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Part 1

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Рецензенти:

Chumachenko V. V. – Doctor of Veterinary Sciences, Senior Researcher of State Scientific Control Institute of Biotechnology and Microbal Strains

Veselskyi S. P. – Doctor of Biological Sciences, Senior Researcher of Taras Shevchenko National University

Zasekin D. A. – Doctor of Veterinary Sciences, Professor of National University of Life and Environmental Sciences of Ukraine

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Textbook «Veterinary clinical biochemistry» prepared for the study of the OS "Master" English training course on the subject "Veterinary clinical biochemistry."

The manual consists of sections: «Carbohydrate metabolism and its diseases»; «Ketogenesis and ketosis»; «Lipid metabolism and its diseases»; «Serum proteins and dysproteinaemia»; «Clinical enzymology».

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INTRODUCTION

At present theoretical and practical veterinary possession, only clinical, physiological and pathological-anatomical indicators are insufficient. It’s important to take into account the data of clinical and laboratory testing of different biological material infected animals, which significantly helps to clarify the pathogenesis of the disease, developing tests early diagnosis, checking the effectiveness of the therapy.

The discipline "Clinical Biochemistry" prepare Master of Veterinary Medicine to work in current market environment where there is a need to independently diagnose a disease and to appoint qualified scheme of treatment or prevention of a particular disease, and using laboratory methods to control the disease. This discipline is the basis for a deeper understanding of the pathological processes occurring in the body during a non-contagious, infectious and parasitic diseases and promotes medical thinking.

Clinical biochemistry – is applied biochemistry section that studies the biochemical processes in animals’ organism at normal state and pathologies for health assessments, diagnosis and reveal the mechanism of the disease, its prognosis and the effectiveness prescribed therapy. This science originated and developed on the brink of biological chemistry and clinical laboratory diagnostics. Using biochemical methods performed up to 75 % of laboratory tests. Preferential application of clinical biochemistry methods due primarily to the fact that the pathogenesis of many diseases is the primary and secondary metabolic disorders.

The manual is written in accordance with the curriculum of the course "Veterinary Clinical Biochemistry" specialty – Veterinary Medicine. It highlights the achievements of modern scientific, educational material presented availably, illustrated by tables and figures. Special attention is paid to the clinical and

biochemical assessment of metabolic disorders of proteins, carbohydrates, lipids, ketone bodies, clinical enzymology.

The authors will be sincerely grateful to readers for constructive comments and suggestions for improving the structure, content and form of the material textbook "Veterinary clinical biochemistry", which will be taken into account at the next reprint.

CHAPTER I
CARBOHYDRATE METABOLISM AND ITS DISEASES

1 INTRODUCTION

The sustenance of animal life is dependent on the availability of chemical energy in the form of food-stuffs. 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 on chlorophyll-containing plants. The photosynthetic process reading to the reduction of CO_2 to carbohydrate 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 nerbivorous animals, which depend on 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 classic division of metabolism into the three major foodstuffs, carbohydrates, lipids, and proteins. The metabolism of the lipids and proteins is discussed in other chapters elsewhere in this volume.

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.

2 DIGESTION

The digestion of carbohydrates in the animal 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 (α-glucosidase). 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 carbohydrate 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 sucrose (sucrose α-glucosidase). The monosaccharide products of enzymatic hydrolysis, glucose, fructose, and galactose, are the principal forms in which absorption occurs.

3 ABSORPTION

Monosaccharides are almost completely absorbed through the mucosa of the small intestine and appear in the portal circulation as the free sugars. Absorption occurs by two methods: (1) facilitated 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 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, rehydrolyzed, and free glucose appears in the portal circulation for transport to the liver.

4 METABOLISM OF ABSORBED CARBOHYDRATES

4.1 General

Liver cells are freely permeable to the absorbed carbohydrates. Within the liver, there are several pathways by which the immediate fate of the 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. I.1). The enzyme, galactose-1-phosphate uridylyl-transferase, which catalyzes the reaction is blocked or deficient in congenital galactosemia of humans.

$$\text{Galactose-1-P} + \text{UDP-glucose} \rightarrow \text{UDP-galactose} + \text{glucose-1-P}$$

The glucose phosphates are then converted to and stored as glycogen, catabolized to CO_2, and water, or, as free glucose, returned to the general circulation. Essentially, intermediate carbohydrate metabolism of animals evolves about the metabolism of glucose, and the liver is the organ of prime importance.

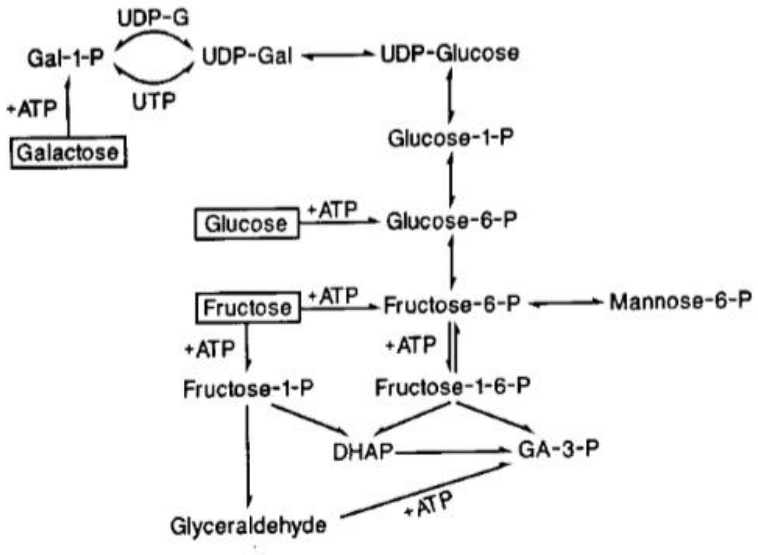


Fig. I.1. Pathways for hexose metabolism. Abbreviations: ATP, adenosine triphosphate; UTP, uridine triphosphate; UDP-G, uridine diphosphoglucose; DHAP, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde 3-phosphate.

4.2 Storage as Glycogen

Glycogen is the chief storage form of carbohydrate in animals and is analogous to the storage of starch by plants. It is found primarily in liver and in muscle, where it occurs at about 3-6 % and about 0.5 %, respectively (Table I.1). 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.

Table I.1

Liver Glycogen Content of Animals

Species	Glycogen in liver (%)
Dog	6.l
Sheep	3.8
Cow (lactating)	1.0
Cow (nonlactating)	3.0
Baby pig	5.2
Baby pig (newborn)	14.8

The result is a complex ramification of chains of glucosyl

units with branch points at the site of the 1-6 links (Fig. I.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. The molecular weights may be as high as 4 x 10^6 and contain about 20.000 glucosyl units.

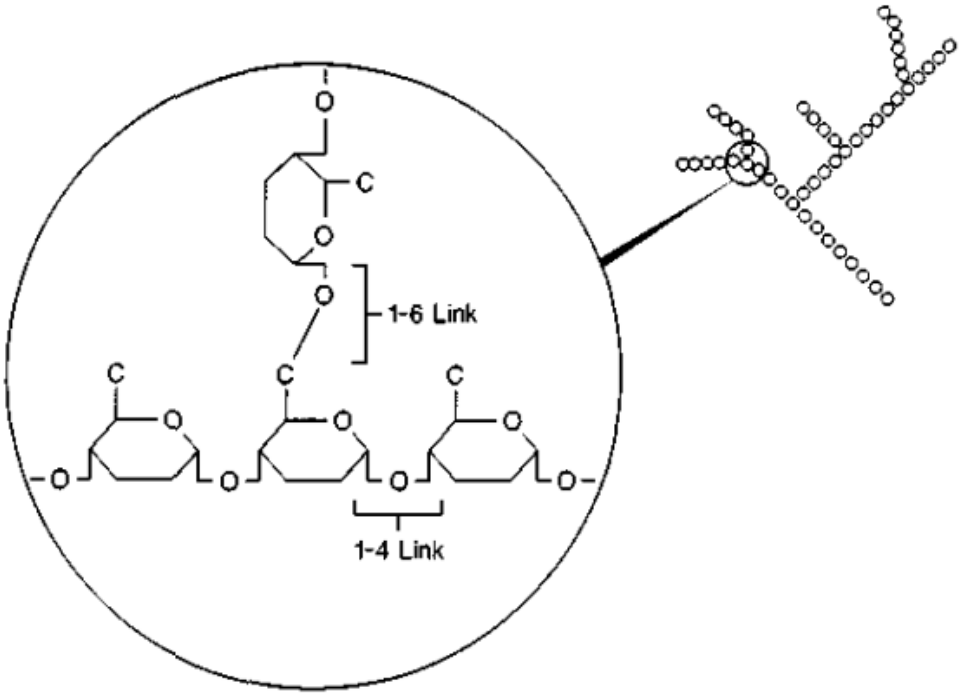


Fig. I.2. Structure glycogen. Adapted from Cori (1954). Note that hydrolysis of a 1 -6 link by the débrancher enzyme yields a mole of free glucose.

In Table I.2, 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 by 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 post-absorptive 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 biological half-time of glycogen which is about a day.

Table I.2

Carbohydrate Content of a Dog*

Muscle glycogen (0.5 %)	25.0 g
Liver glycogen (6 %)	18.0 g
Carbohydrate in fluids [5.5 mmol/liter (100 mg/dl)]	2.2g
	45.2 g
Caloric value (45.2 g x 4 kcal/g) = 181 kcal	
Caloric requirement (70 $kg^{3/4}$ = 70 x 5.6) = 392 kcal/day	
181:392x24 hours = 11 hours	

*Body weight, 10 kg: liver weight, 300 g: muscle weight, 5 kg: volume of blood and extracellular fluid, 2.2 liters.

4.3 Glycogen Metabolism

The processes of glycogenesis and glycogenolysis are now known to proceed by two separate pathways.

4.3.1Glycogenesis

The initial reaction required for the entrance of glucose into the series of metabolic reactions which culminate in the synthesis of glycogen is phosphorylation of glucose at the C-6 position. Glucose is phosphorylated with adenosine triphosphate (ATP) in liver by an irreversible enzymatic reaction catalyzed by a specific glucokinase:

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

Glucokinase (GK) is one of the four hexokinase (HK) coenzymes which occurs in tissues, the nonspecific hexokinase I is found in red cells, brain, and nerve tissue, and hexokinase IV (specific glucokinase) is found in liver.

Liver contains both a nonspecific hexokinase and a glucose-specific glucokinase. The glucokinase is predominant in the liver and has a high Michaelis constant (Km = 2 x 10^{-2} mol g/liter) 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 glucokinase is increased by glucose feeding and by insulin and is decreased during fasting and in insulin lack. i.e., diabetes. The nonspecific hexokinase I. which is found not only in liver, brain, and erythrocytes but in all tissues, has a low Michaelis constant (Km = 5 x 10^{-5} mol G/liter) indicating a high affinity for glucose. Hexokinase-catalyzed phosphorylation in all tissues, therefore, is not controlled by glucose concentration. The activity of hexokinase is not affected by fasting or carbohydrate feeding, diabetes, or insulin.

This unidirectional phosphorylation permits the accumulation of glucose in liver 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 is transferred to the C-l position of the glucose molecule. This reaction is catalyzed by the enzyme phosphoglucomutase (PGM) and involves glucose 1,6-diphosphate as an intermediate:

$$\text{Glucose-6-P} \rightarrow \text{glucose-l-P}$$

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

(UTP-glucose-1-phosphate uridylyltransferase):

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

In the presence of a polysaccharide primer and the enzyme glycogen (starch) synthase (glucosyltransferase) the glucose moiety of UDP-G is linked to the polysaccharide chain by an α l-4 link:

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

Through repeated transfers of glucose, the polysaccharide chain is lengthened. When the chain length of the polysaccharide reaches a critical level between 11 and 16 glucose units, the brancher enzyme, α-glucan glycosyl 4:6 transferase, transfers the terminal 7-residue portion from an ul-4 linkage to a 1-6 linkage. The newly established 1-6 linkage thus becomes a branch point in the glycogen molecule. The remaining stub can again be lengthened by the action of glycogen synthase. Approximately 1 % of the glucose units of the glycogen molecule are involved in these branch points.

4.3.2Glycogenolysis

The breakdown of liver glycogen to glucose, or glycogenolysis, takes place via a separate pathway. The key initiating and regulating factor in glycogenolysis is through the action of the hormones epinephrine on liver and muscle glycogen and of glucagon on liver glycogen only. The mechanism of action of glucagon and epinephrine is through a series of reactions which culminate in phosphorolytic cleavage of the 1-4glucosyl links of glycogen. In liver cells, glucagon and epinephrine stimulate the enzyme adenylate cyclase to form adenosine 3', 5'-cyclic monophosphate (cAMP) from ATP.

cAMP in turn activates a protein kinase which, in its turn, activates liver phosphorylase, the phosphorolytic enzyme. As with many enzymes, liver phosphorylase is present in an inactive form,

dephospho-liver phosphorylase (dLP) which is converted to its active form, liver phosphorylase (LP) by the proteinkinase, phosphorylase kinase.

The action of the phosphorylase is to cleave the 1-4 glycosyl links of glycogen by the addition of orthophosphate in a manner analogous to a hydrolytic cleavage with water, hence the analogous term "phosphorolysis." Phosphate is added to the C-l position of the glucose moiety while H^+ is added to the C-4 position of the other.

Cyclic AMP is also a key regulating factor in cellular processes beyond liver phosphorylase activation. It is required for the conversion of inactive muscle phosphorylase *b* to active muscle phosphorylase, again via phosphorylase *b* kinase. The actions of other hormones known to be mediated by activating adenylate cyclase and cAMP include ACTH, LH, TSH, MSH, T_3, and insulin. From these findings, a general concept of hormone action has evolved in which the hormone elaborated by the endocrine organ is described as the first messenger and cAMP within the target cell as the second messenger.

The action of glucagon on glycogen is confined to the liver while epinephrine acts on both liver and muscle glycogen. In liver, glucagon promotes the formation and release of glucose by increasing glycogenolysis and decreasing glyeogenesis, thus promoting hyperglycemia. An additional factor promoting hyperglycemia is the stimulation of hepatic gluconeogenesis by glucagon. Since the enzyme glucose-6-phosphatase (G-6-Pase) is absent from muscle, however, 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 glucose-l-P (G-l-P) units until a branch point in the glycogen molecule is reached. The residue is a limit dextrin. The débrancher enzymeamylo-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 débrancher enzyme, the glycogen molecule is successively reduced to G-l-P and free glucose.

G-l-P is convened to G-6-P by the reversible reaction catalyzed by phosphoglucomutase. 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. The free glucose formed can, unlike its phosphorylated intermediates, leave the hepatic cell to enter the general circulation, thereby contributing to the blood glucose pool. 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 lactate or Cori cycle. The series of reactions described are illustrated schematically in Fig. I.3.

4.3.3 Hormonal Influences on Glycogen Metabolism

The biochemical basis of the glycogenolytic and hyperglycemic action of glucagon and epinephrine. 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 on carbohydrate metabolism, indicating that metabolism should be considered an integrated concept.

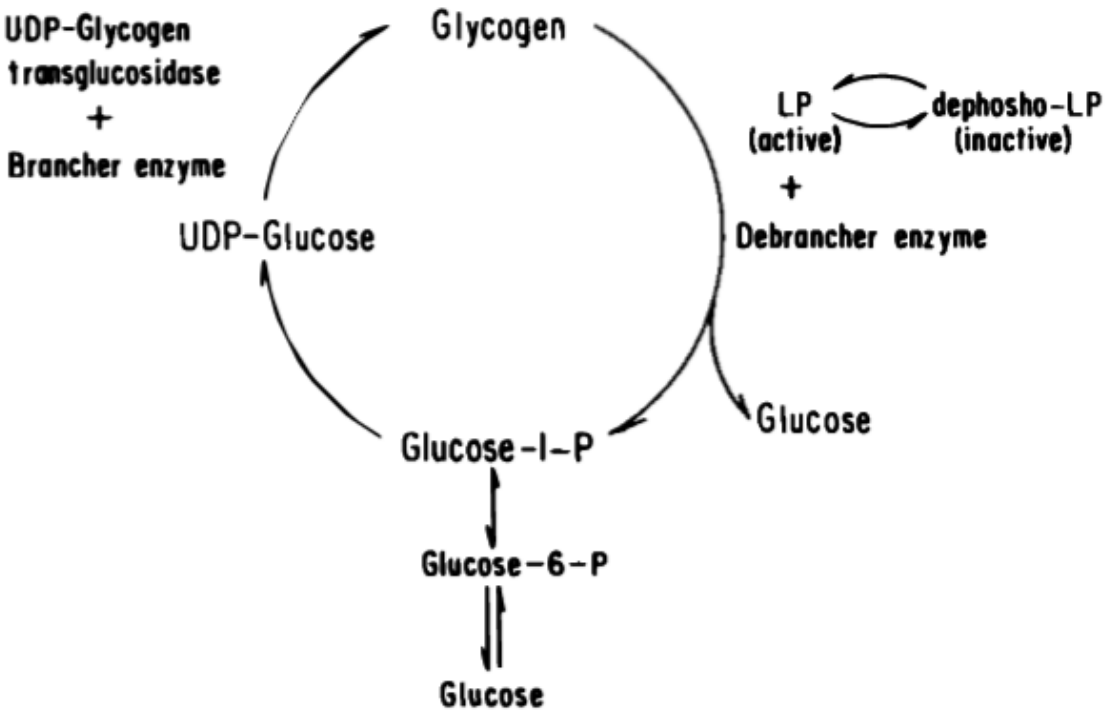


Fig. I.3 Summary of liver glycogen metabolism. In muscle, phosphorylase a is the active form and phospohorylase *b* is the inactive form. UDP, Uridine diphosonate; LP, liver phosphorylase.

One of the results of successful insulin therapy is a restoration of the depleted glycogen reserve. The mechanism of insulin action on carbohydrate metabolism continues to be a subject for intense study and is discussed more fully. 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 certain of its enzyme systems. In either event in the presence of insulin, glucose utilization toward glycogen synthesis or glucose oxidation is favored and a hypoglycemia follows.

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 an overall increase in 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 results of the increased rate of tissue metabolism. That hepatic G-6-Pase activities increased markedly in rats made hyperthyroid is consistent with the view that hepatic glucose production is increased in hyperthyroid states. An additional factor is the stimulation of glucose absorption by the gastrointestinal tract by thyroxine.

4.3.4 Glycogen in Disease

In systemic disease, alterations in glycogen are generally observed as decreases. Depletion of liver glycogen stores is seen in diabetes mellitus, starvation, bovine ketosis, ovine pregnancy toxemia, or in any condition with nutritional carbohydrate deficiency or increased carbohydrate turnover. Pathological increases in liver glycogen occur in the rare glycogen storage diseases (GSD).

5 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 on 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 subsequently 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 emphasis here is placed on the interrelationships of the pathways rather than details of the individual reactions.

5.1 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. 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: production of free glucose, glycogenesis, glycolysis, the hexose monophosphate pathway, and the glucuronate pathway.

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

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

This is essentially an irreversible reaction and opposes the previously described unidirectional glucokinase reactions. These two opposing and independently catalyzed enzyme reactions offer a site of metabolic control whereby the activities of the enzymes play a regulatory role. Significant amounts of G-6-Pase are found only in 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 supply of glucose for the maintenance of blood glucose concentration.

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

b. Glycogenesis. The glycogenesis pathway for G-6-Pase leading to the synthesis of glycogen.

