously secreted Ca has been found (Ramberg *et al.*, 1970; Van'T Klooster, 1976). Balance studies using ^{47}Ca have shown that young horses and ponies excrete about 20 mg Ca per kilogram body weight each day as endogenous fecal Ca regardless of Ca intake (Schryver *et al.*, 1974).

In rats there is evidence that the amount of endogenous fecal Ca is influenced by vitamin D. Thus, Nicolaysen (1937) showed an increased excretion of metabolic fecal Ca in vitamin D-deficient rats on a Ca- and P-free diet.

Inorganic phosphate is also excreted in the urine and digestive secretions. The level of endogenous fecal P was estimated by Kleiber *et al.* (1951), Lofgreen and Kleiber (1953), and Luick and Lofgreen (1957). In lactating cows the value was found to be 10–14 gm P daily, or about twice the amount of endogenous Ca. The site of transfer of endogenous P into the gastrointestinal tract was studied by Smith *et al.* (1955a,b, 1956) and Lofgreen *et al.* (1952). The animals were given injections of ^{32}P and sacrificed at intervals following injection. The specific activity of P was determined in plasma and in various segments of the gastrointestinal tract and their corresponding contents. In swine (Smith *et al.*, 1955a) most of the endogenous P appeared to enter the small intestine, and endogenous P was reabsorbed to a greater extent than dietary P in the posterior portion of the digestive tract. In dairy cows (Smith *et al.*, 1956) and sheep (Smith *et al.*, 1955b) the main site of P excretion was the rumen (saliva).

In ponies fed dietary levels of P varying from 0.2 to 1.2%, the daily endogenous fecal excretion of P, calculated from excretion of ^{32}P in feces, averaged about 10 mg/kg body weight (Schryver *et al.*, 1971). In contrast, fecal endogenous P varies with intake in ruminants (Preston and Pfander, 1964; Young *et al.*, 1966b). The horse excretes less fecal P than sheep and cattle at equivalent P intake.

2. *Urinary Excretion*

The urinary excretion of Ca and P is regulated. Part of the filtered Ca and P is reabsorbed in the renal tubules. Renal P excretion is determined by the balance between the glomerular-filtered load of P and the tubular-reabsorbed phosphorus, the former being the product of plasma P levels and GFR. The renal threshold for Ca excretion is between 6.5 and 8.0 mg/dl, little being eliminated at lower concentrations.

Mineral acids fed to cows increase the urinary Ca excretion (Hart *et al.*, 1931; Stacy and Wilson, 1970). The same is true of nonionized Ca complexes, such as citrate and EDTA. They are not subject to tubular reabsorption in the kidney, and therefore renal Ca excretion is enhanced. In the ruminant, excretion of Ca with the urine generally is of no importance (Van'T Klooster, 1976). Contrary to this, Ca excretion and the kidney seem to

play a unique role in Ca homeostasis in the horse. Horses are able to excrete large amounts of Ca in urine (Schryver *et al.*, 1974), and following nephrectomy they promptly develop a marked hypercalcemia and hypophosphatemia (Tennant *et al.*, 1974), while calves respond by developing hypocalcemia (Watts and Campbell, 1971).

The Sulkowitch test, a rapid method for estimation of urinary Ca, has been considered a valuable clinical tool for serum Ca determination in cases of parturient paresis. Examinations of the reliability of the test (Detweiler and Martin, 1949; Roberts *et al.*, 1951; Hallgren, 1955) have shown it to be of little value or even misleading because of poor correlation between serum Ca and urine Ca and because of the difficulty of achieving a safe estimation of the urinary level of Ca by means of the test. A reliable, 15-minute semiquantitative test for serum Ca has been proposed (Mayer *et al.*, 1965).

Investigations of the renal excretion of acid and base suggest that the renal control of acid and base excretion in the pig, man, and dog is similar (Scott, 1971). Acidosis increases urinary excretion of Ca without increasing the excretion of P. Ingestion of sodium bicarbonate increases urine pH but reduces the excretion of Ca in the urine, thus indicating an inverse relationship between excretion of Ca in urine and urine pH.

In cattle the rate of urinary P excretion amounts to only a fraction of 1% of the rate of P excretion in the feces (Kleiber *et al.*, 1951). Experiments have shown that sheep and cattle excrete more P and acid in urine when fed concentrate diets than when fed roughage diets. This difference in P excretion does not appear to be related to differences in P intake or acid excretion but rather to differences in P reabsorption by the kidney (Scott, 1972). Tomas (1974) found that an inverse relationship exists between the secretion of endogenous P to the gut and the quantity of P excreted in the urine. No direct evidence for any hormonal action could be found; i.e., the renal mechanism seems to function as principal regulator of P homeostasis when the flow of endogenous P to the gut is reduced. Also, Braithwaite (1975b) found sheep P metabolism linked to Ca metabolism in a way indicating that P homeostasis was brought about largely by the control of urinary P excretion.

In the horse there has been little study of factors affecting renal excretion. High-phosphorus diets increase urinary P excretion but decrease excretion of Ca (an alkaline pH seriously limits the possibilities for simultaneous excretion of Ca and P).

3. Mammary Secretion

In lactating animals considerable amounts of Ca and P are secreted in the milk, and several of the constituents are found in concentrations higher than that of the blood. Thus, the concentration of Ca is increased by a factor of 12–13, that of P by about 7, and that of Mg by about 6.

Blood samples drawn from mammary veins may have a P_i level about 1 mg/dl (0.3 mmole/liter) higher than that of blood from the jugular vein (Meigs *et al.*, 1919). The level of serum Ca, on the other hand, is usually lower in the mammary vein than in the jugular vein (Hallgren, 1940). A linear relation between the efficiency of Ca absorption and the secretion of Ca with milk after the onset of lactation suggests that adjustment of absorption is a major adaptation to the lactational Ca loss (Van'T Klooster, 1976).

4. Other Excretions and Secretions

In the horse loss of Ca and P through the skin seems to be of importance. Horses subjected to vigorous and sustained exercise in summer heat often develop hypocalcemia and clinical signs related to hypocalcemia (Mansmann *et al.*, 1973; Lucke and Hall, 1978).

TABLE I

Concentration of Calcium in Blood of Normal Animals

Species	Blood fraction[a]	No. of animals sampled	Concentration (mg/dl) (mean ± 2 SD)	Concentration (mmole/liter)[b] (mean ± 2 SD)	Reference
Shetland pony	S	8	10.2 ± 2.0	2.6 ± 0.5	Eriksen and Simesen (1970)
Horse	S	24	12.8 ± 1.2	3.2 ± 0.3	Simesen (1972)
	S	36	12.6 ± 2.2	3.1 ± 0.6	Wolff *et al.* (1969)
Cattle at parturition	S	31	8.1		Blosser and Smith (1950b)
Cow	S	25	10.2 ± 0.6	2.6 ± 0.2	Mylrea and Bayfield (1968)
Heifer	S	25	10.2 ± 0.6	2.6 ± 0.2	Mylrea and Bayfield (1968)
Calf	S	20	10.1 ± 0.7	2.5 ± 0.2	Mylrea and Bayfield (1968)
	S	13	12.0 ± 1.2	3.0 ± 0.3	Holtenius *et al.* (1970)
Sheep	S	25	9.2 ± 0.8	2.3 ± 0.2	Smith *et al.* (1978)
	S	67	10.9 ± 1.4	2.7 ± 0.4	Simesen (unpublished)
Goat	S	100	10.3 ± 1.0	2.6 ± 0.3	Barakat and El-Guindi (1967)
Pig (6 months old)	S	50	9.7 ± 1.0	2.4 ± 0.3	Simesen (1963a)
Pregnant sow	S	14	10.1 ± 1.1	2.5 ± 0.3	Simesen (1963a)
Cat	S	10	8.2 ± 1.0[c]		Bloom (1957)
Large dog	—	—	10.2 ± 0.4	2.6 ± 0.1	Lane and Robinson (1970)
Beagle (13 months ± 1 month)	—	—	10.8 ± 0.4	2.7 ± 0.1	Stewart and Longwell (1969)

[a] S, serum.

[b] Internationally recommended unit.

[c] Standard deviation was estimated from range and number of observations.

II. SERUM CALCIUM AND PHOSPHORUS LEVELS

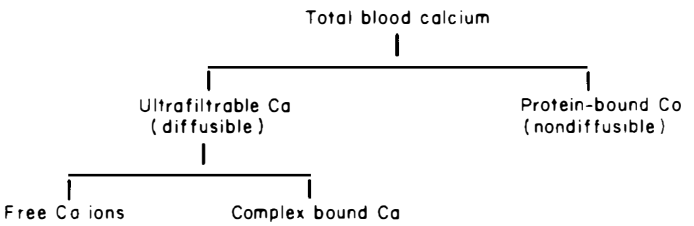
Calcium is one of the more precisely regulated constituents of plasma. Calcium homeostasis is achieved by balancing the absorption of Ca from the alimentary tract with the urinary and endogenous fecal excretions of Ca, as well as the fetal or lactational drain of Ca. If the intake of dietary Ca falls below that required, plasma Ca concentration is maintained by a shift of bone Ca turnover in favor of net resorption.

The serum concentration of Ca in normal adult animals averages 10 mg/dl (or 2.5 mmole/liter). The normal range is usually stated to be 9–12 mg/dl (details in Table I). The serum Ca level of normal young animals is of the same order as that of adults. The quantity of Ca contained in red blood cells (RBC) is negligible.

Extracellular Ca is present in at least three forms: free Ca ions, Ca ions that are ionically bound but ultrafiltrable, and Ca bound to plasma protein. The serum contains several chelators of Ca, including citrate, and there is also probably some interaction between Ca and P or carbonate, which decreases the Ca ion activity. The ionized Ca is diffusible, the complexed Ca is diffusible but not ionized, whereas the protein-bound Ca is neither ionized nor diffusible (Scheme 1). Although all these forms of Ca are in equilibrium with one another, in body fluids only the ionized fraction is under direct control (Copp, 1969). With the invention of the Ca electrode by Ross (1967) and the introduction of membrane cones for ultrafiltration, it has been possible to measure the Ca fractions rapidly and accurately (Raman, 1971). The clinical importance of ionized Ca determinations, however, remains to be shown (Pedersen, 1972).

Ultrafiltrable serum Ca in cattle has been found to constitute from 40 to about 60% of total serum Ca (Boogaerdt, 1954; Smith, 1957; Hallgren *et al.*, 1959).

Most of the P of blood is present as organic esters of P within the RBC; these contain small amounts of P_i at any given moment. Serum contains about 14–15 mg of total P per deciliter, but of this 5–8 mg are lipid P. A trace of the rest is ester P, the most significant portion of the remainder being P_i. The main difficulty in determining serum P_i levels arises from the fact that if a portion of the RBC undergoes hemolysis, the P esters present in RBC may undergo hydrolysis with consequent liberation of P_i. Serum P_i concentration in normal adult animals is 4–7 mg/dl (1.3–2.3 mmole/liter), horses and dogs being exceptions (Table II, Fig. 1). Young animals usually have a higher and more variable concentration [5–9 mg/dl (1.6–2.9 mmole/liter)]. The P_i level appears to be intimately related to carbohydrate metabolism. During increased carbohydrate utilization the level tends to decrease, and during fasting an increase usually is observed [1–2 mg/dl (0.3–0.6 mmole/liter)]. A principal rule, to which there are many exceptions, is that increasing serum P is accompanied by decreasing serum Ca. Generally, the actual concentrations



Scheme 1. Partition of blood calcium. (From Hallgren *et al.*, 1959.)

TABLE II

Concentration of Phosphorus (Inorganic) in Blood of Normal Animals

Species	Blood fraction[a]	No. of animals sampled	Concentration (mg/dl) (mean ± 2 SD)	Concentration (mmole/liter)[b] (mean ± 2 SD)	Reference
Shetland pony	S	8	4.9 ± 2.2	1.6 ± 0.7	Eriksen and Simesen (1970)
Horse	S	36	3.6 ± 1.2	1.2 ± 0.4	Wolff *et al.* (1969)
<2 years	S	7	4.8 ± 1.1	1.6 ± 0.4	Simesen (1972)
2–9 years	S	8	3.4 ± 1.2	1.1 ± 0.4	Simesen (1972)
⩾10 years	S	9	2.8 ± 0.3	0.9 ± 0.1	Simesen (1972)
Cattle	S	25	5.5 ± 0.8	1.8 ± 0.3	Mylrea and Bayfield (1968)
Heifer	S	25	6.2 ± 0.6	2.0 ± 0.2	Mylrea and Bayfield (1968)
Calf	S	20	8.9 ± 0.6	2.9 ± 0.2	Mylrea and Bayfield (1968)
Cattle	S	185	5.6 ± 1.6	1.8 ± 0.5	Crookshank and Sims (1955)
Sheep	P	517	4.3 ± 0.9	1.4 ± 0.3	Marsh and Swingle (1955)
	S	919	5.2 ± 0.1	1.7 ± 0.1	Hackett *et al.* (1957)
	S	67	6.5 ± 2.2	2.1 ± 0.7	Simesen (unpublished)
Goat	S	100	4.3 ± 0.8	1.4 ± 0.3	Barakat and El-Guindi (1967)
Pig (6 months old)	S	43	10.9 ± 1.0	3.5 ± 0.3	Simesen (1963a)
Pregnant sow	S	12	7.9 ± 1.4	2.5 ± 0.5	Simesen (1963a)
Cat	S	10	6.4 ± 1.2[c]	2.1 ± 0.4	Bloom (1957)
Large dog	—	—	3.7 ± 0.5	1.2 ± 0.2	Lane and Robinson (1970)
Beagle (13 months ± 1 month)	—	—	4.5 ± 0.6	1.5 ± 0.2	Stewart and Longwell (1969)

[a] S, serum; P, plasma.

[b] Internationally recommended unit.

[c] Standard deviation was estimated from range and number of observations.

Fig. 1. Normal values for inorganic phosphorus concentration in horses (thoroughbreds and trotters).

depend upon the following conditions: (1) intestinal absorption, (2) uptake from the skeleton, (3) amounts of PTH and calcitonin, and (4) urinary excretion.

Measurement of P_i concentration (preferably heparinized plasma) provides the most readily determinable index of the P status of animals (Newman, 1968; Little *et al.*, 1971).

Allcroft and Godden (1934) showed that calves at birth and for the first 8 weeks of life have rather high levels of serum Ca and P_i. In the adult stage increasing age has been reported to be associated with a slight decline in serum Ca and a marked decline P_i (Payne and Leech, 1964).

Whereas serum Ca concentration is precisely regulated by three major regulatory hormones—PTH, calcitonin, and the active metabolites of vitamin D—there does not appear to be any precise homeostatic regulation of serum P concentration.

A. Parathyroid Hormone

In conditions with poor absorption of Ca from the intestinal tract, the plasma Ca level is maintained primarily by its mobilization from the bones through the action of PTH. Its physiological actions, which are exerted in the metabolism of both Ca and P, can be illustrated by outlining the consequences of (1) removal of the parathyroid glands and (2) injection of PTH.

1. Effect of parathyroidectomy: Decreased urinary output of P_i and increased serum P_i; decreased urinary Ca output and decreased serum Ca level.
2. Effect of injection of PTH: Increased urinary P_i excretion and decreased serum P_i; increased urinary Ca excretion and increased serum Ca.

Early studies on the physiology of the parathyroids led to two conflicting opinions concerning the actual mechanism. One school of thought (Albright *et al.*, 1929; Albright and Reifenstein, 1940) felt that the primary effect of PTH was to increase renal excretion of P by diminishing tubular absorption of the P filtered by the glomeruli. In other words, the hormone would increase the ratio of P clearance to inulin clearance. As a result, the serum P level would decline rapidly, and the serum body fluids would become unsaturated with respect to $Ca_3(PO_4)_2$. Hence, $Ca_3(PO_4)_2$ would be liberated from the bones. Since P would be rapidly excreted, only serum Ca should rise. When serum Ca had increased above the threshold value for renal excretion, more Ca would be excreted in the urine.

Thomson and Collip (1932), on the other hand, proposed that the primary action of PTH was to induce dissolution of bone, possibly by increasing the number and activity of osteoclasts. The increased excretion of P_i followed as a secondary effect.

According to the present view (McLean and Urist, 1955), the skeleton is the chief factor in stabilizing the Ca concentrations of the extracellular fluids. A dual mechanism exists for the PTH control of plasma Ca level. One mechanism involves a simple chemical equilibrium between labile fractions of the bone mineral and plasma. This dynamic equilibrium is adequate to maintain serum Ca concentration at approximately 7 mg/dl (1.75 mmole/liter). The mechanism is independent of PTH.

The second mechanism depends upon the parathyroid gland. The following points have been established:

1. Hypocalcemia is a principal stimulant of secretion of PTH. A feedback system maintains serum Ca concentration constant via changes in PTH secretion mediated through control of bone resorption as well as renal and intestinal Ca transport. Use of sensitive radioimmunoassay techniques has made it possible to demonstrate an inverse relationship *in vivo* between plasma Ca ion concentration and PTH concentration (Sherwood *et al.*, 1966). The rate of PTH secretion has been calculated (Care and Bates, 1970).

2. Without formation of new hormone, a rapidly secreting parathyroid gland would exhaust its content of PTH in less than 1 hour in the cow (Sherwood *et al.*, 1966, 1968).

3. Plasma Mg concentration shows an inverse correlation with PTH secretion rate somewhat similar to that found with Ca (Care *et al.*, 1966; Buckle *et al.*, 1968). The percent change in plasma Mg concentration, however, required to produce a given alteration in PTH secretion rate was greater than that in plasma Ca concentration. A direct effect of plasma P_i concentration on PTH secretion has been suggested, but it seems more likely that the P ion acts indirectly by reducing the concentration of ionized Ca (Sherwood *et al.*, 1968).

4. Intriguing new evidence is accumulating that PTH is linked to vitamin D metabolism. Inappropriately high levels of circulating PTH have been detected in vitamin D deficiency, and it has been shown that both 25-OH-D_3 and 1,25-$(OH)_2D_3$ repress circulating PTH levels and parathyroid secretion *in vivo* and *in vitro* (Cohn and Hamilton, 1976).

B. Calcitonin (Thyrocalcitonin)

Copp *et al.* (1962) showed that perfusion of the thyroid–parathyroid complex with blood of high Ca concentration resulted in liberation of a previously unrecognized hormone, a fast-acting hypocalcemic factor, which they named calcitonin (CT).

The discovery of CT was followed by a rapid elucidation of the chemistry, effects, and metabolism of this hypocalcemic hormone secreted by cells of ultimobranchial origin (C cells) located parafollicularly in the mammalian thyroid.

Unlike PTH secretion, which is largely under divalent cation control, CT secretion is stimulated not only directly by high Ca levels but also by gastrointestinal hormones, particularly gastrin (Cooper *et al.*, 1972). The latter response may be the most important physiological mechanism for CT secretion in mammals and may explain why young, rapidly growing animals deficient in CT develop hypercalcemia and hypercalciuria after oral loading, whereas normal animals do not (Raisz, 1977).

The major direct effect of CT in mammals is inhibition of bone resorption, although

there also are effects on renal and intestinal ion transport. The hypocalcemic response to CT is maximal when bone resorption has been reduced to zero, whereas there is theoretically no upper limit to the response of bone to PTH (Care, 1971). The secretion rate of CT is directly proportional to plasma Ca concentration (Care *et al.*, 1968b). Calcitonin is secreted very rapidly in response to a hypercalcemic stimulus. In contrast to the low concentrations of PTH in the parathyroid glands, the thyroid contains enough calcitonin to maintain a stimulated secretion rate for 2 days without further synthesis of CT. Despite the fact that CT is stored in relatively large amounts in the thyroid, it must be released continuously at least during normo- and hypercalcemic states (Care and Bates, 1970). The sensitivity to CT has been found to decrease with increasing age (Copp and Kuczerpa, 1967; Care and Duncan, 1967).

The action of CT is fast, reaching its peak in a matter of minutes after the hormone is secreted. In contrast, PTH action is relatively slow and lasts for several hours.

C. Miscellaneous Factors

Thyroid hormone has been reported to increase the fecal excretion of Ca in dogs (Logan *et al.*, 1942) and lactating dairy cows (Owen, 1948), but no effect upon serum Ca and P concentrations has been demonstrated.

Estrogens were found to depress the serum Ca level, simultaneously elevating the level of P_i (Folley, 1936; Horváth and Kutas, 1959) in sows, mares, and cows. In bitches, on the contrary, the changes were erratic.

Probably no humoral factors other than PTH, CT, and vitamin D regulate serum ionized Ca concentration directly. However, sex hormones, glucocorticoids, thyroxine, growth hormone, glucagon, and other agents do affect mineral metabolism and may thus indirectly affect regulation.

Growth hormone has been shown to cause a significant increase in the rates of absorption of Ca in the sheep (Braithwaite, 1975a). Earlier, estrogens had been reported to alter Ca metabolism of nearly mature sheep to resemble that of younger animals (Braithwaite *et al.*, 1972). It is possible that the action of estrogens on Ca metabolism might be mediated by increasing the secretion of growth hormone (Braithwaite, 1975b).

III. ABNORMAL CALCIUM AND PHOSPHORUS METABOLISM

A. Hypercalcemia

An increase in serum Ca concentrations is found during hyperparathyroidism and may also be induced via administration or intake of excessive amounts of vitamin D or vitamin D-like substances. Furthermore, hypercalcemia in the horse has been observed after experimental nephrectomy.

1. Hyperparathyroidism

Two types of hyperparathyrodism are known: (1) the primary form caused by parathyroid neoplasia and (2) the secondary form, which has two subtypes, one of renal origin (Section III,E) and one of nutritional origin (Section IV,B,5).

Pseudohyperparathyroidism, a hyperparathyroidism-like syndrome caused by produc-

tion of hormone-like substances in neoplastic tissue of nonendocrine origin, has been reported in dogs (Osborne and Stevens, 1973). Primary hyperparathyroidism is a rare finding in domestic animals. The classical picture of primary hyperparathyroidism is that of hypercalcemia and hypophosphatemia. The excess PTH production increases the amount of P and Ca excreted by the kidney. Minerals are continuously removed from the skeleton and replaced by fibrous connective tissue, causing fractures of long bones following slight traumatic injury. Primary hyperparathyroidism in the dog usually results in a serum Ca level from 12 to 20 mg/dl (3–5 mmole/liter) or greater (Pearson *et al.*, 1965; Capen and Martin, 1971). In case of simultaneous renal failure, the P retention may tend to obscure the picture.

2. Hypercalcemia Due to Vitamin D or Vitamin D-like Substances

In omnivorous animals administration of vitamin D causes primarily a hypercalcemia. In herbivorous animals the same substances cause a hyperphosphatemia and to a lesser degree a hypercalcemia (Dämmrich, 1963).

A series of so-called plant-induced calcinoses in animals has been described: enteque seco (Argentina), espichamento (Brazil), naalehu disease (Hawaii), Manchester wasting disease (Jamaica), enzootic calcinosis (Germany, Austria), and cestrum diurnum poisoning (Florida). They all show great similarities with vitamin D poisoning, i.e., different degrees of hypercalcemia, hyperphosphatemia, and calcifications in the circulatory system, lungs, kidneys, and flexor tendons (Dirksen *et al.*, 1971; Krook *et al.*, 1975; Simesen, 1977a). Recent investigations indicate that all of these diseases most likely are caused by the presence of potent, active vitamin D-like substances in the plants and that these substances are responsible for the abnormalities of mineral metabolism in the animals (Wasserman *et al.*, 1977b).

3. Hypercalcemia in Horses

A clinical syndrome characterized by hypercalcemia, hypophosphatemia, and chronic renal failure was observed in horses (Tennant *et al.*, 1974). Circumstantial evidence suggested that this hypercalcemia was a secondary manifestation of renal insufficiency rather than the primary cause of renal disease. To test this hypothesis, the authors produced acute renal failure in five mature ponies by bilateral nephrectomy. Plasma Ca concentration increased in response to this operation from 12.2 mg/dl (5.1 mmole/liter) to 17.6 mg/dl (4.4 mmole/liter) 48 hours after removal of the second kidney and remained significantly elevated until the time of death. Plasma P_i concentration decreased from 3.2 mg/dl (1.0 mmole/liter) to 1.5 mg/dl (0.5 mmole/liter). The experimental evidence thus confirmed the hypothesis of a special role of the kidney in Ca homeostasis in the horse.

4. Hypercalcitonism in Bulls and Dogs

Hypercalcitonism following excessive dietary Ca intake has been described (Krook *et al.*, 1971; Hedhammer *et al.*, 1974). Clinical chemical data as well as morphological evaluation of the C cells and the parathyroid glands indicated the following sequence of events. Excessive Ca intake caused relative hypercalcemia, resulting in hypercalcitonism and hypoparathyroidism with retarded bone resorption. Isocalcemia was restored, and hypocalcemia resulted eventually from persistent hypercalcitonism, supposedly because of increased gastrin secretion in response to continued high Ca intake. In the dogs these changes were reflected clinically by lameness, pain upon palpation of the skeleton, and

enlargement of the costochondral junctions and the epiphyseal–metaphyseal regions of long bones.

B. Hypocalcemia

Hypocalcemia is one of the most constant and characteristic features of diminished parathyroid function. The clinical and metabolic aspects of hypoparathyroidism are discussed in connection with parturient paresis.

Deficiency of vitamin D often causes pronounced hypophosphatemia in early stages, followed later by a fall in serum Ca. In sheep sudden deprivation of feed and forced exercise may cause marked depression of serum Ca (Asbury, 1962; Herd, 1965; Pierson and Jensen, 1975). Hypocalcemia also occurs in lactating cows after periods of starvation and during bovine ketosis (Allcroft, 1947a; Halse, 1958a,b; Halse and Velle, 1958; Simesen, 1958). During bovine ketosis there is a simultaneous increase in urinary Ca excretion (Sjollema, 1932a).

Moderate hypocalcemia is a common finding during diseases with protein-losing gastroenteropathies, in which it has been shown that the hypocalcemia is due to gastrointestinal loss of Ca bound to albumin (Nielsen, 1966). Hypocalcemia is observed in cases of acute pancreatitis. In these cases the hypocalcemia has been attributed to sudden removal of large amounts of Ca from the blood plasma as a result of its fixation as insoluble calcium soaps by fatty acids in areas of fat necrosis. Hypocalcemia and tetany in the dog have been described. Seizure was associated with a normal or low-normal blood Ca level accompanied by a reduced albumin level or reduced albumin/globulin ratio (Resnick, 1972). It should be emphasized that blood samples should be collected as soon after seizure as possible.

C. Parturient Paresis (Milk Fever)

Milk fever is an afebrile disease which is typically associated with parturition and the very beginning of lactation. It is characterized by a sudden progressive paresis, paralysis, or coma, and, if untreated, it is usually fatal. The main chemical changes in blood serum are a marked decrease in the concentration of total as well as ionized Ca and of total P as well as P_i together with an increase in the concentration of Mg. An excellent review on parturient paresis in dairy cattle was published by Hibbs (1950). Subsequent reviews have been published by Jönsson (1960), Moodie (1965), Mayer *et al.* (1969), Kronfeld (1971), Jönsson and Simesen (1973), and Littledike (1974).

The first epoch in the history of milk fever began with the discovery by the Danish veterinarian Schmidt (1897) that udder insufflation resulted in recovery. This treatment reduced the mortality rate of milk fever from 60–70% to about 15%. Since then students of milk fever have been probing this phenomenon (Fish, 1928, 1929a; Niedermeier and Smith, 1950; Marshak, 1956), and even today udder insufflation is, under certain conditions, the therapy of choice (Marshak, 1956; Mayer *et al.*, 1967).

1. Clinical Biochemical Manifestations

Since Little and Wright (1925) published their first results proving that milk fever in cattle was associated with hypocalcemia, there has been a tendency to regard these two conditions as synonymous. Numerous subsequent reports have been made by Dryerre and Greig (1925, 1928), Little and Wright (1925, 1926), and others. Greig (1930) reported

TABLE III

Biochemical Findings in Cattle with Milk Fever, Cattle with Grass Tetany, and Normal Cattle[a]

Condition	Calcium ion (mg/dl)	Total calcium (mg/dl)	Inorganic phosphorus (mg/dl)	Magnesium (mg/dl)
Milk fever	0.44	4.35	2.16	2.19
Grass tetany	1.18	6.65	4.33	0.46
Normal cattle	1.65	9.35	4.57	1.66

minimal, maximal and average serum Ca values of 3.00, 7.76, and 5.16 mg/dl (0.8, 1.9, and 1.3 mmole/liter), respectively in 82 cases of milk fever. Hypocalcemia was not prevalent in animals suffering from other diseases.

Fish (1929b) reported average blood plasma values of 3.31 mg/dl (0.8 mmole/liter) for Ca and 2.39 mg/dl (0.8 mmole/liter) for P_i, and Sjollema and Seekles (1932) (Table III) drew attention to the difference between the biochemical findings in cows with milk fever, those with grass tetany, and normal cows.

Previously, Palmer and co-workers (Palmer and Eckles, 1930; Palmer *et al.*, 1930) had reported decreased blood levels of both Ca and P in normal cattle at parturition. Wilson and Hart (1932) demonstrated that this decrease was more pronounced in older cattle than in first-calf heifers, and they presented additional evidence of hypocalcemia as an essential factor in milk fever. These findings were confirmed (Allcroft and Green, 1934; Allcroft and Godden, 1934), and an increase in serum Mg was reported to take place in normal cattle at parturition (Table IV).

Barker (1939) recognized three types of hypocalcemia. The clinical symptoms depended on the level of serum Mg, varying from extreme nervousness and tetany when the Mg level was low, to a comatose condition when the Mg level was high. Robertson (1949), however, was unable to confirm this classification of types of milk fever.

Blood Ca exists principally in two fractions: a diffusible ionized form, which accounts for 3.6–7.7 mg/dl (0.9–2.0 mmole/liter) (Hallgren *et al.*, 1959), and a nondiffusible protein-bound fraction. The decrease in total Ca concentration at normal parturition relates almost entirely to the bound Ca fractions. During milk fever a reduction is found in the concentration of ionized calcium as well as of protein-bound Ca (Hallgren *et al.*, 1959),

TABLE IV

Blood Changes at Normal Parturition[a,b]

Group	Number of animals	Serum calcium (% fall)	Inorganic phosphorus (% fall)	Serum magnesium (% rise)
First calvers	7	11.7	33.8	7.7
Second calvers	8	17.6	38.4	24.1
Third calvers	9	22.9	46.4	34.4
Fourth and subsequent calving	8	30.6	48.1	35.9

[a] Robertson *et al.* (1956).

[b] Percent change from 2 days prepartum.

i.e., in the concentration of total and ultrafiltrable Ca (Straub *et al.*, 1959) (Scheme 1).

In normal calving cows, a reduction is found in the concentration of organic P and P_i. During milk fever, the values for P_i are still lower, while the concentration of organically bound P is of the same order of magnitude as in normal cows after calving (Carlström, 1969).

2. *Other Findings*

A decrease in the concentration of blood citric acid (Blosser and Smith, 1950a) has been reported in milk fever cows as compared with healthy cows at parturition. The levels of citric acid seem to reflect those of Ca. This is consonant with the fact that Ca and citrate metabolism are closely related (Lichtwitz *et al.*, 1961). Both Ca and citrate are present in bone salts, and they form a soluble, poorly ionized complex. Therefore, even if citrate concentration influences the disposition and resorption of bone salts, it is impossible to decide whether the altered citric acid levels in parturient paresis only mirror the calcium–citrate reciprocity, or whether they indicate a decreased mobilization from the skeleton (Jönsson, 1960).

A marked increase of blood glucose and pyruvic acid in both normally calving cows and cows developing milk fever has been found, the levels being considerably higher in the latter group (Van Soest and Blosser, 1954; Ward *et al.*, 1953; Littledike *et al.*, 1970). A statistically significant negative correlation between blood glucose and plasma P_i levels and between blood pyruvate and P_i levels was demonstrated.

The degree of hypocalcemia is correlated with the blood glucose level and, in general, with the severity of the clinical signs (Littledike *et al.*, 1970). Relatively low levels of insulin in relation to the hyperglycemia have been reported, and it has been suggested that the very low Ca concentrations may interfere with the insulin secretion from the pancreas. Thus, insulin secretion is immediately restored via Ca administration.

There are reports indicating an association between the activity of the adrenal cortex and milk fever (Garm, 1950; Holcombe, 1953). Neither Merrild and Smith (1954) nor Ward (1956) could find an indication of adrenal involvement in parturient paresis exceeding that associated with the act of parturition. Jonsgård (1963) reviewed a series of experiments on the prophylactic effect of glucocorticoids and ACTH and concluded that there was no evidence that adrenal hypofunction was the cause of the disease. Finally, Littledike *et al.*, (1969) stated that glucocorticoid therapy would be contraindicated in cows with classical parturient paresis.

Minor changes in blood pH during milk fever have occasionally been reported (Hallgren *et al.*, 1959; Aalund and Nielsen, 1960). Little or no changes have been reported in the concentration of plasma Na, K, or AP (Littledike, 1974).

In studies on milk fever, Hallgren (1940) demonstrated a hypotension, the degree of which, however, was negligible and not associated with vasomotor collapse. In 140 healthy cows the systolic blood pressure was 14.7–24.7 kPa (110–185 mm Hg) as compared with a range of 10.6–20.0 kPa (80–150 mm Hg) in 44 cases of milk fever. In the majority of cows with milk fever (39 cases), the pressure was found to be between 14.0–18.6 kPa (105 and 140 mm Hg).

Cholinesterase activity was studied during milk fever (Seekles and Van Asperen, 1949). No alterations could be demonstrated. The estrone and progesterone plasma levels of normal cows and cows with parturient paresis were studied by Edqvist *et al.* (1974). No significant differences between the paretic and the normal cows were revealed.

3. Experimental "Milk Fever"

In an attempt to produce an experimental condition similar to milk fever, several investigators (Seekles *et al.*, 1931; Petersen *et al.*, 1931; Carlström, 1933; Hallgren *et al.*, 1959; Moodie, 1960) injected sodium oxalate into cows and observed the symptoms. Following slow intravenous injection of 20–40 gm oxalate, the concentration of serum Ca was greatly reduced. Clinical symptoms terminating in clonic convulsions and death occurred when serum Ca levels lower than 4 mg/dl (1 mmole/liter) were reached.

The decreased Ca/Mg ratio in milk fever caused Klobouk (1932), Pribyl (1933), and Schulhof (1933) to assume Mg narcosis to be the cause of milk fever, and subsequently magnesium sulfate was injected into calves until a Ca/Mg ratio similar to that found in milk fever was reached. In such cases injections of $CaCl_2$ were demonstrated to be curative.

Hibbs *et al.* (1945, 1946) demonstrated that the anesthetic effect of Mg is due to the Ca/Mg ratio regardless of whether the serum Ca level falls to meet the serum Mg level, as in milk fever or when oxalate is injected, or whether the serum Mg level rises to meet the serum Ca level, as when Mg salts are injected. Hibbs (1950) therefore concluded that the relatively high serum Mg level accounts for the lack of tetanic symptoms in typical milk fever and for the comatose condition which is usually seen in spite of the low blood Ca level.

Mayer *et al.* (1966) studied spontaneously as well as experimentally induced hypocalcemia. From these observations they deduced that the degree of hypocalcemia seemed more crucial to the development of paresis than did duration. This is consistent with the finding that hypocalcemia may exist for hours before symptoms develop (Payne, 1963). The primary cause of hypocalcemic paresis is a depression of neuromuscular transmission (Bowen *et al.*, 1970a). The exact relation of the onset of hypocalcemia to the development of clinical signs, however, is still uncertain.

4. Etiological Considerations

The present concept of milk fever can be outlined on the basis of three theories: (1) the hypoglycemia theory, (2) the parathyroid (hypocalcemia) theory, and (3) the theory of temporary insufficient bone and/or intestinal adaptation of Ca homeostasis to lactation.

Until about 1925 little experimental work had been done concerning the etiology of milk fever. Nocard (1885) had noticed "sugar" in the urine of milk fever cows (at that time it was not possible to distinguish between glucose and lactose). In 1925, two theories were advanced. (1) Widmark and Carlens (1925a,b,c) attributed the disease to glucose deficiency (a hypoglycemic coma) brought on by the intensity of mammary secretion. (2) Dryerre and Greig (1925) in Scotland claimed, without experimental evidence, that "the nature of milk fever may be understood as a parathyroid deficiency resulting in the accumulation of toxic substances such as guanidine, and a fall in blood Ca, the fall in Ca being further accentuated by lactation." These two theories caused an increasing interest in blood chemical analysis, and in the next few years both theories were subjected to intensive scientific investigation.

a. Hypoglycemia Theory. This theory was invalidated when Hayden (1927), by means of the Folin–Svedberg fermentation method (Folin and Svedberg, 1926), was able to demonstrate a hyperglycemia during milk fever.

b. Parathyroid (Hypocalcemia) Theory. Little and Wright (1925) reported that milk fever in cattle was associated with a hypocalcemia. This finding has been repeatedly confirmed, and research has been directed almost exclusively toward the cause of this phenomenon.

The assumption of a guanidine toxemia due to parathyroid deficiency was soon refuted (Hayden, 1929), and PTH was reported to be of dubious value in preventing or curing milk fever (Little and Mattick, 1933). Jackson *et al.* (1962) later confirmed this finding. They concluded that parturient cows were not responsive to PTH extract, and its administration just after calving had no apparent effect on the occurrence or severity of milk fever.

Greig (1930) demonstrated that the blood Ca level in 4–12 hours rose to normal in milk fever cases treated with udder inflation. The recovery followed when a blood serum Ca concentration of about 7 mg/100 ml (1.8 mmole/liter) was reached. Later, Niedermeier and Smith (1950) demonstrated that different cows recover at different serum Ca levels, and they suggested that the relative levels of Ca, P, and Mg may be important in the symptomatology of milk fever.

The question of whether the sudden demand for Ca and P at the onset of lactation might be responsible for the drop in serum Ca and P levels found in parturient paresis has been the subject of considerable interest (Carlström, 1933). However, studies reported by Smith and Blosser (1947) and Jonsgård (1965) showed that prepartum milking had little or no effect on reducing the incidence of parturient paresis, nor did complete milking of cows immediately following parturition increase the incidence (Smith *et al.*, 1948; Owen, 1954). Furthermore, Hibbs (1948) showed that cows with milk fever produced no more colostrum than normal cows during the postparturient period. Ash and Ca content of the colostrum from cows with milk fever was no higher than that of normal postparturient cows. Finally, it was shown that mastectomy in susceptible cows eliminates parturient paresis as well as the usual alterations in blood constituents during parturition (Niedermeier *et al.*, 1949; Robertson *et al.*, 1956).

Calcium equilibrium between skeleton and blood is maintained by a dual mechanism. One is the action of PTH on the skeletal Ca; the other is a chemical equilibrium between bone Ca and the blood. Until recently the arguments for and against PTH insufficiency as an etiological feature in milk fever were almost exclusively based on indirect evidence.

i. Renewed examination of parathyroid function. Garm (1951) reported significantly higher absolute and relative parathyroid weights in cows with parturient paresis than in normal nonpregnant cows. Blosser and Albright (1956) stated that the blood and urine excretion picture during parturient paresis did not conform to that of hypoparathyroidism in man and laboratory animals. Later, Marshak (1957) and Jönsson (1960) in histological studies evaluated the parathyroid function in normal cows and in cows with parturient paresis. Both concluded that there was no indication of parathyroid insufficiency in cows with parturient paresis.

Stott and Smith (1964) reported augmentation of PTH due to an increased amount of secretory tissue in cows just prior to parturition. Capen *et al.* (1965, 1966b) examined the ultrastructure and the histochemical characteristics of the parathyroids from pregnant and nonpregnant cows. Their results suggested an increased secretory activity of the parathyroid glands immediately prior to parturition.

Mayer *et al.* (1966) studied spontaneous and experimentally induced hypocalcemia. It was deduced that the degree of hypocalcemia was more crucial to the development of

paresis than duration. Their results showed that hypocalcemia may exist for hours before symptoms develop.

Stott and Smith (1957) and later Mayer *et al.* (1966) reported results of parathyroidectomy in the adult cow. Following ablation, serum Ca concentration dropped approximately 3 mg/dl (0.8 mmole/liter), with a return to the normal range within 2–3 weeks. Serum P_i concentration decreased to a lesser extent. The cows did not exhibit signs of milk fever, and the absence of the parathyroids did not have any adverse effect on pregnancy or parturiton and did not cause any cessation of lactation. The cows were kept on a normal diet with a high Ca/P ratio.

In calves, thyroparathyroidectomy was followed by hypocalcemic tetany (Stott and Smith, 1957). On thyroparathyroidectomized sheep, Nelson *et al.* (1969) studied the effect of age and Ca-free diet and demonstrated that adult sheep were more tolerant to thyroparathyroidectomy than were young animals. Adult sheep were capable of correcting the hypocalcemia and maintained serum Ca above symptomatic levels, whereas serum Ca levels in young thyroparathyroidectomized sheep declined rapidly. The decline in serum Ca was often followed by fatal tetany.

Rapid intravenous infusion of EDTA into cows resulted in acutely developing hypocalcemia and tetany (Payne *et al.*, 1963; Smith and Brown, 1963). Continuous EDTA infusion into cows at a rate comparable to the Ca outflow into milk elicited a triphasic homeostatic response in plasma calcium. Plasma Ca concentration declined rapidly for a few hours, then remained at a plateau at 6 mg/dl (1.5 mmole/liter) for a few hours, and then fell rapidly again. Parathyroid hormone was continuously secreted at a rate inversely proportional to plasma Ca concentration within the range studied [3.0–10.8 mg/dl (0.8–2.7 mmole/liter)] (Ramberg *et al.*, 1967). A deficient parathyroid response therefore can be ruled out as a primary etiological factor in parturient paresis (Mayer *et al.*, 1969).

ii. Calcitonin. The possible role of CT in the etiology of parturient paresis is not clear. Capen and Young (1967) showed a marked decrease in the CT content of the thyroids of cows with clinical signs of parturient hypocalcemia compared to normal postparturient control cows. They concluded that an abrupt release of a significant amount of CT near parturition might contribute to the development of the hypocalcemia and hypophosphatemia associated with parturient paresis. A hypocalcemic response to infusion of porcine CT was observed in young cows (Barlet, 1967) and cows on low Ca intakes. Negligible response, however, was seen in old cows and in cows on high Ca intakes. Thus, cows expected to have active bone resorption responded, while those expected to have negligible bone resorption did not (Kronfeld, 1971).

Elevation of plasma Ca appears to be the predominant stimulus of thyroid CT (Care *et al.*, 1968a). Despite frequent sampling prior to the onset of hypocalcemia, hypercalcemia has not been observed (Mayer, 1970). Littledike *et al.* (1971) reported increases of CT in the plasma of some cows several days before parturition, but the increase could not conclusively be demonstrated to be associated with the development of clinical parturient hypocalcemia. Therefore, if CT plays a role in parturient paresis, the role remains to be elucidated.

c. Insufficient Adaptation of Calcium Homeostasis. The hypocalcemia observed during milk fever thus appears to be more closely related to the initiation of lactation than to parturition per se. Moodie (1960) and Kronfeld and Ramberg (1970) investigated the

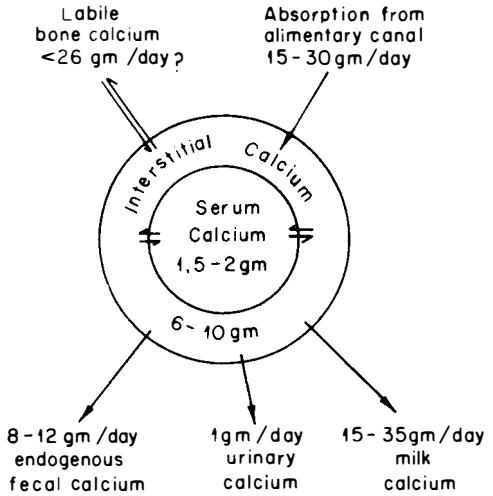


Fig. 2. Exchange of calcium between serum and tissue in lactating cows. (From Moodie, 1960.)

significance of the mammary drain of Ca. They found it to be about the same in normal and paretic cows. They concluded that hypocalcemia in the recently calved cow is essentially a failure to obtain sufficient Ca from the gut and/or bone.

Calcium is transported by the blood and is constantly being exchanged. The total amount of circulating Ca is approximately 1.5–2.0 gm, and the daily turnover of Ca within the body varies from about 10 gm in the nonproductive cow to more than 35 gm in the lactating cow (Moodie, 1960) (Fig. 2). Thus, a quantity of Ca equal to the total amount in the circulation may be removed every 1–5 hours depending on the physiological state of the animal.

From Fig. 2 it can be seen that a cow secreting colostrum at 3–4 gallons a day could not provide the Ca needed for secretion from either the skeleton or the digestive system alone.

i. Temporary insufficient bone adaptation of Ca metabolism to lactation. The amount of mobilizable Ca from bones diminishes as the cow grows older (Hansard *et al.*, 1954). The cow is most susceptible to milk fever during the period from the fourth to the eighth parturition (Jönsson, 1960; Dishington, 1974). Diet, however, also has an influence on the risk of parturient paresis. Boda and Cole (1956) fed a diet high in P and low in Ca and thus reduced the incidence of parturient paresis significantly. Ender *et al.* (1962), on the other hand, were able to induce milk fever on a prepartal diet high in Ca and low or normal in P. Mayer *et al.* (1969) suggested that, as CT appears to inhibit bone resorption, the high Ca prepartal diet may tend to elevate the plasma Ca concentration slightly and to stimulate the release of CT. A continuous or intermittent exposure of bone to the resorption-inhibitory effect of CT might result in a suboptimal response of bone to PTH when the onset of lactation precipitates the development of hypocalcemia.

More recent investigations (Care *et al.*, 1970; Black and Capen, 1973; Mayer *et al.*, 1975), however, failed to provide evidence for increased thyroid C-cell secretory activity either during or preceding the development of parturient hypocalcemia.

The level of hydroxyproline excreted in the urine has been shown to be a valuable index

of bone matrix metabolism. Nurmio (1968) and Black and Capen (1971) investigated bone matrix metabolism in cows which developed parturient paresis as compared to control cows. Alterations of matrix metabolism were correlated with changes in the serum concentration of Ca, P, and Mg. Significant differences were not demonstrated between cows which developed severe hypocalcemia and control cows which maintained their serum Ca concentrations near normal through parturition. Nurmio and Rajakoski (1973a,b) showed that the increase in hydroxyproline excretion on the day of parturition is greater in healthy cows than in cows showing parturient paresis. This would seem to indicate a lower bone resorption rate in cows showing parturient paresis than in healthy cows.

Yarrington *et al.* (1976) demonstrated that cows near parturition developed acute hypocalcemia, hypophosphatemia, and clinical symptoms identical to those associated with naturally occurring parturient paresis when bone resorption was effectively blocked by prepartum administration of a potent inhibitor of bone resorption, disodium ethane-1-hydroxy-1, 1-diphosphonate, even in the presence of a responsive parathyroid gland. Control cows fed the same diet did not develop symptoms. These findings suggest that an interruption of bone resorption and mobilization of Ca from skeletal reserves contributes to the development of spontaneous parturient paresis.

ii. Temporary insufficient intestinal adaptation of Ca metabolism to lactation. Usually dry cows are in positive Ca and P balance, while lactating cows during the first month of lactation are in negative balance (Duncan, 1958). The change from positive to negative balance occurs over a period of a very few days at the time of parturition. About the time of calving the variable nature of the animal's appetite makes it difficult to use conventional balance experiments as a means of determining the quantities of Ca absorbed on an hourly or daily basis. Robertson *et al.* (1960) and Moodie and Robertson (1961, 1962) therefore produced indirect evidence in support of their suggestion that the cow around calving time is dependent not only on release of Ca from bone but also on a continuous uptake of Ca from the alimentary tract. Loss of appetite, reduced fecal Ca excretion, and reduction in alimentary activity were changes reported to take place at parturition in normal calving cows. These findings have been confirmed by Dirksen and Kaufmann (1978). To stress the importance of uninterrupted absorption of Ca from the alimentary tract in heavy-milking cows and cows at time of parturition, Moodie (1960) had previously induced hypocalcemia and milk fever-like symptoms in lactating cows by injection of hyoscine hydrobromide, an alkaloid paralyzing the cholinergic nerve fibers, thus antagonizing acetylcholine. The mechanism responsible for the milk fever-like syndrome was held to be the impaired absorption of Ca brought about through bowel stasis.

A similar milk fever-like picture was induced in fasting lactating cows (Halse, 1958a; Robertson *et al.*, 1960). In both cases it appeared that a loss of Ca through the milk was a prerequisite.

The importance of acid or alkaline diets fed to cows before parturition was illustrated by Ender and Dishington (1970). They performed Ca balance trials on 25 milking cows, age 5–10 years. The results are shown in Table V. Ender and Dishington concluded that the alkaline or acid nature of the prepartal diet reduces or increases intestinal Ca absorption and influences the amount of available bone Ca. More recently, it has been shown that the mineral acid acidifying effect can be replaced by salt mixtures (Dishington, 1975).

In conclusion, parturient paresis is essentially a temporary failure to obtain sufficient Ca from the gut and/or bone.

TABLE V

Results Illustrating the Influence of Acid and Alkaline Diets Fed to Cows before Parturition[a]

Group	Ca and P content of feed before parturition	Diet	Number of milk fever cases
1[b]	140/30	Alkaline[c]	4 + 1 borderline out of 10
2	35/30	Alkaline[c]	1 + 1 borderline out of 3
3[b]	140/30	Acid[d]	0 borderline out of 8
4	35/30	Acid[d]	0 borderline out of 4

[a] From Ender and Dishington (1970).
[b] Groups 1 and 3: high Ca content secured by $CaCO_3$ supplements.
[c] Secured by feeding large amounts of beets and swedes.
[d] Secured by feeding large amounts of mineral acid grass silage.

5. *Prevention of Milk Fever*

Four different methods of prevention have been suggested: (1) use of vitamin D (orally or as injection), (2) dietary adjustment of the Ca/P ratio, (3) oral drench with $CaCl_2$, and (4) use of "acid" diets.

a. Vitamin D. The use of vitamin D_2 as a preventive was first tried by Greig (1930), but it was not until 1955 that Hibbs and Pounden (1955) demonstrated the feasibility of this method of prevention. The daily administration of 30 million IU of vitamin D_2 for at least 4 or 5 days, but no longer than 7 days, prepartum prevented milk fever in mature cows with previous milk fever histories. They reported later that approximately the same protection could be obtained with a dosage level of 20 million IU, whereas 15 million IU daily yielded less protection (Hibbs and Conrad, 1960). The protection increased during the first 3 days of vitamin D_2 feeding, remained at a high level during the third through the seventh day, and dropped precipitously 1 day after cessation of vitamin D_2 feeding. Dell and Poulton (1958) confirmed the reports on the value of vitamin D_2 as a milk fever preventive. In studies with more than 400 cows, the addition of 30 million IU of vitamin D_2 daily to the grain from approximately 72 hours prepartum until 48 hours postpartum reduced the incidence of milk fever by approximately 70% below that of the controls.

The protection provided by vitamin D_2 has been attributed to its calcemic effect, indicated by the relatively high serum Ca level maintained during the critical postpartum period. This apparently is due not to parathyroid stimulation, but to increased absorption of Ca and P from the digestive tract (Conrad *et al.*, 1956; Capen *et al.*, 1966b; Muir *et al.*, 1968).

Harmful effects due to prolonged feeding of vitamin D_2 have been reported (Cole *et al.*, 1957; Payne, 1963). In order to minimize these effects, the use of vitamin D_3 injection was introduced (Seekles *et al.*, 1958). Ten million international units of vitamin D_3 given 2–8 days before calving has been claimed to prevent the disease in 84% of animals compared with 82% when vitamin D_2 is given orally (Seekles *et al.*, 1964). The danger of metastatic calcifications and other harmful effects exists also when vitamin D_3 is used (Manston and Payne, 1964). Pregnant cows are more susceptible to calcifications than are nonpregnant cows (Capen *et al.*, 1966a). Thyroxine administered with vitamin D_3 as well

as adjustment of the dietary Ca/P ratio prior to treatment has been reported to be of value in reducing the risk of calcifications (Payne and Manston, 1967; Manston, 1969). The intramuscular route of vitamin D_3 injection has been recommended (Seekles *et al.*, 1964).

With the discovery of vitamin D metabolites, interest in their possible use in the prevention of milk fever has arisen. Several preparations containing 25-OH-D_3 or 1α-$(OH)_2D_3$ have been investigated (Olson *et al.*, 1973; Jorgensen, 1974; Sansom *et al.*, 1976; Sachs *et al.*, 1977; Wittwer and Ford, 1978; Davies *et al.*, 1978). The results suggest a high degree of protection when injection is given during a period of 8 days just prior to parturition. Serious toxic effects have been reported from Sweden (Björklund *et al.*, 1976). The disadvantage of using vitamin D_3 metabolites and vitamin D is the necessity of accurately estimating the calving date.

b. Dietary Adjustment of Ca/P Ratio. The use of a low-Ca, high-P prepartal diet was suggested by Boda and Cole (1954, 1956). The purpose of this diet was to stimulate the production of endogenous PTH by causing stimulation of the parathyroid glands prior to the onset of lactation and increased demand for Ca mobilization. They divided 69 aged Jersey cows into groups of 4 cows each and fed them on diets containing various Ca/P ratios for various periods of time prepartum. Clinical signs of milk fever were evident in 30% of the cows receiving diets with a Ca/P ratio of 6 : 1, in 15% of those receiving diets with a Ca/P ratio of 1 : 1, and in none of those receiving diets with a Ca/P ratio of 1 : 3.3. In agreement with these figures Ender *et al.* (1956, 1962) found an increased incidence of milk fever in cows on an experimental high-Ca diet (Ca/P ratio between 5 : 1 and 10 : 1). Furthermore, these investigators pointed out that the milk fever-inducing effect of a given fodder also would be strongly influenced by its alkali alkalinity. Feeding fodder containing large quantities of acid (AIV silage), i.e., low alkalinity, resulted in practically no cases of milk fever, and vice versa.

In a study to determine whether cows which have been prefed the diet recommended by Boda and Cole (1954, 1956) are better able to maintain normal serum Ca levels, Luick *et al.* (1957a,b) estimated the reservoirs of mobilizable skeletal Ca. Their results indicated larger Ca reservoirs and a slower turnover in treated cows. Since such cows are in negative Ca balance, the increasing reservoirs (pool size) can be due only to adjustment in the bone itself, i.e., an increase in the pool of mobilizable Ca at the expense of stable Ca. Stable bone thus became vascularized and therefore was identified as part of the mobilizable Ca pool.

Mayer (1972) pointed out that the preventive effect is related to Ca intake and not to P intake or Ca/P ratio of the diet. A low-Ca diet during the 4- to 6-week prepartum period tends to diminish Ca absorption and increase bone resorption. This transposition diminishes reliance upon Ca absorption and thereby lessens the likelihood of hypocalcemia in the event of a reduction in alimentary activity and Ca absorption at parturition. Due to the high Ca content in many roughages, it is difficult to achieve extremely low levels of Ca in the diet. Wiggers *et al.* (1975) concluded that the prepartal Ca intake should not exceed 15–25 gm/day. A preventive effect has been reported for daily Ca intakes of 35–50 gm by Westerhuis (1974), Goings *et al.* (1974), and Pickard (1975). Additional supply of Ca immediately after parturition further improves the maintenance of Ca homeostasis.

c. Drenching with Calcium Chloride. A third method of milk fever prevention appeared when Glawischnig (1962) reported that drenching with $CaCl_2$ had a damping

effect on the initial milk fever attack as well as on relapses. A $CaCl_2$ gel in which changes in viscosity and other properties disguised its salty taste was formulated and its preventive effect investigated (Ringarp *et al.*, 1967). A statistically significant effect was demonstrated. The incidence of milk fever declined in one series of experiments from 57% in the controls to 7.6% in the dosed animals. This finding has been confirmed by Jönsson and Pehrson (1970), Simesen and Hyldgaard-Jensen (1971), and Glawischnig (1974).

It is recommended that the four doses of $CaCl_2$ gel (150 gm) be given from 1 day before calving until 2 days after calving. As with the vitamin D method, the greatest difficulty is to determine the exact time of calving. The treatment can, however, be extended up to 3 weeks if necessary.

Cows which develop milk fever in spite of treatment become ill significantly later than nontreated control cows. This delay in the onset of disease is a positive effect, as the prognosis is better the later the disease commences (Jonsgård, 1972; Alanko *et al.*, 1975).

d. Use of "Acid" Diets. In a series of experiments Ender *et al.* (1962, 1971; Ender, 1964) showed that diets of high alkalinity predispose to milk fever, while acid diets or, more strictly, diets of low alkalinity have a preventive effect. The alkalinity of the diet is determined by the amounts of Na^+ and K^+, on the one hand, and SO_4^{2-} and Cl^-, on the other. An excess of Na^+ and K^+ gives high alkalinity, and vice versa. Most prepartal diets are of high alkalinity, especially when beets are included. The Norwegian experiments were originally carried out with large amounts of beet in the predisposing diet and with mineral acid-preserved grass silage (AIV silage) in the preventive diet. Dishington (1975) produced the acidifying effect by using salt mixtures instead of mineral acid. The most promising results were obtained by dispersing on hay a solution of $CaCl_2$ followed by a solution of aluminum and magnesium sulfates. This "acid" diet was given during the last 4 weeks before parturition. The results are shown in Table VI. By dietary means at alternating parturitions, milk fever could be induced or prevented in the same cow.

Lomba *et al.* (1978), in commenting on the theory of the alkalinity of diets, have pointed out that it is only when cows are absorbing amounts of Ca in excess of their needs that the effect of alkalinity of the diet is observed. There is evidence of a positive effect of "acid" diets (Vagg and Payne, 1970; Verdaris and Evans, 1976). Daily oral administration of 100 gm of NH_4Cl or more, however, is toxic and difficult to carry out since animals refuse the feed (Jorgensen, 1974).

An increase in the grain feeding from 0.5 to 1.0% of body weight was reported to be effective in milk fever prevention (Kendall *et al.*, 1966). The observations, however, were based on a limited number of cows. Milk fever prevention, if any, by increased grain feeding may be due to an acidifying effect of such a diet.

TABLE VI

Data from 5 Cows Tested at 25 Subsequent Parturitions[a,b]

Total	AIV diet	Beet diet	Basic diet with alkaline supplement	Basic diet with "acid" supplement
25/12	4/0	3/2 + 1 B	7/6 + 1 B	11/2

[a] From Dishington (1975).

[b] Total/milk fever cases; B, borderline.

6. *Metabolic Complications in Parturient Paresis*

Some of the metabolic disturbances seen during the development of parturient paresis are found generally in parturient cows, i.e., hypocalcemia, hypophosphatemia, hypermagnesemia, and hyperglycemia. The hypermagnesemia observed in parturient cows as well as in cows with parturient paresis has been suggested to be due to PTH release in response to hypocalcemia (Kronfeld, 1971). The degree and, to a lesser extent, the duration of hypocalcemia appear to dominate the development of the syndrome of parturient paresis (Mayer *et al.*, 1966). Other disturbances may play various roles and thus present clinical as well as clinical biochemical and diagnostic challenges.

In Table VII a survey of 590 cases of clinical parturient paresis classified on the basis of clinical chemical blood analysis is shown. The number of cases which needed more than one treatment is also shown. Only cases appearing during the interval of 48 hours before to 48 hours after parturition are included in the category of parturient paresis. It can be seen that 62.7% of all cases biochemically were uncomplicated cases. Cases with elevated aspartate aminotransferase (AST) values made up the majority of the complicated cases. The incidence of cases associated with normocalcemia, hypophosphatemia, and hypermagnesemia was 2.5%. Hypomagnesemia and ketonemia were found in 2.7 and 0.7% of the cases, respectively. Milk fever-like cases appearing more than 48 hours before or after parturition amounted to 8.5%.

Persistent relief of clinical signs following calcium therapy is associated with the return of plasma Ca and P_i levels to normal. Inadequate response or relapse is sometimes

TABLE VII

Survey of 590 Cases of Clinical Milk Fever-like Paresis Classified on the Basis of Clinical Chemical Analysis[a]

Diagnosis	Number	% of total	Cases needing more than one treatment	
			Number	%
Uncomplicated hypocalcemic parturient paresis	370	62.7	55	14.9
Parturient paresis complicated by muscle damage (ASAT >2 μkat)	50	8.5	23	46.0
Parturient paresis characterized by hypophosphatemia, hypermagnesemia, and normocalcemia	15	2.5	1	6.7
As above, complicated by muscle damage (ASAT >2 μkat)	2	0.3	2	100.0
Hypocalcemic parturient paresis complicated by hypomagnesemia	16	2.7	1	6.3
Hypocalcemic parturient paresis complicated by ketonemia	4	0.7	2	50.0
Alimentary, hypocalcemic milk fever-like paresis (outside ±48 hours from time of parturition)	39	6.6	4	10.3
Hypomagnesemic tetany (paretic)	10	1.7	0	0.0
Milk fever-like ketonemia	1	0.2	0	0.0
Not determined (including contaminated samples, broken samples, etc.)	83	14.1		

[a] From Simesen (1968).

TABLE VIII

Main Causes of the Downer Condition[a,b]

Cause	A (%) (n = 55)		B (%) (n = 74)		
Spreadlegging	32.7		37.8	52.7	
Other muscular injuries		58.2	14.9		70.3
Nerve injuries	25.5		17.6		
Myocardosis	7.3		9.5		
Hepatosis	1.8		8.1		
Persistent hypocalcemia	9.1		5.4		
Persistent hypophosphatemia	10.9		0.0		
Coli mastitis	1.8		0.0		
Not determined	10.9		6.8		

[a] From Jönsson and Pehrson (1969).
[b] Group A: recovered; group B: not recovered.

associated with a normal Ca level and a persistent hypophosphatemia (Marr *et al.*, 1955). This hypophosphatemia per se, however, has no characteristic clinical signs. Such cases usually respond well to Ca-hypophosphite therapy.

The so-called downer cow syndrome in which the animal becomes paretic during the period of susceptibility to parturient paresis but fails to respond to treatment for this disease has been classified as a metabolic disease. However, it should not be classified as such but rather as a complication of parturient paresis (Curtis *et al.*, 1970). In Table VIII are shown the results of a detailed study of 129 downers, i.e., cows unable to rise after two treatments (Jönsson and Pehrson, 1969). The most prominent changes were the increase in AST values indicating muscle injuries. These changes occurred most often in the interval between the first and second or the second and third Ca treatment, indicating that excellent nursing of recumbent cows is needed in connection with the initial treatment with Ca.

D. Condition Similar to Parturient Paresis in Other Animals

A condition similar to parturient paresis occurs in several species. Hypocalcemic paretic syndromes occur quite commonly in sheep but rarely in goats and sows. Greig (1929) and later Leslie (1931) showed that serum Ca concentration is subnormal during lambing sickness of ewes. Hypocalcemia has also been reported in Australia (McClymont, 1947; Franklin *et al.*, 1948), the United States (Asbury, 1962), and Scandinavia (Simesen, 1971; Jonson *et al.*, 1971). A paretic condition of the pre- and postparturient hill ewe, presumably of hypocalcemic origin, the so-called moss-ill, has been described in the north of England (Littlejohn and Hebert, 1969).

The pathogenesis of milk fever in ewes has been discussed by Braithwaite *et al.* (1970). They confirmed the findings of Field and Suttle (1967), who found that there was only moderate change in the demand for Ca at parturition in sheep which had single lambs or twins. This seems consistent with the fact that the ewe is not as susceptible to milk fever as the cow.

Hypocalcemic syndromes have been reported only rarely in sows (Kjeldberg, 1940;

Craige and Beck, 1941), goats (Van Maltits, 1945), and rabbits (Wenger, 1945). Hypocalcemic tetanic syndromes develop in bitches, rarely in cats, usually within 2–4 weeks postpartum (Barto, 1932; Crosfield, 1941; Lawler, 1963; Edney, 1969), and in mares, usually 1 or 2 weeks after foaling or 1 or 2 days after weaning (Montgomery *et al.*, 1929; Baird, 1971; Rach *et al.*, 1972).

E. Hyperphosphatemia

High serum P_i levels are observed during renal failure, 8–25 mg/dl (2.6–8.0 mmole/liter). A slight rise may also occur during the period of healing of fractures.

Hyperphosphatemia due to renal failure may cause secondary hyperparathyroidism (first subtype). This is seen in dogs. When the glomerular filtration rate is reduced, P_i retention emerges. The net effect is an increase in serum P_i accompanied by a decrease in serum Ca and increased parathyroid activity. The renal failure may furthermore cause impaired calcium absorption from the intestine because of an acquired defect in vitamin D metabolism. The final picture is normal to decreased serum Ca values and increased values for P_i.

High P_i levels are also observed in ruminants in connection with vitamin D intoxication or intoxication with vitamin D-like substances (plant-induced calcinosis).

F. Hypophosphatemia

Serum P_i is markedly influenced during periods of inadequate P intake. This condition is described elsewhere (Section IV,B,1,3, and 4.). Hypophosphatemia develops during primary hyperparathyroidism (see Section III,B). Parturient paresis (milk fever) and hypomagnesemic tetany are both accompanied by hypophosphatemia. The degree of hypophosphatemia is more pronounced in parturient paresis than in hypomagnesemic tetany.

G. Postparturient Hemoglobinemia

The relation of postparturient hemoglobinemia to aphosphorosis has not been clearly established. It is a disease mainly affecting heavily producing cows. Recently calved cows are most susceptible. The hemoglobinuria appears suddenly, and in the next 24–48 hours other symptoms occur. These include anemia, rapid loss of condition, listlessness, inappetence, slight icterus, and increased heart rate with a shallow, rapid respiration. Often, milk yield is only slightly disturbed. On autopsy the main findings are anemia, icterus, and central lobular necrosis of the liver (Hjärre, 1930).

The disease was first recognized by Cumming (1853, cited in Hutyra *et al.*, 1946). It occurred on old farms with poor soil, especially following periods of drought. In the United States it is frequently associated with the feeding of beet by-products and alfalfa hay (Madsen and Nielsen, 1940). In Australia it has been observed in herds grazing on winter oats (Parkinson and Sutherland, 1954).

Madsen and Nielsen (1940) first reported the disease to be associated with hypophosphatemia. They produced the disease by feeding a high-producing dairy cow a low-P ration consisting of alfalfa and dried beet pulp (Madsen and Nielsen, 1944). The main

biochemical findings are those of hypophosphatemia (in spite of the hemoglobinemia): pronounced anemia and icterus. Thus, out of 67 blood samples from cattle on farms where postparturient hemoglobinemia occurred, 23 showed blood P_i levels below 1 mg/dl (0.3 mmole/liter) (Mullins and Ramsay, 1959). In the same study the hypotonic resistance of the red blood cells was reported to be within normal range. Otherwise, no consistent alterations have been shown.

Centrilobular necroses of the liver were shown to be secondary to the hemoglobinemia (Hjärre, 1930), and subsequent studies showed that the necroses disappeared rapidly during recovery (Møller and Simesen, 1959).

A consistent finding in herds affected with postparturient hemoglobinemia has been hypophosphatemia. However, herds with extremely low blood P_i levels, where neither anemia nor postparturient hemoglobinemia exists, are well known. Therefore, the existence of a factor able to produce hemoglobinemia in hypophosphatemic animals has been suggested. Freudenberg (1955) produced the disease in three or four dairy cows fed a diet of fresh and ensiled sugar beet leaves and alfalfa hay. The diet had a Ca/P ratio of 4 : 1. In two herds fed the same forage supplemented with concentrates and minerals, no sign of disease occurred. It is concluded that two factors are necessary for the development of postparturient hemoglobinemia: a hypophosphatemia and a hemolytic effect of saponins, present in sugar beet leaves as well as in alfalfa hay. Postparturient hemoglobinemia was reported to appear in buffaloes (Nagpal *et al.*, 1968).

IV. BONE DISORDERS

Failure to assimilate Ca and P normally may be due to a limited supply of these elements or to factors involved in their assimilation, notably vitamin D. Such a failure is reflected differently in immature and adult individuals according to the stage of bone development. Various terms have been used to designate the different conditions. The term *osteoporosis* is used for abnormalities in the formation of the osteoid matrix. There is a net resorption of bone tissue (i.e., mineralized collagen) which is not associated with disturbances in Ca and P metabolism. If the fundamental defect is in the metabolism of Ca and P, the conditions are designated *rickets* and *osteomalacia*.

A. Osteoporosis

The classical definition of osteoporosis by Albright (1941) is "too little bone, but what bone there is, is normal." A more rigorous definition of osteoporosis would be "a pathological decrease in the volume of bone tissue per unit of volume organ" (Fourman *et al.*, 1968).

Today we would call osteoporosis not a disease, but a state of the skeleton at a given time. The bone is usually porous. Anatomically the amount of bone tissue is reduced, but it is qualitatively normal save for some evidence of lacunar resorption giving increased halo volumes. The quantity of mineral ash per unit volume is below normal, but the ratios of ash to matrix and between minerals of the ash are within the normal ranges (Jubb and Kennedy, 1970). Osteoporosis in animals is principally nutritional. Often osteoporosis is a stage in the progression to osteodystrophia fibrosa.

B. Mineral Disorders

I. Rickets

a. Pathophysiology

i. Vitamin D, calcium, and phosphorus deficiency. Rickets is a disease which particularly affects growing animals. The main feature is a failure of Ca salts to be deposited in the newly formed bone matrix. The typical lesion of rickets is defective endochondral mineralization of the "zone of provisional calcification" of the long bones. The cause may be an inadequate supply of Ca and/or P and/or vitamin D. In the mature animal, a similar lack of Ca and/or P and/or vitamin D leads to osteomalacia. Absorption of Ca may be low in spite of a high Ca intake if the body does not contain vitamin D. The vitamin D requirement, on the other hand, is markedly influenced by the Ca/P ratio of the diet. An optimal Ca/P ratio requires minimal vitamin D, and vice versa. Calcium and phosphorus requirements of animals are given in Table IX.

Rickets has been described in a number of different animals. Some authors have used the terms "low-P rickets" and "low-Ca rickets." In both cases, the normal bone mineralization is impeded, causing structural bone abnormalities of a similar nature. Blood analysis has been used extensively in the study of these deficiencies. In general, a decrease in the blood level of P_i, Ca, or both is found. In the diagnosis of severe P deficiency, blood P_i analysis has proved extremely useful (Theiler and Green, 1932). Sometimes, however, the results of blood analysis are difficult to interpret. This applies particularly to data on calcium levels and is due to the hormonal control of the serum Ca concentration. In rickets, therefore, analysis of blood and bone often provides evidence, but a final diagnosis often requires a histopathological examination of epiphyseal cartilage of the long bones, which shows an increased thickness with a deficient provisional calcification. Normal columnar pattern is often maintained in all but the most severe lesions (Fitch, 1943; Nisbet *et al.*, 1966).

ii. Tetany. Tetany is a syndrome characterized by increased neuromuscular excitability. After a variable period of latency, neuromuscular excitability increases to the point where spontaneous manifestations appear. One of the most important biochemical findings during tetany is reduction of the serum Ca concentration to a level that is usually between 7 and 8 mg/dl (1.8–2.0 mmole/liter) in latent and between 4 and 6 mg/dl (1.0–1.5 mmole/liter) in manifest tetany. However, this close parallelism between the severity of symptoms and the degree of hypocalcemia is not constant. Latent tetany may be present at low Ca levels, and manifest tetany may occur at concentrations as high as 8 mg/dl (2.0 mmole/liter). Accordingly, other factors undoubtedly contribute to the pathogenesis of neuromuscular hyperirritability. The most important of these is probably the decreased Ca/P ratio.

Alkalosis, known as one cause of tetany, is usually thought to act by lowering the effective Ca ion concentration at the neuromuscular junctions.

iii. Alkaline phosphatase. Phosphatases are enzymes that are active in the hydrolysis of monophosphoric esters with the liberation of P_i. Alkaline phosphatases have their maximal activity at pH 8.5–9.5, and they are activated by Mg ions (Erdtman, 1928). They are present in practically all tissues (bone, intestinal mucosa, renal tubule cells, and liver

TABLE IX

Calcium and Phosphorus Requirements of Animals[a]

Animal	Calcium	Phosphorus	Optimal Ca/P ratio
Horse[b]			
Mature (500 kg)	27 gm	17 gm	Not less than 1 : 1
Lactating	60 gm	40 gm	
Foal (170 kg)	36 gm	23 gm	
Dairy cow[c]			
Calf (50 kg)	9–10 gm	6 gm	Not less than 1 : 1
Cow (550 kg)	25–28 gm	19–21 gm	
Dairy cow, milk prod—per kg milk (5% fat)	2.90 gm	1.90 gm	
Sheep[d]			
Mature (50 kg)	3 gm	2.8 gm	Not less than 1 : 1
Lactating			
With single lamb	10.9 gm	7.8 gm	
With twins	12.5 gm	8.9 gm	
Pig[e]			
Pig (10–20 kg)	8.1 gm	6.3 gm	1 : 1–1.5 : 1
Bred sow	15 gm	10 gm	
Lactating sow	41 gm	28 gm	
Dog[f]			
Adult—per kg body wt	0.24 gm	0.20 gm	1.2–1.4 : 1
Growing puppy—per kg body wt	0.48 gm	0.40 gm	
Cat[g]			
Per kg diet, dry basis	1%	0.8%	0.9 : 1–1.1 : 1
Mink[h]			
Growing—per kg diet, dry basis	0.4%	0.4%	1 : 1–1.7 : 1
For maintenance—per kg diet, dry basis	0.3%	0.3%	

[a] Data are from the indicated titles published by the National Academy of Sciences, National Research Council, Committee on Animal Nutrition.

[b] "Nutrition Requirements of Horses," 4th rev. ed., No. 6 (1978).

[c] "Nutrition Requirements of Dairy Cattle," 5th rev. ed., No. 3 (1978).

[d] "Nutrition Requirements of Sheep," 5th rev. ed., No. 5 (1975).

[e] "Nutrition Requirements of Swine," 7th rev. ed., No. 2 (1973).

[f] "Nutrition Requirements of Dogs," revised, No. 8 (1974).

[g] "Nutrition Requirements of Cats," revised, No. 13 (1978).

[h] "Nutrition Requirements of Mink and Foxes," 1st rev. ed., No. 7 (1968).

cells), but apparently only the AP of the osteoblasts functions outside the cells. Therefore, some of the osteoblastic phosphatase continuously reaches the plasma and circulates in the bloodstream.

Alkaline phosphatase is a very stable enzyme. It can be frozen and thawed repeatedly with little or no loss of activity.

The concentration of serum alkaline phosphatase (SAP) tends to remain constant. Alkaline phosphatase is normally found in the bile, and SAP activity is increased in obstructive jaundice. In human rickets, there is a marked increase in SAP activity. This is the first apparent evidence of the disease and the last finding to disappear after recovery. In osteomalacia, an increase in SAP activity occurs, whereas no increase is found during osteoporosis. Serum alkaline phosphatase activity in domestic animals has been expressed

in Bodansky, King–Armstrong, Bessey-Lowrey, or international units (see Chapter 5, Section VII).

Allcroft and Folley (1941) found SAP activity to vary between 0.3 and 114.3 and between 3.0 and 166.1 King–Armstrong units/dl in apparently normal cows and ewes, respectively. In the individual animal, however, the activity remained within a fairly narrow range. In both cattle and sheep, SAP activity progressively decreased with advancing age until maturity was reached. During pregnancy a slight increase was observed, although changes in the level during pregnancy did not appear to be correlated with the stage of pregnancy. A marked increase in activity was reported to be associated with the approach of parturition (Wilson, 1955; Ford, 1958). Activity of SAP was found to be low during hypomagnesemic tetany (Halse, 1948). No change in AP was reported to occur in plasma of cows with parturient paresis compared to normal postpartum cows (Littledike *et al.*, 1970).

Wise *et al.* (1958) reported that increased AP activity accompanied decreased serum P_i levels in 3- to 6-month-old calves fed different levels of dietary phosphorus. The AP activity responded rapidly to a change in the phosphorus concentration of the blood, but, after an initial change, the AP activity returned to its "normal" level.

Alkaline phosphatase activity is reduced in serum of Zn-deficient pigs (Miller *et al.*, 1968), calves (Miller *et al.*, 1965), and cows (Kirchgessner *et al.*, 1975). In experimental animals, AP determinations may represent a sensitive indicator of the zinc status of an animal.

Reports have appeared describing AP activity in swine (Luecke *et al.*, 1958; Brown *et al*, 1966; Baustad *et al.*, 1967), horses (Earle and Cabell, 1952; Jennings and Mulligan, 1953; Krook and Lowe, 1964), sheep (blood group-associated polymorphism and serum concentration) (Rendel *et al.*, 1964; Aalund *et al.*, 1965), and dogs (Campbell, 1960; Campbell and Douglas, 1965).

In most animals, perhaps with the exception of the cat, the enzyme is eliminated in its native form by the liver. In cats AP has been reported to be excreted through the kidney. Even ligation of the bile duct does not increase the level (Bloom, 1960). In the dog, the estimation of AP has been found to be one of the most useful tests in the diagnosis of liver disease (Lettow, 1962).

In cases of elevated values, it is difficult to determine the source of the elevation, i.e., separation into its three main components: bone, liver, and intestine. Alkaline phosphatase isozyme determinations may be very useful in identifying the organ(s) responsible for elevated values.

b. Clinical Manifestations

i. Rickets in sheep. In sheep, deficiencies of vitamin D, Ca, and/or P or combinations of P and vitamin D have been described.

Franklin *et al.* (1948) reported that inadequate dietary vitamin D or lack of ultraviolet irradiation caused a drop in serum Ca and P_i levels in the growing sheep in spite of a high dietary intake of Ca and P. Benzie *et al.* (1959) confirmed this observation and showed that the drop in serum Ca was accompanied by skeletal resorption. Quaterman *et al.* (1964) were able to estimate the vitamin D activity directly in the blood of sheep. They showed that the vitamin D activity in the blood was reduced to very low levels in sheep kept indoors during the summer. The normal summer rise was also found much reduced if

the sheep were not clipped in the spring. Quaterman *et al.* (1964) showed that an intramuscular injection of 1 million IU vitamin D raised the blood vitamin D concentration to over normal, and normal levels were maintained for over 4 months.

In lambs kept indoors for more than 3 weeks after birth, hypocalcemia was observed, and lack of vitamin D was suggested to be the main cause (Lode and Øverås, 1968). About 20% exhibited clinical symptoms, namely tetany. An association between rickets in lambs deficient in vitamin D and rates of growth was described by Duckworth *et al.* (1943).

Rations deficient in Ca or with a low Ca/P ratio readily induce a severe fall in the serum Ca level when fed to sheep. The hypocalcemia can be induced with decreasing readiness in lactating ewes, suckling lambs, 3- to 6-month old weaners, in-lamb ewes, and fullmouthed dry ewes (Franklin *et al.*, 1948).

Moderately large depression in serum Ca levels was found by McRoberts *et al.* (1965a,b) in young sheep fed a diet of low Ca content for several months. Reduced ash content of the bone and retarded eruption of teeth with slight hypoplasia of the enamel were observed. During the repair period a tendency toward prognathism was seen.

Diets low in P and vitamin D have been found to cause a marked depression of blood P_i; bone of extremely poor quality, as revealed by ash analysis and radiography; and severe clinical signs of rickets with bent fore and hind legs, swollen joints, and collapse of the digits, resulting in plantigrade instead of digitigrade gait (McRoberts *et al.*, 1965a; Miller, 1946). After about 12 months on this diet low in P and vitamin D, animals developed the clinical condition "open mouth" or "gagged bite." In similar animals fed a control diet or a diet low in Ca, these defects were not observed (McRoberts *et al.*, 1965b). Nisbet *et al.* (1966, 1968) also reported rickets and dental malocclusion in young sheep (hoggs) and osteomalacia in lactating ewes (Nisbet *et al.*, 1970) caused by a deficiency of P and vitamin D. Blood values were reported and compared with normal values provided from sheep with uncomplicated osteoporosis ("cappi" or "double scalp"). Low values for P_i and Ca were found in blood and bone from affected animals. Low blood P_i values were common also from other ewes on the affected farms. Serum alkaline phosphatase activities were inconsistent.

Rickets in goats was studied by Wieland (1940).

ii. Rickets in calves (Hibbs *et al.*, 1945; Colovos *et al.*, 1951). One of the first symptoms of low-vitamin D rickets in calves is a decrease in the serum Ca and/or P_i concentration. Usually the clinical symptoms, difficulty in locomotion, stiffness in gait with swollen and stiff joints, straight pastern, irritability, tetany, and convulsions are preceded by the decrease in serum Ca and P_i concentration.

In young calves a Ca-deficient diet results in failure of normal bone growth. The bones have a low content of Ca and P. The serum Ca levels, however, usually remain normal even in severe deficiency (Wentworth and Smith, 1961). In Sweden, rickets caused by a diet low in vitamin D and Ca was described in calves 2–5 months old and lambs less than 3 months of age (Holtenius *et al.*, 1970). Low blood Ca values were observed only in animals during tetanic convulsions.

Mild rickets caused by low P intake may be seen in calves housed for long periods of the year. The calves seem not to be as sensitive as adults. In herds where cows are severely affected by P deficiency the calves are often normal. A fall in serum P_i values is the first evidence of a P deficiency. A combined P and vitamin D deficiency in 18-

month-old bullocks housed for long periods on an inadequate diet was described (Spratling *et al.*, 1970). Low serum P_i values were reported.

iii. Rickets in horses. There are few reports describing rickets in foals (Åkerblom, 1952). Rachitic changes may be seen in ribs from young animals fed diets able to induce osteodystrophia fibrosa. Groenewald (1952) reported histological evidence of rickets in one out of four fillies fed an experimental diet with a Ca/P ratio of 1 : 5 for 10 months. Since rickets is a disease in which the bones are still growing and osteomalacia is a disease in bones which have ceased to grow, and since all bones do not cease growth at the same time, it is evident that the two diseases may coexist in one skeleton.

iv. Rickets in pigs. Special interest was taken by Pedersen (1940) in studying the role of phytin and phytase in the production of rickets in pigs. It was found that 20% of the phytic acid in cereals that contain phytase is split during digestion. The amount of phytic acid which is not split combines with Ca in the proportion of 1 part Ca to 1.1–1.6 parts phytic acid, thus reducing the amount of utilizable Ca. These findings indicate that the requirement for Ca and P in the pig's diet varies with the type of cereal used. In experiments with dogs, Mellanby (1949) subsequently found that phytate, in the presence of vitamin D either in the food or stored in the body, consistently reduced Ca absorption to a greater extent than an equal amount of P given as phosphate. In the absence of vitamin D, on the other hand, phosphate was as effective as phytate in inhibiting the absorption of Ca.

The concentration of sulfur ions affects the absorption of Ca, P, and Mg. Møllgaard (1943) reported that in balance experiments with pigs as little as 0.5 gm of S fed per day reduced the absorption of Ca and P greatly. In a few months, serum Ca and P_i decreased to low levels. If vitamin D was deficient, simultaneously severe rachitic changes occurred. Calcium and phosphorus requirements of animals are presented in Table IX. Rickets caused by vitamin D deficiency in pigs fed diets with various Ca and P content were accompanied by a fall in serum Ca levels. This drop was rapidly followed by a 100–200% rise in the activity of SAP (Baustad *et al.*, 1967). Doige *et al.* (1975), on the other hand, in a similar experiment designed to evaluate the effects of various levels of dietary Ca and P on growth and skeletal development, found significant changes in the levels of serum Ca and P_i to occur when various levels of Ca and P were fed. However, these changes were not pronounced and would be difficult to recognize and interpret in a given clinical situation. When low levels of P were fed in combination with medium or high levels of Ca, levels of serum P_i were significantly reduced. Serum P_i levels were not changed by low levels of dietary P when the Ca/P ratio was maintained near 1.3 : 1. Levels of SAP were not changed significantly by levels or ratios of Ca and P fed. Significant changes were not found even in pigs that had lesions of rickets. Low dietary levels of Ca or P did not result in reduced bone ash, provided that the Ca/P ratio in the ration was maintained near 1.3 : 1.

Hereditary rickets was shown to occur in pigs (Meyer and Plonait, 1968). No difference between normal pigs and pigs having the inherited disorder could be found in blood levels of Ca, P_i, and AP.

iv. Rickets in dogs and cats. Vitamin D, P, and Ca all play a role in the prevention of rickets in dogs (Campbell and Douglas, 1965). In young, rapidly growing puppies a deficiency of vitamin D is a common cause of rickets. Indian workers (Raghavachari,

1943; Patwardhan *et al.*, 1945) found the serum Ca level to decrease progressively during the development of rickets in dogs fed a normal diet devoid of vitamin D. The P_i values decreased slightly. When vitamin D was given, the serum Ca level rose before that of P_i. When a diet low in P was given, the serum Ca level increased and the P_i level decreased. In dogs diets high in phytates adversely influence the absorption of Ca (Hoff-Jørgensen, 1946). In cats similar results were reported (Gershoff *et al.*, 1957).

vi. Rickets in the silver fox. In the silver fox the serum Ca and P levels were found to be normal when rickets was caused by a diet with a Ca/P ratio of 7 : 1 and a very low vitamin D content (Ott and Coombes, 1941). In fox puppies on a similar diet, but low in P, normal serum Ca values were found. Serum P_i concentration, however, decreased to 2–4 mg/dl (0.6–1.3 mmole/liter) (Ender *et al.*, 1949a). On the other hand, when a low-Ca diet with a Ca/P ratio below 0.85 was given, the serum Ca decreased to 6–7 mg/dl (1.5–1.8 mmole/liter). Coincidently increased muscle tonus and tetany were observed in this group.