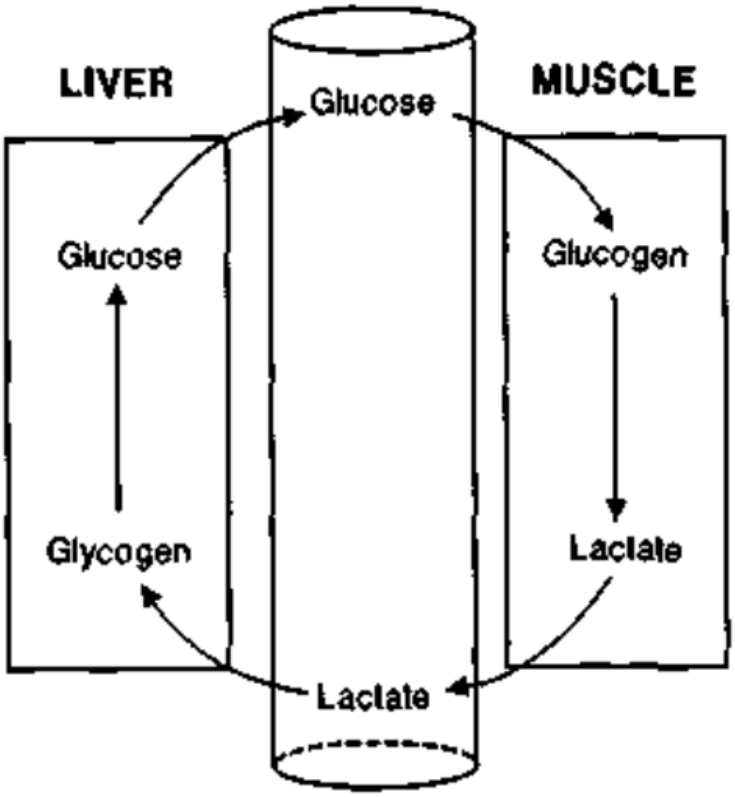


Fig. I.4 The lactate or Cori cycle. Muscle cells are devoid of glucose-6-phosphatase; therefore, muscle glycogen contributes indirectly to blood glucose by this pathway.

c. Glycolysis. One of the three oxidative pathways of G 6-P is the classic anaerobic glycolytic Embden-Meyerhof pathway (EMP). The intermediate steps involved in this pathway of breakdown of G-6-P to three- carbon compounds are summarized in Fig. I.5. A 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_2). 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-P2ase-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 2 mol high-energy phosphates from ATP have been donated to form 1 mol of F-1,6-P_2.

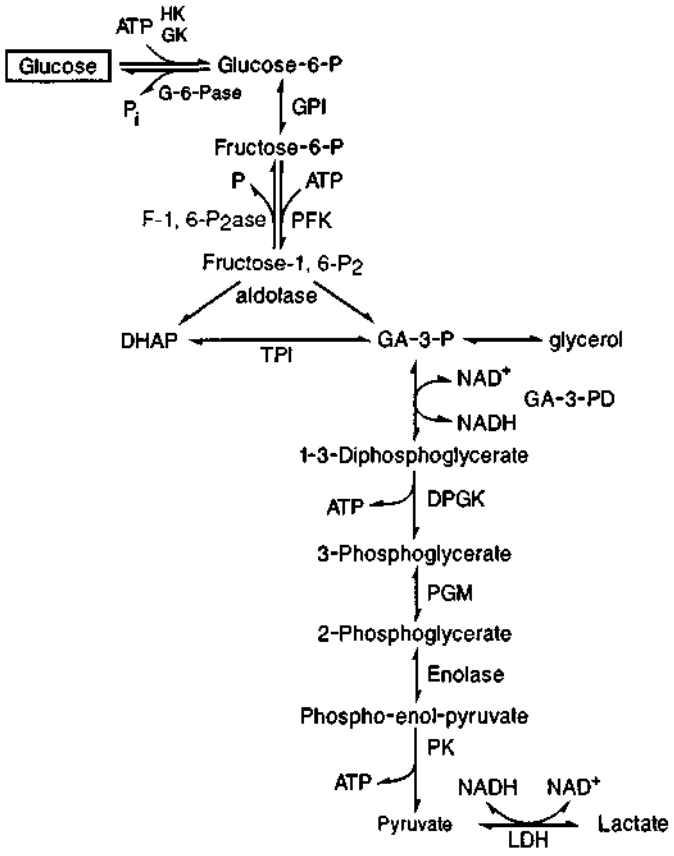


Fig. I.5 The glycolytic or classic Embden-Meyerhof pathway (EMP). Note that 2 mol ATP is used and 4 mol ATP is generated. Abbreviations: ATP, adenosine tri-phosphate; DHAP, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde 3-phosphate; NAD^+, nicotinamide adenine dinucleotide; P_i, inorganic phosphate.

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

A third site of control of glycolysis is the unidirectional formation of pyruvate in the reaction catalyzed by pyruvate kinase

(PK). In its reverse direction. PK is circumvented by two enzymatic reactions. Pyruvate carboxylase (PC) catalyzes the carboxylation of private ate to oxaloacetate (OAA), and the OAA is converted to phosphoenolpyruvate (PEP) by the enzyme PEP carboxykinase (PEP-CK.).

Thus, the over cell conversion of 1 mol glucose to 2 mol pyruvate recuircs 2 mol ATP for the initial phosphorylations, and total of 4 mol ATP are generated in the subsequent dephosphorylations. This net gain of 2 mol ATP represents the useful energy of glycolysis.

For repeated functioning of the glycolytic pathway, supply of NAD^+ must be available for use in the oxidative (GA-3-PD) step. In the presence of molecular O_2, reduced NADH 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 is reoxidized to NAD^+ in a reaction catalyzed by lactate dehydrogenase (LDH), whereby pyruvate is reduced to lactate. Thus, by "coupling" the LDH system to the GA-3-PD system, glucose breakdown may continue in periods of anaerobiosis.

d. Hexose Monophosphate Pathway. The alternate route of G-6-P oxidation has been variously referred to as the pentose phosphate pathway (PPP), direct oxidative pathway, Warburg-Dickens scheme, or the hexose monophosphate pathway (HMP) or "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. I.6. The reaction is catalyzed by elucose-6-phosphate dehydrogenase (G6P-D), and in this pathway, oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$) serves as the hydrogen acceptor. In the second oxidative step, 6-PG is oxidatively decarboxylated by 6-phosphogluconate dehydrogenase (6-PG-D) to yield a pentose phosphate, ribulose

5-phosphate (Rib-5-P) again in the presence of $NADP^+$. Thus, in the initial reactions, which are essentially irreversible, 2 mol of NADPH 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 the relative contributions of the EMP and the HMP have been assessed in domestic animals.

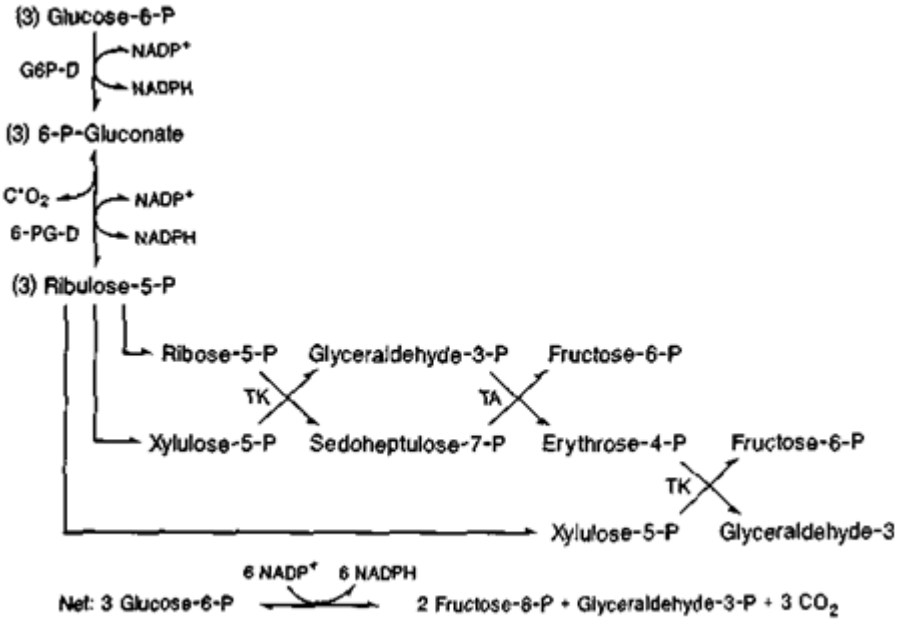


Fig.I.6 The pentose phosphate pathway (PPP) or hexose monophosphate pathway (HMP). Abbreviations: $NADP^+$, nicotinamide adenine dinucleotide phosphate; TK, transketolase; TA, transaldolase; CO_2, derived from C-1 of glucose.

The subsequent metabolism of the Rib-5-P in the HMP is also shown in Fig. I.6. As a result of the series of transformations, F-6-P and GA-3-P are formed which serve as recycling links into the EMP pathway. For continued functioning of the HMP pathway, a supply of $NADP^+$ must be available as the hydrogen acceptor. $NADP^+$ is regenerated from NADPH via the cytochrome system in

the presence of O_2, and thus the HMP pathway is an aerobic pathway of glucose oxidation. Reduced NADPH 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. Accordingly, the availability of NADPH generated in the HMP pathway is essential for use in the synthesis of fat. Generally, the HMP pathway is the major source of the NADPH which is required to maintain the reducing environment in all biosynthetic processes using NADPH as a cofactor.

e. Glucuronate Pathway. The glucuronate pathway is an alternate route of G-6-P oxidation which has been termed the uronate pathway, glucuronate pathway, or the C_6 oxidative pathway (Fig. I.7). Important contributions to its clarification, particularly in relation to L-xylulose metabolism and ascorbic acid synthesis.

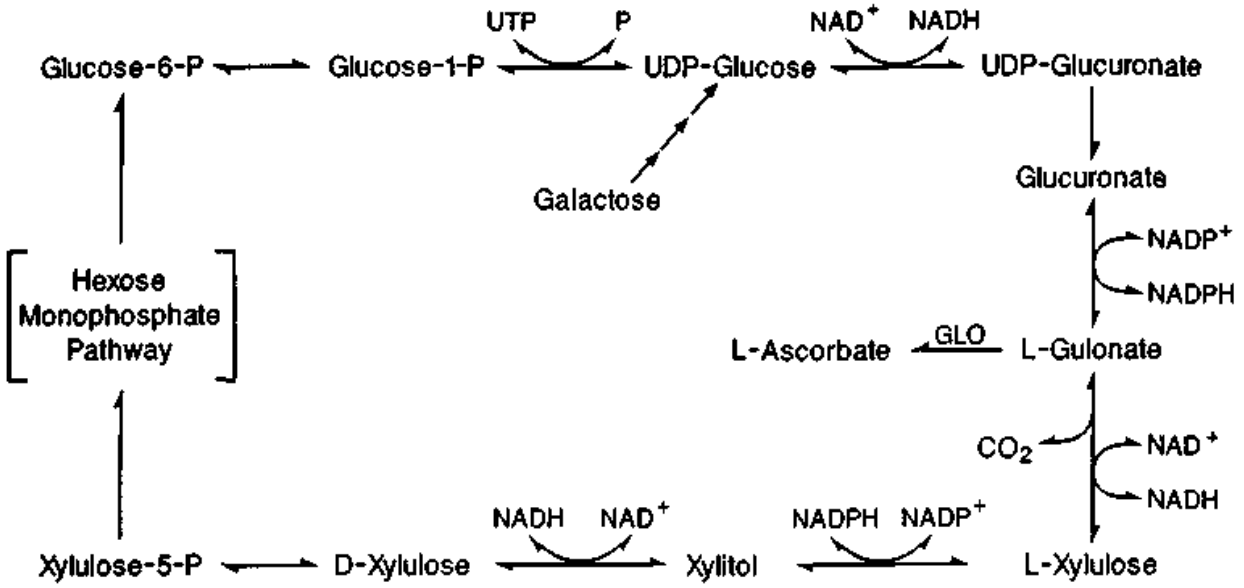


Fig. I.7 The glucuronate pathway or C-6 oxidation pathway. Note that vitamin C is synthesized via this pathway.

The initial steps of this pathway involve the formation of uridine diphosphoglucose (UDP-G), also an intermediate in glycogen synthesis. G-6-Pis first converted to G-l-P, which then reacts with uridine triphosphate (UTP) to form UDP-G. This product is then oxidized at the C-6 position of the glucose moiety

in contrast to the C-l position in the HMP pathway. This reaction requires NAD^+ as a cofactor, and the products of the reaction are uridine diphosphoglucuronic acid (UDPGA) and NADH. This UDPGA is involved in a large number of important conjugation reactions in animals, e.g., bilirubin glucuronide formation, synthesis of mucopolysaccharides chondroitin sulfatel which contain glucuronic acid, and generally in detoxification reactions. UDPGA is cleaved to release D-glucuronate and UDP.

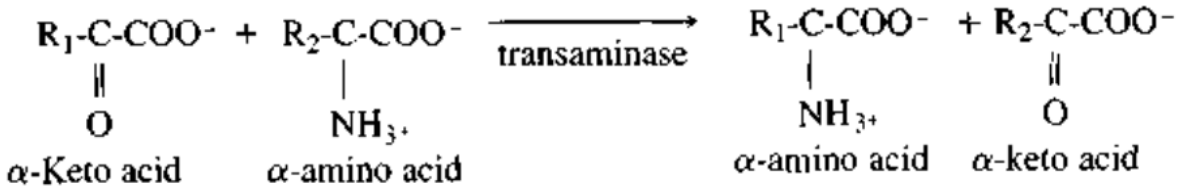
D-Glucuronate is next reduced to L-gulonate in the presence of the enzyme gulonate dehydrogenase GUD), with NADPH as the hydrogen donor. The L- culonate may follow a pathway leading to the synthesis M a pentose, L-xylulose, the compound found in the urine in pentosuria (L-xylulose) of humans. In this reaction, the C-6 carbon of L-gulonate is evolved as CO_2. Next, the reduction of L-xylulose to xylitol is catalyzed the enzyme L-xylulose reductase, the enzyme which deficient or blocked in pentosuria of humans. As shown in Fig. 7, xylitol is converted to D-xylulose which is phosphorylated to D-xylulose-5-P, and a cyclical pathway incorporating the HMP pathway may occur. L-gulonate is also converted by enzyme-catalyzed reactions to L-ascorbate in those species which can synthesize their own vitamin C, i.e. all domestic animals. The enzyme L-gulonolactone oxidase (GLO), is lacking in humans, nonhuman primates, and in guinea pigs. The enzyme is present only in the liver in mice, rats, pigs, cows, and dogs. In dogs, the liver synthetic enzyme activity is low and the ascorbate hydrolytic activity is high so that dogs may have additional needs for vitamin C in times of stress. For the synthesis of L-ascorbic acid, D-galactose may be an even better precursor than D-glucose. This pathway is also indicated in Fig. I.7.

5.2 Terminal Oxidation

The metabolic pathways described thus far are those followed essentially by carbohydrates alone. In analogous

fashion, the breakdown of fats and proteins also 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 a-ketoglutarate from transamination of their corresponding a-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 alt, which also serves as a means for interconversions between them.

a. Pyruvate Metabolism. The pathway for breakdown of glucose to pyruvate. Pyruvate is next oxidatively decarboxylated in *a* complex enzymatic system requiring the presence of lipoic acid, thiamine pyrophosphate, coenzyme A (CoA). NAD^+, and pyruvate dehydrogenase (PD) to form acetyl-CoA and NADH. Pyruvate may follow a number of pathways, as outlined in Fig. I.8. By the mechanism of transamination, pyruvate may be reversibly converted to alanine. The general reaction for transamination may be written as follows:



Where the amino group of an amino acid is transferred to the *α* position of an α-ketoacid and, as a result, the amino acid is converted to its corresponding α-ketoacid. 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 the chapters on liver and muscle elsewhere in this volume.

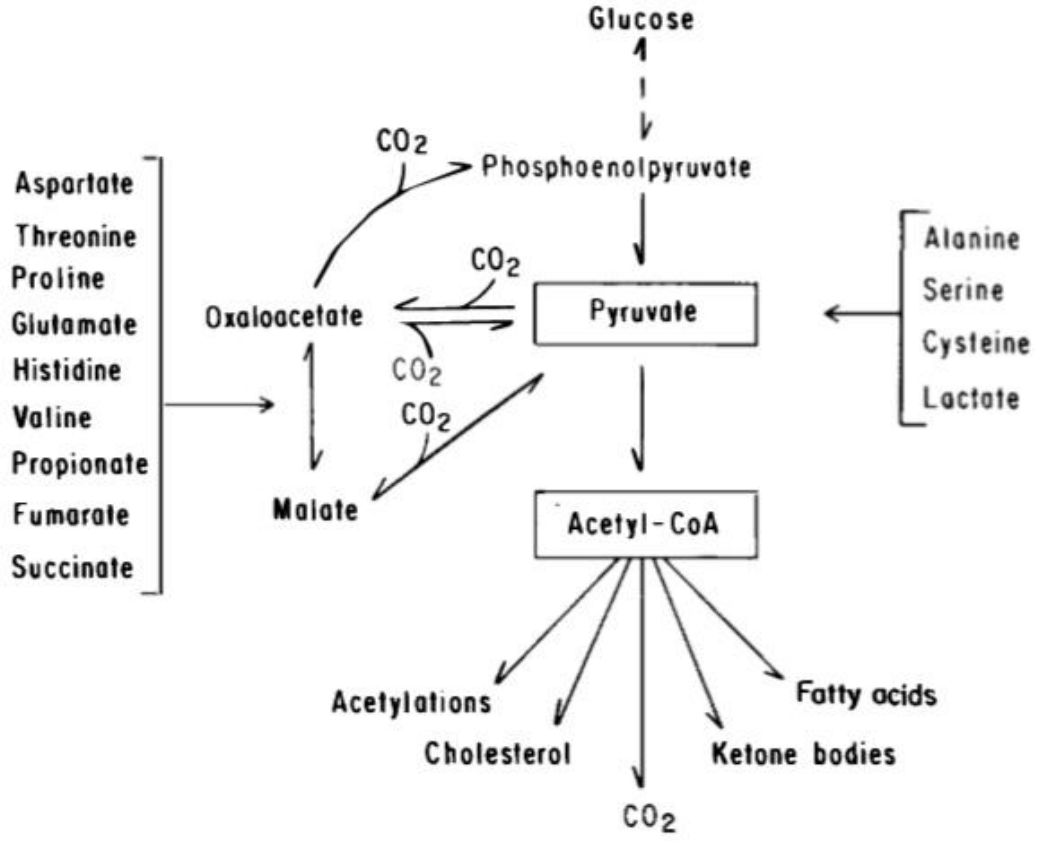


Fig. I.8 Pathways of acetate and pyruvate metabolism.

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

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

b. Tricarboxylic Acid Cycle. Acetyl-CoA formed as a result of oxidative decarboxylation of pyruvate also has a number of possible metabolic fates. This compound occupies a central position in synthetic as well as oxidative pathways, as shown in Fig. I.8. The oxidative pathway leading to the breakdown of acetyl- CoA to CO_2 and H_2O, follows a cyclical pathway called the tricarboxylic acid (TCA) cycle, citric acid cycle, or Krebs cycle. The major steps involved are given in Fig. I.9. In a single turn of the cycle, 1 mol acetyl-CoA enters, 2 mol CO_2 are evolved, and 1 mol oxaloacetate (OAA) is regenerated. The regenerated OAA may then condense with another mole of acetyl-CoA, and the cycle continues. It should be noted that citric acid is a symmetrical molecule which behaves asymmetrically, as shown in Fig. I.9. Also, the CO_2 is derived from the portion of the molecule contributed by oxaloacetate during each turn of the cycle. The expected distribution of carbon atoms from acetyl-CoA in one turn of the cycle is also given in Fig. I.9. During one turn of the cycle, a randomization of carbon atoms occurs at the level of succinate 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. I.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.

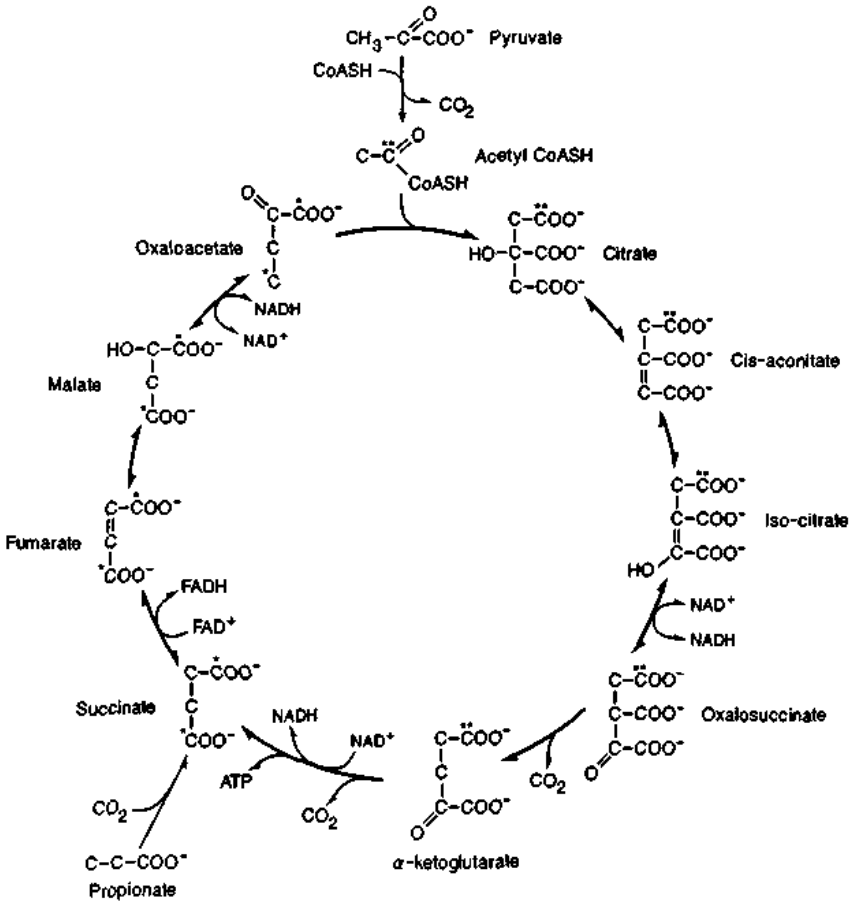


Fig. I.9 The tricarboxylic acid cycle. The pathway tor of propionate into the metabolic scheme is also increased. Asterisks give the distribution of carbon in a single turn of the cycle starting with acetyl-CoA. Note the randomization of carbon atoms at the succinate step.

5.3 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 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 thence other glycolytic intermediates as shown in Fig. I.8. Continued losses of these intermediates to 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

losses of cycle intermediates may be balanced by replacement from other sources (Fig. I.8). The amino acids aspartate and glutamate 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, may also function as important sources of supply. A fourth CO_2-fixing reaction is known, which is of especial importance in ruminant metabolism for maintaining the supply of intermediates in the TCA cycle.

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

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

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

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

Propionate is one of the three major fatty acids, along with acetate and butyrate, involved in ruminant metabolism.

5.4 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 the energy is dissipated as heat. The total available chemical energy in the reaction

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

is about 50 kcal/mol or about 7 % of the 690 kcal/mol 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 mol 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 or NADPH (FAD in the succinate step) is formed. In the presence of molecular O_2, these

compounds are reoxidized to NAD^+ or $NADP^+$ in the cytochrome system. In the sequence of reactions comprising this system, 3 mol ATP are formed per mole of NADH or NADPH oxidized to NAD^+ or $NADP^+$. 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 ($^1/_2O_2$) is conventionally referred to as the P:O ratio, which in this case is 3.

In Table I.3, a balance sheet of the ATPs formed in the various steps are given. The complete oxidation of 1 mol glucose to CO_2 and water yields about 690 kcal, and therefore the net gain of 38 ATPs in the biological scheme represents about 266 kcal for an overall efficiency of 38 %.

Table I.3

ATP Yield in Glucose Oxidalion

Step	ATP
Glucose ATP (2 x)-	 – 2
fructose 1,6 diphosphate NADH – 3 ATP (2 x) ATP (4 x)	 + 6 + 4
2 pyruvate NADH – 3 ATP (2 x)	 + 6
2 acetyl-CoA NADH – 3 ATP (6 x) ATP (2 x) FADH – 2 ATP (2 x) 4 CO_2	 + 18 + 2 + 4
Net: Glucose – 6 CO_2	+ 38 ATP

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

The relationship between carbohydrate and lipid metabolism deserves special mention because the carbohydrate economy and the status of glucose oxidation strongly influence lipid metabolism.

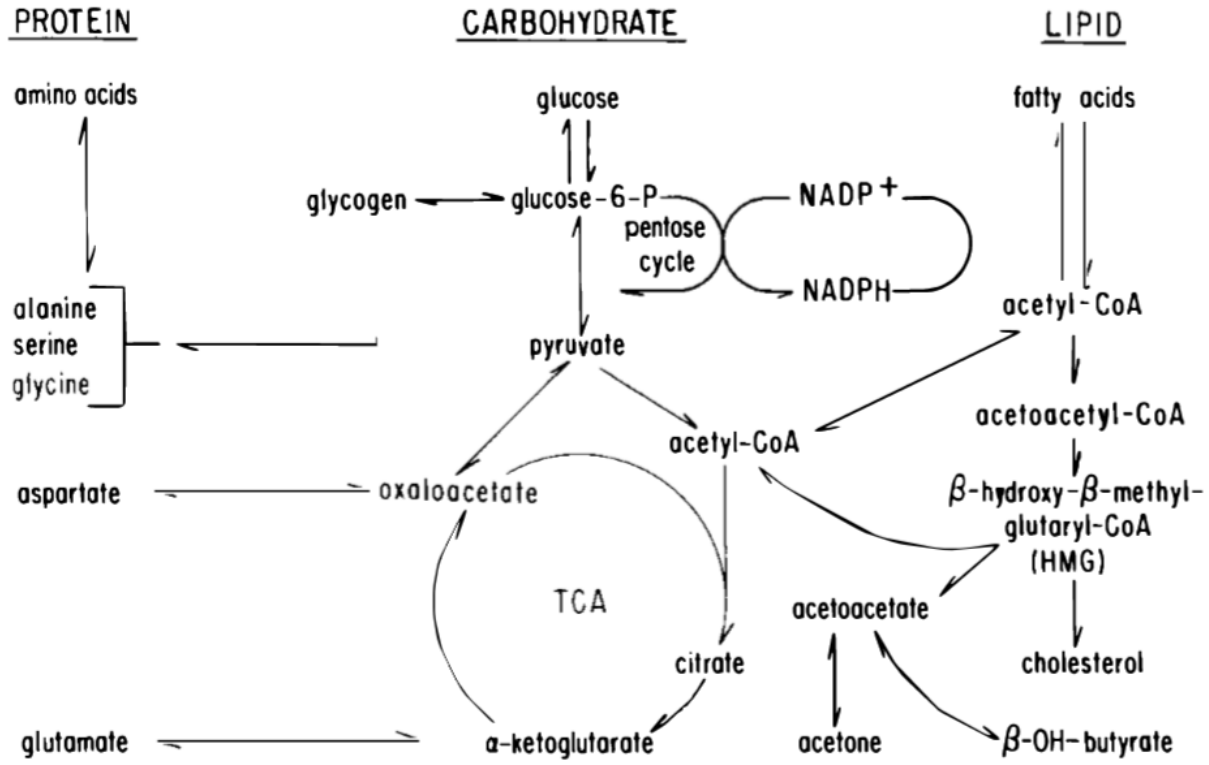


Fig. I.10 Interrelationships of carbohydrate, protein, and lipid metabolism.

6.1 Lipid Metabolism

6.1.1 Oxidation of Fatty Acids

Intracellular 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 into the mitochondria where fatty acyl-CoA is released for imramitochondria) oxidation.

The classic β-oxidation scheme for the breakdown of fatty acids whereby 2-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 ratty acid residue 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^+ and FAD. The further oxidation of acetyl-CoA to CO; and water proceeds in the common pathway of the TCA cycle. In the process, 2 moles of CO_2 are evolved per mole of acetyl-CoA entering the cycle. Therefore, fatty acids could not theoretically lead to a ret synthesis of carbohydrate. Net synthesis of carbo-hydrate from fatty acids would require the direct conversion of acetyl-CoA into some glucose precursor, i.e., pyruvate. Because the reaction

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

is irreversible, however, 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.

6.1.2 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 lipogenesis diverges from that of oxidation. The first point of divergence involves the initial condensation reaction. CO, is a requirement, yet there is no evidence for the incorporation of the CO_2; into fatty acid. This

suggested a pathway involving the initial synthesis of malonyl-CoA. Malonyl-CoA is condensed with an aldehyde or acetyl-CoA, 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 has now been well established.

The second point of divergence involves the requirements for NADPH as the hydrogen donor rather than NADH or FADH. The major source of NADPH is the oxidation of glucose via the HMP pathway. NADPH concentrations are also high in the cytoplasm of tissue cells such as in liver and adipose tissue where HMP activity is also high. The availability of this NADPH is the basis for the linkage of carbohydrate oxidation to lipogenesis.

6.1.3 Synthesis of Cholesterol and Ketone Bodies

Acetyl-CoA is also the precursor of cholesterol and ketone bodies, acetoacetate, β-hydroxybutyrate, and acetone. The synthesis of cholesterol proceeds through a complicated series of reactions beginning with the stepwise condensation of 3 mol acetyl-CoA to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). As shown in Fig. 10. HMG-CoA is a common intermediate for the synthesis of both cholesterol and ketone bodies in the liver cell. In liver, a deacylating enzyme is present which cleaves HMG-CoA to yield acetyl-CoA and free acetoacetate. This is termed the HMG-CoA cycle. The free acetoacetate 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 acetoacetyl-CoA. Increased ketogenesis and ketonemia are the net result of alterations in metabolic pathways and/or enzymes which favor the accumulation of acetoacetyl-CoA as seen in diabetes mellitus.

The increased mobilization and utilization of fatty acids is a well-known requisite for ketogenesis under conditions of

starvation and diabetes. Under these same conditions, lipogenesis from acetyl-CoA is also depressed. The net effect of either or both of these alterations favors the accumulation of acetoacetyl-CoA and thus ketogenesis.

Intrahepatic processes are now recognized as also very important in ketogenesis. 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. A depletion of OAA occurs but that it is the result of an increase in the reductive environment of the cell which is required for synthetic purposes, i.e., gluconeogenesis. An increased NADH/NAD ratio is cited as evidence which would promote 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. In OAA deficiency, acetyl-CoA would then be readily diverted to ketone bodies.

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

6.2 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 whereas the major site of lipogenesis in animals is adipose tissue. The rate of fat synthesis by the liverless animal is comparable to that of the intact animal, and, *in vitro,* adipose tissue converts glucose to fatty acids even faster than 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 (RQ), 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 both to stimulate lipogenesis and to have a sparing effect on fatty acid oxidation. In a condition with relative lack of carbohydrate, i.e., diabetes, with 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, and exercise, there is an increased release of glucose precursors (amino acids) from muscle and free fatty acids from adipose tissue mediated by activated hormone-sensitive lipases. The amino acids and free fatty acids are transported to the liver where hepatic gluconeogenic pathways are activated and fatty acid 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. The net result is an overproduction of glucose and ketones in liver and an underutilization of both in peripheral tissues.

7 INSULIN AND CARBOHYDRATE METABOLISM

The internal secretions of the anterior pituitary, adrenal cortex and medulla, and the pancreas are closely associated with carbohydrate metabolism. The pituitary and adrenal factors have previously accumulated on its role in carbohydrate metabolism. Although the intricate details of insulin action are still being studied, a basic understanding of the major biochemical events which occur in animals with and without insulin has evolved.

7.1 Proinsulin and Insulin

The elucidation of the structure of insulin by Sanger was soon followed by the discovery of its precursor, proinsulin, and the elucidation of its structure. It has been the subject of many reviews, most recently by Kitabchi. Proinsulin is now known to be a single-chain looped polypeptide linked by disulfide bridges. 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 f3 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.

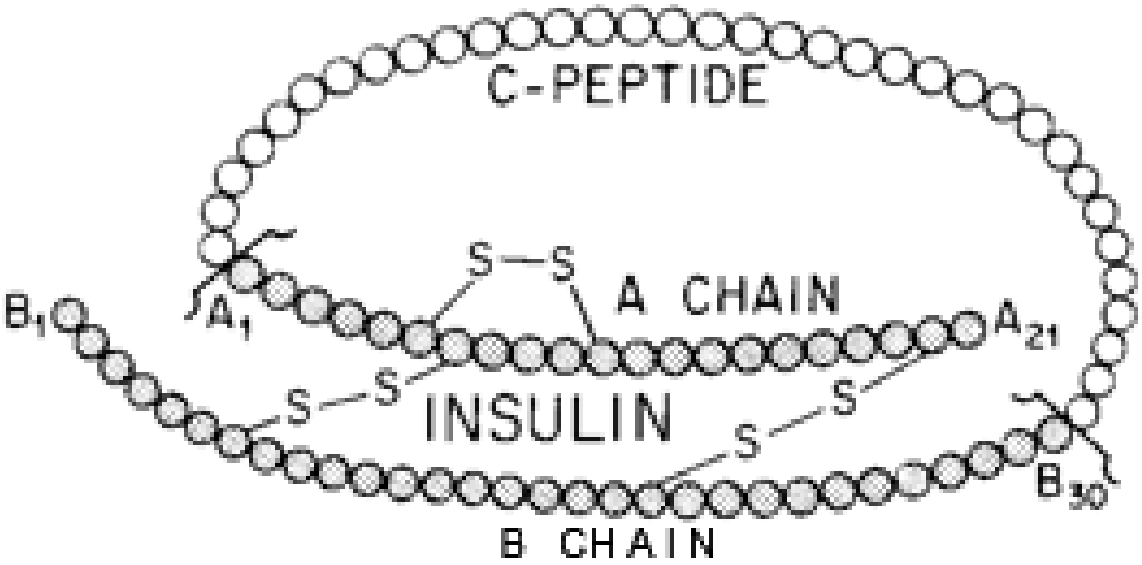


Fig. I.11 Insulin and proinsulin. Proinsulin is the coiled polypeptide. When the connecting C-peptide (open circles), is removed, the insulin molecule (solid circles) is released.

Teased into the circulatory but is devoid of biological activity. Its circulatory concentration is higher than that of insulin mainly due to its slower clearance as evidenced by its half-life of about 20 minutes. C-Peptide is primarily degraded by the kidney, and a portion is excreted in the urine.

As the insulin moiety is released from proinsulin, it crystallizes with zinc for storage in the β-cell granules. The dense central inclusions of these insulin secretory granules consist mainly of crystalline insulin. Insulin release is stimulated by glucose, amino acids, hormones (glucagon, gastrin, secretin, pancreozymin), and drugs (sulfonylureas. isoproterenol). Insulin release is inhibited by hypoglycemia, somatostatin, and many drugs (dilantin. phenothiazines). The liver is the primary site of insulin degradation and the kidney is a secondary site. The half-life of insulin in the circulation is between 5 and 10 minutes.

The A chain of insulin is made up of 21 amino acids and the B chain is made up of 30 amino acid residues (Fig. I.11). The molecular weight is 6.000, and the monomer 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. Differences occur within species also since rats and mice have two nonallelic insulins. The 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 immunologic behavior.

The amount of insulin stored in the pancreas by various species differs. The dog stores about 3.3 U per gram of pancreas, which amounts to about 75 U in a 10 kg dog. This amount, if suddenly released, would be fatal.

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

Table I.4

Species Variation in Amino Acid Sequences of Insulin

Position b							
	A chain				B chain		
Species	A-4	A-8	A-9	A-10	B-3	B-29	B-30
Human	Glu	Thr	Ser	Ile	Asp	Lvi	Thr
Monkey	Glu	Thr	Ser	Ile	Asp	Lys	Thr
Dog	Glu	Thr	Ser	Ile	Asp	Lys	Ala
Pig	Glu	Thr	Ser	Ile	Asp	Lys	Ala
Sperm whale	Glu	Thr	Ser	Ile	Asp	Lys	Ala
Rabbit	Glu	Thr	Ser	Ile	Asp	Lys	Ser
Horse	Glu	Thr	Gly	Ile	Asp	Lys	Ala
Cow	Glu	Ala	Ser	Val	Asp	Lys	Ala
Sheep	Glu	Ala	Gly	Val	Asp	Lys	Ala
Sei whale	Glu	Ala	Ser	Thr	Asp	Lys	Ala
Rat 1	Asp	Thr	Ser	Ile	Lys	Lys	Ser
Rat 2	Asp	Thr	Ser	Ile	Lys	Met	Ser

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 (IVGTT) exhibits two peaks in humans, the early 5-minute peak representing release and the second 10- to 30-minute peak representing *de novo* insulin synthesis. This bimodal curve is not clear in dogs.

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 (RIA). While diabetes studies in humans have focused on all three, 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 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 varying degrees and sufficient to form a basis for a postulated enteroinsular axis. The hormones implicated are secretin cholecystokinin-pancreozymtn (CCK-PZ), gastrin, glucagon-like activity of the gut, (GLA) and gastric inhibitory peptide (GIP). Except for GIP, the evidence for insulin control is tenuous. GIP is now known to be a powerful stimulator of insulin secretion in humans and dogs, and this is associated with changes on blood glucose. Thus, GIP is most likely to be central in any enteroinsularaxis.

Once in the general circulation, insulin is transported to responsive tissues bound to a β-globulin, At the responsive tissue, insulin is bound to receptor sites on the cell membrane. 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.

7.2 Mechanism of Insulin Action

It is well established that the principal site of insulin action is in the initial phases of glucose metabolism. In order for extracellular glucose to gain entry into the metabolic pathways, it must first enter the cell and second be phosphorylated. Insulin acts on its target tissues by first binding to insulin receptors on or in the target cell plasma membranes. Insulin then 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

steps in the metabolism of glucose are enhanced.

Insulin also influences the metabolism of glucose by cells in the liver, the central organ of glucose homeostasis. Liver cells are 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. Additionally, the effect of insulin on other key directional phosphorylative steps directs glucose metabolism toward utilization and fatty acid synthesis. A significant effect of insulin is to increase the activity of the pyruvate dehydrogenase system, thereby effecting increased fatty acid synthesis and oxidation via the Krebs cycle. Thus, there is a dual role for insulin, affecting membrane transport in muscle and adipose tissue and phosphorylation via GK in liver.

In nerve cells, insulin also binds to receptors and affects membrane transport of glucose, but in this case the membrane transport system itself appears to be the limiting factor. Thus, even though the HK system can operate maximum, the limited glucose transport at about 1.5 mmol/ liter (27 mg glucose/dl) induces symptoms of hypoglycemia, i.e., incoordination, disorientation, weakness.

In other cells, such as the red blood cell with the HK system, insulin does not affect glucose metabolism or limit transport. The HK system can operate maximally, and glucose is efficiently utilized by the red blood cell.

7.3 Effects of Insulin

The most characteristic finding following insulin administration is a hypoglycemia. This 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 (RQ) increases toward unity, indicating that the animal is now primarily utilizing carbohydrate.

Table I.5

Effects of Insulin on Animais

Tissue	Increase	Decrease
Whole animal	Anabolism Food intake Respiratory quotient	
Blood		Glucose Ketones Fatty acids Phosphate Potassium Amino acids Ketone bodies
Enzymes	Glucokmase Phosphofructokinase Pyruvate kinase Lipoprotein lipase Acetyl-CoA carboxylase Glycogen synthase	Glucose-6-phosphatase Fructose-1.6-diphosphatase Pyruvate carboxylase PEP carboxykinase Carnitine acyltransferase Serine dehydratase Hormone-sensitive lipase
Liver	Glucose oxidation Glycogen synthesis Lipid synthesis Protein synthesis	Glucose production Ketogenesis
Muscle (skeletal/heart)	Glucose uptake Glucose oxidation Glycogen synthesis Amino acid uptake Protein synthesis Potassium uptake	
Adipose	Glucose uptake Glucose oxidation Lipid synthesis Potassium uptake	

The consequences of this increased utilization of glucose follow 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 while 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.

7.4 Other Pancreatic Islet Hormones

7.4.1 Glucagon

Glucagon is a polypeptide hormone (molecular weight 3485) secreted by the α (A) cells of the islets. Release of glucagon is stimulated by decreased glucose concentrations. Glucagon then increases glucose by stimulating hepatic glycogenolysis and gluconeogenesis. Glucagon does not stimulate muscle glycogenolysis.

7.4.2Somatostatin

Somatostatin is secreted by many cells, including hypothalamus cells, but its major source is the pancreatic Δ (D) cells. It has broad inhibitory effects on the release of many hormones, including glucagon and insulin, and it therefore has a modulating effect on the actions of these two hormones.

8 BLOOD GLUCOSE AND ITS REGULATION

8.1 General

The blood glucose concentration depends on a wide variety of factors, and the concentration at any time is the net result of an equilibrium between the rates of entry and of removal of glucose in

the circulation. As such, all the factors which exert influence on entry or removal become of importance 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 influencing the maintenance of the blood glucose concentration. The blood glucose levels at which this occurs varies between species (Table I.6).

Table I.6

Renal Thresholds for Glucose in Domestic Animals

Species	Threshold	
	mg/dl	mmol/liter
Dog	180-220	10.0-12.2
Horse	180-200	10.0-11.1
Cow	98-102	5.4- 5.7
Sheep	160-200	8.9-11.1
Goat	70-130	3.9- 7.2

8.2 Glucose Supply and Removal

Glucose may 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 hormones epinephrine and glucagon promote the release of glucose from glycogen. The influence of the glucocorticoids is to promote gluconeogenesis and to oppose the hypoglycemic action of insulin.