In foxes fed a ration with a large amount of horsemeat (low Ca, high P) rickets as well as osteodystrophia fibrosa with retarded growth and skeletal deformities were described (Gorham *et al.*, 1970). No blood Ca and P values were given.

vii. Rickets in mink. When mink are fed a diet low in vitamin D and/or with an abnormally low Ca/P ratio (below 1 : 1), they develop rickets (Smith and Barnes, 1941; Bassett *et al.*, 1951). When the diet is deficient in Ca or P, bone development is abnormal. Within 10 days after being placed on a rachitongenic diet, mink kits experience difficulty in walking. Lordosis, bending of leg bones, and enlargements of the costochondral junctions are seen. Ash content of the dry fat-free femurs may decrease to 22–30% compared with 60–64% for normal animals.

2. Osteomalacia

The adult counterpart of rickets is osteomalacia. In the adult animal the growth of the bones has terminated, and therefore the typical lesion of rickets—defective endochondral mineralization of the "zone of provisional calcification" of long bones—cannot occur. Inadequate supply of Ca and/or P and/or vitamin D in the adult animal is manifested by reabsorption of bone already laid down. An excessive mobilization of minerals may be caused by an overfunctioning of the parathyroid and/or a continued body demand for Ca and/or P. Most acute cases occur during pregnancy and lactation, when excessive demands are made upon bones already depleted. Often the depletion develops to a point where the bones break. A characteristic feature of the disease is a negative Ca and P balance. The blood serum concentration may be low in one or both minerals, and tetany may be seen in cases with a low serum Ca.

Although the pathophysiology of osteomalacia is similar in all domestic animals, its etiology varies according to the type of diet used. In bovine and ovine species, it generally occurs as an aphosphorosis, in pigs as an acalcicosis, in dogs as an avitaminosis, and in horses as a hyperphosphorosis.

3. Aphosphorosis

The chronic form of aphosphorosis is best known through the classic work of Theiler and co-workers on "styfziekte" in relation to botulism in South Africa (Theiler *et al.*,

1924, 1927). Since then aphosphoroses have been designated in bovine species by such names as "mjölkhälta" (Svanberg, 1932), "peg-leg" (Turner *et al.*, 1935; Barnes and Jephcott, 1955), "bog-crook" or "bog-lame" (Curran, 1949; O'Donovan and Sheery, 1950; O'Moore, 1950), "cruban" (M. A. Carmichael, 1958, cited in McTaggart, 1959), "stiffs," or "creeps" (Rose, 1954). The chronic form of aphosphorosis in ruminants was reviewed in detail by Theiler and Green (1932). McTaggart (1959) described the syndrome of acute hypophosphatemia in heavily milking cows.

The chief signs of aphosphorosis are bone chewing (allotriophagia) and sudden onset of lameness. Enlargements around the lower end of the metacarpus and metatarsus and a peculiar stiff gait are frequently observed. Loss of body weight and decreased production of milk are usually the first signs to be observed.

Generally it is found that affected animals have very low serum P_i levels, whereas Ca and Mg values are within normal range. The conditions can be reproduced experimentally by feeding animals diets low in P but otherwise adequate. The pastures contain very little P, especially during periods of drought. A high incidence of the disease is frequently associated with such periods. Symptoms may also develop during winter, when animals are being fed a diet low in P (Hutten and Uhlenbruck, 1952). A relative excess of Ca in the diet increases the severity of the disease.

Sheep and horses are not as susceptible to P deficiency as cattle. In New Zealand, osteomalacia in grazing sheep was described as being associated with an anti-vitamin D effect of high carotene levels in green oats, while Ca and P intakes were adequate (Grant, 1955). In the United States low P levels were reported in lame horses (Craige and Gadd, 1941).

4. Osteodystrophia Fibrosa or Osteitis Fibrosa (Nutritional Secondary Hyperparathyroidism)

The nutritional origin of these diseases has gradually been established. It is now generally agreed that osteodystrophia fibrosa or osteitis fibrosa is a result of a secondary hyperparathyroidism. Two types of hyperparathyroidism are known: (1) the primary form, caused by parathyroid neoplasia, and (2) secondary hyperparathyroidism. The latter type has two subtypes, one renal and the other nutritional. In both the compensatory hypertrophy is initiated by hypocalcemia. Quite often the hypocalcemia is found to be secondary to hyperphosphatemia. In animals the nutritional secondary hyperparathyroidism is typically caused by a diet imbalanced in favor of P. The clinical picture of secondary nutritional hyperparathyroidism, however, appears in different animal species so dissimilar that diseases of quite different etiology could easily be expected.

a. Herbivorous Animals. The disease is best known in horses. It was earlier prevalent among army horses fed roughages low in Ca and concentrates high in P. The disease usually manifests in a softening of the bones. In the literature it has been recorded under such names as osteoporosis, osteomalacia, osteitis fibrosa, big head, or "bran" disease (Schmidt, 1940). An identical disease can be produced by feeding an excess of P over Ca. Any Ca/P ratio lower than 0.8 may produce it. The development is rather slow—several months to a year. The disease can be stopped by furnishing an adequate Ca supply, thereby adjusting the Ca/P ratio to 1 or a little above. The development of the disease may be considerably faster if the absolute amount of Ca given is low (Niimi and Kato, 1928; Niimi and Aoki, 1927; Scheunert, 1923).

Affected animals move with a stiff, stilted gait, and often an examination of the head reveals slight bilateral enlargements of the bones of the face or rami of the mandibles. Frequent fractures of the head of the femur have been reported (Hellmich, 1938). Bone analyses reveal a significant decrease in ash content. No change in the Ca/P ratio is found, but a marked decrease in Mg content has been reported. Bardwell (1959) drew attention to the clinical aspects of the disease, especially pointing out that such diseases as navicular disease, ring bones, and spavins could well be local clinical manifestations of a general disease of the skeletal system. So-called mucoid degeneration of the nasal conchae has been classified as an expression of nutritional secondary hyperparathyroidism (Rubarth and Krook, 1968).

In an experiment reported by Krook and Lowe (1964) horses were fed a diet with a Ca/P ratio 1 : 3.6 for 23 weeks, with Ca at an optimal level of 15.8 gm/day. The diet immediately caused hyperphosphatemia, and weekly determinations showed that hyperphosphatemia reached a maximum after 12–20 weeks. Serum Ca dropped to a minimum after 10 weeks and was partly or almost completely compensated after 23 weeks. Clinical symptoms were observed after 12 weeks.

Estimations of serum Ca, P_i, and AP levels are of no great diagnostic value during nutritional secondary hyperparathyroidism (NSH). Estimations of the low urinary Ca and high urinary P excretion during NSH, on the other hand, have been reported to be of greater diagnostic value (Joyce *et al.*, 1971). Calcium infusion tests have also been suggested as diagnostic tools (Argenzio *et al.*, 1974).

In Australia, cases of osteodystrophia fibrosa or NSH were reported to occur in horses grazing pasture grass consisting of *Setaria sphacelata,* which contains a large amount of oxalate (Groenendyk and Seawright, 1974). The Ca/P ratio was about 1 : 1. As no alternative source of fodder was available to the horses, it has been suggested that the comparatively large amount of oxalate in the diet was able to render so much Ca unavailable that this could lead to the bone disorder. The serum Ca level was reported to be normal. The diagnosis was verified by microscopic examination of a bone biopsy from the swollen maxilla.

Osteodystrophia fibrosa has been described in goats (Groenewald *et al.*, 1940; Deckwer, 1950; Hansen *et al.*, 1966). A similar condition with a striking depletion of the bones was produced experimentally in dairy cows by continuously feeding a diet low in Ca and relatively high in P (Becker *et al.*, 1933).

b. Omnivorous Animals. In the swine the effect of imbalanced Ca/P ratios was studied by Marek and Wellmann (1931). With low-Ca and high-P diets, hyperostotic osteitis fibrosa was induced. Diets with Ca/P ratios from 1 : 5.5 to 1 : 11.6 (Liégeois and Derivaux, 1951) produced hyperphosphatemia followed by hypocalcemia in pigs. At necropsy the parathyroids were found to be diffusely hyperplastic. Finally, Brown *et al.* (1966) experimentally induced NSH in pigs and found the morphological and nutritional changes to be identical with those found in spontaneous cases of atrophic rhinitis. The classification of atrophic rhinitis in pigs as an infectious disease, a nutritional disease, or a combination of both, however, has not yet been clarified (Baustad *et al.*, 1967).

Work by Horváth *et al.* (1972; Horváth and Papp, 1972) has indicated that a faulty supply of Ca and P cannot be regarded as a main etiological factor in atrophic rhinitis. On the other hand, they pointed out that determinations of Ca in tail vertebrae from atrophic rhinitic pigs and healthy, identically managed contemporaries showed that atrophic rhini-

tic pigs incorporated less Ca into tail vertebrae than healthy controls. Thus, a diagnostic value of determinations of Ca in tail vertebrae was suggested.

c. Carnivorous Animals. In carnivorous animals the source of imbalanced dietary Ca and P is unsupplemented meat without concomitant access to the bones (Riser, 1961). This causes a disease in young animals known as osteogenesis imperfecta, juvenile osteoporosis, or paper bone disease. The disease is characterized by extreme fragility of the whole skeleton. Fractures occur for no apparent reason and seem to cause little pain. The disease NSH is more pronounced in carnivorous animals than in herbivores. It is a disease of young animals and usually found superimposed on rickets. Enlargements of facial bones are less apparent than in other animals.

The disease was reproduced in young kittens placed on a minced heart diet. Roentgenological signs of generalized osteitis fibrosa developed within 3 weeks, and folding fractures of the long bones developed a few weeks later. Due to the extremely rapid resorption of bone there apparently is not time for fibrous replacement of the resorbed bone, and hence a hypostotic osteitis fibrosa is the result (Krook *et al.*, 1963).

Continuous blood analysis during experimentally induced NSH has not been reported (Krook, 1965). Determinations on single samples drawn during experiments have shown mild hypocalcemia and hyperphosphatemia. However, such determinations are not conclusive in NSH.

When the dietary imbalance is a deficiency of Ca or vitamin D with adequate P, patients have either a mild hypocalcemia [8.0–9.5 mg/dl (2.0–2.4 mmole/liter)] or isocalcemia and normal or slightly reduced serum P_i levels. If the diet contains excessive P, blood P_i concentration may either be elevated or within normal range. Increased SAP activity may be found in animals with overt bone disease.

5. Nutritional Secondary Hypercalcitonism

Osteopetrosis, with ankylosing spondylosis deformance and "degenerative osteoarthrosis," is an exceedingly common manifestation in the bull. The cause of this syndrome has been ascribed to a hypercalcitonism (Krook *et al.*, 1969, 1971; Schiefer, 1970). The daily amounts of Ca and P fed to bulls are often from 3.5 to 5.8 times higher than recommendations. To prevent hypercalcemia, a calcitonin defense mechanism is brought into action, resulting in a blocking of normal resorption of bone. Such a long-standing (several years), monotonous, excessive dietary Ca intake leads to a state of chronic hypercalcitonism with a persistent mild hypocalcemia, arrested bone resorption, and osteopetrosis.

V. MAGNESIUM METABOLISM

Sheep and cattle are the only domestic animals known to be subject to clinical disorders pathophysiologically compatible with Mg deficiency. Magnesium metabolism therefore has been studied most extensively in these species.

A. Distribution of Magnesium in the Body

In terms of amount, Mg is fourth among cations of the body, being surpassed only by calcium, sodium, and potassium. In calves the Mg content of the body is associated with the body weight according to the following equation (Blaxter and McGill, 1956):

$$\text{Mg (gm)} = 0.655 \text{ weight (kg)} - 3.5$$

Magnesium is present in all tissues. About 70% of the total body Mg is located in the skeleton, and approximately one-third of this is available for mobilization to soft tissues when dietary intake is inadequate. Taylor (1959) demonstrated that Mg present in bone exists in at least two different forms. About 70% of the bone Mg could be removed relatively easily with dilute acid, while the remaining 30% was more intimately bound. Taylor therefore suggested that the easily removable Mg is bound to the surface of the bone crystals, probably to the phosphate entity of the apatite crystal, whereas the more firmly bound Mg may be an integral part of the bone crystal lattice.

The Mg of the skeleton acts as a labile source of Mg, but there is a striking difference between the reactions of the young and those of the adult bovine (Blaxter and McGill, 1956). In the immature animal, the entire skeleton may lose 30–60% of its Mg content in exchange reactions, whereas the adult, due to its inability to mobilize Mg from the skeleton, dies in tetany with little or no depletion of its bone Mg (Cunningham, 1936a,b). The existence of this difference between the young and the adult has been confirmed by ^{28}Mg disappearance studies in the bovine (Simesen *et al.*, 1965). In cows the total exchangeable Mg was 20–24% of the total body Mg compared to 30–45% in calves.

Apart from bone, Mg occurs principally intracellularly. The concentration of Mg in cells is about 36 mg/dl (15 mmole/liter) compared with a plasma concentration of about 2.4 mg/dl (1.0 mmole/liter). The mechanism maintaining this concentration gradient of about 1 : 15 across the cell membrane has been the subject of much study (Rogers and Mahan, 1959; MacIntyre, 1959; Rogers *et al.*, 1964). It is now generally held that tissue Mg exists in at least two different forms: one that is free, ionic, and readily exchangeable, and one that is bound and chelated, typically with ATP and the apoenzyme proteins to which Mg^{2+} is attached (Rogers, 1965).

It has been suggested (Stevenson and Wilson, 1963) that the labile, exchangeable form is present in a concentration which is practically identical with the Mg^{2+} concentration in the extracellular fluid. A simple diffusion equilibrium probably exists between these two, while a more complex relation prevails between the two intracellular forms.

The physiological functions of Mg may conveniently be divided into two categories: intracellular and extracellular.

1. Intracellular Function of Magnesium

Magnesium is an activator of numerous enzymes, such as phosphatases and the enzymes catalyzing reactions involving ATP. Since ATP is required in such diverse functions as muscle contraction, synthesis of protein, fat, nucleic acids, and coenzymes, glucose synthesis and utilization, methyl group transfer, sulfate, acetate, and formate activation, and oxidative phosphorylation, it may be inferred that the action of Mg extends to all the major anabolic and catabolic processes involving the main metabolites. Magnesium is an activator for enzymes that require thiamine pyrophosphate as a cofactor. One of them is active in the conversion of pyruvic acid to acetyl-CoA, an example of oxidative decarboxylation. Excellent reviews giving detailed descriptions of the role of Mg in biochemical processes (Aikawa, 1963, 1971) have appeared.

2. Extracellular Function of Magnesium

In the extracellular fluid Mg has an important role in the production and decomposition of acetylcholine, the substance necessary for the transmission of impulses at the

neuromuscular junction (Del Castillo and Engbäk, 1953). A low concentration of Mg, or more particularly a low Mg^{2+}/Ca^{2+} ratio, potentiates the release of acetylcholine. It therefore appears possible that a low concentration of Mg in the extracellular fluid surrounding the muscle end plates may lead to tetany through this mechanism (Blaxter *et al.*, 1954). It is known that cholinesterase activity is unaffected during Mg deficiency (Seekles and Van Asperen, 1949; Todd and Rankin, 1959).

During the hibernation of hedgehogs the serum Mg level was found to increase from a normal of 3 to about 6 mg/dl (1.2–2.4 mmole/liter) (Suomalainen, 1944). Serum Ca concentration remained constant.

B. Absorption and Excretion

The availability of dietary Mg is low compared with that of dietary Ca. The apparent digestibility of Mg (the percentage of dietary Mg which is not excreted in the feces) is 20–25% in ruminants, compared with an average of 39% in monogastric animals (Grönning, 1959). Smith (1959a) found that calves fed a diet consisting basically of whole milk retained 39–54% of their dietary Mg at ages of less than 5 weeks. From about 3 weeks to about 16 weeks of age the mean fecal excretion of Mg increased from 32 to 86% of the dietary Mg (Smith, 1959b). For cattle on a variety of typical winter stall rations, Rook *et al.* (1958) found 62–92% of the Mg fed to be excreted in the feces, compared with 82–83% when fed freshly cut herbage at stall. In pigs 3–5 weeks of age, the average Mg excreted in the feces was 65–84% of the dietary Mg (Bartley *et al.*, 1961). In horses fed a variety of feedstuffs the average apparent digestibility was estimated to be 49.5 ± 10.9% (Hintz and Schryver, 1972).

1. Endogenous Magnesium

The magnesium in the feces is not solely unabsorbed dietary material. The large volumes of digestive secretions produced during digestion contain a considerable amount of endogenous Mg. Knowledge of the amount of this endogenous fecal magnesium is important for determination of the "true" digestibility or availability of dietary Mg. The apparent digestibility,

$$\frac{\text{Food Mg} - \text{fecal Mg}}{\text{Food Mg}}$$

is a valid basis for estimating the "true" digestibility only when the endogenous Mg in the feces is a small part of the total fecal Mg. An increased fecal excretion of Mg may be due either to decreased absorption or to increased production of endogenous Mg. Attempts have been made to determine the endogenous Mg in sheep using ^{28}Mg and methods originally developed for ^{32}P (Visek *et al.*, 1953) and ^{45}Ca (Hansard *et al.*, 1954; Field, 1959; MacDonald *et al.*, 1959), but Field (1961) has questioned the validity of these methods, as he found both the comparative balance technique and the isotope dilution techniques to overestimate the amount of endogenous fecal Mg.

Blaxter and Rook (1954) calculated the total endogenous loss of Mg in milk-fed calves from the relationship between Mg retention and intake. Since the loss via urine is negligible, the above value may be taken as the endogenous fecal Mg (3–4 mg/kg body weight per day). Smith (1959a) estimated the endogenous fecal Mg to be about 0.5 mg/kg body weight per day in milk-fed calves 2–5 weeks of age. This value increased to 2.2 mg/kg body weight per day in calves 26–32 weeks of age. For cows Blaxter and McGill (1956)

reported a figure of 3–5 mg/kg body weight per day. Simesen *et al.* (1962) measured the endogenous Mg in milk-fed calves and in cows fed hay and cereals using intravenous injection of ^{28}Mg. Mean values of 3.5 and 1.5 mg/kg body weight were found for calves and cows, respectively. These results were in agreement with those reported by Blaxter and McGill (1956) for calves but considerably lower than those for cows.

In sheep, endogenous fecal Mg has been estimated by Field *et al.* (1958), MacDonald *et al.* (1959), Care (1960), Hjerpe (1968a), Chicco *et al.* (1972), and Meyer *et al.* (1973). Values from 0.7 to 5.1 mg/kg body weight have been reported.

The amount of endogenous fecal Mg may be increased by a greater flow of saliva stimulated by bulky diets such as roughages and grass (Rook and Storry, 1962). As pointed out by Care (1967), the endogenous fecal excretion of Mg in ruminants is of substantial magnitude. This magnitude was not affected by altering the levels of Na and K in herbage (Powley *et al.*, 1977).

In horses the endogenous fecal Mg excretion has been calculated to be 1.8 mg/kg and 2.2 mg/kg body weight (Hintz and Schryver, 1972, 1973).

2. *Absorption*

Until about 1 month of age, calves absorb Mg via the large and small intestine (Smith, 1959c, 1962). With increasing age, this ability is lost. From about 3–4 months of age the principal site of absorption appears to be the middle third of the small intestine (Field, 1961; Care and Van'T Klooster, 1965). The absorptive efficiency of Mg decreases with increasing Mg concentration when concomitant plasma Mg concentration is normal. It has been shown that Mg and Ca are absorbed as freely diffusing ions (Van'T Klooster, 1967).

From earlier studies on Mg absorption from the digestive tract of ruminants, it was concluded that the intestinal region, particularly the small intestine, was the most important site of Mg absorption (Phillipson and Storry, 1965; Care and Van'T Klooster, 1965; Scott, 1965). However, more recent reports have suggested that net absorption of Mg occurs from the stomach region of cattle (Rogers and Van'T Klooster, 1969) and sheep (Pfeffer *et al.*, 1970; Grace and Macrae, 1972; Axford *et al.*, 1975). Martens *et al.* (1976) demonstrated a significant uptake of Mg by isolated rumen epithelium (*in vitro*), and simultaneously observations were made *in vivo* by Tomas and Potter (1976), who showed conclusively that the rumen is the main site of Mg absorption. A hypothesis that Mg is translocated across the rumen epithelium by an active transport process has been proposed (Martens *et al.*, 1978). Care and Van'T Klooster (1965) and Scott (1965) calculated the conditions for net entry of Mg across the rumen epithelium on the ground that the transfer was passive and reached the conclusion that the Mg ion concentration gradient between lumen and blood was too small to account for net absorption from the rumen.

In ruminants the concentration of Mg ions in the digesta in the small intestine tends to be particularly low because of (1) the presence of materials in the digesta able to bind Mg^{2+} at the intestinal pH (Storry, 1961b; Smith and McAllan, 1966) and (2) the large amount of water which is retained in the ileum (Smith, 1969).

Unlike that of Ca, the absorption of Mg is not influenced by vitamin D (Smith, 1957, 1958). Despite the disparity between the effects of vitamin D on Ca and Mg absorption, a common transport system for absorption of Ca and Mg has been postulated (Alcock and MacIntyre, 1960). Smith failed to find evidence for such a common mechanism for absorption of Ca and Mg in milk-fed calves, but Allcroft and Ivins (1964) found that

further addition of Ca to the diet of milk-fed calves caused a significant depression of serum Mg and bone Mg. Care and Van'T Klooster (1965) showed that Ca interfered with Mg absorption, and vice versa, and suggested that Mg and Ca may be absorbed from the ileum by a process of facilitated diffusion, whereas Ca absorption from the duodenum was thought to be an active process sensitive to vitamin D (Care, 1967).

It has been shown that the chelating agent EDTA, which is known to bind Ca in preference to Mg, inhibits the absorption of Ca from the small intestine of sheep but promotes the absorption of Mg from the proximal third of this region (Van'T Klooster and Care, 1966).

The estimates of availability of dietary Mg for cattle of different ages have been given as 70% up to 5 weeks, 40% from 5 weeks to 5 months, and 20% for animals over 5 months of age (Agricultural Research Council, 1965). It has been shown, however, that the availability of Mg varies considerably and may be very low in some types of feed given to cattle under normal farm conditions (Rook and Campling, 1962). In general, Mg availability is higher for young animals than for old animals. Even in cows on the same diet, however, great individual variation exists.

In horses the primary site of Mg absorption also was found to be the small intestine (Hintz and Schryver, 1972). Absorption of Mg decreased in diets containing high levels of P.

3. Excretion

Magnesium is excreted mainly via three routes: (1) the gastrointestinal tract, (2) the kidney, and (3) the mammary gland during lactation.

a. Gastrointestinal Tract. As already mentioned, the main part of Mg leaving the body appears in the feces. The fecal Mg includes endogenous fecal Mg, the amount of which is relatively higher for ruminants than for monogastric animals. After parenteral injection of ^{28}Mg in man, less than 1% of the injected dose was recovered in feces compared with about 20% in the sheep (Field, 1959; MacDonald *et al.*, 1959). Field (1960) compiled data showing that the daily excretion of Mg into the gastrointestinal tract of sheep is of the order of 60–200 mg Mg, or about the value found for endogenous Mg (Field *et al.*, 1958; MacDonald *et al.*, 1959; Field, 1960).

Estimation of the availability of dietary Mg by use of the ratio of urinary to dietary Mg has been suggested (Field *et al.*, 1958). Such estimations, however, assume constant endogenous fecal Mg, and, even so, the figures obtained are of low accuracy.

b. Kidney. Magnesium absorbed in excess of the body's requirements is excreted by the kidney. Wilson (1960) critically reviewed existing knowledge of renal Mg excretion and concluded "that excretion of Mg is by a filter reabsorption mechanism in which the tubular reabsorption process is acting at or near its maximum rate, and that the excretion of Mg is partly or wholly independent of other ions." Maximal tubular reabsorption rate is usually taken to be a physiological constant and in conjunction with the filtration rate fixes the threshold concentration in the plasma.

Rook *et al.* (1958) established this correlation between serum concentration and urine output of Mg and estimated the renal threshold for Mg to be less than 2.15 mg/dl (0.9 mmole/liter). Later estimates (Storry and Rook, 1962; Rook and Storry, 1962; Meyer, 1963) showed the renal threshold to be about 1.80–1.90 mg/dl (0.7–0.8 mmole/liter) in

TABLE X

Representative Values for the Calcium, Magnesium, and Total Phosphorus Content of Milk

Species	Calcium (gm/liter)	Magnesium (gm/liter)	Total P (gm/liter)
Cow[a]	1.25[b]	0.12[b]	0.96[b]
Sheep	1.93[c]	—	1.00[d]
Goat	1.30[b]	0.16[b]	1.06[b]
Horse	1.02[e]	0.09[e]	0.63[e]
Pig	2.10[f]	—	1.50[f]

[a] Colostrum-milk is usually not considered normal until about the fifth day after calving (Turner, 1930). The content of calcium, magnesium, and phosphorus is high in colostrum, but, as the milk becomes normal, a rapid decline toward a fairly constant level soon sets in (Garrett and Overman, 1940).

[b] Macy *et al.* (1953).

[c] Barnicoat *et al.* (1949).

[d] Basu and Mukherjee (1943).

[e] Holmes *et al.* (1947).

[f] Gregory *et al.* (1952).

the bovine. In two sheep L'Estrange and Axford (1964b) obtained threshold values of 1.37 and 1.90 mg/dl (0.6 and 0.8 mmole/liter). Practically, this means that, if urine Mg is present, no hypomagnesemia exists. Indicator tests taking advantage of this fact have been developed (Halse, 1976; Simesen, 1977b). Thus, theoretically, the kidney plays a significant role in Mg homeostasis.

c. Mammary Gland. The mineral constituents of milk are given in Table X. It appears from the table that a heavily lactating cow may lose about 3 gm Mg per day via this route. This represents a large proportion of the dietary Mg absorbed from the gut; therefore, any factor affecting either the rate of secretion or the concentration of Mg in the milk will modify the requirement for Mg. Robertson *et al.* (1960) and Rook and Storry (1962) provided evidence indicating that no significant fall in the Mg concentration of milk occurs when intake of feed or of Mg is reduced or if hypomagnesemia develops.

About 20% of the Mg in cow's milk is in the ionic form (Van Kreveld and Van Minnen, 1955), 30% is associated with the colloids, and the remaining 50% exists in unknown forms (Alexander and Ford, 1957).

VI. SERUM MAGNESIUM

The method of choice for Mg estimation in biological materials is atomic absorption spectrophotometry. Significant differences have been shown among dye-binding methods (Alcock and MacIntyre, 1966; Teears *et al.*, 1977).

Depending on the species, blood serum normally contains 2–5 mg Mg per deciliter (0.8–2.1 mmole/liter) (Table XI). In animals other than the ox, Mg occurs at a higher concentration in the red blood cells than in plasma (Salt, 1950). There is apparently no exchange of Mg between plasma and RBC in the peripheral circulation (Aikawa, 1963). About 79% of the serum Mg has been found to be an ultrafiltrable fraction independent of the total amount present (Smith, 1957).

TABLE XI

Concentration of Magnesium in Blood of Various Animals

Species	Blood fraction[a]	No. of animals sampled	Concentration (mg/dl) (mean ± 2 SD)	Concentration (mmole/liter)[b] (mean ± 2 SD)	Reference
Shetland pony	S	8	1.5 ± 0.3	0.6 ± 0.1	Eriksen and Simesen (1970)
Horse	S	30	2.5 ± 0.3	1.0 ± 0.1	Jennings and Mulligan (1953)
	S	24	1.9 ± 0.3	0.8 ± 0.1	Simesen (1972)
Cattle	S	90	2.3 ± 0.4	1.0 ± 0.2	Mylrea and Bayfield (1968)
	B	10	2.4 ± 0.3[c]	1.0 ± 0.1	Eveleth (1937)
	P	10	2.8 ± 0.3[c]	1.2 ± 0.1	Eveleth (1937)
	C	10	1.8 ± 0.8[c]	0.7 ± 0.3	Eveleth (1937)
Sheep	S	12	2.5 ± 0.3	1.0 ± 0.1	White *et al.* (1957)
	S	67	2.4 ± 0.3	1.0 ± 0.1	Simesen (unpublished)
Goat	B	3	3.7 ± 0.7[c]	1.5 ± 0.3	Eveleth (1937)
	P	3	3.2 ± 0.4[c]	1.3 ± 0.1	Eveleth (1937)
	C	3	4.5 ± 1.2[c]	1.9 ± 0.5	Eveleth (1937)
Pig	B	10	6.4 ± 0.8[c]	2.6 ± 0.3	Eveleth (1937)
	P	10	3.2 ± 0.5[c]	1.3 ± 0.2	Eveleth (1937)
	C	10	10.5 ± 1.5[c]	4.3 ± 0.6	Eveleth (1937)
Rabbit	B	7	5.4 ± 0.7[c]	2.2 ± 0.3	Eveleth (1937)
	P	7	3.2 ± 0.6[c]	1.3 ± 0.2	Eveleth (1937)
	C	7	9.4 ± 2.6[c]	3.9 ± 1.1	Eveleth (1937)
Cat	S	—	2.2	0.9	Kaneko (1972)
Dog	S[d]	10	2.1 ± 0.3	0.9 ± 0.1	Eichelberger and McLean (1942)

[a] B, whole blood; P, plasma; S, serum; C, cells.
[b] Internationally recommended unit.
[c] Indicates that standard deviation was estimated from range and number of observations.
[d] Fat-free serum.

A. Regulation of Serum Magnesium

Little is known regarding the factors involved in the regulation of the serum Mg content of the blood. There is in some respects a reciprocal relationship between Mg and Ca in the serum; e.g., in oxalate poisoning the decrease in serum Ca is accompanied by an increase in serum Mg (Hallgren *et al.*, 1959). During hypomagnesemia it has clearly been demonstrated that the regulation of serum Mg is critically dependent on the daily Mg intake. In contrast to Ca, no single endocrine gland has been shown to exert a primary regulatory effect on plasma Mg concentration. Three endocrine glands—the adrenal, the thyroids; and the parathyroids—appear to be involved.

About 25 years ago Blaxter and McGill (1956) pointed out that there was no evidence from critical experimentation indicating that the endocrine glands had any specific effect on Mg metabolism. They therefore suggested that the Mg metabolism was regulated by a dynamic equilibrium in which the skeleton acted as a labile source of Mg. Soft-tissue Mg was accepted as being of vital importance and therefore not available for Mg homeostasis. As a first approximation the Mg homeostatis was considered a result of a balance between intake from intestine and renal excretion. In an immature animal the entire skeleton acted

as a labile source of minerals, whereas in an adult a very large part of the skeleton was inert.

1. Adrenal Glands

In man, hyperaldosteronism is associated with a negative Mg balance and hypomagnesemia (Mader and Iseri, 1955). Studies in rats demonstrated a dose-associated function of aldosterone in regard to increased excretion of Mg in urine and feces (Hanna and MacIntyre, 1960). Similar findings were obtained with sheep (Oyaert, 1962; Care and Ross, 1963; Scott and Dobson, 1965).

In adrenal insufficiency, the opposite effect occurs on Mg metabolsm. Both in adrenalectomized laboratory animals and in patients with Addison's disease, serum Mg concentration is increased (Wacker and Vallee, 1958). The effects of mineralocorticoids on Mg homeostatis, however, appear to be secondary to their more important influences on K and Na homeostasis.In contrast to these two ions, no influence of Mg concentration on aldosterone secretion could be detected (Care and McDonald, 1963). Keynes and Care (1967) concluded after a reinvestigation of the possible role of mineralocorticoids in Ca and Mg homeostasis in sheep from which the parathyroids, thyroids, and adrenals had all been removed that adrenal steroids may contribute to Ca and Mg homeostasis in sheep, but their role in the intact animal is subordinate to that of the thyroid and parathyroid hormones.

2. Thyroid Glands

In man, thyrotoxicosis is associated with hypomagnesemia and negative Mg balance, whereas in myxedema the serum concentration tends to be elevated and the balance is positive (Wacker and Parisi, 1968).

Allcroft (1947a) suspected some association between the level of serum Mg and the degree of activity of the thyroid gland. Swan and Jamieson (1956) demonstrated that feeding of thyroprotein to lactating cows led to a decrease in serum Mg. In growing rats Vitale *et al.* (1957) found that the depression of growth rate and the hypomagnesemia, which results from thyroxine feeding, could be partly overcome by supplementing the diet with large amounts of Mg. In balance experiments with calves Meyer and Schmidt (1958) found that parenteral administration of thyroxine caused a transient hypomagnesemia brought about by an increased excretion of Mg in both urine and feces. Thus, most evidence suggests that increased thyroid activity tends to depress the plasma Mg concentration. Hypersecretion of thyroxine, however, does not appear to be one of the primary etiological factors in hypomagnesemic tetany (Todd and Thompson, 1962). Calcitonin, the hormone secreted by the thyroid light cells, has very little or no effect on plasma Mg concentration (Care, 1967).

3. Parathyroid Glands

MacIntyre *et al.* (1963) suggested a homeostatis mechanism for Mg centered around the parathyroid glands. This hypothesis, which is favored from a substantial amount of evidence, proposes that elevation of plasma Mg leads to inhibition of parathyroid secretion, whereas hypomagnesemia stimulates release of PTH. From observations of parathyroid-dependent hypercalcemia it was inferred that hypomagnesemia in Mg-deficient rats stimulated PTH secretion (Heaton, 1965; Gitelman *et al.*, 1968). The

hypothesis was examined and confirmed in experiments in which the PTH concentration in plasma was measured directly by radioimmunoassay (Buckle *et al.*, 1968).

The inverse relationship between Mg concentration and parathyroid gland activity is similar to that between the plasma concentrations of Ca and that of PTH in peripheral plasma of cows (Sherwood *et al.*, 1966). Since PTH exerts a prominent effect on the concentration of Ca in plasma relative to that on Mg, it seems likely that the concentration of Ca in plasma exerts the major physiological control of parathyroid gland function. The PTH release, however, can be specifically influenced by the concentration of Mg in plasma (Rayssiguier *et al.*, 1977).

VII. DISTURBANCES OF MAGNESIUM METABOLISM

Only cattle and sheep are known to be subject to clinical disorders apparently due to Mg deficiency. According to H. H. Green (1948, cited in Allcroft, 1954), the disease can be grossly classified into two types: a rapidly developing and a slowly developing type. In calves and beef cattle the disease is generally of the slow type, whereas in milking cattle and sheep the onset in the majority of cases is rapid. Usually, but not invariably, the occurrence coincides with the first flush grass in the spring.

The fall in serum Mg may take place in a day or two, as seen in the acute type, or the decline may be more gradual, as seen in "outwintered" cattle and stall-fed cattle on Mg poor diets. The depletion of the Mg ion concentration in the extracellular fluid, the fluid bathing neuromuscular junctions and synapses, apparently is responsible for the tetanic syndrome. The main symptoms are restlessness, twitching of the muscles, excitement, staggering gait, increased sensitivity to strange noises, and finally convulsions and death. The onset of symptoms, however, cannot be correlated directly with the concentration of Mg in serum.

Since the very first report assigning this clinical picture to hypomagnesemia, the role of Ca has been discussed (Huffman and Robinson, 1926; Duncan *et al.*, 1935). Duckworth (1939) stated that a "principal effect of Mg deficiency appears to be disturbance of normal Ca metabolism," and Allcroft (1947a) reported that 75% of 406 cows with hypomagnesemia also showed hypocalcemia.

A. Hypomagnesemia in Calves

This syndrome has been intimately associated with the feeding of whole milk for an extended period of time. The subject has been reviewed by Russell (1944), Blaxter and McGill (1956), and Smith (1964).

McCandlish (1923) suggested that the nutritive failure of calves given whole milk for very long periods was related to their metabolism of Mg. Duncan *et al.* (1935) subsequently demonstrated that the Mg concentration of the blood serum of calves declined when they were given rations of whole milk supplemented with iron, copper, manganese, and cod liver oil. Blood Ca and P levels were reported to be within the normal range. The hypomagnesemic nature of some naturally occurring tetanies, however, was not fully recognized until the work of Blaxter and his associates in the early 1950's.

In a series of studies, it was pointed out that the hypomagnesemia in calves is a simple dietary deficiency of Mg (Blaxter and Rook, 1954). Furthermore, it was demonstrated that

the pathological calcification of the endocardium and total vascular system observed in the so-called milk syndrome (Moore *et al.*, 1936, 1938) was not associated with genuine Mg deficiency in calves. The calves were fed an artificial milk-substitute diet containing 0.5 mg Mg per deciliter (0.2 mmole/liter). To this diet was added Mg, so different animals received from 0.5 to 19.0 mg Mg per deciliter (0.2–7.8 mmole/liter). After 6–7 weeks on the experimental diet, three calves showed clinical symptoms of Mg deficiency and died in convulsions. Two of the calves that died had received only the basal diet and the third 1.64 mg Mg per deciliter (0.7 mmole/liter). The concentration of serum Mg had fallen to about 0.7 mg/dl (0.3 mmole/liter) when tetany appeared and to 0.5 mg/dl (0.2 mmole/liter) at death. One calf received a diet containing 5.8 mg Mg per deciliter (2.4 mmole/liter) for about 10 weeks and showed no clinical sign of deficiency, but serum Mg concentration steadily declined to 1.0 mg/dl (0.4 mmole/liter). Calves which received diets containing 12.5 or 19.0 mg Mg per deciliter (5.1 or 7.8 mmole/liter, respectively) were clinically normal, and serum Mg concentration remained between 2.0 and 2.5 mg/dl (0.8–1.0 mmole/liter).

No effect was noted on serum Ca and P_i concentration during tetany, and no depletion of soft-tissue magnesium was found. During the 6 or 7 weeks on the low-Mg diet before death occurred, each calf lost about 2 gm Mg, whereas normal animals gained about 7 gm during the same time. The fastest-growing calves usually showed the greatest depression of serum Mg concentration (Blaxter and Sharman, 1955). One of the most characteristic symptoms observed was constant movement and flapping of the ears (Blaxter and Sharman, 1955; Parr, 1957).

The Mg requirement for a growing calf was estimated to be 16–18 mg/dl (6.6–7.4 mmole/liter), a much lower requirement than suggested by Huffman *et al.* (1941) but still considerably above what can be met via ingestion of cow's milk (Table X). Although applicable to some calves between 8 and 14 weeks, the figures of Blaxter and Rook are usually too high for younger calves and too low for older calves, which may develop hypomagnesemia on diets containing these and higher levels of Mg (Smith, 1957).

1. Bone–Plasma Relationship

As mentioned, bone Mg represents a store which can be called upon under conditions of deficiency. A study of the Mg status of the skeleton during deficiency was made by Smith (1959b). In normal calves the Mg content of the bone ash is about 0.80%. When plasma Mg falls to about 1.6 mg/dl (0.7 mmole/liter), the bone ash contains about 0.60–0.67% and at plasma levels of 0.7 mg/dl (0.3 mmole/liter) the bone ash contains 0.40–0.48% of Mg. During depletion plasma Mg decreases only slowly after it has dropped to 0.7 mg/dl (0.3 mmole/liter), and further depletion of bone Mg is associated with only a slight decrease in the plasma concentration. The bone ash content of Mg decreases with increasing age (Meyer, 1963). In adult cattle, bone ash contains 0.55–0.70% of Mg.

When hypomagnesemia is evident, it may take several weeks of oral Mg supplementation to bring the level back to normal. Repeated subcutaneous injections of 1 gm Mg per day (given as the sulfate) can restore blood as well as bone ash Mg to normal levels, at least in moderately deficient calves (Smith, 1959b).

2. Bone Sample and Its Diagnostic Value

During the final stages of convulsive attacks, the serum Mg concentration is often increased, presumably due to the release of Mg into the blood by rapidly contracting

muscles (British Veterinary Association, 1957). Therefore, a method to assess bone Mg in the living animal as well as postmortem became desirable (Blaxter and Sharman, 1955). For this purpose rib biopsy was used (Thomas and Okamoto, 1958). Later Smith (1959b) suggested the use of vertebrae taken from the tail of the living animal. This method provides a satisfactory way of assessing bone composition and has obvious advantages over rib biopsy. In comparing ash analysis from different bones, Smith's findings are not in agreement with those of Blaxter and McGill (1956). However, the conclusions were based on findings in different bones. Differences might exist between bones at a stage of moderate depletion, and it is possible that bones losing Mg slowly may catch up with bones losing Mg rapidly during severe Mg depletion.

The process of magnesium depletion of the bone apparently takes place as an exchange process in which Mg is replaced by Ca on the bone crystal surface (Blaxter, 1956). Therefore, a diagnostic procedure estimating the Ca/Mg ratio as suggested by Blaxter and Sharman (1955) is clearly preferable under practical field conditions.

3. Balance Experiments

Smith (1958) reported the mean fecal excretion of Mg to be about 32% of the dietary intake at 3 weeks of age compared with about 86% at about 16 weeks of age. Thus, with increasing age the ability to absorb Mg decreases significantly. However, considerable variations among calves were found. In general, the decrease reflects the onset of hypomagnesemia and may thus explain the inexplicable differences in susceptibility to hypomagnesemia found among groups of calves. For example, despite closely similar treatment, the calves of Knoop *et al.* (1939) did not show hypomagnesemia until about 4 months of age, whereas those of Parr and Allcroft (1957) showed hypomagnesemia in 3–4 weeks.

It is not known whether the decreasing ability to utilize dietary Mg is due to a decrease in absorption, an increase in endogenous fecal excretion, or both. Neither vitamin D nor irradiation with ultraviolet light had any effect on this decrease. On the other hand, a marked decrease in Ca retention was also shown. However, this decrease could be prevented by vitamin D, provided that the vitamin D intake was increased beyond that known to be adequate for calves (5–10 IU/kg body weight daily).

4. Vitamin D and Serum Calcium and Magnesium

The convulsions in Mg-deficient calves resemble convulsions due to low Ca levels, and the reports of Parr (1957) and Smith (1958) indicate a constant relationship between convulsions and hypocalcemia.

It has been shown that the addition of vitamin D (5–10 IU/kg body weight daily) to the milk does not prevent hypomagnesemia (Duncan *et al.*, 1935; Huffman *et al.*, 1941; Parr and Allcroft, 1957). Smith (1957), on the other hand, found that, in calves not given vitamin D, the development of hypomagnesemia was followed by a decrease in plasma Ca. The declines began at about the same time and developed at about the same rate. If vitamin D (7000–70,000 IU) or ultraviolet irradiation was given, the blood Ca rapidly returned to normal. Smith (1957) reported a very interesting difference in the clinical picture in hypomagnesemic–normocalcemic calves versus hypomagnesemic and hypocalcemic calves. During the period when the calves were hypocalcemic and moderately hypomagnesemic [1.4–1.5 mg/dl (0.58–0.62 mmole/liter)], they would suddenly collapse and remain almost motionless with their necks and legs stiff for periods varying from a

few minutes to half an hour. Calves having "pure" hypomagnesemia (the calves given ample vitamin D supplement) never started to show clinical signs before the plasma Mg concentration had dropped to below 1 mg/dl (0.4 mmole/liter). The initial signs were nervousness, twitching ears, and staring eyes. Later, the calves were staggering and unable to stand, but convulsions were never observed.

It was shown that plasma CPK activities are considerably elevated for about 24 hours after an acute phase of hypomagnesemic tetany in calves (Todd *et al.*, 1969). After commencement of Mg therapy, the CPK activities decreased rapidly. The CPK activity was found to be closely related to the tetanic syndrome and not to hypomagnesemia per se. The diagnostic value of serum CPK is not yet known. Elevation of CPK activity has also been recorded in calves with muscular dystrophy (Todd and Thompson, 1968).

B. Hypomagnesemia in Adult Cattle

1. Slow Type

A number of reports have appeared concerning a type of hypomagnesemia in which animals exhibit low serum Mg levels for some time, often for months. When serum Mg approaches critically low values, it is generally accompanied by mild clinical symptoms such as nervousness and increased excitability. In such cases, certain conditions, such as fasting or reduced feed intake, may precipitate the tetany–paresis syndrome. Often a combination of several adverse factors is found, but in most cases low dietary Mg together with a low plane of nutrition is essential. Owing to underfeeding, slight ketonemia is often seen. The incidence of the disease seems to increase with age, and there is a tendency for a recurrence in some animals.

a. Seasonal Hypomagnesemia. The first recorded study of seasonal hypomagnesemia in the bovine without clinical symptoms was by Allcroft and Green (1938), when attention was drawn to the winter decline in serum Mg concentration in a herd of Hereford cows at pasture all year round without shelter or additional feeding. From August, the serum Mg fell to minimal levels in December, with a return to normal levels in February. Values as low as 0.5 mg/dl (0.2 mmole/liter) were found for serum Mg, but no changes occurred in the serum Ca levels. The pasture contained 0.20% Mg (dry matter). The additional feeding of 45 gm magnesium oxide per head each day in a mineral mixture alleviated the seasonal fall of serum Mg but did not maintain a normal level. Allcroft (1947b) extended these findings and showed that the low serum Mg values were associated with adverse weather, especially with combined cold, wet, and windy periods. The lowest level of serum Mg might occur any time between December and April. The conditions under which minimal serum Mg was found were such that the heat loss from the body would be the highest. When hay and cabbages were fed, the Mg level rose to normal, only to fall again when supplementary feeding ceased. The clinical cases of hypomagnesemia, all of which showed very low serum concentrations of Mg and various degrees of hypocalcemia, coincided with the time when minimal serum Mg values appeared, i.e., during periods with little or no growth of grass.

b. Norwegian Tetany–Paresis Syndrome. (Ender *et al.*, 1948; Breirem *et al.*, 1949). Special conditions during World War II forced Norwegian farmers to feed rations containing herring meal and fodder cellulose, both very low in Mg. Concentrates and

grain were entirely suspended. The rations may be characterized as deficient in energy with an abundant supply of protein. This fodder caused a marked increase in the frequency of metabolic diseases such as ketosis and tetany. Subnormal serum concentrations of Ca and Mg were found in paretic cows as well as in cows suffering from tetany. Both sets of symptoms often appeared simultaneously in the same herd.

This stimulated feeding experiments in which cows were given an ample supply of protein, Ca, and P, but a supply of energy 10–50% below theoretical requirements and deficient in Mg. In these experiments it was shown that the tetany–paresis syndrome could be reproduced under strictly controlled conditions.

Some cows showed pronounced hypomagnesemia without showing any clinical symptoms. In others the hypomagnesemia was accompanied by muscular irritability, twitchings, or tremor. A marked variation among cows was seen. Fasting periods of 36 hours or abrupt change in the diet brought about a drop in serum Mg as well as in serum Ca. In eight cows, tetany or paresis was induced. Serum analysis revealed a picture similar to that described by Sjollema (1930) for grass tetany, as shown in Table XII. The daily intake of Mg ranged from 4 to 11 gm. Supplements of Mg (28 gm/day given as carbonate) could prevent the hypomagnesemia as well as the nervous symptoms and tetany.

c. Winter Tetany. This type of disorder, seen mainly in beef cattle, may occur in stall-fed cattle (Mershon and Custer, 1958; Mershon, 1959) or in cattle during winter after pasture growth has stopped (Udall, 1947). Generally, the cows appear to be on fairly poor rations during development of the disease. Marshak (1959) stated that the simplest means of controlling winter tetany was improving the nutritional status of the herds. When pasture reappears, the disease disappears.

During winter months persistent low levels of Mg may be found, but this hypomagnesemia is accompanied by few or no clinical symptoms. Certain cows, especially those with recent parturition, may suddenly exhibit clinical symptoms ranging from moderate incoordination to paresis or tetany. Hyperirritability has a dominant place, whereas coma most often is absent. During the clinical stage, it is generally agreed that the hypomagnesemia is associated with various degrees of hypocalcemia (Marshak, 1959). Variation in the clinical behavior was suggested by Mershon and Custer (1958) to be associated with fluctuation in the Ca/Mg ratio.

Leffel and Mason (1959) fed cows hay obtained from two farms which had experienced

TABLE XII

Serum Calcium, Magnesium, and Inorganic Phosphorus Concentrations in Grass Tetany[a]

	Calcium (mg/dl)	Inorganic phosphorus (mg/dl)	Magnesium (mg/dl)
Grass tetany according to Sjollema	6.5	4.5	0.5
Eight cows suffering from experimental tetany or paresis	6.7	3.4	0.7
Same eight cows 2-5 days before onset of tetany or paresis	9.9	5.8	0.7

[a] Modified from Ender *et al.* (1949a).

a high incidence of winter tetany. The hay was given as the sole ration for 4–6 months before calving and in early lactation. The daily intake of Mg ranged from 7 to 12 gm. In both dairy cows and beef cows, severe hypomagnesemia was observed, but only beef cows developed tetany. Plasma Na and K values were reported to be normal.

d. Effect of Underfeeding. The pregnant or recently calved ruminant appears to be especially susceptible to underfeeding. Underfeeding (Swan and Jamieson, 1956; Pehrson, 1963) or fasting (Halse, 1958a, 1960; Robertson *et al.*, 1960; Herd, 1966; Lomba *et al.*, 1972) has been found to be effective in lowering the serum Mg concentration.

Swan and Jamieson (1956) obtained evidence that the following conditions may cause a lowering of the serum Mg concentration in milking cows: (1) feeding thyroprotein alone or in conjunction with underfeeding; (2) a sudden change in quality and quantity of the ration; (3) a sudden increase in the quantity of the ration; (4) calving, especially if accompanied by a reduction in grazing time; (5) estrus, if accompanied by marked homosexual activity and a disturbance of grazing behavior; and (6) strong winds, when there is a reduction of grazing time or, if not, a marked reduction in rumination time.

From Sweden, Pehrson (1963) reported that a number of tetanic conditions in stable-fed cows occurred following the very wet summer of 1960. In all cases the tetany was associated with hypomagnesemia and appeared more than 6 days after calving. Provision of 45 gm MgO daily prevented the hypomagnesemia as well as the tetanic conditions. Careful investigation showed that hypomagnesemia was also present in many symptom-free cows. It was shown that the energy requirements of the milking cows were not satisfied. The supply of Mg was, however, a good deal higher than minimal requirements. The author stated that the minimal requirements for dietary Mg apparently become greater in combination with underfeeding.

The effect of fasting on the mineral metabolism of the lactating cow has been studied (Halse, 1958a, 1960; Robertson *et al.*, 1960; Herd, 1966). These experiments extended and confirmed the experience of Smith *et al.* (1938) that starved cows may develop a clinical syndrome very similar to milk fever. A significant decrease in serum concentrations of Ca and Mg was found. In cows showing symptoms similar to those of milk fever, the minimal Ca levels were no lower than in the cows which remained apparently normal. The Mg levels, on the other hand, were definitely lower just prior to the onset of "milk fever" than they were in other cows at any time during the experiment. In all cows the serum Mg level had a tendency to remain low during the early stages of refeeding (Fig. 3). A similar effect of fasting has been observed in sheep (Christian and Williams, 1960; Herd, 1966).

e. Effect of Artificially Low Magnesium Intakes. Rook (1961, 1963) designed a diet extremely low in Mg and fed it to cows. With this diet the critical low intake of Mg was about 2 gm of Mg daily. The ration, which was otherwise nutritionally adequate, produced hypomagnesemia in four lactating, housed cows in less than 8 days. In two of the cows the fall in Mg was relatively slow and no symptoms appeared. In the other two the fall in serum Mg was more rapid. Both developed tetany, and one died. The day before the final tetanic attack, the serum Ca and Mg values were 9.5 and 0.28 mg/dl (2.4 and 0.1 mmole/liter), respectively. At that time the cow was hyperexcitable and had mild tetany. The final attack, with collapse, violent tonic-clonic convulsions, and opisthotonus with eyeballs suddenly withdrawn almost completely into the socket, lasted only 5–10 minutes.

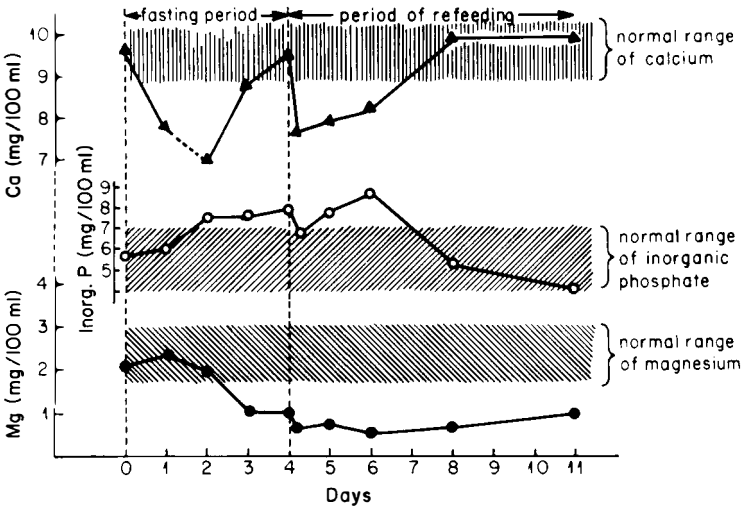


Fig. 3. Change in serum calcium, magnesium, and inorganic phosphorus during a 4-day period of fasting.

2. Rapid Type: Grass Tetany

In 1930 a metabolic disturbance, now known as hypomagnesemic tetany, appeared in veterinary literature. It affected cattle only and was referred to by a variety of synonyms, such as grass staggers (Sjollema, 1930; Sjollema and Seekles, 1930), Hereford disease, and lactation tetany (Lothian, 1931). The disease was probably not a new one, for unexplained, sporadic, sudden death of cattle at pasture had previously been reported and given such names as eclampsia, brain-fever, and bovine hysteria (Robertson, 1960). As the problem became prominent, work was started to elucidate the etiology of this syndrome.

The first form of the disease to be recognized occurred in cows in the spring within a few weeks after they were turned out to pasture following the winter feeding period (Sjollema, 1928; Sjollema and Seekles, 1929). The cases described by Lothian (1931) occurred mainly in "outwintered" cattle, and the clinical syndrome was similar to that described by Sjollema. The clinical chemical findings confirmed this shortly after (Dryerre, 1932).

In nearly all descriptions of hypomagnesemic tetany, young, rapidly growing grass is emphasized as being especially dangerous. Clover and herbs seem to be less dangerous. The disease occurs most frequently during the first weeks of the grazing season. In some years, however, it is also rather frequent in autumn, especially under wet conditions with relatively low temperature levels. In Holland, 95% of the cases reported occurred when the mean temperature was between 5° and 15°C ('tHart and Kemp, 1956). The same seems to be true for "wheat poisoning," tetany on winter grazing of oats in Argentina, and grass staggers in New Zealand. When frost occurs, the number of cases declines rapidly (Inglis, 1960).

a. Clinical Biochemical Findings. Sjollema (1930) first pointed out that a decline in the Mg concentration of the blood was always present during hypomagnesemic tetany,

and he also noted that the pronounced hypomagnesemia in most cases was combined with a moderate hypocalcemia.

Blakemore and Stewart (1934) confirmed these findings and demonstrated that low Mg levels may be found in clinically normal herds in which hypomagnesemic tetany is present. This finding has been repeatedly confirmed. Kemp (1958) demonstrated that the serum Mg levels of 90 milking cows, grazing on pastures where hypomagnesemic tetany occurred, were reduced in nearly all cows. Of the 90 cows, only 1 had a serum Mg level higher than 2.0 mg/dl (0.8 mmole/liter), 38 showed levels between 1.1 and 2.0 mg/dl (0.5 and 0.8 mmole/liter), while 51 cows, almost 60%, had serum Mg levels below 1.1 mg/dl (0.5 mmole/liter).

Todd and Thompson (1960) investigated most of the major blood electrolytes during hypomagnesemia without clinical symptoms. No consistent alterations were found in serum Ca, Na, K, or Cl levels or in pH of the blood. Similar results had been reported previously (Sjollema, 1930; Dryerre, 1932; Bartlett *et al.*, 1957).

Allcroft (1947a) noted that hypocalcemia was present in 76% of 406 clinical cases of hypomagnesemia. Typical cases of hypomagnesemic tetany, however, may be seen in the absence of a fall in serum Ca, and, conversely, a hypomagnesemic animal even with a marked hypocalcemia may remain completely symptom free (Meyer, 1977).

Levels below 1.8 mg/dl (0.7 mmole/liter) are classed as hypomagnesemic, and those below 1.1 mg/dl (0.5 mmole/liter) as severely hypomagnesemic. When blood Mg levels fall below 1.0 mg/dl (0.4 mmole/liter), appetite declines (Kemp, 1971). A pronounced individual susceptibility to hypomagnesemia has been repeatedly reported. Halse (1970) was able to forecast the cows most likely to develop hypomagnesemia on turnout by ranking the cows in order of decreasing serum Mg values on one or two samplings prior to turnout.

Serum P_i levels have been reported to be normal or low (Sjollema, 1930). Values below 2.0 mg/dl (0.6 mmole/liter) have been found (Hvidsten *et al.*, 1959; Simesen, 1959). Van Koetsveld (1955) demonstrated increased sulfate values in serum from tetanic cows. During the tetanic stage a metabolic acidosis and moderate accumulation of lactate in the blood was shown (Hendriks, 1962, 1964a). In sheep serious reductions in plasma Mg were found to be without effect on the proportions of bound Mg and Ca present in plasma (Suttle and Field, 1969).

As a differential diagnostic criterion between milk fever and grass tetany, the proteinuria usually present during grass tetany has been recommended (Sjollema, 1930; Hopkirk *et al.*, 1933; Metzger, 1936). The Mg concentration of the urine, however, offers a much more reliable possibility for a differential diagnostic test. During milk fever the Mg content in urine is about 50 mg/dl (21 mmole/liter) (Sjollema, 1930), whereas during hypomagnesemia the urine is devoid of Mg [i.e., 1.0–3.0 mg/dl (0.4–1.2 mmole/liter)] (Ender *et al.*, 1957; Kemp *et al.*, 1961; Simesen, 1963b). Semiquantitative determinations of urine Mg to be used as a "bedside" test have been developed (Halse, 1976; Simesen, 1977b). It is very important to avoid fecal contamination of the urine sample because of the Mg content in feces. If a suspicion of hypomagnesemia exists before treatment of a paretic cow, a semiquantitative Mg estimation on a urine sample makes it easy to confirm or cancel such suspicion immediately. If hypomagnesemia is confirmed, preventive measures can thus be taken immediately for the whole herd. Postmortem blood samples for diagnostic purposes are of little value, as the Mg level rises rapidly after death

(Burns and Allcroft, 1967). In such cases analysis of a urine sample carefully removed from the bladder, preferably in connection with analysis of several blood samples from susceptible animals in the herd, may supply the diagnosis (Simesen, 1964).

b. Etiological Considerations Sjollema (1932a) suggested that hypomagnesemic tetany had some connection with alteration in the methods of feeding and manuring. This point became evident later when a definite implication of such relationships was demonstrated in well-controlled experiments (Bartlett *et al.*, 1954).

At that time it was known that (1) serum Mg concentration might fall very rapidly, within a few days (Allcroft, 1954); (2) bones of adult animals with hypomagnesemic tetany did not exhibit depletion of Mg (Cunningham, 1936a,b); (3) the condition occurred in cattle given grass with normal Mg content (Sjollema, 1928, 1930); and (4) feeding MgO had a preventive effect (Blakemore and Stewart, 1934). Attempts to correlate the disease with a toxic factor or any special quality of the lush spring grass, such as high concentration of protein, potassium, nitrate (Sjollema, 1932a), or manganese (Blakemore *et al.*, 1937), had been unsuccessful (Green, 1939), and the disease was thought to be of metabolic rather than nutritional origin (Allcroft, 1947b).

c. Chemical and Botanical Composition of Spring Pasture. Compared with hay, young grass has a strikingly high content of P. The Ca content is in the low range, whereas the Mg appears to be adequate. Potassium is usually abundant, while the level of Na is very low. The mineral requirements of a cow milking 20 kg of milk daily compared with the amount of minerals contained in 18 kg of young grass (dry matter) or about 90 kg of fresh grass are shown in Table XIII (modified from Werner, 1960).

Lush spring grass is rich in water (often more than 85%), soluble carbohydrates, and crude protein, whereas the content of crude fiber is low. During the growing season the content of crude fiber increases, while that of crude protein decreases. In autumn, especially during wet and rather cold weather, a second maximum in crude protein content may be reached (Brandsma, 1954). In spring grass a crude protein content of about 25% (dry matter) may be found. On the other hand, clover-rich pastures may produce herbage with protein contents between 25 and 30%. Because hypomagnesemia rarely occurs in cows grazing clover-rich pastures, the crude protein content has sometimes been considered of little importance in relation to hypomagnesemia. This might be due to the high Ca and Mg content of clover. Usually clover and herbs contain at least twice as much Ca and Mg as do grasses.

While the content of P and crude protein decreases during the growing season, the content of Mg and crude fiber slowly increases. The minimal Mg level in pastures thus occurs in early spring and late autumn.

TABLE XIII

Theoretical Mineral Requirements Compared to Actual Mineral Contents of a Day's Ration of Grass

	Phosphorus (gm)	Calcium (gm)	Magnesium (gm)	Potassium (gm)	Sodium (gm)
Theoretical requirement	55	87	10	130	24
Young grass	88	84	29	300-500	12

TABLE XIV

Influence of Different Fertilizer Treatments[a]

Dressing	Potassium	Magnesium	Calcium	Sodium	Phosphorus	Crude protein
Potash (K)	+	−	−	−	0	0
Nitrogen (N)	[b]	+	+	+	0	+
K + N	+	−	−	−	0	+

[a] +, increase; −, decrease; 0, no change.

[b] Nitrogen applied to a sward high in potassium (more than 2%) increases the percentage of potassium in the pasture. Applied to a sward with a potassium content below 2%, on the other hand, it results in a decrease in the potassium content.

d. Influence of Fertilizer Treatments. Fertilizer treatment influences the mineral composition of pasture as well as the botanical composition of the sward. Nitrogen fertilizers often reduce the proportion of clover, whereas application of P and K may quickly produce a clover-dominant sward. Changes in the mineral composition of herbage after different fertilizer treatments are shown in Table XIV (modified from Kemp, 1960).

'tHart and Kemp (1956) demonstrated that herbage from "tetany pastures" had a higher mean K and P content and a lower Na, Ca, and Mg content than herbage from pastures without tetany. The Mg content of "tetany pastures" was about 10% below that of "nontetany pastures." Larvor and Guéguen (1963) studied the relationship between grass composition and hypomagnesemia and found that tetany-prone grasses were significantly lower in Mg and crude fiber content and higher in nonelaborated N than "nontetany grass." Wolton (1963) reviewed the factors affecting herbage chemical composition and concluded that N applications are essential to produce early bite and the bulk of spring growth. She therefore recommended using no more N than is required for immediate herbage production. Potassium applications should be deferred until the danger period is past.

e. Grazing Experiments. The investigations of Bartlett *et al.* (1954) included a series of grazing experiments in which the influence of different fertilizer treatments on the incidence of hypomagnesemia was studied. Dutch workers ('tHart and Kemp, 1956; Kemp, 1958) demonstrated that a high K content of grass promotes the incidence of hypomagnesemia. The incidence was further increased by a simultaneously high N content of the grass, as shown in Table XV.

TABLE XV

Dressing Influence on Serum Magnesium

Dressing	Average serum Mg (mg/dl)	Dressing	Average serum Mg (mg/dl)
Low N, low K	2.43	High N, low K	2.15
Low N, high K	1.74	High N, high K	1.41

Plant Mg uptake is powerfully influenced by the competition of K. Potassium is preferentially absorbed from the soil by most plants. Therefore, a decrease in the plant Mg content may well be an indirect effect of K application.

Some workers do not agree on the importance of K in the development of hypomagnesemia (Ender *et al.*, 1957; Alten *et al.*, 1958; Smyth *et al.*, 1958). On the basis of K-feeding experiments, Brouwer (1951), Kunkel *et al.* (1953), Sjollema *et al.* (1955a), and Van Koetsveld (1964) are of the opinion that K contributes to lower serum Mg, whereas Green and Allcroft (1951), Eaton and Avampato (1952), and Daniel *et al.* (1952) were unable to confirm such findings. Ender *et al.* (1957) suggested the "tetanigen" effect observed to be more dependent on the simultaneously raised dietary level of P than on the high intake of K. Smyth *et al.* (1958) found that treatment of a sward with N alone or K alone did not render it more prone to a lowering of serum Mg levels, whereas treatment with a combined dressing of N plus K resulted in a crop producing a highly significant rapid decline in serum Mg values followed by the onset of tetany. Application of Mg in addition to N plus K resulted in maintenance of serum Mg values within normal range. Blaxter *et al.* (1960) concluded that, if K is in any way associated with hypomagnesemia in cattle, it must be an indirect effect, i.e., via depression of the Mg content of the sward. High K content of the food neither had an influence on serum Mg levels nor had it any influence on the K content of the RBC. However, Suttle and Field (1969) showed that an increase in K intake can reduce plasma Mg and induce hypomagnesemic tetany in sheep if the daily intake of Mg is so low that the minimal Mg requirements are barely met. Meyer and Stehling (1972) have since demonstrated that a high dosage of K under certain conditions may lead to a decreased Mg digestibility (about 12%, absolute), a decrease in renal Mg excretion, and a temporary drop in the blood Mg level.

Sjollema (1932b) stated that the sulfur content of grass parallels the protein content. The importance of the high S content of spring grass was stressed by Van Koetsveld (1955), who demonstrated increased values of sulfate in serum from tetanic cows. Ender *et al.* (1957) reported that various inorganic substances, i.e., sulfates and phosphates, induce marked falls in the level of serum Mg in sheep. In fertilizing experiments, they found that ammonium sulfate-treated pasture, which contained about four times as much S as calcium ammonium nitrate-treated pasture, was more tetanigenic (Havre and Dishington, 1962). Tetany in cows grazing pastures moderate in sulfate content was also reported. By means of balance experiments, Gröning (1959) studied the influence of sulfate on the metabolism of Mg in cattle. High sulfate content of the feed had no effect on the serum Mg levels, whereas the excretion of Mg in the urine decreased. Gröning concluded that even if sulfate proved to possess a slight inhibitory effect upon Mg absorption, a possibility which could not be ruled out by his studies, this inhibition would not be of such magnitude as to explain the etiology of hypomagnesemic tetany.

A high content of nitrate in the grass was suggested as a cause of grass tetany (Rathje, 1958), but a subsequent study of samples from pastures causing tetany did not support this hypothesis. Nor did Seekles and Sjollema (1932), Ender *et al.* (1957), or Trommer and Mehnert (1973) find any indication of such a relationship.

f. Mineral Indices. Several ratios, such as K/(Ca + Mg) (Kemp and 'tHart, 1957), (Na × 100)/(K + Ca + Mg) ('tHart and Kemp, 1957), (K × 100)/(K + Ca + Mg) (Verdeyen, 1953), and alkaline alkalinity (Brouwer, 1957), have been suggested as indices for the relationship between a given sward and the degree of hypomagnesemia to be

expected when the sward is fed to cows. *Alkali alkalinity* is the sum K + Na − Cl − S, expressed in milliequivalents per 100 gm herbage, dry matter. *Alkaline-earth alkalinity* is the sum Ca + Mg − P, expressed in milliequivalents per 100 gm herbage, dry matter. Rook and Wood (1960) demonstrated that no such relationship could be expected between the degree of hypomagnesemia in grazing cattle and the concentration of any particular mineral constituent or the magnitude of any mineral index. Ratios including K and Na are clearly almost entirely dependent on the K and Na content of the sward and are likely to be most markedly influenced by an application of potash fertilizer, which increases the K and decreases the Na content of herbage. For a particular sward and under any given set of grazing conditions, however, an increased severity of hypomagnesemia, brought about by the application of nitrogenous or potash fertilizers, is generally associated with a lowering of the alkaline-earth alkalinity of the grazed herbage. An attempt to use a single sward characteristic as a general diagnostic test for a pasture that is likely to produce a high incidence of hypomagnesemia and tetany in the grazing cow must inevitably fail (Rook and Wood, 1960). Butler (1963), on the other hand, found that, although the K/(Ca + Mg) ratio has not yet been shown to have any physiological or nutritional basis, it does appear to be of particular significance in relation to the occurrence of grass tetany.

Seekles (1964) pointed out that, although the hypothesis of a relationship between the K/(Ca + Mg) ratio in the pasture and the occurrence of hypomagnesemic tetany may be supported by statistical data obtained from farms in certain areas and in particular years, it is not well supported by data obtained over many years from samples collected throughout the Netherlands.

In the spring a level of 0.20% of Mg was found to give protection even in cows grazing "tetany-prone" pastures (Allcroft and Burns, 1968). In the autumn herbage Mg levels of 0.25% seem closer to the critical level (Todd, 1967).

An increase in the Mg content of the herbage brought about by a Mg-containing fertilizer results in higher serum Mg levels in grazing animals (Stewart, 1954; Reith, 1954; Bartlett *et al.*, 1954; Parr and Allcroft, 1957; Smyth *et al.*, 1958), and therefore Stewart and Reith (1956) concluded that grass tetany occurring in cows at pasture must be regarded as a conditioned hypomagnesemic disorder, resulting either from an inadequate intestinal absorption of Mg in the grass or from substances antagonizing the Mg metabolism. Kemp (1960) subjected the relationship which might exist between the Mg content of the herbage and the serum Mg to a close evaluation and demonstrated that the low serum Mg levels occur only with Mg contents of the herbage below 0.20% (dry matter). Furthermore, it was shown that, in cows grazing pasture with Mg content below this value, the low serum levels were associated with a higher percentage of crude protein.

g. Rumen Ammonia. Head and Rook (1955) drew attention to the high ammonia concentration in the rumen of cows during the first days of grass feeding. The level rose from a general level of 10–20 mg/dl (0.6–1.2 mmole/liter) during winter stall-feeding conditions to a continuously high level of 40–60 mg/dl (2.4–3.5 mmole/liter) following transfer to grass feeding. Simultaneously, the urinary excretion fell to negligible amounts, and the serum Mg dropped from normal values to about 1 mg/dl (0.4 mmol/liter). This decrease in urinary excretion of Mg was suggested to reflect a reduced intestinal absorption of Mg. Subsequently, addition of ammonium acetate or ammonium carbonate to the rumen (via a fistula) of cows fed a diet of hay and concentrates produced ruminal ammonia levels comparable to those observed in cows fed grass. The urinary Mg excretion

was reduced, and a moderate reduction in serum Mg levels was seen. Meyer and Rustige (1958), however, could find no indications of a connection between ammonia and the etiology of hypomagnesemia.

h. Balance Experiments. The above-mentioned findings induced a series of Mg balance trials (Rook and Balch, 1958; Field *et al.*, 1958; Kemp *et al.*, 1960; Van der Horst, 1960; Meyer and Steinbeck, 1960). A transient hypomagnesemic effect on blood Mg was reported in connection with the application of a metabolic harness (Hendricks, 1964b). From the experiments it was learned that the change from winter stall feeds to herbage generally involved a decrease in the dietary Mg intake. Head and Rook (1955) in their experiments reported an Mg intake of about 21–25 gm/day in cows on common winter rations compared with about 12.5 gm/day in cows fed rations of cut herbage. However, supposing unaltered "availability" (percentage of ingested Mg not excreted in the feces) of the dietary Mg, even such a daily Mg intake should be sufficient (Ender *et al.*, 1949b). The percentages of ingested Mg excreted in the feces, however, were found to average 71.5–74.8% in cattle fed winter rations compared with 82–83% during grass feeding; i.e., the "availability" of Mg in grass was lowered. This reduction in "availability" might be brought about by a lowering of the "true availability" or by an enlarged endogenous fecal Mg, as pointed out by Field (1960). Under normal dietary conditions the site of maximal Mg absorption was thought to be in the midileum (Care and Van'T Klooster, 1965), i.e., below those parts of the intestinal tract into which the bulk of endogenous Mg is secreted. If the process by which Mg absorption takes place loses efficiency, not only does the ruminant fail to absorb dietary Mg, but it also fails to absorb the Mg secreted into the upper part of the digestive tract. A failure of the system by which Mg is absorbed would thus lead to a reduction in the true absorption rate as well as to an increase in the endogenous fecal Mg excretion (Care, 1967).

The decrease in Mg absorption was at one time considered to be a result of an increased ruminal production of ammonia with subsequent elevation of pH in the upper part of the small intestine and precipitation of Mg as magnesium ammonium phosphate (Simesen, 1959; Kemp *et al.*, 1961). However, significant changes in pH of ruminal, abomasal, or small intestinal contents have been observed in association with diets of spring grass (Simesen, 1963b; Van'T Klooster, 1967). Moreover, it is not usual to find a significant alteration in the ultrafiltrable Mg concentration of digesta obtained from either duodenum or midileum of sheep when the diet is changed from hay to spring grass, despite a hypomagnesemic reaction to this dietary change (Care, 1967).

Feces of cows receiving a grass ration, on the other hand, was found to contain less ultrafiltrable Ca and Mg than the feces of cows fed winter rations (Van'T Klooster, 1967), whereas the percentages of water-extractable Ca and Mg in samples of feces from a normal and a hypomagnesemic cow were found not to be grossly different (Parr, 1961).

In the balance trials the change from winter rations to cut young grass was inevitably followed by an immediate fall in urinary Mg excretion. Since this decrease in the urinary Mg excretion preceded the fall in serum Mg, it was stated that urine Mg is a better measure of the Mg status of an animal than is serum Mg (Kemp, 1960).

Since Ca and Mg are absorbed in the ionic state and since the electrochemical potentials for the absorption from ileum are comparatively small, a relatively small decrease in the concentration of ionized Mg within the ileum would be sufficient to convert a net absorption of Mg to a net secretion.

The findings implicating the rumen as the main site of Mg absorption instead of the small intestine would seem to call for a reevaluation of the etiological considerations as they have hitherto been stated. These considerations are all based on the presumption that the main site of Mg absorption is located below those parts of the intestinal tract into which the bulk of endogenous Mg is secreted. The new evidence makes investigations on the physiochemical status of Mg in the ruminant stomachs under different dietary conditions important.

Regardless of this, however, the greatest single etiological factor of hypomagnesemic tetany is the decrease in the net absorption of Mg from the alimentary tract. Much effort has been directed toward the identification of single dietary factors which could bring about a decrease in the net absorption of Mg. Several factors have been suggested: organic acid chelation [citrate (Swan and Jamieson, 1956; Scholz and Meyer, 1973), *trans*-aconitate, and oxalate (Burt and Thomas, 1961; Burau and Stout, 1965; Lomba *et al.*, 1968)], fatty acids (Kemp *et al.*, 1966), histamine (Fowler, 1963; Wrenn *et al.*, 1963; Geelen *et al.*, 1966; O'Sullivan, 1968), phytate (Evered, 1961), amines (Ashton and Sinclair, 1965; Seekles, 1964), fat supplements (Wind *et al.*, 1966), protein/carbohydrate imbalance (Molloy, 1971), binding of Mg P_i (Smith and McAllan, 1966), and sulfate (Dishington, 1965; Dishington and Tollersrud, 1967) or magnesium ammonium phosphate (Storry, 1961b; Simesen, 1963b), nonprotein nitrogen compounds of "tetany" pastures (Larvor and Guéguen, 1963), and binding of Mg ions by isolated cell walls of rumen bacteria (Fitt *et al.*, 1972). So far no one has been able to reduce Mg to an extent where this alone could explain the etiology of hypomagnesemic tetany. Only under conditions of marginal supply may these factors play a contributing role.

i. Trigger Effect. The final insult that precipitates clinical symptoms of hypomagnesemic tetany in an already susceptible animal may be (1) a suddenly reduced feed intake (Halse, 1958b; Herd, 1966), (2) adverse weather (Allcroft, 1947b), (3) estrus (Swan and Jamieson, 1956), (4) oral application of relatively high doses of Na_2HPO_4, Na_2SO_4, or both (Dishington, 1965), or (5) sudden reductions in dietary supplies of Mg or sudden change of diet (Dishington and Tollersrud, 1967). Even the mere handling of a susceptible cow, the pricking of a needle, or an unexpected noise can sometimes provoke an acute state of tetany. Usually, however, muscular fibrillations are seen for some days prior to the typical spasm.

Todd and Horvath (1970) investigated the relation between Mg and neuromuscular irritability in calves, with particular reference to hypomagnesemic tetany. They concluded that the primary lesion causing tetany is not likely to be located in the muscle fibers or in the central nervous system. It was found that a reduction in the stimulus required to cause contraction occurred within 1 day before tetany, and then the nearer the onset of tetany, the lower the stimulus required. At the point of tetany, about one-third of the normal stimulus caused contraction. Following successful treatment, the stimulus became normal within 24 hours, even though blood Mg concentration was abnormal.

Studies on sheep by Meyer and Scholz (1972) have demonstrated that the development of clinical signs is more closely associated with the Mg content of cerebrospinal fluid (CSF) than with blood Mg levels. A decrease in blood Mg levels is followed by a delayed reaction in CSF. Differences in this delay may explain why clinical signs appear at different Mg levels and, in particular, why some animals with extremely low blood Mg levels can still remain symptom free for extended periods of time (Meyer, 1977).

3. Methods of Prevention

In spite of the variety of opinions on the etiology of hypomagnesemia, there is uniform agreement about the essential aim of prevention: to increase the daily intake of Mg. Extra dietary Mg may be provided by means of drenching (Cunningham, 1934; Blakemore and Stewart, 1934; Allcroft and Green, 1938; R. Allcroft, 1954; Breirem *et al.*, 1954; Jevnaker, 1955; Sjollema *et al.*, 1955b), in a cattle cake mixture (Seekles and Boogaert, 1955, 1956; Line *et al.*, 1958), as a magnesia–molasses mixture (Todd *et al.*, 1966; Horvath *et al.*, 1967; Ross and Gibson, 1969), as foliar dusting of calcined magnesite spread on the pasture (Todd and Morrison, 1964; Poole, 1967; Kemp and Geurink, 1967), or via Mg fertilization of the sward (Parr and Allcroft, 1957; Smyth *et al.*, 1958; Bosch and Harmsen, 1958; Walsh and Conway, 1960), all effective means of prevention.

It has been shown (Herd *et al.*, 1965) that effective control can be achieved by mixing MgO with water and spraying it from a watering can on one or two opened bales of hay and offering this treated hay to the cattle before the remainder of the hay ration is made available [114 gm per cow (4 oz.) every second day; 14 gm per ewe (0.5 oz.)].

Magnesium alloy bullets have been developed to provide supplemental Mg (Ritchie and Hemingway, 1968; Kemp and Todd, 1970). Results of field trials, however, have been conflicting (House and Mayland, 1976), apparently because the rate of decomposition of the bullets in the reticulorumen is highly variable. Foliar dusting with calcined magnesite (34 kg/ha) and access to a magnesia–molasses mixture, on the other hand, are both highly effective methods of preventing severe hypomagnesemia in cows grazing spring and autumn pasture (Rogers and Poole, 1971). These techniques, however, fail to prevent hypomagnesemia and tetany in herds of beef cows at pasture in winter.

Absorption of Mg from the oxide has been shown to be more efficient than that from sulfate or carbonate (Storry and Rook, 1963; Meyer and Grund, 1963) and much better than that from dolomite. For top dressing purposes it has been shown that magnesium sulfate as epsom salt or kieserite is less suitable than calcined magnesite (Allcroft and Burns, 1968). An extensive discussion of suitable methods to ensure an adequate intake under various types of management has been published (Burns and Allcroft, 1967).

C. Miscellaneous Conditions

1. Hypomagnesemia in Sheep

The clinical symptoms of hypomagnesemia (grass staggers) occur in sheep (Blumer *et al.*, 1939; Barrentine, 1948; Stewart, 1954; Pook, 1955; Penny and Arnold, 1955; Inglis *et al.*, 1959). The factors involved are very similar to those in the development of hypomagnesemia in cows (Hjerpe, 1968b; Hemingway *et al.*, 1960). Also, in sheep a striking variation among animals on similar treatment has been observed (L'Estrange and Axford, 1964a). Ewes with twins are more prone to develop hypomagnesemia than sheep with single lambs (Hvidsten, 1967). Pregnancy toxemia, hypocalcemia, and hypomagnesemia in sheep are often seen together. Clinically it is often impossible to distinguish among these three metabolic syndromes (Simesen, 1971).

Oral supplementation of lactating ewes at pasture with Mg, without addition of Ca, has been found to be effective in raising the concentration in serum of both Ca and Mg to normal levels (L'Estrange and Axford, 1964b; Herd, 1966).

2. *Wheat Pasture Poisoning*

"Wheat pasture poisoning" is now usually regarded as a special feature of hypomagnesemic tetany (Sims and Crookshank, 1956). The combined effect of high K and high protein intake characteristic of lush wheat pasture grazing, was studied by means of balance trials (Fontenot *et al.*, 1960). The findings of this study were consistent with the view that such rations may induce tetany by interfering with the absorption or retention of Mg.

3. *Effect of Magnesium Administration*

The effect of orally, intravenously, or subcutaneously administered Ca and Mg was studied by Allcroft (1947c). The anesthetic level of serum Mg in the ruminant was about 14 mg/dl, (5.8 mmole/liter) and the lethal level about 20 mg/dl (8.2 mmole/liter). Similar results were reported in dogs (Moore and Wingo, 1942). When Ca is injected concurrently with Mg, the level of Mg necessary to cause death is considerably higher. It has been questioned (Somjen *et al.*, 1966) whether parenteral administration of magnesium salts can cause narcosis. The anesthetic or lethal effect of Mg ions is due to a block of neuromuscular transmission with little if any depressing effect on the central nervous system (Bowen *et al.*, 1970b). The use of magnesium sulfate as a method of euthanasia should therefore be abandoned. Hypermagnesemia is seen in advanced renal disease associated with uremia (Terkildsen, 1950).

REFERENCES

Aalund, O., and Nielsen, K. (1960). *Nord. Veterinaermed.* **12,** 605.

Aalund, O., Rendel, J., and Freedland, R. A. (1965). *Biochim. Biophys. Acta* **110,** 113.

Agricultural Research Council (1965). "The Nutrient Requirement of Farm Livestock," Ruminants Tech. Rev. Summ., No. 2. Agric. Res. Counc., London.

Aikawa, J. K. (1963). "The Measurement of Magnesium in Biologic Materials," Chap. 6, p. 42. Thomas, Springfield, Illinois.

Aikawa, J. K. (1971). "The Relationship of Magnesium to Disease in Domestic Animals and in Humans." Thomas, Springfield, Illinois.

Åkerblom, E. (1952). *Nord. Veterinaermed.* **4,** 471.

Alanko, M., Cederquist, B., Jonsgård, K., Jönsson, G., Nurmio, P., Pehrson, B., and Simesen, M. G. (1975). *Nord. Veterinaermed.* **27,** 616.

Albright, F., and Reifenstein, E. C. (1948). "The Parathyroid Glands and Metabolic Bone Disease," p. 393. Williams & Wilkins, Baltimore, Maryland.

Albright, F., Bauer, W., Ropes, M. W., and Aub, J. C. (1929). *J. Clin. Invest.* **7,** 139.

Albright, F., Burnett, C. H., Cope, O., and Parson, W. (1941). *J. Clin. Endocrinol.* **1,** 711–716.

Alcock, N., and MacIntyre, I. (1960). *Biochem. J.* **76,** 19.

Alcock, N. W., and MacIntyre, I. (1966). *Methods Biochem. Anal.* **14,** 1.

Alexander, T. G., and Ford, T. F. (1957). *J. Dairy Sci.* **40,** 1273.

Allcroft, R. (1954). *Vet. Rec.* **66,** 517.

Allcroft, R., and Burns, K. N. (1968). *N.Z. Vet. J.* **16,** 109.

Allcroft, R., and Ivins, L. N. (1964). *Rep. Int. Meet. Dis. Cattle, 3rd, Copenhagen. Nord. Veterinaermed.* **16,** Suppl. 1, p. 217.

Allcroft, W. M. (1947a). *Vet. J.* **103,** 2, 30.

Allcroft, W. M. (1947b). *Vet. J.* **103,** 75.

Allcroft, W. M. (1947c). *Vet. J.* **103,** 157.

Allcroft, W. M., and Folley, S. J. (1941). *Biochem. J.* **35,** 254.