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

for conversion to other products (glycogen, pentoses, lipids, amino acids). As such, an outflow from the circulation 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 n 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 of 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; oxidized to lactate or CO_2, and that the remainder goes to glycogen or is released as free glucose. Muscle, on the other hand, does not contain G-6-Pase and is therefore primarily a glucose-utilizing tissue.

8.3 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. It has been demonstrated that at a blood glucose level of approximately 8.33 mmol/liter (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 the mechanisms of normal supply and removal are operating at equal rates. Above 8.33 mmol/ liter (150 mg/dl), glucose removal is greater than supply, and below 8.33 mmol/liter (150 mg/dl), glucose supply is greater than removal. Since the fasting blood glucose level in

most animals is about 5 mmol/liter (90 mg/dl), this means that the liver supplies glucose throughout the day except for the few postprandial periods when blood glucose is greater than 8.33 mmol/ liter (150 mg/dl). Insulin decreases liver glucose output and glycogenolysis and increases glucose uptake by the liver. This directional control results from 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. The opposing G-6-Pase reaction increases in fasting, 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. The increases in activity of G-6-Pase and other gluconeogenic enzymes in the absence of insulin then provides 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 growth hormone. The glucocorticoids increase gluconeogenesis and intracellular G-6-P and by their insulin opposing effect, increase free glucose. An increase also results from the glycogenolytic action of epinephrine and glucagon, and the equilibrium is shifted to favor glucose production. Therefore, the balance of the hormones which directly (insulin) or indirectly (epinephrine, growth hormone, glucagon, cortisol) exert their influence on glucose metabolism sets the homeostatic or glucostatic level of

glucose which controls hepatic glucose uptake or production.

8.4 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. I.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.

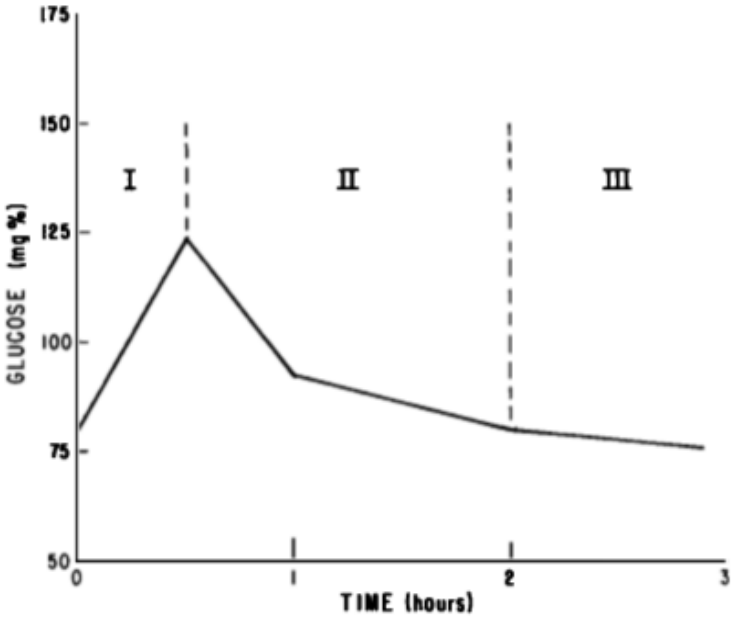


Fig. I.12 Oral glucose tolerance in the dog, I, 11, and III are phases of the curve.

9 METHODOLOGY

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

9.1 Blood Glucose

9.1.1 Methods

All early blood glucose methods were based on the reducing properties of glucose. These methods used an alkaline copper solution containing cupric (Cu^2+) copper which is reduced to the cuprous (Cu^+) form, which subsequently combines with phosphomolybdate or ar-senomolybdate to produce colored compounds. Another color reaction uses the reduction of ferricyanide to ferrocyanide to form Prussian blue. This is the basis for a micromethod of Folin and Malmros. These methods are now replaced by more simple and highly glucose-specific methods.

The o-toluidine method is 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 glucose specific enzyme methods are in use: the glucose oxidase, hexokinase, and glucose dehydrogenase (GD) methods. The glucose oxidase method is coupled with peroxidase and a dye. Glucose oxidase catalyzes the conversion of glucose to gluconic acid:

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

The hydrogen peroxide, with peroxidase, oxidizes a dye to form a colored product. This principle is used in the glucose-specific paper strips for urine glucose. In the hexokinase method, hexokinase catalyzes the phosphorylation of glucose, and the reaction is coupled to another reaction such as G6P-D, for assay:

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

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

In the glucose dehydrogenase method, GD catalyzes the following reaction:

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

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 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 s ample vial containing an anticoagulant.

9.1.2 Blood Glucose Levels in Animals

The normal ranges for blood glucose levels are given in Table I.7. 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.

Table I.7

Blood Glucose Levels in Domestic Animals

Species	Glucose (average)	
	mg/dl	mmol/liter
Horse	75-115 (95 ± 8)	4.2-6.4 (5.3 ± 0.4)
Cow	45-75 (57 ± 7)	2.5-4.2 (3.2 ±0.4)
Sheep	50-80 (68 ± 6)	2.8-4.4 (3.8 ± 0.3)
Goat	50-75 (63 ± 71)	2.8-4.2 (3.5 ± 0.4)
Pig	85-150 (119 ± 171)	4.7-8.3 (6.6 ± 0.9)
Dos	65-118 (90 ± 8)	3.6-6.5 (5.0 ± 0.4)
Cat	50-75 (63 ± 7)	2.8-4.2 (3.5 ± 0.41)
Monkey (*Macaca sp*.)	85-130 (107 ± 13)	4.7-7.3 (5.9 ± 0.7)

This is accomplished in the nonruminant and in the young ruminant by a standard 1 6- 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.

9.2 Tolerance Tests

9.2.1 Glucose Tolerance Tests

Tolerance in its original usage referred to the amount of glucose which could be ingested by an animal without producing a glucosuria-hence, tolerance for glucose. Since in the normal animal, the absence of a glucosuria indicates only a limited rise in blood glucose where the renal threshold is not exceeded, glucose tolerance now refers to the blood glucose level following glucose administration. Accordingly, an animal with an increased glucose tolerance is one which shows a limited rise in blood glucose while the animal with a decreased tolerance shows an excessive rise.

It is important to ascertain the nature of the anima''s diet, especially in omnivores and carnivores, prior to performance of this test. A carbohydrate only diet favors a minimum rise in the tolerance curve while a carbohydrate-free diet (meat only) favors a high or diabetic type of glucose tolerance curve. Therefore, the diet must be standardized by placing the dog. e.g., on a standard diet of 100-200 g carbohydrate plus fat and protein per day for 3 days prior to performance of the test. The tolerance curve is also affected by the status of the intestinal absorptive process, i.e., inflammation, increased motility, thyroxine. 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). 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 a test meal consisting of 4 g glucose/kg 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 (see Fig. I.12). The maximum level. 6.6-7.7 mmol/liter (120-140 mg/dll, was reached within 1 hour and returned to the fasting level, 3.6-5.3 mmol/liter (65-95 mg/dl), in 2-3 hours. The OGTT may be simplified by taking a single sample at 2 hours after giving the glucose, i.e., 2-hour postprandial glucose. A normal blood glucose level at 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 glucose tolerance test.

b. Intravenous Glucose Tolerance Test (IVGTT) and the Insulin Response. Standardization of the IVGTT in animals has been described and is recommended. It has been shown that urinary glucose loss as well as the glucose clearance half-times are directly proportional to the glucose dose, hence the need for standardization in order to compare data to normal values. The recommended method is satisfactory because (1) it does not overload the animal with glucose, (2) the infusion can be given easily within the time limits. (3) the blood glucose level is high enough to give a maximal insulin response, and (4) urinary loss of glucose is minimized. After a standard 16- to 24-hour fast (except for an adult ruminant) a zero-time heparinized blood sample is taken. Next, 0.5 g glucose/kg body weight is infused intravenously as a sterile 50% solution in 30 seconds. Timing of the test is begun at the midpoint, or 15 seconds. In large animals, the glucose 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 are plotted on semilogarithmic coordinates from which the half-time ($T_{1/2}$). the time required for the glucose concentration to fall by one-half is graphically estimated between 15 and 45 minutes postinfusion. From the $T_{1/2}$; the fractional

turnover rate, *k,* can also be calculated:

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

The fractional turnover rate k can also be calculated without graphing the data and using the relationship

$$k = \frac{\ln 1 - \ln 2}{T_2 - T_1} \times 100 = \%/\text{minute}$$

from the k value, the $T_{\frac{1}{2}}$ may be calculated:

$$T_{\frac{1}{2}} = \frac{0.693}{k} \times 100 = \text{minutes}$$

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

The method is equally applicable to and indeed the only practical method in large animals. The *k* value in a spontaneously diabetic cow was 0.38 %/minute ($T_{1/2}$ = 182 minutes) as compared to a normal of 1.98 %/ minute ($T_{1/2}$ = 35 minutes) and was comparable to *k* values obtained using ^{14}C glucose.

Standardization of the IVGTT as described has the advantages that an adequate insulin response is provoked, the influence of urinary glucose loss is minimized, and more reproducible clearance values are obtained (Fig. I.13). Other areas of the IVGTT with observed in hypofunction of the thyroids, adrenals, or pituitary and in hyperinsulinism.

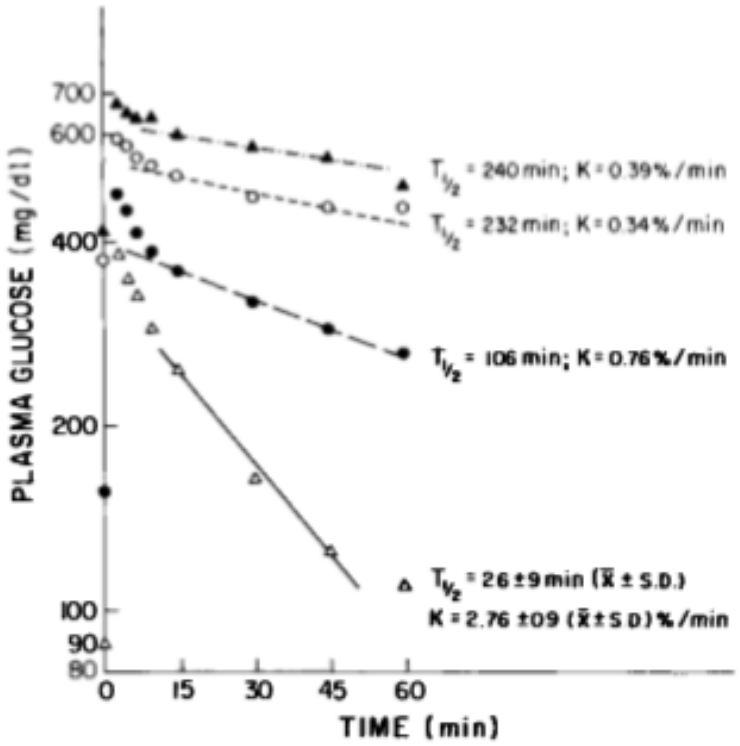


Fig. I.13 Intravenous glucose tolerance test (IVGTT) in normal dogs and in dogs with various types of diabetes mellitus.

9.2.2 Insulin Tolerance Test

The blood glucose response of a normal animal after the administration of a test dose of insulin exhibits a characteristic response (Fig. I.14). After obtaining a fasting blood sample, 0.1 U 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 (I) 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.

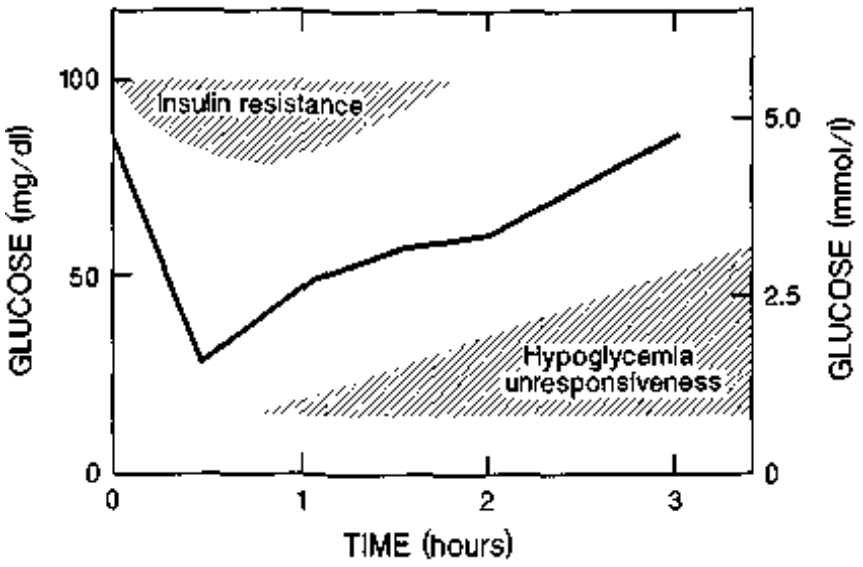


Fig. I.14 Insulin tolerance in the dog. Curves falling in the shaded areas are described as noted.

Two types of abnormal responses are seen. If the blood glucose level does not fall by 50% or requires longer than 30 minutes to reach the maximum hypoglycemic level, the response is described as "insulin insensitive" or "insulin resistant." Insulin resistance is found, though 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 the insulin tolerance test is most often employed in suspected cases of the latter two conditions. In carrying out this test, since hypoglycemia is being induced, a glucose solution should be readily available for injection.

9.2.3Glucagon Tolerance Test

Glucagon has a hyperglycemic effect which in turn evokes an insulin response. On this basis, the glucagon tolerance test is performed by the intravenous injection of 30 glucagon per

kilogram body weight. Samples for blood glucose and insulin are obtained before injection and at 30-minute intervals for 3 hours. An exaggerated 15-minute insulin response followed by a marked hypoglycemia at 2-3 hours or longer is characteristic of pancreatic islet cell tumors, however, found that the glucagon tolerance test was not diagnostic for these cases.

9.2.4Epinephrine Tolerance Test

The response of the blood glucose after the injection of epinephrine is also 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. Tbe test is performed by obtaining a fasting blood sample, injecting 1 ml of 1:1000 epinephrine-HCl (in the dog) intramuscularly, and obtaining blood samples every 30 minutes for 3 hours.

The characteristic increase in blood glucose is 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, concluded that liver glycogen in bovine ketosis was depleted, a finding later confirmed by direct measurement of glycogen in biopsy samples. A lowered glycemic response is a characteristic response in the glycogen storage diseases.

9.2.5Leucine-Induced Hypoglycemia

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 which is released by the tumorous islet cells. The test is performed by the oral administration (stomach tube) of 150 mg L-leucine per kilogram body weight as an aqueous suspension to the 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.

9.2.6 Tolbutamide Test

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

10 DISORDERS OF CARBOHYDRATE METABOLISM

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 on the total interaction of the hormonal influences on carbohydrate metabolism. Further discussions concerning disorders of the pituitary, adrenals, and thyroid are provided in the respective chapters elsewhere in this volume. The following sections discuss the conditions in which the principal manifestations are closely related to derangements in carbohydrate metabolisms.

10.1 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 1:66 (1.52 %) for dogs and 1:800 for cats. Diabetes mellitus in animals has been frequently

reviewed.

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, and it is now known that obesity is the single most important contributing factor to the development of diabetes. 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 humans in which the hereditary predisposition is well known.

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 I.7). 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 Io 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 of Types II and III obese diabetics being the most promising subjects for successful dietary therapy and/or oral hypoglycemic therapy. It is also likely that in the natural history of diabetes. Type III (chemical) diabetes precedes the development of Types I and II depending on the nature of the insulin deficiency, whether absolute (Type I) or relative (Type II). The most frequent contributory factors to the onset of diabetes are pancreatitis, obesity, infection, stress, and estrus. The possibility of a viral etiology has also been reviewed, and isolated a virus from a patient who died which produced diabetes in mice.

Table I.7

Diagnostic Criteria for Types of Diabetes Mellitus in Dogs

Diabetes type	IVGTT			Insulin*		
	G_o (mg/dl)	$T^1_{/2}$ (minutes)	k (%/minute)	I_o	I_p	$\Delta I/\Delta G$
I	>200	>70	<1.0	L	L	L
II	>200	>70	<1.0	N, H	L	L
IIII	100-200	>45	<1.5	N, H	N, D	L, N
Normal	70-110	15-45	>1.5	N	N	N

*Abbrev rations: N, normal; L, low; H, high: D, delayed.

Autoimmunity has also been investigated as a possible cause of diabetes mellitus. An immune response is suggested because lymphocytic infiltration, which is frequently associated with immune processes, is found in spontaneous diabetes of cattle and humans. It is also observed in cattle and or rabbits immunized with bovine insulin. It is reviewed the evidence that Type I diabetes in the biobreeding (BB) rat and the nonobese diabetic (NOD) mouse and Type I diabetes of humans are autoimmune diseases.

In the cat, several studies indicate there is a strong correlation between pancreatic insular amyloidosis and diabetes, though the amyloid does not appear to be the primary cause.

The high estimates of the incidence of diabetes is an indication of its importance as a clinical disease entity. Furthermore, the similarities of the clinical picture of diabetes and other wasting diseases with polyuria and polydypsia attest to the importance of laboratory examinations for 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 spontaneous

diabetic cow by the failure of a test load of glucose to elicit a serum insulin response. More recently, using 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 evident using the glucose tolerance test. 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}C. 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 stages, ketoacidosis.

Several reports have suggested that the development of diabetes mellitus is the result of the interaction of several hormones, principally, insulin and glucagon. While excess glucagon is seen in diabetes and 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. Thus, it is concluded that insulin deficiency is the primary cause of diabetes and that, while glucagon may modify the consequences, it is neither necessary nor sufficient for the development of diabetes.

10.1.1 Hyperglycemia

The finding of a fasting hyperglycemia is one of the most important diagnostic criteria of diabetes mellitus. The homeostatic level of blood glucose is maintained in the normal animal by the equilibrium between glucose supply and removal which in turn is based on the endocrine balance. The effect of insulin tends to lower blood glucose, while the opposing effects of growth hormone, glucagon, and adrenal cortical hormones tend to raise it. In the diabetic with an absolute 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 gluconeogenic enzymes.

In the diabetic, the hyperglycemia itself tends to compensate in part for the decrease in peripheral utilization. This occurs partially as a mass action effect, and glucose flows into the tissues. Thus, the diabetic 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 concentration of blood glucose is established at higher and higher levels and a steady state may never be established. Blood glucose values in canine diabetics have been reported as high as 70 mmol/liter (1250 mg/dl). When the renal threshold of 11.1 mmol/liter (200 mg/dl) for glucose is exceeded, (he diabetic is also faced with excessive loss of glucose in the urine.

In the uncomplicated diabetic, the blood glucose level is exquisitely sensitive to insulin, and this is the mainstay for monitoring the success of insulin therapy. However, a marked posthypoglycemic hyperglycemia, or the Somogyi effect, has long been known in humans, indicating that glucose regulation by insulin is not absolute. This effect is thought to be due to a transient excess of the nsulin antagonists, growth hormone, adrenal cortical hormones, and epinephrine, which were induced by the hypoglycemia.

10.1.2 Glucose Tolerance and the Insulin Response

The glucose tolerance test is the most important test of particular function and is of particular value in those cases of diabetes in which the fasting blood glucose is only moderately elevated and the diagnosis is equivocal. The glucose tolerance curve in diabetes characteristically shows a decreased tolerance for glucose (Fig. I.11) as evidenced by the long $T_{1/2}$ or low k value which reflect the inability of the animal to metabolize the 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 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 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 oversupply glucose, which contributes to the slow 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 normal to elevated insulin. This would mean that the insulin in the plasma of these types is ineffective due to a number of factors including receptor deficiency, receptor blockage, insulin resistance, binding to antibodies or other proteins, or abnormal structure. Thus, the glucose tolerance curve reflects an absolute (Type I) or relative (Types II and III) lack of insulin.

10.1.3 Insulin and the Insulin Response

Serum insulin is characteristically very low or absent in Type I diabetes, whereas it is normal or even very high in Types II or III. Thus, Type I can be readily differentiated on the basis of the fasting insulin level. On the other hand, about 40 % of even severe diabetics have normal to very high insulin levels. The classification of diabetic types is based on the nature of the insulin response curve during the IVGTT. Type II has normal to high insulin with no increment of IRI response to the glucose load. Type III also has normal to high insulin levels; the insulin response is inadequate, and there is a delayed return to preinjection levels. Types II and III have been further subdivided on the basis of obesity or nonobesity, and their insulin levels are given in Table I.8.

Table I.8

Insulin Concentrations in the Various Types of Canine Diabetes

Classification	Serum insulin (μU/ml)
Normal	5-20
Type I	0-5
Type 11, nonobese	5-20
Type II, obese	20-130
Type 111, nonobese	5-20
Type III, obese	8-60

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.

10.1.4 Ketonemia and Lipemia

As the utilization of glucose progressively decreases in the diabetic, 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 owing to increases in hormone-sensitive lipase. This enzyme is different from the lipoprotein lipases.

In severe diabetes, hyperlipemias are often so marked that the blood appears as "tomato soup." A cream layer may separate on storage in the cold owing to hyperchylomicronemia. The plasma is turbid due to the presence of lipoproteins (very low density lipoproteins). On chemical analysis, total triglycerides and cholesterol are elevated. Diabetic hyperlipemia appears to be

caused by impaired lipolysis of chylomicrons secondary to a deficiency of lipoprotein lipase rather than to overproduction of very low density lipoproteins.

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 is generated in excess by the liver from 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 acetyl-CoA. The accumulated acetyl-CoA units are then diverted into alternate pathways, and, with the activation of ketogenic mechanisms, excessive synthesis of ketone bodies and cholesterol results. In the peripheral tissues, there is an underutilization of ketone bodies in the diabetic dog. Ketosis is thus the result of an overproduction of ketone bodies and an 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. This is in keeping with the proposal that diabetes develops through a bihormonal interaction of insulin and glucagon since glucagon levels are high in insulin deficiency.

In the ketoacidotic state, marked cholesterolemias as high as 18 mmol/liter (700 mg/dl) have been observed in clinical diabetes of the dog. Net gluconeogenesis from fatty acid does not occur, and the precursors for gluconeogenesis are the proteins. The relative excesses of glucagon, cortisol, and growth hormone in the diabetic also contribute to protein catabolism and gluconeogenesis. The cofactors which provide the reductive environment required for gluconeogenesis can be provided by increased production of reduced cofactors during the increased fatty acid oxidation. This increase in the reductive environment of (he cell has been proposed the mechanism which stimulates gluconeogenesis, a corollary to the development of ketoacidosis.

10.1.5 Electrolyte Balance and Ketoacidosis

A mild glycosuria of the order of a few grams of glucose Joss 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 ^{o}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^-, $C1^-$, 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 suggests that a similar underutilization of ketone bodies occurs in diabetes.

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.

10.1.6 Urinalysis

Considering that the renal threshold for glucose in the dog is about 11.1 mmol/liter (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. Renal diabetes, though always a consideration, is an extremely rare occurrence. The fluctuations in blood glucose levels following feeding should be considered in the interpretation of the results of urine glucose determinations. Transient glucosurias may occur for 1-1.5 hours after a heavy carbohydrate meal, but a 2-hour postprandial glucosuria and, especially, fasting glucosuria are strong indications of diabetes.

An elevated urinary specific gravity (SG) has been considered 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 (PC_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. Albumin in urine increases the SG 0.003 units for each gram per deciliter, while 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 (140 mmol/liter, 2.5 g/dl) would increase the SG by only 0.010 unit. Therefore, while SG is a valuable measure of renal function, it is of little value with respect to the glucosuria of diabetes or to proteinuria. Conversely, by subtracting the contribution of albumin and glucose from the observed SG, a more accurate measure of renal function in diabetes may be obtained.

Proteinuria is a common sign of renal disease and is often observed in diabetes in dogs. There is doubt whether the proteinuria is associated with chronic nephritis or whether it is due to renal failure as an aftermath of diabetes.

Diabetic nephropathies arising from microangiopathies of the glomerular tufts and 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, but this lesion is not comparable to the Kimmelstiel-Wilson lesion seen in humans. Also, only 1 of 10 diabetic dogs at necropsy had a significant renal lesion although most had some degree of nephritis. In renal function studies in experimental streptozotocin diabetes and in spontaneous diabetes, the urea, creatinine, and phosphate clearances were normal. Also, blood urea and creatinine 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, and their appearance in urine is an early and significant sign of developing ketonemia. It is not, however, diagnostic of diabetes because ketonuria is observed in starvation and is absent in the mild diabetic. Ketonuria of varying degrees is, however, common in the 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, because only in extreme cases does the pH reflect acidosis.

10.1.7 Summary

The alterations in blood plasma which have been described are summarized in Fig. I.15. 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.

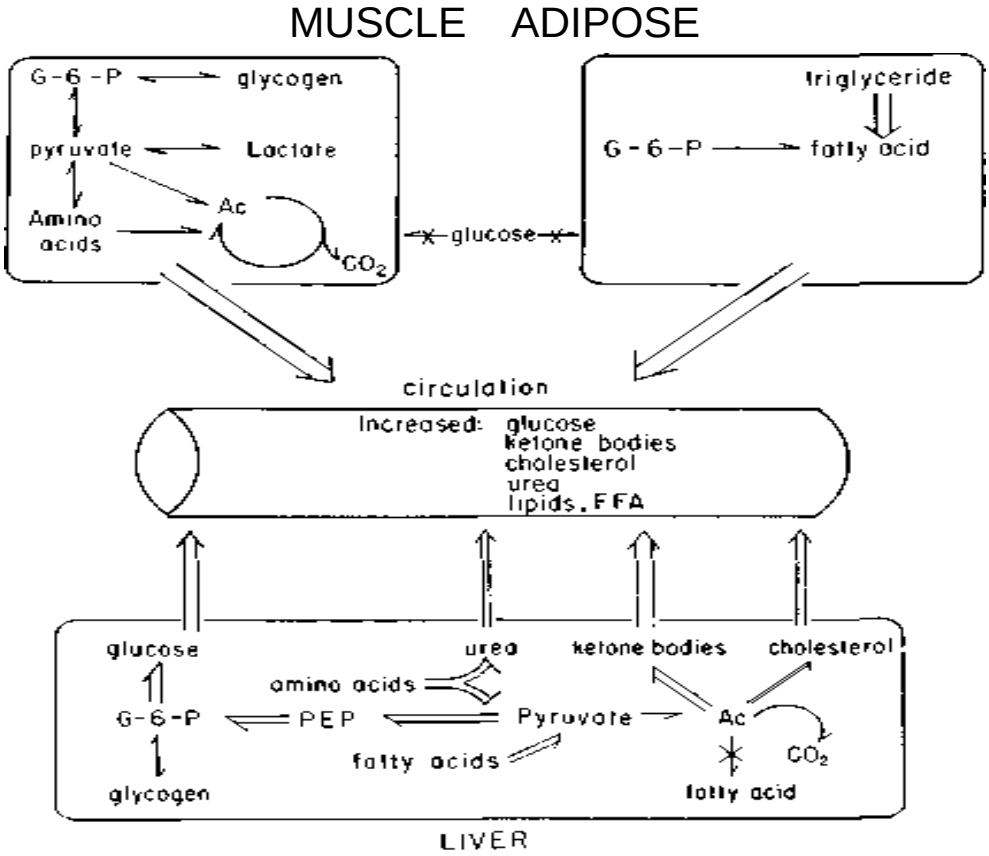


Fig. I.15 Summary of metabolic alterations in tissues of major importance in the diabetic animal. Increased flow in the metabolic pathways is noted by larger arrows. FFA, Free fatty acids.

Increases in the key gluconeogenic enzymes of the liver, G-6-Pase, F-l, 6-P.ase, PEP-CK. and PC, direct glucose metabolism toward an overproduction of glucose. Simultaneously, lipogenesis is suppressed and together with increased mobilization of fatty

acids, acetyl-CoA accumulates 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.

10.2 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 as a result of insulin-secreting islet cell tumors. In the classic case, excess insulin was extracted from metastatic foci in liver as well as from the pancreatic tumor.

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 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. I.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.

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 was employed in studies of patients with islet cell tumors. 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. 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.

Currently, the most useful and direct tests ate the serum insulin and the fasting plasma glucose tests, performed as described above. There is an inappropriately high level of insulin 020 nU/liter) in the presence of hypoglycemia.

10.3 Hypoglycemia of Baby Pigs

The condition occurs during the first few days of life and is characterized by hypoglycemia [glucose <2.2 mmol/liter «40

mg/dl)], apathy, weakness, convulsions, coma, and finally death.

The newborn baby pig is particularly susceptible to hypoglycemia. At birth, the blood glucose level is high [glucose >6 mmol/liter (110 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. In contrast, newborn lambs, calves, and foals are able to resist starvation hypoglycemia for more than a week. The ability of the baby pig to withstand starvation progressively increases from lime of birth, and a 10-day-old pig can be starved up to 3 weeks before symptoms of hypoglycemia occur.

The 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 or enzymes 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 inducibility by feeding would be of great value in understanding the mechanisms 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 baby 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 baby pig. this would explain its inability to withstand starvation as well as the newborn lamb or foal.

10.4 Glycogen Storage Diseases

The glycogen storage diseases (GSD) are characterized by the pathological accumulation of glycogen in tissues. Based on patterns of glycogen accumulation, clinical pathological findings,

and the enzymatic and structural analyses of the glycogen, GSD in humans have been classified into eight types. All have an autosomal recessive mode of inheritance except for GSD VIII which is sex linked. Their glycogen structures are normal except for Types III and IV.

Type I or classic von Gierke's disease is characterized by a high concentration of liver glycogen leading to a marked hepatomegaly. There is a marked hypoglycemia. and the blood glucose response to epinephrine or glucagon is minimal or absent. The liver glycogen structure is normal. The defect in this disease is a deficiency of the enzyme G-6-Pase. Type II or Pompe's disease is a generalized glycogenosis with lysosomal accumulation of glycogen and early death. The defect in this disease is a deficiency of acid-α-glucosidase (AAGase). In Type III or Cori's disease, the debrancher enzyme is deficient, leading to the accumulation of glycogen of abnormal structure. The branches are abnormally short, and there are an increased number of branch points; it is a limit dextrin, and thus the disease is sometimes called a limit dextrinosis. The disease is characterized by variable hypoglycemia, absent epinephrine or glucagon response, hepatomegaly, cardiomegaly and early death. In Type IV or Andersen's disease, the brancher enzyme is deficient, leading to a glycogen with long branches and few branch points. It is clinically similar to Type III. In Type V or McArdle's disease muscle phosphorylase (MPase) is deficient, while in Type VI it is liver phosphorylase (LPase) which is deficient. Type VII or Tarui's disease is characterized by a deficiency of muscle phosphofructokinase (PFK) with accumulation of glycogen in muscle, and Type VIII is deficient in leukocyte or hepatic phosphorylase *b* kinase (PbK). Type VIII disease is uniquely sex linked.

Of these eight types in humans, only Types I, II, Ill and VIII in animals are counterparts of human GSD on the basis of structural and enzymatic criteria. Other forms of glycogen storage in

animals are described as GSD-like based on their patterns of glycogen accumulation. There is also an inherited PFK deficiency in the springer spaniel dog, but, unlike Type VII GSD, there is no muscle pathology or glycogen accumulation in the muscle. The disease is expressed as a hemolytic anemia due to a deficiency of PFK in the erythrocytes and is rightly considered to be an erythrocyte enzyme deficiency rather than a GSD.

A Type I-like syndrome with hepatic glycogen accumulation and no response to epinephrine or to glucagon was reported in dogs. A radiation-induced Type I GSD occurs as an autosomal recessive condition in the C3H mouse and is characterized by hypoglycemia, early death, and a deficiency of liver G-6-Pase.

Type II GSD has been described in Brahman cattle, the Lapland dog, and in the Japanese quail. In Brahman catlle, Type II is characterized by early death, generalized glycogen deposition, and a marked decrease in AAGase activity. It is inherited as an autosomal recessive. In the Lapland dog, there is also early death, generalized glycogen deposition, hepatomegaly, and cardiomegaly; there is also a marked decrease in heart and liver AAGase. The Japanese quail with Type II is also characterized by early death, glycogen deposition in the heart, liver, and muscles, and decreased AAGase.

Type III occurs in the German shepherd dog and is characterized by early death, absent epinephrine or glucagon response, hepatomegaly, and cardiomegaly with glycogen accumulation. The glycogen has a limit dextrin structure, and there is very low debrancher enzyme activity in liver and muscle.

Type VIII GSD is seen in the rat and the mouse. In the rat, the disease is inherited as an autosomal recessive: the animal appears healthy but is hypoglycemic, exhibits hepatomegaly due to glycogen accumulation in the liver, and has a very low liver phosphorylase kinase activity. The affected mouse is also apparently healthy but has increased glycogen accumulation in the muscle with very low muscle phosphorylase *b* kinase. The

inheritance is sex linked

11 DISORDERS OF RUMINANTS ASSOCIATEDWITH HYPOGLYCEMIA

11.1 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, 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 the ketone bodies, acetone, acetoacetate, β-hydroxybutyrate, and isopropanol appear in blood, urine, and in the 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 to the understanding of these metabolic disorders of the ruminant.

11.2 Carbohydrate Balance

11.2.1 Glucose Requirements

The heavy demands for glucose in early lactation and in late pregnancy are well known. Kleiber has calculated that about 60 % of the lactating cow’s daily glucose requirement is for the

production of milk. The balance sheet (Table I.9) indicates a total daily glucose requirement of 1140 g, of which 700 g appear in the milk. For sheep in late pregnancy, Kronfeld calculated that between one-third and one-half of the daily glucose turnover of 100 g was utilized by the fetus.

Table I.9

Carbohydrate Balance Sheet

A. Cow's daily glucose flux	
1. In 12.5 kg milk:	Carbohydrate carbon
610 g lactose	257 g C/day
462 g milk fat with 58 g glycerol	23 g C/day
Carbohydrate carbon in milk/day	280 g C/day
2. Daily glucose catabolism:	
Cow produced daily 3288 liters CO_2 = 1762 g C	
Transfer quotient, plasma glucose → CO_2, is 0.1	
Thus glucose to CO_2 per day	176 g C/day
1 + 2 = daily flux of glucose	456 g C/day
180:72x456=1140 g glucose day	
B. Cow's glucose sources	
Cow secreted daily in urine 34 g N.	
The indicating catabolism of 213 g protein	110 g C/day
In urea	14 g C/day
Maximum available for glucose synthesis from protein	96 g C/day
	456 g C/day
Glucose flow in milk and respiration	360 g C/day
Thus, glucose flow from nonprotein sources	
180:72x360=900 g glucose day glucose daily must have been supplied from nonprotein sourcee	

An alternate approach toward 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, and reports from Kleiber's laboratory using this technique have given estimates of the daily turnover or requirements for glucose by the lactating cows estimated a transfer out of the circulation of about 70 g/houror 1680 g/day in a lactating cow, a figure which they realized may have overestimated the daily glucose turnover. A later report by this group gave an average estimate of 1440 g/day (60 g/hour) in four cows. For sheep, similar techniques gave an average turnover of about 144 g/day in normal pregnant ewes just prior to term.

It would appear that a reasonable estimate of the average daily glucose requirement would be about 50 g/houror 1.20 kg/day for a 454 kg (1000 lb) lactating cow and about one-tenth of this or 120 g/day for the ewe in late pregnancy.

11.2.2 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 carbohydrates (starch, glucose) in the diet escapes this fermentation so that glucose absorption by the digestive tract accounts for little of the daily glucose requirement of ruminants. It is known, however, that the glucose which might have escaped rumen fermentation is readily absorbed.

A possible source of blood glucose is ruminant 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 (Fig. I.4). Normally, blood lactate is derived principally from the breakdown of muscle glycogen. Sodium lactate placed in the rumen, however, results in increased blood lactate and glucose. Therefore, some of the glucose requirement may be met from this source, but it is probably minimal since excesses of lactic acid in the rumen are toxic.

The carbohydrate balance sheet (Table I.9) 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 carbon. These sources are the ruminal volatile fatty acids. The principal products of rumen fermentation are the volatile fatty acids acetate, propionate, and butyrate; 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 estimate the amounts of production and absorption of these acids. These fatty acids are found in blood in the approximate proportion 65:20:10 acetate-propionate-butyrate. In general, carbon atoms of acetate, although they appear in carbohydrate (blood glucose, milk lactose) through the mechanism of rearrangement in the TCA cycle (Fig. I.9). 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 putposes and for Che synthesis of fat. A possible mechanism for the direct incorporation of acetate into a glucose precursor is the so-called glyoxylate pathway, which occurs in plants but has not been demonstrated in animals.

Propionate, on the other hand, is a well-known precursor of carbohydrate. The pathway leading to a net synthesis of glucose from propionate is available via the reaction as shown in Fig. I.9.

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

According to the scheme, 2 mol propionate are theoretically required for the synthesis of a mole of glucose. A more recent refinement of the pathway for the production of glucose from propionate, which separates a mitochondrial pathway from the cytoplasmic route. The overall reaction is, however, the same, and thus 1 g of propionate theoretically can provide 1.23 g of

glucose. The amounts of propionate available from rumen fermentation can theoretically supply at least the glucose requirements not accounted for by protein sources.

Butyrate, the third major fatty acid of rumen fermentation, influences glucogenesis but does not contribute carbon directly to glucose. Butyrate stimulates glucose production by liver by increasing phosphorylase or by increasing gluconeogenesis. β-Oxidation of butyrate yields acetyl-CoA which has been shown to activate pyruvate carboxylase, a key gluconeogenic enzyme.

11.2.3 Utilization of Glucose

The overall utilization of glucose by the ruminant exhibits some significant differences from that observed in other animals. 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 radioactive glucose, were 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 human. The glucose tolerance of the cow and sheep was decreased, but later it was shown to be comparable to that of other animals. The plasma clearance $T_{1/2}$ was 33 minutes, which is similar to that observed in dogs and humans.

There were estimated that about 60 % of the glucose oxidized in the mammary gland of the lactating cow occurred via the hexose monophosphate pathway (Fig. I.6). The same percentage of HMP activity has been observed in the rat mammary gland. HMP activity in the ruminant mammary gland has also been demonstrated by the activities of G6P-D and 6-PG-D in the sheep and cow. 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 of glucose catabolism are essentially similar to those of other animals.

The other major pathway of glucose oxidation is the Embden-Meyerhof pathway and the TCA cycle. The presence of TCA cycle activity in the lactating mammary gland of the cow is established. Through this pathway, carbons from acetate, derived from any source, appear in milk products (Fig. I.16).

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, whereas the higher chain acids of butterfat are derived from blood lipids. The synthetic pathway for fatty acids in the gland is the same as that in other animal tissues.

The major portion of glucose uptake by the mammary gland 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 [1.1 - 4.4 mmol/liter (20-80 mg/dl)], 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. I.16.

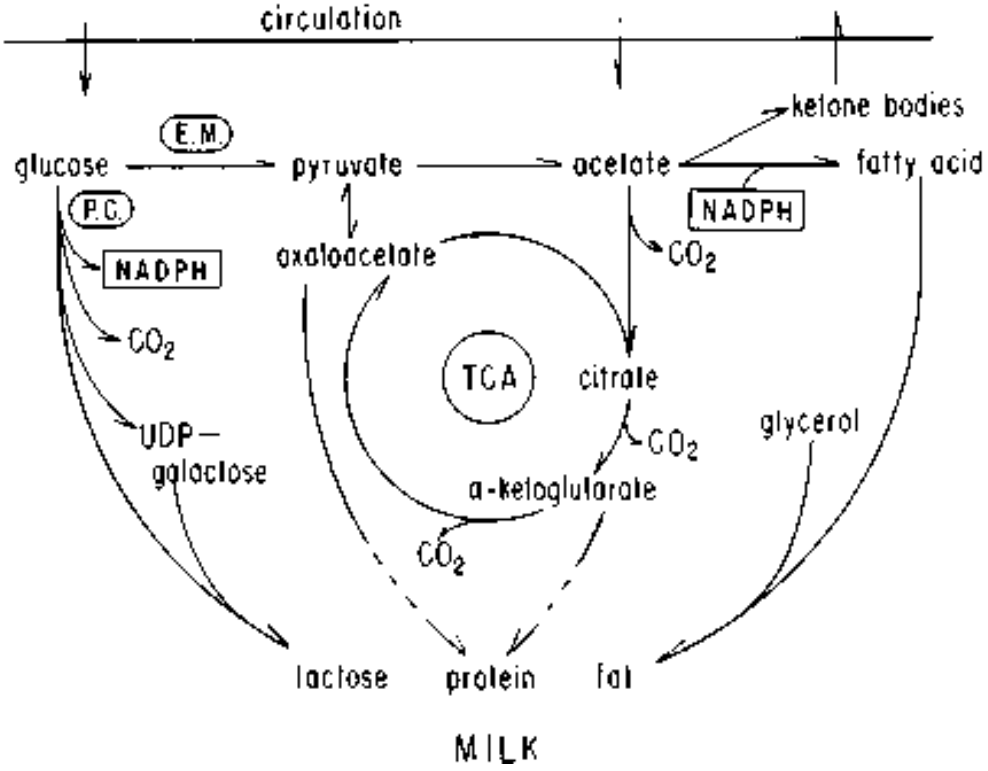


Fig. I.16 Summary of some metabolic pathways in the mammary gland.