Allcroft, W. M., and Godden, W. (1934). *Biochem. J.* **28,** 1004.
Allcroft, W. M., and Green, H. H. (1934). *Biochem. J.* **28,** 2220.
Allcroft, W. M., and Green, H. H. (1938). *J. Comp. Pathol. Ther.* **51,** 176.
Alten, F., Rosenberger, G., and Welte, E. (1958). *Zentralbl. Veterinaermed.* **5,** 201.
Argenzio, R. A., Lowe, J. E., Hintz, H. F., and Schryver, H. F. (1974). *J. Nutr.* **104,** 18.
Asbury, A. C. (1962). *J. Am. Vet. Med. Assoc.* **141,** 703.
Ashton, W. M., and Sinclair, K. B. (1965). *J. Br. Grassl. Soc.* **20,** 118.
Axford, R. F. E., Tas, M. V., Evans, R. A., and Offer, N. W. (1975). *Res. Vet. Sci.* **19,** 333.
Baird, J. D. (1971). *Aust. Vet. J.* **47,** 402.
Barakat, M. Z., and El-Guindi, M. M. (1967). *Zentralbl. Veterinaermed. Reihe A* **14,** 589.
Bardwell, R. E. (1959). *J. Am. Vet. Med. Assoc.* **135,** 72.
Barker, J. R. (1939). *Vet. Rec.* **51,** 575.
Barlet, J. P. (1967). *C. R. Acad. Sci.* **265,** 1075.
Barnes, J. E., and Jephcott, B. R. (1955). *Aust. Vet. J.* **31,** 302.
Barnicoat, C. R., Logan, A. G., and Grant, A. I. (1949). *J. Agric. Sci.* **39,** 237.
Barrentine, B. F. (1948). *J. Anim. Sci.* **7,** 535.
Bartlett, S., Brown, B. B., Foot, A. S., Rowland, S. J., Allcroft, R., and Parr, W. H. (1954). *Br. Vet. J.* **110,** 3.
Bartlett, S., Brown, B. B., Foot, A. S., Head, M. J., Line, C., Rook, J. A. F., Rowland, S. J., and Zundel, G. (1957). *J. Agric. Sci.* **49,** 291.
Bartley, J. C., Reber, E. F., Yusken, J. W., and Norton, H. W. (1961). *J. Anim. Sci.* **20,** 137.
Barto, L. R. (1932). *J. Am. Vet. Med. Assoc.* **80,** 251.
Bassett, C. F., Harris, L. E., and Wilke, C. F. (1951). *J. Nutr.* **44,** 433.
Basu, K. P., and Mukherjee, K. P. (1943). *Indian J. Vet. Sci.* **13,** 231.
Baustad, B., Teige, J., Jr., and Tollersrud, S. (1967). *Acta Vet. Scand.* **8,** 369.
Becker, R. B., Neal, W. M., and Shealy, A. L. (1933). *Fla. Univ. Agric. Exp. Stn. (Gainesville), Tech. Bull.* No. 262.
Benzie, D., Boyne, A. W., Dalgarno, A. C., Duckworth, J., Hill, R., and Walker, D. M. (1959). *J. Agric. Sci.* **54,** 202.
Björklund, N.-E., Jönsson, G., and Pehrson, B. (1976). *Proc. Int. Congr. Dis. Cattle, 9th, Paris* p. 1003.
Black, H. E., and Capen, C. C. (1971). *Metab. Clin. Exp.* **20,** 337.
Black, H. E., and Capen, C. C. (1973). *Horm. Metab. Res.* **5,** 297.
Blakemore, F., and Stewart, J. (1934). *Rep. Director Inst. Anim. Pathol., 4th, Cambridge Univ.* p. 103.
Blakemore, F., Nicholson, J. A., and Stewart, J. (1937). *Vet. Rec.* **49,** 415.
Blaxter, K. L. (1956). *Ciba Found Symp. Bone Struct. Metab., London, 1955* p. 117.
Blaxter, K. L., and McGill, R. F. (1956). *Vet. Rev. Annot.* **2,** 35.
Blaxter, K. L., and Rook, J. A. F. (1954). *J. Comp. Pathol. Ther.* **64,** 176.
Blaxter, K. L., and Sharman, G. A. M. (1955). *Vet. Rec.* **67,** 108.
Blaxter, K. L., Rook, J. A. F., and MacDonald, A. M. (1954). *J. Comp. Pathol. Ther.* **64,** 157.
Blaxter, K. L., Cowlishaw, B., and Rook, J. A. F. (1960). *Anim. Prod.* **2,** 1.
Bloom, F. (1957). *North Am. Vet.* **38,** 114.
Bloom, F. (1960). "The Blood Chemistry of the Dog and Cat." Gamma Publ., New York.
Blosser, T. H., and Albright, J. L. (1956). *Ann. N.Y. Acad. Sci.* **64,** 386.
Blosser, T. H., and Smith, V. R. (1950a). *J. Dairy Sci.* **33,** 81.
Blosser, T. H., and Smith, V. R. (1950b). *J. Dairy Sci.* **33,** 329.
Blumer, C. C., Madden, F. J., and Walker, D. J. (1939). *Aust. Vet. J.* **15,** 24.
Boda, J. M., and Cole, H. H. (1954). *J. Dairy Sci.* **37,** 360.
Boda, J. M., and Cole, H. H. (1956). *J. Dairy Sci.* **39,** 1027.
Boogaerdt, J. (1954). Thesis, State Univ. of Utrecht, Utrecht.
Bosch, S., and Harmsen, H. E. (1958). *Meded. Proefstn. Akker-en Weidebouw. Wageningen* **10,** 9.
Bowen, J. M., Blackmon, D. M., and Heavner, J. E. (1970a). *Am. J. Vet. Res.* **31,** 831.
Bowen, J. M., Blackmon, D. M., and Heavner, J. E. (1970b). *J. Am. Vet. Med. Assoc.* **157,** 164.
Braithwaite, G. D. (1975a). *Br. J. Nutr.* **33,** 309.
Braithwaite, G. D. (1975b). *Br. J. Nutr.* **34,** 311.
Braithwaite, G. D., and Riazuddin, S. (1971). *Br. J. Nutr.* **26,** 215.
Braithwaite, G. D., Glascock, R. F., and Riazuddin, S. (1970). *Br. J. Nutr.* **24,** 661.
Braithwaite, G. D., Glascock, R. F., and Riazuddin, S. (1972). *Br. J. Nutr.* **28,** 269.

Brandsma, S. (1954). *Meded. Landbouwhogesch. Wageningen* **54,** 245.
Breirem, K., Ender, G., Halse, K., and Slagsvold, P. (1949). *Acta Agric. Suec.* **3,** 89.
Breirem, K., Ender, F., Halse, K., and Slagsvold, P. (1954). *Meld. Nor. Landbrukshoegsk.* **34,** 373.
British Veterinary Association (1957). "The Husbandry and Diseases of Calves," p. 20. Br. Vet. Assoc., London.
Brouwer, F. (1951). *Meded. Landbouwhogesch. Wageningen* **51,** 91.
Brouwer, F. (1957). *E.P.A. Proj. Utrecht* No. 204, p. 161 (European Productivity Agency).
Brown, W. R., Krook, L., and Pond, W. G. (1966). *Cornell Vet.* **56,** Suppl. I.
Buckle, R. M., Care, A. D., Cooper, C. W., and Gitelman, H. J. (1968). *J. Endocrinol.* **42,** 529.
Burau, R., and Stout, P. R. (1965). *Science* **150,** 766.
Burns, K. N., and Allcroft, R. (1967). *Br. Vet. J.* **123,** 340 and 383.
Burt, A. W. A., and Thomas, D. C. (1961). *Nature (London)* **192,** 1193.
Butler, E. J. (1963). *J. Agric. Sci.* **60,** 329.
Campbell, J. R. (1960). *Vet. Rec.* **72,** 1153.
Campbell, J. R., and Douglas, T. A. (1965). *Br. J. Nutr.* **19,** 339.
Capen, C. C., and Martin, S. L. (1971). *In* "Hyperparathyroidism in Current Veterinary Therapy IV. Small Animal Practice" (R. W. Kirk, ed.), p. 605. Saunders, Philadelphia, Pennsylvania.
Capen, C. C., and Young, D. M. (1967). *Science* **157,** 205.
Capen, C. C., Koestner, A., and Cole, C. R. (1965). *Lab. Invest.* **14,** 1673.
Capen, C. C., Cole, C. R., and Hibbs, J. W. (1966a). *Pathol. Vet.* **3,** 350 [see *Vet. Bull. (London)* **36,** Abstr. No. 4873 (1966)].
Capen, C. C., Cole, C. R., Hibbs, J. W., and Wagner, A. R. (1966b). *Am. J. Vet. Res.* **27,** 1177.
Care, A. D. (1960). *Br. Vet. Assoc. Conf. Hypomagnesemia, London* p. 8 (discuss.).
Care, A. D. (1967). *World Rev. Nutr. Diet.* **8,** 127.
Care, A. D. (1971). *Rep. Rowett Inst.* **27,** 106.
Care, A. D., and Bates, R. F. L. (1970). *Hormones (Basel)* **1,** 364.
Care, A. D., and Duncan, T. (1967). *J. Endocrinol.* **37,** 107.
Care, A. D., and McDonald, I. R. (1963). *Biochem. J.* **87,** 2P.
Care, A. D., and Ross, D. B. (1963). *Res. Vet. Sci.* **4,** 24.
Care, A. D., and Van'T Klooster, A. T. (1965). *J. Physiol. (London)* **177,** 174.
Care, A. D., Sherwood, L. M., Potts, J. T., and Aurback, G. D. (1966). *Nature (London)* **209,** 55.
Care, A. D., Cooper, C. W., Duncan, T., and Orimo, H. (1968a). *Endocrinology* **83,** 161.
Care, A. D., Cooper, C. W., and Orimo, H. (1968b). *In* "Parathyroid Hormone and Thyrocalcitonin (Calcitonin)" (R. V. Talmage and L. F. Belanger, eds.), p. 417. Excerpta Med. Found., Amsterdam.
Care, A. D., Bates, R. F. L., Phillippo, M., Lequin, R. M., Hackeng, W. H. L., Barlet, J. P., and Larvor, P. (1970). *J. Endocrinol.* **48,** 667.
Carlström, B, (1933). *Skand. Veterinaertidskr.* **23,** 229.
Carlström, G. (1969). Thesis, Research Station, Veterinary College, Skara, Sweden.
Chang, K. Y., and Carr, C. W. (1968). *Biochim. Biophys. Acta* **157,** 127.
Chicco, C. F., Ammerman, C. B., Hillis, W. G., and Arrington, L. R. (1972). *Am. J. Physiol.* **222,** 1469.
Christian, K. R., and Williams, V. J. (1960). *N.Z. J. Agric. Res.* **3,** 389.
Cohn, D. V., and Hamilton, J. W. (1976). *Cornell Vet.* **66,** 271.
Cole, C. R., Chamberlain, D. M., Hibbs, J. W., Pounden, W. D., and Smith, C. R. (1957). *J. Am. Vet. Med. Assoc.* **130,** 298.
Colovos, N. F., Keener, H. A., Teeri, A. E., and Davis, H. A. (1951). *J. Dairy Sci.* **34,** 735.
Comar, C. L., Monroe, R. A., Visek, W. J., and Hansard, S. L. (1953). *J. Nutr.* **50,** 459.
Conrad, H. R., Hansard, S. L., and Hibbs, J. W. (1956). *J. Dairy Sci.* **39,** 1697.
Cooper, C. W., Schwesinger, W. A., Ontjes, D. A., Mahgoub, A. M., and Munson, P. L. (1972). *Endocrinology* **91,** 1079.
Copp, D. H. (1969). *J. Endocrinol.* **43,** 137.
Copp, D. H., and Kuczerpa, A. V. (1967). *Resume Commun. Symp. Eur. Tissus Calcif. 5th* p. 11.
Copp, D. H., Cameron, E. C., Cheney, B. A., Davidson, A. G. F., and Henze, K. G. (1962). *Endocrinology* **70,** 638.
Craige, A. H., and Beck, J. D. (1941). *J. Am. Vet. Med. Assoc.* **98,** 315.
Craige, A. H., and Gadd, J. D. (1941). *Am. J. Vet. Res.* **2,** 227.
Crookshank, H. R., and Sims, F. H. (1955). *J. Anim. Sci.* **14,** 964.

Crosfield, P. (1941). *Vet. Rec.* **53,** 593.
Cunningham, I. J. (1934). *N.Z. J. Sci. Technol.* **15,** 414.
Cunningham, I. J. (1936a). *N.Z. J. Sci. Technol.* **17,** 775.
Cunningham, I. J. (1936b). *N.Z. J. Sci. Technol.* **18,** 419.
Curran, S. (1949). *Eire Dep. Agric. J.* **46,** 60.
Curtis, R. A., Cote, J. F., and Willoughby, R. A. (1970). *Mod. Vet. Pract.* **51,** 25–28.
Dämmrich, K. (1963). *Zentralbl. Veterinaermed.* **10,** 322.
Daniel, O., Hatfield, E. E., Shrewsberry, W. C., Gibson, M. E., and MacVicar, R. (1952). *J. Anim. Sci.* **11,** 790.
Davies, D. C., Allen, W. M., Hoare, M. N., Pott, J. M., Riley, C. J., Sansom, B. F., Stenton, J. R., and Vagg, M. J. (1978). *Vet. Rec.* **102,** 440.
Deckwer, N. (1950). *Berl. Muench. Tieraerztl. Wochenschr.* **63,** 85.
Del Castillo, J., and Engbäk, L. (1953). *J. Physiol. (London)* **120,** 54P.
Dell, J. C., and Poulton, B. R. (1958). *J. Dairy Sci.* **41,** 1706.
Detweiler, D. K., and Martin, J. E. (1949). *Am. J. Vet. Res.* **10,** 201.
Dirksen, G., and Kaufmann, W. (1978). *Fortschr. Veterinaermed.* **28,** 144.
Dirksen, G., Plank, P., Dämmrich, K., and Hänichen, T. (1971). *Vet.-Med. Nachr.* **2/3,** 199.
Dishington, I. W. (1965). *Acta Vet. Scand.* **6,** 150.
Dishington, I. W. (1974). *Nord. Veterinaermed.* **26,** 205.
Dishington, I. W. (1975). *Acta Vet. Scand.* **16,** 503.
Dishington, I. W., and Tollersrud, S. (1967). *Acta Vet. Scand.* **8,** 14.
Doige, C. E., Owen, B. D., and Mills, J. H. L. (1975). *Can. J. Anim. Sci.* **55,** 147.
Dryerre, H. (1932). *Vet. Rec.* **12,** 1163.
Dryerre, H., and Greig, J. R. (1925). *Vet. Rec.* **5,** 225.
Dryerre, H., and Greig, J. R. (1928). *Vet. Rec.* **8,** 721.
Duckworth, J. (1939). *Nutr. Abstr. Rev.* **8,** 841.
Duckworth, J., Godden, W., and Thomson, W. (1943). *J. Agric. Sci.* **33,** 190.
Duncan, C. W., Huffman, C. F., and Robinson, C. S. (1935). *J. Biol. Chem.* **108,** 35.
Duncan, D. L. (1958). *Nutr. Abstr. Rev.* **28,** 695.
Earle, I. P., and Cabell, C. A. (1952). *Am. J. Vet. Res.* **13,** 330.
Eaton, H. D., and Avampato, J. E. (1952). *J. Anim. Sci.* **11,** 761.
Edney, A. T. B. (1969). *J. Small Anim. Pract.* **10,** 231.
Edqvist, L.-E., Ekman, L., Gustafsson, B., and Lindell, J.-O. (1974). *Acta Vet. Scand.* **15,** 587.
Eichelberger, L., and McLean, F. C. (1942). *J. Biol. Chem.* **142,** 467.
Ender, F. (1964). *Nord. Veterinaermed., Suppl.* **1,** 408.
Ender, F., and Dishington, I. W. (1970). *In* "Parturient Hypocalcemia" (J. J. B. Anderson, ed.), p. 71. Academic Press, New York.
Ender, F., Halse, K., and Slagsvold, P. (1948). *Nor. Vet.-Tidsskr.* **60,** 1, 41.
Ender, F., Halse, K., and Slagsvold, P. (1949a). *Rep. Int. Vet. Congr., 14th, London* **3,** 14.
Ender, F., Helgebostad, A., and Bøhler, N. (1949b). *Nord. Veterinaermed.* **1,** 827.
Ender, F., Dishington, I. W., and Helgebostad, A. (1956). *Nord. Veterinaermed.* **8,** 507.
Ender, F., Dishington, I. W., and Helgebostad, A. (1957). *Nord. Veterinaermed.* **9,** 881.
Ender, F., Dishington, I. W., and Helgebostad, A. (1962). *Acta Vet. Scand.* **3,** Suppl. No. 1.
Ender, F., Dishington, I. W., and Helgebostad, A. (1971). *Z. Tierphysiol., Tierernaehr. Futtermittelkd.* **28,** 233.
Erdtman, H. (1928). *Hoppe-Seyler's Z. Physiol. Chem.* **177,** 211, 231.
Eriksen, L., and Simesen, M. G. (1970). *Nord. Veterinaermed.* **22,** 273.
Eveleth, D. F. (1937). *J. Biol. Chem.* **119,** 289.
Evered, D. F. (1961). *Nature (London)* **189,** 228.
Field, A. C. (1959). *Nature (London)* **183,** 983.
Field, A. C. (1960). *Br. Vet. Assoc. Conf. Hypomagnesemia, London* p. 13.
Field, A. C. (1961). *Br. J. Nutr.* **15,** 349.
Field, A. C., and Suttle, N. F. (1967). *J. Agric. Sci.* **69,** 417.
Field, A. C., McCallum, J. W., and Butler, E. J. (1958). *Br. J. Nutr.* **12,** 433.
Fish, P. A. (1928). *J. Am. Vet. Med. Assoc.* **73,** 10.
Fish, P. A. (1929a). *Cornell Vet.* **19,** 147.

Fish, P. A. (1929b). *J. Am. Vet. Med. Assoc.* **75,** 695.
Fitch, L. W. N. (1943). *Aust. Vet. J.* **19,** 2.
Fitt, T. J., Hutton, K., Thompson, A., and Armstrong, D. G. (1972). *Proc. Nutrition Soc. Abstr. Commun.* **31,** 100A.
Folin, O., and Svedberg, A. (1926). *J. Biol. Chem.* **70,** 405.
Folley, S. J. (1936). *Nature (London)* **137,** 741.
Fontenot, J. P., Miller, R. W., Whitehair, C. K., and MacVicar, R. (1960). *J. Anim. Sci.* **19,** 127.
Ford, E. J. H. (1958). *Vet. Rev. Annot.* **4,** 119.
Fourman, P., Royer, P., Levell, M. J., and Morgan, D. B. (1968). "Calcium Metabolism and the Bone," 2nd ed., p. 239. Blackwell, Oxford.
Fowler, H. D. (1963). *Nature (London)* **197,** 619.
Fox, J., and Care, A. D. (1978). *J. Endocrinol.* **77,** 225.
Franklin, M. C., Reid, R. L., and Johnstone, I. L. (1948). *Commonw. Aust., Counc. Sci. Ind. Res.* No. Bull. 240, Parts I–IV.
Freudenberg, F. (1955). *Dtsch. Tieraerztl. Wochenschr.* **62,** 422.
Garm, O. (1950). *Acta Endocrinol. (Copenhagen)* **9,** 413.
Garm, O. (1951). *Proc. Nord. Vet. Congr., 6th, Stockholm* p. 288.
Garrett, O. F., and Overman, O. R. (1940). *J. Dairy Sci.* **23,** 13.
Geelen, M. J. H., van Rheenen, D. L., Hendriks, H. J., and Seekles, L. (1966). *Tijdschr. Diergeneeskd.* **91,** 1577.
Gershoff, S. N., Legg, M. A., O'Connor, F. J., and Hegsted, D. M. (1957). *J. Nutr.* **63,** 79.
Gitelman, H. J., Kukolj, S., and Welt, L. G. (1968). *J. Clin. Invest.* **47,** 118.
Glawischnig, E. (1962). *Int. Tag. Rinderkr., 2nd, Vienna* p. 19.
Glawischnig, E. (1974). *Int. Meet. Dis. Cattle, 8th; Congr. Soc. Ital. Buiatria, 6th, Milan* p. 406. Gallarati, Piacenza, Italy.
Goings, R. L., Jacobson, N. L., Beitz, D. C., Littledike, E. T., and Wiggers, K. D. (1974). *J. Dairy Sci.* **57,** 1184.
Gorham, J. R., Peckham, J. C., and Alexander, J. (1970). *J. Am. Vet. Med. Assoc.* **156,** 1331.
Grace, N. D., and Macrae, J. C. (1972). *Br. J. Nutr.* **27,** 51.
Granström, R. (1908). *Hoppe-Seyler's Z. Physiol. Chem.* **58,** 195.
Grant, A. B. (1955). *Vet. Rev. Annot.* **1,** 115.
Green, H. H. (1939). *Vet. Rec.* **51,** 1179.
Green, H. H., and Allcroft, R. (1951). *Rep. Natl. Inst. Dairying, Reading, Engl.* p. 35.
Gregory, M. E., Kon, K., and Thompson, S. Y. (1952). *Rep. Natl. Inst. Dairying, Reading, Engl.* p. 63.
Greig, J. R. (1929). *Vet. Rec.* **9,** 509.
Greig, J. R. (1930). *Vet Rec.* **10,** 301.
Groenendyk, S., and Seawright, A. A. (1974). *Aust. Vet. J.* **50,** 131.
Groenewald, J. W. (1952). *Rep. Int. Vet. Congr., 14th, London, 1949* 3, 34.
Groenewald, J. W., Thomas, A. D., and Dutoit, B. A. (1940). *Onderstepoort J. Vet. Sci. Anim. Ind.* **15,** 299.
Gröning, M. (1959). Thesis, Tierärzlichen Hochschule Hannover, West Germany.
Hackett, P. L., Gaylor, D. W., and Bustad, L. K. (1957). *Am. J. Vet. Res.* **18,** 338.
Hallgren, W. (1940). Thesis, Lunds Univ., Lund, Sweden.
Hallgren, W. (1955). *Nord. Veterinaermed.* **7,** 433.
Hallgren, W., Carlström, G., and Jönsson, G. (1959). *Nord. Veterinaermed.* **11,** 217.
Halse, K. (1948). *Skand. Veterinaertidskr.* **38,** 567.
Halse, K. (1958a). *Nord. Veterinaermed.* **10,** 1.
Halse, K. (1958b). *Nord. Veterinaermed.* **10,** 9.
Halse, K. (1960). *Proc. Int. Grassl. Congr., 8th, Reading, Engl.* p. 553. Grassl. Res. Inst., Hentley, England.
Halse, K. (1970). *Acta Vet. Scand.* **11,** 394.
Halse, K. (1976). *Nor. Vet.-Tidsskr.* **88,** 11.
Halse, K., and Velle, W. (1958). *Am. J. Vet. Res.* **19,** 575.
Hanna, S., and MacIntyre, I. (1960). *Lancet* **11,** 348.
Hansard, S. L., Comar, C. L., and Plumlee, M. P. (1954). *J. Anim. Sci.* **13,** 25.
Hansen, Aas, M., Flatla, J. L., and Mikkelsen, T. (1966). *Rep. Nord. Vet. Congr., 10th, Stockholm* p. 935.
Hart, E. B., Steenbock, H., Kline, O. L., and Humphrey, G. C. (1931). *J. Dairy Sci.* **14,** 307.
Haussler, M. R., and McCain, T. A. (1977). *N. Engl. J. Med.* **297,** 974.

Havre, G. N., and Dishington, I. W. (1962). *Acta Agric. Scand.* **12,** 298.
Hayden, C. E. (1927). *Cornell Vet.* **17,** 121.
Hayden, C. E. (1929). *Cornell Vet.* **19,** 285.
Head, M. J., and Rook, J. A. F. (1955). *Nature (London)* **176,** 262.
Heaton, F. W. (1965). *Clin. Sci.* **28,** 543.
Hedhammar, Å., Wu, F., Krook, L., Schryver, H. F., de Lahunta, A., Whalen, J. P., Kallfelz, F. A., Nunez, E. A., Hintz, H. F., Sheffy, B. E., and Ryan, G. D. (1974). *Cornell Vet.* **64,** Suppl. 5, 1.
Hellmich, K. (1938). *Tieraerztl. Rundsch.* **44,** 533.
Hemingway, R. G., Inglis, J. S. S., and Ritchie, N. S. (1960). *Br. Vet. Assoc. Conf. Hypomagnesemia, London* p. 58.
Hendriks, H. J. (1962). Thesis, State University, Utrecht,
Hendriks, H. J. (1964a). *Tijdschr. Diergeneeskd.* **89,** 487.
Hendriks, H. J. (1964b). *Nature (London)* **203,** 1306.
Herd, R. P. (1965). *Aust. Vet. J.* **41,** 385.
Herd, R. P. (1966). *Aust. Vet. J.* **42,** 269.
Herd, R. P., Schuster, N., and Coltman, M. (1965). *Aust. Vet. J.* **41,** 142.
Hibbs, J. W. (1948). Thesis, Ohio State Univ., Columbus.
Hibbs, J. W. (1950). *J. Dairy Sci.* **33,** 758.
Hibbs, J. W., and Conrad, H. R. (1960). *J. Dairy Sci.* **43,** 1124.
Hibbs, J. W., and Pounden, W. D. (1955). *J. Dairy Sci.* **38,** 65.
Hibbs, J. W., Krauss, W. E., Monroe, C. F., and Pounden, W. D. (1945). *J. Dairy Sci.* **28,** 525.
Hibbs, J. W., Krauss, W. E., Pounden, W. D., Monroe, C. F., and Sutton, T. S. (1946). *J. Dairy Sci.* **29,** 767.
Hintz, H. F., and Schryver, H. F. (1972). *J. Anim. Sci.* **35,** 755.
Hintz, H. F., and Schryver, H. F. (1973). *J. Anim. Sci.* **37,** 927.
Hjärre, A. (1930). *Acta Pathol. Microbiol. Scand.* Suppl. No. 7.
Hjerpe, C. A. (1968a). *Cornell Vet.* **58,** 193.
Hjerpe, C. A. (1968b). *Am. J. Vet. Res.* **29,** 143.
Hoff-Jørgensen, E. (1946). *Biochem. J.* **40,** 189.
Holcombe, R. B. (1953). *Br. Vet. J.* **109,** 359.
Holmes, A. D., Spelman, A. F., Smith, C. T., and Kuzmeski, J. W. (1947). *J. Dairy Sci.* **30,** 385.
Holtenius, P., Jacobsson, S. O., Jonson, G., and Möllerberg, L. (1970). *Nord. Veterinaermed.* **22,** 463.
Hopkirk, C. S. M., Marshall, D., and Blake, T. A. (1933). *Vet. Rec.* **13,** 355.
Horvath, D. J., Dozsa, L., Kidder, H. E., Warren, J. E., Jr., Bhatt, B., and Croushore, W. (1967). *J. Anim. Sci.* **26,** 875.
Horváth, Z., and Kutas, F. (1959). *Acta Vet. Acad. Sci. Hung.* **9,** 183.
Horváth, Z., and Papp, L. (1972). *Acta Vet. Acad. Sci. Hung.* **22,** 37.
Horváth, Z., Papp, L., and Éliás, B. (1972). *Acta Vet. Acad. Sci. Hung.* **22,** 45.
House, W. A., and Mayland, H. F. (1976). *J. Anim. Sci.* **42,** 506.
Huffman, C. F., and Robinson, C. S. (1926). *J. Biol. Chem.* **69,** 101.
Huffman, C. F., Conley, C. L., Lightfoot, C. C., and Duncan, C. W. (1941). *J. Nutr.* **22,** 609.
Hutten, H., and Uhlenbruck, K. (1952). *Dtsch. Tieraerztl. Wochenschr.* **59,** 5.
Hutyra, F., Marek, J., and Manninger, R. (1946). "Special Pathology and Therapeutics of the Diseases of Domestic Animals," p. 130. Bailliere, London.
Hvidsten, H. (1967). *Z. Tierphysiol., Tierernaehr. Futtermittelkd.* **22,** 210.
Hvidsten, H., Ødelien, M., Baerug, R., and Tollersrud, S. (1959). *Acta Agric. Scand.* **9,** 261.
Inglis, J. S. S. (1960). *Rep. Int. Grassl. Congr., 8th, Reading, Engl.* p. 558. Grassl. Res. Inst., Henley, England.
Inglis, J. S. S., Weipers, M., and Pearce, P. J. (1959). *Vet. Rec.* **71,** 755.
Jackson, H. D., Pappenhagen, A. R., Goetsch, G. D., and Noller, C. H. (1962). *J. Dairy Sci.* **65,** 897.
Jennings, F. W., and Mulligan, W. (1953). *J. Comp. Pathol. Ther.* **63,** 286.
Jevnaker, I. (1955). *Medlemsbl. Nor. Veterinaerforen.* **7,** 118.
Jönsson, G. (1960). Thesis, Uppsala Univ., Uppsala, Sweden.
Jönsson, G., and Pehrson, B. (1969). *Zentralbl. Veterinaermed.* **16,** 49.
Jönsson, G., and Pehrson, B. (1970). *Vet. Rec.* **87,** 575.
Jönsson, G., and Simesen, M. G. (1973). *Aust. Vet. J.* **49,** 252.
Jonsgård, K. (1963). *Nord. Veterinaermed.* **15,** 28.

Jonsgård, K. (1965). *Nord. Veterinaermed.* **17,** 386 [see *Vet. Bull. (London)* **36,** Abstr., 717 (1966)].
Jonsgård, K. (1972). Thesis, Oslo Univ., Oslo.
Jonson, G., Luthman, J., Möllerberg, L., and Persson, J. (1971). *Nord. Veterinaermed.* **23,** 620.
Jorgensen, N. A. (1974). *J. Dairy Sci.* **57,** 933.
Joyce, J. R., Pierce, K. R., Romance, W. M., and Baker, J. M. (1971). *J. Am. Vet. Med. Assoc.* **158,** 2033.
Jubb, K. V. F., and Kennedy, P. C. (1970). "Pathology of Domestic Animals," 2nd ed., Vol. 1, p. 25. Academic Press, New York.
Kaneko, J. J. (1972). "Standard Values in Domestic Animals," 3rd ed. Univ. of California, Davis.
Kemp, A. (1958). *Neth. J. Agric. Sci.* **6,** 281.
Kemp, A. (1960). *Neth. J. Agric. Sci.* **8,** 281.
Kemp, A. (1971). *Magnesium Group-Meet., Dublin* p. 18.
Kemp, A., and Geurink, J. H. (1967). *Agric. Dig.* **12,** 23.
Kemp, A., and 'tHart, M. L. (1957). *Neth. J. Agric. Sci.* **5,** 4.
Kemp, A., and Todd, J. R. (1970). *Vet. Rec.* **86,** 463.
Kemp, A., Deijs, W. B., Hemkes, O. J., and Van Es, A. J. H. (1960). *Br. Vet. Assoc. Conf. Hypomagnesaemia, London* p. 23.
Kemp, A., Deijs, W. B., Hemkes, O. J., and Van Es, A. J. H. (1961). *Neth. J. Agric. Sci.* **9,** 134.
Kemp, A., Deijs, W. B., and Kluvers, E. (1966). *Neth. J. Agric. Sci.* **14,** 290.
Kendall, K. A., Harsbarger, K. E., Hays, R. L., and Ormiston, E. E. (1966). *J. Dairy Sci.* **49,** 720.
Keynes, W. M., and Care, A. D. (1967). *Proc. R. Soc. Med.* **60,** 1136.
Kirchgessner, M., Schwartz, W. A., and Roth, H.-P. (1975). *Z. Tierphysiol., Tierernaehr. Futtermittelkd.* **35,** 191.
Kjeldberg, J. (1940). *Beret. Nord. Vet.-Moede, 5th, Copenhagen, 1939* p. 670 [see *Vet. Bull. (London)* **12,** 230 (1942)].
Kleiber, M., Smith, A. H., Ralston, N. P., and Black, A. L. (1951). *J. Nutr.* **45,** 253.
Klobouk, A. (1932). *Zverolek. Obz.* **6,** 13, 25 [see *Vet. Bull. (London)* **2,** 567 (1932)].
Knoop, C. E., Krauss, W. E., and Hayden, C. C. (1939). *J. Dairy Sci.* **22,** 283.
Kronfeld, D. S. (1971). *Adv. Vet. Sci. Comp. Med.* **15,** 133.
Kronfeld, D. S., and Ramberg, C. F., Jr. (1970). *In* "Parturient Hypocalcemia" (J. J. B. Anderson, ed.), p. 107. Academic Press, New York.
Krook, L. (1965). *Rev. Can. Biol.* **24,** 63.
Krook, L., and Lowe, J. E. (1964). *Pathol. Vet.* **1,** Suppl.
Krook, L., Barrett, R. B., Usui, K., and Wolke, R. E. (1963). *Cornell Vet.* **57,** 224.
Krook, L., Lutwak, L., and McEntee, K. (1969). *Am. J. Clin. Nutr.* **22,** 115.
Krook, L., Lutwak, L., McEntee, K., Henrikson, P.-Ä., Braun, K., and Roberts, S. (1971). *Cornell Vet.* **61,** 625.
Krook, L., Wasserman, R. H., Shively, J. N., Tashjian, A. H., Jr., Brokken, T. D., and Morton, J. F. (1975). *Cornell Vet.* **65,** 26.
Kunkel, H. O., Burns, K. H., and Camp, B. J. (1953). *J. Anim. Sci.* **12,** 451.
Lane, D. R., and Robinson, R. (1970). *Br. Vet. J.* **126,** 230.
Larvor, P., and Guéguen, L. (1963). *Ann. Zootech.* **12,** 39.
Lawler, D. C. (1963). *Vet. Rec.* **75,** 811.
Leffel, E. C., and Mason, K. R. (1959). *Proc. Symp. Magnesium Agric., W. Va. Univ.* p. 182.
Leslie, A. (1931). *Vet. Rec.* **11,** 1148.
L'Estrange, J. L., and Axford, R. F. E. (1964a). *J. Agric. Sci.* **62,** 341.
L'Estrange, J. L., and Axford, R. F. E. (1964b). *J. Agric. Sci.* **62,** 353.
Lettow, E. (1962). *Zentralbl. Veterinaermed.* **9,** 75.
Lichtwitz, A., Hioco, D., Parlier, R., and De Seze, S. (1961). *Presse Med.* **69,** 5, 51.
Liégeois, F., and Derivaux, J. (1951). *Ann. Med. Vet.* **95,** 201.
Line, C., Head, M. J., Rook, J. A. F., Foot, A. S., and Rowland, S. J. (1958). *J. Agric. Sci.* **51,** 353.
Little, D. A., Robinson, P. J., Playne, M. J., and Haydock, K. P. (1971). *Aust. Vet. J.* **47,** 153.
Little, W. L., and Mattick, E. C. V. (1933). *Vet. Rec.* **13,** 238.
Little, W. L., and Wright, N. C. (1925). *Br. J. Exp. Pathol.* **6,** 129.
Little, W. L., and Wright, N. C. (1926). *Vet. J.* **82,** 185.
Littledike, E. T. (1974). *In* "Lactation: A Comprehensive Treatise. Vol. 2: Biosynthesis and Secretion of Milk/Diseases" (B. L. Larson and V. R. Smith, eds.), Chapter 10, p. 355. Academic Press, New York.

Littledike, E. T., Whipp, S. C., and Schroeder, L. (1969). *J. Am. Vet. Med. Assoc.* **155,** 1955.
Littledike, E. T., Whipp, S. C., Witzel, D. A., and Baetz, A. L. (1970). *In* "Parturient Hypocalcemia" (J. J. B. Anderson, ed.), p. 165. Academic Press, New York.
Littledike, E. T., Arnaud, C. D., Schroeder, L., and Jacobson, N. L. (1971). *Proc. Endocrine Soc., 53rd Meet.* No. 49.
Littlejohn, A. I., and Hebert, C. N. (1969). *Vet. Rec.* **84,** 130.
Lode, T., and Øverås, J. (1968). *Medlemsbl. Nor. Veterinaerforen.* **20,** 101.
Lofgreen, G. P., and Kleiber, M. (1953). *J. Anim. Sci.* **12,** 366.
Lofgreen, G. P., Kleiber, M., and Smith, A. H. (1952). *J. Nutr.* **47,** 571.
Logan, M. A., Christensen, W. R., and Kirklin, J. W. (1942). *Am. J. Physiol.* **135,** 419.
Lomba, F., Paquay, R., Bienfet, V., and Lousse, A. (1968). *J. Agric. Sci.* **71,** 181.
Lomba, F., Chauvaux, G., and Bienfet, V. (1972). *Zentralbl. Veterinaermed., Reihe A* **19,** 138.
Lomba, F., Chauvaux, G., Teller, E., Lengele, L., and Bienfet, V. (1978). *Br. J. Nutr.* **39,** 425.
Lothian, W. (1931). *Vet. Rec.* **11,** 585.
Lucke, J. N., and Hall, G. M. (1978). *Vet. Rec.* **102,** 356.
Luecke, R. W., Schmidt, D. A., and Hoefer, J. A. (1958). *J. Anim. Sci.* **17,** 1185.
Luick, J. R., and Lofgreen, G. P. (1957). *J. Anim. Sci.* **16,** 201.
Luick, J. R., Boda, J. M., and Kleiber, M. (1957a). *J. Nutr.* **61,** 597.
Luick, J. R., Boda, J. M., and Kleiber, M. (1957b). *Am. J. Physiol.* **189,** 483.
McCandlish, A. C. (1923). *J. Dairy Sci.* **7,** 94.
McClymont, G. L. (1947). *N.S.W. Dep. Agric., Dis. Anim. Leafl.* No. 52.
MacDonald, D. C., Care, A. D., and Nolan, B. (1959). *Nature (London)* **184,** 736.
MacIntyre, I. (1959). *Proc. R. Soc. Med.* **52,** 212.
MacIntyre, I., Boss, S., and Troughton, V. A. (1963). *Nature (London)* **198,** 1058.
McLean, F. C., and Urist, M. R. (1955). "Bone." Univ. of Chicago Press, Chicago, Illinois.
McRoberts, M. R., Hill, R., and Dalgarno, A. C. (1965a). *J. Agric. Sci.* **65,** 1.
McRoberts, M. R., Hill, R., and Dalgarno, A. C. (1965b). *J. Agric. Sci.* **65,** 11.
McTaggart, H. S. (1959). *Vet. Rec.* **71,** 709.
Macy, I. G., Kelly, H. J., and Sloan, R. E. (1953). *N. A. S.-N. R. C., Publ.* No. 254.
Mader, I. J., and Iseri, L. T. (1955). *Am. J. Med.* **19,** 976.
Madsen, D. E., and Nielsen, H. M. (1940). *North Am. Vet.* **21,** 81.
Madsen, D. E., and Nielsen, H. M. (1944). *J. Am. Vet. Med. Assoc.* **105,** 22.
Mansmann, R. A., Carlson, G. P., White, N. A., and Hoffsis, G. P. (1973). *Proc. Equine Res. Symp., 3rd, Univ. Fla., Gainesville* p. 35.
Manston, R. (1967). *J. Agric. Sci.* **68,** 263.
Manston, R. (1969). *Br. Vet. J.* **125,** 177.
Manston, R., and Payne, J. M. (1964). *Br. Vet. J.* **120,** 167.
Marek, J., and Wellmann, O. (1931). "Die Rachitis," Vol. II. Fischer, Jena.
Marr, A., Moodie, E. W., and Robertson, A. (1955). *J. Comp. Pathol. Ther.* **65,** 347.
Marsh, H., and Swingle, K. F. (1955). *Am. J. Vet. Res.* **16,** 418.
Marshak, R. R. (1956). *J. Am. Vet. Med. Assoc.* **128,** 423.
Marshak, R. R. (1957). *Vet. Ext., Q. Univ. Pa.* No. 146, p. 104.
Marshak, R. R. (1959). *Proc. Symp. Magnesium Agric. W. Va. Univ.* p. 169.
Martens, H., Harmeyer, J., Breves, G., and Scholz, H. (1976). *Z. Tierphysiol., Tierernaehr. Futtermittelkd.* **37,** 44.
Martens, H., Harmeyer, J., and Michael, H. (1978). *Res. Vet. Sci.* **24,** 161.
Mayer, G. P. (1970). *In* "Parturient Hypocalcemia" (J. J. B. Anderson, ed.), p. 177. Academic Press, New York.
Mayer, G. P. (1972). *World Assoc. Buiatrics Congr., London* p. 278.
Mayer, G. P., Raggi, F., and Ramberg, C. F. (1965). *J. Am. Vet. Med. Assoc.* **146,** 839.
Mayer, G. P., Ramberg, C. F., and Kronfeld, D. S. (1966). *J. Am. Vet. Med. Assoc.* **149,** 402.
Mayer, G. P., Ramberg, C. F., Jr., and Kronfeld, D. D. (1967). *J. Am. Vet. Med. Assoc.* **151,** 1673.
Mayer, G. P., Ramberg, C. F., and Kronfeld, D. S. (1969). *Clin. Orthop.* **62,** 79.
Mayer, G. P., Blum, J. W., and Deftos, L. J. (1975). *Endocrinology* **96,** 1478.
Meigs, E. B., Edward, B., Blatherwick, N. R., and Carry, C. A. (1919). *J. Biol. Chem.* **37,** 1.
Mellanby, E. (1949). *J. Physiol. (London)* **109,** 488.

Merrild, W. G., and Smith, V. R. (1954). *J. Dairy Sci.* **37,** 546.
Mershon, M. M. (1959). *J. Am. Vet. Med. Assoc.* **135,** 435.
Mershon, M. M., and Custer, F. D. (1958). *J. Am. Vet. Med. Assoc.* **132,** 396.
Metzger, H. J. (1936). *Cornell Vet.* **26,** 353.
Meyer, H. (1963). Thesis, Tierärztlichen Hochschûle, Hannover, West Germany.
Meyer, H. (1977). *Vet. Sci. Commun.* **1,** 43.
Meyer, H., and Grund, H. (1963). *Tieraerztl. Umsch.* **18,** 181.
Meyer, H., and Plonait, H. (1968). *Zentralbl. Veterinaermed.* **15,** 481.
Meyer, H., and Rustige, J. (1958). *Dtsch. Tieraerztl. Wochenschr.* **65,** 131.
Meyer, H., and Schmidt, P. (1958). *Dtsch. Tieraerztl. Wochenschr.* **65,** 602.
Meyer, H., and Scholtz, H. (1972). *Dtsch. Tieraerztl. Wochenschr.* **79,** 55.
Meyer, H., and Stehling, W. (1972). *Berl. Muench. Tieraerztl. Wochenschr.* **85,** 201.
Meyer, H., and Steinbeck, H. (1960). *Dtsch. Tieraerztl. Wochenschr.* **67,** 315.
Meyer, H., Peters, E., and Scholz, H. (1973). *Dtsch. Tieraerztl. Wochenschr.* **80,** 160.
Miller, C. D. (1946). *J. Am. Diet. Assoc.* **22,** 312 [see *Nutr. Abstr. Rev.* **16,** 329 (1946–1947)].
Miller, E. R., Luecke, R. W., Ullrey, D. E., Baltzer, B. V., Bradley, B. L., and Hoefer, J. A. (1968). *J. Nutr.* **95,** 278.
Miller, W. J., Pitts, W. J., Clifton, C. M., and Morton, J. D. (1965). *J. Dairy Sci.* **48,** 1329.
Møller, T., and Simesen, M. G. (1959). *Nord. Veterinaermed.* **11,** 719.
Møllgaard, H. (1943). *Biedermanns Zentralbl., Abt. B* **15,** 1.
Molloy, L. F. (1971). *N.Z. Soil Bur., Sci. Rep.* No. 5.
Montgomery, R. G., Savage, W. H., and Dodds, E. C. (1929). *Vet. Rec.* **9,** 319.
Moodie, E. W. (1960). *Vet. Rec.* **72,** 1145.
Moodie, E. W. (1965). *Br. Vet. J.* **121,** 338.
Moodie, E. W., and Robertson, A. (1961). *Res. Vet. Sci.* **2,** 217.
Moodie, E. W., and Robertson, A. (1962). *Vet. Sci.* **3,** 470.
Moore, L. A., Sholl, L. B., and Hallman, E. T. (1936). *J. Dairy Sci.* **19,** 441.
Moore, L. A., Hallman, E. T., and Sholl, L. B. (1938). *AMA Arch. Pathol.* **26,** 820.
Moore, R. M., and Wingo, W. J. (1942). *Am. J. Physiol.* **35,** 140.
Morrissey, R. L., and Wasserman, R. H. (1971). *Am. J. Physiol.* **220,** 1509.
Muir, L. A., Hibbs, J. W., and Conrad, H. R. (1968). *J. Dairy Sci.* **51,** 1046.
Mullins, J. C., and Ramsay, W. R. (1959). *Aust. Vet. J.* **35,** 140.
Mylrea, P. J., and Bayfield, R. F. (1968). *Aust. Vet. J.* **44,** 565.
Nagpal, M., Gautam, O. P., and Gulati, R. L. (1968). *Indian Vet. J.* **45,** 1048.
Nelson, T. E., Tavernor, W. D., Jones, E. W., and Tillman, A. D. (1969). *J. Nutr.* **97,** 359.
Newman, D. M. R. (1968). *Aust. Vet. J.* **44,** 443.
Nicolaysen, R. (1937). *Biochem. J.* **31,** 107.
Niedermeier, R. P., and Smith, V. R. (1950). *J. Dairy Sci.* **33,** 38.
Niedermeier, R. P., Smith, V. R., and Whitehair, C. K. (1949). *J. Dairy Sci.* **32,** 927.
Nielsen, K. (1966). Thesis, Royal Veterinary and Agricultural University *Copenhagen*
Niimi, K., and Aoki, M. (1927). *J. Jpn. Soc. Vet. Sci.* **6,** 345.
Niimi, K., and Kato, K. (1928). *J. Jpn. Soc. Vet. Sci.* **7,** 181.
Nisbet, D. I., Butler, E. J., Smith, B. S. W., Robertson, J. M., and Bannatyne, C. C. (1966). *J. Comp. Pathol. Ther.* **76,** 159.
Nisbet, D. I., Butler, E. J., Robertson, J. M., and Bannatyne, C. C. (1968). *J. Comp. Pathol. Ther.* **78,** 73.
Nisbet, D. I., Butler, E. J., Robertson, J. M., and Bannatyne, C. C. (1970). *J. Comp. Pathol.* **80,** 535.
Nocard, E. (1885). *Bull. Soc. Cent. Med. Vet.* **39,** 121.
Nurmio, P. (1968). *Acta Vet. Scand., Suppl.* **26.**
Nurmio, P., and Rajakoski, E. (1973a). *Zentralbl. Veterinaermed.* **20,** 609.
Nurmio, P., and Rajakoski, E. (1973b). *Zentralbl. Veterinaermed.* **20,** 701.
O'Donovan, J., and Sheery, E. J. (1950). *Eire Dep. Agric. J.* **47,** 34.
Olson, W. G., Jorgensen, N. A., Schultz, L. H., and Deluca, H. F. (1973). *J. Dairy Sci.* **56,** 889.
O'Moore, L. B. (1950). *Ir. Vet. J.* **4,** 198, 218.
Osborne, C. A., and Stevens, J. B. (1973). *J. Am. Vet. Med. Assoc.* **162,** 125.
Oslage, H. J., Farries, F. E., Zorita, E., and Becker, M. (1960). *Arch. Tierernaehr.* **10,** 190, 200.
O'Sullivan, M. (1968). *J. Sci. Food Agric.* **19,** 12.

Ott, G. L., and Coombes, A. I. (1941). *Vet. Med. (Kansas City, Mo.)* **36,** 202.
Owen, E. C. (1948). *Biochem. J.* **43,** 243.
Owen, J. R. (1954). *Mo. Univ. Agric. Exp. Stn., Inform. Sheet* No. 497.
Oyaert, W. (1962). *Berl. Muench. Tieraerztl. Wochenschr.* **75,** 323.
Palmer, L. S., and Eckles, C. H. (1930). *J. Dairy Sci.* **13,** 351.
Palmer, L. S., Cunningham, W. S., and Eckles, C. H. (1930). *J. Dairy Sci.* **13,** 174.
Parkinson, B., and Sutherland, A. K. (1954). *Aust. Vet. J.* **30,** 232.
Parr, W. H. (1957). *Vet. Rec.* **69,** 71.
Parr, W. H. (1961). *Res. Vet. Sci.* **2,** 320.
Parr, W. H., and Allcroft, R. (1957). *Vet. Rec.* **69,** 1041.
Patwardhan, V. N., Chitre, R. G., and Sukhatankar, D. R. (1945). *Indian J. Med. Res.* **33,** 195.
Payne, J. M. (1963). *Vet. Rec.* **75,** 848.
Payne, J. M., and Leech, F. B. (1964). *Br. Vet. J.* **120,** 385.
Payne, J. M., and Manston, R. (1967). *Vet. Rec.* **81,** 215.
Payne, J. M., Sansom, B. F., and Manston, R. (1963). *Vet. Rec.* **75,** 588.
Pearson, P. T., Dellmann, H. D., Berrier, H. H., Case, A. A., and Collier, B. L. (1965). *J. Am. Vet. Med. Assoc.* **147,** 1201.
Pedersen, J. G. A. (1940). *Koebenhaven Forsoegslab. Beret.* No. 193 [see *Nutr. Abstr. Rev.* **17,** 694 (1947–1948)].
Pedersen, K. O. (1972). *Scand. J. Clin. Lab. Invest.* **30,** 321.
Pehrson, B. (1963). *Nord. Veterinaermed.* **15,** 937.
Penny, R. H. C., and Arnold, J. H. S. (1955). *Vet. Rec.* **67,** 772.
Petersen, W. E., Hewitt, E. A., Boyd, W. L., and Brown, W. R. (1931). *J. Am. Vet. Med. Assoc.* **79,** 217.
Pfeffer, E., Thompson, A., and Armstrong, D. G. (1970). *Br. J. Nutr.* **24,** 197.
Phillipson, A. T., and Storry, J. E. (1965). *J. Physiol. (London)* **181,** 130.
Pickard, D. W. (1975). *Br. Vet. J.* **131,** 744.
Pierson, R. E., and Jensen, R. (1975). *J. Am. Vet. Med. Assoc.* **166,** 260.
Pook, H. L. (1955). *Vet. Rec.* **67,** 281.
Poole, D. B. (1967). *Ir. Vet. J.* **21,** 10.
Posner, A. S. (1973). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **32,** 1933.
Powley, G., Care, A. D., and Johnson, C. L. (1977). *Res. Vet. Sci.* **23,** 43.
Preston, R. L., and Pfander, W. A. (1964). *J. Nutr.* **83,** 369.
Pribyl, E. (1933). *Zverolek. Obz.* **7,** Suppl., 61, 73 [see *Nutr. Abstr. Rev.* **4,** 88 (1934–1935)].
Quaterman, J., Dalgarno, A. C., and Adam, A. (1964). *Br. J. Nutr.* **18,** 79.
Rach, D. J., Moore, D. W., and Sturm, R. T. (1972). *Can. Vet. J.* **13,** 78.
Raghavachari, K. (1943). *Indian J. Vet. Sci.* **13,** 137.
Raisz, L. G. (1977). *In* "Metabolic Bone Disease" (L. V. Avioli and S. M. Krane, eds.), Vol. 1, p. 1. Academic Press, New York.
Raman, A. (1971). *Clin. Biochem.* **4,** 141.
Ramberg, C. F., Jr., Mayer, G. P., Kronfeld, D. S., Aurback, G. D., Sherwood, L. M., and Potts, J. T. (1967). *Am. J. Physiol.* **213,** 878.
Ramberg, C. F., Jr., Mayer, G. P., Kronfeld, D. S., Phang, J. M., and Berman, M. (1970). *Am. J. Physiol.* **219,** 1166.
Rathje, W. (1958). *Z. Tierphysiol., Tierernaehr. Futtermittelkd.* **13,** No. 3, 155.
Rayssiguier, Y., Garel, J. M., Davicco, M.-J., and Barlet, J. P. (1977). *Horm. Metab. Res.* **9,** 347.
Reid, R. L., Franklin, M. C., and Hallsworth, E. G. (1947). *Aust. Vet. J.* **23,** 136.
Reith, J. W. S. (1954). *Emp. J. Exp. Agric.* **22,** 305.
Rendel, J., Aalund, O., Freedland, R. A., and Møller, F. (1964). *Genetics* **50,** 973.
Resnick, S. (1972). *Vet. Med. Small Anim. Clin.* **67,** 637.
Ricketts, R. E., Campbell, J. R., Weinman, D. E., and Tumbleson, M. E. (1970). *J. Dairy Sci.* **53,** 898.
Ringarp, N., Rydberg, C., Damberg, O., and Boström, B. (1967). *Zentr. Veterinaermed.* **14,** 242.
Riser, W. H. (1961). *J. Am. Vet. Med. Assoc.* **139,** 117.
Ritchie, N. S., and Hemingway, R. G. (1968). *Vet. Rec.* **82,** 87.
Roberts, S. J., Richard, C. G., and Bentick-Smith, J. (1951). *J. Am. Vet. Med. Assoc.* **119,** 380.
Robertson, A. (1949). *Vet. Rec.* **61,** 333.
Robertson, A. (1960). *Br. Vet. Assoc. Conf. Hypomagnesaemia, London* p. 1.

Robertson, A., Marr, A., and Moodie, E. W. (1956). *Vet. Rec.* **68,** 173.
Robertson, A., Paver, H., Barden, P., and Marr, T. G. (1960). *Res. Vet. Sci.* **1,** 117.
Robison, R. (1923). *Biochem. J.* **17,** 286.
Rogers, P. A. M., and Poole, D. B. R. (1971). *Ir. Vet. J.* **25,** 197.
Rogers, P. A. M., and Van'T Klooster, A. T. (1969). *Meded. Landbouwhogesch. Wageningen* **69,** 11.
Rogers, T. A. (1965). *Proc. Symp. Radioisots Anim. Nutr. Physiol., Prague, 1964* p. 285. IAEA, Vienna.
Rogers, T. A., and Mahan, P. E. (1959). *Proc. Soc. Expl. Biol. Med.* **100,** 235.
Rogers, T. A., Simesen, M. G., Lunaas, T., and Luick, J. R. (1964). *Acta Vet. Scand.* **5,** 209.
Rook, J. A. F. (1961). *Nature (London)* **191,** 1019.
Rook, J. A. F. (1963). *J. Comp. Pathol. Ther.* **73,** 93.
Rook, J. A. F., and Balch, C. C. (1958). *J. Agric. Sci.* **51,** 199.
Rook, J. A. F., and Campling, R. C. (1962). *J. Agric. Sci.* **59,** 225.
Rook, J. A. F., and Storry, J. E. (1962). *Nutr. Abstr. Rev.* **32,** 1055.
Rook, J. A. F., and Wood, M. (1960). *J. Sci. Food Agric.* **11,** 137.
Rook, J. A. F., Balch, C. C., and Line, C. (1958). *J. Agric. Sci.* **51,** 189.
Rose, A. L. (1954). *Aust. Vet. J.* **30,** 172.
Ross, E. J., and Gibson, W. W. C. (1969). *Vet. Rec.* **84,** 520.
Ross, J. W., Jr. (1967). *Science* **156,** 1378.
Rubarth, S., and Krook, L. (1968). *Acta Vet. Scand.* **9,** 253.
Russell, F. C. (1944). *Imp. Bur. Anim. Nutr.* (Rowett Res. Inst.), Tech. Commun. No. 15, p. 69.
Saarinen, P. (1950). *Maataloustieteelinen Aikak.* **22** [see *Nutr. Abstr. Rev.* **20,** 1058 (1950)].
Sachs, M., Bar, A., Cohen, R., Mazur, Y., Mayer, E., and Hurwitz, S. (1977). *Am. J. Vet. Res.* **38,** 2039.
Salt, F. J. (1950). *Lab. J. (London)* **8,** 357.
Sansom, B. F., Allen, W. M., Davies, D. C., Hoare, M. N., Stenton, J. R., and Vagg, M. J. (1976). *Vet. Rec.* **99,** 310.
Scheunert, A. (1923). *Landwirtsch. Ztg.* **43,** 351.
Schiefer, B. (1970). *Berlin. Muench. Tieraerztl. Wochenschr.* **83,** 214.
Schmidt, H. (1940). *J. Am. Vet. Med. Assoc.* **96,** 441.
Schmidt, J. (1897). *Maanedsskr. Dyrlaeger* **9,** 225.
Scholz, H., and Meyer, H. (1973). *Dtsch. Tieraerztl. Wochenschr.* **80,** 545.
Schryver, H. F., Hintz, H. F., and Craig, P. H. (1971). *J. Nutr.* **101,** 259.
Schryver, H. F., Hintz, H. F., Craig, P. H., Hogue, D. E., and Lowe, J. E. (1972). *J. Nutr.* **102,** 143.
Schryver, H. F., Hintz, H. F., and Lowe, J. E. (1974). *Cornell Vet.* **64,** 493.
Schulhof, A. (1933). *Klin. Spisy Skoly Zverolek., Brno* **10,** 23 [see *Nutr. Abstr. Rev.* **4,** 699 (1934–1935)].
Scott, D. (1965). *Q. J. Exp. Physiol. Cogn. Med. Sci.* **50,** 312.
Scott, D. (1971). *Q. J. Exp. Physiol. Cogn. Med. Sci.* **56,** 169.
Scott, D. (1972). *Q. J. Exp. Physiol. Cogn. Med. Sci.* **57,** 379.
Scott, D., and Dobson, A. (1965). *Q. J. Exp. Physiol. Cogn. Med. Sci.* **50,** 42.
Seekles, L. (1964). *Rep. Int. Meet. Dis. Cattle, 3rd, Copenhagen* p. 119.
Seekles, L., and Boogaerdt, J. (1955). *Tijdschr. Diergeneeskd.* **80,** 331.
Seekles, L., and Boogaerdt, J. (1956). *Tijdschr. Diergeneeskd.* **81,** 281.
Seekles, L., and Sjollema, B. (1932). *Arch. Wiss. Prakt. Tierheilkd.* **65,** 331.
Seekles, L., and Van Asperen, K. (1949). *Tijdschr. Diergeneeskd.* **74,** 191.
Seekles, L., Sjollema, B., and Van der Kaay, F. C. (1931). *Tijdschr. Diergeneeskd.* **58,** 750.
Seekles, L., Reitsma, P., De Man, T. J., and Wilson, J. H. G. (1958). *Tijdschr. Diergeneeskd.* **83,** 125.
Seekles, L., Wilson, J. H. G., and Philips-Duphar, N. V. (1964). *Vet. Rec.* **76,** 486.
Sherwood, L. M., Potts, J. T., Jr., Care, A. D., Mayer, G. P., and Aurbach, G. D. (1966). *Nature (London)* **209,** 52.
Sherwood, L. M., Mayer, G. P., Ramberg, C. F., Kronfeld, D. S., Aurbach, G. D., and Potts, J. T. (1968). *Endocrinology* **83,** 1043.
Simesen, M. G. (1958). *Rep. Nord. Vet. Congr., 8th, Helsingfors, Sect. B* No. 9, p. 344.
Simesen, M. G. (1959). *Main Pap. Int. Vet. Congr., 16th, Madrid* **2,** 85.
Simesen, M. G. (1963a). *In* "Clinical Biochemistry of Domestic Animals" (C. E. Cornelius and J. J. Kaneko, eds.), p. 441. Academic Press, New York.
Simesen, M. G. (1963b). *Proc. Int. Vet. Congr., 17th, Hannover* **1,** 117.
Simesen, M. G. (1964). *Nord. Veterinaermed.* **16,** Suppl. 1, 167.

Simesen, M. G. (1968). *Medlemsbl. Dan. Dyrlaegeforen.* **51,** 848.
Simesen, M. G. (1971). *Nord. Veterinaermed.* **23,** 99.
Simesen, M. G. (1972). *Nord. Veterinaermed.* **24,** 85.
Simesen, M. G. (1977a). *Nord. Veterinaermed.* **29,** 76.
Simesen, M. G. (1977b). *Nord. Veterinaermed.* **29,** 284.
Simesen, M. G., and Hyldgaard-Jensen, C. (1971). *Nord. Veterinaermed.* **23,** 35.
Simesen, M. G., Lunaas, T., Rogers, T. A., and Luick, J. R. (1962). *Acta Vet. Scand.* **3,** 175.
Simesen, M. G., Rogers, T. A., Lunaas, T., and Luick, J. R. (1965). *Proc. Symp. Radioisot. Anim. Nutr. Physiol., Prague, 1964* p. 721. IAEA., Vienna.
Sims, F. H., and Crookshank. H. R. (1956). *Tex. Agric. Exp. Stn., Bull.* No. 842.
Sjollema, B. (1928). *Tijdschr. Diergeneeskd.* **55,** 1016, 1085, 1121, 1187.
Sjollema, B. (1930). *Vet. Rec.* **10,** 425, 450.
Sjollema, B. (1932a). *Nutr. Abstr. Rev.* **1,** 621.
Sjollema, B. (1932b). *Zentralbl. Tierernaehr.* **3,** 507.
Sjollema, B., and Seekles, L. (1929). *Tijdschr. Diergeneeskd.* **56,** 979.
Sjollema, B., and Seekles, L. (1930). *Biochem. Z.* **229,** 358.
Sjollema, B., and Seekles, L. (1932). *Klin. Wochenschr.* **11,** 989.
Sjollema, B., Grashuis, J., Van Koetsveld, E. E., and Lehr, J. J. (1955a). *Tijdschr. Diergeneeskd.* **80,** 579.
Sjollema, B., Van Koetsveld, E. E., Grashuis, J., and Lehr, J. J. (1955b). *Tijdschr. Diergeneeskd.* **80,** 1111.
Smith, A. H., Kleiber, M., Black, A. L., and Luick, J. R. (1955a). *J. Nutr.* **57,** 497.
Smith, A. H., Kleiber, M., Black, A. L., and Baxter, C. F. (1955b). *J. Nutr.* **57,** 507.
Smith, A. H., Kleiber, M., Black, A. L., and Lofgreen, G. P. (1956). *J. Nutr.* **58,** 95.
Smith, J. A. B., Howat, G. R., and Ray, S. C. (1938). *J. Dairy Res.* **9,** 310.
Smith, M. L., Lee, L., Sheppard, S. J., and Fariss, B. L. (1978). *Am. J. Vet. Res.* **39,** 321.
Smith, R. H. (1957). *Biochem. J.* **67,** 472.
Smith, R. H. (1958). *Biochem. J.* **70,** 201.
Smith, R. H. (1959a). *Biochem. J.* **71,** 306.
Smith, R. H. (1959b). *Biochem. J.* **71,** 609.
Smith, R. H. (1959c). *Nature (London)* **184,** 821.
Smith, R. H. (1962). *Biochem. J.* **83,** 151.
Smith, R. H. (1964). *Nord. Veterinaermed.* **16,** Suppl. 1, 143.
Smith, R. H. (1969). *Proc. Nutr. Soc.* **28,** 151.
Smith, R. H., and McAllan, A. B. (1966). *Br. J. Nutr.* **20,** 703.
Smith, R. H., McAllan, A. B., and Hill, W. B. (1968). *Proc. Nutr. Soc.* **27,** 48A.
Smith, S. E., and Barnes, L. L. (1941). Unpublished observations. Ref. in "Nutrition Requirements of Mink and Foxes," p. 45. Natl. Acad. Sci., Washington, D.C., 1968.
Smith, V. R., and Blosser, T. H. (1947). *J. Dairy Sci.* **30,** 861.
Smith, V. R., and Brown, W. H. (1963). *J. Dairy Sci.* **46,** 223.
Smith, V. R., Niedermeier, R. P., and Hansen, R. G. (1948). *J. Dairy Sci.* **31,** 173.
Smyth, P. J., Conway, A., and Walsh, M. J. (1958). *Vet. Rec.* **70,** 846.
Somjen, G., Hilmy, M., and Stephen, C. R. (1966). *J. Pharmacol. Exp. Ther.* **154,** 652.
Spratling, F. R., Bridge, P. S., Rest, J. R., and Cruickshank, E. M. (1970). *Br. Vet. J.* **126,** 316.
Stacy, B. D., and Wilson, B. W. (1970). *J. Physiol. (London)* **210,** 549.
Stevenson, D. E., and Wilson, A. A. (1963). "Metabolic Disorders of Domestic Animals," p. 75. Blackwell, Oxford.
Stewart, E. V., and Longwell, B. B. (1969). *Am. J. Vet. Res.* **30,** 907.
Stewart, J. (1954). *Scot. Agric.* **34,** 68.
Stewart, J., and Reith, J. W. S. (1956). *J. Comp. Pathol. Ther.* **66,** 1.
Storry, J. E. (1961a). *J. Agric. Sci.* **57,** 97.
Storry, J. E. (1961b). *J. Agric. Sci.* **57,** 103.
Storry, J. E., and Rook, J. A. F. (1962). *J. Sci. Food Agric.* **13,** 621.
Storry, J. E., and Rook, J. A. F. (1963). *J. Agric. Sci.* **61,** 167.
Stott, G. H., and Smith, V. R. (1957). *J. Dairy Sci.* **40,** 897.
Stott, G. H., and Smith, V. R. (1964). *J. Dairy Sci.* **47,** 426.
Straub, O. C., Peoples, S. A., and Cornelius, C. E. (1959). *Cornell Vet.* **49,** 324.
Suomalainen, P. (1944). *Sitzungsber. Finn. Akad. Wiss.* p. 163 [see *Biol. Abstr.* **20,** 12101 (1946)].

Suttle, N. F., and Field, A. C. (1969). *Br. J. Nutr.* **23,** 81.
Svanberg, O. (1932). *K. Lantbruksakad. Handl. Tidskr.* **71,** 41.
Swan, J. B., and Jamieson, N. D. (1956). *N.Z. J. Sci. Technol., Sect. A* **38,** 137, 316, 363.
Talapatra, S. K., Ray, S. C., and Sen, K. C. (1948). *J. Agric. Sci.* **38,** 163.
Taylor, A. N., and Wasserman, R. H. (1972). *Am. J. Physiol.* **223,** 110.
Taylor, T. G. (1959). *J. Agric. Sci.* **52,** 207.
Teears, R. J., Barnes, B. A., Batsakis, J. G., and Bloch, D. M. (1977). *Am. J. Clin. Pathol.* **68,** 159.
Tennant, B., Lowe, J. E., and Tasker, J. B. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 670.
Terkildsen, T. C. (1950). Thesis, Univ. of Copenhagen.
'tHart, M. L., and Kemp, A. (1956). *Tijdschr. Diergeneeskd.* **81,** 84.
'tHart, M. L., and Kemp, A. (1957). *E.P.A. Proj. Utrecht* No. 204, p. 193 (European Productivity Agency).
Theiler, A., and Green, H. H. (1932). *Nutr. Abstr. Rev.* **1,** 359.
Theiler, A., Green, H. H., and DuToit, P. J. (1924). *J. Dep. Agric. Union S. Afr.* **8,** 460.
Theiler, A., Viljoen, P. R., Green, H. H., DuToit, P. J., Meier, H., and Robinson, E. M. (1927). *Director, Vet. Educ. Res. S. Afr. 11th & 12th Rep.* p. 821.
Thomas, J. W., and Okamoto, M. (1958). *U.S. Dep. Agric., Agric. Res. Serv.* **44.**
Thomson, D. L., and Collip, J. B. (1932). *Physiol. Rev.* **12,** 309.
Todd, J. R. (1967). *Vet. Rec.* **81,** Clin. Suppl. VI.
Todd, J. R., and Horvath, D. J. (1970). *Br. Vet. J.* **126,** 333.
Todd, J. R., and Morrison, N. E. (1964). *J. Br. Grassl. Soc.* **19,** 179.
Todd, J. R., and Rankin, J. E. F. (1959). *Vet. Rec.* **71,** 256.
Todd, J. R., and Thompson, R. H. (1960). *Br. Vet. J.* **116,** 437.
Todd, J. R., and Thompson, R. H. (1962). *Res. Vet. Sci.* **3,** 449.
Todd, J. R., and Thompson, R. H. (1968). Unpublished observations. See Todd *et al.* (1969). *Vet. Rec.* **84,** 176.
Todd, J. R., Scally, W. C. P., and Ingram, J. M. (1966). *Vet. Rec.* **78,** 888.
Todd, J. R., Horvath, D. J., and Anido, V. (1969). *Vet. Rec.* **84,** 176.
Tomas, F. M. (1974). *Aust. J. Agric. Res.* **25,** 495.
Tomas, F. M., and Potter, B. J. (1976). *Br. J. Nutr.* **36,** 37.
Trommer, F., and Mehnert, E. (1973). *Monatsh. Veterinaermed.* **28,** 62.
Turner, A. W., Kelley, R. B., and Dann, A. T. (1935). *J. Counc. Sci. Ind. Res.* **8,** 120.
Turner, C. W. (1930). *Mo. Univ. Agric. Exp. Stn., Bull.* No. 285, p. 65.
Udall, R. H. (1947). *Cornell Vet.* **37,** 314.
Vagg, M. J., and Payne, J. M. (1970). *Br. Vet. J.* **126,** 531.
Van der Horst, C. J. G. (1960). *Tijdschr. Diergeneeskd.* **85,** 1060.
Van Koetsveld, E. E. (1955). *Tijdschr. Diergeneeskd.* **80,** 525.
Van Koetsveld, E. E. (1964). *Tijdschr. Diergeneeskd.* **89,** 590.
Van Kreveld, A., and Van Minnen, G. (1955). *Neth. Milk Dairy J.* **9,** 1.
Van Maltits, L. (1945). *J. S. Afr. Vet. Med. Assoc.* **16,** 9.
Van Soest, P. J., and Blosser, T. H. (1954). *J. Dairy Sci.* **37,** 185.
Van'T Klooster, A. T. (1967). *Meded. Landbouwhogesch. Wageningen* **67**, No. 5.
Van'T Klooster, A. T. (1976). *Z. Tierphysiol., Tierernaehr. Futtermittelkd.* **37,** 169.
Van'T Klooster, A. T., and Care, A. D. (1966). *Biochem. J.* **99,** 2.
Vaughan, J. M. (1973). "The Effect of Irradiation on the Skeleton." Oxford Univ. Press, London and New York.
Verdaris, J. N., and Evans, J. L. (1976). *J. Dairy Sci.* **59,** 1271.
Verdeyen, J. (1953). *C. R. Rech. IRSIA* **9,** 87.
Visek, W. J., Monroe, R. A., Swanson, E. W., and Comar, C. L. (1953). *J. Nutr.* **50,** 23.
Vitale, J. J., Hegsted, D. M., Nakamura, M., and Connors, P. (1957). *J. Biol. Chem.* **226,** 597.
Wacker, W. E. C., and Parisi, A. F. (1968). *N. Engl. J. Med.* **278,** 658, 712, 772.
Wacker, W. E. C., and Vallee, B. L. (1958). *N. Engl. J. Med.* **259,** 431, 475.
Walsh, M. J., and Conway, A. (1960). *Proc. Int. Grassl. Congr., 8th, Reading, Engl.* p. 548. Grassl. Res. Inst., Henley England.
Ward, G. M. (1956). *Ann. N.Y. Acad. Sci.* **64,** 361.
Ward, G. M., Blosser, T. H., Adams, M. F., and Crilly, J. B. (1953). *J. Dairy Sci.* **36,** 39.
Wasserman, R. H. (1968). *Calcif. Tissue Res.* **2,** 301.

Wasserman, R. H., and Taylor, A. N. (1973). *J. Nutr.* **103,** 586.
Wasserman, R. H., Corradino, R. A., Feher, J., and Armbrecht, H. J. (1977a). *In* "Vitamin D" (A. W. Norman, K. Schaefer, J. W. Coburn, H. F. DeLuca, D. Fraser, H. G. Grigoleit, and D. v. Herrath, eds.), p. 331. de Gruyter, Berlin.
Wasserman, R. H., Krook, L., and Dirksen, G. (1977b). *Cornell Vet.* **67,** 334.
Watts, C., and Campbell, J. R. (1971). *Res. Vet. Sci.* **12,** 234.
Watts, P. S. (1959). *J. Agric. Sci.* **52,** 244, 250.
Wenger, R. D. (1945). *North Am. Vet.* **26,** 289.
Wentworth, R. A., and Smith, S. E. (1961). *Proc. Cornell Nutr. Conf.* p. 53.
Werner, W. (1960). *Landwirtsch. Forsch.* **12,** 133.
Westerhuis, J. H. (1974). Thesis, State University Utrecht.
White, R. R., Christian, K. R., and Williams, V. J. (1957). *N.Z. J. Sci. Technol. Sect. A* **38,** 440.
Widmark, E., and Carlens, O. (1925a). *Sven. Veterinaertidskr.* **30,** 1 [Engl. transl. by C. A. Nelson, *North. Am. Vet.* **6,** 28 (1925)].
Widmark, E., and Carlens, O. (1925b). *Biochem. Z.* **158,** 81.
Widmark, E., and Carlens, O. (1925c). *Biochem. Z.* **156,** 454.
Wieland, W. (1940). *Tieraerztl. Rundsch.* **46,** 522.
Wiggers, K. D., Nelson, D. K., and Jacobson, N. L. (1975). *J. Dairy Sci.* **58,** 430.
Wilson, A. A. (1955). *Br. Vet. J.* **110,** 233.
Wilson, A. A. (1960). *Vet. Rev. Annot.* **6,** 39.
Wilson, L. T., and Hart, E. B. (1932). *J. Dairy Sci.* **15,** 116.
Wind, J., Deijs, W. B., and Kemp, A. (1966). *Jaarb. Inst. Biol. Scheik. Onderz. LandbGewass.* Pp. 91–100 [*Vet. Bull. (London)* **37,** Abstr. No. 2776 (1967)].
Wise, M. B., Smith, S. E., and Barnes, L. L. (1958). *J. Anim. Sci.* **17,** 89.
Wise, M. B., Ordoveza, A. L., and Barrick, E. R. (1963). *J. Nutr.* **79,** 79.
Wittwer, F. G., and Ford, E. J. H. (1978). *Vet. Rec.* **102,** 442.
Wolff, W. A., Tumbleson, M. E., and Littleton, C. A. (1969). *Adv. Autom. Anal.* **3,** 179.
Wolton, K. M. (1963). *NAAS Q. Rev.* **14,** No. 59, 122.
Wrenn, T. R., Bitman, J., Cecil, H. C., and Gilliam, D. R. (1963). *J. Dairy Sci.* **156,** 1243.
Yarrington, J. T., Capen, C. C., Black, H. E., Re, R., Potts, J. T., and Geho, W. B. (1976). *Am. J. Pathol.* **83,** 569.
Young, V. R., Richards, W. P. C., Lofgreen, G. P., and Luick, J. R. (1966a). *Br. J. Nutr.* **20,** 783.
Young, V. R., Lofgreen, G. P., and Luick, J. R. (1966b). *Br. J. Nutr.* **20,** 795.

16

Iron Metabolism

JIRO J. KANEKO

I. INTRODUCTION

Iron is an element of fundamental importance in the vital respiratory processes of life. It is a constituent of the respiratory pigment hemoglobin, myoglobin, and the heme enzymes---catalase, peroxidase, and cytochromes. While it is one of the most abundant elements in the environment, iron deficiency is the most common cause of anemia in man. Iron deficiency per se is of less importance in the adult domestic animal than in man. It is, however, of equal importance in the young of all species during the period of rapid growth, particularly if they are dependent upon a milk-only diet. Disorders of iron

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

metabolism may have a variety of other causes in domestic animals. Thus, an understanding of iron metabolism is required for the clarification and differentiation of the anemias.

II. IRON BALANCE

A. Distribution

The results of extensive iron balance studies were reviewed by Drabkin (1951) and Granick (1954), and their estimates of iron distribution in the body are in close agreement (Table I). Of a total of 4 gm iron in a 70-kg man, approximately 65% is in hemoglobin, 3% in myoglobin, and 30% in the iron storage compounds ferritin and hemosiderin. The remainder is distributed among the heme enzymes, the nonheme enzymes, and the iron transport protein transferrin (siderophilin). It was estimated (Lintzel and Radeff, 1931) that the body iron content of pups ranges as high as 79 mg/kg body weight. Hemoglobin iron (43 mg/kg)* accounts for greater than one-half. Thus, the percent distribution of iron in the dog is essentially similar to that in man. More recently, Kolb (1963) reviewed and presented extensive data on iron balance in farm animals which indicate that, qualitatively, iron balance is also similar in all species.

B. Function

Iron combines with a variety of proteins in nature, and, depending on the type of combination, various functions are carried out. The most important function of iron is to combine with protoporphyrin (see Chapter 4) to form heme, and the heme combines with various proteins to form the heme proteins. If the protein is a globin, hemoglobin and myoglobin are formed. If the protein is an apoenzyme, heme enzymes are formed. Heme enzymes are of fundamental importance in the vital respiratory processes of life, namely, in the cytochrome system, the ultimate site of O_2 utilization.

Hemoglobin is a protein of 64,458 daltons composed of two α and two β polypeptide chains, four protoporphyrin molecules, and 4 moles of ferrous (Fe^{2+}) iron. Hemoglobin contains 3.34 mg iron per gram. The iron of hemoglobin binds oxygen (O_2) reversibly, and it functions to transport oxygen and CO_2 to and from the tissues. Myoglobin is smaller, a single polypeptide of 17,000 daltons and binds only one molecule of heme. Myoglobin also binds O_2 reversibly and functions as a reserve supply of O_2 in the muscles. The high content of myoglobin in marine mammals, such as the seal and whale, is thought to permit their extended underwater activities (Kendrew *et al.* 1954). Without adequate iron, insufficient hemoglobin is produced, O_2 supply to the tissues is reduced, and all the clinical manifestations of anemia ensue.

The major functions of nonheme iron compounds is iron transport and storage. Iron is transported in the plasma in the ferric (Fe^{3+}) form bound to a specific iron-binding protein, transferrin (siderophilin), which migrates with β_1-globulin under electrophoresis. The storage form of iron is also the Fe^{3+} form and is present in either ferritin or hemosiderin. These nonheme compounds are discussed more fully in Section IV.

*Estimated for 9% blood volume, 14 gm hemoglobin per 100 ml, 0.34% iron in hemoglobin.

TABLE I

Distribution of Iron in Heme and Nonheme[a] Compounds

Species (wt)	Heme iron		Nonheme iron		Reference
	mg	%	mg	%	
Dog (10 kg)	0.43	55	0.36	45	Lintzel and Radeff (1931)
Horse (400 kg)	11.5	67	5.6	33	Obara and Nakajima (1961a)
Cow (386 kg)	9.2	55	7.7	45	Kaneko (1963)
Man (70 kg)	2.67	67	1.33	33	Drabkin (1951)

[a] Approximate nonheme iron distribution is as follows: 12%, hemosiderin; 13%, ferritin; 3%, myoglobin; 1%, transferrin, cytochromes, peroxidase, catalase; 4%, unknown.

There is little established of the iron requirements of animals. The nutritional iron deficiency of calves fed only milk has been known for some time (Knoop *et al.*, 1935; Hibbs *et al.*, 1963), but few balance studies have been conducted (Matrone *et al.*, 1957). More is known of the iron requirements of baby pigs, which are highly susceptible to iron deficiency. If the baby pig on a milk diet is kept in clean-floored pens without access to iron sources, anemia can develop within a few weeks. Dietary iron requirements for young pigs can be met with iron contents in the feedstuffs of 60–125 mg/kg feed (Matrone *et al.*, 1960; Ullrey *et al.*, 1960). Kolb (1963) estimated the iron requirements of farm animals, as shown in Table II. The requirement for the cat is only an estimate based on its more rapid iron turnover as compared to the dog and taking its size into account.

TABLE II

Estimated Dietary Iron Requirements for Domestic Animals[a]

Species	Iron (mg/day)
Calves	25-30
Yearling cattle	40-50
Milking cows	50-60
Pregnant cows	60-80
Sheep	10-15
Horses	50-80
Feeder pigs	30-40
Sows	40-60
Laying hens	2-3
Dogs	10-30
Cats[b]	5-15

[a] Modified after Kolb (1963).

[b] Estimated on the basis of its turnover data and body size.

III. DIETARY IRON

The recommended (National Research Council, 1962) minimal daily requirement (MDR) for iron for the dog is 1.3 mg/kg body weight. However, a large percentage of this food iron is tightly bound to phytates and phosphates and is unavailable for absorption. It has been estimated (National Research Council, 1962) that approximately one-half of the food iron is available for absorption. The actual amount absorbed varies with the composition of the diet. Table III gives the iron contents and estimated percent absorption for some foods. Highest iron contents (5–18 mg/100 gm) are found among the organ meats, eggs, and legumes, whole milk and milk products, starches, and fruits generally contain less

TABLE III

Iron Content of Some Foods and Its Intestinal Absorption

Food source[a]	Iron content (mg/100 gm)	% absorbed[b]	Reference for absorption
Liver	6.6–18.0		
Brain	3.6		
Heart	4.6		
Kidney	7.9		
Spleen	8.9		
Pancreas	6.0		
Red meats	2.2–5.1	10.4–38.3 (20.3)	Layrisse *et al.* (1969)
Poultry	1.5–3.8		
Hemoglobin (uncooked)	340 mg	6.7–30.8 (15.6)	Layrisse *et al.* (1969)
		1.0–21.0 (19)	Callender *et al.* (1957)
Hemoglobin (cooked)	340 mg	0–16 (7)	Callender *et al.* (1957)
Fish	0.4–1.1	1.9–42.4 (18.3)	Layrisse *et al.* (1969)
Fish (cnd)	0.9–2.7		
Egg yolk	7.2		
Eggs, whole	2.7	0.5–5.0 (1.4)	Chodos *et al.* (1957)
Nuts	1.9–5.0		
Dried fruits	1.4–6.9		
Wheat	3.0–4.3	1.1–7.4 (4.5)	Hussain *et al.* (1965)
		0.4–22.5 (7.9)	Layrisse *et al.* (1969)
Wheat germ	8.1		
Wheat bran	16.7		
Legumes	4.7–8.0	0.7–6.4 (3.2)	Layrisse *et al.* (1969)
Soybean flour	13	1.7–42.2 (17.9)	Layrisse *et al.* (1969)
Green vegetables	1.6–3.0	1.7–5.8	Layrisse *et al.* (1969)
		0.5–2.3	Chodos *et al.* (1957)
Root vegetables	0.5–1.0	0.5–2.3	Chodos *et al.* (1957)
Cornmeal	1.0–2.7	0.2–14.8 (5.9)	Layrisse *et al.* (1969)
Milk, whole	0.07		
Coarse hays	14–22[c]		
Grains	3.0–14[c]		
Blood meal	311[c]		
Fish meal	21–80[c]		

[a] Compiled from various nutritive and feed tables.

[b] Numbers in parentheses are means.

[c] Milligrams per 100 gm dry weight.

than 1 mg iron per 100 gm. The amount of the iron absorbed from these foods varies from less than 10% for egg and liver iron to as high as 30% for muscle and hemoglobin iron (Moore, 1961; Moore and Dubach, 1951). This indicates that the actual absorption of iron is considerably less than one-half of the MDR of 1.3 mg/kg. It has been estimated from radioiron feeding studies that normal dogs absorb 5–10% of ingested iron (Hahn *et al.*, 1939; Stewart and Gambino, 1961). In general, anemia increases iron absorption. Thus, iron absorption is increased in hemolytic anemia, iron deficiency pregnancy, and blood loss and is decreased in transfusional polycythemia, iron loading, and aplastic anemia. During early life and during periods of rapid growth, iron absorption also increases.

IV. IRON METABOLISM

A. Absorption

The central role of the absorptive mechanism in iron metabolism (Fig. 1) has long been recognized but has not been clearly defined. There has recently been extensive and renewed interest in control of iron absorption as evidenced by the number of excellent reviews (Conrad, 1967; Callender, 1967; Bothwell, 1968; Van Campen, 1974). A critique of iron absorption studies has been published by Cook *et al.* (1969). Despite the many years of intensive study, the precise regulatory mechanism of iron absorption is still not known.

1. Luminal Factors

Dietary iron is principally in the Fe^{3+} form and occurs either as organic or inorganically bound iron. As a general rule, as the amount of iron in the diet increases, on a percentage basis the amount of iron absorbed decreases. A large percentage of food iron is bound to phytates and phosphates. These forms are unavailable for absorption, and a number of luminal factors affect this availability. Dietary iron must first enter the acidic environment of the stomach. The acidity in the stomach is sufficient to release iron from proteins and

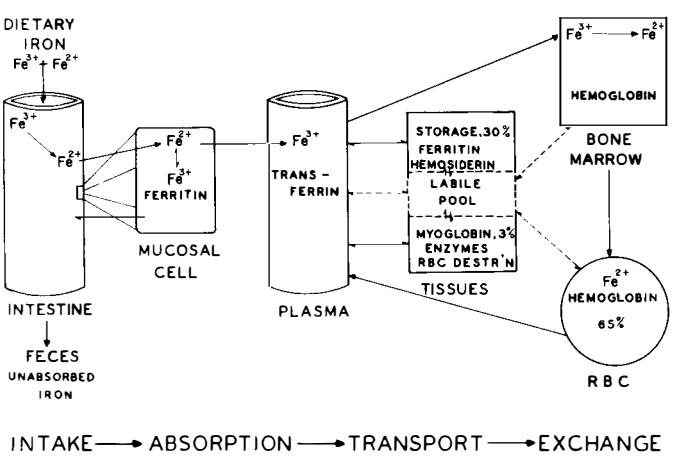


Fig. 1. Pathways of iron metabolism. Single arrows show a unidirectional flow of iron to emphasize the limited excretion of iron and its "closed" cycle in the body. (From Kaneko, 1964; by permission.)

also to maintain the available Fe^{3+} and Fe^{2+} in solution (Cook *et al.*, 1964). In this way, gastric HCl appears to potentiate Fe^{2+} absorption (Jacobs *et al.*, 1964).

The iron next passes into the alkaline environment of the small intestine. In the upper duodenum, the pH and the redox potential appear to be optimal, and reducing substances, such as ascorbic acid, reduce Fe^{3+} to Fe^{2+}, which is more soluble in the alkaline condition. These factors also help to maintain iron in the Fe^{2+} state that is available for absorption. Foods containing large amounts of oxalates, phytates, or phosphates, which bind more of this iron in the duodenum, may further decrease the availability of the iron. Heme iron is apparently absorbed differently than inorganic iron; it is rather efficient, is not bound to phytates, and tends to be constant. Thus, the composition of the diet as well as the amount of iron affects the availability of iron for absorption in the intestine. The pancreatic enzymes, however, do not appear to play a role (Balcerzak *et al.*, 1967).

Other luminal factors of importance may be gastroferrin, an iron-binding protein found in gastric juice (Davis *et al.*, 1966), and an undefined gastric factor in gastric juice (Murray and Stein, 1968). Gastroferrin is proposed to contribute to iron absorption regulation by binding iron and thus preventing its absorption. The gastric factor is suggested to actively promote iron absorption. Neither factor has yet been established as the primary regulator of iron absorption.

2. *Mucosal Factors*

Iron in the Fe^{2+} form is known to be absorbed throughout the intestine, but the transfer of iron to the plasma occurs mainly in the duodenum (Manis and Schachter, 1962). The control of body iron content is unique in that it appears to be controlled by the rate of absorption rather than by excretion. McCance and Widdowson (1937, 1938) in their well-known studies demonstrated the limited excretion of iron by animals. Later studies of Hahn *et al.* (1943) and Granick (1946) led to the "mucosal block" theory of control of iron absorption by the intestinal mucosal cell. This concept held that, when all the apoferritin in the mucosal cell was saturated with iron to form ferritin, no further uptake could occur.

Therefore, the amount of apoferritin and its degree of saturation would control iron absorption (Granick, 1954). This concept did not receive experimental support (Brown *et al.*, 1958; Chodos *et al.*, 1957) but did provide impetus for seeking other control mechanisms for iron absorption. It has become clear from metabolic studies that iron absorption in general varies (1) inversely with the body iron stores and (2) directly with the rate of erythropoiesis. This would imply a mechanism of "feedback" control by which the absorptive process in the duodenum is governed by the body's need for iron. This concept also places the mucosal cell in a central role in the control of iron absorption and has caused renewed interest in its role.

Conrad *et al.* (1964) studied the incorporation of ^{59}Fe into intestinal lumen cells by autoradiography. Their studies suggested that iron is incorporated into the intestinal cells as they are being formed in the crypts of Lieberkühn. The iron stays in these cells as they migrate upward toward the top of the intestinal villus and is lost when the cell is exfoliated, if it was not transferred to the plasma. The total iron content of the cell appears to control iron uptake in an inverse manner. Since the intestinal cell turnover is about 2–3 days (Creamer, 1967), this also serves as a means of excretion of body iron. Thus, the mucosal cell once again appears to be the site of regulatory control of iron absorption and excretion, although not as a "mucosal block."

Iron in the mucosal cell is thought to occur in two forms: a storage or slowly turning over pool of Fe^{3+} iron, which is probably ferritin (Charlton *et al.*, 1965), and a more rapidly turning over "labile" pool of iron. The content of ferritin in the mucosal cell is thought to be about 8% of the total protein-bound Fe^{3+} (Pinkerton, 1969). The intake of iron occurs in two phases: (1) absorption into the mucosal cell and (2) its transfer into the plasma (Wheby *et al.*, 1964). The iron transfer step appears to be rate limiting because the cell increases iron absorption during periods of high luminal content but the transfer to plasma remains constant (Manis and Schachter, 1962). The iron transfer system is an active transport process which requires ATP and oxygen (Manis and Schachter, 1964; Jacobs *et al.*, 1966).

Conrad and Crosby (1963) and Conrad (1967) proposed that iron absorption is regulated primarily through the mucosal cell. According to this concept the iron deposits within the mucosal cells regulates, within limits, the amount of iron which enters the body. Mucosal cell iron may enter the body or remain in the cell to be lost when the cell is exfoliated. In iron deficiency, little iron is in the cell because of increased body requirements and increased iron transfer to plasma. Therefore, absorption is enhanced, and very little iron is excreted. In iron overload, iron from the body iron pool loads the mucosal cell and blocks further uptake from the intestine. When the cell is exfoliated, the excess iron in the cell is lost with it, and excretion is thereby also enhanced. Pinkerton (1969) speculated that an unknown carrier substance and a hypothetical "iron transferase" enzyme participate in the transfer to the plasma at the cell membrane.

The absorption of intact heme by the mucosal cell appears to be a separate and distinct phenomenon. Heme can be absorbed and transferred to the plasma intact, or it can be broken down and its Fe^{2+} transported to the plasma (Conrad *et al.*, 1966; Weintraub *et al.*, 1968). Both mechanisms appear to operate in the dog.

3. Metabolic Factors

As mentioned, the rate of erythropoiesis and the state of the tissue iron stores are important stimuli for iron absorption. Accelerated erythropoiesis, whether caused by hemorrhage, hemolysis, or hypoxia, always seems to be associated with increased iron absorption. Conversely, decreased erythropoiesis diminishes absorption. Numerous studies, however, have failed to uncover a humoral or other messenger substance which links erythropoiesis or iron stores to the gut (Beutler and Buttenweiser, 1960).

B. Transport

After the movement of iron through the mucosal cell, it is rapidly and tightly bound to a specific iron-binding protein in the plasma. This protein has been called transferrin or siderophilin, has an electrophoretic mobility of β-globulin, and is found in Cohn's fraction IV_7. The transferrins are actually a group of proteins distinguishable by their electrophoretic mobility on starch and are under genetic control (Smithies and Hiller, 1959). The various types of transferrins do not show differences in their iron transport functions and can therefore be considered in this discussion as a single entity with respect to iron transport.

Transferrin is a colorless protein with a molecular weight of about 90,000 and an ability to bind tightly 2 moles of ferric iron. The Fe-transferrin complex, however, has a characteristic salmon pink color (Schada and Caroline, 1946), which is used for the measure-

ment of unbound transferrin in plasma. This is usually referred to as the unbound iron-binding capacity (UIBC) and expressed as micrograms Fe per deciliter serum or plasma. Normally, approximately two-thirds of the transferrin is unbound to iron. The other one-third is bound to Fe^{3+}, and therefore serum iron concentration is a measure of the bound transferrin. The total iron-binding capacity is the sum of the serum iron concentration and the UIBC. The major role of transferrin in iron metabolism is the transport of iron to and from "compartments" or acceptor sites in the body. Thus, it occupies a central position in the body exchange mechanism shown in Fig. 1. Iron is given up by transfer at the surface of the cell membrane of receptor cells, principally those of the bone marrow for hemoglobin synthesis (see Chapter 4, Fig. 7).

C. Turnover

The scheme in Fig. 1 summarizes the internal iron exchange or turnover of iron in the animal body. It was evident early (McCance and Widdowson, 1937, 1938) that iron was uniquely confined or trapped in an essentially closed cycle within the body with little loss by the normal routes of excretion. It is generally accepted that about 1 mg/day is absorbed and excreted. A large percentage of the total body iron, such as that contained in the intact erythrocytes, ferritin, or hemosiderin, is not a readily available part of the metabolic pool. A smaller and more readily available pool of iron, which is called the "labile iron pool," is also shown in Fig. 1. The concept of this "labile iron pool" has arisen largely as a result of study of radioiron metabolism. By analysis of radioiron turnover data, it was postulated that a small portion of the total body iron pool was in sufficiently rapid flux with major iron metabolic sites in the body to be considered a single pool. This pool was called the "labile iron pool," and Pollycove and Maqsood (1962) identified a pool of this type on the cell membranes of the immature erythrocytic bone marrow cells in the dog.

The flux of iron among the various compartments, as shown in Fig. 1, is most readily studied by (1) labeling the plasma transferrin with ^{59}Fe, and (2) following the plasma disappearance and appearance of radioactivity in the other compartments. Sophisticated analyses of these complex curves result in a description of erythropoiesis and iron metabolism in quantitative terms (Pollycove and Mortimer, 1961). The figures for the distribution of iron (Fig. 1, Table I) are in part a result of analysis of these curves. From these curves, the flux of iron in the plasma and among pools may also be calculated, and some ferrokinetic data on iron turnover derived from the curves are given in Table IV.

D. Storage

In the tissues, iron that is not required for hemoglobin synthesis is transferred to the reticuloendothelial cells of the liver, spleen, and bone marrow for incorporation into the iron storage compounds ferritin and hemosiderin. Ferritin is a soluble ferric hydroxide–phosphoprotein complex consisting of the protein apoferritin and iron. It is a large compound (450,000 daltons) which contains about 23% Fe in the Fe^{3+} form. It is colorless and soluble and cannot be detected by light microscopy. Ferritin is one of the main storage forms of iron in the body.

Ferritin is synthesized in small quantities by nearly all mammalian cells, but the major site is the liver cell. It was once thought that ferritin was a multimeric molecule consisting of about 24 subunits each of 18,000–19,000 daltons. More recent data indicate that ferritin

TABLE IV

Plasma Iron Turnover (Ferrokinetics) in Domestic Animals[a]

Animal	Half-time $T_{1/2}$ (minutes)	Fractional transfer rate k (day^{-1})	Transfer rate Tr (mg/day)	Tr or plasma iron turnover rate		Maximum erythrocyte ^{59}Fe uptake (% dose)	Reference
				Tr/kg body wt (mg/kg day)	Tr/100 ml plasma (mg/100 ml day)		
Horse	75–103		111–153	0.45–0.65	0.77–1.48	74–77	Obara and Nakajima (1961b)
	(88.8)	(10.6)	(132)	(0.55)	(1.18)	(76)	
Cow	187	5.3	106	0.27	0.27	55	Kaneko (1963)
Calf	88–137	8.0–11.3	45–104	0.50–0.64	1.18–1.49	66–82	Kaneko and Mattheeuws (1966)
	(117)	(8.7)	(74)	(0.57)	(1.30)	(73)	
Sheep	85–110	9.9–11.7		0.42–0.65	1.91–2.26	74–87	Baker and Douglas (1957b)
	(94)	(10.3)		(0.56)	(1.99)	(78)	
Pig	43–100	2.3–10.0		0.40–1.66	1.30–4.13	72–100	Bush *et al.* (1956b)
	(71.4)	(14.0)		(1.11)	(1.57)	(92)	
Dog	39–63	16–40			1.71–2.22	58–93	Kaneko (1964)
	(56)	(18.2)			(1.96)	(75)	
Cat	(40)	(24.8)			1.75–1.86	19–21	Kaneko (1969)
					(1.80)	(20)	

[a] Values have been recalculated when necessary to maintain consistency. Numbers in parentheses are means.

might be composed of only two subunits (Drysdale and Alpert, 1975). Ferritin can also exist as a multiple of isoferritins analogous to the polymorphism of transferrin.

Electron density studies indicate that the ferritin molecule is a hollow globular shell with an outside diameter of 12.4–13.0 nm and an internal diameter of 7.0–8.0 nm. Iron in the Fe^{2+} state is thought to enter the hollow sphere through channels in the shell. Inside the sphere, Fe^{2+} is oxidized to Fe^{3+} and incorporated into the ferric hydroxide phosphoprotein complex.

After being formed in the liver, ferritin is stored in the reticuloendothelial cells of the liver, spleen, and bone marrow. Ferritin is now known to be in the circulation, and its assay by radioimmunoassay (RIA) is one of the exciting new developments in iron metabolism (Jacobs *et al.*, 1972). Using RIA, Pollock *et al.* (1978) found a plasma clearance $T_{1/2}$ of 5.8–8.7 ($\bar{x} = 7.1$) minutes. They reported the normal geometric mean of ferritin in dogs to be 24 ng/ml (range 7.0–75 ng/ml). Determining the serum ferritin concentration is presently the most accurate means of assessing body iron stores.

Hemosiderin is the other major storage form. The chemical nature of hemosiderin is unclear, although there is evidence that is contains ferritin (Richter, 1958). Hemosiderin differs from ferritin in that it is insoluble and contains more Fe^{3+} (25–33%) and the granules are coarse enough to be seen with the light microscope. The iron of hemosiderin is visible microscopically as blue granules after staining with the Prussian blue stain for iron. The amount of stainable iron in bone marrow smears is generally accepted as an accurate index of iron stores. The iron of ferritin and hemosiderin appears to be readily available when need exists, but the repletion of stores occurs slowly.

E. Excretion

As previously mentioned, actual excretion of iron in the normal body amounts to no more than 1 mg/day. The large amounts of iron normally in the feces are almost entirely unabsorbed dietary iron. The normal routes for excretion of body iron are through the major excretory routes in the body. The very small amounts lost in exfoliated skin cells, hair, nails, milk, and urine remain relatively constant. As mentioned in Section IV, A, control of excretion may be exercized by the amount of iron contained in the mucosal cells when they are sloughed at the end of their life span. In times of need, little iron is contained in the mucosal cell, and therefore little iron is lost by this route. In iron overload, body iron moves into the mucosal cell to block further uptake, and, when the mucosal cell is sloughed, its iron load is also lost (Conrad and Crosby, 1963). Thus, the mucosal cell participates in iron metabolism as a major controlling mechanism of iron excretion as well as absorption.

V. TESTS OF IRON METABOLISM

The number of laboratory tests of iron metabolism is not extensive, and the most useful of these are (1) hematology, (2) serum iron, (3) iron-binding capacity, (4) stainable iron in the bone marrow, and (5) serum ferritin. Ferrokinetic studies utilizing ^{59}Fe permit the quantitation of iron absorption, turnover, and excretion, but the technique continues to be most useful in its research applications. It will become apparent that a combination of these techniques should be employed to evaluate suspected disorders of iron metabolism.

A. Hematology

The hematological techniques employed for the examination of animal blood have been described in detail by Schalm *et al.* (1975). Evidence of anemia should be sought in the hemoglobin (Hb) determination, packed cell volume (PCV), and erythrocyte (RBC) count. The stained blood film should always be examined in particular for cell size (anisocytosis, macrocytosis, microcytosis), shape (poikilocytosis, leptocytosis), and Hb pigmentation (polychromasia, hypochromasia). From the Hb, PCV, and RBC, the following useful erythrocyte indices can be empirically calculated:

$$\text{Mean corpuscular hemoglobin (pg)} = \frac{\text{Hb (gm/dl)}}{\text{RBC } (10^6/\mu\text{l})} \times 10$$

$$\text{Mean corpuscular volume (fl)} = \frac{\text{PCV (\%)}}{\text{RBC } (10^6/\mu\text{l})} \times 10$$

$$\text{Mean corpuscular hemoglobin concentration (gm/dl)} = \frac{\text{Hb (gm/dl)}}{\text{PCV (\%)}}$$

On the basis of mean corpuscular volume (MCV), or cell size, and mean corpuscular hemoglobin concentration (MCHC), a morphological classification of the anemias has been devised, as shown in the following tabulation:

MCV	Anemia	MCHC	Anemia
High	Macrocytic	—	—
Normal	Normocytic	Normal	Normochromic
Low	Microcytic	Low	Hypochromic

Any combination of the above can occur, and the finding of a microcytic hypochromic anemia is most commonly associated with iron deficiency and more rarely as a result of a block in heme synthesis. Thus, it can also occur in pyridoxine-responsive anemia or in copper deficiency.

B. Serum Iron

The abundance of iron in the environment and the microgram quantities of iron in the serum require that extra care be taken in the serum iron determination. An accurate measurement of iron is dependent upon freedom from contamination from time of sampling to its final determination. Specially cleaned iron-free glassware should be used throughout, with extra care taken during sampling, and the use of disposable sampling equipment is recommended.

Most procedures for the determination of serum or plasma iron are based on the separation of the Fe^{3+} from transferrin, its reduction to Fe^{2+}, and the colorimetric determination of Fe^{2+} using such reagents as thiocyanate, *o*-phenanthroline, or 2,2′-dipyridyl. The method of Peters *et al.* (1956) has proved to be satisfactory in our laboratories, and some representative data are given in Table V.

C. Unbound Iron-Binding Capacity

The iron-binding capacity of the serum is most often referred to as the unbound iron-binding capacity, or the latent iron-binding capacity. This refers to that portion of the

TABLE V

Serum Iron and Unbound Iron-Binding Capacity in Domestic Animals[a,b]

	Horse	Cow	Calf	Sheep	Pig	Dog	Cat
Serum iron	73–140	57–162	114–170	166–222	91–199	94–122	68–215
	(111±11)	(97±29)	(148)	(193±7)	(121±33)	(108)	(140)
Unbound iron-binding capacity	200–262	63–186	139–264	(141±10)	100–262	170–222	105–205
	(218±21)	(131±36)	(218)		(196±39)	(200)	(150)

[a] Compiled from the following: horse, cow, pig: Kolb (1963), Planas and De Castro (1960), Obara *et al.* (1957). Calf (4–13 months): Kaneko and Mattheeuws (1966). Sheep: Baker and Douglas (1957a,b). Dog and cat: Kaneko (1964, 1969).

[b] Micrograms per 100 ml. Numbers in parentheses are mean ±SD.

plasma transferrin molecule which is not bound to Fe^{3+}, hence unbound or latent. The UIBC is measured by the amount of iron that the plasma can bind and is therefore expressed as micrograms Fe per deciliter. Since the serum iron (SI) determination represents the portion of the transferrin which was bound to Fe^{3+}, the total iron-binding capacity is the sum of the SI and the UIBC.

The Fe–transferrin complex has a characteristic salmon pink color (Schade and Caroline, 1946), and its colorimetric determination is the basis of several methods of determination (Cartwright and Wintrobe, 1949; Rath and Finch, 1949). A method (Ressler and Zak, 1958) based on iron determination is now more commonly employed. The UIBC in some domestic animals is also given in Table V.

D. Bone Marrow Stain for Iron

The amount of hemosiderin in the body is about one-half of the total body storage iron. It contains more iron than ferritin (30 versus 23%), and it forms granules large enough to be seen with the light microscope. Hemosiderin appears as a dark yellowish brown pigment. After staining with Prussian blue, these granules are blue. The amount of hemosiderin is graded qualitatively from 0 to 4+ on a bone marrow smear, with the normal amount being 1+ to 2+.

The stain consists of 4 gm potassium ferrocyanide and 20 ml distilled water (Davidsohn and Nelson, 1969). Concentrated HCl is added until a white precipitate is formed, and then filtered. A marrow smear is fixed in formalin vapor for 10 minutes in a Coplin jar. The smear is flooded with the filtered stain for 30 minutes. The iron of ferritin and hemosiderin is stained blue, and the amount is graded 0 to 4+.

The amount of stainable iron in bone marrow is an accurate index of iron stores. The sample is not difficult to obtain in animals, but the results are subjective and imprecise.

E. Serum Ferritin

With the exception of hemoglobin, ferritin is the most abundant iron-containing protein in animals. It is the principal labile iron storage form from which iron can be mobilized in response to need, such as in anemia, dietary iron deficiency, pregnancy, or growth. Thus, a means of quantifying body iron stores would be of great clinical significance. Until recently, the staining of bone marrow (BM) iron was the only means available of estimating the size of the body iron stores. Now, direct quantitation of serum ferritin offers an accurate and convenient measure of storage iron, a means of diagnosing iron deficiency, and a method of distinguishing between this and other anemias such as the anemias of chronic disease, liver disease, and renal disease. Low serum ferritin concentration is virtually diagnostic of iron deficiency.

Sensitive RIA techniques have been developed to measure serum ferritin (Jacobs *et al.*, 1972). New enzyme immunoassay methods for serum ferritin hold additional promise for simplication of the assay (Watanabe *et al.*, 1979). A major advantage of serum ferritin assay is that it is the most sensitive indicator of storage iron, much more so than other indicators (anemia, serum iron, RBC indices, BM iron). There is a direct linear correlation between serum ferritin and storage iron, about 1 ng ferritin per milliliter serum to 8 mg storage iron (Walters *et al.*, 1973; Jacobs *et al.*, 1972). Serum ferritin is also directly correlated with the BM stainable iron (Lipshitz *et al.*, 1974).

Serum ferritin was determined in dogs by Pollock *et al.* (1978), who reported a normal geometric mean of 24 ng/ml (range 7.0–75 ng/ml). This is about one-third the concentration for human beings, 59 ng/ml (range 12–302 ng/ml) (Lipschitz *et al.*, 1974) (see also Section IV,D). No reports on the clinical application of serum ferritin, determination in animals have yet been published.

F. Ferrokinetics

1. Iron Turnover

The ferrokinetic technique originally introduced by Huff *et al.* (1950) is now widely employed to study iron metabolism. In this method, the recipient's own transferrin is labeled with ^{59}Fe *in vitro* by incubation of its plasma with ^{59}Fe and then reinjection of the labeled plasma intravenously. The serum iron and iron-binding capacity are determined before addition of isotope, and the dosage of ^{59}Fe which does not exceed the binding capacity is added and incubated. At intervals after injection, blood samples in heparin for plasma radioactivity are obtained. The time course of the plasma radioactivity is then plotted on semilogarithmic coordinates using appropriate scales (Fig. 2).

The disappearance rate during a few minutes or hours can be used to determine the plasma volume by isotope dilution after extrapolation to time zero.

$$\text{Plasma volume (ml)} = \frac{\text{total activity injected}}{\text{activity at time zero/ml plasma}}$$

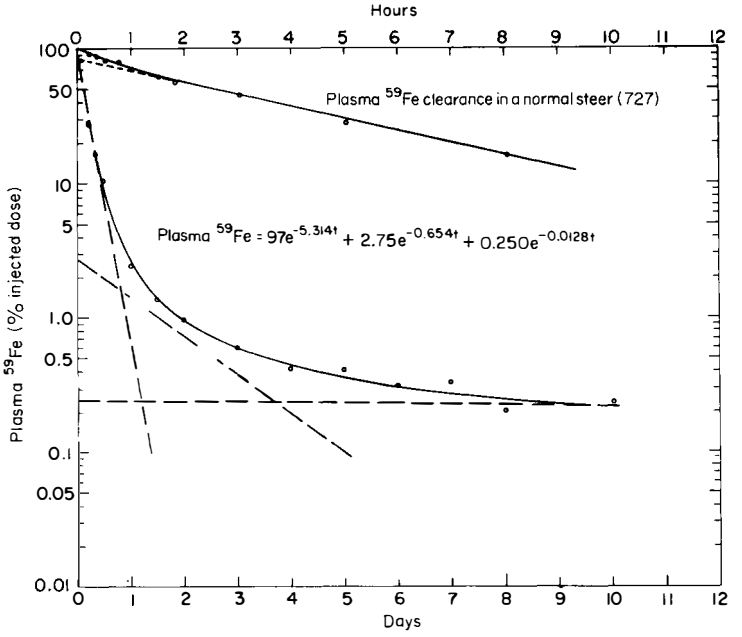


Fig. 2. Plasma ^{59}Fe clearance in a normal steer. The formulation was derived by logarithmic analysis. (From Kaneko, 1963; by permission.)

In actuality, the rate of plasma disappearance is an extremely complex curve. This curve can be resolved by logarithmic analysis as in the example in Fig. 2, in which the formula of the curve is given by a three-component polynomial expression. Each component presumably represents the relative rate of change of all ^{59}Fe atoms which are in sufficiently rapid equilibrium so as to represent a single pool of these atoms. Pollycove and Mortimer (1961) proposed an anatomical compartmentation of these iron pools in man based on theoretical and experimental information of this type. Their mathematical formulations of the curves were of even greater complexity.

A more simplified approach to ferrokinetics has been to use only the initial rapid component of the plasma iron disappearance curve which follows the mixing period. From the half-time $T_{\frac{1}{2}}$, the slope of the line k is calculated:

$$k = 0.693/T_{\frac{1}{2}}$$

The slope k represents the relative rate of change or the fraction (or number of times if greater than 1) of a pool which is transferred or replaced per unit time. This is termed the fractional transfer rate k. The reciprocal of k, $\alpha\ 1/k$, is then the turnover time T_t, the time required for a complete replacement or turnover of the iron pool. The slope k is also proportional to the rate of transfer Tr divided by the pool size:

$$k\ (\text{day}^{-1}) = \frac{Tr}{\text{pool size (mg Fe)}}$$

The plasma iron pool size is given by the plasma iron concentration times the plasma volume. From the slope, k the Tr into or out of the plasma can then be calculated and is given in milligrams Fe per day. If the body weight is known, it can be standardized by division:

$$Tr/\text{kg} = \frac{k\ (\text{day}^{-1}) \times \text{pool size (mg Fe)}}{\text{body wt (kg)}} = \text{mg Fe/kg} \times \text{day}$$

This is the plasma iron turnover rate, or PITR as used by Huff *et al.* (1950).

A more convenient standardization is to express Tr in milligrams Fe per 100 ml plasma per day. The advantage of this standardization is that the plasma volume is not required and the calculation reduces to:

$$Tr/100\ \text{ml} = k\ (\text{day}^{-1}) \times \frac{\text{mg Fe}}{100\ \text{ml plasma}} = \text{mg Fe/100 ml} \times \text{day}$$

It should be apparent also that its accuracy is largely dependent on an accurate determination of SI. Table IV gives the plasma iron turnover data in a number of domestic animals. These turnover data are probably the most useful quantitative indices of erythropoiesis.

2. Red Blood Cell Iron Incorporation

Together with plasma ferrokinetic studies, the rate of iron incorporation into erythrocytes (RBCII) can also be measured by following the time course of radioactivity in erythrocytes (Fig. 3). Pollycove and Mortimer (1961) evaluated RBCII rate curves in their extensive mathematical treatment of this type of data. Under usual conditions, however, a measure of the percent incorporation into the RBC suffices for most purposes. These values are also shown in Table IV

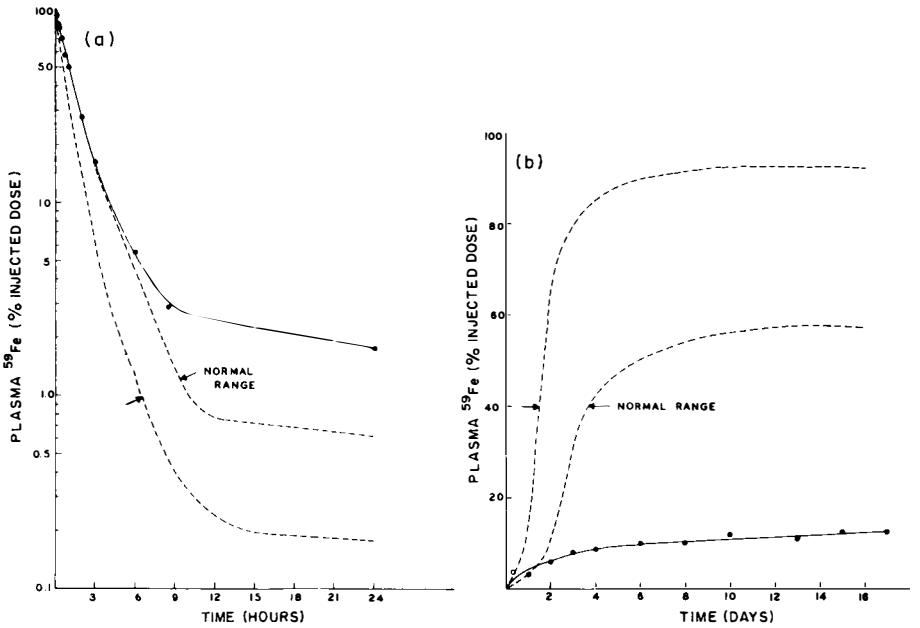


Fig. 3. Iron metabolism in dogs. The dashed lines show the normal range in dogs for plasma ^{59}Fe clearance (a) and RBC ^{59}Fe uptake (b). The delayed clearance and low incorporation are evident in refractory anemia (solid lines). (From Kaneko, 1964; by permission.)

VI. DISORDERS OF IRON METABOLISM

Disorders of iron metabolism are a worldwide problem and the most common causes of anemia in man. In animals, dietary disorders of a similar nature are of great importance in the young, especially those on a milk-only diet. There are no indications that an animal's need for iron is grossly dissimilar to man's, and the variety of disorders listed in Table VI provides some indication of the clinical biochemical significance of iron metabolism. Iron deficiency, whether from a milk-only diet in the young or from other causes, e.g., chronic hemorrhage, is by far the most common iron metabolic disorder. Rarely does iron overload occur.

A. Iron Deficiency

Iron deficiency is defined as a reduction in total body iron stores and, depending on the degree of reduction, can be mild, moderate, or severe. Iron deficiency can be differentiated from other nutritional anemias by the finding of small, pale RBC in the blood smear or a microcytic hypochromic anemia based on the RBC indices. The extent of the morphological change depends on the degree and severity of the deficiency. In mild iron deficiency, the iron stores may be depleted and the serum iron concentration low, but morphological and hemoglobin changes have not yet occurred. In moderate and severe deficiencies, iron stores have been depleted, and, with insufficient iron to meet the replacement needs for hemoglobin, anemia occurs. Thus, differentiation from other causes

TABLE VI

Iron Metabolism in Various Disease States

Disease	Serum iron	Unbound iron-binding capacity	B.M. iron stores	Serum ferritin	Iron transfer rate	RBC iron incorporation
Microcytic hypochromic anemias, iron deficiency (chronic blood loss, dietary, etc.)	Decrease	Increase	Decrease	Decrease	Increase	Increase
Macrocytic anemia, vitamin B_{12}/folate deficiency (nutritional malabsorption)	Increase	Normal	Increase	Increase	Decrease	Decrease
Pyridoxine-responsive anemia	Increase	Normal	Increase	Increase	Decrease	Decrease
Chronic inflammation	Decrease	Decrease	Normal	Normal	Decrease	Decrease
Renal disease	Decrease	Decrease	Increase	Increase	Decrease	Decrease
Hemolytic anemia	Increase	Decrease	Increase	Increase	Increase	Increase
Refractory anemia	Increase	Decrease	Increase	Increase	Decrease	Decrease
Iron overload	Increase	Decrease	Increase	Increase	Decrease	Decrease
Liver disease	Increase	Decrease	Increase	Increase	Increase	Increase
Hypoproteinemia	Decrease	Decrease	—	—	—	—
Late pregnancy	Decrease	Increase	Decrease	Decrease	Normal	Normal
Hypothyroid	Decrease	—	—	—	Decrease	Decrease

of anemia can be made by the finding of a decreased SI, increased UIBC, and decreased iron stores together with microcytic hypochromic anemia.

The mechanism for iron incorporation into hemoglobin is described in Chapter 4. Hemoglobin synthesis has a high priority for tissue iron stores, and these are depleted to maintain normal hemoglobin levels.

Since iron is absorbed in only limited amounts, factors which induce excessive loss of iron also readily induce iron deficiency. In fact, in most cases, iron deficiency anemia in animals must be considered to be evidence of bleeding until another cause is demonstrated. Since hemoglobin contains 3.34 mg Fe per gram, the loss of 1 ml of blood (15 gm Hb per 100 ml) represents the loss of about 0.5 mg Fe from the body:

$$3.34 \text{ mg Fe/gm} \times \frac{0.15 \text{ gm}}{\text{ml}} = 0.51 \text{ mg Fe/ml}$$

This 0.5 mg Fe per day represents about one-half or more of the normal daily iron absorption, and thus chronic losses of a few milliliters per day can easily lead to iron deficiency. Bleeding may have a wide variety of intestinal and external causes. Intestinal hemorrhage or loss can occur with lesions of the wall, intestinal parasites, or coagulation defects. Hookworm infestation can cause severe anemia. Conversely, failure of absorption, such as in the rare malabsorption syndromes (Kaneko *et al.*, 1965), induces iron deficiency in addition to all the other manifestations of absorptive failure.

Microcytic hypochromic anemias may also be caused by factors which block the heme synthetic pathway. Thus, in addition to iron deficiency, the anemia of copper deficiency and pyridoxine-responsive anemia are usually microcytic hypochromic. A number of conditions associated with iron deficiency are given in Table VII.

Ceruloplasmin, a copper-containing compound in the plasma, functions as a ferroxidase, oxidizing Fe^{2+} to Fe^{3+} (Frieden, 1970) and thereby promoting the transfer of iron to transferrin. Mucosal cells, hepatic cells, and reticuloendothelial (RE) cells of swine deficient in copper can take up iron but do not release iron to plasma transferrin (Roeser *et al.*, 1970). Microcytic hypochromic anemia morphologically undistinguishable from iron deficiency occurs in copper deficiency.

The anemia of chronic (inflammatory) disease is probably one of the most common forms of anemia encountered in animals. The anemia is usually normocytic normochromic

TABLE VII

Conditions Associated with Iron Deficiency

1. Excessive iron loss—chronic hemorrhage
   a. Tumors associated with hemorrhage
   b. Intestinal parasites
   c. Coagulation defects
2. Deficient iron intake
   a. Dietary iron deficiency
   b. Protein deficiency
   c. Copper deficiency
   d. Malabsorptive syndromes
3. Increased iron requirements
   a. Growth
   b. Pregnancy
4. Chronic inflammation and infection

and progresses to microcytic in its advanced stages. There is a pattern of decreased serum iron in the presence of large iron stores as demonstrated by B.M. stains or serum ferritin. This is a pattern compatible with a defect in the transfer of RE iron–ferritin to developing RBC in the B.M. (Haurani *et al.*, 1965). This phenomenon has been termed the "RE block" (Erslev and Gabuzda, 1975). Feldman (1978), in extensive studies of the anemia of inflammatory disease in the dog, demonstrated that (1) the availability of iron to the developing RBC is the limiting factor in erythropoiesis and (2) based on increased superoxide dismutase activity, the superoxide anion (O_2^-) was increased. These findings suggested that the O_2^- released by cell oxidative activities during inflammation is rapidly converted to H_2O_2. Hydrogen peroxide promotes the oxidation of ferritin to hemosiderin, which is a less labile form of storage iron and a poor source of iron for erythropoiesis (Feldman, 1978).

Finally, ferrokinetic studies permit a quantitation of iron turnover. In general, increased iron turnover is associated with iron deficiency, increased erythropoiesis, or both. Decreases are observed in depression of erythropoiesis (Table VI).

B. Iron Overload

Frank iron overload is a relative rarity among animals. Idiopathic hemochromatosis with hereditary implications as seen in man has not been reported to occur in animals. Iron overload may, however, result from excessive iron therapy or as a transfusion hemosiderosis.

C. Other Disorders

Iron metabolic studies have been carried out in a number of naturally occurring and experimental disease conditions in animals, particularly in those associated with anemia. Rapid iron turnover with a degree of ineffective erthropoiesis, i.e., bone marrow hemolysis, has been observed in equine infectious anemia (Obara and Nakajima, 1961b). In cattle, increased iron turnover has been observed in experimental anaplasmosis (Hansard and Foote, 1959), erythropoietic porphyria (Kaneko, 1963; Kaneko and Mattheeuws, 1966), polycythemia vera (Fowler *et al.*, 1964), and familial polycythemia (Kaneko *et al.*, 1968). In studies with copper-deficient swine, Bush *et al.* (1956a) demonstrated an increased iron turnover, which was in keeping with their conclusion that iron and copper metabolism were closely related in erythropoiesis. Bush *et al.*, (1956a) also studied iron metabolism in experimental hemolytic anemia, pyridoxine-responsive anemia, and pteroylglutamic acid-deficient anemia in pigs. Iron turnover was increased in all these conditions, and the rate of erythropoiesis, as might be expected, was markedly increased in hemolytic anemia, slightly increased in pteroylglutamic acid-deficient anemia, and decreased in pyridoxine deficiency. Many iron metabolism studies have been carried out on an experimental basis, but relatively few have been conducted in naturally occurring disease states in dogs. Ferrokinetic studies demonstrated increased iron turnover in hemolytic anemia and decreased iron turnover in depression anemia (Kaneko, 1964). In a polycythemic cat, it was shown that iron turnover was increased (Kaneko, 1969). As pointed out earlier, ferrokinetic studies are likely to remain research tools in the laboratory, but their use in experimental and naturally occurring disease has provided a sounder basis for evaluation of the other, more readily utilized parameters of iron metabolism.

REFERENCES

Baker, N. F., and Douglas, J. R. (1957a). *Am. J. Vet. Res.* **18,** 295.

Baker, N. F., and Douglas, J. R. (1957b). *Am. J. Vet. Res.* **18,** 142.

Balcerzak, S. P., Peternel, W. W., and Heinle, E. W. (1967). *Gastroenterology* **53,** 257.

Beutler, E., and Buttenweiser, E. (1960). *J. Lab. Clin. Med.* **55,** 274.

Bothwell, T. H. (1968). *Br. J. Haematol.* **14,** 453.

Brown, E. B., Jr., Dubach, R., and Moore, C. V. (1958). *J. Lab. Clin. Med.* **52,** 335.

Bush, J. A., Jensen, W. N., Athens, J. W., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M. (1956a). *J. Exp. Med.* **103,** 701.

Bush, J. A., Jensen, W. N., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M. (1956b). *J. Exp. Med.* **103,** 161.

Callender, S. T. (1967). *Br. Med. Bull.* **23,** 263.

Callender, S. T., Mallett, B. J., and Smith, N. O. (1957). *Br. J. Haematol.* **3,** 186.

Cartwright, G. E., and Wintrobe, M. M. (1949). *J. Clin. Invest.* **28,** 86.

Charlton, R. W., Jacobs, P., Torrance, J. D., and Bothwell, T. H. (1965). *J. Clin. Invest,* **44,** 543.

Chodos, R. B., Ross, J. F., Apt, L., Pollycove, M., and Halkett, J. A. (1957). *J. Clin. Invest.* **36,** 314.

Conrad, M. E. (1967). *Borden Rev. Nutr. Res.* **28,** 49.

Conrad, M. E., and Crosby, W. H. (1963). *Blood* **22,** 406.

Conrad, M. E., Weintraub, L. R., and Crosby, W. H. (1964). *J. Clin. Invest.* **43,** 963.

Conrad, M. E. Weintraub, L. R., Sears, D. A., and Crosby, W. H. (1966). *Am. J. Physiol.* **211,** 1123.

Cook, J. D., Brown, G. M., and Valberg, L. S. (1964). *J. Clin. Invest.* **43,** 1185.

Cook, J. D., Layrisse, M., and Finch, C. A. (1969). *Blood* **33,** 421.

Creamer, B. (1967). *Br. Med. Bull.* **23,** 226.

Davidsohn, I., and Nelson, D. A. (1969). *In* "Todd-Sanford Clinical Diagnosis by Laboratory Methods" (I. Davidsohn and J. B. Henry, eds.), 14th ed., p. 203. Saunders, Philadelphia, Pennsylvania.

Davis, P. S., Luke, C. G., and Deller, D. J. (1966). *Lancet,* **2,** 1431.

Drabkin, D. L. (1951). *Physiol. Rev.* **31,** 345.

Drysdale, S. W., and Alpert, E. (1975). *Ann. N.Y. Acad. Sci.* **259,** 427.

Erslev, A. J., and Gabuzda, T. G. (1975). "Pathophysiology of Blood." Saunders, Philadelphia, Pennsylvania.

Feldman, B. F. (1978). Thesis, Univ. of California, Davis.

Fowler, M. E., Cornelius, C. E., and Baker, N. F. (1964). *Cornell Vet.* **54,** 154.

Frieden, E. (1970). *Nutr. Rev.* **28,** 87.

Granick, S. (1946). *Science* **103,** 107.

Granick, S. (1954). *Bull. N.Y. Acad. Med.* **30,** 81.

Hahn, P. F., Bale, W. F., Lawrence, E. O., and Whipple, G. H. (1939). *J. Exp. Med.* **69,** 739.

Hahn, P. F., Bale, W. F., Ross, J. F., Balfour, W. M., and Whipple, G. H. (1943). *J. Exp. Med.* **78,** 169.

Hansard, S. L., and Foote, L. E. (1959). *Am. J. Physiol.* **197,** 711.

Haurani, F. I., Burke, W., and Martinez, E. J. (1965). *J. Lab. Clin. Med.* **65,** 560.

Hibbs, J. W., Conrad, H. R., Vandersall, T. H., and Gale, C. (1963). *J. Dairy Sci.* **46,** 1118.

Huff, R. L., Hennessy, T. G., Austin, R. E., Garcia, J. R., Roberts, B. M., and Lawrence, J. H. (1950). *J. Clin. Invest.* **29,** 1041.

Hussain, R., Walker, R. B., Layrisse, M., Clark, P., and Finch, C. A. (1965). *Am. J. Clin. Nutr.* **16,** 464.

Jacobs, A., Miller, F. M., Worwodd, M., Beamish, M. R., and Wardrop, C. A. (1972). *Br. Med. J.* **4,** 206.

Jacobs, P., Bothwell, T. H., and Charlton, R. R. (1964). *J. Appl. Physiol.* **19,** 187.

Jacobs, P., Bothwell, T. H., and Charlton, R. W. (1966). *Am. J. Physiol.* **210,** 694.

Kaneko, J. J. (1963). *Ann. N.Y. Acad. Sci.* **104,** 689.

Kaneko, J. J. (1964). *Gaines Vet. Symp., 13th Athens, Georgia,* p. 2.

Kaneko, J. J. (1969). Unpublished observations.

Kaneko, J. J., and Mattheeuws, D. R. G. (1966). *Am. J. Vet. Res.* **27,** 923.

Kaneko, J. J., Moulton, J. E., Brodey, R. S., and Perryman, V. D. (1965). *J. Am. Vet. Med. Assoc.* **146,** 463.

Kaneko, J. J., Zinkl, J., Tennant, B. C., and Mattheeuws, D. R. G. (1968). *Am. J. Vet. Res.* **29,** 949.

Kendrew, J. C., Parrish, R. G., Marrack, J. R., and Orlans, E. S. (1954). *Nature (London)* **174,** 946.

Knoop, C. E., Krauss, W. E., and Washburn, K. G. (1935). *J. Dairy Sci.* **18,** 337.

Kolb, E. (1963). *Adv. Vet. Sci.* **8,** 49.

Layrisse, M., Cook, J. D., Martinez, C., Roche, M., Kuhn, I. N., Walker, R. B., and Finch, C. A. (1969). *Blood* **33,** 430.

Lintzel, W., and Radeff, T. (1931). *Arch. Tierernaehr. Tierz.* **6,** 313.
Lipshitz, D. A., Cook, J. D., and Finch, C. A. (1974). *N. Engl. J. Med.* **290,** 1213.
McCance, R. A., and Widdowson, E. M. (1937). *Lancet* **2,** 680.
McCance, R. A., and Widdowson, E. M. (1938). *J. Physiol. (London)* **94,** 148.
Manis, J. G., and Schachter, D. (1962). *Am. J. Physiol.* **203,** 73.
Manis, J. G., and Schachter, D. (1964). *Am. J. Physiol.* **207,** 893.
Matrone, G., Conley, C., Wise, G. H., and Waugh, L. K. (1957). *J. Dairy Sci.* **40,** 1437.
Matrone, G., Thomason, E. L., and Bunn, C. R. (1960). *J. Nutr.* **72,** 459.
Moore, C. V. (1961). *Harvey Lect.* **55,** 67.
Moore, C. V., and Dubach, R. (1951). *Trans. Assoc. Am. Physicians* **64,** 245.
Murray, M. J., and Stein, N. (1968). *Lancet* **1,** 614.
National Research Council (1962). "Nutrient Requirements of Dogs," Publ. No. 989. Washington, D.C.
Obara, J., and Nakajima, H. (1961a). *Norinsho Kachiku Eisei Shikensho Kenkyu Hokoku* **42,** 45.
Obara, J., and Nakajima, H. (1961b). *Jpn. J. Vet. Sci.* **23,** 247.
Obara, J., Yamamoto, H., and Nakajima, H. (1957). *Norinsho Kachiku Eisei Shikensho Kenkyu Hokoku* **33,** 103.
Peters, T., Giovannaiello, T. J., Apt, L., and Ross, J. F. (1956). *J. Lab. Clin. Med.* **48,** 280.
Pinkerton, P. H. (1969). *Ann. Intern. Med.* **70,** 401.
Planas, J., and De Castro, S. (1960). *Nature (London)* **187,** 1126.
Pollock, A. S., Lipschitz, D. A., and Cook, J. P. (1978). *Proc. Soc. Exp. Biol. Med.* **157,** 481.
Pollycove, M., and Maqsood, M. (1962). *Nature (London)* **194,** 152.
Pollycove, M., and Mortimer, R. (1961). *J. Clin. Invest.* **40,** 753.
Rath, C. E., and Finch, C. A. (1949). *J. Clin. Invest.* **28,** 79.
Ressler, M., and Zak, B. (1958). *Am. J. Clin. Pathol.* **30,** 87.
Richter, G. W. (1958). *J. Biophys. Biochem. Cytol.* **4,** 55.
Roeser, H. P., Lee, G. R., Noeht, S., and Cartwright, G. E. (1970). *J. Clin. Invest.* **49,** 2408.
Schade, A. L., and Caroline, L. (1946). *Science* **104,** 340.
Schalm, O. W., Jain, N. C., and Carroll, E. J. (1975). "Veterinary Hematology," 3rd ed. Lea & Febiger, Philadelphia, Pennsylvania.
Smithies, O., and Hiller, O. (1959). *Biochem. J.* **72,** 121.
Stewart, W. B., and Gambino, S. R. (1961). *Am. J. Physiol.* **201,** 67.
Ullrey, D. E., Miller, E. R., Thompson, O. A., Ackerman, I. M., Schmidt, D. A., Hoefer, J. A., and Leucke, R. W. (1960). *J. Nutr.* **70,** 187.
Van Campen, K. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 100.
Walters, G. O., Miller, F. M., and Worwood, M. J. (1973). *Clin. Pathol.* **26,** 770.
Watanabe, N., Niitsu, Y., Ohtsuka, S., Koseki, J.-i., Kohgo, Y., Urushizaki, I., Kato, K., and Ishikawa, E. (1979). *Clin. Chem.* **25,** 80.
Weintraub, L. R., Weinstein, M. B., Huser, H. J., and Rafal, S. (1968). *J. Clin. Invest.* **47,** 531.
Wheby, M. S., Jones, L. G., and Crosby, W. H. (1964). *J. Clin. Invest.* **43,** 1433.

17

Hemostasis and Coagulation*

W. JEAN DODDS

I. INTRODUCTION

Hemostasis, the process by which bleeding is arrested, comprises a complex series of physiological and biochemical events which terminate in the formation of a stable plug that seals the blood vessel. The process involves the following sequence of events: interaction between the blood vessel wall and platelets, blood coagulation, and fibrinolysis. The pathophysiological mechanisms involved with each of these categories are summarized below. More detailed descriptions can be found elsewhere (Dodds and Kaneko, 1971; Mustard and Packham, 1971; Biggs, 1972; Dodds, 1974a; Owen *et al.*, 1975; Belamarich, 1976; Ogston and Bennett, 1977a; DeGaetano and Garattini, 1978; Day *et al.*, 1978; Wester *et al.*, 1978).

*Supported in part by NIH research grants HL09902 and HL07173 awarded by the National Heart, Lung, and Blood Institute, PHS/DHEW.

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

II. MECHANISMS OF HEMOSTASIS

A. The Blood Vessel

When a blood vessel is injured or severed, a brief local reflex vasoconstriction occurs which reduces blood flow at the site. Vascular contraction is maintained by the release of vasoactive compounds from adjacent platelets and surrounding tissues. At about the same time, platelets in the vicinity rapidly adhere to exposed subendothelial collagen fibers. This interaction with collagen causes a "release reaction" whereby platelet constituents, such as adenosine diphosphate (ADP), serotonin, epinephrine, and histamine, are released into the surrounding medium. The role of platelets in continuing and perpetuating the hemostatic process is described in Section II,B.

Other constituents of the blood vessel wall and its intimal lining cells, the endothelium, play important roles in the formation and dissolution of hemostatic plugs, thrombi, and atherogenic lesions. The mechanism of interaction among these components has been elucidated only recently. An excellent review of the subject has been written by Thorgeirsson and Robertson (1978).

One of the basic functions of the intact, normal vascular endothelium is to provide a thromboresistant surface to the flowing blood. Intact endothelium does not promote platelet or leukocyte adherence, nor does it activate coagulation. Both active and passive mechanisms apparently play a role in maintaining thromboresistance. Recent experimental evidence strongly supports an active contribution by endothelial cells to their thromboresistance. Endothelium, particularly in the pulmonary microcirculation, actively removes from the circulation compounds which promote platelet aggregation. These include prostaglandin $F_{1\alpha}$, serotonin, adenine nucleotides, bradykinin, and angiotensin I. In addition, an exciting discovery by Moncada and associates (1976) is that all layers of blood vessels, but especially the intima and endothelium, synthesize and release a potent inhibitor of platelet aggregation, first called prostaglandin X and now known as prostacyclin, or PGI_2. Thus, it is currently believed that the capacity of endothelium to produce PGI_2 is the fundamental mechanism of thromboresistance (Moncada and Vane, 1978; Thorgeirsson and Robertson, 1978; Ross and Harker, 1978).

Passive mechanisms of thromboresistance also exist but are less contributory. These include protection of the cell surface by its carbohydrate-rich cell coat, the glycocalyx; the negative surface charge of the endothelial lumen, which repels similarly charged cells such as platelets; continuous, nonspecific renewal of cell membranes; and presence at the cell surface of the protease inhibitor α_2-macroglobulin (Thorgeirsson and Robertson, 1978).

In addition to its role in thromboresistance, which is discussed further in Section II,B, the endothelium has important synthetic, metabolic, and pathological functions (Thorgeirsson and Robertson, 1978). Endothelial cells synthesize factor VIII-related antigen and von Willebrand's factor (Jaffe, 1977) (discussed in Section II,C,2,d); basement membrane, type III and IV collagens, and perhaps elastin; cold-insoluble globulin (CIg), also known as plasma fibronectin or α_2-opsonic protein (Blumenstock *et al.*, 1978); a large number of enzymes which play active roles in various anabolic and catabolic reactions; and PGI_2, as mentioned above. The capacity of endothelium to synthesize large amounts of factor VIII-related antigen and CIg has made it possible for these proteins to be

used as markers for readily identifying endothelial cells in tissue specimens and cell cultures.

The endothelium plays an important role in such pathological processes as atherosclerosis, thrombosis, and disseminated intravascular coagulation (DIC), defective hemostasis, inflammation, immune disorders, vascular neoplasia, and metastasis (Thorgeirsson and Robertson, 1978). The initiating event in atherogenesis has long been attributed to endothelial injury. Following injury, there is increased entrapment of plasma lipoprotein components followed by proliferation of arterial smooth muscle cells and/or deposition of free cholesterol and cholesterol esters, *de novo* synthesis of connective tissue, and intracellular and extracellular deposition of lipids in variable amounts. This reaction-to-injury hypothesis incorporates both the myogenic and lipogenic mechanisms of atherogenesis. Other causes of endothelial injury are infectious agents, especially viruses, gram-negative bacteria, and rickettsia; prolonged hypotension and acidosis or hypoxia; dysproteinemias; primary and secondary immune mechanisms; and acute inflammation. These predispose to either thrombosis and DIC, increased vascular permeability, or defective hemostasis with purpura or hemorrhage depending on the nature of the incitant.

B. The Platelet

Blood platelets are cellular particles produced by megakaryocytes mainly of the bone marrow. The lung and spleen are also sources of platelets. The megakaryocyte is a large (25–50 μm) cell with a polyploid nucleus which is extremely pleomorphic. As the cell matures, pseudopods form and platelets bud off at the extremities. The normal platelet seen in a Wright's-stained blood film is from 1 to 4 μm in diameter and is spherical, oval, or rod-shaped. The cytoplasm is pale blue and contains reddish granules. Circulating platelets are disc-shaped.

The role of the blood platelet in hemostasis is as important as that of the coagulation mechanism. Platelets are involved together with the blood vessel wall and the contact-activated coagulation factors (XII and XI) in the initiation of the hemostatic process. A decrease in the number of circulating platelets (thrombocytopenia) or the presence of abnormal nonfunctioning platelets (thrombasthenia, thrombopathia) will impair hemostasis. In some instances, an excess of platelets (thrombocytosis or thrombocythemia) can produce inadequate hemostasis, but usually this condition promotes clotting and may predispose a patient to thrombosis. The multifaceted role of platelets in biochemical, physiological, and pathological processes has been the subject of extensive literature in recent years. Examples are Mustard and Packham (1970, 1971), Caprino and Rossi (1974), Owen *et al.* (1975), Holmsen *et al.* (1977), Day *et al.* (1978), DeGaetano and Garattini (1978), Dodds (1978b, 1979b), Lüscher and Pfueller (1978), Moncada and Vane (1978), Nachman (1978), and Ross and Harker (1978).

Platelets promote hemostasis in several ways. When a blood vessel is injured, they accumulate at the site of injury. They adhere to the vessel wall and then to each other, and finally become involved in the intrinsic coagulation pathway. Upon exposure of platelets to collagen fibers of the vessel wall, certain active constituents are released from the platelet (serotonin, histamines, ADP). The release of ADP into the ambient fluid causes the adherence and aggregation of surrounding platelets to the area. The aggregation

process, which also requires calcium, fibrinogen, and metabolic energy, forms an initial hemostatic plug of platelets that seals the vessel. In addition to mediating platelet aggregation, ADP makes the platelet surface phospholipoprotein, platelet factor 3, available for its thromboplastic or accelerating affect on blood coagulation (see below). Platelets also undergo a series of reactions mediated by thrombin and connective tissue which produce marked structural changes. This process has been called "viscous metamorphosis." The thrombin associated with viscous metamorphosis and evolved from tissue injury rapidly activates the coagulation mechanism. Once fibrin has formed around the platelet aggregate, a stable hemostatic plug is produced.

Platelets are metabolically active in biochemical, physiological, and pathological processes. In addition to the basic adhesion, aggregation, and release (secretion) reactions, these cells agglutinate in the presence of a variety of compounds including bovine factor VIII, ruminant plasma, ristocetin, polylysine, snake venoms, and polybrene (Holmsen *et al.*, 1977; Dodds, 1978b). Platelets actively synthesize proteins, lipids, carbohydrates, and nucleotides. Among the proteins synthesized by or contained in platelets are fibrinogen; the components of the contractile system, actomyosin, myosin, actin, tropomyosin–troponin, and sarcoplasmic reticulum (relaxing factor); acid hydrolases and other enzymes; and a series of factors including the growth, permeability, bactericidal, and chemotactic activity factors (Holmsen *et al.*, 1977). The lipids synthesized by platelets include fatty acids; prostaglandins; glycerides; a series of phosphatides; the splingolipids, sphingomyelin, cerebrosides, and gangliosides; and sterols. The carbohydrates synthesized by or contained within platelets are glycogen, glycosaminoglycans, and glycoproteins, such as factor VIII-related antigen and CIg. Platelet glycolytic pathways synthesize sugars and fatty acids by means of an active hexose monophosphate shunt. The adenine nucleotides of platelets are compartmentalized into the storage and metabolically active pools. Other active nucleotides of platelets include the cyclic AMP and GMP systems, which regulate the production of platelet endoperoxides; thromboxanes; and prostaglandins. Other important components of platelets are serotonin and metal ions (Ca^{2+}, Mg^{2}, Zn^{2+}, K^{+}).

The generation of metabolic energy is an active function of platelets (Holmsen *et al.*, 1977). The net effect is to produce ATP from platelet glycolysis, glycogenolysis, oxidation of citric acid cycle intermediates, fatty acid oxidation, and mitochondrial oxidative phosphorylation, which is then utilized to maintain the many functions of the cell.

Platelets have other essential functions, including the ability to phagocytize such particles as viruses, latex, immune complexes, and iron (Mustard and Packham, 1970, 1971); maintenance of vascular integrity by filling gaps that form in the endothelium and by directly supporting endothelial cells (DeGaetano and Garattini, 1978); synthesis and release of major components of the factor VIII complex (factor VIII-related antigen and von Willebrand's factor)(Jaffe, 1977; Bloom and Peake, 1977) and glycocalyx (Blumenstock *et al.*, 1978; Thorgeirsson and Robertson, 1978); production and release of potent smooth muscle and endothelial cell proliferating factor(s) (Ross and Harker, 1978; Mustard *et al.*, 1978); and retraction of clots, a process which stabilizes the initial hemostatic plug and activates clot lysis. The mechanism of clot retraction is not completely understood, but it is known to require calcium, metabolic energy, and thromboasthenin, the platelet contractile protein.

A final and extremely important function of platelets, as alluded to above, is their regulatory role via prostaglandin pathways in promoting hemostasis and thrombosis and

maintaining the thromboresistance of intact endothelium (Moncada *et al.*, 1976; Holmsen *et al.*, 1977; Thorgeirsson and Robertson, 1978; Day *et al.*, 1978; DeGaetano and Garattini, 1978; Moncada and Vane, 1978). This involves metabolism of arachidonic acid released from platelet phospholipids. A potent but unstable platelet aggregating agent and vasoconstrictor, thromboxane A_2 (TXA_2), is produced from cyclic endoperoxides by the action of cyclooxygenase on arachidonic acid. The potent aggregating effect of TXA_2 in platelets is inhibited by the production of prostaglandins E_1 and D_2 (PGE_1 and PGD_2) from linolenic and linoleic acids. Thus, platelet phospholipid and lipid metabolism plays a crucial role in the stimulation and inhibition of platelet reactivity (DeGaetano and Garattini, 1978; Mustard *et al.*, 1978). Aspirin is an effective inhibitor of the platelet release reaction because it acetylates cyclooxygenase (Caprino and Rossi, 1974; Kelton *et al.*, 1978; Moncada and Vane, 1978).

A key component of the platelet prostaglandin regulatory mechanism of hemostasis and thrombosis is the production of prostacyclin, or PGI_2, from endothelium and other vascular tissues. Prostacyclin is estimated to be about 30 times more potent than PGE_1 and PGD_2 in inhibiting platelet aggregation and also causes relaxation of vascular smooth muscle. Like platelet TXA_2, PGI_2 is formed in blood vessels from cyclic endoperoxides by the action of cyclooxygenase on arachidonic acid. Production of PGI_2 from endogenous or exogenous arachidonic acid is effectively blocked by aspirin and indomethacin, although production from other prostaglandin endoperoxides is not (Moncada and Vane, 1978). The dose of aspirin required to inhibit vascular PGI_2 formation is apparently much greater than that required to prevent platelet synthesis of TXA_2 (Kelton *et al.*, 1978). This observation has considerable significance because the promising antithrombogenic properties of aspirin, as mediated by inhibition of platelet TXA_2, could potentially be reversed at high aspirin dosage by inhibition of vessel wall PGI_2. In fact, high-dose aspirin has been shown by Kelton *et al.* (1978) to be thrombogenic in rabbits.

The final point to emphasize about platelets is the marked species differences encountered in the morphological and functional expression of these cells. A recent review summarizes the comparative aspects of platelets in animals and man (Dodds, 1978b).

C. The Coagulation Mechanism

1. General

Blood coagulation is a complex series of reactions involving various coagulation factors, designated by the International Committee for the Nomenclature of Blood Clotting Factors (1962) by roman numerals (Table I). The process of blood coagulation becomes active in hemostasis after the initial interactions of the platelets with the vessel wall and with one another. Blood flowing through an injured vessel encounters exposed foreign surfaces, and the injured cells release tissue thromboplastins. These surfaces and released thromboplastins activate coagulation.

Coagulation proceeds by an intrinsic or intravascular pathway and by an extrinsic or tissue juice pathway, both of which convert prothrombin to thrombin. Thrombin converts fibrinogen to soluble fibrin monomer, which, with factor XIII and calcium, becomes converted to the fully polymerized, insoluble fibrin clot. This final stage of coagulation is also the end point measured in most coagulation tests. A simplified scheme of coagulation is shown in Fig. 1.

TABLE I

Blood Clotting Factors and Synonyms

Factor[a]	Synonym
I	Fibrinogen
II	Prothrombin
III	Tissue thromboplastin, tissue factor
IV	Calcium
V	Proaccelerin, labile factor, accelerator globulin
VII	Proconvertin, serum prothrombin conversion accelerator, stable factor, autoprothrombin I
VIII	Antihemophilic factor (AHF), antihemophilic globulin (AHG), platelet cofactor I
IX	Christmas factor, plasma thromboplastin component (PTC), platelet cofactor II, autoprothrombin II
X	Stuart factor, Stuart-Prower factor
XI	Plasma thromboplastin antecedent (PTA)
XII	Hageman factor, contact factor
XIII	Fibrin-stabilizing factor (FSF), fibrinase, Laki-Lorand factor

[a] As recommended by the International Committee for the Nomenclature of Blood Clotting Factors (1962). There is no designated factor VI.

Once initiated, activation of the intrinsic and extrinsic systems continues sequentially, forming the end products plasma thromboplastin and prothrombinase, respectively. These prothrombin-converting principles convert factor X to Xa, which initiates the final common pathway of prothrombin conversion. This pathway involves factor V, prothrombin (factor II), fibrinogen (factor I), and factor XIII. The phospholipid necessary for the interaction of factors IX and VIII and for the activation of factors X and V is supplied *in vivo* primarily by platelet factor 3 but also by red cells and tissue juices. *In vitro,* it can be provided by various phospholipid preparations or their substitutes.

The clotting mechanism can be best described, therefore, as a series of sequential activating steps that produce a plasma thromboplastic and/or a tissue thromboplastic component, both of which can convert prothrombin to thrombin in the presence of calcium ions. The enzymatically active factors are protein in nature, and, in general, each of the sequential activations are enzymatic hydrolyses of the inactive precursor forms. It should be noted that factor III (tissue thromboplastin) does not take part in the intrinsic system and that there is no factor VI designated in the nomenclature. Several new clotting factors have recently been described that participate in the contact phase of intrinsic clotting (factors XII and XI) and are part of the plasma kallikrein–kinin system responsible for capillary permeability and smooth muscle contraction. These have not been given numerical designations and include Fletcher factor (prekallikrein) and Fitzgerald factor, Williams factor, and Flaujeac factor (high molecular weight kininogens).

The fibrin produced by blood coagulation forms at the periphery of the initial hemostatic plug of platelets. The platelet–fibrin mass continues to grow and becomes covered with a cap of fibrin. This stabilized hemostatic plug then contracts to seal the vessel permanently.

Thrombin generated during the coagulation process also plays an important part in growth and stabilization of the initial platelet plug. Very low concentrations of thrombin, insufficient to produce visible fibrin formation, cause aggregation of platelets, induce the process of viscous metamorphosis and the release reaction, and make the platelet factor 3

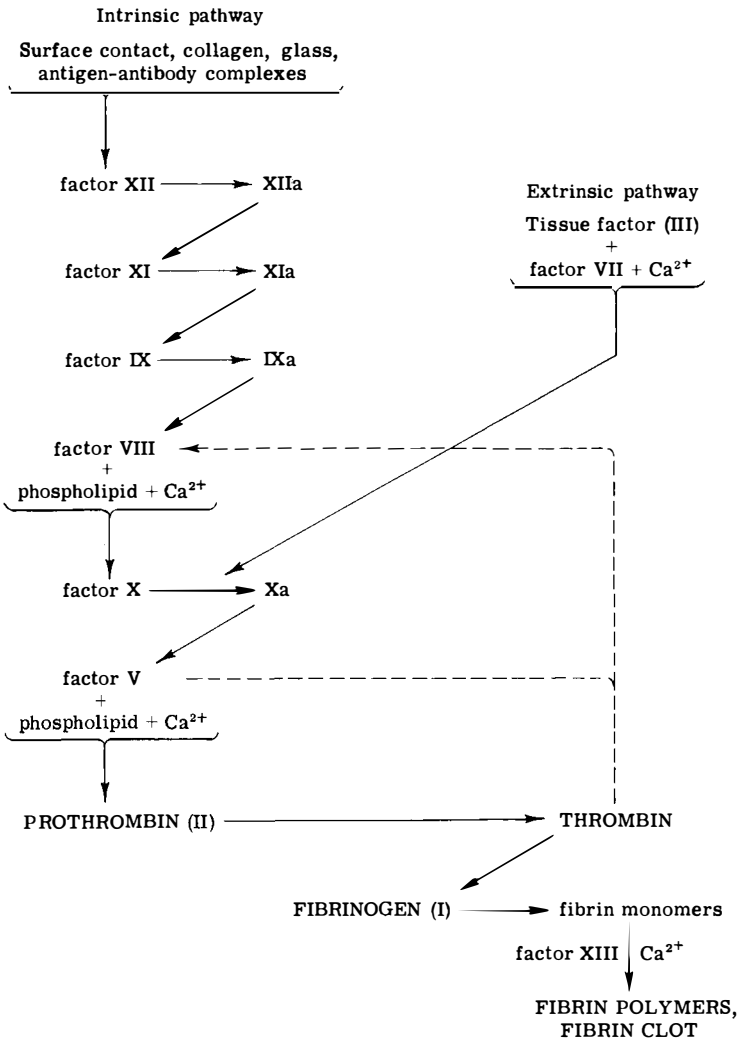


Fig. 1. Intrinsic and extrinsic systems of blood coagulation. (See Table I for nomenclature.)

on the platelet surface available for coagulation. Thus, the processes of platelet aggregation, release, and contraction, as discussed earlier, are directly enhanced by products evolved following activation of the coagulation mechanism.

The coagulation mechanisms of man, other animals, and lower forms are remarkably similar (Belamarich, 1976). Although comparative studies have been extensive and there is a large body of literature, much of it is obsolete, nonspecific, and contradictory. Few of these studies have systematically compared platelet function, coagulation, and fibrinolysis among species or have provided a reliable comparison using human beings as the reference species. Tables II and III have been compiled from the literature cited separately at the end of this chapter (p. 717).

Another major consideration in the understanding of coagulation, which really applies to hemostasis in general, is the varied effects of physiological stress on coagulation,

TABLE II

Comparison of Hemostatic Parameters in Animals with Those in Normal Human Beings[a,b]

Species	Platelet/thrombocyte				Extrinsic clotting	Intrinsic clotting	Fibrinolysis
	Number	Retention	Aggregation[c]	Release			
Nonhuman primate	E	E or D	E	E	E or I	E or I	E or I
Rabbit	I	I	D[d]	E	E	I	E or D
Guinea pig	I	I	D[c]	E or D	D	E	E
Rat	Markedly I	I	D[d]	D	E or I	I	D, E, or I
Mouse	Markedly I		D[d]	D	E or I	E or I	E or I
Hamster	I		D[d]		E or I	I	E
Cat	E or D	E	E	E	E	I	E or I
Dog	I	I	E[c]	E	I	I	I
Pig	I	E or I	D or E[d]	E	E or D	I	E or D
Sheep	I	E or I	D[d]	E	I or E	I	E or D
Goat	I	E	D[d]		E	I	E or D
Cow	I	E	D[d] or E[d]	E	D or E	D, E, or I	E or D
Horse	D	E	E[d]	E	D or E	D	E
Bird and reptile	E		D[f]		E[g] or D	Markedly D	D or Absent

[a] See References for Comparative Study for papers from which data were compiled.
[b] Abbreviations: E, equivalent; I, increased; D, decreased.
[c] For specifics see Dodds (1978).
[d] No response to Adrenalin.
[c] Occasional response to Adrenalin.
[f] Response to thrombin only.
[g] With hologous thromboplastin.

TABLE III

Comparison of Coagulation Factor Activities in Animals with Those in Normal Human Beings[a,b]

Species	Factor									
	I (fibrinogen)	II (prothrombin)	V	VII	VIII	IX	X	XI	XII	XIII
Nonhuman primate	E or I	E or I	I or E	I	I or E	E	E	E	I or E	E or D
Rabbit	E or I	D	Markedly I	D or E	I	I	I	I	E	I
Guinea pig	I or E	D	I	D	I	E	D	E	E	I
Rat	E	E or I	I	E	I	D or E	D	D or E	I	E
Mouse	E	E or D	I	I	E	E	I	E	E	E or I
Hamster	E	E		D	I	E	E	E	E	E or I
Cat	I or E	D or E	Markedly I	E	I	I	Markedly D	I	I	E
Dog	I or E	E or D	Markedly I	I	Markedly I	I	I	I	E or I	I
Pig	I or E	E or D	I or E	E or I	I	I	E	E or I	I	D
Sheep	I	D	I	D	Markedly I	I	D	D	I	D
Goat	I or E	E or D	I	E or D	I	E	D or E	D	I	E
Cow	I	D or E	Markedly I	E or D	E	E	E	E or D	I	Markedly I
Horse	I	E or I	I	E	E or D	D or I	I	D	D	D
Bird	E	D	Markedly D	Markedly D	D or E	Markedly D	E	Markedly D	Absent	E

[a] See References for Comparative Study for papers from which data were compiled.

[b] Abbreviations: E, equivalent; I, increased; D, decreased.

fibrinolysis, and platelet behavior. An extensive literature documents these physiological variations and has been reviewed in depth by Bennett and Ogston (1977). Briefly, alterations from the normal, resting level of hemostasis occur during fetal and neonatal life and with other age and sex differences, hormonal changes, especially during pregnancy, ethnic background, exercise, mental and physical stress, diurnal variation, obesity, smoking, alimentary lipemia, arteriovenous differences, and blood groups. It should be emphasized that the significance of many of these changes is unknown. Some of these differences, however, can affect the interpretation of diagnostic test results or place the patient at risk to bleed or thrombose. For example neonates have reduced levels of the prothrombin complex, vitamin K-dependent clotting factors, and individuals who are pregnant, markedly stressed, heavy smokers, or grossly overweight may have enhanced hemostasis and be at risk for thrombotic problems.

The individual clotting factors are briefly discussed in the following sections. Each has been the subject of extensive research in the past decade, and the reader is referred elsewhere for greater detail (Biggs, 1972; Nemerson and Pitlick, 1972; Hershgold, 1974; Owen *et al.*, 1975; Ogston and Bennett, 1977a; Ratnoff, 1977b; Robbins, 1977; Brozović, 1977; Bouma and van Mourik, 1977; Bloom and Peake, 1977). The reader should also be aware that many details concerning the mechanisms of action of these clotting factors have only recently been elucidated. Current knowledge has refuted several earlier theories about the enzymatic and nonenzymatic pathways involved, including some found in the previous edition of this book.

2. The Intrinsic System

a. Hageman Factor (Factor XII). Hageman factor is involved in the initial stage of clotting through a surface-mediated or "contact activation" process. Recent reviews on this subject include those of Ratnoff (1977b) and Ratnoff and Green (in Dodds, 1979b). Factor XII has a molecular weight of 80,000, is not absorbed by $Al(OH)_3$, $BaSO_4$, or $Ca_3(PO_4)$, is absorbed with kaolin and celite, is stable at 56°C and various pH values, and is a sialoglycoprotein. It is activated by glass, collagen, skin, stearate, vascular basement membranes, ellagic and uric acids, and most foreign surfaces. Factor XII activation is inhibited by cytochrome c, lysozyme, ribonuclease, spermine, and C1-esterase inhibitor. Hageman factor migrates near the γ-globulins upon electrophoresis; is present in plasma at about 15–45 μg/ml; is split by trypsin or plasma kallikrein into two fragments, a 28,000-dalton active component and a larger one of 52,000 daltons; and contains about 15% carbohydrate and no lipid. The site of synthesis is unknown, although its concentration is reduced in hepatic disease. The half-life of factor XII is 52–60 hours (Brozović, 1977).

The functions of activated Hageman factor are numerous and include an important role in conversion of plasma kininogens to kallikrein, the enzyme that elaborates plasma kinins; enhancement of vascular permeability; activation of the fibrinolytic mechanism; triggering of the Schwartzman reaction; possible role in the extrinsic coagulation pathway; interaction with the Fletcher (prekallikrein) and Fitzgerald (high molecular weight kininogen) factors in surface-mediated reactions; and mediation of inflammatory and complement (C1-esterase) reactions (Ratnoff, 1977b).

It should be emphasized here that most of the properties and functions of Hageman factor apply to plasma thromboplastin antecedent (factor XI), which also plays an important role in the initial activation stages of coagulation.

b. Plasma Thromboplastin Antecedent (Factor XI). Plasma thromboplastin antecedent (PTA) is involved in the early stages of coagulation along with Hageman factor (Ratnoff, 1977b). It does not appear to require Ca^{2+} for its activity. Factor XI is a β_2-globulin the molecular mass of which is estimated to be 100,000–200,000 and the half-life of which is 30–84 hours (Brosović, 1977). Factor XI is present in both serum and plasma and is stable when frozen at −40°C or lower, or at room temperature for over 4 months. Factor XI levels in plasma from patients with either mild or moderate deficiency tend to increase on storage but not if the patient is very severely deficient in factor XI. The increase in PTA activity of human and canine deficient plasmas upon storage tends to make them unreliable as a reagent for factor XI assay. Fortunately, however, several useful artificial factor XI-deficient substrates have been developed by celite adsorption of normal plasma, and bovine factor XI-deficient plasma has turned out to be an excellent substrate (Dodds, 1979b). There is some disagreement concerning the adsorbability of factor XI to such products as $Al(OH)_3$, $BaSO_4$, kaolin, and celite. Recent evidence suggests that it can be adsorbed by varying the concentration of adsorbing agent used.

Biochemical studies of factor XI have shown it to resemble prekallikrein (Fletcher factor) and the precursor of Hageman factor cofactor. Its site of synthesis is unknown, but, like those of factor XII, factor XI levels are reduced in liver disease. Normal plasma contains about 0.7 μg/ml of PTA (Ratnoff, 1977b).

c. Christmas Factor (Factor IX). Christmas factor is a single-chain glycoprotein of molecular mass of 60,000–80,000 daltons that is present in both plasma and serum. Its activity increases in serum over that in plasma and is also increased by contact with glass. It is relatively heat labile, has an amino-terminal tyrosine, and is absorbed from plasma or serum by $Al(OH)_3$, $BaSO_4$, and $Ca_3(PO_4)_2$. Factor IX is a β-globulin, stable to storage, and synthesized by the liver. The plasma concentration of factor IX is low and has been estimated to be about 0.3 μg/ml. Factor IX activation takes place in the presence of activated factor XI, and Ca^{2+} accelerates the reaction but is not an absolute requirement. Activated factor IX (factor IXa) is a two-chain glycoprotein and acts as a serine protease, which, together with factor VIII, phospholipid, and calcium ions, forms a complex that converts factor X to its active form, factor Xa. Factor VIII acts as the accelerator of this reaction. One of the actions of heparin is to bind to factor IXa and thus inhibit further activation of the coagulation cascade. The turnover time (18–36 hours) of factor IX in the circulation is longer than that of factor VIII (9–16 hours)(Biggs, 1972). A review of the biochemistry of factor IX was published by Bouma and van Mourik (1977).

d. Antihemophilic Factor (Factor VIII). There is a voluminous clinical and research literature concerning the antihemophilic factor (AHF) which is well outside the scope of this chapter. The following summarizes some of the salient points of factor VIII. For additional reading, consult Hershgold (1974), Benson and Dodds (1976a,b), Hoyer (1976), Bouma and van Mourik (1977), and Bloom and Peake (1977).

Factor VIII is a large protein with a molecular mass estimated to be as high as 2,000,000. It is found in normal plasma at levels of about 50–200 units/ml or 0.10 μg/ml. Reduction of factor VIII reveals one subunit band on sodium dodecyl sulfate-polyacrylamise gel electrophoresis with a molecular weight of 200,000–260,000. The factor VIII level in plasma fluctuates greatly upon exercise, epinephrine response, pregnancy, central nervous stimulation, and use of oral contraceptives (Biggs, 1972; Bennett

and Ogston, 1977). Factor VIII migrates in the α_2- and β_2-globulin fractions. In plasma, it is closely associated with fibrinogen so that many purified factor VIII fractions are still contaminated with trace amounts of fibrinogen. Factor VIII is stable for only short periods of time at room temperature or at 4°C. For maximal stability it should be quick-frozen and kept at −40°C or lower. It is stable to heat of 56°C for up to 15 minutes, but this thermolability is extremely variable. Factor VIII is not adsorbed by $Al(OH)_3$ or $BaSO_4$, is activated by thrombin, and is inactivated by plasmin. This factor has a short survival time *in vivo* (9–16 hours) and is synthesized primarily in the liver but also in extrahepatic sites.

The biochemistry of factor VIII has been and is being intensively studied. As mentioned above, factor VIII is the accelerator protein which, together with factor IXa, phospholipids, and Ca^{2+}, forms a complex that activates factor X. Thrombin activates factor VIII, apparently without altering its electrophoretic mobility or subunit structure. Factor VIII forms a complex with the von Willebrand's factor (VWF). At high ionic strength this complex dissociates to form a high molecular weight fragment with VWF activity and a small molecular weight fragment with factor VIII coagulant activity. In contrast, at low ionic strength, factor VIII dissociates into at least two components but loses both coagulant and VWF activities (Bouma and van Mourik, 1977). In homozygous von Willebrand's disease, a naturally occurring low molecular weight form of factor VIII coagulant exists in plasma in the absence of VWF activity (Benson *et al.*, 1980).

Much of our present knowledge of the molecular structure of factor VIII has come from immunological studies (Hershgold, 1974; Hoyer, 1976; Benson and Dodds, 1976a,b; Ratnoff, 1977a; Bloom and Peake, 1977). Factor VIII is currently thought to circulate in plasma as two major components, factor VIII coagulant (FVIII-C) and factor VIII-related antigen (FVIII-RAg), complexed with VWF. The FVIII-C component has biological activity modified during blood coagulation; is deficient or abnormal in hemophilia A; is synthesized at the same or different site(s) as FVIII-RAg, which is required for its formation and release; and does not influence platelet retention, ristocetin-induced platelet aggregation, or the bleeding time. Production of FVIII-C is controlled by the X chromosome. The FVIII-RAg component, on the other hand, is biologically inactive during clotting; represents antigenic determinants of the protein that affects platelet retention in glass bead columns and ristocetin-induced platelet aggregation; is the major factor VIII fraction isolated by gel filtration of normal plasma; is present in large amounts in platelets; is responsible for the immunoprecipitin line produced in agarose by reactions with heterologous antifactor VIII; is synthesized and released by endothelial cells except in von Willebrand's disease, when these functions are reduced or abnormal; is stable during coagulation and present in serum; and may be required for stabilization, formation, or release of FVIII-C. Production of FVIII-RAg is controlled by an autosomal locus or loci (Bloom and Peake, 1977).

The genetics of factor VIII, another fascinating and much studied subject (Ratnoff, 1972, 1977a; Dodds, 1974b; Bloom and Peake, 1977; Brinkhous, 1978), is discussed later in the respective sections dealing with its two deficiency states, hemophilia A and von Willebrand's disease.

3. The Extrinsic System

a. Tissue Factor (Factor III). Tissue factor is a species-specific thromboplastin extracted from tissue juice and is a potent activator of the extrinsic coagulation mechanism. The functions of tissue factor in the extrinsic pathway have been reviewed by

Nemerson and Pitlick (1972) and Jetsy and Nemerson (in Ogston and Bennett, 1977a). The species specificity of tissue extract disappears if it is first incubated with homologous serum. Purified human brain thromboplastin is more effective for use as a universal activator in coagulation tests than either bovine or rabbit brain thromboplastins, but for practical purposes these are satisfactory. Tissue factor accelerates the activation of factor X in the presence of factor VII and Ca^{2+}, thus forming factor Xa according to the scheme in Fig. 1. The precise mechanism of action of tissue factor is unknown.

The structure of tissue factor includes a protein and a lipid component, neither of which is active by itself. The most active lipid in restoring activity to the protein component of tissue factor is phosphatidylethanolamine, although lecithin has some activity as well (Nemerson and Pitlick, 1972). Unsaturated fatty acid side chains are also required, and mixed lipids are as effective as purified compounds in restoring activity.

b. Proconvertin (Factor VII). Factor VII is a stable, extrinsic coagulation factor that is apparently not required for intrinsic coagulation; it is present in both plasma and serum in trace amounts (0.01 μg/ml). The name proconvertin is rarely used. Factor VII has the most rapid turnover rate of any coagulation factor and a half-life of about 2–7 hours (Brozović, 1977). It seems surprising that a factor not essential for primary hemostasis would be synthesized and metabolized so rapidly. On the basis of extensive studies on the structure, metabolism, and function of factor VII its molecular mass has been estimated to be 48,000 in serum and 63,000 in plasma (Nemerson and Pitlick, 1972; Jesty and Nemerson, in Ogston and Bennett, 1977a).

Factor VII is the second component along with tissue factor which rapidly accelerates the coagulation of blood via the extrinsic pathway. Calcium ions are a requirement for this reaction, which proceeds by activating factor X to form factor Xa. Thus, many of the early stages of coagulation in the intrinsic pathway are bypassed. The extrinsic system needs only factors VII, X, and V, prothrombin, and fibrinogen to form fibrin.

The biochemistry of factor VII has received considerable attention in recent years (Jesty and Nemerson, in Ogston and Bennett, 1977a). Bovine factor VII consists of about 85% protein and 15% carbohydrate. There is marked homology in the amino-terminal sequences of factor VII, prothrombin, and the light chain of factor X. The bulk of factor VII in plasma is thus similar to the other vitamin K-dependent coagulation factors (II, IX, and X). Despite structural similarities, factor VII differs significantly in its metabolic behavior. The single-chain form of factor VII as found in plasma, once enzymatically cleaved, becomes a two-chain form with 60–80 times the coagulant activity. It is not known which of these forms initiates activation of the extrinsic pathway.

Factor VII activity can be increased in a variety of ways, including storage at 0°–4°C, contact activation with glass, kaolin, or celite, and either intrinsic or extrinsic coagulation. Enhancement of factor VII by contact activation requires not only factor XII, but also plasmin and factors XI and IX, and probably proceeds via factor IXa. Formation of factor Xa by either pathway causes a 400-fold increase in the rate of factor VII activation in the presence of phospholipid and Ca^{2+}. Thus, factor Xa is the positive feedback regulator enzyme for factor VII activity during coagulation.

4. The Common System

a. Stuart Factor (Factor X). Factor X is activated by the action of factor IXa, phospholipid, and Ca^{2+}, is accelerated by factor VIII in the intrinsic system or by the

action of factor VIII and Ca^{2+}, and is accelerated by tissue factor in the extrinsic system. The factor Xa formed activates factor V. Thus, the intrinsic and extrinsic pathways of coagulation converge at the step involving the activation of factor X to proceed via a common pathway to fibrin formation. Factor X is an α-globulin of about 55,000–80,000 daltons in plasma and 36,000 daltons in serum and is synthesized by the liver in the presence of vitamin K. It is present in both plasma and serum at levels of about 0.10 μg/ml. A number of compounds, such as Russell's viper venom (Stypven), promote the activation of factor X. The half-life is about 20–40 hours (Brozović, 1977). Factor X is destroyed by heating to 56°C and is stable when frozen at −40°C or lower for several months. Its activity disappears from blood that is clotted with an excess of brain extract and can be adsorbed out of plasma by $Al(OH)_3$, $BaSO_4$, and $Ca_3(PO_4)_2$.

Because of its pivotal role in both coagulation pathways, there has been considerable emphasis on the biochemistry and function of factor X (Nemerson and Pitlick, 1972; Esnouf, 1977; Jesty and Nemerson, in Ogston and Bennett, 1977a). Factor X is an inactive zymogen converted to its active form during clotting. Factor X has two N-terminal amino acids, and activation involves cleavage of a specific peptide bond, yielding factor Xa. The activated form has very potent coagulant and esterolytic activities, which are inhibited by plasma antithrombin III (heparin cofactor) and a variety of reagents that inhibit this and other serine proteases. The major substrate for the enzyme factor Xa is prothrombin. In the presence of factor V, a high molecular weight cofactor that accelerates the reaction, phospholipid, and Ca^{2+}, factor Xa readily converts prothrombin to thrombin.

The majority of factor X in plasma (85–90%) is present in monomeric form, and the remainder exists as a dimer. Bovine factor X is a glycoprotein with two disulfide-linked polypeptide chains: a heavy chain of 38,000 daltons and a light chain of 17,000 daltons. The heavy chain contains all the carbohydrate, which accounts for 10% of the molecular weight. The light chain is highly acidic and remains intact on activation of the parent molecule. The two-chain feature of factor X is unique, as other zymogens of the serine proteases and prothrombin complex clotting factors all exist as a single chain (Esnouf, 1977).

Activation of factor X can be accomplished by intrinsic and extrinsic clotting, Stypven, insolubilized trypsin, and 25% sodium citrate. During prolonged activation the coagulant activity of factor Xa is reduced, but the esterase activity remains unchanged. Factor Xa, as mentioned earlier, is under positive feedback control via its activation of factor VII. Additional feedback control of factor X activation includes the activation of factor IX by factor Xa, and thus the intrinsic pathway may prime the extrinsic pathway but not initiate it (Jesty and Nemerson, in Ogston and Bennett, 1977a).

b. Proaccelerin (Factor V). This factor is required for both the intrinsic and extrinsic coagulation pathways. The term "proaccelerin" is rarely used. Factor V is an extremely labile material and disappears from plasma as it ages. The factor V of bovine plasma is more stable and found in much higher concentrations than in human plasma. This has permitted isolation and biochemical characterization of bovine and, more recently, human factor V (Colman, 1976; Esnouf, 1977). In human plasma, factor V levels are 5–10 μg/ml.

In recent years, the original theory that both factors V and VIII were enzymes converted to active forms during coagulation has been proven to be incorrect (Ogston and Bennett,

1977a). It is now known that factor V is a high molecular weight glycoprotein which, like factor VIII, is nonenzymatic and acts as an accelerator or cofactor of specific coagulation reactions. In the presence of factor Xa, phospholipid, and Ca^{2+}, factor V converts prothrombin to thrombin. Various molecular masses have been obtained for factor V, including about 300,000 and 60,000 daltons for one or two active components and 60,000, 100,000, 180,000, and 200,000 for other fractions. It has been suggested that this variety of molecular weights reflects multiples of a 60,000-dalton subunit and that native factor V contains six of these subunits. Calcium is thought to play a significant role in maintaining the integrity of the structure, as chelating agents rapidly and irreversibly inactivate factor V (Esnouf, 1977). Factor V has extreme affinity for hydrophobic surfaces such as phospholipid, and its reactivity is increased about tenfold with reduction in molecular weight upon reaction with thrombin. Factor V also reacts with other proteolytic enzymes, especially Stypven.

Bovine factor V is an α-globulin with about 15–20% carbohydrate and high sialic acid content. It requires lipid for its coagulant properties and avidly binds lipids. In the presence of phospholipid and Ca^{2+}, but not prothrombin, factor V increases the esterase activity of factor Xa by more than threefold. Thus, factor V directly or indirectly, via binding to phospholipid, regulates factor Xa activity. Procoagulant phospholipids, such as phosphatidylethanolamine, are much more active in complexing with factor V than are other phospholipids (phosphatidylcholine, phosphatidylserine, or cardiolipin). A surprising feature of factor V in contrast to prothrombin and factor Xa is the ability to bind to phospholipid micelles in the absence of calcium. Recent data, however, have shown that each factor V molecule possesses a single, tightly bound calcium that is important for the interaction with phospholipid (Colman, 1976).

Factor V appears to be synthesized in the liver and is tightly bound to platelet membranes, an association regulated by its carbohydrate moiety. Platelet factor V could originate from plasma factor V or, alternatively, could be synthesized by megakaryocytes. Other coagulation factors, such as fibrinogen and factors VIII and XIII, exist in both platelets and plasma. The half-life of factor V has been reported to be between 12 and 36 hours (Colman, 1976; Brozović, 1977). One study located factor V antigen on endothelial cells, but whether this represents synthesis or adsorption from plasma is unknown (Colman, 1976).

c. Prothrombin (Factor II) and Thrombin. Prothrombin is a protein of molecular mass about 68,000. It is an α-globulin and present in the plasma at a concentration of 300 Iowa units/ml or 15 μg/ml. It is converted to the active enzyme thrombin by the action of several factors known as plasma or tissue thromboplastins (Fig. 1). These prothrombin activators are a complex mixture of substances. Prothrombin is synthesized by the liver and has a half-life reported to be as short as 10–12 hours and as long as 60 hours, with the average about 36 hours (Brozović, 1977; Hemker, in Ogston and Bennett, 1977a). Both the biosynthesis and release of prothrombin require the action of vitamin K. Warfarin (a coumarin derivative), the ingredient found in rat poison, blocks prothrombin synthesis at an intermediate step, which can be overcome by the administration of vitamin K_1. Vitamin K is also a known requirement for three other clotting factors, factors VII, IX, and X. The complete amino acid sequence of prothrombin has been defined.