Ruminant nervous tissue, i.e., brain, is similar to that of other animals in being an obligatory glucoseutilizing tissue. The HK *activity of* sheep brain, however, *is significantly* lower than in rat brain. This suggests that in spite of the glucose requirement, its utilization by nervous 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 rat intestine. Ruminant muscle also utilizes less glucose than muscle tissue of another species.

With respect to hepatic enzymes of gluconeogenesis and ketosis, highest G-6-Pase activities were found in liver *as* compared to other organs of sheep and were only about two-thirds of the activities in rats. Hepatic G-6-Pase activities in older calves and lactating cows were slightly higher than G-6-Pase activities of rats and could be reduced by intraduodenal infusions of glucose. 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 were 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.

There were reported their studies of a number of gluconeogenic enzymes of cow liver. There was found that cow liver PEP-CK, a key gluconeogenic enzyme, is already very high in comparison to that reported in rat liver. This supports the concept that the high producing dairy cow, which has been genetically selected for these qualities, is already synthesizing glucose maximally under normal conditions.

11.3 Biochemical Alterations in Body Fluids

11.3.1 Hypoglycemia

Hypoglycemia is such a consistent finding in bovine ketosis and in ovine pregnancy toxemia that "hypoglycemia" has been suggested as another name for bovine ketosis. This hypoglycemia has played an important role in ketosis, as a rationale for therapy, and 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.

To summarize, the ruminant appears to be an animal well adapted to a carbohydrate economy based on the endogenous synthesis of glucose from noncarbohydrate sources (gluconeogenesis). The enzymatic mechanisms for gluconeogenesis are already operating at near 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 partitioning of oxidation may be different, the pathways by which this oxidation is accomplished

are similar to those of other animals (Fig. I.16). Considering that the endocrine relationships of ruminants are also qualitatively similar to those of nonruminants, the normally low blood glucose concentrations of ruminants might also be considered to be a reflection of their degree of influence rather than of type. For example, the blood glucose response of the ruminant to insulin as compared to that of the dog has a slower rate of fall, i.e. insulin resistance.

11.3.2 Ketonemia

Ketonemia is another consistent feature of bovine ketosis and pregnancy toxemia of sheep. The ketone bodies are the same as those previously mentioned, acetoacetate, β-hydroxybutyrate, and acetone. A fourth compound, isopropanol, should also be included for the ruminant, and interconversions can occur between these ketone bodies. The fundamental mechanism of ketosis is covered elsewhere in this volume and a brief outline is presented here.

a. Site of Ketone Body Production. Increased ketogenesis occurs under conditions which 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 is not the sole source of ketone bodies. Rumen epithelium and mammary gland are also sources of ketone bodies. The extent of their contribution to the ketone body pool, however, is uncertain, but it could be considerable in the ketotic animal.

b. Hyperketonemia. Hyperketonemia is influenced by a number of conditions, all of which relate to the carbohydrate economy of the ruminant. Starvation is the most well-known method of producing ketosis. Mild to moderate ketonemia is often seen without detrimental effects during early lactation, late pregnancy, underfeeding, and feeding high fat diets. In these

states, the continuing demands of the body for carbohydrate are not adequately met.

Hyperketonemia is a consistent finding in bovine ketosis and pregnancy toxemia, though 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 172 mmol/liter (10 mg/dl).

11.4 Ruminant Ketosis

The finely balanced carbohydrate economy of the ruminant plays a central role in the development of ketosis in cows and sheep. In the cow, large amounts of glucose must be produced by the liver to meet the heavy demands for lactose, particularly in early lactation, when the demand is highest. Similarly, in the pregnant ewe, especially when carrying twins, there is a large obligatory demand for hexoses (fructose) near term. The mechanisms whereby internal metabolic imbalances occur in trying to meet these demands and manifest as ketosis, however, are uncertain and have been extensively investigated. The fundamental imbalance is a failure of hepatic gluconeogenesis leading to a hypoglycemia, followed by hyperketonemia and, finally, development of clinical ketosis.

Test questions for Chapter I

1. Clinical value of the research of glucose concentration in blood. Causes of hypo- and hyperglycemia.
2. Clinical value of glycoproteins and glycosaminoglycans content research.
3. Diagnostic value of glycosuria.

CHAPTER II
KETOGENESIS AND KETOSIS

1 INTRODUCTION

The ketones or ketone bodies, which are composed of acetoacetic acid, 3-hydroxybutyric acid (also known as β-hydroxybutyric acid), and acetone, are important compounds in the metabolism of birds and mammals. Ketosis simply means that ketones are present in body fluids in elevated concentrations. Ketones are important clinically and have a rather sinister and unsavory reputation. The terms ketosis and ketoacidosis connote maladies with which even experienced clinicians would rather not deal.

Even as late as 1968, the biological usefulness of ketones to the body as a whole was considered uncertain. Thus, the view was expressed. “Clearly it is not obvious in what ways ketogenesis in fasting is a good thing for the whole animal; should the liver be regarded as providing manna for the extrahepatic tissues or does it simply leave them to eat up its garbage?” Since that time, the survival value of ketogenesis has become clearer, and although increased levels of ketones in biological fluids will continue to be regarded a pathological sign in many situations, perhaps the beneficial aspects of ketogenesis will be more widely appreciated.

2 CHEMISTRY OF KETONES

2.1 Structure and Properties

The ketones acetone, 3-hydroxybutyric acid, and acetoacetic acid are relatively simple chemical structures. Of the three, only 3-hydroxybutyric acid can exist as stereoisomers, having L-(+) and D-(-) forms. Only the d-(-) form is produced in a tree state in intermediary metabolism. The l-(+) form exists only as its CoA thioester produced and destroyed in β-oxidation.

Acetone is relatively volatile, whereas the other two ketones are not. Acetone has a characteristic organic solvent odor which may be detectable in the exhaled breath of animals with elevated blood ketone levels. Anecdotal evidence indicates that people vary greatly in their olfactory sensitivity for acetone.

Acetone does not ionize appreciably, whereas 3-hydroxybutyric acid and acetoacetic acid do readily ionize. Acetoacetic acid has a pH, of 3.58, and 3- hydroxybutyric acid has a pH, of 4.41. Consequently, at normal plasma pH of 7.40, 99.98% of acetoacetic acid exists as acetoacetate, and 99.89 % of 3-hydroxybutyric acid exists as 3-hydroxybutyrate. Because these acids exist predominately in their ionized form at physiological pH, they will usually be referenced by the names of their ions whenever their metabolism is discussed.

Acetoacetic and 3-hydroxybutyric acids are more powerful acids than the volatile fatty acids (VFA; acetic. propionic, and butyric acids), which have pH, values ranging from 4.76 to 4.87. Acetoacetic acid is more powerful and 3-hydroxybutyric acid is less powerful as an acid than lactic acid, which has a pH, of 3.86.

Acetone and acetoacetic acid are miscible in water in all proportions, and 3-hydroxybutyric acid is exceedingly soluble, but not in all proportions. The common metallic salts of acetoacetic acid and 3-hydroxybutyric acid are soluble in water. Acetone and 3-hydroxybutyric acid and its salts are relatively stable compounds. Acetoacetic acid spontaneously decomposes to acetone and carbon dioxide. This reaction occurs readily without catalysis, and its rate is accelerated by increased temperature and hydrogen ion concentration- Apparently, there can be some nonspecific catalysis of acetoacetate decarboxylation by cellular proteins. The lithium, sodium, and potassium salts of acetoacetic acid are relatively stable if stored in dry form below 0°C.

2.2 Detection and Assay

2.2.1. Qualitative

The most common qualitative test for ketones is the alkaline nitroprusside test, which is also known as the Rothera test. This test has been used for decades in clinical practice and is still exceedingly useful. The test relies on the reaction of nitroprusside with acetone or acetoacetate to produce a purple chromogen. The nitroprusside test has been used for virtually every body fluid imaginable including whole blood, serum, plasma, urine, and milk. The test is most sensitive for acetoacetate (0.5 mM can be detected) and gives only a slight response to acetone. The test is completely insensitive to 3-hydroxybutyrate. It has been reported, albeit with little detail or confirming data, that, for urinalysis, 3 hydroxybutyrate can be converted to acetoacetate by adding a few drops of hydrogen peroxide to the sample. If this technique truly works, it would greatly enhance the sensitivity of the test because 3-hydroxybutyrate is usually in higher concentration than acetoacetate in urine.

The nitroprusside test is available commercially in the form of strips, tablets, and powders. The maximum sensitivity of all three forms is approximately 0.5 mM, although specific formulations may have a sensitivity less than this value. The strip form is commonly used for urine. The powder form and strips are both commonly used for milk. The tablet form is used for serum, plasma, and whole blood and can be used for milk and urine as well. The test is often used in a semi-quantitative manner with the result expressed in adjectival form (negative, weak, strong) or as a series of pluses (−, +, + +, etc.).

There are a number of drugs or other substances that may appear in urine and give a false positive with the nitroprusside test. Some compounds react with nitroprusside to yield a purple or near purple color. Included in this group are phenylketones, levodopa, methyldopa, acetaldehyde, paraldehyde, cysteine,

cysteamine, penicillamine, and mesna. In general, substances with keto, aldehyde or sulfhydryl groups have the potential for reacting with nitroprusside.

Since the nitroprusside test is performed in an alkaline medium, some substances which may exist in urine and are otherwise colorless may yield a purple or near purple color simply due to the alkaline pH. These substances include sulfobromophthalein and phenolsulfonphthalein.

Another qualitative test which has been used to detect acetoacetate is the Gerhardt or ferric chloride test. This test is relatively insensitive, is subject to interference by a wide range of substances, and should not be used.

2.2.2 Quantitative

Commonly used means of quantitative assay for ketone concentrations in biological fluids fall into three categories; (a) microdiffusion method, (b) enzymatic methods, and (c) chromatographic methods. Other less commonly used methods include a radiochemical method and isotachophoresis.

Regardless of the method to be used for analysis, proper handling of the samples prior to analysis is crucial for obtaining representative results. In particular, the volatility of acetone and instability of acetoacetate must be respected.

Blood samples should be cooled immediately after collection. Ketones can be determined in whole blood or plasma. Serum is not recommended because of losses, particularly of acetoacetate, that may occur during the time required for clotting. Any of the common anticoagulants (heparin, fluoride, oxalate, citrate, or EDTA) may be used. If whole blood is to be used, it should be mixed with perchloric acid immediately after collection to precipitate proteins. The tube should be chilled on ice until centrifuged which should be performed within a few hours. The supernatant should be frozen until analyzed. If plasma is to be used, the red cells should be spun down within a few hours, and

the plasma proteins precipitated with perchloric acid. The supernatant should be frozen until analyzed.

a. Microdiffusion Method. The microdiffusion method can be used to determine the concentration of acetone or acetone plus acetoacetate in any biological fluid. The reagents are relatively simple and inexpensive although rather corrosive. The diffusion step requires specialized, but inexpensive, apparatus and adds to the complexity and time to complete the assay.

The method relies on the reaction of acetone with vanillin to produce divanillalacetone which absorbs light at 415 nm. The chromogenic reactant used in the original description of the method was salicylaldehyde; however, vanillin provides more consistent results and sensitivity. The method determine acetone; it would also detect acetoacetate simultaneously. In fact, the method is specific for acetone. It has been found in my laboratory that to use the method for acetone plus acetoacetate, it is necessary to preincubate the sample with an equal volume of 10 *N* sulfuric acid for 4 hours at 50 °C in a sealed container to decarboxylate all of the acetoacetate. The method can be adapted to measure 3-hydroxybutyrate as well by introducing a step in which 3-hydroxybutyrate is oxidized to acetoacetate with potassium dichromate.

b. Enzymatic Method. The enzymatic method can be used to determine the concentration of acetoacetate or 3-hydroxybutyrate in biological fluids. The method is accurate and precise and is probably the most common method used for quantitative assay of ketone concentrations. The method has been successfully adapted to a variety of automated analysis systems. It is a relatively straightforward spectrophotometric or fluorometric method, but the reagents are relatively expensive.

The method relies on the reversible reaction catalyzed by 3-hydroxybutyrate dehydrogenase;

$$\text{3-hydroxybutyrate} + NAD^+ \longleftrightarrow \text{acetoacetate} + NADH + H^+$$

The reaction is run in the forward direction by including an excess of NAD^+ in (the reaction mixture to assay 3-hydroxybutyrate and in the backward direction by including an excess of NADH in the reaction mixture to assay acetoacetate.

The equilibrium constant of the reaction is 1.42×10^{-9} and therefore, is highly favorable toward the reduction of acetoacetate at pH 7.0. In order to force the reaction to completion in the direction of oxidizing 3-hydroxybutyrate, hydrazine is used as a trapping agent to remove acetoacetate as it is formed, and the reaction mixture is buffered at an alkaline pH. The change in NADH concentration is measured by the change in absorbance at 340 nm in either case. Alternately, a fluorometer can be used to measure the change in NADH concentration. To avoid interference from lactate or pyruvate in the sample, the 3-hydroxybutyrate dehydrogenase should be free of lactate dehydrogenase, or, alternately, the lactate dehydrogenase inhibitor, oxamic acid, can be added to the reaction mixture.

c. Chromatographic Methods. Thin-layer (and paper chromatography have been used to separate ketones. Both methods are rather slow, and quantitation is cumbersome and not as precise as with other methods.

Gas chromatography requires expensive equipment but can yield precise results. Gas chromatographic methods generally select only acetone; however, 3-hydroxybutyrate can be oxidized with dichromate or 3-hydroxybutyrate dehydrogenase to acetoacetate, and acetoacetate will decarboxylate on treatment with acid or due to the heat of the chromatographic column. Thus, by appropriate pretreatment of the sample, it is possible to determine all three ketones by gas chromatography.

d. Normal Values. Table II.1 lists normal blood and plasma concentrations for several domestic species.

Table II.1

Blood and Plasma Ketone Concentrations of Domestic Animals

Species	Butyrate (mmol/liter)	Acetoacetate (mmol/liter)	Sample
Cow (lactating)	0.41±0.03 0.95±0.18	0.043±0.001 0.13±0.03	Plasma Blood
Cow (nonlactating)	0.27±0.04	0.011±0.003	Plasma
Dog	0.033±0.015 0.030±0.006	0.018±0.010	Blood Plasma
Goose	0.042±0.015	0.023±0.003	Plasma
Horse	0.11±0.01 0.064± 0.006	 0.029±0.003	Blood Plasma
Sheep	0.27±0.04 0.55±0 04	0.051±0.005 0.030±0.002	Blood Blood

The values are for healthy fed animals. It is assumed that plasma and blood ketone concentrations should be similar because of the generally high permeability of cell membranes to ketones and lack of protein binding of ketones: however, reports of definitive studies on this problem are not apparent in the literature. For clinical purposes, there is no lower normal limit for ketone concentrations.

3 ORIGIN OF KETONES

Ketones are primarily products of intermediary metabolism. Only under unusual circumstances would more than trace amounts be absorbed from the contents of the gastrointestinal tract. The real source of ketones is fatty acids including those with short (1-4 carbons), medium (5-11 carbons) and long (>11 carbons) chains. Of course, any compound (glucose, lactate, glycerol, amino acids, etc.) that can be converted to fatty acids can be considered as a source of ketones, but for the purposes of

this discussion the origin of ketones will be considered to be fatty acids, either esterified or non-esterified.

3.1 Lipolysis and Long Chain Fatty Acids

The predominant source of ketones in healthy animals is long chain fatty acids (LCFA) released during lipolysis in adipose tissue. The LCFA themselves originated by being absorbed as such from the gastrointestinal tract or by being synthesized in tissues.

The synthetic precursors of LCFA are a function of the diet and fermentative processes occurring in the gastrointestinal tract. In carnivores and omnivores, fermentative processes play a minimal role in supplying precursors to long chain fatty acids. Thus, in these species, amino acids and/or carbohydrates are the precursors, and their use for LCFA synthesis is determined by the dietary content of protein, carbohydrate, and triglyceride.

In herbivores, fermentative processes usually play a significant role in altering the gut contents. In species, such as ruminants and other herbivores having significant fermentation in the stomach, short chain fatty acids are produced in abundance and are the predominant contributors to LCFA and ketone synthesis. The main VFA absorbed from the rumen is acetate which is metabolized to only a slight extent by the rumen wall or liver but is removed from the plasma by many other organs including skeletal muscle, adipose and mammary gland. Adipose and mammary convert much of the acetate to LCFA which are esterified to glycerol to form triglycerides.

When plasma insulin levels decrease and plasma glucagon levels increase. cAMP levels in adipose cells increase. Increased cAMP levels lead to activation of hormone-sensitive lipase which hydrolyzes triglycerides to LCFA and glycerol. The LCFA bind to plasma albumin for transport to other tissues, whereas glycerol freely dissolves in plasma water.

3.2 Ketogenesis by Liver

The liver has an enormous capacity to remove LCFA from plasma. LCFA unbind from albumin, diffuse through the hepatocyte plasma membrane, and bind to fatty acid-binding protein in the cytosol. Before the LCFA can be processed further it must be esterified to CoA which is accomplished by the following reaction:

$$\text{LCFA} + \text{ATP} + \text{CoA} \leftrightarrow \text{LCFA-CoA} + \text{AMP} + \text{PP}$$

The reaction is catalyzed by LCFA-CoA synthase (EC 6.2.1.3), an enzyme bound to the endoplasmic reticulum and the outer mitochondrial membrane. The pyrophosphate (PP) is rapidly hydrolyzed, so the reaction effectively consumes two ATPs. The activation of LCFA is not rate limiting for either re-esterification or catabolism.

In the cytosol, LCFA-CoA can be esterified to glycerol phosphate to form triglycerides. Hormonal changes that favor lipolysis inhibit triglyceride formation, however, under conditions where plasma LCFA levels remain increased for days, the rate of esterification may exceed the rate at which the liver can export triglyceride as lipoprotein, and fatty liver results.

In order for LCFA-CoA to be catabolized, it must pass into the mitochondrion, which is a problem because the inner mitochondrial membrane is impermeable to it. The CoA must be exchanged for a carnitine moiety, a reaction catalyzed outside the mitochondrion by carnitine acyltransferase I (CAT I; EC 2.3.1.21): LCFA-CoA + carnitine ↔ LCFA-carnitine + CoA LCFA-carnitine passes readily through the inner mitochondrial membrane and is acted on by carnitine acyltransferase II which converts the LCFA-carnitine back to LCFA-CoA.

CAT I appears to be controlled by inhibition by malonyl-CoA. Malonyl-CoA is produced from acetyl-CoA by acetyl-CoA carboxylase (EC 6.4.1.2) and is the first intermediate in the

pathway to LCFA synthesis. Acetyl-CoA carboxylase activity is stimulated by increased plasma insulin concentrations. It is logical that when lipogenesis is stimulated, the LCFA that are produced should be prevented from entering the mitochondrion where they will be catabolized.

In the mitochondrion, LCFA-CoA undergoes β-oxidation which cleaves it into acetyl-CoA units. β- Oxidation appears to be controlled only by substrate availability. The acetyl-CoA units can be oxidized in the citric acid cycle provided there is sufficient oxaloacetate to condense with them to form citrate. Alternatively, acetyl-CoA units can be recondensed to form ketones, which will occur when there is not sufficient oxaloacetate for citrate formation or when citrate synthetase is inhibited by high levels of citrate.