Within recent years, there has been an explosion of information about the biochemistry and control of the prothrombin–vitamin K synthetic pathway. The critical discovery which

precipitated this interest was the demonstration by Stenflo and associates (1974; Stenflo, 1975) and others that the vitamin K-dependent coagulation factors are synthesized from inactive precursor proteins by the action of vitamin K, which converts their glutamic acid groups to active γ-carboxyglutamic acid residues. The clotting proteins are then anchored by the dicarboxyl groups on these residues, via Ca^{2+}, to a phospholipid surface in the process of coagulation.

Prothrombin is a metal-binding protein containing two high-affinity and several lower-affinity metal-binding sites. The presence of ten γ-carboxyglutamic acid residues near the amino terminus of the molecule is an integral part of its metal-binding properties. These amino acids have unique properties and participate as metal ligands in prothrombin (Furie *et al.*, 1978). Metal ions play a key role in the activation of prothrombin to thrombin, and the interaction of all the vitamin K-dependent clotting proteins is metal dependent.

Thrombin, the active enzyme formed from prothrombin by the action of factor Xa, factor V, phospholipid, and Ca^{2+}, has two chains of unequal size (α and β) held together by a disulfide bond. The active site of thrombin, located on the α chain, includes a serine residue which, like similar serine proteases, can readily be inhibited by diisopropyl fluorophosphate. The molecular mass of thrombin varies from 32,000 to 37,000. Heparin acts as an effective anticoagulant, primarily by inhibiting the action of thrombin on fibrinogen and, to a lesser extent, by preventing activation of factor IX by factor XIa.

Thrombin has many critical functions in hemostasis, including not only the formation of fibrin from fibrinogen but also regulation of FXIIIa (fibrinoligase) production, activation of factors V, VII, and VIII, and a variety of roles in platelet reactions. Thrombin is inhibited by antithrombin III (heparin cofactor), fibrin degradation products, α_1-antitrypsin, and α_2-macroglobulin. These inhibitory reactions are discussed further in Section II, E.

d. Fibrinogen (Factor I) and Fibrin. Fibrinogen is the substrate for thrombin and the precursor of fibrin. It circulates in plasma at levels of 2–4 mg/ml. The molecular mass of the human and bovine proteins is 340,000 ± 5%; they have a dimeric structure composed of three pairs of peptide chains (Aα, Bβ, and γ) bonded together by disulfide bonds (Gaffney, 1977). Most vertebrate fibrinogens share a similar structure. Each unit of the dimer is held together by disulfide bridges between the two Aα chains and between the two γ chains near the N-terminus of the molecule. Following the action of thrombin upon fibrinogen, two major fibrinopeptides (A and B) are released from the N-termini of the Aα and Bβ chains, respectively. The remaining fibrin monomers are then polymerized to form insoluble, cross-linked fibrin by the interaction of fibrin-stabilizing factor (factor XIII) and Ca^{2+} (Curtis and Lorand, 1977). The details of this polymerization step are discussed below.

Fibrinogen is believed to be synthesized as a heterogeneous group of molecules. This degree of heterogeneity perhaps explains the large number of abnormal or dysfibrinogens known to occur as inherited disorders, in fetal life, or in the presence of underlying conditions, especially liver disease (Ratnoff, 1977b). The Aα chain is especially heterogeneous and has been shown to vary in size during evolutionary development. Except in the orangutan, there is a continuous decrease in the size of the α chains with a slight increase in the size of the β chains during development up the evolutionary scale from closely related higher primates to man (Gaffney, 1977).

Fibrinogen has a low carbohydrate content, although it is thought to have biological

importance. Fibrinogen is also an important cofactor in mediating platelet aggregation responses *in vivo* and *in vitro,* and platelets contain relatively large amounts of this protein. The site of fibrinogen biosynthesis is the hepatic parenchymal cell, and the half-life is about 36 hours. This turnover rate can be markedly accelerated by ACTH.

Additional properties of fibrinogen and fibrin include their interactions with plasmin, discussed below, and other enzymes, such as trypsin, chymotrypsin, brinase, Pronase, elastase, and collagenase, and their homology, as demonstrated by the high degree of immuno-cross-reactivity among individual vertebrate classes, especially mammals and birds.

e. Fibrin-Stabilizing Factor (Factor XIII). Discovered in 1948, this factor is the latest one to be given a roman numeral according to the international committee classification. A very small amount (2–10%) of factor XIII is sufficient for adequate hemostasis. The half-life of this factor appears to be 3–7 days (Brozović, 1977). Fibrin-stabilizing factor (FSF) is a thrombin-labile protein, requires Ca^{2+} for its activity, and an α_2-globulin found in plasma and platelets, but only a trace is left in serum. It can be inhibited by several metals (Ag, Pb, Zn) and by snake venoms. The molecular mass is estimated to be 160,000 or 320,000, and the molecule contains two subunits. The similarity of the molecule to fibrinogen has been studied extensively (Gaffney, 1977; Curtis and Lorand, 1977). The function of factor XIII is to convert unstable, soluble fibrin monomer in the presence of calcium ions to insoluble, stable fibrin polymer. It is thought to do this by forming cross-linkages between adjacent fibrin strands, a process which has been examined in detail by Lorand and associates among others (Curtis and Lorand, 1977).

The biochemistry of the fibrin-stabilizing process is rather complex. Fibrin–fibrin bonding is strengthened by intermolecular γ-glutamyl–ϵ-lysine bridges, the formation of which is entirely dependent on the FSF system. Factor XIII circulates as an inactive precursor in plasma and serves as the zymogen for factor XIIIa, or fibrinoligase, a transamidase formed and controlled by the action of thrombin and Ca^{2+} in a precise, sequential manner. Thus, thrombin has a dual regulatory function, as it controls not only the rate of fibrin network formation but also the generation of fibrinoligase. Factor XIIIa activity can be measured by its effect on fibrin as assayed by clot solubility in 1% monochloroacetic acid or 5 *M* urea, by direct or indirect quantitation of the number of γ-glutamyl–ϵ-lysine peptides formed, or by evaluating the electrophoretic profiles of reduced and nonreduced fibrin in sodium dodecyl sulfate gels (Curtis and Lorand, 1977).

The FSF zymogen of human and bovine plasma has a heterologous protomeric structure, with two subunits differing in amino acid composition and size (a, 75,000 daltons; b, 80,000 daltons). The native protein is said to exist in an equilibrium of $2\,ab \rightleftharpoons a_2b_2$. The platelet zymogen, in contrast, is composed of two identical a subunits and contains no b. The FSF activity of platelets represents about 20% of that in whole blood (Curtis and Lorand, 1977).

D. The Fibrinolytic Mechanism

1. General

The counterpart to the coagulation mechanism of plasma is a group of zymogens and enzymes that form the fibrinolytic system. Fibrinolysis involves a series of events critical

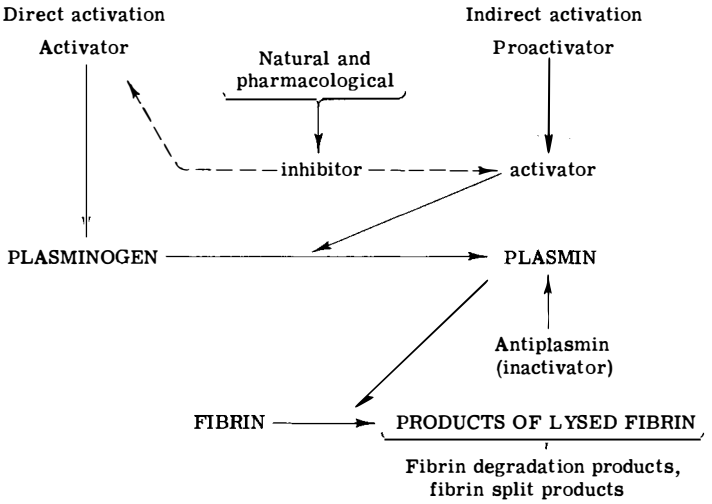


Fig. 2. Scheme of the fibrinolytic mechanism.

to the removal of an established hemostatic plug in support of the process of vessel healing and repair. A simplified scheme of the fibrinolytic mechanism is shown in Fig. 2; more details can be found elsewhere (Biggs, 1972; Robbins, 1977).

After direct or indirect activation of the fibrinolytic system, plasminogen is converted to plasmin. Plasmin actively digests not only fibrin but also fibrinogen and factors V and VIII. This mechanism is quite similar to that of coagulation, as the active enzyme (plasmin) is formed by a series of activation steps from its precursor (plasminogen).

The fibrinolytic system is kept under physiological control by natural fibrinolytic inhibitors. *In vivo,* fibrinolysis is usually restricted to the area in and around a deposit of fibrin. Effective plasmin levels within a hemostatic plug are provided by the action of fibrin in absorbing plasminogen, which becomes activated by materials released from the vessel wall and the cellular elements of the blood. Plasmin and activator escaping from a plug are immediately diluted by the flowing blood and neutralized by antiplasmins.

2. *Plasminogen and Plasmin*

Mammalian plasminogens, when isolated from either plasma or serum, are single-chain monomeric proteins with multiple isoelectric forms. The native molecule is a β-globulin with a molecular mass of about 85,000 in either the Glu or Lys form and has been reported to be synthesized in the kidney.

Many naturally occurring substances are plasminogen activators (Ogston and Bennett, 1977a). These include the tissue activators, or cytokinases—urokinase from urine and those in tears, saliva, milk, bile, and prostatic, cerebrospinal, synovial, and amniotic fluids; streptokinase from β-hemolytic streptococci; niacin and vitamin B_1; vascular endothelial and circulating activators; erythrokinase isolated from erythrocyte hemolysates; neutrophilic granulocyte activator; and the factor XII-dependent activator, which requires factor XII, prekallikrein (Fletcher factor), and Hageman factor cofactor for its activity. Thus, activators of plasminogen are present in blood, the vessel wall, body fluids, and most tissues.

Plasmin, the active enzyme formed from plasminogen, is a serine protease with

trypsin-like specificity and two chains: a heavy (A) chain and a light (B) chain. These chains also have multiple isoelectric forms and molecular masses of about 49,000 and 26,000, respectively. The Lys form of native plasmin has a molecular weight of about 77,000 (Robbins, 1977). Plasmin readily hydrolyzes a variety of proteins, such as factor V, gelatin, casein, arginine esters, and lysine, in addition to fibrinogen and fibrin.

In contrast to plasminogen, plasmin is normally absent from blood and body fluids since a group of circulating antiplasmins readily inactivates free plasmin. These include α_2-macroglobulin, α_1-antitrypsin, $C\bar{1}$ inactivator, and antithrombin III and are discussed below.

E. Inhibitors of Coagulation and Fibrinolysis

1. Coagulation Inhibitors

Much less is known about the naturally occurring inhibitors of coagulation than about the procoagulants of blood. However, without this inhibitor mechanism to neutralize the effects of thromboplastins and thrombin, widespread intravascular coagulation and thrombosis could result from a simple vascular injury (Biggs, 1972).

The known types of natural anticoagulants include the circulating antithrombins (designated I through VI), heparin, and anticoagulants to factors IXa, Xa, and XIa. The most widely studied antithrombins are I (a reversible inhibitor) and III (a heparin cofactor, progressive irreversible inhibitor, and an α_2-globulin). Plasma α_2-macroglobulin, α_1-antitrypsin, and $C\bar{1}$ inactivator are also components of blood known to bind thrombin *in vitro* and *in vivo*.

Of the six major protease inhibitors of blood, four have inhibitory action against one or more clotting factors. These are antithrombin III, $C\bar{1}$ inactivator, α_2-macroglobulin, and α_1-antitrypsin (Ogston and Bennett, 1977b).

Human antithrombin III (AT III) is a relatively heat stable α_2-globulin with a molecular mass of about 65,000 and a carbohydrate content of about 15%. It is the principal physiological thrombin inhibitor of human plasma and constitutes about 50% of the total progressive antithrombin activity, while the remaining activity is shared by α_2-macroglobulin and α_1-antitrypsin. The total progressive antithrombin activity of human plasma is decreased in inherited disorders of AT III and in certain disease states, such as disseminated intravascular coagulation (DIC). Raymond and Dodds (1979b) have observed similar decreases in animals with DIC. Other functions of AT III are neutralization of factor Xa, which is markedly accelerated in the presence of heparin, and heparin-potentiated inhibition of factors VII, IXa, and XIa.

a. $C\bar{1}$ Inactivator. This is a neuraminoglycoprotein capable of inhibiting its natural substrate $C\bar{1}$-esterase, as well as plasma kallikrein (Fletcher factor), factors XIa and XIIa but not factor Xa, and plasmin. It has an estimated molecular weight of 105,000 and 35% carbohydrate content.

b. α_1-Antitrypsin. The major trypsin inhibitor of plasma, α_1-antitrypsin, is a glycoprotein of molecular mass 50,000 daltons. In addition to trypsin, this proten inhibits other proteases, such as chymotrypsin, plasmin, and elastase, in a stoichiometric, irreversible reaction and is the major inhibitor of factor XIa. The question about whether

thrombin is inhibited by α_1-antitrypsin has not been resolved (Ogston and Bennett, 1977b).

c. α_2-Macroglobulin. This is a high molecular mass (650,000–725,000) glycoprotein, which complexes with a number of proteases, including thrombin, kallikrein, and plasmin. The binding of α_2-macroglobulin to thrombin is slow, whereas binding to plasmin is rapid. The subunit chains of the molecule have a molecular mass of 185,000.

d. Factor XIa Inhibitor. The fourth known inhibitor of factor XIa, this factor has a molecular mass of 65,000 and is an α-globulin.

e. Lipoprotein Factor Xa Inhibitor. This is contained in the low-density lipoprotein fraction of plasma. Its action is facilitated by Ca^{2+}.

2. Fibrinolytic Inhibitors

The naturally occurring inhibitors of the fibrinolytic system have been summarized by Ogston and Bennett (1977b). These can be categorized as inhibitors of plasmin, plasminogen activator inhibitors, inhibitors of the factor XII-dependent fibrinolytic pathway, and tissue inhibitors of fibrinolysis.

a. Plasmin Inhibitors. These include α_2-macroglobulin, α_1-antitrypsin, $C\bar{1}$ inactivator, antithrombin III as discussed above, as well as other antiplasmins of plasma and platelets, as yet uncharacterized.

b. Plasminogen Activator Inhibitors. Inhibitors of various molecular masses have been identified, including those of 75,000 and 20,000 daltons. There is a coagulation-dependent inhibitor and one released from platelets. α_2-Macroglobulin also possesses plasminogen activator inhibitory activity, but α_1-antitrypsin and AT III do not.

c. Factor XII-dependent Inhibitors. These inhibitors are discussed in Section II,C,2,a.

d. Tissue Inhibitors. Inhibitors of fibrinolysis have been isolated from placenta and have molecular masses of 105,000 and 43,000.

III. LABORATORY DIAGNOSIS OF BLEEDING DISORDERS

A. General

The following briefly outlines the major points to consider for the diagnosis of bleeding disorders. More details can be found elsewhere (Dodds and Kaneko, 1971; Hall, 1972; Biggs, 1972; Dodds, 1974a; Owen *et al.*, 1975; Dodds, 1977b, 1978a).

1. Sample Collection and Preparation

The importance of proper collection and preparation of blood samples for laboratory diagnosis of coagulation disorders cannot be overemphasized. Scrupulous cleanliness and

avoidance of rough handling or rough surfaces must be adhered to in order to obtain satisfactory results. Smooth surfaces are necessary to prevent activation of factor XII (Hageman factor) and to inhibit spontaneous platelet clumping. Therefore, plastic or siliconized glassware, syringes, and test tubes should be used in all sample preparations and testing procedures.

Blood must be taken by careful venipuncture to avoid contamination with tissue juices, which will activate the coagulation system in about 10 seconds. A small clot in a blood sample will activate and consume enough clotting activity to invalidate the interpretation of results. Because most animal blood coagulates more rapidly than human blood, it is best to add the anticoagulant to the syringe beforehand and then draw the sample.

After collection, blood is centrifuged immediately for platelet-poor plasma (PPP) or platelet-rich plasma (PRP). Platelet-poor plasma is obtained for routine studies by centrifugation at 3000–4000 rpm (800–1000 *g*) for 15 minutes; this plasma contains about 30,000–80,000 platelets per cubic millimeter. If relatively platelet free ($<10{,}000/mm^3$) or hard-spun plasma is desired for specialized or research tests, centrifugation at speeds of 3500–5000 *g* for about 30 minutes or, preferably, 10,000–20,000 *g* for 10–15 minutes is required. After centrifugation the PPP is drawn off with siliconized or plastic pipets, or with plastic syringes and tubing, into plastic or siliconized glass tubes. It is then mixed, dispensed in small aliquots, and tested immediately or frozen at −20°C or lower (−70°C is preferable) for assay in the near future. Frozen plasma should be thawed only once and tested immediately; clotting factors are labile, and repeated freezing and thawing denatures proteins and destroys their biological activity.

Platelet-rich plasma for platelet function tests (250,000–450,000 platelets per cubic millimeter) can be prepared at room temperature by either rapid centrifugation for a short time (1500 rpm or 300 *g* for 2–3 minutes) or slow centrifugation (900 rpm or 150 *g* for 5–10 minutes). Platelets undergo shape changes in the cold, and improper freezing disrupts their membranes. After preparation, PRP is drawn off as described above and put into plastic tubes (preferably polycarbonate), allowed to sit undisturbed for 30 minutes for equilibration of intra- and extracellular constituents, and used within 2 hours.

2. *Anticoagulants*

Trisodium citrate is the anticoagulant of choice for coagulation studies. A standard technique is to use 1 part 3.8% trisodium citrate to 9 parts blood. Sodium oxalate is also used, but less frequently. Heparin, which inhibits thrombin and factor IX activation, interferes with the assay of coagulation factors, while EDTA inhibits platelet interactions so that neither of these anticoagulants is applicable to coagulation studies.

3. *Standard Reference Plasmas and Controls*

Because of the many effects of physiological factors (Bennett and Ogston, 1977) and species specificities (Tables II and III; see also Dodds, 1978b) on hemostatic parameters, it is extremely important that the appropriate reference and control plasmas be used for diagnostic evaluation.

Reference and control reagents include fresh-frozen, pooled, citrated plasmas deficient in specific clotting factor activities (either congenitally deficient or artificially depleted), which are used as substrates in specific coagulation assays and as reference blanks, and fresh-frozen, pooled plasma from healthy animals of the species being studied. This homologous, pooled plasma should be obtained from at least eight animals, four of each

sex, and should be age-matched, whenever possible, with the patient sample. In some cases, both age- and sex-matched reference plasma is desirable.

The deficient substrate plasmas need not be obtained from the same species as the reference standard or test samples. In fact, heterologous deficient substrates have been used successfully for many years in the quantitation of specific coagulation factor activities (Dodds, 1974b, 1979a,b). Examples are the use of canine factor VII-, VIII-, and IX-deficient plasmas for the measurements of their respective clotting factor activities in all mammalian plasmas including human; bovine factor XI-deficient plasma as an excellent reagent for the measurement of factor XI in all species; and coagulation factor-deficient plasmas from human patients to measure clotting factor levels in other animal species. Thus, deficient substrate plasmas are interchangeable, provided that the reference control plasma used to quantitate or compare with the patient sample is a pool from the same species.

For practical purposes, general screening tests of coagulation and assays of specific coagulation factors are best performed on fresh-frozen patient and reference plasmas. Frozen reference plasmas should be stored in small aliquots in plastic tubes at −40°C or lower. The comparison of fresh patient samples with an individual, fresh control plasma is not advisable, as mild or moderately abnormal results can easily be masked. There is a relatively wide normal range for most coagulation assays, and the range for the specific method and laboratory involved should be established by testing of at least 20 normal, healthy individuals. Results obtained with fresh plasma differ from those obtained with frozen samples, and so comparison of fresh patient plasma with a frozen standard plasma is misleading. Most laboratories find it impractical to bleed a fresh, homologous plasma pool on a daily basis. It is often advisable when test samples are shipped frozen to a diagnostic laboratory to request an individual control sample as well, which will serve as a monitor of sample collection and processing.

B. Evaluation of Platelet Function

1. Quantitative Disorders

Thrombocytopenic states and thrombocytosis are diagnosed by direct or indirect platelet counting. These disorders are discussed in detail in Section IV,B.

a. Platelet Count. The first assessment of platelet function is a direct or indirect platelet count. The direct method uses diluents, such as 1% ammonium oxalate, 2% disodium EDTA in saline, or commercial counting fluids. The sample is then counted by a hemacytometer, preferably with phase-contrast microscopy, or by an electronic cell counter. The normal range for most animal species except rodents is 175,000–500,000/mm^3, and less than 100,000/mm^3 can be considered significant. Rodents usually have platelet counts of at least 900,000/mm^3. The indirect method of platelet counting is more practical for routine screening purposes and gives a satisfactory estimate. The platelets per oil immersion field on a stained blood smear are counted and compared with the number of red or white cells. For example, the number of platelets per 100 white blood cells, multiplied by the absolute white count, is an estimate of the platelet count.

b. Clot Retraction. The phenomenon whereby a whole blood or PRP clot retracts from the sides of a glass container depends on normal platelet quantity and function. Clot

retraction may be reduced in anemia and accelerated in polycythemia because the hematocrit tends to influence the retraction of whole blood clots. A variation of this test that is more sensitive to differences in the platelet count employs blood diluted 1 : 10 with cold buffered saline and clotted with a standardized concentration of thrombin. Specifically, 0.5 ml of fresh whole blood is taken by plastic syringe and added to 4.5 ml of the saline. Two milliliters of this diluted mixture is added in duplicate to two small glass test tubes, each containing 1 unit of bovine thrombin* (0.1 ml of a 10 unit/ml solution) and mixed by inversion. The tubes are kept in an ice bath or refrigerator for 30 minutes and transferred to a 37°C water bath. They are examined for retraction every 30 minutes for up to 2 hours. For most animal species, normal samples are maximally retracted by 1–2 hours, after which time the clot commences to lyse. Clot lysis time can also be recorded here by monitoring the sample until lysis is complete.

c. Russell's Viper Venom Time (RVVT). The Russell's viper venom, or Stypven, assay measures extrinsic clotting and is described in more detail in Section III,C. However, it also assesses platelet function, as the venom needs a source of phospholipid for its activation. In PPP or PRP the phospholipid is supplied by an adequate number of functional platelets. Normal animals have an 8- to 14-second RVVT. The assays of thrombocytopenic or thrombopathic patients are prolonged by 3–10 seconds.

d. Bleeding Time. The bleeding time measures hemostasis from a standardized skin incision. Meaningful results can be obtained only if the test is performed under controlled conditions which regulate the size and depth of the incision. Choice of skin sites is complicated in animals by their relatively thick, hairy skin, but the tip of the ear, the lip, and the inner thigh can be used. Normal bleeding times vary from 1 to 5 minutes; prolonged times occur with quantitative and qualitative platelet defects, capillary fragility, vascular lesions, and in von Willebrand's disease.

e. Whole Blood Clotting Time (WBCT). The WBCT in plastic or silicone-coated tubes may be prolonged in thrombocytopenic states, while the glass tube clotting time is usually normal.

2. Qualitative Disorders

See sections (a) through (d) above.

a. Platelet Aggregation and Release. The effect of various aggregating agents, such as ADP, collagen, Adrenalin, and thrombin, on PRP or platelet suspensions is used to evaluate platelet function. The assay measures the change in optical density of standardized platelet preparations kept at 37°C and stirred at 1000 rpm after the addition of an aggregating agent. The optimal concentration of these agents varies with the species from which the platelets were prepared. In general, low doses of ADP cause reversible aggregation, whereas higher doses trigger the release reaction and a secondary wave of irreversible aggregation. Platelet aggregation with collagen or connective tissue suspensions results from adherence of platelets to collagen fibers, followed by the release of platelet ADP, which causes secondary aggregation and is irreversible. Epinephrine- and

*Parke-Davis, Detroit, Michigan.

thrombin-induced aggregation are also mediated by platelet ADP release. It should be emphasized here that there are tremendous species variations in the responses to aggregating agents (Table II; see also Dodds, 1978b).

The platelet release reaction can be assessed by the degree of secondary ADP-mediated aggregation. It can be quantitated by measuring the amount of platelet factor 3, platelet factor 4, nucleotide, [^{14}C]serotonin, or other materials released by optimal concentrations of agents that induce release.

b. Platelet Retention (Adhesiveness). Retention of platelets in a glass bead column or filter of standard size measures their ability to adhere to foreign surfaces. When normal animal blood is passed through these columns, over 75% of the platelets are retained; the platelet count in the filtrate is therefore low. In animals with platelet function defects or von Willebrand's disease, platelet retention is abnormally low, varying from 0 to 50%. A similar finding occurs with human blood, except that platelet retention in normal subjects varies with the assay method and is generally lower (25–80%) than in animals.

The most widely used platelet retention assays are the Salzman method, which employs native whole blood collected directly from the patient's vein and passed through a glass bead column by vacuum, and the Bowie technique, in which blood is taken by syringe into heparin and them pumped or drawn by vacuum through a bead column. In animal species a modification of the Salzman technique has given the best results. Six milliliters of native whole blood are collected as quickly as possible into a plastic syringe. A 1-ml aliquot is immediately added to and mixed in a plastic tube containing 0.1 ml of 3.8% trisodium citrate. The remainder is passed by syringe pump at an even flow rate of 6–8 ml/minute over a standardized 1-gm glass bead column and collected in four successive 1-ml aliquots in a series of plastic tubes containing 0.1 ml of the citrate anticoagulant. The percentage of platelet retention is calculated as follows:

$$\frac{\text{Initial platelet count minus average count for last 3 aliquots from beads}}{\text{Initial paltelet count}} \times 100$$

C. Evaluation of Coagulation

1. General Screening Tests

A great variety of tests are available for the diagnosis of hemostatic and thrombotic disorders. Table IV lists the practical screening tests for evaluating these conditions.

The tests listed in Table IV are designed primarily for screening purposes and as such are not very sensitive to minor abnormalities. This fact should be kept in mind, especially when a diagnostic work-up is performed on patients with only mild bleeding tendencies. If a defect is not found and the history strongly suggests a hemorrhagic problem, more specialized coagulation tests should be performed. As pointed out by Owen *et al.* (1975), it is ironic that mild bleeders are usually evaluated by screening tests of intrinsic and extrinsic clotting and platelet functions, whereas severe bleeders are extensively studied with more specific tests. Obviously the screening assays may not demonstrate an abnormality in a mildly affected patient but are often diagnostic in severe deficiencies.

The methods for platelet counting and the whole blood clot retraction and lysis test are discussed in Sections III,B,1,a and b above.

TABLE IV

Practical Screening Tests for Bleeding Disorders

Platelet count
Dilute whole blood clot retraction and lysis test
One-stage prothrombin time
Activated partial thromboplastin time or activated coagulation time
Thrombin clotting time
Fibrinogen assay
Assay for fibrin–fibrinogen split products[a]

[a] Performed for definitive diagnosis of suspected intravascular coagulation.

a. One-Stage Prothrombin Time (OSPT). The OSPT or Quick time measures clotting factors I, II, V, VII, and X in plasma after activation with tissue thromboplastin and recalcification with calcium chloride. The most commonly used commercial thromboplastins are made from crude extracts of rabbit brain, although human brain extract is also used by many specialized coagulation laboratories because it gives good results for all species. Regardless of the brain extract used, it is essential the *homologous* normal plasmas serve as the assay control.

Animal plasmas frequently clot very rapidly (< 10 seconds) when activated by commercial rabbit brain extract. For this reason, it is difficult to detect minor deficiencies that prolong the assay by only 1 or 2 seconds. This problem can be overcome by using human brain thromboplastin because the OSPT is longer (12–15 seconds for dog and human plasmas, 20–30 seconds for ruminant and guinea pig plasmas). An alternative is to dilute the commercial reagent until the clotting time of the control becomes 12–15 seconds. With this adjusted assay system or with the human reagent, mild deficiencies usually produce a 3- to 6-second longer clotting time.

b. Activated Partial Thromboplastin Time (APTT). The APTT performed on kaolin- or ellagic acid-activated plasma is a reliable indicator of intrinsic clotting. The test with activated plasma gives shorter clotting times and is more widely used and reliable than that in which plasma is not exposed to surface activators. The APTT varies in different species and with the source of partial thromboplastin used to activate clotting. Pooled, homologous plasma is therefore essential as a control. With commercial partial thromboplastins of rabbit origin, the APTT of dog and human plasmas are 14–25 and 24–45 seconds, respectively. Human brain partial thromboplastin gives an APTT of 25–45 seconds with either dog or human plasma.

If the APTT is abnormal, differential mixing tests can be used qualitatively to determine which clotting factor(s) is reduced. An equal mixture of test plasma with plasma from a human being or animal known to have a specific coagulation abnormality is measured by the APTT; failure to correct the APTT to normal identifies the defect.

A simplified variation of the APTT, which measures the intrinsic clotting activity of whole blood, is the *activated coagulation time* (ACT). The advantage of using this test is that it requires only whole blood, activator, and calcium and depends for activation on the

platelets supplied by the sample. Thus, the ACT is sensitive to significant changes in the platelet count. It has been used successfully in small and large animals as a screening test.

c. Thrombin Clotting Time. The time required for a standardized thrombin solution to clot plasma is a measure of the rate of conversion of fibrinogen to fibrin. This test provides important information on fibrinogen clottability and is abnormal in hypofibrinogenemic states, in the presence of heparin or heparin-like anticoagulants, in fibrinolytic disease, and in dysfibrinogenemia. The thrombin time is thus an indicator of quantitative and/or qualitative fibrinogen disorders. In the syndrome of intravascular coagulation with secondary fibrinolysis, the presence of fibrinogen and/or fibrin breakdown products prolongs the thrombin time. Cases of dysfibrinogenemia caused by an abnormal fibrinogen molecule are often detectable only by this test, as fibrinogen concentration is normal.

d. Fibrinogen Assays. Fibrinogen concentrations can be quantitated by several methods, including biological, physical, and immunological assays. Physical methods include an estimation of fibrinogen levels by determining the amount of precipitate formed when plasma is heated to 56°C or treated with 25% ammonium sulfate. A practical method for clinical use in human and veterinary medicine is based on the *heat precipitability* of fibrinogen at 56°C. Plasma samples are placed in microhematocrit tubes, and the amount of precipitate formed upon heating the tubes for 3–9 minutes at 56°C is quantitated by ocular micrometry (Blaisdell and Dodds, 1977).

More accurate, biological methods are the quantitative *Ratnoff–Menzie assay* and its modifications and the measurement of *clot weight.* The first assay is based on thrombin clottability; the last method determines the weight of dried recalcified plasma clots.

Another common technique is the immunological assay of fibrinogen, which quantitates the immunoprecipitate formed with specific antifibrinogen antisera.

e. Assays for Fibrin–Fibrinogen Split Products (FSP). A commercially available latex-agglutination method for measuring FSP* in human blood[+] can also be used for several animal species, including dogs. The test, although expensive, is extremely helpful and reliable for confirming suspected cases of intravascular coagulation.

Other tests include the *ethanol gel test,* which depends upon the separation of FSP from fibrinogen by ethanol, and the *protamine paracoagulation test,* which detects fibrin monomers and early FSP by their ability to cause gelation or form fibrin strands when mixed with a 1% solution of protamine sulfate at pH 6.5.

More specialized quantitative tests are the *staphylococcal clumping test,* in which FSP induce clumping of most strains of *Staphylococcus aureus* by activating their cell wall coagulase, and the very sensitive *hemagglutination inhibition assay,* which measures FSP in the presence of antifibrinogen serum and tanned, formalized, fibrinogen-coated, homologous erythrocytes. Details of these methods are given in Dodds (1974a).

f. Other Assays. The *Russell's viper venom time* or Stypven time, as discussed earlier, is a measure of extrinsic clotting like the OSPT, except that it is insensitive to factor VII and requires phospholipid (supplied by platelet factor 3) for its activation. A long OSPT and normal RVVT are diagnostic of factor VII deficiency. The Stypven assay

*Also called fibrin–fibrinogen degradation products (FDP).

[+]Thrombo-Wellcotest, Burrough-Wellcome, Research Triangle Park, North Carolina.

gives essentially the same clotting time in all species and so eliminates the problems encountered with species specificities of brain thromboplastin. Stypven is quite expensive, however, and may not be practical for routine use.

The *whole blood clotting time* is measured on a small (1.5- to 2-ml) blood sample collected by careful venipuncture into a plastic syringe. The syringe is tilted back and forth until a solid clot forms, or the blood is rapidly dispensed into small glass and/or plastic or siliconized tubes and the clotting time recorded. The results are more referable to the *in vivo* situation if the tubes are kept at 37°C for this determination. The clotting time in plastic syringes and tubes or in siliconized tubes is usually about twice that obtained in glass tubes because glass contact activates factor XII and enhances clotting. Canine whole blood clots in about 2–10 minutes in glass and 4–20 minutes in plastic or silicone. Many laboratories do not routinely perform this test because it is difficult to standardize and the variable results do not readily contribute much diagnostic information.

The *recalcification or plasma clot time* is a more sensitive measure of intrinsic thromboplastin formation than is the WBCT. It times the fibrin end point which forms at 37°C when calcium chloride is added to plasma. Since the recalcification time varies inversely with platelet count, the speed and time of centrifugation for plasma collection must be standardized for control and test samples. Normal plasma clots in 65–100 seconds; abnormal results occur in thrombocytopenic states, with deficiencies of the intrinsic system, or in the presence of circulating anticoagulants. Inhibitors can be ruled out by showing correction of the defect in the test plasma after it has been mixed with normal plasma and incubated for 30–60 minutes at 37°C; a double aliquot of normal plasma incubated in parallel serves as the control.

The *prothrombin consumption or serum clot time* measures the amount of prothrombin remaining after a clot has formed and is actually the prothrombin time of serum. To be reliable, the assay must be performed immediately on the serum obtained from fresh control and test blood incubated at 37°C for 1 or 2 hours. A source of factor V and fibrinogen is required for the test and is supplied by aluminum hydroxide-adsorbed normal rabbit or bovine plasma. The test serum and adsorbed plasma are activated by rabbit or human brain tissue extract and recalcified. In normal individuals, coagulation is complete, and there is very little prothrombin left in the serum. The prothrombin consumption time is thus long (>40 seconds). Patients with defective intrinsic clotting, thrombocytopenia, or thrombopathia cannot consume prothrombin properly, and an excess is present in their serum. The time in these cases is short; in severe defects, such as hemophilia, it may be less than 10 seconds. Thrombocytopenia or thrombopathia can be ruled out by allowing the whole blood for this test to clot in the presence of a source of PF3 activity such as Inosithin.

2. *Specific Assays*

a. Functional Activity Assays. *Specific quantitative assays of intrinsic clotting factors* are usually reserved for experienced coagulation laboratories because they are relatively difficult, time-consuming, and costly to perform. These assays measure factors VIII, IX, XI, and XII and are based on either one-stage (PTT) or two-stage (thromboplastin generation) techniques. Both methods can be accurate and reproducible when performed by experienced personnel, although some investigators prefer two-stage assays despite their complexity.

The clotting factor in question is measured in dilutions of test plasma mixed with standard aliquots of substrate plasma specifically deficient in the factor. The clotting activities of the diluted test plasma are compared by double-logarithmic plots to those obtained for a standard pool of normal plasma *from the same species* and are reported as the percentage of normal. As mentioned before, as long as the control and test plasmas are homologous, the deficient substrate plasma can be heterologous. An added advantage of these substrates is that animals from the same mutant strain can be used to provide large volumes of a standard deficient reagent for use nationwide. The potential of this approach to coagulation assays has not yet been fully realized.

Specific quantitative assays of extrinsic clotting factors (V, VII, and X) are based on the OSPT and/or RVVT tests and are performed by experienced coagulation laboratories. Animal or human plasma known to be deficient in one of these factors is used as the substrate and mixed with dilutions of test plasma. Equivalent dilutions of a normal, pooled homologous plasma serve as the control. The results are compared by double-logarithmic plot, and the patient's sample is reported as the percentage of normal.

Factor X assays usually use either brain tissue extract or Stypven for activation but may use both of these activators. Factor V and VII assays use the OSPT technique, although factor V can also be measured by RVVT and by a more complex two-stage technique (Colman, 1976).

Quantitative assays of prothrombin (factor II) are either one-stage methods, based on specific activation of factor II by snake venoms, or two-stage clottability assays. Although the one-stage assay is more easily performed, availability of the Taipan or tiger snake venom needed for the reaction is limited, and the reagent is expensive.

Fibrinogen assays were discussed earlier in Section III,C,1,d.

The *clot solubility time* depends on the concentration of factor XIII and is thus a measure of its activity. Recalcified PRP or PPP clots are put into a solution of 5 *M* urea or 1% monochloroacetic acid and left at room temperature for 48 hours. Normal clots do not dissolve, whereas factor XIII-deficient clots usually dissolve within 24 hours.

b. Immunological Assays. The most widely used immunological assays for diagnosis of coagulation disorders measures *factor VIII-related antigen*. The functions of this protein component of factor VIII are discussed in detail in Section II,C,2,d. FVIII-RAg is usually quantitated in one or two ways: the Laurell electroimmunoassay (Ratnoff, 1972; Hoyer, 1976; Benson and Dodds, 1976a; Bloom and Peake, 1977; Bennett, 1977) and the more sensitive radioimmunoassay (Hoyer, 1976). When used in conjunction with the assay for factor VIII coagulant activity, the level of FVIII-RAg and the ratio of FVIII-C to FVIII-RAg in man and animals are diagnostic of hemophilia A, are usually diagnostic of von Willebrand's disease (VWD), and often identify hemophilic carrier females (Bloom and Peake, 1977; Bennett, 1977; Dodds, 1977a,b, 1978a,c). Further details of this relationship are discussed later in Section IV,B.

Immunological assays have also been developed for the antigens related to factors V (Colman, 1976), IX (Brozović, 1977; Kasper *et al.*, 1977; Yang, 1978), VII and X (Biggs, 1972; Brozović, 1977), and XI, XII, and XIII (Brozović, 1977) and for prothrombin and fibrinogen (Brozović, 1977; Crum, in Ogston and Bennett, 1977a). These assays include neutralizing, precipitating, and radioimmunoassays and have been used to examine whether the plasma of patients with specific functional deficiencies of clotting factors contain normal, reduced, or undetectable amounts of the related antigens.

c. Other Assays. Quantitative assays for the von Willebrand's factor, also called the *ristocetin cofactor* of the factor VIII–VWF complex, include various measurements of ristocetin- and venom-induced platelet aggregation and macroscopic platelet aggregation tests. These have been the subject of considerable research in recent years (Hoyer, 1976; Fass *et al.*, 1976; Bennett, 1977; Bloom and Peake, 1977; Bowie and Dodds, 1979; Johnson *et al.*, 1979). There are many demonstrable species specificities of the interaction of mammalian plasmas with ristocetin (Brinkhous, 1978; Dodds, 1978b). Some of these differences have necessitated the modification of existing assays designed for measuring ristocetin cofactor in human plasma to permit its quantitation in animal plasmas (Brinkhous, 1978; Johnson *et al.*, 1979).

At present, several new assays or modifications of existing techniques are being developed to measure canine VWF for research and clinical diagnosis of canine VWD. The new assays utilize such reagents as Polybrene (Rosborough *et al.*, 1980) or specific snake venoms (Brinkhous, 1978) to measure VWF-induced platelet agglutinations. Attempts to modify the standard human ristocetin-induced agglutination system have been disappointing or difficult to standardize (Dodds, 1978b; Brinkhous, 1978; Johnson *et al.*, 1979; Rosborough *et al.*, 1980). Thus, it is important to recognize that specific assays used for the platelet–VWF component of the human factor VIII complex may not be applicable to other species.

D. Evaluation of Fibrinolysis

1. General Screening Tests

Tests of the fibrinolytic system are used along with the appropriate coagulation and platelet function assays to distinguish between primary fibrinolysis, a rare entity, and the fibrinolysis commonly seen secondary to intravascular coagulation and consumption coagulopathy (Kwaan, in Ogston and Bennett, 1977a).

a. Clot Lysis Time. The time required for whole blood clots to lyse at 37°C is proportional to the plasmin activity of the blood. It is also dependent on the degree of clot retraction and fibrinogen content of the sample. Poor clot retraction retards clot lysis, whereas hypofibrinogenemic clots are friable. A simple, sensitive method for measuring clot retraction and lysis in dilute whole blood is described in Section III,B,1,b.

Clot lysis of whole blood is measured in glass tubes incubated at 37°C for up to 48 hours. Normal human blood clots take about 16–36 hours to lyse. Most animals have more active fibrinolytic mechanisms, and their blood is usually lysed by 8–20 hours. Clot lysis tends to be more active in females than males and to increase with age and stress situations.

b. Euglobulin Lysis Time. The euglobulins of plasma, such as fibrinogen, plasminogen, plasmin, and plasminogen activator, precipitate upon dilution in water. Fibrinolytic inhibitors, such as antiplasmin and antiactivator, do not. The plasma euglobulin lysis test measures the time for redissolved, thrombin-clotted euglobulin precipitates to be lysed by endogenous plasmin. The assay should be performed on fresh PPP prepared at 4°C since platelets contain fibrinolytic inhibitor activity and chilling retards the inactivation of plasminogen activator. The use of a tourniquet or other trauma at the site of venipuncture should be minimized because it enhances fibrinolysis.

c. Fibrin Plate Test. The ability of plasma to lyse standardized fibrin plates is a measure of its lytic capacity and depends on the plasminogen and plasminogen activator concentration. A standard fibrinogen solution (250 mg/ml) is clotted uniformly with bovine thrombin* (50 NIH units/ml) in a petri dish. The fresh test plasma or its euglobulin precipitate and a standard urokinase solution+ are applied to duplicate fibrin plates and incubated for 18 hours at 37°C. The same precautions apply to plasma collection and preparation as described for the euglobulin lysis test. The area of lysis on each fibrin plate is expressed in square millimeters as the product of its two perpendicular diameters.

2. Specific Assays

a. Functional Activity Assays. The most accurate measures of the *plasminogen content* of plasma is a caseinolytic assay, based on the ability of plasmin to digest a standard solution of α-casein.‡ The plasma is first acidified to decrease its antiplasmin content and then converted by streptokinase (for human plasma) or urokinase (for animal plasmas) to plasmin. The caseinolytic activity of the resultant plasmin is quantitated and compared to that of a known plasmin standard.§

b. Other Assays. A variety of other assays are used to measure the plasminogen level of blood by quantitating the plasmin generated. These include rate assays of plasmin activity using basic amino acid ester substrates, specific active-site titration, fluorometric and radioimmunoassays, and protein inhibitor and affinity chromatographic methods (Robbins, 1977).

IV. BLEEDING DISORDERS

A. General

Over the past 40 years a variety of hemorrhagic diseases have been recognized and studied in animals and man. Historically, these disorders in animals have been one of the most successfully exploited areas of biomedical research in comparative medicine. Today, reliable and useful animal models exist for nearly all of the inherited and acquired hemostatic and thrombotic disorders (''Animal Models,'' 1976; Brinkhous, 1978; Dodds, 1974b, 1977d, 1979b). Only factor V and XIII (FSF) deficiencies have yet to be discovered in domestic or other animals. Numerous reviews exist that describe the clinical, pathophysiological, genetic, management, and treatment aspects of these diseases in man and animals (Hall, 1972; Dodds, 1974a,b, 1975a,b, 1977a,b,c, 1978c, 1979a,b, 1980; Owen *et al.*, 1975; Greene, 1975, 1980; Kociba, 1977; Hamilton *et al.*, 1978). The following section, therefore, is brief, and the reader is referred to the above citations for more detail.

1. Medical History

A list of the common causes of hemorrhage in animals is given in Table V. A complete medical history should point to one or more of these causes of bleeding. This should

*Parke-Davis, Detroit, Michigan.
+Sigma Chemical Co., St. Louis, Missouri.
‡Worthington Biochemicals, Freehold, New Jersey.
§Michigan Dept. Health, East Lansing, Michigan.

TABLE V

Some Causes of Hemorrhage in Animals

Hereditary defects
- Coagulation disorders
  - X-Chromosome-linked recessive traits: hemophilia A, hemophilia B (Christmas disease)
  - Autosomal traits: factor VII deficiency, factor X deficiency, factor XI deficiency, factor XII deficiency, von Willebrand's disease, fibrinogen deficiency (afibrinogenemia and hypofibrinogenemia), prothrombin deficiency (dysprothrombinemia and hypoprothrombinemia)
- Platelet disorders
  - Autosomal traits: thrombasthenia (Glanzmann's disease), thrombopathia (storage-pool disease, thrombocytopathy)

Acquired defects
- Trauma: accidental or from surgical intervention
- Poisoning: warfarin, moldy sweet clover, cottonseed meal, aspirin overdose
- Vitamin deficiency: scurvy, absorptive failure, vitamin K deficiency
- Liver disease: obstructive jaundice, infectious canine hepatitis, tumors, liver failure
- Thrombocytopenia: idiopathic, virus-induced, autoimmune disease, septicemia, splenomegaly, aplastic anemia
- Intravascular coagulation and fibrinolysis syndrome: obstetric complications, sepsis, malignancy, shock, liver disease, heat stroke, incompatible transfusions, heartworm disease
- Platelet function defects: uremia, hyperestrogenism, allergies, drugs, chronic disease, malignancy
- Drug-induced defects: aspirin, steroids, phenylbutazone, live-virus vaccines, furacins, sulfonamides, antihistamines, local anesthetics, tranquilizers

include information about the current bleeding problem, previous bleeding problems, family history, environmental influences, and drugs. Careful questioning of the owner about the patient's present and past bleeding episodes often provides the key to accurate diagnosis, as bleeding problems, especially when internal, can mimic a variety of other disease states. It is therefore important to reevaluate previous illnesses in light of possible clinical signs of bleeding. More details about taking the medical history are given in Dodds (1977b).

2. Physical Examination

A thorough physical examination is essential to determine the location, severity, and nature of the bleeding and to identify the underlying disease that may be present. The type and site of bleeding should be considered carefully. Is the hemorrhage superficial or deep? Is there epistaxis, hematuria, melena, petechiation, or ecchymosis on mucosal surfaces, or hematoma formation in soft tissues or body cavities? Chronic mucosal surface bleeding suggests a platelet disorder or von Willebrand's disease, whereas large hematomas are more common in clotting factor disorders. Other findings, which suggest acquired rather than inherited causes, are splenomegaly, hepatomegaly, and lymphadenopathy.

3. Management and Treatment

Specific details of the management and treatment of bleeding disorders in animals are not given here. Several other articles explain this in depth (Dodds, 1974a,b, 1975a, 1977a,c, 1979a, 1980).

An appropriate physiological and physical environment for hemostasis, tissue repair, and prevention of recurrence are important adjuncts to management and treatment of bleeding disorders. Drugs known to interfere with hemostasis are contraindicated for patients with moderate and severe hemostatic defects since they impair platelet function

and further compromise the stability of the hemostatic plug. These are also discussed in Section IV,C and include aspirin, promazine tranquilizers, phenylbutazone, nitrofurans, sulfonamides, penicillins, phenothiazines, antihistamines, local anesthetics, estrogens, antiinflammatory drugs, plasma expanders, and live-virus vaccines. The latter, like virus infections, affect platelet and/or endothelial function during the viremic phase (5–10 days after vaccination or exposure). Elective surgical procedures, such as ear cropping, spaying, castration, or dentistry, should be performed within 48 hours after vaccination or postponed for 10–14 days (Dodds, 1977c).

Adequate replacement therapy of the correct type is essential for the control of moderate or severe hemostatic defects. Specific details can be found elsewhere (Dodds, 1974a, 1977c, 1979a). Because platelets and coagulation factors are relatively labile, whole blood, plasma, or platelet concentrates should be transfused as soon as possible after collection. Although plasma can be freshly frozen and stored at −20°C or preferably at −70°C for long periods, the techniques for freezing and storing platelets have not yet been perfected. Recent studies with frozen human platelets indicate, however, that successful, practical methods will soon be available; these techniques could then be adapted for animal platelets.

An efficient method of obtaining maximal utilization of blood products involves removing and freezing the plasma from fresh units of whole blood soon after collection and storing the packed red cells at 4°C for 4–6 weeks. These packed cells are readily reconstituted for transfusion purposes. Experience with the storage and utilization of whole blood and packed red cells from animal species indicates that they are best collected and kept under conditions similar to those used for human blood. Plastic bags are preferable to vacuum bottles for collection, and red cells are more viable in citrate–phosphate dextrose (CPD) anticoagulant than in acid–citrate dextrose (ACD).

Although blood-typing sera are not commercially available for animal species, matched blood is definitely preferable because animals with serious bleeding disorders will most likely require repeated transfusions during their lifetimes. Whole blood or packed red cells resuspended in plasma should therefore be used only when absolutely necessary to maintain the patient's hematocrit at or above 20%. The risk of transfusion incompatabilities is particularly applicable to canines because 60% of the random population carries one of the two red cell A antigens (A_1 and A_2*), while the remaining 40% do not. The A_1 antigen (DEA-1 · 1) is strongly hemagglutinating, compared to other canine red cell antigens, and can be responsible for severe anaphylactic reactions in sensitized, mismatched recipients. The A_2 (DEA-1 · 2) antigen is a weaker agglutinin. Several research groups and veterinary institutions now produce their own canine blood-typing sera and maintain banks of A-negative "universal-donor" dog blood. Another canine red cell antigen, Tr (DEA-7), has recently been described and should be considered when transfusing blood because of its presence in half of the random dog population. The ideal canine blood donor is A-negative, Tr-negative (Dodds and Bull, 1979).

Whole blood should be collected in CPD- or ACD-filled plastic blood bags. For treatment of platelet defects, whole blood should be used within 8 hours or, preferably, processed at room temperature into PRP concentrates and infused immediately. If platelets are not required, the blood is centrifuged at 4°C and frozen in practical-sized aliquots in

*New nomenclature for these canine erythrocyte antigens was CEA-1 · 1 and CEA-1 · 2 and has been changed once more to DEA-1 · 1 and DEA-1 · 2 (dog erythrocyte antigens).

sterile plastic bags or bottles. This plasma may be stored for up to 1 year and should be thawed only once before use. The packed red cells are kept at 4°C and reconstituted when needed with an equal volume of fresh-frozen plasma or dextrose in saline, depending on the patient's needs.

B. Hereditary Disorders

Table V contains a list of the inherited coagulation and platelet function defects currently recognized in animals. The inheritance of coagulation deficiencies and the species in which these diseases have been reported are presented in Table VI.

1. Fibrinogen (Factor I) Deficiencies

Hereditary fibrinogen deficiencies have been recognized in goats and dogs. These can be caused by a complete lack of fibrinogen (afibrinogenemia), a reduced level of fibrinogen (hypofibrinogenemia), or an abnormal fibrinogen (dysfibrinogenemia). These defects produce a mild to severe bleeding diathesis and have an autosomal inheritance pattern (Ratnoff, 1972; Gaffney, 1977; Dodds, 1979b).

Fibrinogen defects, quantitative and qualitative, result in a complete failure of plasma or whole blood to clot in any coagulation test or when thrombin is added to it, and plasma does not form a precipitate when heated to 56°C or treated with 25% ammonium sulfate. Some abnormalities of platelet function, a prolonged bleeding time, and abnormal clot

TABLE VI

Inherited Coagulation Defects in Animals

Clotting factor deficiency	Disease	Inheritance[a]	Species
I	Afibrinogenemia, hypofibrinogenemia	AID	Caprine, canine
II	Hypoprothrombinemia, dysprothrombinemia	AID	Canine
VII	Hypoproconvertinemia[b]	AID	Canine
VIII	Hemophilia A, classic hemophilia	X-Chromosome-linked	Canine (purebred and mongrel), feline, equine
IX	Hemophilia B, Christmas disease	X-Chromosome-linked	Canine, feline
VIII complex[c]	Von Willebrand's disease	AID	Porcine, canine, lapine
X	Stuart factor deficiency	AID	Canine
XI	Plasma thromboplastin antecedent (PTA) deficiency	AID	Bovine, canine
XII	Hageman trait, Hageman factor deficiency	AR in cats[d]	Feline, marine mammals, reptiles, birds

[a] Abbreviations: AID, autosomal, incomplete dominance; AR, autosomal recessive.
[b] Not commonly used.
[c] Factor VIII + platelets + endothelium.
[d] Normal physiological event for the other species listed.

retraction also may occur. Although biological assays of fibrinogen are abnormal, fibrinogen may be detected immunologically.

Caprine afibrinogenemia was recognized in the Netherlands in a large family of Saanen dairy goats (Dodds, 1974b, 1979b). It is characterized by a severe hemorrhagic diathesis in newborn and young kids, incompletely dominant inheritance, umbilical bleeding, recurrent hemarthroses, and bleeding into subcutaneous tissues and from mucous membranes. Fibrinogen was undetectable by bio- and immunoassays. Heterozygotes had hypofibrinogenemia.

Canine hypofibrinogenemia was recognized in a family of St. Bernards from West Germany. Both biological and immunological assays of fibrinogen were decreased. The propositus succumbed to a severe hemorrhagic crisis. Related dogs appeared to be heterozygous for the trait.

Inherited dysfibrinogenemia has not yet been reported in animals, although one case in a collie was studied by the author (Dodds, unpublished observations).

2. *Prothrombin (Factor II) Deficiency*

Prothrombin defects prolong the extrinsic system screening tests (OSPT and RVVT), while intrinsic coagulation tests are normal. Both hypo- and dysprothrombinemia are recognized as clinical entities in man. These are autosomal characteristics that produce mild to moderately severe bleeding problems (Ratnoff, 1972, 1977b).

Canine hypoprothrombinemia is the only inherited prothrombin abnormality recognized to date in animals. The affected family of boxers came from Texas, and two generations are currently being studied by Dodds (1979b) and associates. The defect is characterized by epistaxis and umbilical bleeding in newborn puppies and mild mucosal surface bleeding in young adults. Although the disease appeared at first to resemble dysprothrombinemia with reduced prothrombin activity and normal antigen (Dodds, 1979b), current studies point to a metabolic or kinetic problem with turnover of all the vitamin K-dependent clotting factors. These animals are also warfarin sensitive and have a considerably delayed warfarin turnover time. The exact nature of this defect remains to be determined.

3. *Factor VII Deficiency*

This disorder is quite rare in man but is commonly reported worldwide in beagle dogs from large commercial breeding colonies. The apparent high incidence of the defect in this breed probably reflects the widespread use of beagles in biomedical research. One case was also described in an Alaskan malamute. In each case the disorder was discovered fortuitously during routine hematological screening from drug testing, research, or clinical work-up (Dodds, 1974b, 1979b).

Canine factor VII deficiency is a mild disease with no overt bleeding tendency except for easy bruisability and an apparent predisposition to systemic demodicosis, presumably because the animals' defective extrinsic clotting provides an ideal, moist environment for mange mites (Dodds, 1978c). The homozygote and heterozygote are both readily detected by the presence of a long OSPT especially with human brain thromboplastin, normal RVVT, and reduced factor VII activity. Affected dogs have less than 5% factor VII, whereas heterozygotes have about 35–65% of normal levels. The prothrombin consumption time of factor VII-deficient animals is usually quite prolonged, as factor VII is absent from their serum.

Factor VII-deficient dogs have served as important models for biomedical research (Dodds, 1979b). Plasma from affected dogs is an excellent standard source of deficient substrate for quantitating factor VII in man and other species and for quality-control studies of various tissue thromboplastins. The model has been used to investigate the role of factor VII in mediating endotoxin-induced intravascular coagulation and shock. Cross-circulation studies between normal and factor VII-deficient dogs confirmed the rapid turnover and short half-life of this clotting factor as predicted from studies in human beings (Dodds, 1974b, 1977b).

4. Hemophilia A (Factor VIII Deficiency, Classic Hemophilia)

The most common of the severe inherited coagulopathies, hemophilia A, occurs in human beings, dogs, horses, and cats. Clinical signs and laboratory studies have shown the disease to be similar if not identical in these species. Mild, moderate, and severe forms of hemophilia have been recognized in man and dogs, whereas the equine defect has always been severe and the feline defect quite mild. The canine disease has been reported in nearly every purebreed of dogs and in mongrels. Smaller breeds tend to be less severely affected than larger breeds, which appears to be related to weight bearing and is consistent with the severe expression in horses and the mild problem in cats. More details about the clinical signs, management, and treatment of hemophilia can be found in numerous reviews (Ratnoff, 1972; Dodds, 1974b, 1977a,c, 1978c, 1979a; Bennett, 1977; Cotter *et al.*, 1978).

Hemophilia is an X-chromosome-linked recessive trait in man and other animals. Affected males are hemizygous for the trait, whereas carrier females are heterozygous, and affected females (the product of a hemizygous and heterozygous mating) are homozygotes. Affected individuals have markedly prolonged screening tests of intrinsic clotting and very low levels of FVIII-C activity but normal or elevated levels of FVIII-RAg. In accord with the Lyon hypothesis, whereby heterozygous females have one active and one inactivated X chromosome, hemophilic carriers have about half of the normal amount of FVIII-C. They synthesize a full complement of FVIII-RAg, however, and so carrier females can be identified by their moderately reduced FVIII-C (40–60% of normal) and normal or elevated FVIII-RAg. This finding, which resulted from intensive research on factor VIII in normal and deficiency states (see Section II,C,2,d), has been used effectively for carrier detection in both man and animals (Ratnoff, 1972; Bloom and Peake, 1977; Dodds, 1978c, 1979b). The pros and cons of this predictability have generated considerable discussion and differences of opinion (Bloom and Peake, 1977; Bennett, 1977).

Studies of animals with hemophilia A have contributed significantly to current medical knowledge of the analogous human disease and to knowledge of the biochemical and physiological roles of factor VIII (Dodds, 1974b; Benson and Dodds, 1976a,b; Dodds, 1977d; Brinkhous, 1978). Hemophilic dogs have been used in physiological, organ perfusion, and transplantation research to study hemostatic plug formation and the *in vivo* half-life and sites of synthesis of factor VIII. They have provided an extremely valuable standard source of deficient substrate for quantitating FVIII-C in all species and helped in the development of the PTT assay method. Affected animals have also been used to investigate the biochemical nature and molecular structure of factor VIII and to test the *in vivo* efficacy of new homologous and heterologous factor VIII concentrates. Genetic studies with hemophilic dogs have characterized the hemophilic phenotype and genotype,

provided the proof of X-chromosome-linked recessive inheritance, and examined linkage of other closely or distantly located X-chromosomal markers (e.g., the production of canine double hemophilia, hemophilia AB; see Section IV,B,10,b).

5. *Hemophilia B (Factor IX Deficiency, Christmas Disease)*

Hemophilia B, also an X-chromosome-linked recessive trait, is much rarer than hemophilia A and occurs in man, six breeds of dogs, and a family of British shorthair cats (Ratnoff, 1972; Dodds, 1974b; Kasper *et al.*, 1977; Yang, 1978; Dodds, 1978c; Peterson and Dodds, 1979). Christmas disease occurs as a mild to moderate disorder of small breeds (Cairn terrier, American cocker spaniel, and French bulldog) and cats and as a severe diathesis in larger breeds (black and tan coonhounds, St. Bernards, and Alaskan malamutes). As in classic hemophilia, carrier females can be identified by reduced (40–60%) levels of factor IX. However, in contrast to hemophilia A, in which mild, moderate, and severe factor VIII deficiencies exist, all reported cases of hemophilia B in animals have less than 1% factor IX. Recent studies with the human disease have identified two types of patients: those with undetectable or very low levels of factor IX antigen and a few with normal amounts of antigen (Kasper *et al.*, 1977; Yang, 1978). Parallel studies of factor IX antigen in the canine model have not yet been made.

It is important to perform accurate diagnostic tests to differentiate between the hemophilias, as both disorders have markedly prolonged intrinsic system clotting tests. The propositi in the malamute and British shorthair cat families were referred for confirmation of suspected hemophilia A and found to have hemophilia B. The plasma defect of the latter is corrected by addition of fresh normal serum, whereas that of the former is not, because serum contains factor IX but not factor VIII activity.

Like hemophilia A dogs, hemophilia B dogs are important models for biomedical science (Dodds, 1979b). Their plasma is an excellent deficient substrate of standard source for quantitating factor IX activity, and they were used in organ transplantation studies which established the liver to be the sole site of factor IX synthesis. Genetic cross-breeding studies with hemophilia A dogs produced a double hemophiliac, and infusion studies determined the half-life of factor IX.

6. *Von Willebrand's Disease*

Von Willebrand first described this complex, multifaceted syndrome in 1926. The bleeding diathesis, usually more mild than the hemophilias, involves primarily mucous membranes and skin, with gastrointestinal and urogenital bleeding and epistaxis as common symptoms (Hoyer, 1976; Bloom and Peak, 1977; Bennett, 1977). The prolonged bleeding time also results in excessive surgically induced hemorrhage. Von Willebrand's disease is the most common inherited bleeding disorder of man and has been recognized in swine, six breeds of dogs, and a family of laboratory rabbits (Dodds, 1978c; Bowie and Dodds, 1979; Fass *et al.*, 1979). The six affected breeds are German shepherds, miniature schnauzers, golden retrievers, Scottish terriers, Doberman pinschers, and Pembroke Welsh corgis. Isolated cases have occurred in several other breeds (Johnson *et al.*, 1980). The homozygous form of VWD is relatively rare and, in some cases, may be a lethal entity. The heterozygous state may or may not be clinically expressed depending on the mutant or variant and the depth of penetrance of the incompletely dominant trait. Variable penetrance and expressivity is a characteristic of this disorder. The disease in pigs, Scottish terriers, and Chesapeake Bay retrievers, closely

resembles the classic recessive form of human VWD, with the homozygote manifesting moderate to severe hemorrhagic tendency, and the heterozygote being detected by laboratory tests but otherwise asymptomatic (Fass *et al.*, 1979; Johnson *et al.*, 1980). The expression of VWD in dogs varies among breeds and seems to be milder that that in pigs. Thus, canine VWD more closely resembles the wide spectrum of the condition in man (Miller *et al.*, 1979a,b; Rosborough *et al.*, 1980).

Only severely affected individuals have significantly prolonged screening tests of intrinsic clotting, although the bleeding time is more often prolonged. The classic disease has variable reduction in FVIII-C levels (from mild to severe) and parallel reduction in FVIII-RAg levels. The homozygous VWD patient has very low or undetectable FVIII-C and undetectable FVIII-RAg. In addition, the platelet component of the factor VIII complex (FVIII–VWF) is abnormal and produces reduced platelet retention in a glass bead column and low ristocetin-induced platelet agglutination or platelet-aggregating factor. Further details are given in Sections II,C,2,d and III,C,2,b. The other phenomenon observed in classic VWD is a paradoxical increase in FVIII-C, which does not parallel the behavior of FVIII-RAg, FVIII-VWF, or bleeding time, following transfusion of normal or hemophilic plasma. Correction of the other components of the factor VIII complex is usually more immediate and transient, whereas the increase in FVIII-C is delayed (Hoyer, 1976; Bloom and Peake, 1977; Bowie and Dodds, 1979). The mechanism of this response has been studied intensively. The FVIII-RAg present in the infused material is thought to stimulate synthesis of active FVIII-C or else to provide the missing antigenic component which is transformed by the patient to FVIII-C at some remote site yet to be identified (Bouma *et al.*, 1976).

In contrast to the classic type of VWD, there are variants recognized in man, the dog, and the rabbit in which FVIII-RAg is quantitatively normal or else qualitatively altered. Some of these variants have reduced carbohydrate associated with the factor VIII subunit (Bloom and Peake, 1977; Ratnoff, 1977a).

The genetics of VWD are complex and have been studied in depth, but without resolution (Fass *et al.*, 1979; Miller *et al.*, 1979b). The problem concerning the molecular genetics of factor VIII stems from the current theory that the molecule is a combination of two gene products, one coded at an autosomal (VWD) locus and the other at an X chromosome (hemophilia) locus. Both loci are apparently necessary to produce FVIII-C (Bloom and Peake, 1977). The question of true homozygosity versus completely penetrant heterozygosity in VWD has been debated because of studies of families in which there is severe clinical disease. It now seems clear that different types of VWD exist and that true homozygotes are a rareity (Miller *et al.*, 1979a,b; Fass *et al.*, 1979). The classic homozygote is an individual with undetectable or very low FVIII-C and no measurable FVIII-RAg or FVIII–VWF, two heterozygous parents, and a very long bleeding time. However, some heterozygotes also have undetectable FVIII-RAg and/or FVIII–VWF, which is lower than expected on the basis of a simple structural gene defect (Miller *et al.*, 1979a,b; Fass *et al.*, 1979; Rosborough *et al.*, 1980).

A final area of intense interest is the relationship of FVIII–VWF to atherogenesis (Fuster and Bowie, 1978; Thorgeirsson and Robertson, 1978). Studies of normal and VWD swine showed that the affected pigs had significantly fewer vascular lesions and less atherosclerosis than did control swine fed the same diet and raised in the same environment. These exciting observations led to the theory that platelet components of the FVIII complex are involved in the endothelial and vessel wall proliferation of atherogenesis.

7. Factor X Deficiency

This rare coagulation disorder was first described in the mid-1950's in Great Britain and America. It has since been recognized in other human families and in a large inbred family of American cocker spaniels (Dodds, 1974b, 1978c, 1979b). Homozygotes and strongly penetrant heterozygotes have clinically expressed disease, whereas weakly penetrant heterozygotes are usually asymptomatic. Severely affected humans have from <1 to 10% factor X, while affected dogs with less than 20% factor X do not survive neonatal life. Complete absence of factor X is thought to be a lethal mutation because of the central role of factor X in coagulation.

In addition to reduced factor X levels, clinically affected individuals have mild to moderately prolonged APTT, OSPT, and RVVT. In heterozygotes these screening tests may be slightly prolonged. Two types of disease have been recognized in human beings: one in which there is no demonstrable antigen related to factor X (classic form) and another in which normal or reduced amounts of mutant protein are detectable by immunoassays (Ratnoff, 1972).

In affected newborn pups, like infants, serious bleeding occurs. This disease mimics the "fading puppy syndrome," as severely affected pups are stillborn or fade and die in the first 2 weeks of life. Autopsies show massive internal bleeding. Signs in mature adults are mild and referable to mucosal surfaces (Dodds, 1979b).

8. Factor XI (PTA) Deficiency

Factor XI deficiency is a rare disorder in man which occurs in over 90% of cases in persons of Jewish background. Clinical signs are mild (hematuria, bruising, epistaxis, menorrhagia) unless the patient is subjected to surgical procedures. Bleeding usually starts 12–24 hours after surgery and can be severe and protracted. Lethal bleeding has also been reported after minor procedures, such as biopsies and tonsillectomy. The disorder has also been recognized in Holstein cattle (Ratnoff, 1972), English springer spaniels, and Great Pyrenees dogs (Dodds, 1979b).

In cattle and dogs, PTA deficiency is similar to that of man, as there is protracted bleeding after surgery. Clinically affected animals have very low factor XI levels, prolonged APTT and recalcification times, and abnormal prothrombin consumption. Heterozygotes are asymptomatic and have 25–50% factor XI. The propositus in the springer spaniel family is now deceased, and her heterozygous relatives are not available for breeding. Affected cattle are still being maintained, and the value of this model is to provide large quantities of deficient substrate plasma for measuring factor XI levels in man and other species (Dodds, 1979b). Factor XI activity is also deficient in several lower mammalian and nonmammalian species, such as fowl and cetaceans. This is a normal phenomenon, however, and the significance of the apparent absence of PTA-like activity is unknown. Many of these species also lack Hageman and Fletcher factor activities (see Sections IV,B,9 and IV,B,11,c).

9. Factor XII Deficiency (Hageman Trait)

Hageman trait is an asymptomatic coagulation deficiency recognized in man and cats (Ratnoff, 1972, 1977b; Dodds, 1979b). The first feline case involved one animal discovered fortuitously by prolonged screening tests of intrinsic clotting; it had less than 5% factor XII and died without progeny. The second case, also discovered by screening tests, had less than 1% factor XII and has produced five kittens, some of which are heterozygous

for the trait and are being bred to propagate the defect (Green, in Dodds, 1979b). Affected individuals of either species have significantly prolonged APTT, recalcification times, and WBCT in glass tubes.

In addition to these defects, the absence of detectable biological or immunological factor XII is a normal phenomenon of a variety of lower vertebrates and invertebrates (Ratnoff, 1977b). Among these are whales, birds, including the common domestic fowl and waterfowl, reptiles, and, possibly, fish. Other uncommon mammalian and nonmammalian species that do contain Hageman activity are seals, echidnas, marsupials, and amphibians (Ratnoff, in Dodds, 1979b). However, the difficulties in interpreting results of clotting factor tests on nonmammalian plasmas measured with human factor XII-deficient substrate should be considered.

10. Platelet Function Defects

a. Thrombasthenia (Glanzmann's Disease). The term "thrombasthenia" literally means "weak thrombocytes" and was coined by Glanzmann to describe an autosomal recessive bleeding disorder in which there was no clot retraction. There may be low-normal platelet counts or mild thrombocytopenia, but platelet morphology is essentially normal. Affected patients have long bleeding times, defective platelet aggregation with standard aggregating agents, especially ADP, and abnormal platelet retention. In most cases, ristocetin cofactor is normal, but a few patients have been described with low ristocetin response. Platelet fibrinogen, PF3 availability, and platelet membrane glycoproteins IIb and IIIa are usually significantly reduced. The latter abnormalities are thought to be responsible for the defective aggregation and clot retraction (Dodds, 1979b). The bleeding diathesis is severe and of the purpuric type; epistaxis is common and profuse. In severely affected human beings, low-level estrogen treatment has reduced and controlled bleeding. In 1967, Dodds (1974, 1978b, 1979b) described a family of otterhounds with an inherited platelet function defect similar to human Glanzmann's disease except for a large proportion of bizarre giant platelets. The morphological abnormality resembles that of human Bernard–Soulier syndrome. Thus, this canine defect appears to be mixed (*thrombasthenic–thrombopathia*). The function defect is essentially identical to that of thrombasthenia, including the platelet membrane glycoprotein abnormalities (Raymond and Dodds, 1979IIIa; Dodds, 1979b). The disease is inherited as an autosomal dominant trait with variable expression. Both homozygotes and heterozygotes are readily identified by specific platelet function tests.

b. Thrombopathia. A group of inherited thrombopathias have been described in man, many of which have unique characteristics. These include primary or function thrombopathy, Bernard–Soulier syndrome, storage-pool disease or deficit thrombopathy, and other platelet release defects. The specific features of each of these disorders are described elsewhere (DeGaetano and Garattini, 1978; Dodds, 1979b).

Animal thrombopathias have been recognized in fawn-hooded rats (Raymond and Dodds, 1975), basset hounds, Simmental cattle, and cats (Dodds, 1978c, 1979b). The defect in fawn-hooded rats is like human storage-pool disease, as there is reduced platelet serotonin and release. Interesting concomitant findings are a focal glomerulonephritis and proteinuria associated with aging, and docile behavior, thought to be related to reduced content of brain serotonin. These rats are thus useful models for hemostatic, immunological, pathological, and behavioral studies (Dodds, 1979b).

The other reported animal thrombopathias are not as well characterized but appear to have both platelet aggregation and release defects. Homozygotes have a mild to moderate bleeding diathesis primarily involving mucosal surfaces, and heterozygotes are asymptomatic. Both genotypes can be detected by specific platelet function tests.

11. Other Defects

a. Complement Deficiencies. A variety of inherited complement deficiencies are recognized in human beings (Amiraian and Pickering, in Dodds, 1979b). There are, however, only three known complement deficiencies of other species. These are C4 deficiency in guinea pigs and rats, C5 deficiency in mice, and C6 deficiency in hamsters and rabbits (Dodds, 1979b). Of these, the hemostatic mechanism has been evaluated in depth only in the C4-deficient guinea pig and C6-deficient rabbit. Intrinsic coagulation was found to be reduced in both models relative to species-specific controls. These animals have proved to be useful models for pathophysiological studies of hemostasis and thrombosis. The studies were prompted by the variety of interrelationships that exist between the complement and hemostatic mechanisms (Lüscher and Pfueller, 1978; Nachman, 1978; Dodds, 1979b).

b. Double Hemophilia (Hemophilia AB). A planned cross-breeding study between dogs with hemophilia A and hemophilia B was undertaken to study possible linkage between these two X-chromosomal genes. Dogs with double hemophilia were readily produced, thus demonstrating that the loci for hemophilia A and B are not linked but are located far apart on the canine X chromosome (Dodds, 1974b; Brinkhous, 1978).

c. Coagulopathies Not Recognized in Animals. These include *factor V and XIII (FSF) deficiencies,* both rare autosomal traits. The former produces a moderate to severe bleeding diathesis, prolonged APTT, OSPT, and WBCT, short serum prothrombin consumption time, and very low factor V activity. Factor XIII-deficient patients have poor would healing, bleed at delivery, bruise easily, and bleed after minor surgery, especially dental extractions. FSF-Deficient clots dissolve within 24 hours when placed in 5 *M* urea or 1% monochloroacetic acid.

Other defects not yet recognized in animals are the *prekallikrein* (Fletcher factor) and *high molecular weight kininogen* (Fitzgerald or Williams traits) *deficiencies*. Several nonmammalian species, however, lack these activities as a normal characteristic. Prekallikrein activity has been recognized in apes, swine, guinea pigs, mice, goats, sheep, and horses but is absent or minimal in dogs, cats, cattle, rabbits, whales, ducks, and chickens (Ratnoff, in Dodds, 1979b). These results must be interpreted with caution, as human Fletcher factor-deficient plasma was used to make these measurements. For example, rabbit plasma without prekallikrein activity does contain a prekallikrein-like protein.

Similarly, high molecular weight kininogen activity is detectable in primate, dog, and whale plasmas, is very low in rabbit and cattle plasmas, and is absent in fowl, reptilian, and amphibian plasmas. It is still possible, however, that high molecular weight kininogen can be identified or isolated from these plasmas by other types of techniques (Ratnoff, in Dodds, 1979b).

C. Acquired Disorders

The causes of acquired bleeding are numerous, and such conditions are more common than are the inherited deficiencies of clotting factors and platelets (Table V). The major

causes are briefly discussed below. Additional information can be found elsewhere (Dodds, 1974b; Greene, 1975; Owen *et al.*, 1975; Ratnoff, 1977b; Dodds and Wilkins, 1977; Kociba, 1977; Day *et al.*, 1978).

1. Platelet Function Defects

a. Quantitative (Thrombocytopenic States and Thrombocytosis). Quantitative platelet defects are listed by cause in Table VII. Of these, immune-mediated thrombocytopenia accounts for about 70% of cases. The immunological basis has been thoroughly examined in man and to a lesser extent in the dog and horse (Dodds and Wilkins, 1977). Primary immunological thrombocytopenia, of unknown etiology, has been termed idiopathic thrombocytopenic purpura. The majority of cases, however, appear secondary to a variety of underlying conditions (Table VII; see also Owen *et al.*, 1975; Greene, 1980; Dodds, 1979b).

Nonimmunological cases of thrombocytopenia are less common and have a better prognosis if the causative agent or disease can be eliminated. Several useful diagnostic tests exist for detecting platelet antibody. These tests, developed for human use, are all based on assays to detect circulating humoral or cell-mediated antibodies directed against autologous or homologous platelets. Methods include a variety of platelet release tests ([C^{14}]serotonin and PF3 release) and platelet migration inhibition, radioimmunoassays, complement fixation, and hemagglutination inhibition assays (DeGaetano and Garattini, 1978). Experience with animal cases has shown the PF3 release test to be a reliable assay for detecting platelet autoantibody in canine and equine immune thrombocytopenia

TABLE VII

Quantitative Platelet Function Defects[a]

Thrombocytopenia
Increased platelet destruction, utilization, or sequestration
Immunological: primary (ITP); secondary to incompatible blood transfusions, autoimmune diseases (SLE, AIHA, RA), lymphoproliferative disorders, allergies, drugs, toxic agents, live-virus vaccines
Nonimmunological: hemolytic disease of newborn, severe infections, malignancy, drugs (heparin), DIC, hemolytic-uremic syndrome, splenomegaly, hypothermia
Decreased platelet production
Congenital or hereditary: neonatal virus disease, X-Chromosome-linked trait (Wiskott-Aldrich syndrome), autosomal traits (various diseases such as Bernard-Soulier syndrome)
Acquired: Marrow depression (drugs, anemias), marrow infiltration, infections especially viral, drugs (diuretics, estrogens), cyclic thrombocytopenia, paroxysmal nocturnal hemoglobinuria
Thrombocytosis
Increased platelet production
Myeloproliferative disorders: polycythemia vera, chronic leukemia, thrombocythemia
Chronic inflammatory disorders: autoimmune diseases (RA), cirrhosis, granulomatosis, tuberculosis, sarcoidosis, chronic pneumonitis, osteomyelitis
Malignancy: lymphomas, carcinomas
Miscellaneous: acute infection, acute hemorrhage, iron deficiency, postoperative rebound, osteoporosis
Release from tissue stores (spleen, lung)
Response to exercise
Drugs: Adrenalin, vincristine

[a] Abbreviations: ITP, idiopathic thrombocytopenic purpura; SLE, systemic lupus erythematosis; AIHA, autoimmune hemolytic anemia; RA, rheumatoid arthritis; DIC, disseminated intravascular coagulation.

(Dodds and Wilkins, 1977; Wilkins, 1977). This assay is based on the fact that immune complexes formed between antibody isolated from the patient's plasma globulin fraction and antigenic receptors on the surface of normal, homologous platelets alter the membrane and trigger the release reaction. One of the platelet constituents released by antigen–antibody complexes is PF3, and this causes a shortening of the contact-activated clotting time of the normal PRP. Antibody-positive samples significantly shorten the clotting time in the assay, whereas antibody-negative cases and a control, homologous plasma globulin fraction do not.

b. Qualitative. The major causes of qualitative platelet function defects are listed in Table VIII. In addition to the hereditary defects discussed earlier, there are a group of diseases and a large number of drugs currently known to produce thrombopathias. Most of these act by inhibiting the adhesion of platelets to subendothelium (e.g., aspirin, which blocks platelet cyclic endoperoxides, as discussed earlier) and/or the platelet release reaction (e.g., phenylbutazone, antiinflammatory drugs, promazine tranquilizers). Details of the mechanisms of drug interactions with platelets can be found in Caprino and Rossi (1974) and DeGaetano and Garattini (1978). As mentioned in Section IV,A,3, drugs that interfere with platelet function are contraindicated or must be used with caution in moderate or severely affected individuals. Similarly, elective surgery should be avoided during the viremic phase (5–10 days) after live-virus vaccination or viral exposure.

The most common diseases manifesting a bleeding tendency attributable to platelet dysfunction are *uremia* and *liver disease*. Less common causes are the dysproteinemias, such as myelomas and macroglobulinemia, and estrogen toxicity. The classic clinical case of uremic bleeding is that of the old dog with compensated chronic interstitial nephritis, which has inflammed gums and chronic periodontal disease. Dentistry on such a patient frequently results in excessive and prolonged gingival bleeding.

2. Disseminated Intravascular Coagulation with Fibrinolysis

The combined syndrome of intravascular coagulation and secondary fibrinolysis is an important acute, subacute, or chronic disease process of human beings and other animals. Many reviews have been written on the subject, and the reader is directed to recent articles for discussion of the pathophysiology of this syndrome (Owen *et al.*, 1975; Greene, 1975, 1980; Dodds *et al.*, 1977; Kociba, 1977; Hamilton *et al.*, 1978; Kociba, in Dodds, 1979b). The major causes of DIC and thrombosis in man and animals include viral,

TABLE VIII

Qualitative Platelet Function Defects

Hereditary
Thrombasthenia (Glanzmann's disease)
Thrombopathia: Bernard-Soulier syndrome, storage-pool disease, albinism, Ehlers-Danlos syndrome, Wiskott-Aldrich syndrome, osteogenesis imperfecta, others
Acquired
Uremia, myeloproliferative disorders, macroglobulinemia, liver disease, fibrinolysis, thrombocytopenia, systemic lupus erythematosis, congenital heart disease, anemias, leukemias
Drugs: aspirin, phenylbutazone, promazine tranquilizers, estrogens, plasma expanders, nitrofurans, sulfonamides, antiinflammatory drugs, local anesthetics, phenothiazines, live-virus vaccines

bacterial, protozoal, and parasitic *infections; neoplasia; obstetric complications;* and *miscellaneous* conditions, such as trauma, shock, heat stroke, burns, drowning, liver disease, and canine heartworm disease (Greene, 1975; Kociba, 1977; Hamilton *et al.*, 1978; Day *et al.*, 1978; Mustard *et al.*, 1978; Kociba, in Dodds, 1979b).

a. Infections. A number of spontaneous and induced viral infections cause DIC in animals and serve as models for research. Among these are hog cholera, infectious canine hepatitis, fowl plague, epizootic hemorrhagic disease of deer, and equine viral arteritis.

Bacterial infections include leptospirosis in cattle and guinea pigs, gram-negative sepsis, and the endotoxin-mediated Shwartzman reaction of rabbits, horses, rats, guinea pigs, and dogs.

Malaria in monkeys and trypanosomiasis and babesiosis of dogs and cattle also produce DIC. Some dogs with the pulmonary or vena caval lesions of complicated or treated heartworm disease succumb to DIC.

b. Neoplasia. This is a common cause of DIC in animals, perhaps because many neoplasms are not diagnosed early or else the owner elects not to treat the animal. Naturally occurring adenocarcinomas of the mammary gland, testicles, and thyroid gland and carcinomas of the lymphatic system, liver, and spleen have been the common tumors involved.

c. Obstetric Complications. A life-threatening complication of pregnancy in women, DIC is also associated with obstetric problems in animals. These include dystocias, eclampsia, and retained fetus(es). Other causes are amniotic fluid embolism and abruptio placentae.

d. Heat Stroke. Spontaneous heat stroke in dogs and pigs has been associated with DIC and is often fatal.

e. Atrial Thrombosis. A high incidence of atrial thrombosis and concomitant DIC has been reported to occur with aging in Syrian hamsters (Dodds *et al.*, 1977).

The diagnosis of DIC is complicated by the various stages of the syndrome, which include initial activation of hemostatic elements, the so-called hypercoagulable state, followed by thrombosis and consumption, which depletes hemostatic elements. This results in the "consumption coagulopathy" phase of the syndrome with secondary enhancement of fibrinolysis and bleeding. The pathophysiological process is dynamic and proceeds, without treatment, to death of the host or becomes reversible once healing and repair have restored homeostatic balance. The selection of the most appropriate management and treatment is often a crucial decision. Procoagulants given at the wrong time may promote further thrombosis, whereas antiplatelet drugs or anticoagulants may facilitate bleeding. In many cases, removal of the primary cause is the only way to manage the patient effectively.

Diagnostic tests for establishing the presence of ongoing DIC were described earlier (Section III,C,1,e). Classically, the consumption phase of DIC shows thrombocytopenia, prolonged APTT, OSPT, and thrombin time; reduced factor V and VIII activities; and enhanced fibrinolysis with elevated FSP. Details of management and treatment are discussed elsewhere (Greene, 1975; Owen *et al.*, 1975; Kociba, 1977; Dodds, 1977c, 1979a;

Hamilton *et al.*, 1978). There may also be reduced antithrombin activity (Raymond and Dodds, 1979b).

3. Liver Disease

As the liver is the primary site of clotting factor synthesis, acute or chronic generalized hepatic disease often results in a bleeding tendency (Ratnoff, 1977b; Dodds, 1977b). Hepatitis may also produce DIC and platelet dysfunction. Usually the most significant findings in liver disease are a prolonged OSPT with low factor VII levels early in the course of the disease, followed by reduction of other prothrombin-complex clotting factors as the disease progresses and variable prolongation of APTT and thrombin time.

4. Vitamin K Deficiency and Warfarin Toxicity

The Vitamin-K dependent clotting factors (II, VII, IX, and X) are reduced in warfarin toxicity (accidental from ingestion of rodenticide or from therapeutic overdosage to control venous thrombosis), in malabsorption syndromes, and in sterilization of the gut by prolonged use of antibiotics (Owen *et al.*, 1975; Dodds, 1977b). Diagnosis is confirmed by identifying the probable cause and by finding a moderate to markedly prolonged OSPT, variable APTT, normal thrombin time, and low-normal platelet count or mild to moderate thrombocytopenia. A corrective response to treatment with whole blood or plasma transfusion and/or treatment with vitamin K_1 are diagnostic of vitamin K antagonism or depletion.

5. Other Causes

Monoclonal gammopathies, such as myelomatosis and macroglobulinemia, produce a nonspecific bleeding tendency characterized by such problems as epistaxis, oozing from surface abrasions, and mucosal surface bleeding. The cause is thought to be coating of platelets and clotting factors with the abnormal protein, which impairs hemostasis (Dodds, 1974b).

Amyloidosis is also associated with an acquired coagulopathy, but this has been restricted in most cases to isolated factor X deficiency, although some patients also have factor IX deficiency or platelet defects (Dodds *et al.*, 1977).

REFERENCES

"Animal Models of Thrombosis and Hemorrhagic Diseases" (1976). DHEW Publ. 76-982. NIH/USPHS, Washington, D.C.

Belamarich, F. A. (1976). *In* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), Vol. 3, p. 191. Grune & Stratton, New York.

Bennett, B. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 377. Wiley, New York.

Bennett, B., and Ogston, D. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 354. Wiley, New York.

Benson, R. E., and Dodds, W. J. (1976a). *Blood* **48,** 521.

Benson, R. E., and Dodds, W. J. (1976b). *Proc. Soc. Exp. Biol. Med.* **153,** 339.

Benson, R. E., Johnson, G. S., and Dodds, W. J. (1980). *Blood* (in press).

Biggs, R., ed. (1972). "Human Blood Coagulation, Haemostasis and Thrombosis." Davis, Philadelphia, Pennsylvania.

Blaisdell, F. S., and Dodds, W. J. (1977). *J. Am. Vet. Med. Assoc.* **171,** 340.

Bloom, A. L., and Peake, I. R. (1977). *Semin. Hematol.* **14,** 319.

Blumenstock, F. A., Saba, T. M., Weber, P., and Laffin, R. (1978). *J. Biol. Chem.* **253,** 4287.
Bouma, B. N., and van Mourik, J. A. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 56. Wiley, New York.
Bouma, B. N., Dodds, W. J., van Mourik, J. A., Sixma, J. J., and Webster, W. P. (1976). *Scand. J. Haematol.* **17,** 263.
Bowie, E. J. W., and Dodds, W. J. (1979). *In* "Spontaneous Animal Models of Human Disease" (E. J. Andrews, B. C. Ward, and N. H. Altman, eds.), Vol. I, p. 272 Academic Press, New York.
Brinkhous, K. M. (1978). *Adv. Exp. Med. Biol.* **102,** 123.
Brozović, M. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 390. Wiley, New York.
Caprino, L., and Rossi, E. C., eds. (1974). "Platelet Aggregation and Drugs." Academic Press, New York.
Colman, R. W. (1976). *In* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), Vol. 3, p. 109. Grune & Stratton, New York.
Cotter, S. M., Brenner, R. M., and Dodds, W. J. (1978). *J. Am. Vet. Med. Assoc.* **172,** 166.
Curtis, C. G., and Lorand, L. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 186. Wiley, New York.
Day, H. J., Molony, B. A., Nishizawa, E. E., and Rynbrandt, R. H., eds. (1978). *Adv. Exp. Med. Biol.* **102.**
DeGaetano, G., and Garattini, S., eds. (1978). "Platelets: A Multi-disciplinary Approach." Raven, New York.
Dodds, W. J. (1974a). *In* "Handbook of Laboratory Animal Science" (E. C. Melby and N. H. Altman, eds.), Vol. 2, p. 85. Chem. Rubber Publ. Co., Cleveland, Ohio.
Dodds, W. J. (1974b). *In* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), Vol. 2, p. 215. Grune & Stratton, New York.
Dodds, W. J. (1975a). *In* "Textbook of Veterinary Internal Medicine" (S. J. Ettinger, ed.), Vol. 2, p. 1679. Saunders, Philadelphia, Pennsylvania.
Dodds, W. J. (1975b). *J. Am. Anim. Hosp. Assoc.* **11,** 366.
Dodds, W. J. (1977a). *In* "Current Veterinary Therapy VI" (R. W. Kirk, ed.), p. 438. Saunders, Philadelphia, Pennsylvania.
Dodds, W. J. (1977b). *Mod. Vet. Pract.* **58,** 680.
Dodds, W. J. (1977c). *Mod. Vet. Pract.* **58,** 756.
Dodds, W. J. (1977d). "First International Registry on Animal Models of Thrombosis and Hemorrhagic Diseases," *ILAR News,* **21,** A1.
Dodds, W. J. (1978a). *Small Anim. Weekly Update Ser.* **3.**
Dodds, W. J. (1978b). *In* "Platelets: A Multidisciplinary Approach" (G. DeGaetano and S. Garattini, eds.), p. 45. Raven, New York.
Dodds, W. J. (1978c). *Canine Pract.* **5,** 49.
Dodds, W. J. (1979a). *Small Anim. Weekly Update Ser.* **20.**
Dodds, W. J. (1979b). *In* "Spontaneous Animal Models of Human Disease" (E. J. Andrews, B. C. Ward, and N. H. Altman, eds.), Vol. I. Academic Press, New York.
Dodds, W. J. (1980). *In* "Pathophysiology in Small Animal Surgery" (M. J. Bojrab and W. E. Wingfield, eds.), Lea & Febiger, Philadelphia, Pennsylvania (in press).
Dodds, W. J., and Bull, R. W. (1979). *Pure-Bred Dogs Am. Kennel Gazette* **96,** 68.
Dodds, W. J., and Kaneko, J. J. (1971). *In* "Clinical Biochemistry of Domestic Animals" (J. J. Kaneko and C. E. Cornelius, eds.), 2nd ed., Vol. 2, p. 179. Academic Press, New York.
Dodds, W. J., and Wilkins, R. J. (1977). *Am. J. Pathol.* **86,** 489.
Dodds, W. J., Moynihan, A. C., Raymond, S. L., and McMartin, D. N. (1977). *Thromb. Haemost.* **38,** 457.
Esnouf, M. P. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 76. Wiley, New York.
Fass, D. N., Brockway, W. J., Owen, C. A., Jr., and Bowie, E. J. W. (1976). *Thromb. Res.* **8,** 319.
Fass, D. N., Bowie, E. J. W., Owen, C. A., Jr., and Zollman, P. E. (1979). *Blood* **53,** 712.
Furie, B., Provost, K. L., Blanchard, R. A., and Furie, B. C. (1978). *J. Biol. Chem.* **253,** 8980.
Fuster, V. D., and Bowie, E. J. W. (1978). *Adv. Exp. Med. Biol.* **102,** 187.
Gaffney, P. J. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 105. Wiley, New York.
Greene, C. E. (1975). *J. Am. Anim. Hosp. Assoc.* **11,** 674.
Greene, C. E. (1980). *In* "Pathophysiology in Small Animal Surgery" (M. J. Bojrab and W. E. Wingfield, eds.), Lea & Febiger, Philadelphia, Pennsylvania (in press).

Hall, D. E. (1972). "Blood Coagulation and its Disorders in the Dog." Williams & Wilkins, Baltimore, Maryland.
Hamilton, P. J., Stalker, A. L., and Douglas, A. S. (1978). *J. Clin. Pathol.* **31,** 609.
Hershgold, E. J. (1974). *In* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), Vol. 2, p. 99. Grune & Stratton, New York.
Holmsen, H., Salganicoff, L., and Fukami, M. H. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 239. Wiley, New York.
Hoyer, L. W. (1976). *In* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), Vol. 3, p. 231. Grune & Stratton, New York.
International Committee for the Nomenclature of Blood Clotting Factors (1962). *J. Am. Med. Assoc.* **180,** 733.
Jaffe, E. A. (1977). *N. Engl. J. Med.* **296,** 377.
Johnson, G. S., Benson, R. E., and Dodds, W. J. (1979). *Thromb. Res.* **15,** 835.
Johnson, G. S., Lees, G. E., Benson, R. E., Rosborough, T. K., and Dodds, W. J. (1980). *J. Am. Vet. Med. Assoc.* **176,** 1261.
Kasper, C. K., Osterud, B., Minami, J. Y., Shonick, W., and Rappaport, S. I. (1977). *Blood* **50,** 351.
Kelton, J. G., Hirsh, J., Carter, C. J., and Buchanan, M. (1978). *J. Clin. Invest.* **62,** 892.
Kociba, G. J. (1977). *In* "Current Veterinary Therapy VI" (R. W. Kirk, ed.), p. 448. Saunders, Philadelphia, Pennsylvania.
Lüscher, E. F., and Pfueller, S. L. (1978). *In* "Platelets: A Multidisciplinary Approach" (G. DeGaetano and S. Garattini, eds.), p. 261. Raven, New York.
Miller, C. H., Graham, J. B., Goldin, L. R., and Elston, R. C. (1979a). *Blood* **54,** 117.
Miller, C. H., Graham, J. B., Goldin, L. R., and Elston, R. C. (1979b). *Blood* **54,** 137.
Moncada, S., and Vane, J. R. (1978). *In* "Platelets: A Multidisciplinary Approach" (G. DeGaetano and S. Garattini, eds.), p. 239. Raven, New York.
Moncada, S., Gryzlewski, R. J., Bunting, S., and Vane, J. R. (1976). *Nature (London)* **261,** 663.
Mustard, J. F., and Packham, M. A. (1970). *Pharmacol. Rev.* **22,** 97.
Mustard, J. F., and Packham, M. A. (1971). *In* "Inflammation, Immunity and Hypersensitivity" (H. Z. Movat, ed.), p. 528. Harper, New York.
Mustard, J. F., Packham, M. A., and Kinlough-Rathbone, R. L. (1978). *Adv. Exp. Med. Biol.* **102,** 7.
Nachman, R. L. (1978). *In* "Platelets: A Multidisciplinary Approach" (G. DeGaetano and S. Garattini, eds.), p. 199. Raven, New York.
Nemerson, Y., and Pitlick, F. A. (1972). *In* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), Vol. 1, p. 1. Grune & Stratton, New York.
Ogston, D., and Bennett, B., eds. (1977a). "Haemostasis: Biochemistry, Physiology and Pathology." Wiley, New York.
Ogston, D., and Bennett, B. (1977b). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), pp. 202, 230. Wiley, New York.
Owen, C. A., Bowie, E. J. W., Didisheim, P., and Thompson, J. H., eds. (1975). "The Diagnosis of Bleeding Disorders," 2nd ed. Little, Brown, Boston, Massachusetts.
Peterson, M. E., and Dodds, W. J. (1979). *J. Am. Vet. Med. Assoc.* **174,** 1326.
Ratnoff, O. D. (1972). *In* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), Vol. 1, p. 39. Grune & Stratton, New York.
Ratnoff, O. D. (1977a). *J. Lab. Clin. Med.* **89,** 1159.
Ratnoff, O. D. (1977b). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), pp. 1, 25, 446. Wiley, New York.
Raymond, S. L., and Dodds, W. J. (1975). *Thromb. Diath. Haemorrh.* **33,** 361.
Raymond, S. L., and Dodds, W. J. (1979a). *J. Lab. Clin. Med.* **93,** 607.
Raymond, S. L., and Dodds, W. J. (1979b). *Proc. Soc. Exp. Med. Biol.* **161,** 464.
Robbins, K. C. (1977). *In* "Haemostasis: Biochemistry, Physiology and Pathology" (D. Ogston and B. Bennett, eds.), p. 208. Wiley, New York.
Rosborough, T. K., Johnson, G. S., Benson, R. E., Swaim, W. R., and Dodds, W. J. (1980). *J. Lab. Clin. Med.* (in press).
Ross, R., and Harker, L. (1978). *Adv. Exp. Med. Biol.* **102,** 135.
Stenflo, J. (1975). *In* "Prothrombin and Related Coagulation Factors" (H. C. Hemker and J. J. Veltkamp, eds.), p. 152. Leiden Univ. Press, Leiden.
Stenflo, J., Fernlund, P., and Egan, W. (1974). *Proc. Nat. Acad. Sci. U.S.A.* **71,** 2730.

Thorgeirsson, G., and Robertson, A. L., Jr. (1978). *Am. J. Pathol.* **93,** 801.
Wester, J., Sixma, J. J., Geuze, J. J., and vander Veen, J. (1978). *Lab. Invest.* **39,** 298.
Wilkins, R. J. (1977). *In* "Current Veterinary Therapy VI" (R. W. Kirk, ed.), p. 445. Saunders, Philadelphia, Pennsylvania.
Yang, H. C. (1978). *Thromb. Res.* **13,** 97.

REFERENCES FOR COMPARATIVE STUDIES FOR TABLES II AND III

Abildgaard, C. F., and Link, R. P. (1965). *Proc. Soc. Exp. Biol. Med.* **119,** 212.
Abildgaard, C. F., Harrison, J., and Johnson, C. A. (1971). *J. Appl. Physiol.* **30,** 400.
Abildgaard, C., Harrison, J. Espana, C., Spangler, W., and Gribble, D. (1975). *J. Trop. Med. Hyg.* **24,** 537.
Adaval, S. C. (1973). *Diss. Abstr.* **43,** 459B.
Addonizio, V. P., Jr., Edmonds, L. H., Jr., and Colman, R. W. (1978). *J. Lab. Clin. Med.* **91,** 989.
Archer, R. K. (1970). *Symp. Zool. Soc. London* **27,** 121.
Barkhan, P., Tomlin, C., and Archer, R. K. (1957). *J. Comp. Pathol. Ther.* **67,** 358.
Bell, T. G., Meyers, K. M., Prierer, D. J., Fauci, A. S., Wolff, S. M., and Padgett, G. A. (1976). *Blood* **48,** 175.
Bell, W. N., and Tomlin, S. C. (1955). *J. Comp. Pathol.* **65,** 255.
Birndorf, N. I., Pearson, J. D., and Wredman, A. (1971). *Comp. Biochem. Physiol. A* **38,** 157.
Blaisdell, F. S., and Dodds, W. J. (1977). *J. Am. Vet. Med. Assoc.* **171,** 340.
Bowie, E. J. W., Owen, C. A., Jr., Zollman, P. E., Thompson J. H., Jr., and Fass, D. N. (1973). *Am. J. Vet. Res.* **34,** 1405.
Breukink, H. J., Hart, H. C., van Arkel, C., van der Velden, N. A., and van der Watering, C. C. (1972). *Zentralbl Veterinaermed., Reihe A* **19,** 661.
Cliffton, E. E., and Mootse, G. (1967). *Thromb. Diath. Haemorrh.* **18,** 291.
Didisheim, P., Hattori, K., and Lewis, J. H. (1959). *J. Lab. Clin. Med.* **53.** 866.
Dodds, W. J. (1974). *In* "Progress in Hemostasis and Thrombosis" (T. H. Spaet, ed.), Vol. 2, p. 215. Grune & Stratton, New York.
Dodds, W. J. (1975). *Proc. Int. Symp. Equine Hematol., 1st* p. 202. Am. Assoc. Equine Pract., Golden, Colorado.
Dodds, W. J. (1978). *In* "Platelets: A Multidisciplinary Approach" (G. DeGaetano and S. Garattini, eds.), p. 45. Raven, New York.
Dodds, W. J., Raymond, S. L., Moynihan, A. C., and McMartin, D. N. (1977a). *Thromb. Haemost.* **38,** 457.
Dodds, W. J., Raymond, S. L., Moynihan, A. C., Pickering, R. J., and Amiraian, K. (1977b). *Proc. Soc. Exp. Biol. Med.* **154,** 382.
Fantl, P. (1961). *Aust. J. Exp. Biol.* **39,** 403.
Gajewski, J., and Povar, M. L. (1971). *Am. J. Vet. Res.* **32,** 405.
Gallimore, M. J., Nulkar, M. V., and Shaw, J. J. B. (1965). *Thromb. Diath. Haemorrh.* **14,** 145.
Hall, D. E. (1972). "Blood Coagulation and its Disorders in the Dog." Williams & Wilkins, Baltimore, Maryland.
Hampton, J. W., and Matthews, C. (1966). *J. Appl. Physiol.* **21,** 1713.
Hathaway, W. E., Hathaway, H. S., and Belhasen, L. P. (1964). *J. Lab. Clin. Med.* **63,** 784.
Hawkey, C. (1970). *Symp. Zool. Soc. London* **27,** 133.
Hawkey, C. (1976). *In* "Animal Models of Thrombosis and Hemorrhagic Diseases," DHEW Publ. 76-982, p. 69. NIH/USPHS, Washington, D.C.
Hofmann, W., El Amrouse, S., and Schultze, H. (1964). *Berl. Muench. Tieraerztl. Wochenschr.* **4,** 70.
Holman, H. H., and Dew, S. M. (1965). *Res. Vet. Sci.* **6,** 510.
Hurt, J. P., and Krigman, M. R. (1970). *Am. J. Physiol.* **218,** 832.
Kociba, G. J., Ratnoff, O. D., Loeb, W. F., Wall, R. L., and Heider, L. E. (1969). *J. Lab. Clin. Med.* **74,** 37.
Lewis, J. H. (1976). *Am. J. Vet. Res.* **37,** 601.
Lopacuik, S., McDonagh, R. P., and McDonagh, J. (1978). *Proc. Soc. Exp. Biol. Med.* **158,** 68.
Mason, R. G., and Read, M. S. (1971). *J. Biomed. Mater. Res.* **5,** 121.
Meier, H., Allen, R. C., and Hoag, W. C. (1961). *Am. J. Physiol.* **201,** 375.
Ollendorff, P. (1960). *Thromb. Diath. Haemorrh.* **4,** 45.
Osbaldiston, G. W., Stowe, E. C., and Griffith, P. R. (1970). *Br. Vet. J.* **126,** 512.

Raymond, S. L., and Dodds, W. J. (1975). *Thromb. Diath. Haemorrh.* **33,** 361.
Rowsell, H. C. (1968). *Adv. Vet. Sci.* **12,** 337.
Rowsell, H. C., and Mustard, J. F. (1966). *Can. J. Med. Technol.* **23,** 2.
Seaman, M. J., and Malinow, M. R. (1968). *Lab. Anim. Care* **18,** 80.
Stopforth, A. (1970). *J. Comp. Pathol.* **80,** 525.
Stormorken, H. (1958). *Acta Physiol. Scand.* **41,** 301.
Todd, M. E., McDevitt, E., and Goldsmith, E. I. (1972). *J. Med. Primatol.* **1,** 132.
Tsao, C. H., Lo, R., and Raymond, J. (1976). *Am. J. Clin. Pathol.* **65,** 518.
White, J. G., Matlack, K. L., Mundschenk, D., and Rao, G. H. R. (1975). *Proc. Int. Symp. Equine Hematol., 1st* p. 209. Am. Assoc. Equine Pract., Golden, Colorado.

18

Cerebrospinal Fluid

EMBERT H. COLES

I. INTRODUCTION

Diseases involving the central nervous system (CNS) in domesticated animals often present a diagnostic problem to the clinician. Considerable information relative to the lesion and its effects on the animal can be obtained by a careful, systematic physical examination and the elucidation of a complete history. Confirmation of a tentative diagnosis may be dependent upon the utilization of other techniques. Examination of cerebrospinal fluid (CSF) may provide information relative to the type of lesion; it can be

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

of assistance in the confirmation of a diagnosis and the selection of appropriate regimens of treatment and may enable the clinician to make a more accurate prognosis.

In order to interpret the results of a CSF examination properly, one must be aware of the normal physiology of CSF, factors governing its composition, the components of normal CSF, methodologies for the examination of CSF, and the interpretation of alterations in the composition of CSF.

As research on CSF physiology continues, the basis for the interpretation of alterations increases. Much of the basic knowledge concerning CSF has been determined in animals, but there remains a lack of definitive information concerning CSF alteration in diseases of the CNS in animals.

II. SECRETION OF CSF

It is generally considered that CSF is formed in the ventricles of the brain and drains via the arachnoid villi into the venous system. Any interruption of this drainage pathway results in an accumulation of fluids and thus an increase in ventricular size. In such a clinical state the pressures exerted by the fluids undoubtedly exceed that of capillary hydrostatic pressure. Therefore, if CSF were produced by passive ultrafiltration, its production would cease in hydrocephalus. Since this is not the case, there is little doubt that most CSF production is a secretory process that depends on active transport mechanisms. In addition, it has been confirmed that distribution ratios for ions between CSF and plasma are markedly different from those of a normal plasma dialyzate (Davson, 1955).

A. Site of Formation of CSF

Most of the CSF appears to be produced in the ventricles. The two sites where CSF production is thought to occur are the choroid plexuses and ependymal lining of the ventricles.

Choroid plexuses play a significant role in CSF production. Proof that these plexuses secrete CSF has been obtained by a variety of techniques. Welch (1963) measured arteriovenous hematocrit differences and confirmed that fluid was lost from blood on passage through the plexuses. Absolute proof that the choroid plexuses secrete CSF was obtained by direct observation (Ames *et al.*, 1964). The choroid plexus of a cat was exposed and covered with oil, and drops of CSF were recovered as they coalesced on the surface of the epithelium. Chemical analysis of fluid collected in this fashion demonstrated that it had a different chemical composition than did a plasma ultrafiltrate. Newly formed CSF had a different chemical composition than did CSF collected from the cisterna magna.

Experiments to demonstrate extrachoroidal secretion of CSF are difficult, as most require surgical intervention that may produce an abnormal situation. Milhorat *et al.* (1971) found that, following plexectomy, CSF production was reduced by approximately 30%. Sato *et al.* (1975), using perfusion techniques, measured the total CSF formation in dogs and reported that 58.5% of the total amount of CSF formed came from extraventricular CSF space. Estimates for intraventricular and extraventricular formation of CSF in the cat were 59% intraventricular CSF formation and 41% extraventricular formation (Bering, 1959).

It has been demonstrated (Cserr, 1975) that there are currents of bulk fluid flow in brain perivascular spaces. The precise source of this fluid has not been identified, although the mitochondrial content of brain capillary endothelial cells is greater than in other body cells (Oldendorf, 1977). Oldendorf (1977) suggested that this apparent metabolic work capability may be used to energize the ionic pump required to maintain blood–brain ionic gradients and could provide a mechanism for generating extrachoroidal CSF.

Segal and Pollay (1977) suggested that the concept that cerebral endothelial cells have a transport role be reexamined, as there is a possibility that this layer may contribute to total CSF production. These authors concluded that, "while there can be no doubt that the choroid plexuses are the site of the bulk of CSF formation, the ependyma and the cerebral endothelial cells may also contribute to the total production of CSF. The role of these extrachoroidal sources of CSF and brain [extracellular fluids] in the total turnover of fluid within the brain must, however, await further quantitative data."

B. Rate of Secretion of CSF

The rate of CSF production in various animal species has been measured, as summarized in Table I.

TABLE I

Rate of CSF Secretion in Various Species

Species	Rate of secretion (μl/min)	Rate of secretion (μl/min/mg of choroid plexus)	Reference
Dog (12–17 kg)	65		Sato *et al.* (1975)
Dog	49 ± 20		Deck *et al.* (1972)
	50	0.625	Oppelt *et al.* (1963)
	50	0.77	Bering (1959)
	47	0.96	Sahar (1972)
Dog (newborn)	4.5–6.4		Holloway and Cassin (1972)
Cat	20	0.50	Davson *et al.* (1962)
	20 ± 65		Graziani *et al.* (1965)
	15		Hammerstad *et al.* (1968)
	21		Snodgrass and Lorenzo (1972)
	22.7 ± 3.2		Wald *et al.* (1976)
Calf	290		Calhoun *et al.* (1967)
		0.29 ± 0.11	Frier *et al.* (1972)
Goat	154	0.36	Oppelt *et al.* (1963)
	164		Heisey *et al.* (1962)
	116	0.27 ± 0.13	Frier *et al.* (1974)
Sheep		0.13	Pollay *et al.* (1972)
Monkey	97		Heisey *et al.* (1962)
Rhesus monkey	19.2 ± 2.2		Milhorat *et al.* (1971)
Man	350	0.18	Cutler *et al.* (1968)
	300		Lorenzo *et al.* (1970)
Boy (12 years of age)	500		Heisey *et al.* (1962)
Rabbit	10.1	0.43	Bradbury and Davson (1964)
	7.8	0.37	Welch (1963)

C. Mechanism of Secretion of CSF

Although the precise mechanism by which CSF secretion is achieved by choroid plexuses is unresolved, some facets have been identified. Studies on CSF flow in the exposed choroid plexus of the rabbit have shown that the flow continues against an osmotic gradient and is isosmotic with plasma (Welch *et al.*, 1966). Such an observation would support the concept of osmotic movement of water possibly linked to the movement of sodium, as inhibitors of sodium transport cause the rate of secretion to be reduced and the fluid to become hypertonic. Replacement of sodium with choline chloride in artificial CSF used for ventriculocisternal perfusion caused a marked fall in the rate of secretion and the rate of entry of sodium. It would therefore appear that for full functioning of the secretory mechanism for CSF it is necessary to have sodium on both sides of the epithelium (Davson and Segal, 1970).

Anion transport in the CSF secretory processes has been studied less than has cation transport. The presence of an active chloride pump has been postulated by Abbot *et al.* (1971) and Bourke *et al.* (1970). In the cat, Domer (1969) demonstrated that furosemide, an agent that acts as a chloride inhibitor, inhibited CSF secretion. This would support the concept of an active chloride pump. Thionine, another chloride inhibitor, had no action on CSF secretion (Segal and Pollay, 1977). Studies on the frog choroid plexuses have shown that chloride and bicarbonate transport are linked (Wright, 1972) and that anion transport in the opposite direction is dependent upon the presence of sodium and potassium (Wright, 1974).

Although there is no direct proof that hydrostatic pressure is part of the mechanism of CSF secretion, data collected following the use of inhibitors would support this possibility. In an intact rabbit in which CSF secretion was inhibited with Diamox (acetazolamide), CSF took on the characteristics of plasma ultrafiltrate. Such information has been interpreted as indirect evidence to support the hypothesis that hydrostatic forces act as a component of CSF secretion (Segal and Pollay, 1977).

A number of metabolic and transport inhibitors have been utilized to provide information relative to the nature of the secretory process. Ouabain, a potent inhibitor of Na/K-activated ATPase, inhibits sodium transport and secretory processes in epithelium (Keynes, 1969). This compound also inhibits CSF secretion in most species and in the dog inhibits the Na/K-activated ATPase in the choroid plexuses (Vates *et al.*, 1964).

Diamox inhibits the enzyme carbonic anhydrase and also inhibits secretion of CSF and aqueous humor. Inhibition of CSF secretion by Diamox is accompanied by a reduction in the rate of entry of sodium from the blood (Davson and Segal, 1970). In perfused sheep choroid plexuses, Diamox caused no change in either blood pressure or blood flow but reduced both secretion rate and the flux of sodium from blood to CSF (Segal and Pollay, 1977).

The effect of carbon dioxide on CSF secretion is an area of controversy. An increase in blood CO_2 was reported to cause an increase in CSF secretion by some authors (Ames *et al.*, 1965), but others found no change (Oppelt *et al.*, 1963) or inhibition (Davson and Segal, 1970). The differences could be explained in part by the use of an exposed choroid plexus, as the intracellular acidosis caused by raised CO_2 levels might have the same effect as that caused by Diamox (Segal and Pollay, 1977).

Amiloride, which is thought to inhibit entry of sodium into cells at their transport site,

depresses both the rate of CSF secretion and the entry of sodium when used *in vivo* (Davson and Segal, 1970).

Segal and Pollay (1977) summarized the available data and suggested that the following factors play a key role in the CSF secretion mechanism. (1) There is some relationship with the metabolic energy supply of the cell, probably via the sodium pump. (2) Secretion is related to the active transport of sodium coupled to the passive transfer of water. The precise location of this process and direction of pumping are still a matter of speculation. (3) There is a possibility that a chloride pump exists which may be either an independent process or coupled to the active transport of sodium. (4) There is some interrelation of the transport process with bicarbonate movement, but this may be a separate process and related more to acid–base exchanges. (5) The enzyme Na/K-activated ATPase is involved in all sodium transport processes, but its relation to fluid secretion is not known nor is its function in the sodium pump. Segal and Pollay (1977) concluded that "the basic coupling between sodium transport and water movement is reasonably certain, but how this is achieved and the location of the processes is unknown. The use of inhibitors of transport processes enables comparisons to be made with other systems, but until the mechanism of this sodium pump is understood, and until such factors as the direction of pumps and the permeability of cell faces is known, no real advance in this field can be made."

In addition to evaluating the physiological characteristics of the choroid plexuses, one must also consider their morphology. Unlike endothelial cells of blood vessels supplying most body organs, those within the CNS are linked together by tight junctions forming continuous belts around contiguous endothelial cells (Brightman, 1977). The tight junctions between choroidal epithelial cells resemble those between endothelial cells. The epithelial tight junctions originally were considered to be barriers to free diffusion, but recent data, obtained by electrical techniques, have shown great variability in the values for transepithelial resistance among epithelia (Frömter and Diamond, 1972). By the use of small molecules such as lanthanum it has been demonstrated that certain types of tight junctions are permeable to ions. This has been demonstrated in the choroid plexuses (Castel *et al.*, 1974). This would suggest that the choroid epithelium is "leaky." This was substantiated by Brightman (1977). When the choroid plexus from an untreated animal is fractured, the "leaky" tight junctions appear as parallel rows of strands or ridges, with only occasional anastomotic strands connecting adjacent parallel ridges except where two cells meet, and the ridges are usually no more than four or six rows in dept. In contrast, the number of such strands in very tight junctions have more rows and more anastomoses. A generalization has been drawn that a complex network of multilayered strands is associated with a high degree of impermeability, whereas a simpler array of ridges is associated with "leaky" tight junctions. Thus, the configuration of the cleaved junctions of the choroid plexuses denotes the presence of a "leaky" tight junction.

Segal and Pollay (1977) summarized the processes occurring during CSF secretion by the choroid plexuses. Cerebrospinal fluid consists of various salts and water, and, as cells of the choroid plexuses do not shrink or swell, the sum of the processes occurring in the lateral cell walls and basal infoldings must be balanced by an equal movement at the apical microvilli face and by means of the "leaky" tight junctions. They suggested that this process could be visualized as occurring in two phases: the entry phase and the exit phase.

The entry phase occurs when salts and water can be made to enter choroidal intercellular clefts by capillary hydrostatic pressure and by withdrawal of water and salt from within

the cleft. Minor ions pass through the tight junctions under control of hydrostatic pressure or, alternatively, have individual transport mechanisms. Protein is reabsorbed into the vesicle system of the cell. Intercellular clefts contain salt "pumps," which pump sodium into the cell, generating a local osmotic gradient that draws water into the cell. Chloride can enter the cell in association with these sodium pumps as it is moving up its electrochemical gradient or can pass through the tight junctions as a shunt pathway. Bicarbonate is formed within the cell by carbonic anhydrase, and the hydrogen ion is fed back into the sodium pump as a counterion with potassium. The hydrogen ion in the cleft produces CO_2 from extracellular bicarbonate, and CO_2 enters the choroidal cell.

Segal and Pollay (1977) visualize the exit processes as involving sodium pumps located at the microvilli face of the cell, where they extrude sodium accompanied by water in a local osmotic fashion. Chloride diffuses out down its electrochemical gradient, and CSF potassium forms the pump counterion. Hydrogen in the CSF is closely controlled by this mechanism, but some sort of hydrogen exchange must also be present. These authors cautioned that this scheme is presented only as a hypothesis, not as an absolute explanation for CSF secretion by the choroid plexuses.

III. OUTFLOW OF CSF

Since CSF production is continual and there is a rather constant quantity of total CSF, it is only logical to assume that there must be a reabsorptive process that approximates the rate of formation (Domer, 1977).

The mechanism whereby CSF is removed from the CNS has interested researchers for some time. The importance of the arachnoid villi in the removal of CSF was first confirmed by Weed (1914). Weed, using a solution of potassium ferrocyanide infused under relatively low pressure, concluded that CSF passed into the venous sinus by filtration through the arachnoid villi and that a minor amount was lost to lymphatic drainage. Weed systematically studied the arachnoid villi histologically and was unable to demonstrate the presence of any valves that might control movement of CSF back into the bloodstream. Welch and Friedman (1960) studied arachnoid villi from African green monkeys and found them to consist of a number of tubes that varied in diameter from 4 to 12 μm and were interconnected to form a maze. They found that reduced CSF pressure resulted in a collapse of the channels in the arachnoid villi. When CSF pressure was elevated, the villi became distended, thus increasing the outflow of CSF.

Size limitation of the channel was substantiated by the work of Welch and Pollay (1961), who perfused particles of various sizes through the arachnoid villi in dura mater removed from monkeys. They found that small particles in the range of 0.2–7.5 μm readily passed the villi, while polystyrene microspheres in the range of 6.4–12.8 μm were excluded.

There was a difference of opinion between anatomists, who felt that arachnoid villi were covered by an intact membrane, and physiologists, who felt that their evidence indicated a bulk flow mechanism for CSF transfer back into the blood. Subsequent research supported both views. Dye-labeled plasma proteins injected into the cisterna magna of cats appeared for the most part in the circulation, with less than 0.5% in the thoracic ductal lymph (Courtice and Simmonds, 1951). It was felt that this exchange

occurred across the arachnoid villi, as proposed by Weed. Erythrocytes labeled with radioactive chromium were injected into the cisterna magna of dogs by Adams and Prawirohardjo (1959). Forty-eight hours following the injection, approximately one-fourth of the intact erythrocytes were found in the systemic circulation and the remaining were found in the brain, in CSF, or (most) enmeshed in the arachnoid villi. These authors assumed that the villi served as the greatest point of restriction to flow between the two fluid compartments.

Mayer *et al.* (1960) injected a number of drugs into the cisterna magna of rabbits and measured their disappearance. Highly lipid soluble substances almost disappeared within an hour, whereas poorly lipid soluble substances left the CSF more slowly. These authors interpreted these results to mean that the drugs had exited via the arachnoid villi, in agreement with Weed's (1914) idea. Domer (1977) suggested that it was more likely that the movement of these substances from the CSF involved a transport system now known to exist for organic anions and cations. Experimental work on the reabsorption of CSF continued and was supplemented by investigations of the arachnoid villi using the electron microscope. Early results with this approach were also contradictory; some workers reported no channels, while others were able to demonstrate the presence of naturally occurring openings on the dural side of the endothelium.

Some investigators reported that endothelial cells showed marked pinocytotic activities and concluded that the passage of CSF across the endothelium was an active transcellular process occurring by pinocytosis (Shabo and Maxwell, 1971; Alksne and Lovings, 1972). Vacuoles or macrovesicles within endothelial cells lining the villi were demonstrated in monkeys (Tripathi, 1974; Tripathi and Tripathi, 1974). Such vesicles had frequent openings leading to the subendothelial space, and occasional openings leading to the venous aspect of the endothelium could be demonstrated. These were interpreted as being a system of transcellular channels for CSF passage across endothelium.

Gomez and Potts (1977) studied the ultrastructural characteristics of villi in monkeys in which there was an increase in pressure. Examination of the villi in such animals showed a uniform distention of those located in the lacuna laterales. These villi were covered by a smoother stretched endothelium containing fewer clear spaces than villi in the group with normal pressure. Electron microscope examination of the villus endothelium revealed a number of irregularly widened intercellular spaces, some of which were very wide, giving the appearance of large ovoid vesicles.

Domer (1977) proposed a hypothesis to explain how the drainage from CSF into the bloodstream is effectively a bulk flow phenomenon. He explained that the membranes of endothelial cells that cap arachnoid villi are very active. They can form temporary channels through the cell, which permits drainage of CSF. Pinocytosis and transepithelial transport also occur continually. When intracranial pressure increases, this activity increases by means of channel formation and pinocytosis. Domer contended further that the pressure gradient between the two body compartments is reduced by extrusion of the villus farther into the venous sinus, a thinning (flattening) of the endothelial cell, and separation of the interdigitation of cells. An absolute determination of the precise mechanism for CSF outflow must await the development of additional information.

In the dog, a significant leakage of CSF into the nasal cavity and submucosal lymphatics has been demonstrated by use of radioisotope cisternograms and ventriculograms (DiChiro *et al.*, 1972). Cerebrospinal fluid rhinorrhea was demonstrated in normal dogs

and in three dogs with noncommunicating hydrocephalus. The identification of nasal fluid as CSF is readily accomplished by demonstrating the presence of glucose in the fluid. This can be achieved by soaking a urinalysis dipstick with the nasal fluid.

IV. FACTORS GOVERNING CSF COMPOSITION

The chemical composition of CSF, under normal conditions, is closely regulated. Various processes associated with the metabolic activity of tissues of the CNS, the exchange of molecules between CSF and blood and the turnover of CSF, act together or independently to regulate the fluid chemical composition.

The existence of specific transport processes within the CSF compartments was first demonstrated in 1961, when Pappenheimer and his group showed that Diodrast and phenosulfonphthalein were extracted from a perfusate and transferred to blood by an active transport process (Pappenheimer *et al.*, 1961). These authors compared the organic acid transport system within the CSF to that present in the kidney tubules and suggested that this system could affect the concentration of drugs within the CSF.

The failure to achieve adequate drug levels within the brain and CSF is a serious problem in the treatment of diseases of the CNS. Several factors tend to limit the rate of drug penetration into the CSF. These include factors that affect blood concentration and consequently the amount of drug presented to the brain and the CSF as well as the physical properties of the drug, such as its degree of dissociation, lipid solubility, and protein binding. The morphological and biochemical properties of cerebral blood vessels also influence drug levels. The drug concentration achieved within CSF is also affected by processes which remove or clear drugs from CSF. For example, there is an efflux transport system for penicillin that is very effective in reducing CSF concentration. With some other drugs, such as gentamicin, salicylic acid, morphine, and methylatropine, the process is relatively or completely ineffective (Lorenzo, 1977). This efflux transport system may provide a secondary defense mechanism which influences the concentration of potentially toxic substances in the brain and CSF. In addition, the efflux process is important in the regulation of endogenous substances and the removal of water-soluble end products of brain metabolism the accumulation of which could prove deterimental to the function of the CNS (Lorenzo, 1977).

In addition to the control of drugs and other chemical substances, the concentration of amino acids between blood and CSF is markedly different. The CSF concentration of amino acids is approximately one-third that of plasma, and the concentration of individual amino acids in CSF differs vastly from that of plasma. In the rabbit, for example, the concentration of glycine in blood is 200 times greater that that in CSF, while that of serine is only twice as great (Lorenzo, 1977). Carrier mechanisms by which amino acids are cleared from CSF have been demonstrated. The clearance of amino acids from CSF is not restricted solely to ventricular cavities, as it has been demonstrated that amino acids injected into the rat cranial subarachnoid space were transported to blood.

Changes in the blood concentration of glucose are slowly reflected in the CSF. It has been suggested (Lorenzo, 1977) that the glucose concentration in CSF under normal conditions is maintained within rather narrow limits. Various mechanisms probably influence glucose levels in CSF. It has been suggested that glucose entering the CSF may have

its concentration reduced by metabolic utilization in tissues surrounding the CSF compartment. It is also probable that part of the glucose filtering into the CSF compartment is transported back to the blood. Welch *et al.* (1970) reported that the glucose concentration of nascent rabbit CSF was approximately 60% that of blood and suggested that glucose taken up by the choroid plexus from blood was secreted into the CSF at a low concentration.

The control of ions in CSF is also considered to be an active process. It is known that the concentrations of potassium, calcium, and magnesium are remarkably stable in CSF in the face of severe and prolonged disturbances in the concentration of these ions in blood plasma (Bradbury and Sarna, 1977). The concentration of CSF potassium remains relatively constant in animals with prolonged hypokalemia or hyperkalemia (Bekaert and Demeester, 1951a,b). More recent work has confirmed the tight homeostasis of potassium and magnesium in CSF with less perfect control of calcium and hydrogen ions (Bradbury and Sarna, 1977). There is evidence that the ionic composition of brain interstitial fluid is equally as well controlled and that these control mechanisms protect the central neurons from the effects that electrolyte blood disturbances might have.

In explaining CSF ionic homeostasis, Bradbury and Sarna (1977) suggested that two mechanisms might be considered: "(1) A static extracellular fluid in association with particular relationships between unidirectional ion fluxes across the barriers and the concentrations of the ion in plasma and extracellular fluid, (2) secretion of extracellular fluid of constant composition together with bulk flow and drainage of the secreted fluid before a significant change in its composition has occurred." They further suggested that the homeostasis of calcium and magnesium in CSF and brain interstitial fluid may be largely explained by secretion by the choroid plexuses of a fluid of near constant composition in the presence of a relatively impermeable brain barrier to these ions. With potassium, active mechanisms at the blood–brain barriers must be invoked. These may be associated with the astrocytic component of the barrier.

V. BLOOD–BRAIN BARRIER

A concept of a barrier that prevented an exchange between blood and brain (blood–brain barrier) originated when it was discovered that injected polar dyes were not distributed to the brain, while all other body tissues were quickly colored. Today it is known that this barrier to dyes occurs because they are bound to macromolecules and their passage into the brain is stopped at the capillary level by the tight junction between vascular endothelial cells. The term "blood–brain barrier" (BBB) was later used to reflect the restriction of passage of other water-soluble substances, many of them of small molecular size, from the blood to the brain. It would be anticipated that such molecules would follow common routes to other tissues by being transported across cellular membranes. Thus, the BBB was more than just a blocking mechanism that prevented entry of large molecules.

The term "blood–brain barrier" would suggest that capillaries in the brain are completely impermeable, but it is obvious that although they are impermeable to some plasma solutes they must be freely permeable to others (Oldendorf, 1977). Some drugs that affect the CNS function immediately following intravenous injections. In addition, it must also be assumed that the brain receives substantial amounts of its metabolic substrates from the

blood. Therefore, Oldendorf (1977) suggested that the BBB is more correctly considered to be selectively permeable in that there is an extremely wide range of permeability to various plasma solutes.

In most capillary beds all blood plasma solutes of low molecular mass diffuse freely between plasma and their adjacent extracellular fluid, largely through narrow clefts between adjacent capillary endothelial cells. In brain capillaries these intracellular clefts are sealed, and fenestrae in the capillary cells are virtually absent. Thus, exchange across brain capillary walls must take place directly through the cells rather than between cells (Crone and Thompson, 1970). A solute, to penetrate the BBB, must penetrate the two (inner and outer) plasma membranes and survive transit through the interposed endothelial cell cytoplasm. Thus, the BBB might be considered a continuous double sheet of cell membrane through which material must pass in order to enter the brain. The interface between the blood and brain occurs at the inner capillary cell membrane, and the permeability of the barrier to a given plasma constituent is dependent upon the entrance of that molecule into the membrane as it leaves the plasma. In order to enter the membrane a plasma molecule must have a fraction which is unattached to plasma protein and is in free solution in plasma water, as protein has virtually no chance of entering the membrane. Such a water-bound solute must either enter the lipid portion of the membrane or attach itself to membrane protein (Oldendorf, 1977). Membrane proteins exhibiting specific affinities for molecules are carrier proteins that specifically manipulate solutes out of adjacent water. When an animal loses consciousness within a few seconds after receiving an intravenous injection of thiopental, it is the drug's lipid solubility that permits it to enter brain capillary membranes from the plasma. Having entered the brain capillary membranes, it then moves into the brain because cell membranes are no great obstacle to its diffusion down concentration gradients and into the brain (Oldendorf, 1977).

The lipid solubility of some solutes explains their penetration of the BBB, but highly polar solutes, such as glucose, have little lipid solubility and a great affinity for water and therefore must have some other mechanism by which they penetrate the barrier. Removal of glucose from plasma water requires a membrane protein that has a high glucose affinity. This is the glucose carrier protein, which creates a solubility of glucose in the membrane much greater than that in the lipid portion of the membrane (Oldendorf, 1977). Several other carriers within the BBB transport system have been identified experimentally. Independent carrier systems thus far identified have been for certain hexoses (Crone, 1965); for neutral, basic, and acidic amino acids (Richter and Wainer, 1971; Oldendorf and Szabo, 1976); two for nucleic acid precusors (Cornford and Oldendorf, 1975); and one for choline (Oldendorf, 1977).

The function of these carriers is to accelerate the BBB flux of their carrier substrate. The barrier in effect shuts off the blood–brain interface to an indiscriminate exchange of solutes but permits the passage of other classes of molecules (Oldendorf, 1977). Oldendorf suggested that this strategy benefits brain function under normal conditions in a number of ways but may compromise the brain's ability to adapt to certain pathological circumstances. The role that the barrier plays in the pathophysiology of disease is relatively unknown, although it is recognized that regionally the barrier may be lost in association with a range of brain pathologies. An increase in CSF protein may well reflect an abnormally permeable BBB but may also represent CSF stagnation (Oldendorf, 1977).

The barrier also serves as a mechanism for homeostatically stabilizing brain extracellular fluid. Many foreign solutes that appear in the blood plasma are excluded from the brain

by the BBB, including a variety of polar toxic substances that have been introduced into the general extracellular fluids of the body. A major function of the BBB may be to maintain the brain extracellular fluid electrolytes at concentrations optimal for neurons but substantially different from their concentrations in blood plasma (Oldendorf, 1977). It is known, for example, that the potassium level in brain extracellular fluid is approximately 40% less than that in plasma. Such a reduced concentration requires metabolic energy within the capillary cells and may well correlate with the observation that brain capillary endothelial cells contain approximately five times as many mitochondria as do corresponding cells in skeletal muscle (Oldendorf and Brown, 1975).

VI. COMPOSITION OF CSF

Cerebrospinal fluid has a different composition than blood plasma or plasma filtrate. As previously indicated, this is because of the presence of transport mechanisms which tend to accelerate the removal of certain molecules and ions while retarding that of others. The end result of these processes is a fluid having a composition that serves as an environment for tissues that have a close relationship with the fluid. To some extent the chemical composition of CSF is independent of chemical concentrations within plasma, whereas with other chemical substances it appears to be directly related to the plasma concentration. Any increase in blood osmolarity causes an abrupt withdrawal of water from the CSF, which effectively lowers intracranial pressure and raises CSF osmolarity.

The chemical composition of CSF of animals is summarized in Table II. As with any other list of normal values these should serve only as a guide, and normal values should be established in each laboratory. The data in this table represent a summary of published values.

Although the number of cells in the CSF of most animals is small, established normal values must be considered in the interpretation of laboratory results. The normal cellular elements found in the CSF of animals are summarized in Table III.

VII. REMOVAL OF CSF

Cerebrospinal fluid examination is indicated when there is clinical evidence of pathology involving the CNS, and occasionally it is used as a prognostic method to determine the state of disease and the response to therapy. Removal of CSF may be of value for the relief of abnormally high CSF pressures and for drainage of blood or exudates from the subarachnoid space. Penetration of the subarachnoid space may also be used in initiating radiographic procedures and for the injection of medicaments into the spinal canal.

Removal of CSF is contraindicated if an animal is suspected of having increased intracranial pressure. In such animals fluid removal from the cisterna magna will decrease the pressure caudal to the cranial vault and increase the chances of tentorial herniation (Oliver and Knecht, 1975).

Lumbar or cisternal puncture of the spinal canal is a relatively safe and simple procedure, although it should not be performed unless there are definite indications for the procedure. In order to remove CSF safely, the clinician must be aware of the associated anatomy and techniques for performing a puncture. These techniques have been discussed

TABLE II

Chemical Composition of CSF in Animals[a,b]

Species	Total protein (mg/dl)	Albumin (mg/dl)	Glucose (mg/dl)	Calcium (mg/dl)	Phosphorus (mg/dl)	Sodium (mEq/liter)	Potassium (mEq/liter)	Chloride (mEq/liter)	Mg (mg/dl)	Urea N (mg/dl)
Dog	15–34.8	13.97–27	74–75	5.6–6.5	1.1–3.9	151.6–155	2.98–3.11	131–138	—	10–11
Cat	20–27	19–25	85	5.2	—	158	3.0–5.9	144	1.33	10–11
Horse	32–48	15–39	48–57	4.2–6.2	0.83–1.44	145	2.9–3.2	109–126	1.98	11.8–13
Cow	20–33	10–20	35–70	5.1–6.3	0.9–2.5	110–123	2.9–3.5	111–123	2.1–2.4	8.2–11.0
Sheep	29–42	—	52–85	5.1–5.5	1.2–2.0	145–157	3.0–3.3	128–148	2.2–2.8	—
Goat	12	—	70	4.6	—	131	2.96	116–130	2.3	—
Pig	24–29	17–24	45–87	—	—	134–144	—	—	—	—

[a] Ranges of mean values from the literature.

[b] References: Dog: Altman and Dittmer (1974), Long *et al.* (1973), Heywood *et al.* (1973), Jordan (1977), Fridman and Petrova (1935), Chazan *et al.* (1969), Bito and Davson (1966), Kaneko (1973). Cat: Altman and Dittmer (1974), Kaneko (1973), Hochwald and Wallenstein (1967), Ames *et al.* (1964). Horse: Kristensen and Firth (1977), Mayhew *et al.* (1977), Kirk *et al.* (1974), Altman and Dittmer (1974), Kaneko (1973). Cow: Soliman *et al.* (1965), Fankhauser (1962), Altman and Dittmer (1974), Kaneko (1973). Sheep: Altman and Dittmer (1974), Kaneko (1973), Fankhauser (1962), Adamesteanu *et al.* (1944), Scholz and Meyer (1972), El Amrousi *et al.* (1966). Goat: Altman and Dittmer (1974), Kaneko (1973), Held *et al.* (1964). Pig: Altman and Dittmer (1974), Kaneko (1973), Osweiler and Hurd (1974).

TABLE III

Cellular Elements in CSF of Animals

Species	Lymphocytes	Other cells	Total cell count (cells/μl)
Dog	5–40% large; 15–95% small[a]		0–8[b]
Cat	All lymphocytes		0–1[b]
Horse	50% lymphoid[b]	50% histiocytes[b] and degenerate cells	1–7[b] 1.41 ± 1.74[c]
Cattle	90–100% large[d]	0–10% endothelial cells[d]	10–20[d]
Swine	All lymphocytes[e]		0–7 up to 20[b]
Sheep	Large and small[b]	Mononuclear cells and endothelial cells[b]	0–5[b]
Goat	All lymphocytes[e]		0–1[e]

[a] Bindrich and Schmidt (1952).
[b] Fankhauser (1962).
[c] Mayhew *et al.* (1977).
[d] Soliman *et al.* (1965).
[e] Altman and Dittmer (1974).

in detail by Fankhauser (1962), DeLahunta (1977), Mayhew (1975), and Oliver *et al.* (1975) and are not repeated here.

In any procedure involving entering the subarachnoid space for the collection of fluid, strict aseptic precautions must be taken. The areas should be shaved and washed and the skin disinfected prior to fluid removal. The cisternal approach is recommended for most species but may be extremely difficult in pigs weighing in excess of 180 lb. The lumbosacral approach may be utilized in swine, cattle, sheep, and horses. Consideration should be given to collecting fluid from this source if the suspected lesion is thought to be located in the thoracolumbar cord region (Oliver *et al.*, 1975). Although general anesthesia is not usually necessary for cattle or sheep or for removal of fluids from the lumbosacral region, it is recommended for removal of cisternal fluid from all other animal species. The operator should remove only the amount of fluid necessary for analysis.

VIII. EXAMINATION OF CSF

Examination of CSF should include physical, cytological, and chemical evaluations. The extent of the chemical examination depends upon clinical observations and normally includes only protein. However, under circumstances that suggest the presence of a bacterial infection or other lesion that might alter the chemical composition, additional tests may be indicated.

A. Physical Examination

Normal CSF is clear and colorless. If hemorrhage occurs at the time of puncture, fresh blood may appear in the fluid. Another common discoloration is xanthochromia, in which the fluid is yellow and clear. This discoloration may appear following hemorrhage into the

arachnoid space and is probably due to the presence of free bilirubin resulting from the chemical breakdown of heme in the spinal canal. In advanced cases of icterus, various amounts of bilirubin may be present in the CSF. Xanthochromia may also be present if the CSF protein concentration is high (>400 mg/dl), in hydrocephalics, in association with some acute inflammation, abscessation, or neoplasia, and in some cases of spinal block.

If there has been recent hemorrhage into the subarachnoid space or if blood has contaminated the specimen during collection, the sample may be centrifuged to detect color changes. If contamination has occurred as a result of hemorrhage at the time of collection, the supernatant fluid is usually clear following centrifugation. If the hemorrhage is over 48 hours old, the supernatant is xanthochromic.

Normal CSF is completely transparent. Turbidity usually occurs because of the presence of cells but does not become grossly evident until there are 500 or more cells per microliter. In acute meningeal infection the fluid may be slightly cloudy or frankly purulent.

Normal CSF does not coagulate. Coagulation occurs only if fibrinogen is present, although occasionally a fibrin net containing blood cells may result from a meningeal reaction. Heavy coagulation may occur with acute suppurative meningitis. If the CSF has been contaminated with large quantities of blood, coagulation may also occur.

Wilson and Stevens (1978) reported that the range of CSF specific gravity, as determined by a refractometer, in 71 normal dogs was 1.004–1.006 with a mean of 1.005. They reviewed 124 CSF analyses of animals hospitalized for suspected neurological disease and found that the CSF gravity exceeded 1.006 in 30 samples (24.2%). They concluded that CSF specific gravity had little diagnostic or prognostic value. Although increased specific gravity is a nonspecific alteration, it would appear, from these results, that a specific gravity greater than 1.006 may be an indication of CNS disease, while a specific gravity in the normal range does not eliminate the possibility of disease.

B. Cytological Examination

Although the number and types of cells in CSF are limited, detection of the total number and cell type may be of value in arriving at a diagnosis. If cells are found in normal CSF, they are lymphocytes or rarely mononuclear phagocytes. As cells in CSF degenerate rapidly following collection, total and differential counts should be completed as quickly as possible (within 30 minutes after collection). If a delay is anticipated in completing the count, the specimen may be preserved with an equal quantity of 50% ethanol and a smear prepared later.

If the total nucleated cell count is 500 per microliter or greater, a direct smear of the CSF should be prepared for cytological examination. If the total cell count is less than 500 per microliter or the specimen has been diluted with ethanol to preserve the cells, the sample can be centrifuged and smears prepared from sediment. Care should be taken to treat such specimens gently, and centrifugation at a slow speed (600 rpm or less) should be used for concentration. Membrane filtration of CSF has certain advantages: Morphological features of the cells are retained, a small quantity of fluid may be used, and the technique is relatively simple (Roszel, 1972; Gondos and King, 1976). Depending upon the method of concentration, smears are prepared in the usual fashion and an appropriate stain, such as Wright's, Giemsa, or new methylene blue, can be used. A wet-mount coverslip method may be preferable as less cellular distortion and disintegration and fewer artifacts are observed with this technique than with the dried-flim method

(Cornelius, 1963). Techniques for completing total cell counts on CSF are described elsewhere (Coles, 1974; Cornelius, 1963).

As peripheral blood contamination of CSF is not uncommon, it has been suggested that the following formula be used to adjust the total count and total protein (Krieg, 1969):

$$WBC_{CSF} = WBC_0 - \left(\frac{WBC_b \times RBC_0}{RBC_b}\right)$$

Where WBC_{CSF} = leukocyte count (or total protein) presumably present in CSF prior to peripheral contamination
WBC_0 = leukocyte count (or total protein) observed in CSF
WBC_b = leukocyte count (or total protein) of peripheral blood
RBC_0 = erythrocyte count observed in CSF
RBC_b = erythrocyte count of peripheral blood

In utilizing the above formula, it is assumed that no erythrocytes were present in the CSF prior to collection. This formula is applicable only when it is impossible to obtain needed information in any other fashion. Wilson and Stevens (1977) studied the effects of blood contamination on CSF analysis and were unable to validate this formula. They commented that blood contamination had little effect on the number of leukocytes or protein content of CSF samples. Thus, this formula should be used with caution.

If physical examination of CSF reveals turbidity or other abnormality, direct smears for identification of microorganisms should be made. If bacteria are present, bacteriological cultures should be initiated as soon as possible.

C. Chemical Examination

1. Protein

Determinations of protein content in CSF can be conducted by a variety of techniques. Qualitative procedures, such as the Pandy and Nonne–Apelt tests, are used for a preliminary examination and, if positive, indicate the presence of globulins. It must be remembered that these are relatively insensitive tests and should be used only as a screening procedure to rapidly identify the presence of globulins. Quantitation of CSF protein can be based on ultraviolet absorption, micro-Kjeldahl reaction, the Folin–Ciocalteau reaction, the development of turbidity with trichloroacetic acid, or immunochemical methods.

Recently there has been an increase in the study of cerebrospinal proteins using electrophoretic and immunoelectrophoretic techniques. Kirk *et al.* (1974) used cellulose polyacetate to electrophoretically separate the protein fractions in equine CSF. They found this a reliable method for identifying the albumin and the α_1-, α_2-, β-, and γ-globulins. They suggested that electrophoresis of the CSF protein might be a diagnostic aid for diseases of the CNS. In a similar study, Kristensen and Firth (1977) used agarose as the supporting matrix for electrophoresing CSF samples. They identified a prealbumin fraction and a shoulder on the cathodal aspect of the albumin fraction. These authors suggested that CSF electrophoresis should be included in the investigation of neurological problems in the horse and other animals.

Cutler and Averill (1969) electrophoresed CSF from dogs with canine distemper encephalitis. They compared their results with those from normal individuals. The identity of the fractions was confirmed by immunoelectrophoresis. In a similar study (Long *et al.*,

1973) the CSF protein fractions were electrophoretically separated on cellulose acetate. The results confirmed the previous observations (Cutler and Averill, 1969) of an increase in CSF globulin in dogs with encephalitis. It was suggested that inflammatory processes may alter the BBB, making it more permeable to large protein molecules.

Baltch *et al.* (1969) experimentally produced aseptic meningitis in dogs by intracisternal administration of streptokinase-streptodornase (SK-SD). The greatest pleocytosis and total protein increases occurred 48 hours following intracisternal injection of SK-SD. At the peak of pleocytosis and total protein increase, the greatest CSF protein increase was in the albumin fraction. A prompt return to normal albumin levels occurred within 4 weeks. The immunoglobulin G (IgG) level increased 14-fold during acute inflammation and remained elevated as long as 19 weeks. The total amount of CSF protein returned to normal within 3–8 weeks. These authors suggested that during acute aseptic meningeal inflammation there was a significant change in the blood–CSF–brain barrier for protein which caused a marked increase in albumin and later in IgG without a concomitant change in the serum. Their data also suggested that altered concentrations of CSF protein components may occur in the presence of a normal CSF total protein.

The mechanism of exchange of γ-globulins between blood, CSF, and brain was studied by Hochwald and Wallenstein (1967). They found that the clearance of ^{131}I-labeled cat γ-globulin during steady-state ventriculocisternal perfusion was dependent on bulk absorption of CSF but was independent of protein concentration. When ^{131}I-labeled γ-globulin was injected intravenously, the total amount of this protein that entered the ventricular system was 0.7 μg/minute. The transfer of γ-globulin was similar to, but did not exceed, that of inulin clearance. Similar results were obtained in the study of the efflux of albumin from the CSF.

MacPherson (1967) studied the γ_c-globulin in bovine CSF withdrawn from newly born, colostrum-deprived calves and older animals. She demonstrated that the average γ_c-globulin concentration in bovine CSF from newborn calves was 1.4 mg/dl. This level decreased sharply for a few days after birth, and the average value for 4-month-old animals was 0.44 mg/dl. The CSF of 1-year-old steers contained 0.22 mg/dl. The presence of γ_G-globulin was demonstrated in the CSF of newborn, colostrum-deprived calves, but quantitation was not possible because of the small volume of available CSF and the low concentration of this globulin. The level of γ_G-globulin increased with age so that the CSF of adult animals contained 2.22 mg/dl.

The significance of the presence of these protein fractions in the CSF of domestic animals must be determined by continued research into the changes occurring in disease.

2. Glucose

Cerebrospinal fluid glucose levels are approximately 60–80% of blood levels in most animal species. Mayhew *et al.* (1977) reported that in the horse the CSF glucose level was from 35 to 75% of serum values. In man, it has been reported that with very high glucose levels (800 mg/dl and over) the differences become more marked, and CSF glucose is only about 30–40% of blood levels (Krieg, 1969). With an increase or decrease in blood glucose, CSF glucose undergoes a corresponding change within 1–3 hours. This time lag probably reflects the slow transport of glucose across the CSF–blood barrier. Because of this relationship between blood glucose and CSF glucose levels, blood glucose concentration should be estimated simultaneously with CSF glucose concentration.

Hypoglycorrhachia is most commonly associated with bacterial meningitis, although it

may also be seen in association with marked hypoglycemia. The decreased CSF glucose associated with bacterial meningitis is undoubtedly due to glycolysis by the microorganisms and leukocytes present. There is evidence to suggest that the glucose fall is a result of synergism between bacteria and leukocytes (Petersdorf *et al.*, 1960a,b). There is also a suggestion that meningeal neoplasia may cause a decrease in CSF glucose (Krieg, 1969).

3. Chloride and Sodium

The fact that sodium and chloride concentrations in CSF are slightly greater than those in plasma suggests the presence of an active secretory mechanism for these substances into CSF. Although the concentration difference is realistic, Davson (1967), in speaking of sodium chloride, remarks that "these ions constitute such an important fraction of the total osmolality of the cerebrospinal fluid and plasma that it seems unlikely that their concentrations in the cerebrospinal fluid could be held at levels that were independent of the plasma levels." Thus, it might be considered impossible to completely separate serum and plasma levels of these two substances from levels that occur in the CSF.

Alterations in chloride levels undoubtedly do occur but, other than in the hypochloremia associated with meningitis and that which may occur as a result of protracted vomiting and in advanced pneumonia, are probably of little diagnostic significance.

Friedman *et al.* (1963) produced hypochloremia of various degrees in dogs by surgically creating a gastric fistula. In dogs that were only slightly depleted the CSF chloride/plasma chloride ratio went from a control value of 1.11 to 1.16 in one dog and from 1.20 to 1.32 in another. In more severely depleted dogs the difference in ratios was considerably greater, with CSF chloride/plasma chloride ratios ranging from 1.54–1.83 following depletion to 1.15–1.18 before depletion. Total CSF chloride levels were reduced from control values of 128–135 mEq/liter to 81–97 mEq/liter after depletion. In the same experiments plasma sodium decreased from 144–149 to 122–132 mEq/liter after depletion, while the CSF sodium levels decreased from 149–154 to 124–134 mEq/liter following depletion.

4. Calcium and Magnesium

The normal concentration of calcium in CSF is much lower than that in plasma. The principal reason for this difference is the great degree of protein binding of calcium that occurs in plasma. When a comparison is made between plasma dialyzate and CSF, the discrepancy is not large, but the concentration of ionized calcium in plasma is greater than that in CSF. According to Davson (1967), studies on human beings and experimental animals in which plasma calcium concentration was lowered or raised demonstrated that the concentration of calcium in CSF is virtually independent of its concentration in plasma.

The concentration of magnesium in the CSF of most species is slightly greater than that in plasma. Although it was thought that CSF magnesium levels were virtually independent of plasma magnesium concentration, it has been demonstrated (Meyer and Scholz, 1972) that CSF magnesium content decreases in sheep on a magnesium-deficient diet. The effects of hypomagnesemia on blood and CSF calcium levels in sheep have also been studied (Scholz and Meyer, 1972). With a progressive hypomagnesemia the calcium concentration in blood fell from a control value of 9.96 ± 1.41 mg/dl to 6.81 ± 1.91

mg/dl. At the same time the CSF calcium level fell from 5.23 ± 0.54 to 4.95 ± 1.13 mg/dl.

Alterations in CSF calcium and magnesium concentrations in other disease states have not been extensively studied, and their clinical significance has not been determined.

5. *Potassium*

The potassium concentration in CSF is considerably less than that of a plasma dialyzate. Increases in plasma potassium do not produce a significant increase in the concentration of CSF potassium. Bekaert and Demeester (1951a,b) maintained hypokalemic and hyperkalemic states in dogs and demonstrated a remarkable stability of CSF potassium concentration in the presence of prolonged disturbances in blood plasma. Scholz and Meyer (1972) demonstrated an increase in both plasma and CSF potassium in sheep with hypomagnesemic tetany. It has been suggested (Cornelius, 1963) that potassium alterations in CSF would be likely to occur when whole plasma or blood escapes into the CSF.

6. *Acid–Base Balance*

Cerebrospinal fluid is slightly more acidic than arterial plasma. It has been suggested that CSF is more likely to be in equilibrium with venous than with arterial plasma, and, as venous blood is more acidic than arterial, this may explain the difference between CSF pH and that of arterial plasma. Davson (1967) suggested that this is only a partial explanation of the variation.

The response of CSF pH to changes in the blood is of considerable physiological interest. It has been established (Davson, 1967) that the pH of CSF does not accurately follow that of blood. In the case of metabolic acidosis the pH of the two fluids may move in opposite directions (Cestan *et al.*, 1925). Following the injection of HCl into dogs, it was found that, although the blood became more acidic, the CSF became more alkaline. The difference in pH was thought to be due to the fact that, although the bicarbonate concentration of plasma fell rapidly, the bicarbonate concentration of CSF fell more slowly. Collip and Backus (1920) demonstrated that there is a low permeability of the blood–CSF barrier to HCO_3^-. They reported that the injection of $NaHCO_3$ had no measurable effect on the concentration in the CSF until 1 hour after injection. In contrast to the slow movement of HCO_3^-, there was rapid transport of CO_2 between blood, brain, and CSF. Coxon and Swanson (1965) studied the passage of labeled CO_2 from blood to CSF and concluded that passage of this labeled material into CSF was quite rapid, with the maximal peak occurring approximately 15 minutes following the injection of labeled CO_2 into the bloodstream. Maren (1977) suggested that HCO_3^- transport from the blood to CSF is limited by the rate of blood flow to the choroid plexus.

The effects of respiratory alkalosis and acidosis in dogs have been studied (Leusen, 1954, 1965; Leusen and Demeester, 1960). There was a shift of blood and CSF pH in the same direction without an appreciable alteration in the concentration of HCO_3^- in the fluids. Bureau and Bouverot (1975) subjected dogs to hypoxia in a hypobaric chamber for up to 4 weeks. Within the first 30 minutes of acute hypoxia the pH of arterial blood and CSF increased by 0.05 unit in both compartments. During a second stage, between 30 minutes and 3 hours of hypoxia, the CSF pH remained at a high value, while arterial pH continued to increase. In a third stage, beyond 3 hours of high-altitude exposure, the pH progressively decreased similarly in both compartments. At a low altitude, HCO_3^- concentration was slightly lower in arterial blood than in CSF. In the course of exposure

to a higher altitude, HCO_3^- decreased similarly in both compartments. The lowest H^+ in blood and CSF was observed when ventilation reached its maximal plateau value at 3 hours of high altitude exposure; thereafter blood and CSF pH slowly returned toward control values.

In chronic metabolic acidosis, produced by prolonged administration of HCl to dogs, there was no significant change in CSF hydrogen ion activity and the CSF bicarbonate concentration changed in a linear fashion (Chazan *et al.*, 1969). With chronic metabolic alkalosis there was a significant fall in hydrogen ion activity that was about one-half as large as that which occurred in plasma. The increase in CSF bicarbonate was smaller than that in plasma water (Chazan *et al.*, 1969).

Posner *et al.* (1965) studied arterial and CSF pH, pCO_2, and HCO_3^- in 35 control and 48 human patients with an acid–base disorder. They found that metabolic acidosis was associated with only a slight shift in CSF pH, as the concentration of bicarbonate in CSF did not fall proportionately to that in blood. They also found that the difference in pCO_2 of venous blood, with which the CSF apparently was in approximate equilibrium, had become closer to that of arterial blood. They suggested that this might have been due to an increased blood flow following the fall in blood pH.

Comparable studies have not been completed in animals, and consequently no definite conclusions can be reached, although the mechanisms would appear to be similar in human beings and animals.

7. Urea

The concentration of urea in CSF apparently occurs in a purely passive manner in response to the concentration gradient between blood and CSF. Thus, we might expect that any animal having an increase in blood urea nitrogen would have a corresponding increase in the concentration of urea nitrogen in CSF. Cockrill (1931) found, in experimental uremia in cats, that the concentration of urea in blood and CSF increased at about the same rate, with the urea concentration in CSF always slightly lower than that in the blood.

8. Enzymes

As enzymes are relatively large protein molecules, usually of the globulin type, the concentration of enzymes in CSF will be small if their entry and exit are controlled in a fashion similar to those of the main protein constituents. Some enzymes, however, may be derived from the CNS and under certain conditions may be present in higher proportions in CSF (Davson, 1967). One of the difficulties encountered in interpreting increased enzyme activity in CSF is in determining whether an increase is due to release from the CNS or whether it represents enzyme leakage into the CSF from plasma. If there is a dramatic increase in CSF proteins, enzyme increases must be interpreted accordingly.

Cerebrospinal fluid levels of aspartate aminotransferase (glutamic oxaloacetic transaminase, GOT) and alanine aminotransferase (glutamic pyruvic transaminase, GPT) were studied by Hibbs and Coles (1965). In a group of 37 dogs, the mean CSF GOT (Reitman-Frankel units) was 20.1 units with a range of 9–46 units. In the same animals, the CSF GPT was 13.7 units with a range of 2–32 units. Hibbs (1962) also estimated the CSF levels of GOT and GPT in dogs with distemper. Eleven of 14 dogs had increased enzymatic activity in the CSF. The mean CSF GOT was 81.5 units, and the mean CSF GPT was 30.7 units.

Belsey (1966) produced purulent meningitis in a group of dogs by intracisternal and intravenous inoculation of *Diplococcus pneumoniae* type III. In six of seven dogs that died as a result of meningitis, the CSF GOT was significantly elevated. In animals that developed meningitis but recovered, the CSF GOT was normal or rose slightly, followed by a return to normal. No elevation in CSF GOT was noted in animals without meningitis. Belsey assumed that the increased CSF GOT activity during meningitis was the result of brain injury. This assumption was based on the fact that there was no evidence of changes in blood–CSF permeability as determined by the use of radioactive ^{131}I-labeled human serum albumin intracisternally and intravenously.

Wakim and Fleisher (1956) produced cerebral infarctions in dogs by intracarotid injection of vinyl acetate and found that following infarction there was an increase in both serum GOT activity, which doubled, and in CSF GOT activity, which rose severalfold. At the same time, the brain tissue lost more than 80% of its activity. This suggested that the CSF GOT was derived from brain tissue.

Creatine phosphokinase (CPK) is found in measurable quantities in the CSF of man and other animal species (Wilson, 1977). The normal level in the dog is <1 Sigma unit (SU) per milliliter (Wilson and Wiltrout, 1976). In the horse CPK activities in the CSF are similar, with a mean of 1.08 IU and a range of 0–8 (Mayhew *et al.*, 1977).

Wilson (1977) reported on the CPK activity in CSF samples from 126 animals suspected of having CNS disease. Values greater than 1 SU/ml were obtained on 32 samples, and values less than 1 SU/ml on 94 samples. Increased values (>1 SU) were reported in some animals with neoplasia, degenerative diseases, inflammatory diseases including toxoplasmosis, distemper, feline infectious peritonitis, and meningoencephalitis. Wilson felt that evaluation of CPK activity in CSF was a diagnostic aid in feline toxoplasmosis and feline infectious peritonitis, as all cats with these two diseases had increased CSF CPK activity. With the other diseases the increase in CSF CPK activity was not constant, and some animals with distinctive CNS diseases did not have increased CPK CSF activity.

β-Glucuronidase activity in the CSF was significantly elevated in dogs with distemper encephalitis when compared with controls (Long *et al.*, 1973). Increased β-glucuronidase activity in CSF is associated with neurological diseases of man characterized by rapid mobilization or proliferation of cells and tissue necrosis. The results of Long *et al.* (1973) suggested that the major portion of the increased β-glucuronidase activity was associated with the inflammatory response in the CNS rather than with leakage of this enzyme into the CSF from the blood. The CSF β-glucuronidase activity was highest in dogs with the most extensive brain tissue destruction. There was a high degree of correlation between β-glucuronidase and total protein values in the CSF.

Increased levels of CSF lactate dehydrogenase (LDH), with a normal serum LDH concentration has been described in man in association with bacterial meningitis, metastatic carcinoma, subarachnoid hemorrhage, and cerebral infarction (Krieg, 1969). Lactate dehydrogenase has been demonstrated in the CSF of horses (Mayhew *et al.*, 1977). Normal levels in equine CSF were 1.54 IU with a range of 0–8.

IX. CSF IN DISEASE

Knowledge concerning CSF alterations in diseases of domesticated animals is somewhat limited, although recent publications indicate an increasing interest in CSF. Accurate

interpretation of CSF alterations and their relation to diseases of the CNS awaits the accumulation of more data.

Fankhauser (1962) divided CSF findings into three groups:

1. Cerebrospinal fluid having slight alterations or which is almost normal. Such fluid can be found in intoxications, acute serous meningoencephalitis (acute distemper, infectious canine hepatitis, leptospirosis, posttraumatic encephalopathy, tetanies, congenital hydrocephalus, as well as traumatic, neoplastic, and inflammatory lesions concerning only the spinal cord, and disc herniation). Changes noted include pleocytosis (up to 30 cells per microliter) and a slightly elevated protein concentration.
2. Cerebrospinal fluid with xanthochromia, erythrocytes, protein levels from 90 to 500 mg/dl, and strongly positive Nonne-Aspelt and Pandy tests. This type of fluid is usually associated with inflammatory processes such as toxic encephalopathies and acute meningoencephalitides accompanied by severe vascular lesions and hemorrhages.
3. Cerebrospinal fluid which is clear or slightly turbid (when the total cell count reaches 200 per microliter or more), sometimes having either white flakes or a fibrinous network, with the number of cells ranging from 10 to 1000 per microliter.

A. Dog

In canine distemper, the CSF is characterized by an increase in cell number and protein content (Fankhauser, 1962; Bindrich and Schmidt, 1952; Markiewicz, 1965; Vandevelde and Spano, 1977).

In a group of 25 dogs (Vandevelde and Spano, 1977) the mean total white blood cell count was 51.8 per microliter with a range of 0–501. The majority of the cells were mononuclear with a mean of 61% lymphocytes and 25% monocytes. Similar findings were noted in the CSF of 47 dogs with a nervous form of distemper in which the cellular increase was mainly in lymphoid cells (Markiewicz, 1965).

The increase in CSF protein in canine distemper is not great and usually parallels the increase in cells (Fankhauser, 1962; Markiewicz, 1965). Total CSF protein values in these studies ranged from 40 to 150 mg/dl, and Nonne-Apelt and Pandy reactions were usually positive, although occasionally they were negative. The mean CSF protein level in the Vandevelde and Spano (1977) study was 36.8 mg/dl with a range of 4.3–136. The glucose concentration was usually within normal range (Fankhauser, 1962) but could be decreased or increased. Markiewicz (1965) reported a decrease in CSF glucose in dogs with a nervous form of distemper. The CSF chloride level was reported to be increased (Markiewicz) or normal (Fankhauser). Croft (1955) reported only slightly lower glucose levels in the CSF of 17 dogs with distemper. These dogs also had an elevated CSF total protein (130–15,000 mg/dl) and uric acid concentration. Cutler and Averill (1969) reported an increase in IgG in the CSF of dogs with clinical canine distemper. Immunoglobulin M was present in the CSF in 7 of 16 infected animals. The mean total protein concentration was increased. Six dogs had erythrocyte and leukocyte counts that exceeded normal.

Long *et al.* (1973) found an increased β-glucuronidase activity in CSF of dogs with distemper encephalitis. There appeared to be a positive correlation between the degree of brain tissue destruction and the level of this enzyme in the CSF. These workers also reported a marked increase in total protein, with a mean of 117.24 ± 90.5 mg/dl, and an increase in CSF globulin content.

Pleocytosis is a constant finding with encephalitis. The cell increase in viral infections

is due largely to an increase in mononuclear cells, while bacterial and mycotic infections are characterized by a strong participation of polymorphonuclear cells (Vandevelde and Spano, 1977). This difference assists in distinguishing between viral and mycotic or bacterial encephalitis.

In general, diseases of the meninges produce greater alterations in CSF than do lesions of CNS parenchyma. Thus, if neutrophil counts, protein values, and total cell counts are greatly increased, involvement of the meninges should be suspected.

Belsey (1966) experimentally produced meningitis in dogs by the injection of *Diplococcus pneumoniae* type III. A significant pleocytosis with cell counts of up to 2500 per microliter were recorded. In addition, there was a significant increase in CSF GOT activity in infected animals. Belsey suggested that CSF GOT might be useful in clinical studies of meningitis.

Changes in CSF were recorded in five cases of meningoencephalitis in dogs (Bullmore and Sevedge, 1978). The CSF protein, total cell counts, and neutrophils were increased, while CSF glucose levels were variable. The authors commented that the early stages of viral infection may be characterized by the appearance of neutrophils. In the later stages of disease the pleocytosis is predominantly lymphocytic.

Changes in CSF in the majority of dogs with spinal cord trauma of a variety of causes were not severe (Vandevelde and Spano, 1977). In spinal cord compressions of prolonged duration there was a moderately high protein content and usually a small increase in total cell count. In the differential count there was usually a relatively high neutrophil percentage (Vandevelde and Spano, 1977). The authors did not think it was possible to relate the CSF changes to the extent of spinal cord damage and felt that the diagnostic and prognostic value of CSF examination was limited. They did indicate, however, that in some instances, in which the nature and cause of the spinal disorder were not clear on clinical, neurological, or radiologic examination, CSF changes could support the diagnosis of physical damage to the spinal cord and differentiate it from muscular, neurogenic, or functional disorders clinically presented as spinal ataxia.

In the same study (Vandevelde and Spano, 1977), the CSF of eight animals with cerebrovascular disease was examined. There was a high protein content in two dogs with vasculitis, while in three dogs with cerebral infarcts many polymorphonuclear cells were present in the CSF. In dogs with nontraumatic hemorrhage in the CNS, the finding of many erythrophagocytes in the CSF was considered diagnostic. Xanthochromia was present in one dog with cerebral hemorrhage.

Several dogs with brain tumors had a high CSF protein content (Vandevelde and Spano, 1977). In some tumor cases there was no pleocytosis, while in others the cell count was moderately to markedly high. The authors felt that pleocytosis was due mainly to secondary effects of the tumor rather than to exfoliation of tumor cells. Two dogs in this group had an oligodendroglioma that invaded the CSF pathways. The authors were not, however, able to confirm that any of the cells in the CSF were malignant oligodendrocytes. Two dogs had carcinoma metastases characterized by strong pleocytosis. In neither dog was the presence of malignant cells demonstrated in the CSF. In two dogs with central lymphosarcoma the CSF cytological features were diagnostic, as a large number of lymphoblastic cells were recognized.

Monlux (1948) studied CSF in experimental leptospirosis of the dog. He found CSF xanthochromia in dogs with icterus. In most experimental cases, the fluid was free from pleocytosis and an elevation in protein. Monlux was unable to find erythrocytes in the

CSF, although there were gross and microscopic lesions indicating hemorrhage. In naturally occurring cases of leptospirosis, however, erythrocytes were found.

Infection with rabies virus produced a severe meningial reaction in animals with total cell counts ranging from 30 to 1200 cells per microliter, of which 60–98% were lymphocytes (Durand, 1951).

B. Cat

Although much experimental work on the physiology of CSF has been reported in the cat, there is little information concerning alterations in CSF in diseases of the CNS.

In cerebellar ataxia due to encephalitis of the cerebellum and marked atrophy, there was a positive Pandy reaction, increased protein, and a pleocytosis with a cell count of 900 per microliter. The cells were small and large lymphocytes, mononuclear cells, and endothelial cells (Fankhauser, 1962). In a case of head injury with hemorrhage and softening in the hypothalmus, Fankhauser (1962) found a positive Pandy test and a blood-stained fluid with 10,000 erythrocytes and approximately 120 lymphocytes per microliter. He also reported the presence of some granulocytes and gitter cells. Two Siamese cats with congenital tremor and a cat experimentally infected with *Leptospira pomona* had normal CSF (Fankhauser, 1962).

Wilson (1977) reported a CSF CPK activity of greater than 1 SU in cats with toxoplasmosis and feline infectious peritonitis. The CPK values in these animals ranged from 8 to 23 SU. Toxoplasmosis is also accompanied by pleocytosis with an increase in neutrophils and a total CSF protein concentration of greater than 35 mg/dl (Farrow and Love, 1975). In three cases of feline infectious peritonitis meningoencephalitis there was a mononuclear pleocytosis with total cell counts of from 500 to 1144/microliter. The pleocytosis was accompanied by a marked increase in CSF protein with values of from 185 to 498 mg/dl (DeLahunta, 1977).

In a case of cuterebra encephalitis in a cat there was a neutrophilic pleocytosis with a total white cell count of 258 per microliter and a total protein content of 89 mg/dl (DeLahunta, 1977).

C. Horse

In equine encephalomyelitis, Meyer *et al.* (1931, 1934) found that there was a greater pleocytosis (600–25,000 per microliter) in infection with the eastern strain than with the western strain of the encephalomyelitis virus (12–30 cells per microliter). In horses affected with Russian equine encephalomyelitis, abnormal quantities of albumin and globulin, an increase in sugar, and a decrease in alkali reserve were noted. The pH and calcium content were both high (Fridman *et al.*, 1935). In a later study (Fridman, 1938) a considerable decrease in vitamin C content was reported in horses experimentally infected with the virus of Russian meningoencephalitis.

The encephalitides accompanying arborvirus infection produce a pleocytosis that can be variable in nature. With Venezuelan equine encephalomyelitis and eastern equine encephalomyelitis, during the acute phase, a high neutrophil count can be expected. This changes to a predominantly small mononuclear pleocytosis over several days (DeLahunta, 1977). Western equine encephalomyelitis produces a less marked pleocytosis that is predominantly small mononuclear cells. The CSF protein concentration is elevated with arborvirus infections.

Infection with equine herpes virus I (rhinopneumonitis) vasculitis/myelopathy results in xanthochromic CSF with an elevated protein content and little or no cellular response (DeLahunta, 1977).

DeLahunta (1977) commented that, as a general rule, injury to the CNS results in a xanthochromic CSF with a slightly elevated protein concentration and occasionally a definitely bloody sample. Large hemorrhages may be present epidurally with very little change in the CSF. The CSF changes depend in part on the site of collection. If a brain or cranial cervical lesion is suspected, an atlantooccipital sample should be taken. For spinal cord problems located caudally, a lumbosacral sample is preferred. Other conditions with an associated xanthochromia and elevated protein content with a few mononuclear cells include intracarotid injections of drugs such as promazine, deep cerebral abcess, vertebral osteomyelitis, and acute wobbler syndrome (DeLahunta, 1977).

In purulent leptomeningitis, the CSF was turbid, viscous, and yellow. The cell count was greater than 350 per microliter, and almost all cells were neutrophils (Schulze, 1953). The CSF glucose concentration was 27 mg/dl as compared to a blood glucose concentration of 123 mg/dl. Both the Pandy and Nonne-Apelt reactions were positive. Fankhauser (1962) reported rapid coagulation of CSF from a horse with severe purulent leptomeningitis. The CSF in a case of disseminated septic meningitis in a mare, from which an *Actinomyces* sp. was cultured, was cloudy in appearance, had a marked increase in total protein content (513–1912 mg/dl), and a pleocytosis characterized by the presence of predominantly neutrophils (Rumbaugh, 1977).

D. Cattle

The most exhaustive study of CSF alterations in diseases involving the CNS was reported by Fankhauser (1962). As a result of his studies he was able, by examination of the CSF, to differentiate various clinical symdromes involving the CNS. In tuberculosis of the CNS he distinguished two groups.

1. The typical alterations of the CSF in acute tuberculous leptomeningitis or meningoencephalitis included the presence of a fibrinous clot, a high content of cells and protein, low glucose content, and markedly pathological colloidal tests. He concluded, however, that a positive diagnosis could be provided only by a positive bacteriological test.
2. Atypical alterations occurred in CSF in intracerebral tuberculosis with only a slight meningeal reaction or in space-occupying tuberculous growths originating from the surrounding tissues. In these cases CSF alterations were less pronounced but seldom absent. In two cases of this type involving what was termed "tuberculoma of the cerebellum" the CSF was clear and colorless with 5 and 23 cells per microliter and protein concentrations of 40 and 70 mg/dl, the Nonne–Apelt and Pandy reactions were slightly positive to negative, and the CSF glucose concentrations were 47 and 59 mg/dl respectively.

In purulent meningoencephalitis (Fankhauser, 1962) the CSF was opaque, whitish, or yellowish, with fibrinous filaments or flakes, often coagulating. The total cell count ranged from 75 to several thousand per microliter, and most of the cells were neutrophils. The total protein content was increased, and both the Pandy and Nonne–Apelt tests were distinctly positive. In these cases, the CSF glucose concentration ranged from 50 to 78 mg/dl.

In nonpurulent meningoencephalitis and meningomyelitis the CSF was usually clear and colorless and occasionally had a slight opalescence with fine fibrinous filaments. Total cells, most of which were lymphocytes and mononuclear cells, ranged from 10 to 140 per microliter. Total protein content was up to 300 mg/dl with an average of 88 mg/dl. Protein content and cell count generally were parallel, although this was not always true. The Pandy test was negative to 3+, and the Nonne–Apelt test was 1+ to 2+.

Fankhauser also reported the CSF findings in a steer with traumatic encephalopathy. In this animal, the CSF was clear and colorless; the pressure was noticeably increased; the cell count was 70 per microliter, and the cells were predominantly lymphocytes and histiocytes; the total protein content was 42 mg/dl, of which 6.6 mg was globulin and 35.4 mg albumin; and the Pandy and Nonne–Apelt reactions were negative. In a second case of a cow with endogenous intoxication and edema of the brain, the total cell count was not increased; the total protein content was 50 mg/dl; the Pandy reaction was 1+; the Nonne–Apelt reaction was negative; and the CSF glucose concentration was 55 mg/dl. In a cow with extensive brain abscesses in the left cerebral hemisphere, Fankhauser (1962) found that the CSF formed a fibrinous clot after a few minutes; the fluid was slightly turbid and contained 20 cells per microliter; total protein content was 500 mg/dl; the Nonne–Apelt and Pandy reactions were positive; and the sugar concentration was 70 mg/dl. The presence of a high protein concentration in the absence of a high cell count enabled him to rule out the presence of a purulent leptomeningitis.

Howard (1969) summarized methods for neurological disease differentiation in cattle and pointed out that examination of CSF provides information concerning the type and degree of inflammatory or degenerative process present. He emphasized that diseases causing severe inflammatory reactions in tissue contacting CSF (i.e., thromboembolic meningoencephalitis and other bacterial infections) result in escape of blood proteins into the CSF and strong Pandy's test reaction. In contrast, bacterial infections limited to deep brain tissues are also exudative, but the proteins are filtered by the surrounding tissues so that the intensity of the Pandy reaction is reduced or negative. This, Howard reported, is true of listeriosis and may also occur in other focal bacterial encephalitides. Lesions that do not affect blood vessel contiguity may not result in a positive Pandy test (polioencephalomalacia, coccidial encephalopathy, idiopathic myelopathy, and viral infections).

Howard (1969) reported that total cell counts in viral degenerative and toxic conditions ranged from less than 20 to 100 per microliter. Cell counts in listeriosis and streptococcosis ranged between 100 and 200 per microliter. Cell types were also characteristic, as mononuclear cells accompany viral infections, polioencephalomalacia, idiopathic myelopathy, coccidial encephalopathy, and listeriosis. Neutrophils accompany purulent diseases, such as thromboembolic meningoencephalitis, and staphylococcal, *Corynebacterium,* and *Pseudomonas* encephalitis and meningitis.