There are four enzymes involved in ketogenesis from acetyl-CoA: acetoacetyl-CoA thiolase (EC 2.3.1.9), hydroxymethylglutaryl-CoA (HMG-CoA) synthase (EC 4.1.3.5). HMG-CoA lyase (EC 4.1,3.4), and D-3-hydroxybutyrate dehydrogenase (EC 1.1. 1.30). These four enzymes catalyze the following four reactions, respectively:

$$2\ \text{Acetyl-CoA} \leftrightarrow \text{acetoacetyl-CoA} + \text{CoA}$$
$$\text{Acetoacetyl-CoA} + \text{acetyl-CoA} \rightarrow \text{HMG-CoA} + \text{CoA}$$
$$\text{HMG-CoA} \rightarrow \text{acetoacetate} + \text{Acetyl-CoA}$$
$$\text{Acetoacetate} + \text{NADH} + \text{H+} \leftrightarrow \text{3-hydroxybutyrate} + \text{NAD}^{+}$$

However, there is some HMG-CoA synthase in the cytosol, and the HMG-CoA which is synthesized there is used for cholesterol synthesis. The first three enzymes are in the mitochondrial matrix, whereas 3-hydroxybutyrate dehydrogenase is in the inner membrane of the mitochondrion, and membrane lipids are required for full activity of the enzyme. Interestingly, livers of ruminants have lower apparent activities of 3-hydroxybutyrate dehydrogenase than the livers of other species,

a situation which has not been explained adequately. Since ruminant liver is continuously presented with 3-hydroxybutyrate synthesized by the rumen epithelium, the low hepatic activity of 3-hydroxybutyrate dehydrogenase may be beneficial because more of the compound will reach peripheral tissues in an unoxidized state.

Acetoacetyl-CoA thiolase and 3-hydroxybutyrate dehydrogenase catalyze reactions that are at or near equilibrium, i.e., the rates of these reactions are controlled by the concentrations of the substrates and products. HMG-CoA synthase and lyase catalyze reactions that are far removed from equilibrium, and these enzymes may be subject to regulatory controls other than the concentrations of substrates and products. The synthase is considered to be the rate-limiting enzyme and appears to be restricted almost exclusively to the liver. It is not known whether the enzyme has physiological allosteric controls: however, the substrate, acetoacetyl-CoA, is an inhibitor at high concentrations. A summary of ketogenesis in the liver is depicted diagrammatically in Fig. II.1.

Because of the relative abundance of 3-hydroxybutyrate dehydrogenase, the ratio of acetoacetate to 3-hydroxybutyrate produced by the liver is proportional to the ratio of NAD^+ to NADH concentrations in the liver mitochondrion. The plasma ratio of the two ketones reflects not only the ratio of mitochondrial NAD^+ to NADH in the liver but also reflects the ratio in other tissues as well and is affected by different rates of blood flow to various tissues.

Ketogenesis can occur from VFA and medium chain fatty acids. Medium chain fatty acids are in quite low concentration in the diet or in triglycerides of mammals and, therefore, are not usually quantitatively important m ketogenesis. Except in unusual circumstances, nonherbivores do not absorb large quantities of VFA from the gastrointestinal tract. Among the herbivores, the metabolism of VFA has been studied most thoroughly in ruminants.

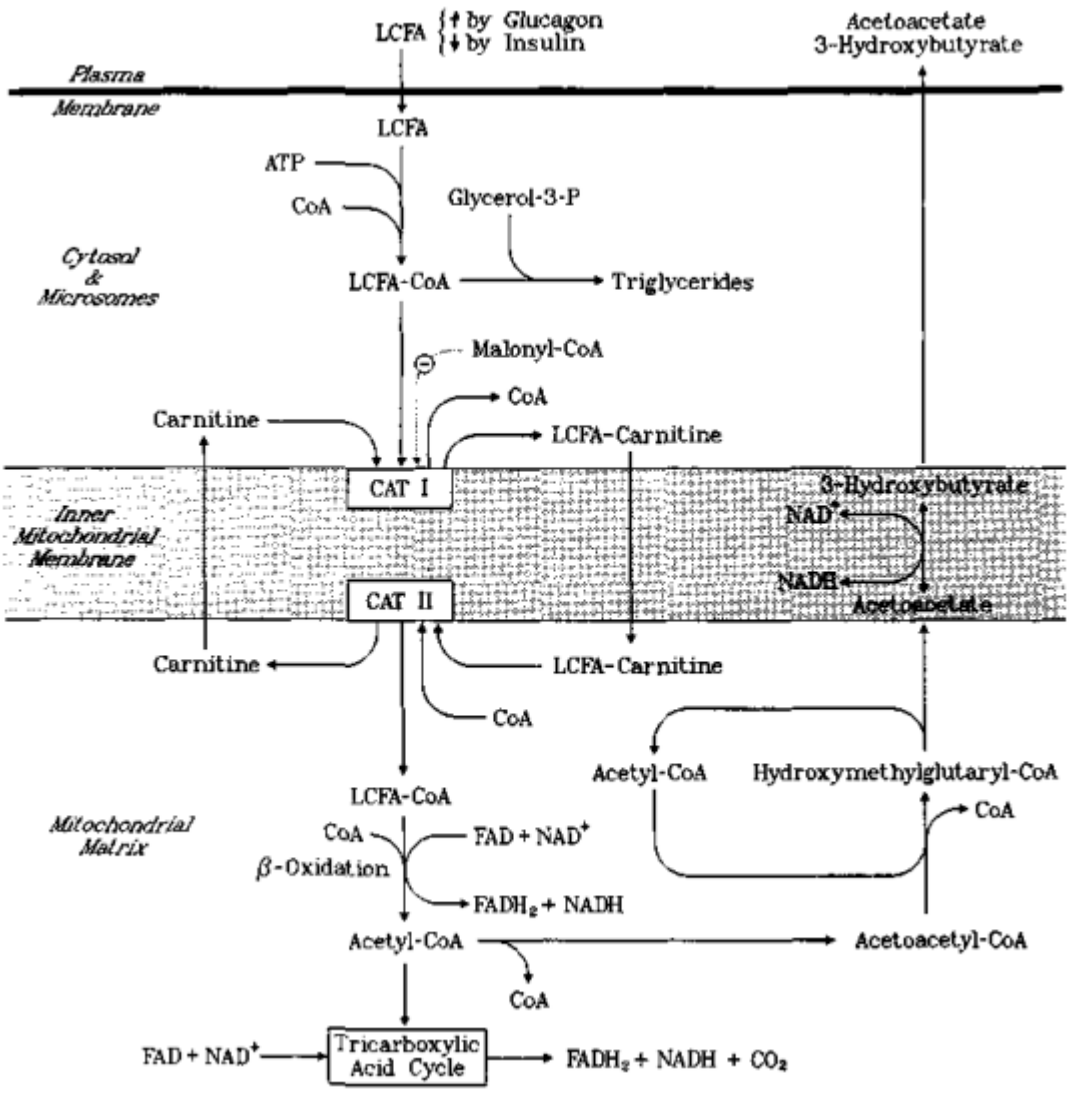


Fig. II. 1. Ketogenesis in the liver. Abbreviations: CAT, carnitine acyltransferase; LCFA, long chain fatty acids.

Propionate is the major gluconeogenic precursor and is not an important precursor of ketones. Butyrate is converted to 3-hydroxybutyrate by the rumen epithelium and is discussed later.

Like LCFA, acetate must be covalently bound to CoA before it can be catabolized further. The enzyme responsible for catalyzing the reaction for acetate is acetyl-CoA synthase. This enzyme is found in the cytosol and mitochondria of most cells of most organs. In ruminants, the liver has a relatively low concentration of acetyl-CoA synthase, and most absorbed acetate passes through the liver and is removed from the plasma

by other tissues, particularly heart, skeletal muscle, kidney, and mammary gland.

3.3 Ketogenesis by the Alimentary Tract

Butyrate which is produced during fermentation of feedstuffs in the rumen is readily absorbed by the rumen wall. The rumen epithelial cells possess high activities of butyryl-CoA synthase which can convert butyrate to butyryl-CoA. By β-oxidation, butyryl-CoA is converted to L-3-hydroxybutyryl-CoA, which is oxidized to acetoacetyl-CoA followed by cleavage of the CoA and reduction of the resulting acetoacetate to 3-hydroxybutyrate. Rumen epithelium does have HMG-CoA synthase. HMG-CoA lyase, and 3-hydroxybutyrate dehydrogenase activities, although in lesser concentration than in liver. It is possible that rumen epithelium can also cleave CoA from acetoacetyl-CoA by means other than the HMG-CoA cycle. Rumen epithelium does contain acetoacetyl-CoA deacylase which can directly hydrolyze the compound. This enzyme can catalyze the transfer of CoA from acetoacetyl-CoA to succinate, thus liberating acetoacetate. This latter route may be the predominant pathway in rumen epithelium.

3-Hydroxybutyrate appears in portal blood. At least 50 % of absorbed butyrate is oxidized to ketones in the rumen wall, and of the butyrate that does appear in portal blood, nearly all of it is removed on the first pass through the liver. Ruminal production of 3-hydroxybutyrate is probably the main reason why fed ruminants normally have a higher plasma concentration of this compound than fed non-ruminants.

3.4 Ketogenesis by Other Organs

It has been claimed that mammary gland may synthesize appreciable ketones in ketotic dairy cows. The evidence for appreciable ketone synthesis by mammary gland is weak. One study using intramammary injection of [^{13}C] acetate showed some transfer of label to milk ketones which was greater in ketosis.

However, that study used only one normal and one ketotic cow, so no statistically significant conclusions could be drawn. Furthermore, the specific radioactivity of milk acetate was not determined, so it was not possible to estimate the rate of conversion of acetate to ketones.

In another study, arteriovenous concentration differences and mammary blood flow were used to estimate mammary ketone production and uptake in dairy cows. It was found that the mammary gland utilized small quantities of acetoacetate and larger quantities of 3-hydroxybutyrate in healthy cows, whereas the mammary gland of ketotic cows produced large quantities of acetoacetate. The increased uptake of 3-hydroxybutyrate by mammary in ketotic cows equaled almost exactly mammary production of acetoacetate. There was no significant difference in mammary uptake of acetate between healthy and ketotic cows.

In yet another study on ketotic cows, arteriovenous concentration differences across the mammary glands of acetoacetate and 3-hydroxybutyrate were observed. A positive arteriovenous difference was noted for 3-hydroxybutyrate which was almost equal in magnitude to the negative arteriovenous difference noted for acetoacetate. The foregoing results point toward mammary conversion of 3-hydroxybutyrate to acetoacetate which increases in ketosis. This process cannot really be called ketogenesis: perhaps ketoconversion would be the appropriate nomenclature.

4 FATE OF KETONES

4.1 Reduction

Reduction is a possibility for acetoacetate, and. of course, the reduction product is 3-hydroxybutyrate. 3-Hydroxybutyrate is a metabolic cul-de-sac because it can be metabolized only by being reconverted to acetoacetate. In comparison to acetoacetate, 3-hydroxybutyrate should be viewed as a means by

which the liver can export reducing power (hydrogen) to the peripheral tissues for combustion and energy generation there.

Both acetoacetate and 3-hydroxybutyrate can be reduced by being converted to LCFA. This fate is more likely to occur in mammary gland than in adipose tissue. Plasma 3-hydroxybutyrate has been shown to be a milk fat precursor in cows, goats, and rabbits. A substantial portion of 3-hydroxybutyrate used for milk fat synthesis in ruminants is incorporated as a four-carbon unit.

4.2 Oxidation

4.2.1 Acetoacetate and 3-Hydroxybutyrate

Ultimately, the fate of most 3-hydroxybutyrate and acetoacetate is oxidation in the peripheral tissues. Once 3-hydroxybutyrate has been oxidized to acetoacetate, the acetoacetate is converted to acetoacetyl-CoA by the following reaction:

$$\text{Acetoacetate} + \text{succinyl-CoA} \longleftrightarrow \text{acetoacetyl-CoA} + \text{succinate}$$

The above reaction is catalyzed by 3-ketoacid CoA-transferase, and, viewed from the point of converting succinvl-CoA to succinate, it effectively bypasses the succinyl-CoA synthase reaction of the citric acid cycle. Since the reaction catalyzed by succinyl-CoA synthase produces one GTP from GDP, the 3-ketoacid CoA-transferase reaction effectively consumes 1 mol of ATP. The reaction also pushes succinyl-CoA toward oxaloacetate in the citric acid cycle, and oxaloacetate will be needed to form citrate from the acetyl-CoA derived from the acetoacetyl-CoA.

3-Ketoacid CoA-transferase is found in spleen, skeletal muscle, brain, adipose, heart, lung, and kidney of rodents and sheep although the activities are very low in sheep heart and brain. In general, though, the highest activities are in the heart

and kidney. Activity of 3-ketoacid CoA- transferase is absent in liver. The absence of the enzyme from liver is logical because if there were a need for NADH for combustion in the liver, it could be obtained directly from acetyl-CoA in the citric acid cycle rather than shunting the acetyl-CoA units into ketones and back again.

All tissues have thiolase, and it is in greatest activity in heart, liver, and kidney. Heart and brain of sheep have significantly less thiolase than in rodents. The activities of 3-ketoacid CoA-transferase and acetoacetyl-CoA thiolase are relatively stable in fasting, fed state, high fat diet and diabetes except that in rodents, thiolase increases on feeding a high fat diet. In general, it appears that the ketoneutilizing capacity of the body is relatively constant, and ketone availability controls ketone oxidation.

4.2.2 Acetone

For many years, acetone was viewed as a metabolic dead end, a substance destined to be excreted in the urine or exhaled in the breath. Although much acetone does indeed wind up in the breath and urine, evidence has accumulated that indicates that some acetone is metabolized. Furthermore, evidence for the catabolic pathway for acetone metabolism indicates that it is metabolized to pyruvate. Thus, by this mechanism, it is possible that small amounts of fat can be converted to glucose.

Labeling patterns of milk glutamate carbons following injection of [2-^{14}C] acetone into cows indicated that acetone was metabolized via pyruvate. Labeling patterns of glucose in humans injected with radiolabeled acetone also indicate metabolism via pyruvate. In rats, however, labeling patterns of glucose following radiolabeled acetone injection indicate that acetone can be metabolized via pyruvate and acetate, but that the latter pathway predominates. Thus, there appear to be real species differences in acetone metabolism. In humans, at least, the fraction of acetone that is metabolized versus excreted varies inversely with

acetone concentration, so it appears that the catabolizing pathways for acetone are not capable of handling large quantities.

4.3 Renal Metabolism and Excretion

The kidney cannot synthesize ketones to any appreciable extent, but it is a voracious consumer of ketones as an energy source in ruminants and non-ruminants. It is interesting though that in fasting sheep the kidney removes from the plasma and catabolizes both acetoacetate and 3-hydroxybutyrate, whereas in fasting humans there is substantial removal of 3-hydroxybutyrate and a slight production of acetoacetate.

Ketones are freely filterable in the glomerulus. There appears to be in humans and dogs, at least, a direct or indirect energy-consuming tubular transport system for acetoacetate and 3-hydroxybutyrate which approaches saturation at relatively low plasma concentrations of ketones such as encountered in the fed state or a 1-day fast. Neither ketone is excreted at these lower concentrations, but they do begin to appear in the urine as plasma levels begin to rise. However, as the ketone concentrations increase in the glomerular filtrate, the primary mode of reabsorption is by diffusion down a concentration gradient as water is reabsorbed from the tubular lumen. Acetone begins to appear in the urine as soon as it begins to appear in the plasma; presumably, this effect is due to the great lipid solubility of acetone which allows it to penetrate cell membranes with relative ease.

Renal excretion and reabsorption of ketones is approximately proportional to their filtration rates (or plasma concentrations if glomerular filtration rate remains constant) at concentrations found after more than a 1-day fast in humans and in ruminants. At least some parts of the nephron, probably beyond the proximal tubule, are less permeable to ketones than to water because when plasma ketone levels are substantially

increased, the urinary concentration exceeds the plasma concentration.

The dual mode of ketone reabsorption has an advantage in that none of this valuable energy source is lost at lower plasma concentrations; however, there is no transport maximum for the kidney as a whole, so 80-90 % of filtered ketones are reabsorbed regardless of how concentrated ketones become in the plasma during pathological conditions or prolonged starvation. Mammals presumably could have evolved a greater activity of the energy-consuming ketone transport system. However, the energy cost of continuously maintaining the system at a higher activity probably outweighed the survival value of having the system available during rare periods of prolonged starvation.

5 PATHOPHYSIOLOGY OF KETONEMIA

5.1 Acid-Base Balance

As discussed earlier, acetoacetate and 3-hydroxybutyrate are more powerful acids than the VFA, and. in the case of acetoacetate, more powerful than lactic acid. Not surprisingly, then, a high concentration of ketones in plasma results in a metabolic acidosis known as ketoacidosis. The most significant ketoacidoses encountered in domestic animals are in diabetes mellitus and ovine pregnancy toxemia. The ketoacidosis encountered in these syndromes may cause plasma bicarbonate to drop below 10 m*M* and is a chief contributor to mortality.

The ketoacidosis in diabetes of dogs and cats can be severe with blood pH being 7.2 or less. Since plasma ketone concentrations in diabetic dogs have been reported to average 3.2 mM with some individuals having levels of 7-8 mM, the base deficit in extracellular fluids would be greater than that concentration for two reasons. First, the distribution space of the ketones is greater than that of extracellular fluid, and, second, some acetoacetate and 3-hydroxybutyrate anions may have been

lost in the urine without equal losses of hydrogen ion (a mineral ion, such as sodium or potassium would have balanced the electrical charge). Base deficits of more than 15 mM have been reported in spontaneously diabetic dogs.

As the metabolic acidosis of diabetes progresses in dogs, there is increased catabolism of muscle protein. Much of the nitrogen from protein degradation is diverted to ammonia rather than urea, and it is ammonium ion that balances most of the electrical charge on excreted acetoacetate and 3-hydroxybutyrate.

5.2 Energy Balance

Ketones are really an alternate form of lipid, comparable to triglycerides, LCFA, or VFA. and should be considered as such in caloric balance. VFA and ketones are water-soluble forms of lipids; however, only the ketones can be produced in large quantities in tissue metabolism.

In fed animals, only a nominal caloric production is derived from oxidation of ketones; however, in fasted animals or in some pathological conditions, ketone oxidation accounts for a substantial quantity of expended calories. For example, only 3-4 % of expired carbon dioxide is derived from 3-hydroxybutyrate in fed cows, whereas 30 % of expired carbon dioxide is derived from ketones in fasted pregnant ewes.

5.3 Other Effects

In isolated rat hepatocytes, acetoacetate has been shown to decrease the rate of gluconeogenesis from lactate, whereas 3-hydroxybutyrate has been shown to stimulate it. The inhibitory effect of acetoacetate was capable of overpowering the stimulatory effect of 3-hydroxybutyrate. It was hypothesized that acetoacetate had its effect by competing with pyruvate for transport into the mitochondrion, and 3- hydroxybutyrate had its effect by increasing mitochondrial NADH levels. However, *in vivo*

infusion of acetoacetate into rats has been shown to decrease plasma glucose and LCFA concentrations and to increase plasma insulin concentrations. Presumably, the increase in plasma insulin inhibited hepatic gluconeogenesis and hormone-sensitive lipase in adipose, leading to a lower rate of release of LCFA.

It has been demonstrated in canine perfused liver and *in vivo* in humans and dogs by infusing acetoacetate or 3-hydroxybutyrate that both ketones inhibit gluconeogenesis. In most of these experiments, there has been evidence of increased plasma insulin concentrations which could account for the diminution in plasma glucose concentration. The survival value of having ketones inhibit gluconeogenesis is that in starvation, as concentrations increase and the ketones become available for tissue energy needs, the rate at which body protein must be catabolized to supply glucose precursors can decrease.

In some of the experiments mentioned above, decreased plasma levels of LCFA were observed. Not surprisingly in view of the increased insulin levels usually observed, decreased levels of LCFA were observed during ketone infusions. Thus, increased ketone levels may serve a negative feedback on rate of lipolysis in adipose and, therefore, on the plasma levels of ketones themselves.

6 FASTING KETOSIS

During fasting, hormonal changes occur which pro mote lipolysis. Most important, as less glucose is available from the gut or from gluconeogenesis in the liver, plasma glucose concentrations will decrease. Responding to the hypoglycemia, pancreatic islet cells will release less insulin and more glucagon, so that plasma insulin concentrations will decrease and plasma glucagon concentrations will increase. These hormonal changes will increase cAMP concentrations in adipose cells, which leads to the activation of hormone-sensitive lipase.

Through the action of hormone-sensitive lipase, triglycerides are hydrolyzed with release of LCFA and glycerol. LCFA are utilized directly by tissues for energy but are also taken up by the liver in proportion to their plasma concentration. During fasting, hepatic concentrations of malonyl-CoA are relatively low, so CAT I activity is relatively high, and LCFA-CoA are quickly converted to LCFA-carnitine which is translocated into the mitochondrion. Once in the mitochondrion LCFA-carnitine is converted to LCFA-CoA again.

Following β-oxidation of ketones, some acetyl- CoA is combusted in the citric acid cycle. During fasting, however, gluconeogenesis is quite active in the liver, and much of the mitochondrial oxaloacetate is used for that purpose and is unavailable for citrate formation with acetyl-CoA. Consequently, large quantities of acetyl-CoA are shunted into ketogenesis.