The CSF in bovine spastic paralysis was reported to have a low phosphorus and normal calcium concentration (Bijleveld and Binkhorst, 1973). There was also a decrease in ascorbate concentrations in the CSF and serum of these spastic animals. De Ley and De Moor (1975) studied the CSF concentrations of homovanillic acid and 5-hydroxyindoleacetic acid in normal and spastic calves. The concentrations of homovanillic acid were significantly lower in spastic calves, whereas differences in 5-hydroxyindoleacetic acid were not found between normal and spastic calves. These authors suggested that such findings indicated that a lower dopaminergic metabolism took place in the CNS of spastic calves.

E. Swine

In experimental Teschen disease (Fankhauser, 1962) the total cell count ranged from 16 to 70 per microliter, and the cells consisted predominantly of small and large lymphocytes and mononuclear cells. The total protein content was not over 50 mg/dl, and Pandy and Nonne–Apelt reactions were not always positive. Lax (1961) studied the CSF in 74 pigs that were either experimentally or spontaneously infected with Teschen's disease. In about half the cases, the CSF developed a fibrinous precipitation within 24 hours. Globulin tests ranged from negative to positive, and there was generally an increase in the number of lymphocytes and in albumin content. Similar findings were reported by Fischer and Starke (1951). They found the fluid to be clear and colorless, and slightly turbid when the pleocytosis reached 170 per microliter; sometimes they noted the presence of fibrinous filaments forming a fine "spider web."

Rauch (1961) studied CSF from pigs prior to and following subcutaneous injections of crystal violent vaccine. Inoculation of the vaccine was followed by a moderate to average lymphocytosis and an increase in protein content of the CSF, which reached its highest concentration 3–9 days following the second dose of vaccine.

Fankhauser (1962) reported on the CSF findings in a boar with paraplegia of some days' duration. A lumbosacral puncture revealed a turbid, yellowish CSF which coagulated after a few minutes. The fibrinous clot retracted, and the supernatant fluid was xanthochromic, had 1 cell per microliter, and a protein concentration of 2500 mg/dl with very strong Nonne–Aspelt and Pandy reactions.

F. Sheep

Data concerning pathological alterations in the CSF of sheep are scarce, in spite of the fact that neurological disorders of sheep occur which are of considerable economic importance.

Sigurdsson (1954) and Sigurdsson *et al.* (1957) reported some data on the CSF in *rida,* a chronic encephalitis, and *visna,* a subacute demyelinating encephalomyelitis and meningitis of sheep in Iceland. A long-lasting pleocytosis was said to be characteristic of *visna* and often began months before the onset of clinical signs. The increase in protein varied in experimental cases from 31 to 295 mg/dl. Sigurdsson believed that the presence of pleocytosis permitted the differentiation of *visna* from *rida.*

Buck (1964) induced convulsions in sheep by inhalation of carbon dioxide, subcutaneous injection of insulin, and oral administration of heptachlor. He found a twofold increase in CSF protein, GOT, and LDH following insertion of a catheter into the cisterna magna. During CO_2 inhalation, induced seizures were very acute. Both blood and CSF glucose levels doubled during seizures, and there were definite elevations in serum and, to a lesser extent, CSF potassium values within 6 minutes which returned to nearly normal within 1 hour. Both blood and CSF pH decreased by 0.5–0.7 unit within 1 minute and returned to normal within 1 hour. The GOT activities were elevated in both blood and CSF of the two sheep from which analyses were made. Buck also found that the CSF calcium and magnesium concentration increased inconsistently during CO_2-induced seizures.

Convulsive seizures were induced in five sheep by injecting insulin and in four sheep by giving heptachlor orally. Physiological and biochemical changes were much less acute than during CO_2-induced seizures. Blood and CSF glucose concentrations decreased to below 10 mg/dl during insulin shock seizures but, after treatment with glucose or DL-

glutamic acid, returned to above control levels. Blood and CSF glucose levels increased markedly in sheep given heptachlor prior to the time that clinical signs of toxicity were evident. Slight elevations in blood serum GOT were noted in the sheep given insulin, and both blood and CSF elevations were noted in those given heptachlor.

Migration of the adult nematode *Parelaphostrongylus tenuis* through the spinal cord and brain of sheep and goats can produce a variety of clinical signs involving the CNS. The CSF frequently has a pleocytosis greater than 200 white cells per microliter that consists of mononuclear cells and eosinophils. Hemorrhage occurs frequently, and protein levels are usually increased moderately to higher than 100 mg/dl. The presence of eosinophils is helpful in arriving at a diagnosis, as they rarely occur in CSF without a parasitism (DeLahunta, 1977).

G. Goat

Little work has been done on CSF alterations in diseases of the goat, although a great deal of research has been conducted on the physiological relationship between CSF and respiration.

Mould and Dawson (1965) studied the free and esterified cholesterol in CSF of goats affected with experimental scrapie. They found that the total cholesterol in CSF remained relatively constant, but a small increase occurred in clinically affected animals. The proportions of free cholesterol increased in all goats when only slight histological damage to brain tissue was present. The mean value for total cholesterol was 4.0 mg/liter in experimental animals, and of this 48% was free cholesterol when the terminal elevations were ignored.

Millson *et al.* (1960) reported that the concentrations of total protein, glucose, chloride, and urea in CSF of goats affected with scrapie showed no deviation from normal. Viral leukoencephalomyelitis occurs in kids between 2 and 4 months of age. The CSF has a mononuclear pleocytosis of between 100 and 18,000 leukocytes per microliter and a variable protein elevation with a positive Pandy test (Cork, 1976).

REFERENCES

Abbot, J., Glen, I., Davson, H., and Grant, N. (1971). *Brain Res.* **29,** 185.
Adamesteanu, J. E., Stefanuscu, E., and Nicolau, A. (1944). *Arch. Wiss. Prakt. Tierheilkd.* **78,** 382.
Adams, J. E., and Prawirohardjo, S. (1959). *Neurology* **9,** 561.
Alksne, J. F., and Lovings, E. T. (1972). *Arch. Neurol.* **27,** 371.
Altman, D. L., and Dittmer, D. S. (Eds.) (1974). *In* "Biology Data Book," 2nd ed., Vol. 3, p. 1978. Fed. Am. Soc. Exp. Biol., Bethesda, Maryland.
Ames, A., III, Sakanoue, M., and Endo, S. (1964). *J. Neurophysiol.* **27,** 672.
Ames, A., III, Higashi, K. G., and Nesbett, F. B. (1965). *J. Physiol. (London)* **181,** 506.
Baltch, A. L., Fuller, T., and Osborne, W. (1969). *J. Lab. Clin. Med.* **73,** 883.
Bekaert, J., and Demeester, G. (1951a). *Arch. Int. Physiol.* **59,** 262.
Bekaert, J., and Demeester, G. (1951b). *Arch. Int. Physiol.* **59,** 391.
Belsey, M. A. (1966). *Antimicrob. Agents Chemother.* p. 19.
Bering, E. A., Jr. (1959). *Am. J. Physiol.* **197,** 825.
Bijleveld, K., and Binkhorst, G. J. (1973). *Tijdschr. Diergeneeskd.* **98,** 803.
Bindrich, H., and Schmidt, D. (1952). *Arch. Exp. Veterinaermed.* **6,** 162.
Bito, L. Z., and Davson, H. (1966). *Exp. Neurol.* **14,** 264.
Bourke, R. S., Gabelnick, H. L., and Young, O. (1970). *Exp. Brain Res.* **10,** 17.

Bradbury, M. W. B., and Davson, H. (1964). *J. Physiol.* (*London*) **170,** 195.
Bradbury, M. W. B., and Sarna, G. S. (1977). *Exp. Eye Res.* **24,** Suppl., p. 249.
Brightman, M. W. (1977). *Exp. Eye Res.* **24,** Suppl., p. 1.
Buck, W. B. (1964). *Ann. N.Y. Acad. Sci.* **3,** 751.
Bullmore, C. C., and Sevedge, J. P. (1978). *J. Am. Anim. Hosp. Assoc.* **14,** 387.
Bureau, M., and Bouverot, P. (1975). *Respir. Physiol.* **24,** 203.
Calhoun, M. C., Hurt, H. D., Eaton, H. D., Rousseau, J. E., Jr., and Hall, R. C., Jr. (1967). *Univ. Conn. Agric. Exp. Stn., Bull.* No. 401.
Castel, M., Sahar, A. G., and Erlÿ, D. (1974). *Brain Res.* **67,** 178.
Cestan, R., Sandrail, M., and Lassalle, H. (1925). *C. R. Soc. Biol.* **93,** 475.
Chazan, J. A., Appleton, F. M., London, A. M., and Schwartz, W. B. (1969). *Clin. Sci.* **36,** 345.
Cockrill, J. R. (1931). *AMA Arch. Neurol. Psychiatry* **25,** 1297
Coles, E. H. (1974). "Veterinary Clinical Pathology," 2nd ed., p. 392. Saunders, Philadelphia, Pennsylvania.
Collip, J. B., and Backus, P. L. (1920). *Am. J. Physiol.* **51,** 551.
Cork, L. C. (1976). *J. Am. Vet. Med. Assoc.* **169,** 1303.
Cornelius, C. E. (1963). *In* "Clinical Biochemistry of Domestic Animals" (C. E. Cornelius and J. J. Kaneko, eds.), p. 385. Academic Press, New York.
Cornford, E. M., and Oldendorf, W. H. (1975). *Biochim. Biophys. Acta* **394,** 211.
Courtice, F. C., and Simmonds, W. J. (1951). *Aust. J. Exp. Biol. Med. Sci.* **29,** 255.
Coxon, R. V., and Swanson, A. G. (1965). *J. Physiol.* (*London*) **181,** 712.
Croft, P. G. (1955). *Vet Rec.* **67,** 872.
Crone, C. (1965). *J. Physiol.* (*London*) **181,** 103.
Crone, C., and Thompson, A. H. (1970). *In* "Capillary Permeability" (C. Crone and N. Lassen, eds.), p. 457. Academic Press, New York.
Cserr, H. F. (1975). *In* "Fluid Environment of the Brain" (H. F. Cserr, J. D. Fenstermacher, and V. Fencl, eds.). Academic Press, New York.
Cutler, R. W. P., and Averill, D. R., Jr. (1969). *Neurology* **19,** 1111.
Cutler, R. W. P., Page, L., Galicich, J., and Watters, G. V. (1968). *Brain* **71,** 707.
Davson, H. (1955). *J. Physiol.* (*London*) **129,** 11.
Davson, H. (1967). "Physiology of the Cerebrospinal Fluid." Churchill, London.
Davson, H., and Segal, M. B. (1970). *J. Physiol.* (*London*) **209,** 131.
Davson, H., Kleeman, C. R., and Levin, E. (1962). *J. Physiol.* (*London*) **161,** 126.
DeChiro, G., Stein, S. C., and Harrington, T. (1972). *J. Neuropathol. Exp. Neurol.* **31,** 447.
Deck, M. D. F., Deonarine, V., and Potts, D. G. (1972). *J. Neurosurg.* **37,** 177.
DeLahunta, A. (1977). "Veterinary Neuroanatomy and Clinical Neurology," Saunders, Philadelphia, Pennsylvania.
De Ley, G., and De Moor, A. (1975). *Am. J. Vet. Res.* **36,** 227.
Domer, F. R. (1969). *Exp. Neurol.* **24,** 54.
Domer, F. R. (1977). *Exp. Eye Res.* **24,** Suppl., p. 323.
Durand, P. (1951). *Arch. Inst. Pasteur, Tunis* **30,** 55.
El Amrousi, S., Soliman, M. K., and Youssef, L. B. (1966). *Ind. Vet. J.* **43,** 106.
Fankhauser, R. (1962). *In* "Comparative Neuropathology" (J. R. M. Innes and L. Z. Saunders, eds.), p. 21. Academic Press, New York.
Farrow, B. R. H., and Love, D. N. (1975). *In* "Textbook of Veterinary Internal Medicine—Diseases of the Dog and Cat" (S. J. Ettinger, ed.), p. 226. Saunders, Philadelphia, Pennsylvania.
Fischer, K., and Starke, G. (1951). *Exp. Veterinaermed.* **5,** 38.
Fridman, A. P. (1938). *Sov. Vet.* **15,** No. 1, p. 20.
Fridman, A. P., and Petrova, V. V. (1935). *Arkh. Biol. Nauka* **39,** 209.
Fridman, A. P., Shokhor, N. I., Glinka-Cernorucka, E. L., Petrova, V. V., and Arkina, R. (1935). *Sov. Vet.* **12,** No. 12, p. 39.
Friedman, S. B., Austen, W. G., Rieselbach, R. E., Block, J. B., and Rall, D. P. (1963). *Proc. Soc. Exp. Biol. Med.* **114,** 801.
Frier, H. I., Gallina, A. M., Rousseau, J. E., Jr., and Eaton, H. D. (1972). *J. Dairy Sci.* **55,** 339.
Frier, H. I., Gorgacz, E. J., Hall, R. C., Jr., Gallina, A. M., Rousseau, J. E., Jr., Eaton, H. D., and Nielsen, S. W. (1974). *Am. J. Vet. Res.* **35,** 45.
Frömter, E., and Diamond, J. (1972). *Nature (London), New Biol.* **235,** 9.

Gomez, D. G., and Potts, D. G. (1977). *Exp. Eye Res.* **24,** Suppl., p. 117.
Gondos, B., and King, E. B. (1976). *Acta Cytol.* **20,** 542.
Graziani, L. J., Kaplan, R. K., Escriva, A., and Katzman, R. (1965). *Am. J. Physiol.* **208,** 1064.
Hammerstad, J. P., Lorenzo, A. V., and Cutler, R. W. P. (1968). *Neurology* **18,** 296.
Heisey, S. R., Held, D., and Pappenheimer, J. R. (1962). *Am. J. Physiol.* **203,** 775.
Held, D., Fencl, V., and Pappenheimer, J. R. (1964). *J. Neurophysiol.* **27,** 942.
Heywood, R., Osborne, B. E., and Street, A. E. (1973). *Lab. Anim.* **7,** 85.
Hibbs, C. M. (1962). M.S. Thesis, Kansas State Univ. Manhattan.
Hibbs, C. M., and Coles, E. H. (1965). *Proc. Soc. Exp. Biol. Med.* **118,** 1059.
Hochwald, G. M., and Wallenstein, M. C. (1967). *Exp. Neurol.* **19,** 115.
Holloway, L. S., and Cassin, S. (1972). *Am. J. Physiol.* **223,** 503.
Howard, J. R. (1969). *J. Am. Vet. Med. Assoc.* **154,** 1174
Jordan, J. E. (1977). *Am. J. Vet. Res.* **38,** 509.
Kaneko, J. J. (1973). "Standard Values in Domestic Animals," 3rd ed. Univ. of California, Davis.
Keynes, R. D. (1969). *Q. Rev. Biophys.* **2,** 177.
Kirk, G. R., Neate, S., McClure, R. C., and Hutcheson, D. P. (1974). *Am. J. Vet. Res.* **35,** 1263.
Krieg, A. F. (1969). *In* "Clinical Diagnosis by Laboratory Methods" (I. Davidsohn and J. B. Henry, eds.), p. 1161. Saunders, Philadelphia, Pennsylvania.
Kristensen, F., and Firth, E. C. (1977). *Am. J. Vet. Res.* **38,** 1089.
Lax, T. (1961). *Sb. Cesk. Akad. Zemed. Ved., Vet. Med.* **6,** 147.
Leusen, I. (1954). *Am. J. Physiol.* **176,** 513.
Leusen, I. (1965). *In* "Cerebrospinal Fluid and the Regulation of Respiration" (C. McC. Brooks *et al.*, eds.), p. 58. Davies, Philadelphia, Pennsylvania.
Leusen, I., and Demeester, G. (1960). *Pfluegers Arch. Gesamte Physiol. Menschen Tiere* **270,** 390.
Long, J. F., Jacoby, R. O., Olson, M., and Koestner, A. (1973). *Acta Neuropathol.* **25,** 179.
Lorenzo, A. V. (1977). *Exp. Eye Res.* **24,** Suppl., p. 205.
Lorenzo, A. V., Page, L. K., and Walters, G. V. (1970). *Brain* **93,** 679.
MacPherson, C. F. G. (1967). *J. Immunol.* **98,** 1039.
Maren, T. H. (1977). *Exp. Eye Res.* **24,** Suppl., p. 157.
Markiewicz, K. (1965). *Med. Welt* **21,** 528.
Mayer, S. E., Maickel, R. P., and Brodie, B. B. (1960). *J. Pharmacol. Exp. Ther.* **128,** 41.
Mayhew, I. G. (1975). *Cornell Vet.* **65,** 500.
Mayhew, I. G., Whitlock, R. H., and Tasker, J. B. (1977). *Am. J. Vet. Res.* **38,** 1271.
Meyer, H., and Scholz, H. (1972). *Dtsch. Tieraerztl. Wochenschr.* **79,** 49.
Meyer, K. F., Wood, F., Harding, C. M., and Howitt, B. (1931). *Science* **74,** 227.
Meyer, K. F., Harding, C. M., and Howitt, B. (1934). *Proc. Soc. Exp. Biol. Med.* **32,** 56.
Milhorat, T. H., Hammock, M. K., and Fenstermacher, J. D., (1971). *Science* **173,** 330.
Millson, G. C., West, L. C., and Dew, S. M. (1960). *J. Comp. Pathol. Ther.* **70,** 194.
Monlux, W. S. (1948). *Cornell Vet.* **38,** 109.
Mould, D. L., and Dawson, A. McL. (1965). *Berl. Muench. Tieraerztl. Wochenschr.* **66,** 117.
Oldendorf, W. H. (1977). *Exp. Eye Res.* **24,** Suppl., p. 177.
Oldendorf, W. H., and Brown, W. J. (1975). *Proc. Soc. Exp. Biol. Med.* **149,** 736.
Oldendorf, W. H., and Szabo, J. (1976). *Am. J. Physiol.* **230,** 94.
Oliver, J. E., Jr., and Knecht, C. D. (1975). *In* "Textbook of Veterinary Internal Medicine—Diseases of the Dog and Cat" (S. J. Ettinger, ed.), p. 357. Saunders, Philadelphia, Pennsylvania.
Oliver, J. E., Jr., Redding, R. W., and Knecht, C. D. (1975). *In* "Textbook of Veterinary Internal Medicine—Diseases of the Dog and Cat" (S. J. Ettinger, ed.), p. 235. Saunders, Philadelphia, Pennsylvania.
Oppelt, W. W.,Maren, T. H., Owens, E. S., and Rall, D. P. (1963). *Proc. Soc. Exp. Biol. Med.* **114,** 86.
Osweiler, G. D., and Hurd, J. W. (1974). J. Am. Vet. Med. Assoc. **165,** 165.
Pappenheimer, J. R., Heisey, S. R., and Jordan, E. F. (1961). *Am. J. Physiol.* **200,** 1.
Petersdorf, R. G., Swarner, D. R., and Garcia, M. (1960a). *Proc. Soc. Exp. Biol. Med.* **103,** 380.
Petersdorf, R. G., Swarner, D. R., and Garcia, M. (1960b). *Proc. Soc. Exp. Biol. Med.* **104,** 65.
Pollay, M., Stevens, A., Estrada, E., and Kaplan, R. (1972). *J. Appl. Physiol.* **32,** 612.
Posner, J. B., Swanson, A. G., and Plum, F. (1965). *Arch. Neurol.* **12,** 479.
Rauch, H. (1961). *Arch. Exp. Veterinaermed.* **15,** 1147.
Richter, J. J., and Wainer, A. (1971). *J. Neurochem.* **18,** 613.

Roszel, J. F. (1972). *J. Am. Vet. Med. Assoc.* **160,** 720.
Rumbaugh, G. E. (1977). *J. Am. Vet. Med. Assoc.* **171,** 452.
Sahar, A. (1972). *J. Neurol. Sci.* **16,** 49.
Sato, O., Bering, E. A., Jr., Yagi, M., Tsugane, R., Hara, M., Amano, Y., and Asai, T. (1975). *Acta Neurol. Scand.* **51,** 1.
Scholz, H., and Meyer, H. (1972). *Dtsch. Tieraerztl. Wochenschr.* **79,** 613.
Schulze, J. (1953). *Monatsh. Veterinaermed.* **15,** 1147.
Segal, M. B., and Pollay, M. (1977). *Exp. Eye Res.* **24,** Suppl., 127.
Shabo, A. L., and Maxwell, D. S. (1971). *J. Neuropathol. Exp. Neurol.* **30,** 506.
Sigurdsson, B. (1954). *Br. Vet. J.* **110,** 341.
Sigurdsson, B., Pálsson, P., and Grímsson, P. (1957). *J. Neuropathol. Exptl. Neurol.* **16,** 389.
Snodgrass, S. R., and Lorenzo, A. V. (1972). *Am. J. Physiol.* **222,** 1524.
Soliman, M. K., El Amrousi, S., and Youssef, L. B. (1965). *Zentralbl. Veterinaermed., Reihe A* **12,** 769.
Tripathi, B. J., and Tripathi, R. C. (1974). *J. Physiol. (London)* **239,** 195.
Tripathi, R. C. (1974). *Brain Res.* **80,** 503.
Vandevelde, M., and Spano, J. S. (1977). *Am. J. Vet. Res.* **38,** 1827.
Vates, T. S., Jr., Bonting, S. L., and Oppelt, W. W. (1964). *Am. J. Physiol.* **206,** 1165.
Wakim, K. G., and Fleisher, G. A. (1956). *Proc. Staff Meet. Mayo Clin.* **31,** 391.
Wald, A., Hochwald, G. M., and Malhan, C. (1976). *Exp. Neurol.* **50,** 304.
Weed, L. H. (1914). *J. Med. Res.* **31,** 51.
Welch, K. (1963). *Am. J. Physiol.* **205,** 617.
Welch, K., and Friedman, V. (1960). *Brain* **83,** 454.
Welch, K., and Pollay, M. (1961). *Am. J. Physiol.* **201,** 651.
Welch, K., Sadler, K., and Gold, G. (1966). *Am. J. Physiol.* **210,** 232.
Welch, K., Sadler, K., and Hendee, R. (1970). *Brain Res.* **19,** 465.
Wilson, J. W. (1977). *J. Am. Vet. Med. Assoc.* **171,** 200.
Wilson, J. W., and Stevens, J. B. (1977). *J. Am. Vet. Med. Assoc.* **171,** 256.
Wilson, J. W., and Stevens, J. B. (1978). *J. Am. Vet. Med. Assoc.* **172,** 911.
Wilson, J. W., and Wiltrout, S. K. (1976). *Am. J. Vet. Res.* **37,** 1099.
Wright, E. M. (1972). *J. Physiol. (London)* **226,** 545.
Wright, E. M. (1974). *J. Physiol. (London)* **240,** 535.

19

Synovial Fluid

VICTOR PERMAN

I. INTRODUCTION

Articular diseases rank high among the crippling diseases of animals and man. Most types of articular disease tend to increase the volume of synovial fluid in the affected joint. In the case of superficial joints, the synovial fluid is easily aspirated for analysis. The study of the origin and nature of joint fluids has allowed for a more accurate interpretation of abnormalities encountered in pathological synovial effusions. Chemical and cellular analysis of synovial fluid has received more attention in recent years. A clear understanding of what information can be obtained from synovial fluid analysis and the limitations of the procedure must be understood. Positive diagnosis is likely in infectious arthritis and crystal-induced synovitis. Useful information will be obtained to aid in determining whether the effusion is inflammatory or noninflammatory.

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed.

ISBN 0-12-396350-8

Synovial fluid appears to have two main functions: the nutrition of joint tissues and lubrication of articulating surfaces. These functions are related to the synovial membrane, which regulates the volume of fluid and its macromolecular composition. Knowledge of joint structure is important for understanding synovial fluid.

The synovial joint is a mobile structure separating articulating bones of the skeleton by a cavity. Articular cartilage covers the bearing surfaces. The segments are connected by a fibrous envelope, of which the inner surface is covered by the synovial membrane. A tough fibrous capsule is external. The articular surfaces of the synovial joint are covered by small quantities of viscous synovial fluid. The joint tissues develop from mesenchymal cells and are composed of many different types of extracellular matrices. These matrices are composed of collagen, proteoglycans, and glycoproteins, which vary with tissue location. Studies on synovial membranes (Woodward *et al.*, 1969), on those of sheep (Cutlip and Cheville, 1973), and on those of the horse (Shively, 1975; Johansson and Rejno, 1976; Shively and Van Sickle, 1977) define three types of synovial membranes with variability in form and surface. The surface of the synovial membrane varies from fibrillar mats with small-diameter (1- to 5-μm) holes to surfaces of a cellular nature. The surface cells of all species are of two main cell types. One is called the type A cell, which contains prominent Golgi complexes and many vesicles and vacuoles but little rough endoplasmic reticulum (Ghadially and Roy, 1969). Another cell, called type B, has a well-developed rough endoplasmic reticulum but a poorly developed Golgi complex. It is suggested that the type A and B cells have both phagocytic and secretory potential (Ghadially, 1978). The synovial membrane has a rich blood supply (Ropes and Bauer, 1953). The capillaries have a fenestrated structure. The synovial lining cells do not form a discrete layer over all the synovial membrane, resulting in the contact of the extracellular matrix and the joint cavity.

Early investigators suggested that the origin of synovial fluid was as follows: (1) synovial membrane glands (Havers, 1691); (2) disintegration of the synovial membrane and transudation (Freichs, 1850); (3) destruction of the cartilage from constant use (Ogston, 1876); (4) gland secretion and transudation from capillaries and lymphatics (Mayeda, 1920); and (5) synovial fluid as a fluid matrix with a specialized connective tissue lining a large tissue space (Hueck, 1920). Most early theories were based primarily upon histological studies prior to the time physiological studies indicated the synovial fluid to be a modified dialyzate of plasma.

The distribution of electrolytes and nonelectrolytes between plasma and the synovial fluid is in accord with its origin as a protein-containing dialyzate of blood plasma (Ropes *et al.*, 1939). Nettebladt *et al.* (1963) showed that sedimentation of plasma through hyaluronic acid produced an ultrafiltrate with a composition similar to that of synovial fluid. The concept of a modified dialyzate is in keeping with the resemblance of synovial fluid to other body transudates. The major difference between synovial fluid and other body fluids derived primarily from plasma is the high mucin content, resulting in exceptionally high colloid osmotic pressure and elevated ionic calcium levels due to the high base-combining power of mucin. Synovial fluid is derived from blood and modified by constituents secreted by the joint tissues.

The articular cartilage obtains its nutrition via the synovial fluid. The cartilage covering the articular surfaces of synovial joints is usually of the hyaline variety (Barnett *et al.*, 1961). The glassy-looking matrix contains many fibers, fibrils, and filaments. The chon-

drocytes are set in the matrix and occupy about 0.01–0.1% of the volume of the tissue (Hamerman and Schubert, 1962). Articular cartilage is aneural, alymphatic, and avascular. It is nourished by the synovial fluid.

The surface of the articular cartilage appears smooth to the naked eye. Since the advent of the scanning electron microscopy, the ridges and undulations demonstrated appear to result from shrinkage and distortion of the cartilage (Ghadially *et al.*, 1977). Scanning electron microscopy shows innumerable pits on the surface of adult human cartilage similar to the surface of a golf ball (Clarke, 1973; Ghadially *et al.*, 1974). The humps demonstrated (Redler, 1974) are found most frequently on the surface of young articular cartilage. The humps appear to be due to underlying chondrocytes, while in adult animals the pits reflect their presence.

Joint lubrication is a controversial subject. Numerous mechanisms have been proposed (McCutchen, 1978). Charnley (1959) showed that hydrodynamic lubrication was impossible in animal joints because of slow oscillation and stopping at each reversal of motion. Dintenfass (1963) showed that hyaluronic acid could be depolymerized without loss of lubrication of cartilage. The mechanism was not viscosity dependent. McCutchen (1966) showed that the lubricating property of synovial fluid resided with large molecules and suggested that the reaction between molecules and the surface is similar to boundary lubrication. McCutchen (1978) concluded that joints are lubricated by the complementary action of weeping and boundary lubrication.

II. CHARACTERISTICS AND COMPOSITION OF SYNOVIAL FLUID

The characteristics of normal synovial fluid of the dog (Bennett, *et al.*, 1932; Sawyer, 1963), horse (Davies, 1945; Andreev, 1948; Van Pelt, 1962, 1967d, 1974), cow (Bauer *et al.*, 1930, 1933, 1940; Blair *et al.*, 1961; Van Pelt, 1965; Van Pelt and Conner, 1963a,b,c; Preston *et al.*, 1965; Smith and Frame, 1965; Dabich and Neuhaus, 1966a,b), pig (Crimmins and Sikes, 1965), and man (Labor and von Balogh, 1919; Key, 1929; Forkner, 1930; McEwen, 1935; Blair *et al.*, 1961; Shaw and Martin, 1962; Tanner, 1966) provide substantial data on normal mammalian synovial fluid. The availability of large quantities of synovial fluid from cattle for physicochemical studies has extended our knowledge of basic properties of these fluids.

Examination of synovial fluid includes both physical and chemical properties as follows:

1. Physical characteristics: quantity, turbidity, color, specific gravity, clot formation, and viscosity
2. Cell counts and types: total leukocyte count, red blood cell count, and differential cell count
3. Chemical examination: total protein concentration, glucose, enzymes, and isoenzymes
4. Immunological examination: immunoglobulins and complement
5. Bacteriological examination: aerobic and anaerobic culture, mycoplasma culture, chlamydial culture, viral culture, and stains
6. Crystals: phase-contrast, light, and polarized-light examination.

A. Physical Characteristics

Studies on the physical and chemical characterization of synovial fluid indicate a close similarity among mammals, with minor notable exceptions. The cow and man have been studied extensively, the former in part because of availability of synovial fluid, and the latter with emphasis on pathogenesis of joint diseases.

Normal bovine synovial tarsal joint fluid is clear, colorless to straw-colored, viscous fluid which does not clot after standing (Ropes *et al.*, 1939; Van Pelt and Conner, 1963b). According to Van Pelt and Conner (1963a), the volume of synovial fluid obtained from tibiotarsal joints of 77 adult cattle was 12.6 (4–20)* ml. Amrousi and associates (1966) found an average of 15 (12–30) ml of synovial fluid in the tibiotarsal joint of 50 cattle 1–2 years old and 20 (10–29) ml for 60 bulls over 7 years of age. The specific gravity of bovine synovial fluid averages 1.010, with a range of 1.008–1.015. The average content of solids is 2.084% with a range of 1.672–3.886%. The freezing point of the fluid is between −0.509° and −0.556°C. According to Ropes and associates (1939), the average relative viscosity of fluid from the hock joint of cattle at 25°C is 3.72 (2.84–4.15) and is due chiefly to the presence of mucin, a macromolecular acidocglycoprotein which contains hyaluronic acid. Van Pelt and Conner (1963b) obtained an average relative viscosity of 3.79 (1.57–13.01) with an inverse relationship between relative viscosity and volume of fluid; i.e., the highest relative viscosity occurs with the lowest volume. Furthermore, the relative viscosity increased significantly with age of animal. The relative viscosity of human and horse synovial fluid is higher than that for cattle. The average osmotic pressure for bovine synovial fluid against Ringer–Locke's solution is 150 mm water. Albumin and globulin, according to Ropes *et al.* (1939), accounts for only 57 mm water pressure with the remainder of the osmotic pressure due to the mucin. Mucin has a calculated value of nine times the osmotic pressure of albumin. The average pH of cattle fluid is 7.31 (Bauer *et al.*, 1940) and 7.4 (Amrousi *et al.*, 1966), compared to 7.4 for postmortem human synovial fluid (Bauer *et al.*, 1940). The mucin clot quality after precipitation with acetic acid was normal for 92–95% of cattle and fair for remaining animals (Van Pelt and Conner, 1963b; Amrousi *et al.*, 1966).

Horse synovial fluid is a clear, straw-colored, viscous, tenacious, and adhesive liquid which becomes gelatinous (thixotropism) 2–3 hours after its removal but returns to normal consistency on agitation (Muller, 1929; Wheat, 1955; Van Pelt, 1962, 1967d). Van Pelt (1962) examined synovial fluid from radiocarpal, intercarpal, metacarpophalangeal, tibiotarsal, and metatarsophalangeal joints of 29 horses. The total volume obtained averaged 4.2, 6.2, 3.3, 10.3, and 4.6 ml, and the relative viscosity at 37°C averaged 12.4, 16.8, 21.6, 4.4, and 46.7 for the respective joints. The quality of the mucin clot was normal for the majority of specimens (88.5–100%). The liquid did not clot for 48 hours; however, thixotropism occurred occasionally in fluids left unagitated. Rejno (1976a) examined synovial fluid from horse carpal, stifle, and hock joints for rheological properties. The analysis showed that synovial fluids have complex rheological properties. The viscosity varied with the shear rates, except for synovial fluids from joints with infectious arthritis. Rejno (1976a) found that flow anomalies rather than single observations or viscosity values may be representative of different joint diseases.

Sawyer (1963) studied various joints of 46 normal dogs. The volume of synovial fluid obtained from normal articulations averaged 0.24 ml (0.01–1.0). The pH range was 7.0–

*Numbers in parentheses refer to the range of values obtained.

7.8, and the mucin clot quality was normal for most subjects. McCarty *et al.* (1966) found that normal synovial fluid pH from the dog stifle was identical to that of blood. Olson *et al.* (1967) compared the osmotic activity of dog synovial fluid to that of arterial plasma and found no significant differences between means of total osmotic activity. Muller (1929) found a slightly negative pressure in normal human and dog joint cavities, ranging from -2 to -12 cm of water pressure and varying with muscle tone and position. Investigations concerning the viscosity of sheep synovial fluid as well as sedimentation measurements of the hyaluronic acids from various fluids have been made (Fessler *et al.*, 1954). Particle weights for hyaluronic acid from the synovial fluid of sheep were $5–6 \times 10^6$ as compared to 10^7 for the ox and $1–4 \times 10^6$ for human fluid. Preston *et al.* (1965) found molecular weight estimates of hyaluronic acid to vary from $8–10 \times 10^6$ based on sedimentation and viscosity to $12–14 \times 10^6$ by light-scattering methods. The authors suggested the existence of inter and intraspecies differences in specific biochemical properties of synovial hyaluronic acid. Crimmins and Sikes (1965), in a limited number of adult swine, found a volume of 4.0 ml (1.3–9.2) for stifle joints. The pH was 7.069 (7.0–7.2), the specific gravity was 1.015 (1.010–1.018), and normal mucin clot tests were recorded. Normal joints of smaller animals, such as the cat and small dogs, contain small quantities of fluid, and rarely can more than a drop or two be aspirated (Rhinelander *et al.*, 1939; Warren *et al.*, 1935). Hardy and Wallace (1974) described arthrocentesis techniques for the dog and cat.

B. Cellular Composition

1. Total Cellularity

Normal cell counts for synovial fluids of animals (Table I) vary among species, and within species they tend to vary considerably among joints. In general, low counts are the order, and higher counts probably should be log-transformed to account for skewness, thus giving more realistic values for comparison.

Normal cell counts for bovine synovial fluid (Ropes *et al.*, 1939; Warren *et al.*, 1935; Van Pelt and Conner, 1963a; Amrousi *et al.*, 1966) are similar, considering that variation among fluids from different joints exists. In six series of studies of bovine tarsal fluids, an average of 112, 131, 182, 224, 316, and 103 nucleated cells per microliter were reported, and in two series of carpal fluids, 213 and 222 nucleated cells per microliter were observed on average. The range for nucleated cell counts varies among series, particularly at the higher end. Highest counts reported were 575 and 725 cells per microliter. Erythrocyte counts on bovine synovial fluid (Ropes *et al.*, 1939) were 194 (0–1540) per microliter. Average total cell counts in the horse vary greatly from joint to joint (Davies, 1945; Van Pelt, 1962, 1967d). Davies (1945) reported average cell counts (per microliter) as follows: atlantooccipital, 594 (348–1162); elbow, 207 (107–336); knee, 671 (390–1638); radiocarpal, 234 (40–453); and temporomandibular, 983 (412–2350). Van Pelt (1962, 1967d) listed average leukocyte counts (per microliter) for equine synovial fluid as follows: radiocarpal, 124 (25–300); intercarpal, 91 (9–555); metacarpophalangeal, 226 (44–1350); tibiotarsal, 167 (25–464); metatarsophalangeal, 164 (66–611); and tibiotarsal, 79 (11–178). The mean red blood cell count for horse tibiotarsal fluid was 1314 (0–16,500) (per microliter), the high value probably a result of contamination. For dogs (Warren *et al.*, 1935; Sawyer, 1963; McCarty *et al.*, 1966) average cell counts (per microliter) of 963 (327–1450), 430 (0–2900), and 633 (50–2725)

TABLE I

Total Cell Counts and Differential Cell Counts for Synovial Fluid from Domestic Animals

Animal	No. of specimens	Cells/μl		No. of specimens	Differential[a] (%)							Reference
		Total cells	Range		N	L	M	E	Macro	Clasm	Other	
Cattle												
Tibiotarsal	21	90		21	1	1.3	87.0			3.7	7.0	Bauer *et al.* (1930)
Tibiotarsal	25	181.8	55–575	25	2.2	40.1	36.4			15.0	6.1	Warren *et al.* (1935)
Tibiotarsal	15	131	65–250									Ropes *et al.* (1939)
Tibiotarsal	21	194.2	50–316									Davies (1945)
Tibiotarsal[b]	50	224.0	—	50	7.2	47.8	38.4	0.6	6.0			Amrousi *et al.* (1966)
Tibiotarsal[c]	60	316.0	—	60	6.8	46.8	39.8	0.8	5.8			Amrousi *et al.* (1966)
Tarsal	76	103.5	0–725	60	6.0	49.1	38.2	0.8	5.9			Van Pelt and Conner (1963a)
Carpometacarpal	5	170.4	—	5	0.6	1.6	79.8			6.4	6.8	Bauer *et al.* (1930)
Carpometacarpal	12	213.3	100–555	12	2.2	40.1	36.4			15.0	6.1	Warren *et al.* (1935)
Radiocarpal	20	246.2	130–530									Davies (1945)
Atlantooccipital	22	783.9	542–1208									Davies (1945)
Atlantoepistrophial	21	888.4	549–1604									Davies (1945)
Temporomandibular	21	1337.5	800–1756									Davies (1945)
Stifle	41	246.6	86–483									Davies (1945)
Elbow	15	197.3	53–500									Davies (1945)
Horses												
Tibiotarsal	18	192	72–368									Davies (1945)
Tibiotarsal	34	166.8	25–466	23	7.2	32.2	48.1	0.6	4.6			Van Pelt (1962)
Tarsal	14	79	11–178	79	3.0	66.9	27.5	0.2	2.4			Van Pelt (1967d)
Radiocarpal	17	234	50–453									Davies (1945)

Radiocarpal	17	123.8	25–300	11	7.6	48.5	33.2	0.7	9.3			Van Pelt (1962)
Intercarpal	26	91.2	9–555	18	7.6	56.5	31.4	1.0	4.1			Van Pelt (1962)
Metacarpophalangeal	11	266.0	44–1350	7	4.1	33.9	56.9	0.3	4.9			Van Pelt (1962)
Metacarpophalangeal	4	163.8	66–411	4	0.0	54.0	40.5	0.0	5.5			Van Pelt (1962)
Atlantooccipital	5	594	358–1162									Davies (1945)
Atlantoepistrophial	10	534	346–678									Davies (1945)
Temporomandibular	14	983	412–2350									Davies (1945)
Stifle	16	671	390–1638									Davies (1945)
Elbow	12	207	107–336									Davies (1945)
Dogs												
Stifle	14	963.8	327–1450	14	1.7	15.7	68.5			6.5	8.2	Warren *et al.* (1935)
Stifle	58	716	50–2725	42	32.4	28.7	17.7				20.2	McCarty *et al.* (1966)
Average of six[d]	55	430	0–2900	64	1.4	44.2	39.7			4.2		Sawyer (1963)
Swine												
Unspecified	5	220	50–450	5	32	49	17			0	2	Crimmins and Sikes (1965)
Sheep												
Tibiotarsal	24	207.3	81–405									Davies (1945)
Radiocarpal	15	157.5	73–298									Davies (1945)
Atlantooccipital	49	926.9	228–1974									Davies (1945)
Atlantoepistrophial	14	1060.7	728–1960									Davies (1945)
Temporomandibular	13	993.5	347–1478									Davies (1945)
Stifle	16	254.7	113–519									Davies (1945)
Elbow	19	200.1	73–411									Davies (1945)

[a] Differential: N, neutrophils; L, lymphocytes; M, monocytes; E, eosinophils; Macro, macrophages; Clasm, clasmatocytes; other, synovial cells, histiocytes, unclassified cells.

[b] Tibiotarsal of male calves.

[c] Tibiotarsal of bulls.

[d] Carpal, elbow, shoulder, hip, stifle, hock.

for different joints indicate greater variability for this species. Total cell counts in sheep vary considerably from joint to joint. Average values have been reported (cells per microliter) for atlantooccipital (200) and elbow joints (1100) (Davies, 1945). Crimmins and Sikes (1965) recorded nucleated cell counts for swine as 220 (50–450) cells per microliter. An explanation for the apparent differences among nucleated cell counts of synovial fluid taken from various anatomical locations is lacking. However, since the cells involved participate in the inflammatory process, variations among sites may be anticipated even in apparently normal animals. Hasselbacher *et al.* (1978) demonstrated that leukocyte sediment within joint spaces can significantly alter the cell count. Palmer (1968) used hyaluronidase in the initial step of performing total white cell counts. The total cell count was regularly increased by a factor of 2.5 in patients with rheumatoid arthritis. Smaller increases were found in fluids from patients with osteoarthritis. The high-viscosity fluid sticks to the stem of the diluting pipet and is not drawn into the bulb.

2. Cellular Composition

There is considerable variation in terminology concerning cell types among investigators. The term ''clasmatocytes'' has been used to categorize mononuclear cells other than lymphocytes, monocytes, and macrophages. These cells are more appropriately classified as type A and type B cells when arising from the synovial membrane. Both type A and type B cells are known to have phagocytic potential. Bauer and co-workers (1930) used graphite suspension for differential studies on the basis of phagocytosis. Eggers (1959) examined synovial fluid from 20 healthy horses and reported many specific cell types other than those coming exclusively from the bloodstream. Andreev (1948) reported the presence of lymphocytes, monocytes, histiocytes, and epitheloid cells of the synovial membrane in a study concerning the joint fluid of 50 stabled horses. The cell classification used by early workers may be modified to encompass newer knowledge concerning the origin of monocytes and macrophages in body fluids. The lymphocyte is the most numerous cell in synovial fluid (Table I). Both type T and B lymphocytes are found in synovial fluid. Monocytes have their origin in bone-marrow-derived cells. Maturation of monocytes to macrophages is a continuum and dependent upon the need for phagocytic cells. Large mononuclear cells resembling macrophages, except lacking ingestion vacuoles, have been termed histiocytes. The histiocyte classification term has little significance and should be avoided.

Normal synovial fluid from domestic animals should have less than 10% polymorphonuclear leukocytes present; however, the absolute number must also be considered. Studies have revealed that much variation in the total number of cells normally occurs in the mononuclear cell counts. Erythrocytes have been reported as absent or in low number. It is probable that red blood cells are introduced into synovial fluid during the process of aspiration.

The mononuclear cells derived from local tissues may be difficult to identify. The type A synovial lining cells have prominent Golgi complexes but little endoplastic reticulum. The type B synovial cells have a poorly developed Golgi complex and well-developed rough endoplastic reticulum. Reactive macrophages may possess these features, and precise identification is impossible. Aspiration of synovial fluid may include cell clusters directly from the synovial membrane, and features of type A and B synovial cells are more apparent.

Chondrocytes are sometimes found in pathological fluids. The chondrocyte is identified

by location within lacunae, in fragments of articular cartilage. Erosive joint disease may expose bone and release osteoblasts and osteoclasts. The latter cells are multinucleate and may be difficult to distinguish from multinucleate forms of chondrocytes. Osteoblasts resemble the type A cells of the synovial membrane.

C. Chemical Characteristics

1. Normal Synovial Mucin

The lubricating synovial mucin is an acidoglycoprotein, the acid polysaccharide portion of which is hyaluronic acid. Mucin was first prepared by Freichs (1850) by the addition of acetic acid to synovial fluid. By this procedure, the mucin normally separates into a tough, ropey mass. Mucin is precipitated by common protein precipitants, but denaturation results in the polysaccharide moiety becoming partly depolymerized. Mucin can also be salted out with 60% ammonium sulfate. In most methods of serum protein fractionation, it appears in the globulin fraction. Mucin concentrations vary greatly among the joints in the same animal. Van Pelt and Conner (1963b) reported the highest viscosity of synovial fluid in cattle with the smallest volume of fluid. Average concentrations of tarsal and carpal joint mucin have been reported to be 140 and 600 mg/dl, respectively. Human fluids have been found to contain on an average over ten times as much mucin as in synovial fluid from cattle (Fisher, 1929). Ropes *et al.* (1939) reported that only one-eighth of the fluid protein (0.138 gm/dl) is mucin. Freichs (1850) found higher levels (0.326 gm/dl) of mucin in the fluid of newborn calves. Cows maintained in stalls for a long period of time had mucin levels of 0.24 gm/dl; white cattle free to graze on pastures had levels of 0.5 gm/dl mucin in their fluid. von Holst (1904) determined the level of mucin in normal cattle to be 0.5 gm/dl. Mucin nitrogen levels in the bovine astrogalotibial joint (Ropes *et al.*, 1939), bovine carpometacarpal joint (Bauer *et al.*, 1940), and canine carpal joint (Bauer *et al.*, 1930) were 22 (5–40), 96 (63–202), and 43–58 mg/dl, respectively. Carpal fluid from cattle and horses was reported to contain 200 ± 57 and 136 ± 77 mg/dl of hyaluronic acid, respectively (Levine and Kling, 1956). Others reported levels in cattle tarsal joints as low as 20–25 mg/dl (Meyer, 1947). Hyaluronic acid levels in the tempomandibular joint of the horse and knee joint of the rabbit (Sundblad, 1953) were reported to be 56 and 389 mg/dl, respectively. The carpal synovial fluid of the horse exhibits a lower degree of polymerization of the hyaluronic acid, as evidenced by its lower intrinsic viscosity. The lower viscosity of horse synovial fluid as compared to that of cattle may be due to both the lower concentration of hyaluronic acid and the lesser degree of its polymerization. Differences in the concentration and polymerization, and hence viscosity, of hyaluronic acid of synovial fluid vary according to age, species, and the specific joint examined. Fessler *et al.* (1954) suggested that hyaluronic acid differs chemically within species (sheep, man) as well as among species.

Early studies on whether polysaccharides and proteins exist combined as mucin or as independent substances were not clear. Electron microscopic studies of Smith and Frame (1965) suggest that the washed mucin clot contains no appreciable amount of free protein, and it is regarded as a hyaluronate–protein complex. The protein of synovial fluid is of vascular origin (Curtain, 1955). Shaw and Martin (1962) suggested that hyaluronic acid arose from articular cartilage. The studies of Kling *et al.* (1955) of synovial tissue in tissue culture indicated formation of this viscous material. It is now known that the hyaluronate, a glycosaminoglycan, is synthesized by the type B cells lining the synovial

membrane. These molecules are linked covalently with a protein to form very large proteoglycan molecules.

2. *Hyaluronic Acid*

Studies upon the physical and chemical properties of hyaluronic acid in bovine synovial fluid have been quite interesting (Blumberg and Ogston, 1956; Curtain, 1955; Platt *et al.*, 1956; Blair *et al.*, 1961; Smith and Frame, 1965; Preston *et al.*, 1965). Bovine fluids, in addition to containing one-third of the nitrogen concentration of human synovial fluids, also show differing hyaluronic acid components (P_1, P_2, and P_3), which migrate faster electrophoretically than serum albumin. Seven of 11 bovine synovial fluids had only the P_1 component. The other fluids contained P_2 and P_3 components with an absence of the P_1 component (Platt *et al.*, 1956). Preston and associates (1965) described measurements of refractive properties, osmotic pressure, light-scattering, viscosity, sedimentation, and optical rotatory dispersion. Ox synovial fluid hyaluronic acid, prepared by filtration and deproteinized, contained 21% of protein in the ultrafilter residue. Measurements of light-scattering and viscosity suggest that the structure of hyaluronic acid may be some degree of branching and of cross-linkage. Optical rotatory dispersion measurements support a structure less simple than a linear random coil. Preston *et al.* (1965) found hyaluronic acid to contain glucosamine (1.51–1.90 mEq/gm, total acetyl (1.76–2.31 mEq/gm), *O*-acetyl (0.04–0.05 mEq/gm), sialic acid (0.049–0.052 mEq/gm), glucuronic acid (1.98–2.59 mEq/gm), total nitrogen (3.74–5.91%), and ash (3.2–12.9%). A comparison of data on hyaluronic acids has been compiled by Preston *et al.* (1965). Synovial fluid hyaluronic acid from cattle is composed as follows: (1) percent recovery, 95.7–97.5; (2) acetyl-glucosaminyl, 36.4–46.9%; (3) glucuronyl, 36.6–45.5%; (4) protein, 3.0–21.9% and about 1.4–2.1% based on grams per 100 gm dry weight.

Trichloroacetic acid treatment of synovial fluid gives higher hyaluronic acid values than pretreatment with protease (Marsh *et al.*, 1976). Protease treatment may remove small molecular weight components of blood such as glucose which enchance peaks obtained on spectra analysis.

The chemical structure of hyaluronic acid is a linear nonsulfated polysaccharide composed largely of 2-acetamido-2-deoxy-3-*O*-β-D-glycopyranosyluronic acid–D-glucose linked by 1,4-β-glycosidic linkages (Meyer, 1958; Jeanloz, 1956). The levels of organization or types of extracellular hyaluronic acid have been defined (Swann, 1978). Type I has a molecular mass of up to 12×10^6 and is a macromolecular hyaluronic acid complex composed of polysaccharide chains with a tertiary and quaternary structure involving interaction with adjacent chains and/or other matrix constituents. Type II hyaluronic acids have a molecular weight of $1\text{–}1.5 \times 10^6$. The polysaccharide chains have a random coil configuration. Type III hyaluronic acids has a molecular mass of $2\text{–}8 \times 10^4$. It is produced by treatment of type II with ascorbic acid and is the type found in normal bovine vitreous fluid (Sundblad and Balazs, 1966). The viscosity of hyaluronic acid is dependent upon three major factors: the length of the polysaccharide chains, the structure of these chains, and the interaction of adjacent chains and molecules. There is little or no direct evidence to indicate that the viscosity drop in altered hyaluronic acid is due to a depolymerization (Swann, 1978). Considerable data exist to indicate that the polysaccharide chains can interact with other matrix components (Hardingham and Muir, 1972; Hascall and Heinegard, 1974; Swann *et al.*, 1976). Hyaluronic acid has the potential of assuming ordered stacking arrangements (Sheehan *et al.*, 1975). From these studies it appears that

the hyaluronic acid macromolecules in synovial fluid are not composed of linear polysaccharide chains (n = 25,000) but linear structures (n = 2500) organized with specific interactions to form macromolecules (Fessler and Fessler, 1966). Hyaluronic acid is synthesized by the B-type lining cells of the synovial membrane.

3. *Glycoproteins*

Coagulation of synovial fluid mucin with weak acid in the clot test leaves a fluid with viscosity similar to that of water. Dintenfass (1963) showed that hyaluronic acid could be depolymerized without loss of lubricating ability of cartilage. The mechanism was not viscosity dependent. Linn and Radin (1968) demonstrated this experimentally in canine tarsal joints using bovine synovial mucin treated with hyaluronidase to lower the viscosity. Tryptic digestion destroyed the ability to lubricate without reducing the viscosity. A glycoprotein with lubricating ability has been isolated (Radin *et al.*, 1970).

The glycoproteins have been characterized (Swann and Radin, 1972; Swann *et al.*, 1977). The lubricating fraction isolated contained three recognizable components: lubricating glycoprotein 1 (LGP-1), lubricating glycoprotein 2 (LGP-2), and a small amount of protein similar to γ-globulin.

Swann *et al.* (1977) analyzed purified LPG-1 and showed that it was composed of approximately equal quantities of amino acid and carbohydrates with a molecular mass of 227,500. The second glycoprotein (LPG-2) has a relative molecular mass of 70,000 daltons based on motility. The amino acid composition and electrophoretic characteristics are similar to those of a glycoprotein present in articular cartilage (Swann *et al.*, 1975).

Immunological studies have shown the presence of a new component (''new component'') in human synovial fluid (Sandson, 1967). ''New component'' is a normal constituent of synovial fluid and is synthesized by cells of the synovial membrane. Immunological studies indicate cross-reaction with species-specific component of proteoglycan and a glycoprotein-link fraction (Keiser and Sandson, 1974).

Detailed studies correlating the lubricating ability of synovial fluid fractions with chemical composition have been performed only with normal bovine synovial fluid fractions. A specific glycoprotein, LGP-1, appears to be uniquely involved in the lubrication of articular cartilage (Swann, 1978).

4. *Proteins*

Bovine synovial fluid was reported to have an albumin/globulin ratio of 1.21 ± 0.02 with the presence of albumin, α_{-1}-, β-, and γ_{-2}-globulins. A few fluids contained α_{-1}- and α_{-2}-globulins (Platt *et al.*, 1956). Eggers (1959) reported averages in the synovial fluid of ten normal horses (%) as follows: albumin, 39.5 ± 4.47; α-globulins, 10.5 ± 2.3; β-globulins, 21.3 ± 4.2; and γ-globulins, 28.7 ± 4.0. Prior to the separation of proteins electrophoretically, albumin/globulin ratios as high as 3.9 : 1 were reported (Butler and Montgomery, 1932). Van Pelt (1967d) recorded total synovial fluid protein for the horse as 1.37 gm/dl (0.83–2.17), and albumin/globulin ratio 3.9 (0.31–8.06), by a modified biuret procedure. Amrousi *et al.* (1966) found bovine synovial protein (0.9 gm/ml) to be less than that for horses and considerably less than that for man. The average protein concentration, excluding mucoproteins, in cattle fluid was reported to be 0.88 gm/dl (Bauer *et al.*, 1940). Fisher (1929) indicated that nonmucin protein levels in cattle were on an average 0.92 gm/dl. Total protein levels for equine carpal fluid (Bywaters, 1937) and knee joint fluid in the rabbit (Sundblad, 1953) were reported to be 1.4 (0.88–1.95)

and 3.6 gm/dl, respectively. Synovial fluid protein for swine was recorded as 3.9 (3.1–5.4) gm/dl (Crimmins and Sikes, 1965) higher than the normal for man (Neuhaus, 1962).

Except for a few, the protein constituents are derived directly from plasma, and the relative proportions of the constituents depend on the molecular weight of the protein and the vascular permeability of the synovial membrane. Schur and Sandson (1963) conducted immunoelectrophoretic studies of proteins found in human synovial fluid and found that all proteins identified were present in plasma. Kushner and Somerville (1971), using a radioimmunodiffusion technique, studied the relationship of serum proteins present in synovial fluid and found that the ratio of the concentration of α_1-glycoprotein, transferrin, ceruloplasmin and α_2-macroglobulin in synovial fluid to that in serum was directly related to the molecular weight. They studied these proteins in rheumatoid arthritis and found a threefold increase in the concentration of ceruloplasmin, α_1-acid glycoprotein, and transferrin compared to a tenfold increase for α_2-macroglobulin. The ratio of the synovial fluid proteins to plasma proteins was determined in patients with osteoarthritis, rheumatoid arthritis, and gout. The authors concluded that the transfer of plasma proteins to synovial fluid was quantitatively related to the size and shape of the molecule and the degree of inflammation. Stone and Deyoe (1974) compared the serum and synovial fluid proteins and immunoglobulin levels against *Brucella* in calves before and after colostrum feeding. They found the total protein of serum to be considerably higher than that of the synovial fluid. The serum protein did not correlate with synovial protein levels. A good correlation existed between *Brucella* antibody titer of serum and synovial fluid after the feeding of colostrum. The data of Kushner and Sommerville (1971) indicate that the mechanisms regulating the passage of plasma proteins are insensitive to molecular mass changes below 100,000. When permeability is altered by inflammatory change, the synovial fluid protein constituents depend on the degree of alteration and the size and shape of high molecular mass proteins. A mechanism for immunoglobulin accumulation in synovial fluid has been demonstrated (Smiley *et al.*, 1968) to occur by *in situ* production in the synovial membrane.

Another method that may influence the type and quantity of plasma protein found in synovial fluid is alteration through modification and/or catabolism as the proteins pass through the synovial membrane. Lunney and Ashwell (1976) showed that modification of glycoprotein by removal of the terminal sialic acid increases hepatic uptake. A selective uptake of glycoprotein by synovial cells may account for variability in the concentrations of glycoproteins (Sundblad, 1965).

The enzymes of synovial fluid studied by West *et al.* (1963) are lactate dehydrogenase (LDH), aldolase, phosphohexase, isomerase, malate dehydrogenase, isocitrate dehydrogenase, glutathione reductase, glutamic pyruvic transaminase. In addition, diatase, lipase, and protease were detected in synovial fluid (Podkaminsky, 1931). The enzyme levels of synovial fluid are lower than corresponding serum enzyme levels and correlate with leukocyte number, suggesting origin from the latter. However, enzymes of small molecular weight may enter directly from plasma or be produced locally. Van Pelt (1967d) reported horse tibiotarsal synovial fluid enzymes as follows: alkaline phosphatase, 1.02 (0.28–2.05) Sigma units/ml; acid phosphatase, 0.09 (0.00–0.27) Sigma units/ml; LDH, 44 (0–94) LDH units/ml; aldolase, 9 (1–29) Sibley-Lehninger units/ml; glutamic oxaloacetic transaminase, 26 (8–60) Sigma-Frankel units/ml; and glutamic pyruvic transaminase, 10 (0–23) Sigma-Frankel units/ml. Comparison with serum levels reveals significantly lower values for all enzymes except for aldolase.

Alkaline phosphatase activity has been reported to be high in human synovial fluid

(Barnett *et al.*, 1961). Van Pelt (1967d) reported lower than serum levels of alkaline phosphatase for horse synovial fluid. Dabich and Neuhaus (1966a,b) studied the origin of alkaline phosphatase in bovine synovial fluid since the specific activity is often 100 times higher than the serum value (Bauer *et al.*, 1940). Dabich and Neuhaus (1966a,b) concluded that the synovial enzyme differs from the serum enzyme in electrophoretic motility and is similar to the enzyme of cartilage and bone extracts.

Timoney (1976) studied synovial fluid of pigs and found the following: lysozyme, 5.79 ± 0.9 μg/ml; acid phosphate (ACP), 0.41 ± 0.7 Sigma units/ml; and LDH, 515 ± 38 LDH units/ml. Timoney (1976) concluded that LDH, a cytoplasmic enzyme, was increased in proportion to ACP, indicating that cell death was an important mechanism of enzyme release in arthritis joints. Lysozyme activities were often increased above serum levels, indicating a special role in the joint fluid.

Rejno (1976b) reported on LDH and LDH isoenzymes of synovial fluid in the horse. Synovial fluid from clinically healthy joints had a mean total LDH activity of 88 (50–109) U/liter. The total LDH activity [88 (50–109) U/liter] was found to consist of LDH_1 [47 (29–69) U/ml], LDH_2 [17 (18–32) U/ml], LDH_3 [31 (10–40) IU/ml], and to a small extent LDH_4 [1 (0–9) U/ml]. Synovial membrane examined contained mainly LDH_2 (11%), LDH_3 (48%), LDH_4 (38%), and in two of five animals a small amount of LDH_5 (3%). Articular cartilage contained LDH_3 (29%), LDH_4 (48%), and LDH_5 (22%). Erythrocytes in the material contained LDH_1 (71%) and LDH_2 (29%), while leukocytes contained LDH_2 (9%), LDH_3 (38%), LDH_4 (37%), and LDH_5 (16%). Plasma levels were LDH_1 (18%), LDH_2 (27%), LDH_3 (43%), LDH_4 (11%), and LDH_5 (1%). The LDH levels of synovial fluid increased over companion serum levels even in mildly diseased joint fluids.

Fibrinogen and most coagulation factors are not present, and normal synovial fluid characteristically does not clot. Proteins of the plasmin system may be found in variable quantity.

Complement can be measured in synovial fluid. The complement in normal synovial fluid is usually 10% of the companion serum level for man (Yehia and Duncan, 1975). In inflammation, the level of complement activity parallels the degree of inflammation, as reflected by the total leukocyte count. In rheumatoid arthritis, the synovial fluid complement level is low, reflecting utilization (Pekin and Zvaifler, 1964). The complement level in synovial fluid of systemic lupus erythematosus (SLE) in man is low, similar to that of rheumatoid arthritis. Studies of complement in synovial fluid of rheumatoid patients indicate complement levels to be of prognostic significance (Bunch *et al.*, 1974).

5. *Electrolytes*

The concentration and distribution of synovial fluid electrolytes agree with those expected from the Gibbs–Donnan theory of membrane equilibrium. The values obtained from cattle fluid agree, in general, with studies of "in vivo dialysates" (Greene and Power, 1931) and also with values reported for lymph, edema, and ascitic fluid.

Values for synovial fluid chloride and bicarbonate are slightly higher than those for serum, while the concentrations of magnesium, sodium, calcium, and potassium are slightly lower in synovial fluid. Total inorganic phosphate is equal in both fluids. Distribution ratios for cattle synovial fluid are as follows (fluid/serum): Cl, 1.01; HCO_3, 1.065; PO_4, 1.00; Na, 0.93; K, 0.75; Ca, 0.83; and Mg, 0.88. Theoretical ratios for Donnan's equilibrium would be near 0.93 for electrolytes. The average ratio for total anions of serum to total anions of synovial fluid is 0.99 (Bauer *et al.*, 1940). Calculations concerned with the ratios of ionic calcium in the synovial fluid to that in the serum give values

on an average of 1.18. This ratio is obviously higher than would be expected from membrane equilibration phenomena and is most likely due to the high base-combining power of the synovial mucin.

The theory that synovial fluid is a dialyzate of blood plasma explains many of its physical and chemical properties. The presence of serum albumin and globulins, which are derived from capillary permeability to protein, and of mucin, which is produced most likely from adjacent connective tissue, in no way invalidates this concept.

6. *Nonelectrolytes*

The distribution of nonelectrolytes has been found to be similar to that found in a dialyzate of blood plasma. Urea concentrations in cattle fluid (8.2 mg/100 ml) are on an average the same as in serum (8.5 mg/100 dl). This would be expected if the membrane of separation were permeable to urea (Bauer *et al.*, 1940). Distribution ratios of the total nonprotein nitrogen (0.87) and uric acid (0.84) between the synovial fluid and serum of cattle are somewhat lower but are still of the same general magnitude. Similar distribution ratios have been found in the synovial fluid of horses (Hare and Cohen, 1929). It can be assumed that the average distribution ratios for most nonelectrolytes are only slightly under 1.00, since in individual cases their concentration in serum and synovial fluid has been found to be nearly equal.

Average glucose concentrations of bovine synovial fluid have been found to be slightly lower than the serum glucose levels. Occasionally, however, samples approach a distribution ratio of 1.00. Bauer *et al.* (1940) suggested that the struggling prior to sampling could easily elevate the blood glucose concentration from hepatic glycogenolysis, and insufficient time would lapse for its equilibration between plasma and the synovial fluid. Nonfasting conditions could also account in part for any differences in the blood and synovial glucose concentration.

Van Pelt and Conner (1963a) studied the blood sugar level of tarsal fluid from cattle on full feed and slaughtered cattle from which feed had been withheld for 18 hours. With cattle on full feed, no significant differences among blood, plasma, or synovial fluid sugar levels were noted, nor among bulls, cows, and steers. However, with the slaughtered fasted cattle, blood plasma and synovial sugar levels were greater than those of the nonfasted group. In addition, plasma sugar levels for cows were significantly greater than for bulls and steers. Van Pelt (1967d) reported horse synovial fluid levels similar to those of blood; however, the range in synovial fluid sugar levels [70 (33–105) mg/dl] is great. Bacterial joint infections and increased cell counts are responsible for low concentrations of monosaccharides in the joint fluid from their glycolytic activities. The absence of such reducing substances in infectious joint effusions is a common finding. Owen (1978) found that synovial fluids with leukocyte counts greater than 50,000 cells per microliter reduced the synovial fluid to 30 mg/dl or less within 1 hour. While low levels may imply infectious arthritis, in immune-mediated arthritis, it reflects glycolytic activity by leukocytes.

III. EXAMINATION OF SYNOVIAL FLUID

Laboratory examination of synovial fluid may include the following:

1. Physical characteristics: volume, appearance, clot formation, and viscosity

2. Cell counts and types: total leukocyte count, red blood cell count, differential cell count, and morphological study
3. Chemical examination: total protein concentration, glucose, enzymes, and isoenzymes
4. Immunological examination: immunoglobulins and complement
5. Bacteriological examination: aerobic and anaerobic culture, mycoplasma culture, chlamydial culture, viral culture, and stains
6. Crystals: phase-contrast, light, and polarized-light examination

A. Arthrocentesis

Synovial fluid may be obtained by needle aspiration from many diseased joints even though there may not be much clinical evidence of effusion. Complete sterile technique is critical not only to avoid possible infection but to have sterile specimens to culture. Knowledge of volume requirements for various laboratory procedures is necessary prior to arthrocentesis so rational judgment may be made in determining the priority of tests. This is important with small joints, in which the total volume of synovial fluid obtained may be of the order of 1 ml or less. When small quantities of synovial fluid are obtained, the nature of the disease process suspected will invariably indicate the order of tests to be done (Hardy and Wallace, 1974).

Aseptic technique must be used in withdrawal of synovial fluid to guard against contamination of the joint cavity and the sample for microbial study. The skin, subcutaneous tissues, and joint capsule should first be infiltrated with a local anesthetic. The dog and cat are usually restrained with a combination of products producing analgesia and sedation or a short-acting barbiturate.

The needle size must conform to the joint to be sampled. An 18- to 20-gauge needle is advised. A needle with large bore will plug less readily with thick exudates. The use of short-beveled needles with a stylet is recommended. The syringe size should relate to the amount of fluid anticipated. A large-volume syringe should be avoided with small-quantity specimens. Synovial fluid should always be collected in an anticoagulant appropriate for the determination. Ethylenediaminetetraacetate (EDTA), 1 mg/ml, is preferable for leukocyte studies but may interfer with biochemical, immunological, and mucin clot test analysis. Heparinized samples are needed for biochemical and immunological tests. When small-volume specimens (less than 0.2 ml) are aspirated, glass slide smears should be made for morphological study.

B. Physical Characteristics

Gross examination of synovial fluid frequently indicates the nature of the pathological process altering the synovial joint. The selection of the most important tests may be best made on the basis of gross examination of the synovial fluid and related clinical findings.

1. Quantity

The quantity of synovial fluid from any joint generally varies directly with the size of the joint and its communication with another joint. Normal synovial joints of the dog yield, on the average, 0.24 ml of fluid (Sawyer, 1963). Van Pelt and Conner (1963a) obtained 12.6 (4–20) ml from the tibiotarsal joints of cattle. Van Pelt (1962) examined

synovial fluid from several joints of horses, and the total volume ranged from 3.3 to 10.3 ml.

The volume of synovial fluid is not appreciably increased in many forms of degenerative joint disease (Van Pelt, 1974). In inflammatory conditions, the amount of fluid present in the affected joint is generally at a maximum when signs of inflammation are at their height. This is not true for all cases, as active inflammation may be accompanied by a small effusion in a large joint.

2. *Appearance*

The gross appearance of normal synovial fluid is clear, pale yellow, highly viscous, and free of flocculent material. The pale yellow color varies with the species of animal. When print cannot be read easily through the fluid, the effusion is cloudy, suggesting an inflammatory process.

Degenerative joint disease synovial fluid samples may vary from pale yellow and clear to pale yellow and opaque. The opacity is due to floccuent material, and markedly flocculent fluids may be associated with cartilage degeneration. In acute traumatic joints, the sample may be altered with blood. Streaking of the sample with blood is generally associated with hemorrhage resulting from capillary damage on aspiration. Cytological examination of such samples to demonstrate platelets may be helpful in distinguishing hemorrhage that occurred prior to aspiration from needle puncture since platelets are invariably absent from normal and disease-related samples.

Dark yellow or pale amber fluids result from chronic hemorrhage and subsequent erythrocyte breakdown and formation of bilirubin compounds. Turbid samples of varied color are most commonly associated with inflammatory joint disease, which may be septic or nonseptic.

3. *Clotting*

Normal synovial fluid does not clot. The fluid exhibits thixotrophy on standing; however, the samples return to their normal liquid state on shaking. Synovial fluid from degenerative joint disease may not clot. The synovial fluid from a traumatic and inflammatory joint disease usually clots rapidly, even in the syringe during aspiration.

4. *Mucin Clot Test*

The mucin clot formed in weak acid is a reliable indicator of synovial fluid hyaluronic acid polymerization and a quantitative measure of its concentration in synovial fluids. The test is accomplished by carefully adding 1 part synovial fluid to 4 parts 2.5% glacial acetic acid. The total volume may be varied for small samples. The clot formed is characterized immediately, although Van Pelt (1974) recommended that the tube be left standing at room temperature for 1 hour before reading.

On the basis of the characteristics of the clot (Ropes and Bauer, 1953), the results are graded as good, fair, poor, and very poor. If a compact, large clot forms surrounded by clear solution with normal synovial fluid, this is graded as good. If a soft clot forms in turbid solution, this is graded as fair. A friable clot with cloudy surrounding fluid is graded as poor. No clot formation with flakes in a cloudy suspension is graded as very poor. The tube or vessel should be agitated to observe the character of the clot. Poor clots tend to break up, while good clots remain in tight masses. The mucin clot test characterizes the quality and quantity of hyaluronic acid in synovial fluid.

Poor-quality clot formation is the result of depolymerization of the hyaluronic acid and is equated to the degree of inflammation, a concept that is misleading. Poor-quality clot formation results from hyaluronidase activity from pathogenic bacteria. It is not a reflection of total leukocyte number since good-quality clots have been observed in synovial fluids containing more than 100,000 neutrophils per microliter.

It has been reported that EDTA causes a decrease in synovial fluid viscosity by degradation of the hyaluronic acid component of mucin (Ogston and Sherman, 1959). The mucin clot test should be performed from the sample in the collection syringe.

5. Viscosity

Normal synovial fluid is viscous, and, when allowed to drop from a needle, the drop will have difficulty separating from the point of the needle, and a long connecting strand will result. Viscosity is a function of the concentration and quality of hyaluronic acid. Viscosity can be determined by manipulating a few drops of fluid between thumb and forefinger. When separated, a connecting strand of fluid several inches long will be seen with normal hyaluronic acid. Hasselbacher (1976) proposed measuring relative viscosity with the use of a white blood cell diluting pipet as a viscometer. The technique is reproducible with 2% accuracy. Perhaps the greatest significance of viscosity determination is to provide a basis for the use of hyaluronidase to decrease the synovial fluid viscosity before other tests are performed (Schumacher, 1977) in which high viscosity interfers.

C. Cell Counts and Types

1. Total Cell Counts

The cells in synovial fluid, undiluted or diluted with physiological saline, are counted. An acid diluent precipitates mucin in the field. The cells are best counted under high-power magnification, and the number of fields counted should be sufficient to insure accurate counts. In obvious exudative conditions, prior examination of a stained smear will usually indicate whether cell counts are practical on the basis of excessive numbers, clumping, clots, and degenerative cellular changes.

Leukocyte counts have been made with particle counters (Liberg *et al.*, 1977). Erythrocyte and leukocyte counts performed with particle counters are subject to great error when counts are in the normal range. Hemocytometer counts should be made on low-cellularity synovial fluids.

Palmer (1968) reported that total leukocyte counts made on pathological synovial fluid with high viscosity were subject to great variation. Using 0.1% methylene blue in 0.3% sodium chloride solution, a central stream of colored diluent and a layer of clear synovial fluid adherent to the internal stem was seen with viscuous fluids, indicating incomplete mixing. Pretreatment with hyaluronidase increased the leukocyte count by a factor of 1.5–3.9 in synovial fluids from rheumatoid arthritis patients. Significant differences were noted in samples from patients with osteoarthritis. The 0.3% saline solution is used to lyse erythrocytes.

2. Differential Counts

Coverglass smears are preferred to glass slides since the fluid films are thinner. Slow smearing of glass slide films gives rise to satisfactory smears. Wright's or Giemsa stains

are practical. New methylene blue stain on dried film (Schalm, 1961) gives excellent tinctorial properties to cells. Fibrin strands and alcohol-soluble crystals are clearly seen with the use of the water-soluble new methylene blue stain. Sabin (1923) used supravital stains to differentiate the various mononuclear cells. As mentioned earlier, there is variation in terminology concerning cell types among investigators. The term "clasmatocyte" has been used to classify mononuclear cells other than lymphocytes, monocytes, and macrophages (Table I). Identification of these cells as to origin from synovial membranes, articular cartilage, and bone has not always been possible in routine cytological study. In light of recent knowledge of the origin of macrophages of synovial fluid, most mononuclear cells of synovial fluid are derived from bone marrow cells and classified as lymphocytes, monocytes, or macrophages (Yehia and Duncan, 1975; Currey and Vernon-Roberts, 1976; Schumacher, 1977).

In degenerative joint disease, the most significant change may be the proportion of mononuclear cells with phagocytic vacuoles. The number of phagocytic cells increases in fluids left standing for even short periods; hence, preparation of smears for cytological study should be made at the time of collection.

Morphological study of cells and cytoplasmic inclusions is useful for the clinical characterization of a disease process. The high leukocyte counts of immune-mediated polyarthritis are characterized by normal-appearing mature neutrophils. In septic arthritis, degenerative changes in neutrophils characterized by karolysis, pyknosis, and unusual clumping are more common. In untreated patients, microorganisms are readily demonstrated with common blood stains.

The terms "ragocyte," "inclusion body cells," and "RA cells" denote neutrophils examined as unstained wet preparations and containing numerous dark intracytoplasmic granules 0.5–1.5 μm in diameter (Hollander *et al.*, 1965). These were initially thought to be quite specific for rheumatoid arthritis but are now found in various disease conditions. Immunofluorescent studies (Zvaifler, 1973) have shown these intracellular inclusions in neutrophils to be combinations of IgG, IgM, complement, and rheumatoid factor in various proportions.

In Wright's-stained smears of dog synovial fluid, intracytoplasmic DNA particles and LE cells have been illustrated in SLE polyarthritis. Hunder and Pierre (1970) found LE cells in several cases of SLE in man. The neutrophils with DNA particles have been described in man (Pekin *et al.*, 1966) and correlated best with the presence of rheumatoid factor and long-standing, moderately advanced rheumatoid arthritis. Miller *et al.* (1974) illustrated neutrophils containing particulate DNA and LE cells in the effusions of dogs with systemic lupus polyarthritis. The neutrophils with particulate DNA were erroneously termed ragocytes.

In nonseptic joint effusions, the neutrophils found are mainly intact, healthy cells. Cells with pyknotic nuclei are present. The intact neutrophils may contain lipid vacuoles, a feature common to effusions of immune-mediated joint diseases.

Lymphocytes are found in normal synovial fluid and in effusions of degenerative and immune-mediated joint diseases. The synovial fluid of rheumatoid and systemic lupus arthritis contain T and B lymphocytes and other subpopulations (Utsinger, 1975). Blastogenesis of lymphocytes is not uncommon in these effusions. Currey and Vernon-Roberts (1976) indicated that no useful diagnostic information can be obtained by classifying the lymphocytes.

Identification of chondrocytes, osteoblasts, or osteoclasts may indicate erosive cartilage and exposed osteoid.

Differential cell counts may be of value when the total cell count is elevated. When a differential cell count is predominantly neutrophils, examination for microorganisms and culture are indicated. In the dog, rheumatoid and systemic lupus polyarthritis total and differential cell counts may be similar to those in infectious arthritis.

3. Erythrocytes

Erythrocytes are present in all synovial fluids to various degrees. Contamination of fluids with blood at arthrocentesis accounts for the high number found in some cases of degenerative joint disease. Acute traumatic joint disease and inflammatory joint disease are accompanied by varied numbers of erythrocytes. The presence of a high number of erythrocytes, such as in hemarthrosis, should arouse suspicion of congenital or acquired bleeding or coagulation disorders.

4. Cytological Study

Most cytological studies of animal synovial fluid cells has been made with Wright's type of staining. Criteria for the specific diagnosis of several arthrites of man, on the basis of exfoliative cytology and the Papanicolaou staining method, have been developed (Broderick *et al.*, 1976; Naib, 1973). The synovial fluid remaining after other tests are performed is spun at 2000 rpm for 20 minutes. The sediment is streaked, wet-fixed, and stained by the Papanicolaou method. Synovial fluid concentrate smears can be made with a centrifuge designed for the preparation of cytology specimens. Thin smears with clear cellular features are a distinct advantage in cytological examination.

Techniques have been reported for biopsy of the synovial membrane (Hardy and Wallace, 1974; Van Pelt, 1967b). Cytology and histology of synovial membranes prove useful for categorizing the nature of the inflammatory response.

D. Biochemical Examination

1. Protein

The concentration of synovial fluid protein derived from plasma is a function of molecular size of the protein, its concentration in plasma, and the permeability of the vascular. The synovial fluid protein may vary with age, sex, and activity of the animal (Persson, 1971). As synovial protein is derived chiefly from plasma and may vary greatly from joint to joint, the serum and synovial proteins should be examined in parallel and ratios calculated for diagnostic purposes (Liberg *et al.*, 1977).

The biuret method is used for synovial fluid protein determination. The refractive index of synovial fluid gives an estimate of protein concentration. The electrophoretic pattern of synovial fluid protein differs from that of serum protein due to the semipermeability of the synovial membrane. Hyaluronic acid in the perisynovial tissues has an exclusion effect on protein normally found in plasma (Nettebladt *et al.*, 1963; Laurent *et al.*, 1963).

2. Enzymes

Enzyme determinations of synovial fluid have been studied (Van Pelt, 1974; Rejno, 1976b; Barthel *et al.*, 1971) for diagnostic purposes. The most commonly studied en-

zymes are alkaline phosphatase, LDH, and glutamic oxaloacetic transaminase (GOT/AST). The levels of enzymes arise from tissue damage to joint structures, plasma, and cellular constituents in the inflammatory joint. Total leukocytes and erythrocyte counts should be made in parallel with enzyme studies.

Rejno (1976b) studied the isoenzymes of LDH in normal and diseased joint fluids. The large amount of LDH_3 isoenzyme in articular cartilage, when compared to erythrocytes, white blood cells, and plasma, provides a useful measure of joint disease. Rejno (1976b) demonstrated differences between healthy and diseased joint fluids in a horse with arthrosis by determination of LDH isoenzymes.

3. Glucose

In normal synovial fluid, the glucose level is approximately equal to the glucose level in serum. The glucose level in synovial fluid in noninflammatory joint diseases is essentially the same as or up to 10 mg/dl lower than that in serum. High cellularity and glycolysis on standing may account for marked reductions in synovial fluid glucose. Thus, low or zero glucose level is not necessarily evidence of bacterial inflammation.

4. Acidity

Ward and Steigbigel (1978) determined a close correlation between decreasing pH and increasing white blood cell counts. The low pH of septic joint fluid with high leukocyte number may contribute to the poor response of gram-negative septic arthritis treated with aminoglycoside antibiotics. Brook *et al.* (1978) measured synovial fluid lactic acid by gas–liquid chromatography and found diagnostically elevated levels in septic arthritis except for gonococcal infections.

E. Immunological Examination

Immunological tests carried out on serum can equally well be applied to synovial fluid (Currey and Vernon-Roberts, 1976). Complement, rheumatoid factor, antinuclear factors, and LE tests have been conducted on synovial fluids (Zvaifler, 1975). There appears to be no distinct advantage of conducting immunological studies on synovial fluid since similar findings are obtained from studies of serum. An exception may be with complement, low levels of which are found in rheumatoid arthritis and systemic lupus erythematosus polyarthritis (Pekin and Zvaifler, 1964).

F. Microbiological Examination

Microbiological examination follows the standard principles of staining and culture applicable to biological fluids. When an infected agent is suspected, every effort must be made to obtain an adequate sample of synovial fluid prior to initiation of antimicrobial therapy.

Smears of synovial fluid should be examined immediately to define whether the cellular constituents are consistent with a microbial etiology and for morphological study of organisms which may be present. Aerobic and anaerobic cultures should be made whenever possible. Mycoplasma and viral isolation studies are required for completeness of the microbial examination.

G. Crystals

Various crystals have been identified in synovial fluid, and crystal-induced synovitis has been described for man. Both gout and pseudogout are related to the presence of crystals within the joint, and crystal identification is considered pathognomonic of each disease. Cholesterol-rich synovial effusions have been reported for man (Meyers and Watermeyer, 1976). Corticosteroid intraarticular injections may result in multiple crystal forms, depending on the corticosteroid preparation. Other crystals that may be found are calcium phosphate crystals associated with calcific lesions.

The demonstration of crystals in synovial fluid is accomplished by the use of wet-mount preparations of synovial fluid or water-soluble stains. Ordinary light microscopy with proper light adjustment for viewing is adequate for the demonstration of crystals. Phase microscopy is superior to ordinary light microscopy for the demonstration of crystals.

Identification of crystals is accomplished by the use of the polarized-light microscope (McCarty *et al.*, 1961). The optical principles involved have been described (Phelps *et al.*, 1968; Fagan and Lidosky, 1974). The crystals are examined for size and shape. The angle of extinction and the sign of birefringence are determined for precise identification of crystals.

Techniques are available for precise chemical analysis of crystals and particulates found in biological fluids. Crocker *et al.* (1977) described methods for correlating light microscopy with electron microscopy and X-ray energy spectroscopy for positive identification of crystals of calcium pyrophosphate dehydrate and hydroxyapatite in synovial fluids. Gaucher (1978) conducted microcrystal analysis in synovial fluids by scanning electron microscopy and X-ray diffraction.

IV. PATHOLOGICAL CHANGES IN SYNOVIAL FLUID

A. Classification

The classification of synovial fluids into groups (Bauer, *et al.*, 1940; Ropes *et al.*, 1941, 1947; Van Pelt, 1965) has been used for man and animals. The group classifications are broad, with some diseases showing characteristics of more than one group. Such a classification is as follows: group I: fluids produced by effusion from traumatic origin; group II: intermediate fluids; group III: septic fluids (infectious arthritis).

Pedersen *et al.* (1976b) proposed a classification for arthropathies of the dog. The comprehensive nature of the classification serves a useful clinical purpose in evaluating arthritis and is based on etiological mechanisms. Parts of this scheme were used to form the following classification (V. Perman, L. J. Wallace, and A. J. Lipowitz, unpublished observations). This classification divides synovial fluid types into broad groups based on characteristics used in evaluating the composition of normal synovial fluid.

Type I. Normal
Type II. Inflammatory–Nonpurulent
- A. Degenerative joint disease
- B. Traumatic joint disease
- C. Neoplastic joint disease

Type III. Inflammatory–Purulent
- A. Noninfectious
  1. Immunological
  2. Idiopathic
  3. Crystal-induced
  4. Chronic hemarthrosis
  5. Neoplastic
- B. Infectious
  1. Bacterial
  2. Mycoplasmal
  3. Viral
  4. Fungal
  5. Protozoal

Synovial fluids in specific diseases may exhibit characteristics of more than one classification group. Changes in the course of a disease may be associated with significant alterations in the composition of synovial fluid.

B. Normal Synovial Fluid

Table I lists total leukocyte counts and differential cell counts on synovial fluid from domestic animals. Mean total counts in different species of greater than 500 cells per microliter are reported (Davies, 1945; McCarty *et al.*, 1966; Warren *et al.*, 1935). The high synovial fluid leukocyte counts lack explanation, except for origin from synovial joints of certain anatomical areas. Clinical laboratory studies of normal synovial fluids of animals taken from synovial joints of the limbs usually have total leukocyte counts of a few hundred cells or less, and seldom do the counts exceed 500 cells per microliter. The leukocytes are mainly lymphocytes, monocytes, macrophages, and unspecified mononuclear cells (Figs. 1, 2, 3). Neutrophils form a small population in normal synovial fluid, and proportions of up to 10% of the cells are considered normal. The data in Table II represent general values applied to synovial fluid findings for purposes of classification.

C. Inflammatory–Nonpurulent Synovial Fluid

The term "inflammatory–nonpurulent" is introduced to encompass the spectrum of primary and secondary joint diseases. Classification of these entities as noninflammatory, as is frequently done, is inconsistent with tissue, cellular, and biochemical changes of the synovial joint and the related synovial fluid.

1. Degenerative Joint Disease

Degenerative joint disease is a chronic disease of synovial joints, characterized by degeneration of articular cartilage in association with secondary changes in synovial membranes, cartilage, and bone (Van Pelt and Langham, 1966). Examples of specific entities which may be classified in this grouping are osteochrondrosis dissecans (Johansson and Rejno, 1976; Grondalen, 1974), ankylosing spondylitis of the dog (Archibald and Cawley, 1959), chronic traumatic arthritis of the horse (Van Pelt, 1974), and mucopolysaccharidosis (Cowell *et al.*, 1976). Tarsal hydraarthrosis (Van Pelt and Riley, 1967; Van Pelt, 1967a,b,c, 1968a,b) is grouped with degenerative joint disease. Hydraarthrosis and serous arthritis have major increases in the volume of fluid and resultant

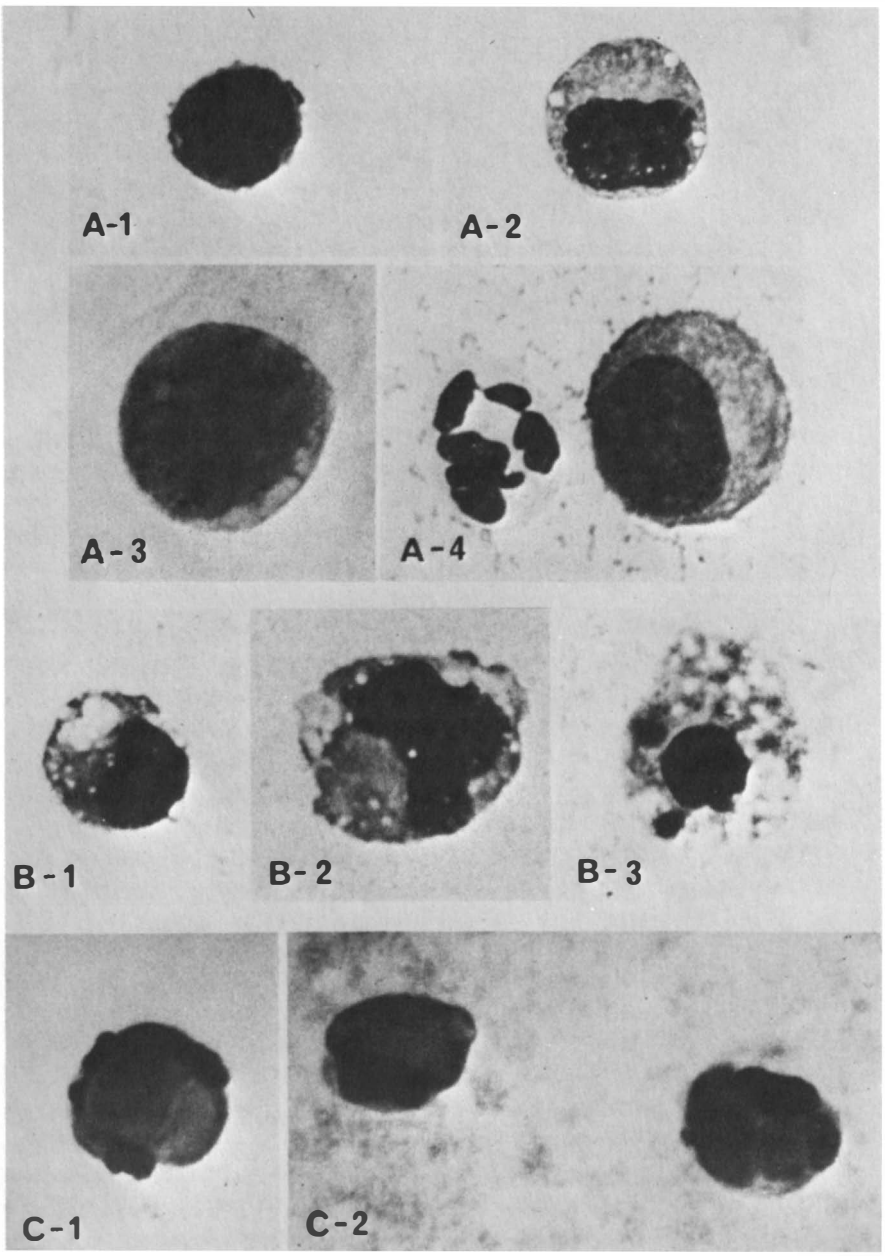


Fig. 1. (A) Equine synovial fluid from stifle joint (Wright's stain). This normal-appearing fluid of low cellularity contained predominantly mononuclear cells. (1) Small lymphocyte; (2) and (3) large mononuclear cells; and (4) segmented neutrophil and large mononuclear cell. (B) Equine traumatic carpitis (Wright's stain). This cloudy, straw-colored fluid contained 400 leukocytes per microliter. The mononuclear cells are (1) and (2) phagocytes and (3) phagocytic and degenerate. (C) Canine polyarthritis associated with canine systemic lupus erythematosus, stifle fluid (Wright's stain). This pinkish fluid contained 30,000 leukocytes per microliter with 17% lymphocytes, 72% neutrophils, and 11% LE cells. Classical LE cells (1) and (2) present on direct smear.

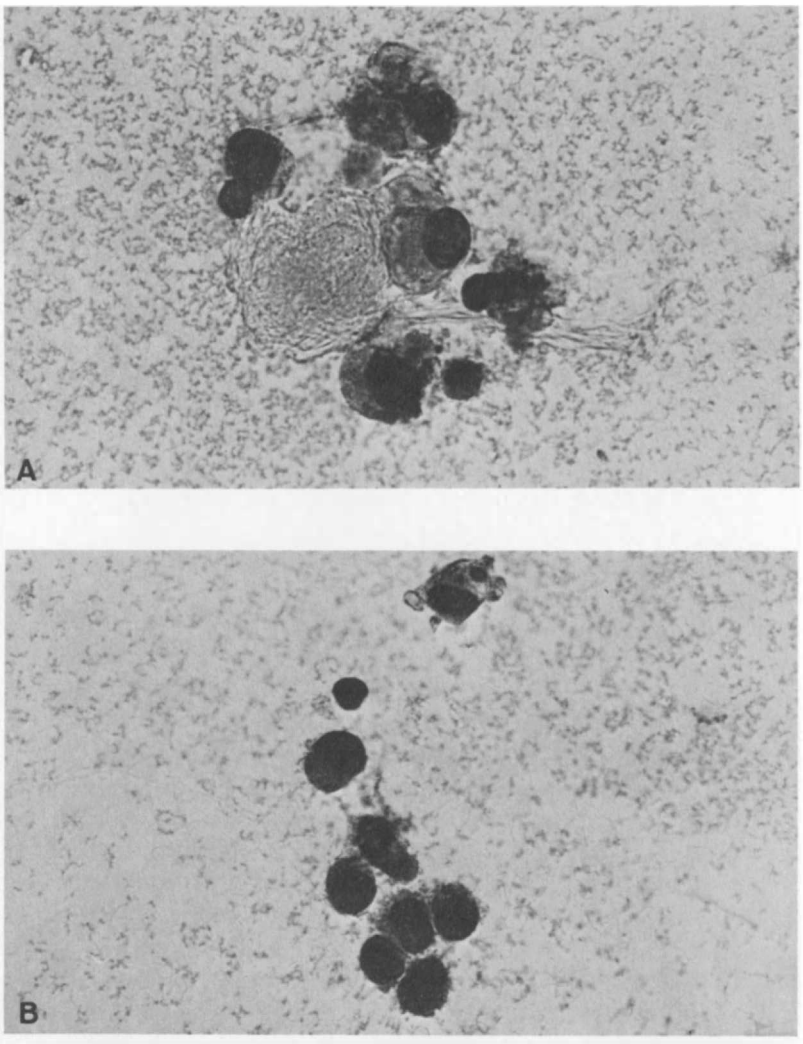


Fig. 2. (A) Equine synovial fluid from a carpal joint (new methylene blue stain). The large size of the monocyte-macrophage cells is contrasted with the small lymphocyte present. The cluster of cells is related to the fibrin strands and coils readily apparent with this water-soluble stain. (B) Equine synovial fluid illustrated in A (Wright's stain). The large monocyte-macrophage cells are contrasted with a small lymphocyte. The cluster of phagocytes may be fibrin trapping. Fibrin does not stain with alcohol-based stains and is not apparent. Traumatic joint disease.

dilution of the macromolecular composition of the fluid. The viscosity of the fluid is decreased, and the quality of the mucin clot is fair to poor.

The fluid in degenerative joint disease is usually clear. Slight haziness or flocculents may be found due to cartilage detritus and cellular constituents. The fluid does not clot. The mucin precipitated by the acetic acid in the mucin clot test is ropey, and the resultant solution is clear. Reduction in viscosity is minimal except for serous arthritis and hydraarthrosis, in which the quality of the mucin clot is altered by dilution. The cellular characteristics may differ from those of normal synovial fluid. When synovial fluid from degen-

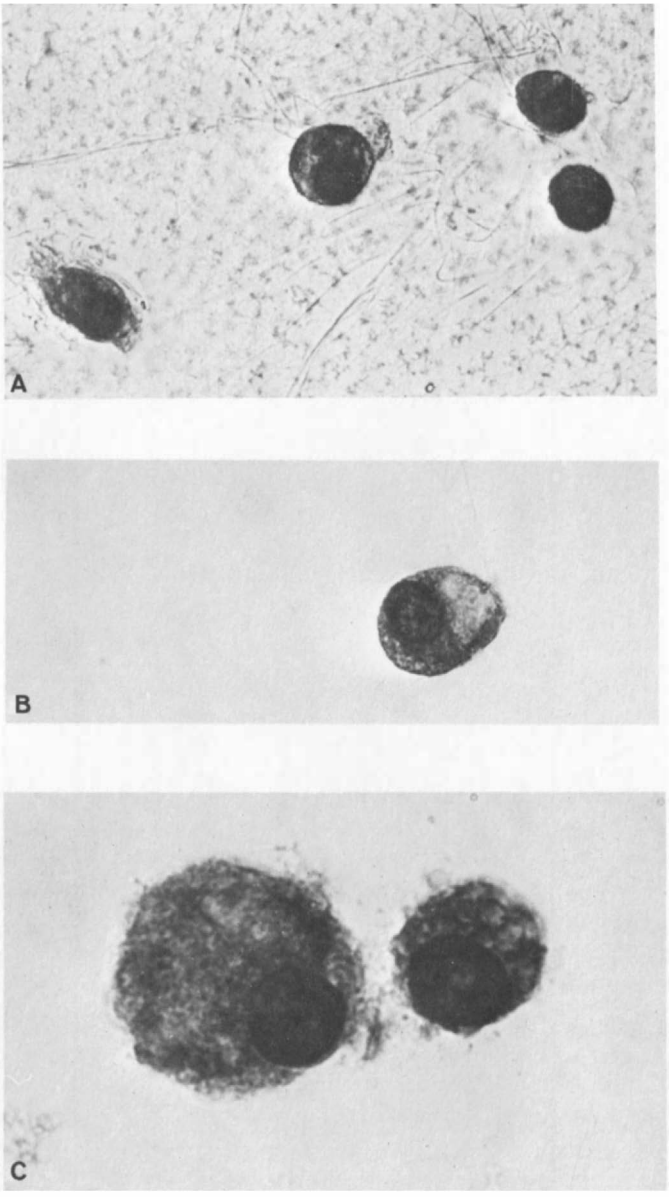


Fig. 3. Equine synovial fluid from a carpal joint. (A) The granular background of precipitated mucin is laced with fibrin strands from the exudative process. The round cells are monocyte–macrophage type of cells and are typical of 90% of the cells (3500 per microliter) in this traumatic joint. (B) and (C) Large mononuclear cells (Wright's stain) located in a thin portion of the smear. The density of mucin precipitate in the background is a useful indication of smear thickness.

TABLE II

Synovial Fluid Properties in Health and Disease

Type	Gross appearance		Viscosity	Acetic acid clot	White blood cells[a] (number/μl)	Red blood cells	Phagoyctes	Micro-organisms
	Color	Clarity						
Normal	Pale yellow	Clear	High	Good	<500, 10% N	Rare	Few, 10% white cells	Absent
Inflammatory–nonpurulent								
Degenerative joint disease	Pale yellow to yellow	Clear to hazy	High; decreased in hydraarthrosis	Good	<5000, 10% N	Rare	Few to many	Absent
Traumatic joint disease	Pale yellow, amber to bloody	Hazy to bloody	High; decreased in serous effusion	Good	<5000, 25% N	Variable	Few to many	Absent
Inflammatory–purulent								
Noninfectious	Pale yellow to sanguineous	Hazy, turbid to bloody	High; decreased in serous effusion	Variable	>5000, 50% N	Variable	Few to many	Absent
Infectious	Yellow, gray, creamy to bloody	Turbid to bloody	Variable; DNA of damaged cell is sticky	Fair to poor	>5000, 50% N	Variable	Increased	Usually present

[a] N, neutrophils.

erative joint diseases is compared with normal synovial fluid, many cases of degenerative joint disease have normal synovial fluid (Van Pelt, 1974). Conversely, alterations from normal synovial fluid are indicative of active change in the synovial joint. Total leukocyte counts greater than 500 per microliter should be considered increased. Total leukocyte counts seldom exceed 5000 cells per microliter in degenerative joint disease. The increase in cells is in both lymphocyte and monocyte–macrophages. On properly prepared and stained thin synovial smears, the monocyte–macrophage compartment of cells must be assessed for change. The majority of cells with phagocytic properties form a transitional state between small lymphocyte-type cells and monocyte-macrophages. These cells in thick smears are easily classed as lymphocytes, whereas in thin areas the large size and phagocytic vacuoles in the cytoplasm define their macrophage potential. The viscous nature of synovial fluid prevents the cells from flattening during smear preparation. Comparison of these cells with neutrophils or erythrocytes in the same field provides a useful and simple means of assessing size of cells. Small lymphocytes range from 6 to 10 μm in diameter, while spread neutrophils are 14 μm in diameter. Smears must be made within minutes of collection to minimize *in vitro* development of phagocytic vacuoles.

Glucose and protein values are unchanged in most cases of degenerative joint disease. Slight reduction in synovial fluid glucose occurs in fluids of increased cellularity by the glycolytic action of cells. Delays in analysis must be considered in the interpretation of low glucose levels. Parallel blood glucose levels are required. Enzyme determinations commonly made are usually normal. Cellular fluids may be reflected in increased enzyme levels for enzymes derived from cellular cytosol. The studies of Rejno (1976b) on isoenzymes of LDH provide a means for assessing articular cartilage damage in degenerative joint disease.

2. Traumatic Joint Disease

Traumatic joint disease of the acute form alters synovial fluid by the addition of blood components through damage of vascular structures. The changes may be slight and indistinguishable from normal synovial fluid to markedly altered bloody synovial fluid. When the fluid is sanguineous, the tendency for clotting is readily apparent. In chronic traumatic joint disease, evidence for past bleeding may be manifest by discoloration of the fluid with hemoglobin degradation products. Acute traumatic joint disease may progress to chronic states and appear as degenerative joint disease on analysis of synovial fluid.

The color of synovial fluid in traumatic joint disease may range from pale to light amber or reddish. The clarity may be reduced due to erythrocytes. The volume of fluid in traumatic joint disease may be increased. The viscosity of the synovial fluid is high unless altered by copious effusion. The quality of the mucin clot is good.

Leukocyte numbers are normal to slightly increased in degeneration joint disease, and counts rarely exceed 5000 per microliter. Wheat (1955) found sterile, clear, viscous synovial fluids containing less than 100 mononuclear cells in some forms of traumatic equine joint effusions. When cellularity is increased, the principal cells are mononuclear. Monocyte–macrophage type of cells with phagocytic vacuoles are found.

Mitotic figures may be found in active cellular reactions. Neutrophils should not exceed 25% of the total number of cells and are usually scarce in resolution stages. The number of erythrocytes is reflected in the color of the fluid. Glucose levels may be reduced in cellular effusions, and protein levels are increased in sanguineous fluids.

Making the distinction between hemorrhage on aspiration and bleeding in traumatic

joint disease requires careful observation of the fluid at arthrocentesis. Streaking of the synovial fluid with blood is usually associated with hemorrhage induced by arthrocentesis. The presence of platelets on smears of synovial fluid is indicative of contamination of synovial fluid with blood at arthrocentesis.

3. Neoplastic Joint Disease

Neoplasia of synovial joints occurs as primary and metastatic disease. Primary neoplasms are synoviomas or sarcomas of structural components of the synovial joint. Sarcomas seldom exfoliate cells. Hemorrhage is a prominent feature of neoplastic tissue growth in the synovial cavity, and the fluids assume characteristics similar to those of traumatic joint disease. Synovial fluids with alterations in patterns of mononuclear cells, such as a major clumping phenomenon or unidentifiable cells, should be subjected to detailed cytological study.

D. Inflammatory–Purulent Synovial Fluid

Purulent arthritis may involve one or more joints. The distinguishing feature of purulent arthritis is the presence of an increased number of neutrophils in the synovial fluid. The total number of leukocytes is generally in excess of 5000 cells per microliter. The neutrophils usually number 50% or more of the cells. The presence of neutrophils in the purulent synovial fluid results from chemotactic factors of complement activation. Septic and nonseptic mechanisms may activate complement, resulting in the purulent synovial joint. With the exception of septic arthritis due to penetration of the synovial membrane, the synovium or surrounding structures of the joint must be involved before alteration of the synovial fluid takes place (Atcheson and Ward, 1978).

1. Noninfectious Purulent Synovial Fluid

The characteristic feature of noninfectious purulent synovial fluid is the presence of an increased number of neutrophils and the inability to establish an infectious etiology. The principal diseases are SLE (Lewis and Hathaway, 1967; Miller *et al.*, 1974; Pedersen *et al.*, 1976b) and rheumatoid arthritis (Halliwell *et al.*, 1972; Newton *et al.*, 1976; Pedersen *et al.*, 1976a; Alexander *et al.*, 1976; Krum *et al.*, 1977). These diseases may present in early stages with minimal changes in synovial fluid.

Several infectious diseases of synovial joints may progress to immunological stages where distinction from immune-mediated arthritis is not possible. Mycoplasma infections in several species (Sokoloff, 1973; Bennett and Jasper, 1978; Switzer, 1964) have been proposed as models of rheumatoid arthritis of man. Similarly, erysipelas of swine appears to be a rheumatoid-like disease (Crimmins and Sikes, 1965; Sikes, 1960, 1968). Infectious diseases of diverse nature are associated in the development of noninfectious arthritis (Van Pelt and Langham, 1966). Joint inflammation occurs frequently in man with chronic bacterial infections and may occur in the dog (Bennett *et al.*, 1978). These diseases may have circulating immune complexes which lodge in many tissues, causing immune-mediated responses simulating rheumatoid and SLE polyarthritis (Pederson *et al.*, 1976b).

Criteria of the American Rheumatism Association have been used to differentiate canine rheumatoid arthritis and SLE polyarthritis. Distinctions based on the established

criteria appear to be less definitive than division into erosive (rheumatoid) and nonerosive (SLE) joint disease (Pederson *et al.*, 1976a,b).

The distinction between known forms of immunological polyarthritis and idiopathic noninfectious purulent arthritis is usually not apparent from routine examination of synovial fluid alone.

The appearance of synovial fluid in immune-mediated polyarthritis may vary considerably from normal fluid to typical inflammatory–purulent exudates. The color is related to the number of erythrocytes and leukocytes. The fluid is hazy to turbid with increased cellularity and may contain flocculent material. The viscosity of the fluid ranges from normal to decreased and is dependent on the volume of effusion. The mucin clot test ranges from good to poor (Pederson *et al.*, 1976a). The diminished quality of mucin clot is attributed to inflammatory cell constituents and is normally suggestive of septic arthritis. Hyaluronidase required for alteration of the mucin clot quality is considered to be of bacterial origin. The nature of the diminished mucin clot quality in some cases of immune-mediated arthritis remains unclear.

The total leukocytic count in rheumatoid arthritis and SLE of dogs ranges up to several hundred thousand cells (Pedersen *et al.*, 1976a,b). The cells are mainly neutrophils. In low-cellularity effusions, large numbers of lymphocytes, monocytes, and macrophages are present. Ragocytes have not been definitively defined in animals. Particles of DNA and LE cells have been observed in synovial fluids of dogs with SLE (Perman and Cornelius, 1971; Miller *et al.*, 1974) (Fig. 4). Similar particles in human synovial fluid neutrophils have been associated with rheumatoid arthritis (Hunder and Pierre, 1970; Pekin *et al.*, 1966).

Glucose, protein, and enzyme levels in synovial fluid are frequently altered and relate to the quality and quantity of inflammatory exudate. Complement levels in immune-mediated arthritis are reduced. Measurement of complement components has been demonstrated to be of value in the prognosis of rheumatoid arthritis of man (Bunch *et al.*, 1974).

Crystal-induced polyarthritis occurs in man. Demonstrations of urate, pyrophosphate, cholesterol, and steroid crystals in synovial fluid of man give specificity for gout, pseudogout, cholesterol effusions, and reactions to steroid therapy, respectively. The frequency of natural occurrences in animals is unknown. Inflammation of synovial joints characterized by a large number of neutrophils was induced by injection of monosodium urate (McCarty and Hollander, 1961), calcium pyrophosphate (McCarty *et al.*, 1962), and esters of adrenocorticosteroid (McCarty and Hagan, 1964) into the synovial space. In further studies, Phelps *et al.* (1966) and Phelps and McCarty (1966) concluded that the influx of neutrophils is necessary for the inflammatory response, suggesting care in interpretation of neutrophil number.

Chronic hemarthrosis in hemophilia of man is well documented in standard texts. Similar changes occur in animals with hemophilia and related bleeding disorders (Dodds, 1975). The quality of the synovial fluid reflects the degree and frequency of bleeding. Wide variation in synovial fluid composition is possible. Neoplasia of synovial membrane and joint surfaces may be associated with an influx of neutrophils.

2. *Infectious Purulent Synovial Fluids*

The literature on infectious synovial joint diseases of animals is extensive. The pathogenesis of infectious arthritis and changes in synovial joints is multifactoral. Dis-

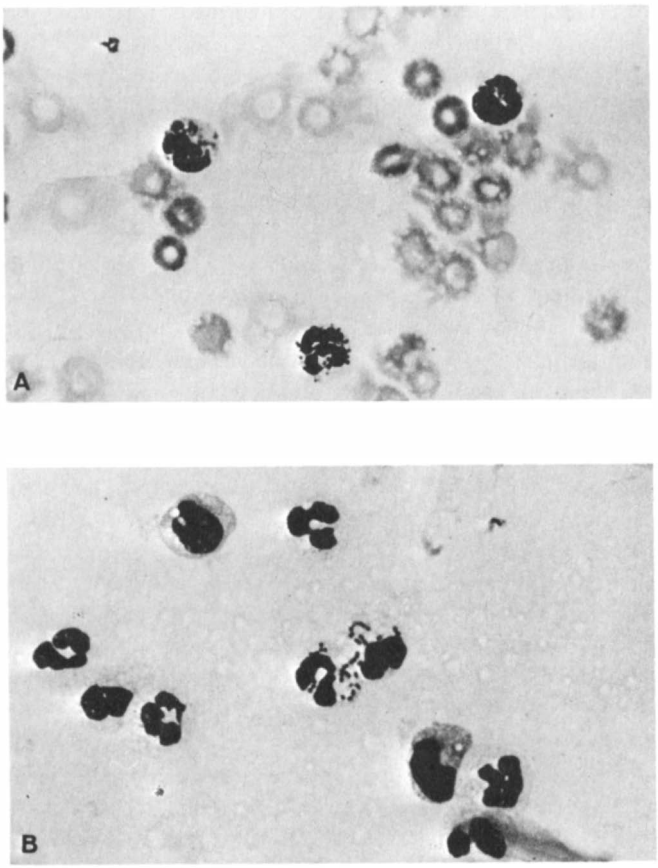


Fig. 4. (A) Synovial fluid from a stifle joint (Wright's stain) of a dog with systemic lupus erythematosus polyarthritis. The particles in the neutrophils are DNA. Typical LE cells were found in adjacent fields (Fig. 1, C-1 and C-2). (B) Synovial fluid from a carpal joint of a 2-week-old pig. The majority of cells are neutrophils. Two monocyte–macrophages with phagocytic vacuoles are present. A short-chained streptococcus is located within neutrophils.

eases associated with mycoplasmal, bacterial, viral, and fungal organisms result in hematogenous spread of infections to synovial joint cavities. The organisms are trapped in platelet clusters or contained within phagocytic cells and may localize in the vascular of the synovial membrane. The spread may occur during acute stages of the disease associated with septicemia. Spread occurs from focal infections such as omphalitis and endocarditis. Extension of infection from the synovial membrane may be generalized to most synovial joints. Predisposition of synovial joints to localization of infection may be related to pressure points and traumatized tissue.

Septic osteomyelitis does not result in septic arthritis in the young, in which the avascular epiphyseal cartilage in the growing bone is an effective barrier to the spread of infection (Atcheson and Ward, 1978). Spread of osteomyelitis to septic arthritis does occur in turkeys (Nairn, 1969).

The nature of the organism determines the nature of the inflammatory exudate in septic arthritis. Andreev (1948) observed that synovial fluid from horses with septicemia con-

tained many desquamated mesothelial cells, tissue histiocytes, monocytes, small lymphocytes, and plasma cells. He indicated that the lymphoid reaction in the joints was associated with a neutrophilia in the peripheral blood. Warren *et al.* (1935), on the other hand, reported that synovial cellular components were not affected by variations in the blood cytology of dogs and cows in experimental leukocytosis.

Bacterial-induced septic arthritis is a well-documented entity. Probably the first recorded advance in clarifying the etiology of purulent polyarthritis in foals was made by Bollinger (1873), who made an association between joints and umbilical abscesses. Joint ill in foals associated with naval infections was studied (Magnusson, 1919; Snyder, 1925; Dimock *et al.*, 1928).

The description of these fluids are those documented for *Actinobacillus equuli* infections. Van Pelt (1965, 1967a,c) and Van Pelt and Langham (1968) studied several causes of synovial fluid alteration of cattle. They classified infectious arthritis with respect to duration and, depending on its pathogenesis, as primary, secondary, or tertiary. Infectious arthritis synovial effusions were primarily exudative. Excessive volume of fluid with reduced viscosity and poor-quality mucin clots were characteristic. The synovial fluid glucose level was approximately one-half the blood glucose level. The alkaline phosphatase levels were reported to exceed the blood levels. The synovial fluid leukocyte counts were variable, with generally markedly increased values. *Corynebacterium pyogenes,* whether singly or in combination with *Escherichia coli, Streptococcus fecalis,* or an α-hemolytic *Streptococcus* were not necessarily the most common isolate, however, were associated with the most severe reaction. Van Pelt (1967b) described the use of punch biopsy of the synovial membrane as an additional means studying joint disease associated with synovial effusions. Synovial disease is common in swine and of varied etiology (Switzer, 1964). Crimmins and Sikes (1965; Sikes, 1960, 1968) described synovial fluid in a rheumatoid-like arthritis caused by *Erysipelothrix rhusiopathiae.* The synovial fluid was described as less viscous than normal, with the mucin clot formation thick, white, and ropey, or web-like, white, and friable. The nucleated cell counts were greatly elevated, with neutrophils in preponderance. Roberts *et al.* (1968) described synovial membrane changes in *Streptococcus equisimilis* infection of swine; however, no qualitative or quantitative changes in synovial fluid were recorded.

Potentially, all pathogenic bacteria may localize in or gain access to synovial joints and cause alteration of the synovial fluid. Infections localizing outside the joints, such as subacute bacterial endocarditis of man, may cause sterile arthritis resulting from immune-mediated mechanisms (Ward and Atcheson, 1977). Similar mechanisms are important for the development of rheumatoid arthritis of swine (Crimmins and Sikes, 1965; Sikes, 1960, 1968). Pedersen *et al.* (1976a) reported arthritis of the nonerosive type in dogs associated with a number of chronic infectious disease processes. Synovial fluid leukocytic counts ranged from 14,000 to 179,000 cells per microliter, and the percentage of neutrophils ranged from 52 to 95. Microorganisms were cultured from primary infectious foci but could not be identified in synovial fluid or synovial membranes of inflamed joints.

Joint inflammation in man with chronic bacterial infections occurs frequently, and the severity of the synovial joint process is often proportional to the severity of the chronic bacterial disease (Coggeshall *et al.*, 1941). The chronic bacterial infection may result in immune complexes composed of tissue and antigens, immunoglobulins and complement, to be deposited in many tissues. Complement fixation results in local inflammation and an

influx of neutrophils to the tissue. The release of lysosomal enzymes from neutrophils causes a progression of the inflammatory disease. These mechanisms are associated with immune-complex-mediated glomerulonephritis and are postulated as a mechanism for synovial joint disease in chronic infectious disease processes (Pederson *et al.*, 1976b).

Mycoplasmal-induced polyarthritis occurs in most species of animals (Sokoloff, 1973). The organisms induce arthritis that closely resembles rheumatoid arthritis of man, for whom it is a frequently studied animal model. The synovial joint in acute stages is involved with fibrinopurulent exudation. The amount of synovial fluid may be great. Similar changes occur in the polyarthritis associated with chlamydial-type organisms (Story, 1966).

The occurrence of fungal and protozoal infections of synovial joints has been demonstrated (Pederson *et al.*, 1976a). Extension of fungal osteomyelitis to the synovial joint is possible.

The changes in synovial fluids that typify an infectious etiology are exudation of plasma proteins and neutrophils. The quantity of fluid may be copious in bacterial disease and scanty when fibinopurulent exudate is abundant. Demonstration of microorganisms in the cells and fluid is frequently made with common stains. Cultural methods are necessary for definitive identification of bacterial, mycoplasmal, viral, and fungal agents. Distinction between infectious inflammatory disease and noninfectious inflammatory joint disease is difficult unless microorganisms are identified.

REFERENCES

Alexander, J. W., Begg, S., Dueland, R., and Schaltz, R. D. (1976). *J. Am. Vet. Med. Assoc.* **12,** 727.

Amrousi, S. E., Soliman, M. K., and Youssef, L. B. (1966). *Can. J. Comp. Med. Vet. Sci.* **30,** 251.

Andreev, P. P. (1948). *Veterinariya (Moscow)* **25,** 20.

Archibald, J. A., and Cawley, A. J. (1959). *Vet. Scope* **4,** 3.

Atcheson, S. G., and Ward, J. R. (1978). *Arthritis Rheum.* **21,** 1978.

Barnett, C. H., Davies, D. V., and MacConnaill, M. A. (1961). "Synovial Joints; Their Structure and Mechanics." Thomas, Springfield, Illinois.

Barthel, C. H., Duncan, J. R., and Ross, R. F. (1971). *Am. J. Vet. Res.* **32,** 2011.

Bauer, W., Bennett, G. A., Marble, A., and Claflin, D. (1930). *J. Exp. Med.* **52,** 835.

Bauer, W., Short, C. L., and Bennett, G. A. (1933). *J. Exp. Med.* **57,** 419.

Bauer, W., Ropes, M. W., and Waine, H. (1940). *Physiol. Rev.* **20,** 272.

Bennett, D., Gilbertson, E. M. M., and Grennan, D. J. (1978). *J. Small Anim. Pract.* **19,** 185.

Bennett, G. A., Bauer, W., and Maddock, S. J. (1932). *Am. J. Pathol.* **8,** 499.

Bennett, R. H., and Jasper, D. E. (1978). *J. Am. Vet. Med. Assoc.* **172,** 484.

Blair, M. G., Pigman, W., and Holly, H. L. (1961). *Arthritis Rheum.* **4,** 612.

Blumberg, B. S., and Ogston, A. G. (1956). *Biochem. J.* **63,** 715.

Bollinger, O. (1873). *Arch. Pathol. Anat. Physiol.* **58,** 329.

Broderick, P. A., Corvese, N., Pierik, M. G., Pike, R. F., and Marcorengi, A. L. (1976). *J. Bone J. Surg. Am. Vol.* **58,** 396.

Brook, I., Reza, M. J., Bricknell, K. A., Bluestone, R., and Finegold, S. M. (1978). *Arthritis Rheum.* **21,** 774.

Bunch, T. W., Hunder, G. G., Offord, K., and McDuffie, F. C. (1974). *Ann. Intern. Med.* **81,** 32.

Butler, A. M., and Montgomery, H. (1932). *J. Biol. Chem.* **99,** 173.

Bywaters, E. G. L. (1937). *J. Pathol. Bacteriol.* **44,** 247.

Charnley, J. (1959). *Proc. Inst. Mech. Eng. London, Symp. Biomech.* p. 12.

Clarke, I. C. (1973). *J. Microsc. (Oxford)* **97,** 309.

Coggeshall, H. C., Bennett, G. A., Warren, C. F., and Bauer, W. (1941). *Am. J. Med. Sci.* **202,** 486.

Cowell, K. R., Jezyk, P. F., Haskins, M. E., and Patterson, D. F. (1976). *J. Am. Vet. Med. Assoc.* **169,** 334.

Crimmins, L. T., and Sikes, D. (1965). *Can. J. Comp. Med. Vet. Sci.* **29,** 312.

Crocker, P. R., Duppe, P. A., Tyler, G., Chapman, S. K., and Willoughby, D. A. (1977). *J. Pathol.* **121,** 37.
Currey, H. L. F., and Vernon-Roberts, B. (1976). *Clin. Rheum. Dis.* **2,** 149.
Curtain, C. C. (1955). *Biochem. J.* **61,** 688.
Cutlip, R. C., and Cheville, N. F. (1973). *Am. J. Vet. Res.* **34,** 45.
Dabich, D., and Neuhaus, O. W. (1966a). *J. Biol. Chem.* **241,** 415.
Dabich, D., and Neuhaus, O. W. (1966b). *Proc. Soc. Exp. Biol. Med.* **123,** 584.
Davies, D. V. (1945). *J. Anat.* **19,** 66.
Dimock, W. W., Edwards, P. R., and Bullard, J. F. (1928). *J. Am. Vet. Med. Assoc.* **73,** 163.
Dintenfass, L. (1963). *Nature (London)* **197,** 496.
Dodds, W. J. (1975). *In* "Textbook of Veterinary Internal Medicine" (S. J. Ettinger, ed.), Vol. 2, p. 1679. Saunders, Philadelphia, Pennsylvania.
Eggers, H. (1959). *Wien. Tieraerztl. Monatsschr.* **46,** 24.
Fagan, T. J., and Lidosky, M. D. (1974). *Arthritis Rheum.* **17,** 256.
Fessler, J. H., and Fessler, L. I. (1966). *Proc. Natl. Acad. Sci. U.S.A.* **56,** 141.
Fessler, J. H., Ogston, A. G., and Stanier, J. E. (1954). *Biochem. J.* **58,** 656.
Fisher, A. G. T. (1929). "Chronic (Non-Tuberculous) Arthritis, Pathology and Principles of Modern Treatment." Macmillan, New York.
Forkner, C. E. (1930). *J. Lab. Clin. Med.* **15,** 1187.
Freichs, D. D. (1850). *In* "Handwörterbuch der Physiologie," Vol. 3, p. 463.
Gaucher, A. (1978). *Biomedicine* **27,** 242.
Ghadially, F. N. (1978). *In* "The Joints and Synovial Fluid" (L. Sokoloff, ed.), p. 105. Academic Press, New York.
Ghadially, F. N., and Roy, S. (1969). "Ultrastructure of Synovial Joints in Health and Disease." Butterworth, London.
Ghadially, F. N., Ailsby, R. L., and Aryschak, A. F. (1974). *Ann. Rheum. Dis.* **33,** 327.
Ghadially, F. N., Ghadially, J. A., Aryschak, A. F., and Yong, N. K. (1977). *J. Anat.* **123,** 527.
Greene, C. H., and Power, M. H. (1931). *J. Biol. Chem.* **91,** 183.
Grondalen, T. (1974). *Acta Vet. Scand.* **15,** 1.
Halliwell, R. E. W., Lavelle, R. B., and Butt, K. M. (1972). *J. Small Anim. Pract.* **13,** 239.
Hamerman, D., and Schubert, M. (1962). *Am. J. Med.* **33,** 555.
Hardingham, T. E., and Muir, H. (1972). *Biochim. Biophys. Acta* **279,** 401.
Hardy, R. M., and Wallace, L. J. (1974). *Vet. Clin. North Am.* **4,** 449.
Harer, T., and Cohen, H. (1929). *Proc. R. Soc. Med.* **22,** 1121.
Hascall, V. C., and Heinegard, D. (1974). *J. Biol. Chem.* **249,** 4232.
Hasselbacher, P. (1976). *Arthritis Rheum.* **19,** 1358.
Hasselbacher, P., Passero, F. C., and Ludivico, C. L. (1978). *Pa. Med.* **8,** 54.
Havers, C. (1691). "Osteologia Nova, or Some New Observations on the Bones and the Parts Belonging to Them, with the Manner of Their Accretion and Nutrition," communicated to the Royal Society in several discourses. S. Smith, London.
Hollander, J. L., McCarty, D. J., Jr., Astorga, G., and Castro-Murillo, E. (1965). *Ann. Intern. Med.* **62,** 271.
Hueck, W. (1920). *Beitr. Pathol. Anat. Allg. Pathol.* **66,** 330.
Hunder, G. G., and Pierre, R. V. (1970). *Arthritis Rheum.* **13,** 448.
Jeanloz, R. W. (1956). *Proc. Int. Congr. Biochem., 3rd, Brussels, 1955,* p. 65.
Johansson, H. E., and Rejno, S. (1976). *Acta Vet. Scand.* **17,** 153.
Keiser, H., and Sandson, J. I. (1974). *Arthritis Rheum.* **17,** 219.
Key, J. A. (1929). *J. Bone J. Surg.* **11,** 705.
Kling, D. H., Levine, M. G., and Wise, S. (1955). *Proc. Soc. Exp. Biol. Med.* **89,** 261.
Krum, S. H., Cardinett, G. H., Anderson, B. C., and Holliday, T. A. (1977). *J. Am. Vet. Med. Assoc.* **61,** 61.
Kushner, I., and Somerville, J. A. (1971). *Arthritis Rheum.* **14,** 560.
Labor, M., and von Balogh, E. (1919). *Wien. Klin. Wochenschr.* **32,** 535.
Laurent, T. C., Bjork, I., Pietruszkiewics, A., and Persson, H. (1963). *Biochim. Biophys. Acta* **78,** 351.
Levine, M. G., and Kling, D. H. (1956). *J. Clin. Invest.* **35,** 1419.
Lewis, R. M., and Hathaway, J. E. (1967). *J. Small Anim. Pract.* **8,** 273.
Liberg, P., Magnusson, L. E., and Schowgaard, H. (1977). *Equine Vet. J.* **9,** 87.
Linn, F. C., and Radin, E. L. (1968). *Arthritis Rheum.* **11,** 674.
Lunney, J., and Ashwell, G. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 341.

McCarty, D. J., Jr., and Hagan, J. M. (1964). *Arthritis Rheum.* **7,** 359.
McCarty, D. J., Jr., and Hollander, J. L. (1961). *Ann. Intern. Med.* **54,** 452.
McCarty, D. J., Jr., Kohn, N. N., and Faires, J. S. (1962). *Ann. Intern. Med.* **56,** 738.
McCarty, D. J., Jr., Phelps, P., and Pyenson, J. (1966). *J. Exp. Med.* **124,** 99.
McCutchen, C. W. (1966). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **25,** 1061.
McCutchen, C. W. (1978). *In* "The Joints and Synovial Fluid" (L. Sokoloff, ed.), Academic Press, New York.
McEwen, C. (1935). *J. Clin. Invest.* **14,** 190.
Magnusson, H. (1919). *J. Comp. Pathol. Ther.* **32,** 143.
Marsh, J. A., Hallet, F. R., and Owen, R. (1976). *Can. J. Comp. Med.* **40,** 202.
Mayeda, T. (1920). *Mitt. Med. Fak. Tokio* **23,** 393.
Meyer, K. (1947). *Physiol. Rev.* **27,** 335.
Meyer, K. (1958). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **17,** 1075.
Meyers, O. L., and Watermeyer, G. S. (1976). *S. Afr. Med. J.* **50,** 973.
Miller, J. B., Perman, V., Osborne, C. A., Hammer, R. F., and Gambardella, P. C. (1974). *J. Am. Anim. Hosp. Assoc.* **10,** 392.
Muller, W. (1929). *Dtsch. Z. Chir.* **218,** 395.
Naib, Z. M. (1973). *Acta Cytol.* **17,** 299.
Nairn, M. E. (1969). Ph.D. Thesis, Univ. of Minnesota, St. Paul.
Nettelbladt, E., Sundblad, L., and Johsson, E. (1963). *Acta Rheumatol. Scand.* **9,** 28.
Neuhaus, O. W. (1962). *Mich. State Med. Soc.* **61,** 458.
Newton, C. D., Lipowitz, A. J., Halliwell, R. E., Allen, H. L., Biery, D. N., and Schumacher, H. R. (1976). *J. Am. Vet. Med. Assoc.* **168,** 113.
Ogston, A. (1876). *J. Anat. Physiol.* **10,** 49.
Ogston, A. G., and Sherman, T. F. (1959). *Biochem. J.* **72,** 301.
Olson, M. E., Kubicek, W. C., Sampson, G. R., Bilka, P. J., and Kottke, F. J. (1967). *Arthritis Rheum.* **10,** 180.
Owen, D. S. (1978). *J. Am. Med. Assoc.* **239,** 193.
Palmer, D. G. (1968). *Am. J. Clin. Pathol.* **49,** 812.
Pedersen, N. C., Pool, R. C., Castles, J. J., and Weisner, K. (1976a). *J. Am. Vet. Med. Assoc.* **169,** 295.
Pedersen, N. C., Weisner, K., Castles, J. J., Ling, G. V., and Weisner, G. (1976b). *J. Am. Vet. Med. Assoc.* **169,** 304.
Pekin, T. J., and Zvaifler, N. J. (1964). *J. Clin. Invest.* **43,** 1372.
Pekin, T. J., Malinin, T. I., and Zvaifler, N. J. (1966). *Ann. Intern. Med.* **65,** 1229.
Perman, V., and Cornelius, C. E. (1971). *In* "Clinical Biochemistry of Domestic Animals" (J. J. Kaneko and C. E. Cornelius, eds.), 2nd ed., p. 233. Academic Press, New York.
Persson, L. (1971). *Acta Vet. Scand., Suppl.* **35,** 29.
Phelps, P., and McCarty, D. J., Jr. (1966). *J. Exp. Med.* **124,** 115.
Phelps, P., Prockop, D. J., and McCarty, D. J. (1966). *J. Lab. Clin. Med.* **68,** 433.
Phelps, P., Steele, A. D., and McCarty, D. J. (1968). *J. Am. Med. Assoc.* **203,** 508.
Platt, D., Pigman, W., Holly, H. L., and Patton, F. M. (1956). *Arch. Biochem. Biophys.* **64,** 152.
Podkaminsky, N. A. (1931). *Langenbecks Arch. Chir.* **165,** 383.
Preston, B. N., Davies, M., and Ogston, A. G. (1965). *Biochem. J.* **96,** 449.
Radin, E. L., Swann, D. A., and Weisser, P. A. (1970). *Nature (London)* **228,** 377.
Redler, I. (1974). *J. Bone J. Surg., Am. Vol.* **52,** 1395.
Rejno, S. (1976a). *Acta Vet. Scand.* **17,** 169.
Rejno, S. (1976b). *Acta Vet. Scand.* **17,** 178.
Rhinelander, F. W., Jr., Bennett, G. A., and Bauer, W. (1939). *J. Clin. Invest.* **18,** 1.
Roberts, E. D., Ramsey, F. K., Switzer, W. P., and Layton, J. M. (1968). *Am. J. Vet. Res.* **29,** 253.
Ropes, M. W., and Bauer, W. (1953). "Synovial Fluid Changes in Joint Diseases," Harvard Univ. Press, Cambridge, Massachusetts.
Ropes, M. W., Bennett, G. A., and Bauer, W. (1939). *J. Clin. Invest.* **18,** 351.
Ropes, M. W., Coggeshall, H. C., and Bauer, W. (1941). *J. Clin. Invest.* **20,** 455.
Ropes, M. W., Robertson, W. van B., Rossmeisl, E. C., Peabody, R. B., and Bauer, W. (1947). *Acta Med. Scand., Suppl.* **196,** 700.
Sabin, F. R. (1923). *Bull. Johns Hopkins Hosp.* **34,** 277.
Sandson, J. (1967). *Science* **155,** 839.
Sawyer, D. C. (1963). *J. Am. Vet. Med. Assoc.* **143,** 609.

Schalm, O. W. (1961). *Calif. Vet.* **15,** 28.
Schumacher, H. R. (1977). *In* "Rheumatic Disease'.' (W. A. Katz, ed.), p. 279. Lippincott, Philadelphia, Pennsylvania.
Schur, R. H., and Sandson, J. (1963). *Arthritis Rheum.* **6,** 115.
Shaw, N. E., and Martin, B. F. (1962). *J. Anat.* **96,** 359.
Sheehan, J. K., Atkins, E. D. T., and Nieduszynski, I. A. (1975). *J. Mol. Biol.* **91,** 153.
Shively, J. A. and Van Sickle, D. C. (1977). *Am. J. Vet. Res.* **38,** 681.
Shively, M. J. (1975). M.S. Thesis, Purdue Univ., Lafayette, Indiana.
Sikes, D. (1960). *Can. J. Comp. Med. Vet. Sci.* **24,** 347.
Sikes, D. (1968). *Am. J. Vet. Res.* **29,** 1719.
Smiley, J. D., Sachs, C., and Ziff, M. (1968). *J. Clin. Invest.* **47,** 624.
Smith, J. B., and Frame, J. (1965). *Nature (London)* **208,** 867.
Snyder, E. M. (1925). *J. Am. Vet. Med. Assoc.* **66,** 481.
Sokoloff, L. (1973). *Am. J. Pathol.* **73,** 261.
Stone, S. S., and Deyoe, D. L. (1974). *Am. J. Vet. Res.* **35,** 1259.
Story, J. (1966). *J. Comp. Pathol.* **76,** 351.
Sundblad, J., and Balazs, E. A. (1966). *In* "The Amino Sugars" (E. A. Balazs and R. W. Jeanloz, eds.), Vol. 2B, p. 229. Academic Press, New York.
Sundblad, L. (1953). *Acta Soc. Med. Ups.* **58,** 113.
Sundblad, L. (1965). *In* "The Amino Sugars" (E. A. Balazs and R. W. Jeanloz, eds), Vol. 2A, p. 229. Academic Press, New York.
Swann, D. A. (1978). *In* "The Joints and Synovial Fluid" (L. Sokoloff, ed.), p. 407. Academic Press, New York.
Swann, D. A., and Radin, E. L. (1972). *J. Biol. Chem.* **274,** 8069.
Swann, D. A., Caulfield, J. B., and Ceselski, A. (1975). *Ann. Rheum. Dis.* **34,** Suppl. No. 98.
Swann, D. A., Powell, S., Broadhurst, J., Sordillo, E., and Sotman, S. (1976). *Biochem. J.* **157,** 503.
Swann, D. A., Satman, S., Dixon, M., and Brooks, C. (1977). *Biochem. J.* **161,** 473.
Switzer, W. P. (1964). *In* "Diseases of Swine" (H. W. Dunne, ed.), p. 498. Iowa State Univ. Press, Ames.
Tanner, R. I. (1966). *Phys. Med. Biol.* **2,** 119.
Timoney, J. F. (1976). *Am. J. Vet. Res.* **37,** 295.
Utsinger, P. D. (1975). *Arthritis Rheum.* **18,** 595.
Van Pelt, R. W. (1962). *J. Am. Vet. Med. Assoc.* **141,** 1051.
Van Pelt, R. W. (1965). *Diss. Abstr.* **26,** 3258.
Van Pelt, R. W. (1967a). *Am. J. Vet. Res.* **28,** 421.
Van Pelt, R. W. (1967b). *Am. J. Vet. Res.* **150,** 1121.
Van Pelt, R. W. (1967c). *Am. J. Vet. Res.* **151,** 590.
Van Pelt, R. W. (1967d). *Can. J. Comp. Med. Vet. Sci.* **31,** 342.
Van Pelt, R. W. (1968a). *Am. J. Vet. Res.* **29,** 569.
Van Pelt, R. W. (1968b). *J. Am. Vet. Med. Assoc.* **153,** 446.
Van Pelt, R. W. (1974). *J. Am. Vet. Med. Assoc.* **165,** 91.
Van Pelt, R. W., and Conner, G. H. (1963a). *Am. J. Vet. Res.* **24,** 112.
Van Pelt, R. W., and Conner, G. H. (1963b). *Am. J. Vet. Res.* **24,** 537.
Van Pelt, R. W., and Conner, G. H. (1963c). *Am. J. Vet. Res.* **24,** 735.
Van Pelt, R. W., and Langham, R. F. (1966). *J. Am. Vet. Med. Assoc.* **149,** 505.
Van Pelt, R. W., and Langham, R. F. (1968). *Am. J. Vet. Res.* **29,** 507.
Van Pelt, R. W., and Riley, W. F. (1967). *J. Am. Vet. Med. Assoc.* **151,** 328.
von Holst, G. (1904). *Hoppe-Seyler's Z. Physiol. Chem.* **43,** 145.
Ward, J. R., and Atcheson, S. G. (1977). *Med. Clin. North Am.* **61,** 313.
Ward, T. T., and Steigbigel, R. T. (1978). *Am. J. Med.* **64,** 933.
Warren, C. F., Bennett, G. A., and Bauer, W. (1935). *Am. J. Pathol.* **11,** 953.
West, M., Poske, R. M., Black, A. B., Pilz, C. G., and Zimmerman, H. J. (1963). *J. Lab. Clin. Med.* **62,** 175.
Wheat, J. D. (1955). *J. Am. Vet. Med. Assoc.* **127,** 64.
Woodward, D. H., Gryle, A., and Gardner, D. L. (1969). *Experientia* **25,** 1301.
Yehia, S. K., and Duncan, H. (1975). *Clin. Orthop.* **107,** 11.
Zvaifler, N. J. (1973). *Adv. Immunol.* **16,** 265.
Zvaifler, N. J. (1975). *J. Rheumatol.* **2,** 221.

Appendixes

JIRO J. KANEKO

APPENDIX I—SI UNITS

The Système International d'Unités (SI), or the International System of Units, was recommended for use in the health professions by the World Health Assembly (WHA 30.39) in May, 1977. The SI is the culmination of more than a century of effort to develop a universally acceptable system of units of measure. A brief description is given here in order to help the reader acquire familiarity with SI and to facilitate conversions to SI from the conventional units used in this volume.

TABLE A

SI Base Units

Quantity	Name of unit	Symbol
Length	meter	m
Mass	kilogram	kg
Time	second	s
Electric current	ampere	A
Thermodynamic temperature	kelvin	K
Luminous intensity	candela	cd
Amount of substance	mole	mole

CLINICAL BIOCHEMISTRY OF DOMESTIC ANIMALS, 3d ed

ISBN 0-12-396350-8

TABLE B

Some SI-Derived Units

Quantity	Name of derived unit	Symbol
Area	square meter	m^2
Volume	cubic meter	m^3
Speed	meter per second	m/s
Acceleration	meter per second squared	m/s^2
Substance concentration	mole per cubic meter	$mole/m^3$
Pressure	pascal	Pa
Work; energy	joule	J
Celsius temperature	degree Celsius	°C

TABLE C

SI Prefixes

Factor	Prefix	Symbol	Factor	Prefix	Symbol
10^{18}	exa	E	10^{-1}	deci	d
10^{15}	peta	P	10^{-2}	centi	c
10^{12}	tera	T	10^{-3}	milli	m
10^{9}	giga	G	10^{-6}	micro	μ
10^{6}	mega	M	10^{-9}	nano	n
10^{3}	kilo	k	10^{-12}	pico	p
10^{2}	hecto	h	10^{-15}	femto	f
10^{1}	deca	da	10^{-18}	atto	a

TABLE D

Non-SI Units Still in General Use

Quantity	Unit	Symbol	Value in SI
Time	minute	—	60 s
	hour	—	3600 s
	day	—	86,400 s
Volume	liter	—	10^{-3} m^3
Mass	tonne	t	1000 kg
Length	angstrom	Å	10^{-10} m (0.1 nm)
Pressure	bar	bar	10^5 Pa
	atmosphere	atm	101,325 Pa
Radioactivity	curie	Ci	3.7×10^{10} Bequerel
	roentgen	R	2.58×10^{-4} C/kg
	rad	rad	10^{-2} Gy
Enzyme activity	International unit	U	μmole/min

TABLE E

SI Conversion Factors

Component	Conventional "old" unit	×	Factor	=	"New" SI unit
Acetone	mg		17.22		μmole
Albumin	gm/dl		10		g/l
Ammonia (NH_3, NH_4^+)	μg/dl		0.5872		μmole/l
Bicarbonate (HCO_3^-)	mEq/liter		1		mmole/l
Bilirubin	mg/dl		17.10		μmole/l
Calcium	mg/dl		0.2495		mmole/l
CO_2, total	mEq/liter		1		mmole/l
pCO_2	mm Hg		0.1333		kPa
Cholesterol	mg/dl		0.02586		mmole/l
Chloride (Cl^-)	mEq/liter		1		mmole/l
Cortisol	μg/dl		0.02759		μmole/l
Creatinine	mg/dl		88.40		μmole/l
Copper	μg/dl		0.1574		μmole/l
Fibrinogen	mg/dl		0.01		g/l
Glucose	mg/dl		0.05551		mmole/l
Hemoglobin	gm/dl		0.6206		mmole/l
Insulin	μU/ml		7.175		pmole/l
Iron	μg/dl		0.1791		μmole/l
Lead	μg/dl		0.04826		μmole/l
Methemoglobin	gm/dl		620.6		μmole/l
Magnesium	mg/dl		0.4114		mmole/l
Mercury	μg/liter		4.985		
Myoglobin	mg/dl		0.5848		μmole/l
pO_2	mm Hg		0.1333		kPa
Phosphate (P_i)	mg/dl		0.3229		mmole/l
Potassium	mEq/liter		1		mmole/l
Protein	gm/dl		10		g/l
Sodium	mEq/liter		1		mmole/l
Thyroxine (T_4)	μg/dl		12.87		nmole/l
Triiodothyronine (T_3)	ng/dl		0.01536		nmole/l
Urate	mg/dl		59.48		μmole/l
Urea	mg/dl		0.1665		mmole/l
Urea nitrogen (UN)	mg/dl		0.3570		Urea, mmole/l
Zinc (Zn)	μg/dl		0.1530		μmole/l
Enzymes[a]	U/liter		0.01667		μkat/l

[a] There is as yet no definitive recommendation for the use of the katal (1 kat = 1 mole/s in place of the more commonly used international unit (U = 1 μmole/min). The U/liter should be used for all enzyme activities.

APPENDIX II. CONVERSION FACTORS OF SOME CONVENTIONAL SERUM ENZYME UNITS TO INTERNATIONAL UNITS[a]

Serum enzyme	Conventional unit(s)	× Factor	= International units
Aldolase (Ald)	Sibley-Lehninger unit (SLU) (mg DNP/hour × ml)	0.75	U/liter
Amylase (Amyl)	Somogyi unit (SU) (mg glucose/30 min)	1.85	U/liter
Glutamic oxaloacetic transaminase (GOT), (AST)	Sigma-Frankel unit (SFU)	0.48	U/liter
	Karmen unit (KU)		
	Reitman-Frankel unit (RFU) (0.001 OD[b]/min × ml)		
Glutamic pyruvic transaminase (GPT), (ALT)	Sigma-Frankel unit	0.48	U/liter
	Karmen unit		
	Wroblewski-LaDue unit (WLU)		
	Reitman-Frankel unit (0.001 OD/min × ml)		
Isocitrate dehydrogenase (ICD)	Wolfson-Williams Ashman unit (WWAU) (nmole/hour × ml)	0.0167	U/liter
Lipase	Roe-Byler unit (RBU) (μmole/hour × ml)	16.7	U/liter
Phosphatase, acid	Cherry-Crandall unit (50 μmole/3 hours × ml)	2.77	U/liter
	King-Armstrong unit (KAU) (mg phenol P/30 min)	1.85	U/liter
Phosphatase, alkaline (AP)	King-Armstrong unit (mg Phenyl P/30 min)	7.10	U/liter
	Bodansky unit (BU) (mg P/hour)	5.4	U/liter
Sorbitol dehydrogenase (SDH)	Sigma-Frankel unit (nmole/hour × ml)	0.0167	U/liter

[a] U = 1 μmole/min = 16.67 nmole/s = 16.67 nkat/s = 0.01667 μkat/s.

[b] OD = optical density units.

APPENDIX III. TEMPERATURE CORRECTION FACTORS FOR SOME COMMON ENZYMES[a,b]

Temperature of reaction mixture (°C)	AP	CPK	LDH	SDH	AST SGOT	ALT SGPT
20	2.61	2.05	2.10	1.48	2.29	2.29
21	2.37	1.82	1.96	1.42	1.85	1.85
22	2.15	1.70	1.80	1.37	1.71	1.71
23	1.95	1.59	1.67	1.32	1.59	1.59
24	1.77	1.49	1.55	1.27	1.45	1.45
25	1.61	1.39	1.45	1.22	1.37	1.37
26	1.46	1.31	1.33	1.17	1.29	1.29
27	1.33	1.23	1.26	1.12	1.21	1.21
28	1.21	1.15	1.16	1.08	1.12	1.12
29	1.10	1.07	1.07	1.04	1.05	1.05
30	1.00	1.00	1.00	1.00	1.00	1.00
31	0.90	0.93	0.93	0.96	0.95	0.95
32	0.81	0.87	0.86	0.93	0.89	0.89
33	0.73	0.81	0.80	0.89	0.85	0.85
34	0.66	0.75	0.74	0.85	0.80	0.80
35	0.59	0.70	0.68	0.82	0.77	0.77
36	0.53	0.65	0.64	0.79	0.73	0.73
37	0.48	0.60	0.59	0.76	0.70	0.70

[a] Multiply observed temperature values by temperature correction factor to correct to a standard temperature of 30°C.

[b] Abbreviations: AP, alkaline phosphatase; CPK, creatine phosphokinase; LDH, lactate dehydrogenase; SDH, sorbitol dehydrogenase; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

APPENDIX IV. STABILITY OF ENZYMES IN SERUM UNDER VARIOUS STORAGE CONDITIONS[a]

Enzyme[b]	25°C (room temp)	0°-4°C (refrigeration)	−25°C (frozen)
Acid phosphatase	4 hours[c]	3 days[d]	3 days[d]
Alkaline phosphatase	2-3 days[e]	2-3 days	1 month
Aldolase	2 days	2 days	Unstable[f]
α-Amylase	1 month	7 months	2 months
Cholinesterase	1 week	1 week	1 week
CPK			
"Nonactivated"	2 hours	6 hours	Unstable[f]
"Activated"	2 days	1 week	1 month
GGT	2 days	1 week	1 month
GDH	1 day	2 days	1 day
GOT (AST)	3 days	1 week	1 month
GPT (ALT)	2 days	1 week	Unstable[f]
α-HBDH	Unstable	3 days	Unstable[f]
ICDH	5 hours	3 days	3 weeks
LAP	1 week	1 week	1 week
LDH	1 week	1-3 days[g]	1-3 days[g]
MDH	Unstable	3 days	3 days
SDH	Unstable	1 day	2 days

[a] No more than 10% of original activity lost during specified time.

[b] Abbreviations: CPK, creatine phosphokinase; GGT, γ-glutamyltransferase; GDH, glutamate dehydrogenase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; α-HBDH, α-hydroxybutyrate dehydrogenase; ICDH, isocitric dehydrogenase; LAP, Leucine amino peptidase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; SDH, sorbitol dehydrogenase.

[c] At pH 5-6.

[d] With added citrate or acetate.

[e] Activity may increase.

[f] Enzyme does not tolerate thawing well.

[g] Depending on isoenzyme pattern in the serum.

APPENDIX V. TEMPERATURE CONVERSIONS FROM DEGREES FAHRENHEIT TO DEGREES CELSIUS

°F	°C	°F	°C	°F	°C	°F	°C
−40.0	−40	24.8	−4	89.6	32	154.4	68
−38.2	−39	26.6	−3	91.4	33	156.2	69
−36.4	−38	28.4	−2	93.2	34	158.0	70
−34.6	−37	30.2	−1	95.0	35	159.8	71
−32.8	−36	32.0	0	96.8	36	161.6	72
−31.0	−35	33.8	+1	98.6	37	163.4	73
−29.2	−34	35.6	2	100.4	38	165.2	74
−27.4	−33	37.4	3	102.2	39	167.0	75
−25.6	−32	39.2	4	104.0	40	168.8	76
−23.8	−31	41.0	5	105.8	41	170.6	77
−22.0	−30	42.8	6	107.6	42	172.4	78
−20.2	−29	44.6	7	109.4	43	174.2	79
−18.4	−28	46.4	8	111.2	44	176.0	80
−16.6	−27	48.2	9	113.0	45	177.8	81
−14.8	−26	50.0	10	114.8	46	179.6	82
−13.0	−25	51.8	11	116.6	47	181.4	83
−11.2	−24	53.6	12	118.4	48	183.2	84
−9.4	−23	55.4	13	120.2	49	185.0	85
−7.6	−22	57.2	14	122.0	50	186.8	86
−5.8	−21	59.0	15	123.8	51	188.6	87
−4.0	−20	60.8	16	125.6	52	190.4	88
−2.2	−19	62.6	17	127.4	53	192.2	89
−0.4	−18	64.4	18	129.2	54	194.0	90
+1.4	−17	66.2	19	131.0	55	195.8	91
3.2	−16	68.0	20	132.8	56	197.6	92
5.0	−15	69.8	21	134.6	57	199.4	93
6.8	−14	71.6	22	136.4	58	201.2	94
8.6	−13	73.4	23	138.2	59	203.0	95
10.4	−12	75.2	24	140.0	60	204.8	96
12.2	−11	77.0	25	141.8	61	206.6	97
14.0	−10	78.8	26	143.6	62	208.4	98
15.8	−9	80.6	27	145.4	63	210.2	99
17.6	−8	82.4	28	147.2	64	212.0	100
19.4	−7	84.2	29	149.0	65	213.8	101
21.2	−6	86.0	30	150.8	66	215.6	102
23.0	−5	87.8	31	152.6	67	217.4	103

APPENDIX VI. NORMAL CONCENTRATIONS OF BLOOD CONSTITUENTS IN DOMESTIC ANIMALS[a]

Constituent[b]	Unit	Horse	Cow	Sheep	Pig	Dog	Cat	Goat	Monkey
Acetylcholinesterase (AChE) (R)	U/liter	450-790	1270-2430	640	930	270	540	270	
Butyrylcholinesterase (ButChE) (P)	U/liter	2000-3100	70	0-70	400-430	1210-3020	640-1400	110	
Ammonia (HP, S)	μg/dl	13-108				19-120			523-1711
		(61 ± 29)				(53 ± 25)			(1089 ± 481)
Amylase (S, HP)	U/liter	75-150				185-700			
	Caraway unit					318-1050			
	Somogyi unit	139-278				100-400			283-925
									(589 ± 260)
Arginase (ARG) (S, HP)	U/liter	0-70	1-30	0-4.5		0-4.7			
		(10.7 ± 18.1)	(8.3 ± 5.8)	(5.0 ± 0.9)		(0.5 ± 0.2)			
Bicarbonate (HCO_3^-) (S, P)	mmole/liter	20-28	17-29	20-25	18-27	18-24	17-21		
Bilirubin (S, P, HP)									
Direct	mg/dl	0-0.4	0.04-0.44	0-0.27	0-0.3	0.06-0.12			0.00-0.35
		(0.1)	(0.18)	(0.12)	(0.1 ± 0.1)				(0.04 ± 0.04)
Indirect	mg/dl	0.2-2.0	0.03	0-0.12	0-0.3	0.01-0.49			0.00-0.22
		(1.0)							(0.20 ± 0.18)
Total	mg/dl	0-2.0	0.01-0.47	0.1-0.42	0-0.6	0.1-0.3	0.15-0.20	0-0.1	0.10-0.57
		(1.0)	(0.21)	(0.23 ± 0.09)	(0.2 ± 0.2)	(0.2 ± 0.1)			0.25 ± 0.05
Bromide	μg/dl					0-1.0			
Calcium (S, HP)	mg/dl	11.2-13.6	9.7-12.4	11.5-12.8	7.1-11.6	9.0-11.3	6.2-10.2	8.9-11.7	9.1-11.8
		(12.4 ± 0.58)	(11.08 ± 0.67)	(12.16 ± 0.28)	(9.65 ± 0.99)	(10.2 ± 0.6)	(8.22 ± 0.97)	(10.3 ± 0.7)	(10.2 ± 6.0)
	mmole/liter	2.8-3.4	2.4-3.1	2.8-3.2	1.9-2.9	2.2-2.8	1.5-2.6	2.2-2.9	2.3-3.0
		(3.1)	(2.8)	(3.0)	(2.4)	(2.6)	(2.0)	(2.6)	(2.5 ± 1.5)
Chloride (Cl^-) (S, HP)	mmole/liter	99-109	97-111	95-103	94-106	105-115	117-123	99.0-110.3	97.5-113.5
		(104 ± 2.6)	(104)					(105.1 ± 2.85)	(105 ± 4.0)
Cholesterol (Chol) (S, P, HP)									
Total	mg/dl	75-150	80-120	52-76	36-54	135-270	95-130	80-130	97-186
		(111 ± 18)		(64 ± 12)		(178 ± 38)			(147 ± 34)
Free	mg/dl	(15.7)	22-52		5.7-10.9	31-71	20-40		7.4-41.7
			(37 ± 15)			(51 ± 20)	(30 ± 10)		(22 ± 10)
Ester	mg/dl	(81.1)	58-88		28-48	40-78	40-86		
			(73 ± 15)			(59 ± 19)	(63 ± 23)		
CO_2, total (S, P)	mmole/liter	24-32	21.2-32.2	21-28		17-24	17-24	25.6-29.6	9.6-25.9
		(28)	(26.5)	(26.2)		(21.4)	(20.4)	(27.4 ± 1.4)	(18.6 ± 4.0)
CO_2 pressure (pCO_2) (B)	mm Hg	38-46	35-44	(41.3 ± 4.7)		(38)	(36)		
		(42.4 ± 2.0)							
Copper (S)	μg/dl		32.8-35.2	58-160	133-278	100-120			
					(206)				
Coproporphyrin (Copro) (R)	μg/dl		Trace						
Coproporphyrin (HP)	μg/dl		Trace						
Cortisol (C-CPB) (S, HP)	μg/dl	1-14							
Cortisol (C-RIA) (S, HP)	mg/dl								

Creatine phosphokinase (CPK) (S, HP)	U/liter	2.4–23.4 (12.89 ± 5.25)	4.8–12.1 (7.4 ± 2.4)	8.1–12.9 (10.3 ± 1.6)	2.4–22.5 (8.9 ± 6.0)	1.15–28.40 (6.25 ± 2.06)	7.2–28.2 (19.5 ± 6.7)	0.8–8.9 (4.5 ± 2.8)	
Creatinine (S, P, HP)	mg/dl	1.2–1.9	1–2	1.2–1.9	1–2.7	0.5–1.5	0.8–1.8	1.0–1.8 (1.6 ± 0.06)	
Fibrinogen (Fibr) (P, HP)	mg/dl	200–400	200–500			200–400	100–400		
Folic acid (S, HP)	ng/ml					4.5–22			
Free fatty acid (FFA) (HP)	mg/dl		3–10						
	mmole/liter	0.5–0.75		0.1–0.4					
Glucose (G) (S, P, HP)	mg/dl	75–115 (95.6 ± 8.5)	45–75 (57.4 ± 6.8)	50–80 (68.4 ± 6.0)	85–150 (119.0 ± 17.0)	65–118 (91 ± 12)	70–110 (91.1 ± 7.5)	50–75 (62.8 ± 7.1)	85–131 107 ± 12.9
Glutamate dehydrogenase (GDH) (S, HP)	U/liter	0–11.8 (5.6 ± 4.2)							
γ-Glutamyltransferase (GGT) (S, HP)	U/liter	4.3–13.4 (7.6 ± 1.5)				1.2–6.4 (3.5 ± 1.8)	1.3–5.1		
Glutathione (GSH) (B)	mg/dl		76–113 (89 ± 14)						
Hemoglobin (Hb) (B)	g/dl	11–19 (15)	8–14 (11)	8–16 (12)	10–16 (13)	12–18 (15)	8–14 (12)	8–14 (11)	
Icterus index (II) (P, HP)	Units	5–20	5–15	2–5	2–5	2–5	2–5	2–5	
Insulin (S, HP)	μU/ml		<5			(5.8 ± 1.6)			31.9 ± 2.6
Iodine, total (S)	μg/dl	5–12				5–20 (6.0 ± 3.5)			
Iron (S)	μg/dl	73–140 (111 ± 11)	57–162 (97 ± 29)	166–222 (193 ± 7)	91–199 (121 ± 33)	94–122 (108)	68–215 (140)		
Iron-binding capacity, unbound (UIBC) (S)	μg/dl	200–262 (218 ± 21)	63–186 (131 ± 36)	(141 ± 10)	100–262 (196 ± 39)	170–222 (200)	105–205 (150)		
Isocitrate dehydrogenase (ICD) (S, HP)	Wolfson-Williams Ashman unit	290–1080 (600 ± 200)	560–1310 (1000 ± 170)	26–480 (280 ± 170)		27–440 (180 ± 100)	120–700 (320 ± 190)		
	U/liter	4.8–18.0 (10.0 ± 3.3)	9.4–21.9 (16.7 ± 2.8)	0.4–8.0 (4.7 ± 2.8)		0.4–7.3 (3.0 ± 1.7)	2.0–11.7 (5.3 ± 3.2)		
Ketones (HP)									
Acetone (Ac)	mg/dl		0–10	0–10					
Acetoacetate (AcAc)	mg/dl		0.0–1.1 (0.5)						
β-OH-Butyrate (β-OHB)	mg/dl		0.0–9.0 (4.0)						
Lactic acid (Lac) (B)	mg/dl	10–16	5–20	9–12		2–13 (11)			
Lactate dehydrogenase (LDH) (S, HP)	U/liter	162–412 (252 ± 63)	692–1445 (1061 ± 222)	238–440 (352 ± 59)	380–634 (499 ± 75)	45–233 93 ± 50	63–273 (137 ± 59)	123–392 (281 ± 71)	173–275 (232 ± 31)
LDH isozymes (S, HP)									
LDH-1 (heart, fast)	%	6.3–18.5 (11.5 ± 4.0)	39.8–63.5 (49.0 ± 5.4)	45.7–63.6 (54.3 ± 6.5)	34.1–61.8 (50.8 ± 10.1)	1.7–30.2 (13.9 ± 9.5)	0–8.0 (4.5 ± 2.8)	29.3–51.8 (41.0 ± 8.0)	2.7–38.2 (17.2 ± 8.4)
LDH-2	%	8.4–20.5 (14.8 ± 3.2)	19.7–34.8 (27.8 ± 3.4)	0–3.0 (0.8 ± 1.2)	5.9–9.2 (7.3 ± 1.2)	1.2–11.7 (5.5 ± 4.2)	3.3–13.7 (6.1 ± 3.4)	0–5.4 (2.4 ± 1.8)	4.3–39.7 (19.8 ± 9.4)
LDH-3	%	41.0–65.9 (50.2 ± 7.2)	11.7–18.1 (14.5 ± 1.9)	16.4–29.9 (23.3 ± 4.0)	5.7–11.7 (7.4 ± 1.9)	10.9–25.0 (17.1 ± 5.7)	10.2–20.4 (13.3 ± 3.4)	24.4–39.9 (31.2 ± 6.2)	12.8–50.4 (24.5 ± 7.2)

(continued)

APPENDIX VI—*Continued*

Constituent[b]	Unit	Horse	Cow	Sheep	Pig	Dog	Cat	Goat	Monkey
LDH-4	%	9.5-20.9 (16.2 ± 3.8)	0-8.8 (4.4 ± 2.4)	4.3-7.3 (5.3 ± 1.0)	6.9-15.9 (10.9 ± 3.1)	11.9-15.4 (13.0 ± 1.2)	11.6-35.9 (23.6 ± 8.6)	0-5.5 (2.5 ± 2.5)	0.8-38.0 (17.7 ± 10.6)
LDH-5 (liver, slow)	%	1.7-16.5 (7.3 ± 4.0)	0-12.4 (4.3 ± 3.4)	10.5-29.1 (16.3 ± 6.2)	16.3-35.2 (23.6 ± 6.5)	30.0-72.8 (50.5 ± 16.9)	40.0-66.3 (52.5 ± 9.3)	14.1-36.8 (20.9 ± 9.4)	4.7-36.3 (18.6 ± 8.3)
Lead (B)	μg/dl	5-25	0-24 (10 ± 6)	5-25		0-50		5-25	
Lipase (S)	U/liter					13-200	0-83		
Magnesium (S, HP)	mg/dl	2.2-2.8 (2.5 ± 0.31)	1.8-2.3 (2.05 ± 0.25)	2.2-2.8 (2.5 ± 0.3)	2.7-3.7 (3.2 ± 0.49)	1.8-2.4 (2.1 ± 0.3)	(2.2)	2.8-3.6 (3.2 ± 0.35)	
Nonprotein nitrogen (NPN) (B)	mg/dl					20-36	30-48	22-38 (30 ± 3.65)	
Ornithine carbamyltransferase (OCT) (S, HP)	U/liter	(3.3 ± 4.2)	(4.7 ± 0.3)			(2.7 ± 0.7)	(3.8 ± 1.0)		
O_2 pressure (pO_2) (B)	mm Hg					85-100	78-100		
pH (B)		7.32-7.44 (7.38 ± 0.03)	7.31-7.53 (7.38)	7.32-7.54 (7.44)		7.31-7.42 (7.36)	7.24-7.40 (7.35)		
Phosphatase, acid (S, HP)	U/liter					5-25	0.5-24		
Phosphatase, alkaline (S, HP)	U/liter	143-395 (244 ± 101)	0-488 (194 ± 126)	68-387 (178 ± 102)	118-395 (194 ± 84)	20-156 (66 ± 36)	25-93 (50 ± 25)	93-387 (219 ± 76)	(100-277) (171 ± 55)
Phosphate (P_i) (S, HP)	mg/dl	3.1-5.6	5.6-6.5	5.0-7.3 (6.4 ± 0.2)	5.3-9.6	2.6-6.2 (4.3 ± 0.9)	4.5-8.1 (6.2)	(6 5)	4.4—5 .5 (5.0 ± 0.4) (S)
	mEq/liter	1.8-3.2	3.2-3.8	8.0-4.0 (3.7 ± 0.2)	3.1-5.6	2.3-5.2	2.6-4.7	1.7-4.3 (3.1 ± 71)	
Potassium (S, HP)	mole/liter	2.4-4.7 (3.51 ± 0.57)	3.9-5.8 (4.8)	3.9-5.4 (4.8 ± 0.4)	4.4-6.7	4.37-5.65	4.0-4.5 (4.3)	3.5-6.7	3.5-6.5 (4.7 ± 0.6)
Potassium (R)	mmole/liter	(88)	10-45 (24 ± 7.0)	(64 or 18)	(100)	(9)	(6)		
Protein-bound iodine (PBI) (S)	g/dl	1.5-3.5 (2.2 ± 0.6)	2.7-4.1	3.6-4.0 (3.8 ± 0.1)	(2.7 ± 0.1)	1.8-4.5 (2.5 ± 0.7)	2.5-6.0 (3.5)	2-5 (4)	
Protein (S) (cellulose acetate electrophoresis)									
Total	g/dl	5.20-7.90 (6.35 ± 0.59)	6.74-7.46 (7.10 ± 0.18)	6.00-7.90 (7.20 ± 0.52)	7.90-8.90 (8.40 ± 0.50)	5.40-7.10 (6.10 ± 0.52)	5.40-7.80 (6.60 ± 0.70)	6.40-7.00 (6.90 ± 0.48)	7.80-9.60 (8.72 ± 0.73)
Albumin	g/dl	2.60-3.70 (3.09 ± 0.28)	3.03-3.55 (3.29 ± 0.13)	2.40-3.00 (2.70 ± 0.19)	1.8-3.30 (2.59 ± 0.71)	2.60-3.30 (2.91 ± 0.19)	2.10-3.30 (2.70 ± 0.17)	2.70-3.90 (3.30 ± 0.33)	3.13-5.30 (4.21 ± 0.20)
Globulin	g/dl	2.62-4.04 (3.33 ± 0.71)	3.00-3.48 (3.24 ± 0.24)	3.50-5.70 (4.40 ± 0.53)	5.29-6.43 (5.86 ± 0.57)	2.70-4.40 (3.40 ± 0.51)	2.60-5.10 (3.90 ± 0.69)	2.70-4.10 (3.60 ± 0.50)	3.05-5.22 (4.14 ± 0.20)
α_1	g/dl	0.06-0.70 (0.19 ± 0.26)			0.32-0.44 (0.38 ± 0.06)	0.20-0.50 (0.30 ± 0.03)	0.20-1.10 (0.70 ± 0.02)		0.10-0.49 (0.27 ± 0.03)
α	g/dl		0.75-0.88 (0.79 ± 0.02)	0.30-0.60 (0.50 ± 0.10)				0.50-0.70 (0.60 ± 0.06)	
α_2	g/dl	0.31-1.31 (0.65 ± 0.13)			1.28-1.54 (1.41 ± 0.13)	0.30-1.10 (0.60 ± 0.21)	0.40-0.90 (0.70 ± 0.02)		0.25-0.80 (0.47 ± 0.05)

β_1	g/dl	0.40-1.58 (0.92 ± 0.30)		0.70-1.20 (1.00 ± 0.14)	0.13-0.33 (0.23 ± 0.10)	0.70-1.30 (0.82 ± 0.23)	0.30-0.90 (0.70 ± 0.03)	0.70-1.20 (0.90 ± 0.10)	
β	g/dl		0.80-1.12 (0.96 ± 0.08)						0.96-2.72 (1.89 ± 0.17)
β_2	g/dl	0.29-0.89 (0.57 ± 0.11)		0.40-1.40 (0.70 ± 0.26)	1.26-1.68 (1.47 ± 0.21)	0.60-1.40 (0.89 ± 0.33)	0.60-1.00 (0.70 ± 0.02)	0.30-0.60 (0.40 ± 0.02)	
γ_1	g/dl			0.70-2.20 (1.60 ± 0.41)		0.50-1.30 (0.80 ± 0.25)	0.30-2.50 (1.60 ± 0.77)		
γ	g/dl	0.55-1.90 (1.00 ± 0.14)	1.69-2.25 (1.97 ± 0.14)		2.24-2.46 (2.35 ± 0.11)			0.90-3.00 (1.70 ± 0.44)	0.73-2.84 (1.51 ± 0.14)
γ_2	g/dl			0.20-1.10 (0.80 ± 0.30)		0.40-0.90 (0.70 ± 0.14)	1.40-1.90 (1.70 ± 0.36)		
A/G Ratio		0.62-1.46 (0.96 ± 0.17)	0.84-0.94 (0.89 ± 0.05)	0.42-0.76 (0.63 ± 0.09)	0.37-0.51 (0.44 ± 0.07)	0.59-1.11 (0.83 ± 0.16)	0.45-1.19 (0.71 ± 0.20)	0.63-1.26 (0.95 ± 0.17)	0.72-1.21 (0.94 ± 0.16)
Protoporphyrin (Proto) (HP)	μg/dl		Trace						
Protoporphyrin (R)	μg/dl		Trace		118	35			
Sodium (S, HP)	mmole/liter	132-146 (139 ± 3.5)	132-152 (142)	139-152	135-150	141.1-152.3	147-156 (151)	142-155 (150.4 ± 3.14)	142-160 (149 ± 5.0)
Sodium (R)	mmole/liter		52-96 (72 ± 9.0)	(16 or 84)	(11)	(107)	(104)		
Sorbitol dehydrogenase (SDH) (S, HP)	U/liter	1.9-5.8 (3.3 ± 1.3)	4.3-15.3 (9.2 ± 3.1)	5.8-27.9 (15.7 ± 7.6)	1.0-5.8 (2.6 ± 1.6)	2.9-8.2 (4.5 ± 1.9)	3.9-7.7 (5.4 ± 1.3)	14.0-23.6 (19.4 ± 3.6)	
Thyroxine (T_4-RIA) (S)	μg/dl	0.9-2.8 (1.9)				0.6-3.6 (2.3 ± 0.8)			
Thyroxine (T_4-CPB: Murphy-Pattee) (S)	μg/dl	0.9-2.8 (1.9)	4.2-8.6 (6.4)			0.3-2.5 (1.3 ± 0.5)	0.1-2.5 (1.0 ± 0.5)		
Thyroxine, free (T_4f) (S)	ng/dl					(3.53 ± 0.34)			
Triiodothyronine (T_3-RIA) (S)	ng/dl					82-138 (107 ± 18)			
Transaminase (SGOT, AST) (S, P, HP)	U/liter	226-366 (296 ± 70)	78-132 (105 ± 27)	(307 ± 43)	32-84 (61 ± 26)	23-66 (33 ± 12)	26-43 (35 ± 9)	167-513	13-37 (22 ± 8)
Transaminase (SGPT, ALT) (S, P, HP)	U/liter	3-23 (14 ± 11)	14-38 (27 ± 14)	(38 ± 4)	31-58 (45 ± 14)	21-102 (47 ± 26)	6-83 (26 ± 16)	24-83	0-82 (27 ± 28)
Urea nitrogen (UN) (B, P, HP, S)	mg/dl	10-24	20-30	8-20	10-30	10-28 (17 ± 4.0)	20-30	10.0-20 (15 ± 2)	8-20 (15 ± 3.3) (S)
Uric acid (S, P, HP)	mg/dl	0.9-1.1	0-2	0-1.9		0-2	0-1	0.3-1	
Vitamin A (S)									
Carotenol	μg/dl	9-16 (12)	10-30 (24)	20-45	10-35 (20)	0-5 (3)	50-194		
Carotene	μg/dl	20-175 (100)	25-950 (40)	0-20 (10)		35-90	188		
Vitamin B_{12} (S)	pg/ml					170-180			
B_{12}-Binding capacity (S)	pg/ml					886			
Zinc	μg/dl								(79 ± 4)

[a] Means and their standard deviations (in parentheses) and observed ranges.

[b] Abbreviations: B, whole blood; S, serum; P, plasma; HP, heparinized plasma; R, red blood cells.

APPENDIX VII. NORMAL CONCENTRATIONS OF URINE CONSTITUENTS IN DOMESTIC ANIMALS[a]

Constituent[b]	Unit	Horse	Cow	Sheep	Pig	Dog	Cat	Goat
Allantoin	mg/kg × day	5–15	20–60	20–50	20–80	35–45	80	
Arsenic	μg/dl					30–150		
Bicarbonate (HCO_3^-)	mEq/kg × day					0.05–3.2 (1.3)		
Calcium	mg/kg × day		0.10–1.40	2		1–3	0.20–0.45	1.0
Chloride (Cl^-)	mEq/kg × day		0.1–1.1 (0.6)			0–10.3 (2.0)		
Coproporphyrin (Copro)	μg/dl	5–14		8.8		16–28		
Creatinine	mg/kg × day		15–20	10	20–90	30–80	12–20	10
Cystine	mg/gm creatinine					(67 ± 15)		
Lead	μg/dl					20–75		
Lysine	mg/gm creatinine					(21 ± 6)		
Magnesium	mg/kg × day		3–7			1.7–3.0	3.12	
Mercury	μg/dl					1–10		
Nitrogen								
Urea N (UN)	mg/kg × day		23–28	98	201	140–230	374–1872	107
Total N	mg/kg × day	100–160	40–450	120–350	40–240	250–800	500–1100	120–400
Ammonia N	mg/kg × day		1–17			30–60	60	3–5
pH		Alkaline	7.4–8.4	Alkaline	Acid or alkaline	5–7	5–7	
Phosphorus	mg/kg × day			0.2		20–30	108	1.0
Porphobilinogen (PBG)		Neg.		Neg.				
Potassium	mEq/kg × day		0.08–0.15 (0.12)			0.1–2.4 (1.0)		
Sodium	mEq/kg × day		0.2–1.1 (0.7)			0.04–13.0 (1.9)		
Specific gravity		1.020–1.050 (1.035)	1.025–1.045 (1.035)	1.015–1.045 (1.030)	1.010–1.030 (1.015)	1.015–1.045 (1.025)	1.020–1.040 (1.030)	1.015–1.045 (1.030)
Sulfate (SO_4^{2-}) (total)	mg/kg × day		3–15			30–50		
Uric acid	mg/kg × day	1–2	1–4	2–4	1–2			2–5
Urine volume	ml/kg × day	3–18	17–45 (30)	10–40	5–30	17–45	10–20	10–40
Uroporphyrin (Uro)	mg/dl	1.5–7.0		3.8		5.0		

[a] Means and standard deviations (in parentheses) and observed ranges.

[b] Abbreviations: **B**, whole blood; **S**, serum; **P**, plasma; **HP**, heparinized plasma; **R**, red blood cells.

APPENDIX VIII. NORMAL CONCENTRATIONS OF CONSTITUENTS OF CEREBROSPINAL FLUID IN DOMESTIC ANIMALS[a]

Constituent	Unit	Horse	Cow	Sheep	Pig	Dog	Cat	Goat
Calcium	mg/dl	4.2-6.2	5.1-6.3	5.1-5.5		5.6-6.5	(5.2)	(4.6)
Chloride	mEq/liter	109-126	111-123	128-148		131-138	144	116-130
Glucose	mg/dl	48-57	35-70	52-85	45-87	74-75	(85)	(70)
Magnesium	mg/dl	2.0	2.1-2.4	2.2-2.8		(3.1)	(1.3)	(2.3)
pH		7.13-7.36 (7.25)	7.22-7.26	(7.35)		(7.42)		
Phosphorus	mg/dl	0.8-1.4	0.9-2.5	1.2-2.0		1.1-3.9		
Potassium	mEq/liter	2.9-3.2	2.9-3.5	3.0-3.3		3.0-3.1 (2.98)	3.0-5.9	(3.0)
Pressure	mm H_2O	272-490 (379)				24-172 (86.5)		
Protein								
Total	mg/dl	32-48	20-33	29-42	24-29	15-35	20-27	(12)
Albumin	mg/dl	15-39	10-20		17-24	14-27	19-25	
Globulin								
Nonne-Apelt test		1+	Neg.	Neg.	Neg.	Trace		
Pandy test		1+ to 3+ (2+)	Neg.	Neg.	Neg.	Trace		
Ross-Jones test		1+	Neg.	Neg.	Neg.	Neg.		
Sodium	mg/dl	(145)	110-123	145-157	134-144	151.6-155	(158)	(131)
Specific gravity		1.004-1.008 (1.006)	1.005-1.008			1.004-1.006 (1.005)		
Urea nitrogen (UN)	mg/dl	12-13	8-11			10-11	10-11	
Viscosity		1.00-1.05 (1.00)	1.019-1.029					
Cells	number/μl	4.23 (11)	0-10	0-15	1-20	0-25 (6)	0-1	0-1
Small lymphocytes	%					15-95 (65)		
Large lymphocytes	%					5-40 (21)		
Degen. cells	%					0-40 (14)		

[a] Observed ranges; means in parentheses.

Index

C

D

I

M

S