Acetoacetate and 3-hydroxybutyrate can be utilized by most extrahepatic tissues. Since peripheral tissues can also use LCFA, the utility of hepatic production of ketones from LCFA was not dear originally. However, many tissues have as great or greater capacity for utilizing plasma ketones as for utilizing plasma LCFA. Among these tissues are heart and kidney. In some species, such as the rat and human, ketones constitute a major energy source for the brain during fasting. In some other species, though, it appears that the brain prefers glucose and utilizes only small quantities of ketones in the fed or fasted state in sheep, dog, and pig. Resting skeletal muscle utilizes ketones preferentially as a fuel during short-term starvation; however, LCFA are preferred during long-term starvation (or exercise.

The ketones are quite soluble, require no protein carrier, and diffuse (in their unionized form) or are transported rapidly through biological membranes including the blood-brain barrier. The liver has an advantage over other tissues regarding uptake of LCFA from plasma albumin because of its unique sinusoidal vascular system. Therefore, the liver can be regarded as a machine that

can rapidly remove LCFA from plasma and convert them to a form, the ketones, that other tissues can utilize rapidly.

Because LCFA must be bound to albumin if they are to be nontoxic, the maximum safe plasma concentration of LCFA is fixed by the albumin concentration. Furthermore, in prolonged fasting, albumin concentration decreases which decreases the number of LCFA carriers. Generally, LCFA concentrations do not rise above 2 mM in fasting, whereas ketone concentrations can increase to 3-4 mM or more. Thus, ketones can have a greater concentration gradient to allow their entry into the cell.

Although the acid nature of ketones has received much attention in the clinical literature, less well recognized is the toxic potential of LCFA. If LCFA are released into the plasma in excess of hepatic uptake, albumin binding capacity will be exceeded. Unbound fatty acids have a detergent action and may damage cell membranes, particularly the membranes of endothelial cells which line the blood vessels. Such damage to endothelial cells has been proposed as a mechanism in the development of atherosclerotic plaque. There is some evidence in humans and guinea pigs that high levels of LCFA within the heart may predispose it to arrhythmias.

Ketogenesis in fasting should be viewed as an evolved mechanism with specific survival value for peripheral tissues and not a burden that the liver is placing on the rest of the body. It is important to remember that fasting animals should be expected to have a degree of ketonemia, ketonuria, and ketolactia. Thus, any disease condition which causes anorexia will usually be accompanied by increased ketone levels in body fluids which have no significance other than the fact that the animal has a subnormal caloric intake.

7 DIABETIC KETOSIS

In experimental diabetes in dogs, plasma total ketone concentrations are 3.2 mM as compared with 0.1 mM *i*n healthy dogs. Diabetes is accompanied by hyperglycemia, whereas most other ketotic syndromes occurring in domestic animals are usually accompanied by normoglycemia or hypoglycemia. The ketonemia in diabetes is due to increased lipolysis in adipose plus accelerated hepatic gluconeogenesis, both brought about by a lack of insulin. Thus, there are abundant plasma LCFA as ketogenic substrates and metabolic conditions in the liver that favor ketone synthesis.

8 KETOSIS ASSOCIATED WITH PREGNANCYAND LACTATION

Ketoses associated with pregnancy and lactation, are most commonly observed in ruminants although they have been documented in dogs and humans. Before specific syndromes are discussed, a general picture of ketogenesis in pregnancy and lactation will be presented.

Fetal demands for glucose are high, and the placenta can transport glucose from maternal to fetal plasma. When an imbalance occurs between the maternal ability to synthesize or absorb glucose and fetal consumption. hypoglycemia results. Under these circumstances, hypoglycemia will lead to lipolysis in adipose tissue and release of LCFA as discussed earlier. The LCFA will be taken up by the liver and converted to ketones with resulting ketosis.

Ketosis in lactation is somewhat more complex than ketosis occurring during pregnancy. The volume of milk produced is almost totally dependent on the rate of lactose synthesis by the mammary gland because milk volume formation is an osmotic phenomenon, and lactose is the predominant molecular species

in milk. There is virtually only one precursor of lactose and that precursor is plasma glucose. Therefore, a female that is in heavy lactation will have a heavy drain on plasma glucose. There are two sources of plasma glucose, absorption from the gut and gluconeogenesis.

In ruminants, little glucose is absorbed from the gut so the overwhelming bulk of it is synthesized. Most (approximately 90%) of this synthesis occurs in the liver with the remainder occurring in the kidney. The chief substrates are propionate and amino acids, with the former being most important in animals on a high grain diet. Other precursors are branched chain VFA and lactate absorbed from the rumen and glycerol released during lipolysis. If there is a mismatch between mammary drain of glucose for lactose synthesis and gluconeogenesis in the liver, hypoglycemia will result. Under these circumstances, hypoglycemia will lead to ketosis as explained in the discussion on fasting ketosis. It is important to note, however, that in dairy cows, at least, ketonemia can occur without significant hypoglycemia. This phenomenon is discussed below.

8.1 Bovine Ketosis

Bovine ketosis is actually at least three different syndromes which occur in cows during lactation. The syndromes are characterized by anorexia, depression (usually), ketonemia, ketolactia, ketonuria, hypoglycemia, and decreased milk production. The three syndromes are underfeeding ketosis, alimentary ketosis, and spontaneous ketosis.

8.1.1 Underfeeding Ketosis

Underfeeding ketosis occurs when a dairy cow receives insufficient calories to meet the demands of lactation plus body maintenance. This version of ketosis can be conveniently divided into nutritional underfeeding ketosis and secondary (or complicated) ketosis. The former occurs when the cow has a

normal appetite but is given an insufficient quantity of feed or a diet with low metabolic energy density. The latter occurs when a cow has some other disease which suppresses appetite, and the cow fails to consume adequate nutrients. The most common complicating diseases are hypocalcemia, mastitis, and metritis. In most respects, underfeeding ketosis resembles starvation ketosis explained above, except that there is the additional caloric and glycemic burden of milk production.

8.1.2 Alimentary Ketosis

Alimentary ketosis occurs when cattle have been fed spoiled silage that contains excessive amounts of butyric acid. As discussed previously, the rumen epithelium has a high capacity to activate butyrate to butyryl-CoA, to oxidize the butyryl-CoA to acetoacetyl-CoA. and to convert the acetoacetyl-CoA to acetoacetate and 3-hydroxybutyrate. Under conditions where excessive butyrate is presented to the rumen epithelium, large amounts of 3-hydroxybutyrate will be produced and released to the circulation with resulting ketosis. Alimentary ketosis then is really butyrate toxicosis.

8.1.3 Spontaneous Ketosis

Spontaneous ketosis is probably the most common, the most researched, the most controversial, and the least understood form of bovine ketosis. It occurs in high-producing dairy cows that are near the peak of lactation, that have access to abundant high quality feed, and that have no other disease. The disease is not accompanied by severe acidosis, and spontaneous recovery is common although there is a large decrease in milk production. There are several schemes proposed for the molecular pathogenesis of the syndrome. As these schemes are discussed, it will be seen that they are not necessarily mutually exclusive, and more than one of them may be correct and may be present simultaneously in the same animal.

The most widely accepted theory of bovine ketosis is the hypoglycemia theory. In this theory, hypoglycemia is the driving force in the syndrome and ultimately causes the ketonemia. Dairy cows are selected for remaining in the herd more for milk production that for any other factor. Thus, dairy cows have been selected for many generations to have a metabolically aggressive mammary gland. Cows are selected secondarily for remaining in the herd on other factors such as conformation, behavior, and freedom from health and reproductive problems.

These selection forces have dictated that the mammary gland produce a maximum amount of milk with secondary regard for the metabolic consequences for the rest of the animal. It is not surprising, therefore, that occasionally the mammary gland might withdraw glucose from the plasma more rapidly than the liver can resupply it, which leads to hypoglycemia even in a well-fed animal. The hypoglycemia will lead to ketonemia by mechanisms discussed earlier and below. The hypoglycemia and ketonemia may cause the cow to be ill enough that she will decrease her feed intake. At this point, the syndrome will resemble underfeeding ketosis.

As explained above, high milk production equates to a high rate of plasma glucose utilization by the mammary gland which equates to a high rate of hepatic gluconeogenesis. In a lactating cow, the plasma glucose concentration represents the balance point between hepatic glucose production and mammary glucose utilization. If mammary gland glucose utilization should leap ahead of hepatic glucose production, hypoglycemia will result. In theory, hypoglycemia under these circumstances should lead to a decrease in plasma insulin and an increase in plasma glucagon levels. Lower plasma insulin and higher plasma glucagon should increase the activity of hormone-sensitive lipase in adipose tissue which will lead to increase plasma levels of LCFA. Consequently, more LCFA will reach the liver and exceed its capacity to oxidize

them completely or to re-esterify them, and increased ketogenesis will result.

What evidence supports this theory? First, the vast majority of cows with clinical spontaneous ketosis are indeed hypoglycemic. Second, cows with spontaneous ketosis usually are hypoinsulinemic. Third, postparturient dairy cows have been found to have elevated levels of plasma immunoreactive glucagon. Whether spontaneously ketotic cows have even greater plasma glucagon levels remains unknown. Fourth, ketotic cows have elevated levels of plasma LCFA.

Some investigation of molecular mechanisms of ketogenesis in the liver ketotic cows has been performed. In particular, there has been interest in hepatic mitochondrial oxaloacetate levels. The acetyl-CoA can be oxidized to carbon dioxide provided there is sufficient oxaloacetate to permit entry into the citric acid cycle as citrate. In order for the citric acid cycle to operate, there must also be a sufficient amount of ADP available for phosphorylation as well or accumulation of NADH will slow the cycle. If acetyl-CoA accumulates, the excess will be diverted into ketogenesis.

Two studies have attempted to investigate oxaloacetate concentrations in the livers of ketotic cows. Unfortunately, different methodologies were used to estimate oxaloacetate concentrations. One study concluded that there was no change in oxaloacetate concentration during ketosis, and the other concluded that oxaloacetate concentrations were lower in ketotic than in healthy cows. Actually, both studies measured total hepatic oxaloacetate rather than mitochondrial oxaloacetate which may be critical in ketogenic control. However, there has been no evidence to indicate that the ruminant liver should be any different from the non-ruminant liver with regard to the concept that if the liver is presented with sufficient LCFA ketogenesis will result. There has been insufficient research on the control of lipolysis in adipose in ruminants. In particular, there has been insufficient research in differences in plasma levels of lipogenic

and lipolytic hormones and sensitivity of adipose to these hormones in cow populations that are susceptible and non-susceptible to ketosis. No matter how low mitochondrial oxaloacetate levels might be in the liver, ketogenesis will not occur at a significant rate without sufficient precursor in the form of LCFA, and. conversely, ketogenesis could occur with normal oxaloacetate levels if the liver were presented with a sufficiently high concentration of LCFA.

It has been noticed, however, that dairy cattle can become ketonemic without the presence of significant hypoglycemia. This is often the case with subclinical ketosis in which ketonemia exists without other signs of ketosis. It has been postulated that there is a signal for lipolysis to meet mammary demand for LCFA which is independent of plasma glucose concentration. The increased plasma LCFA lead directly to increased hepatic ketogenesis. The identity of this postulated signal is unknown.

Based on this theory, it has been proposed that there should be a decrease in ketosis incidence which occurs when protected fats are fed in the diet. Protected fats are triglyceride micelles which are protected from attack by rumen microbes by being encased within a coating of formaldehyde-treated protein. The protected fats will be absorbed from the small intestine as chylolmicrons which will be removed from the plasma by the mammary gland and incorporated into milk fat. Under these circumstances, there would be less need for lipolysis and release of LCFA from adipose. Feeding protected fats does decrease plasma concentrations of ketones in healthy dairy cows in the first half of lactation. Whether feeding more fat will reduce the incidence of spontaneous ketosis remains to be seen. Although the theory of feeding more fat to lessen the incidence of ketosis is attractive from a viewpoint of mass action, the controlling mechanisms have yet to be established.

When it was first observed that glucocorticoids appeared to be an effective treatment for spontaneous ketosis. it was

hypothesized that the disease was due to adrenal cortical insufficiency. This theory has fallen into disfavor since it has been shown that ketotic cows have higher plasma levels of glucocorticoids than healthy cows. Glucocorticoids are efficacious and probably have their effect by stimulating proteolysis and inhibiting glucose use in muscle, thereby providing gluconeogenic precursors and glucose.

The efficacy of glucose or glucose precursors as ketosis treatments favor the hypoglycemic theory. Parenteral glucose provides nearly immediate relief although relapses are common. Gluconeogenic precursors, such as propylene glycol, glycerol, and sodium propionate, have been shown to be efficacious. Low doses of insulin have been proposed as a treatment, on the theory that a low dose of insulin will aid utilization of parenteral glucose and will inhibit lipolysis without significantly inhibiting gluconeogenesis. In a well-designed study, however, insulin was not shown to be of value when added to glucocorticoid therapy. Insulin has the potential to exacerbate hypoglycemia.

8.2 Ovine Pregnancy Toxemia

Ovine pregnancy toxemia occurs in pregnant ewes that are carrying more than one fetus and which have been subjected to caloric deprivation or stress. Because of intense genetic selection for twinning, the syndrome is. to a large extent, a man-made disease. Susceptibility increases as ewes' approach term because fetal glucose demands increase with increasing body size. The ovine placenta is capable of extracting glucose from maternal plasma at concentrations below 1 mM and readily does so. It might seem biologically useless for the fetuses to cause a fatal hypoglycemia in the ewe which will also lead to their own demise, but the fetuses are highly dependent on glucose as a caloric and synthetic source and would expire without it anyway.

Fetal lambs normally maintain a very low plasma glucose concentration of approximately 0.6 mM compared to 2.7 mM in an

ewe. Thus, the transplacental glucose gradient greatly favors movement from dam to fetus. Curiously, the most concentrated carbohydrate in fetal sheep plasma is fructose (5.1 mM) which is synthesized from glucose in the placenta by reducing glucose to sorbitol followed by oxidation to fructose. Despite the abundance of fructose in the plasma of the fetal sheep, glucose constitutes its primary energy supply, and the fetuses normally consume 60-70 % of maternal glucose production.

The ovine placenta appears to have a low permeability for acetoacetate. When acetoacetate loads have been infused into pregnant sheep, the concentrations in fetal blood have remained low. Further, *in vitro* experiments with perfused sheep placenta have also demonstrated a low permeability for acetoacetate. Thus, it appears that maternal acetoacetate, and perhaps 3-hydroxybutyrate, cannot be a major energy source for the ovine fetus.

The disease is characterized by depression and weakness in the ewes which is associated with hypoglycemia, ketonemia, and ketonuria. The ketonemia is severe enough to cause acidosis which can be severe. There is also considerable fatty deposition in the liver to the extent that it may interfere with liver function. Eventually, the ewes are unable to rise, become comatose, and die if untreated.

Mild cases respond to intravenous glucose, glucocorticoids, or glucose precursors such propylene glycol or glycerol coupled with removal of stress and improved nutrition. Severe eases, in which the ewes are unable to rise, usually respond only to delivery of the lambs, and even then, a high mortality will occur.

8.3 Syndromes in Other Species

Ketosis associated with lactation can occur in dairy goats. The syndrome has also been reported in beef cows with caloric deprivation and nursing two calves.

Pregnancy toxemia has been reported in goats carrying multiple fetuses. The syndrome can be produced with calorie deprivation, particularly if coupled with stress, and almost always occurs in does carrying more than one fetus. Obesity also may be a predisposing factor in does. Generally, the syndrome in does appears entirely similar to that in ewes.

Pregnancy toxemia has been reported in beef cows in the last two months of gestation. The disease occurs predominantly in cows that are carrying twins. The cows may be in good or even obese body condition, but sudden food deprivation, decrease in food quality, or imposition of stress such as water deprivation may precipitate the syndrome. The disease resembles pregnancy toxemia in sheep in many respects: there is ketonemia. ketonuria. and fatty infiltration of the liver. Severe cases (unable to rise and severely depressed or comatose) usually do not respond well to any treatment other than delivery of the fetuses. Mild cases will respond to improved nutrition, parenteral glucose, and steroids, although relapses are common.

Pregnancy toxemia has been reported in pregnant bitches and appears similar to the disease in sheep. Hypoglycemia is severe in canine cases, and the animals respond readily to intravenous glucose. If the animals will eat a carbohydrate-containing diet, a relapse is unlikely: otherwise, removal of the fetuses is required for a cure.

Pregnancy toxemia occurs in pregnant guinea pigs, and, like in pregnant ewes, the syndrome can be precipitated by inadequate calories and stress. The syndrome in guinea pigs is quite similar to that in sheep. The animals become weak and depressed with eventual coma. There is marked ketonemia and acidosis.

9 POSTEXERCISE KETOSIS

Postexercise ketosis, which was first documented in 1909, has been investigated most extensively in humans and rats.

Neither trained nor untrained humans or rats show much increase in ketones during exercise, but only untrained individuals exhibit a significant ketonemia and ketonuria after exercise. The greater enzymatic capacity of muscles of trained rats to catabolize ketones. It also appears that trained athletes have a greater capacity to oxidize LCFA in muscle than nonathletes. A high carbohydrate diet in conjunction with training also decreases the magnitude of postexercise ketosis.

From the foregoing, it appears that a number of factors are involved in postexercise ketosis. During exercise, all forms of fuel, including LCFA, ketones, and glucose, are oxidized. After exercise, there is a diminution of LCFA release, although plasma LCFA concentrations decrease little at first because of an even greater diminution in LCFA oxidation by the heart and skeletal muscle, and more LCFA may be converted to ketones by the liver. Ketone oxidation by muscle is decreased postexercise which will allow ketones to accumulate. In the postexercise period, there is gluconeogenesis as lactate is cycled back into glucose and glycogen which may lead to decreased mitochondrial oxaloacetate levels and increased ketogenesis. Finally, compared to the exercise period, in the postexercise period relatively more of the cardiac output will flow through the portal system, and the rate at which LCFA are presented to the liver may increase.

Postexercise ketosis undoubtedly occurs in most mammalian species but, among the domestic species, has been best documented in dogs and horses. Postexercise increases in plasma levels of ketones have been observed in racing sled dogs. Postexercise ketosis has been reported several times in the horse. In these studies, horses were subjected to endurance rides of 80-160 km. Plasma 3-hydroxybutyrate concentrations increased two- to three-fold 5-60 minutes postexercise compared to pre-exercise levels. Plasma LCFA concentrations increase five-fold or more in horses during exercise and decrease little during 30-60 minutes postexercise. Thus, abundant LCFA are available

to the liver postexercise when muscle utilization of LCFA and ketones is decreased, a situation which results in ketonemia.

Test questions for Chapter II

1. Chemical structure of ketone bodies.
2. Quantitative and qualitative methods for determining ketone bodies.
3. Diagnostic value of ketonemia and ketonuria.

CHAPTER III
LIPID METABOLISM AND ITS DISEASES

1 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. 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 carbohydrates 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 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. Earnest students of this field are referred to a recently published comprehensive overview of lipid chemistry, biochemistry, and nutrition.

Lipids have special importance physiologically as the hydrophobic constituents of membranes and as the most concentrated source of energy (9 cal/g) 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). Such a definition is not all-inclusive; for example, lecithin is insoluble in acetone and yet is considered a lipid.

2 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 recently is useful. The three major classes are simple lipids, phospholipids, and sphingolipids.

2.1 Simple Lipids

Compounds that are not degraded by alkaline or acid hydrolysis or that on 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.

2.2 Phospholipids

Compounds that on hydrolysis yield derived lipids plus inorganic phosphate, glycerol, and usually a third water-soluble product are classified as phospholipids. The 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. Variations, of course, do occur. One variant, a lecithin containing two saturated fatty acids, deserves special mention. Scientists have shown that the surfactant secreted by certain cells in the lung 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.

Even though phophoinositides are the least common phospholipids in most cell membranes, they have taken on new significance as part of a second messenger system. It is not appropriate to review this messenger function in the context of this chapter, but many reviews are available. Suffice to say here that specific agonists acting at the cell surface can affect the release of inositol triphosphate and arachidonate from the membrane phospholipid and lead to the formation of diacylglycerol. Each of these can act in a message-transducing system: inositol tris-phosphate mobilizes calcium ion as part of calcium-mediated cell responses: arachidonate can be oxidized to a host of intracellular messengers including the prostaglandins, thromboxanes, and leukotrienes; and diacylglycerol acts as a cofactor for protein kinase C.

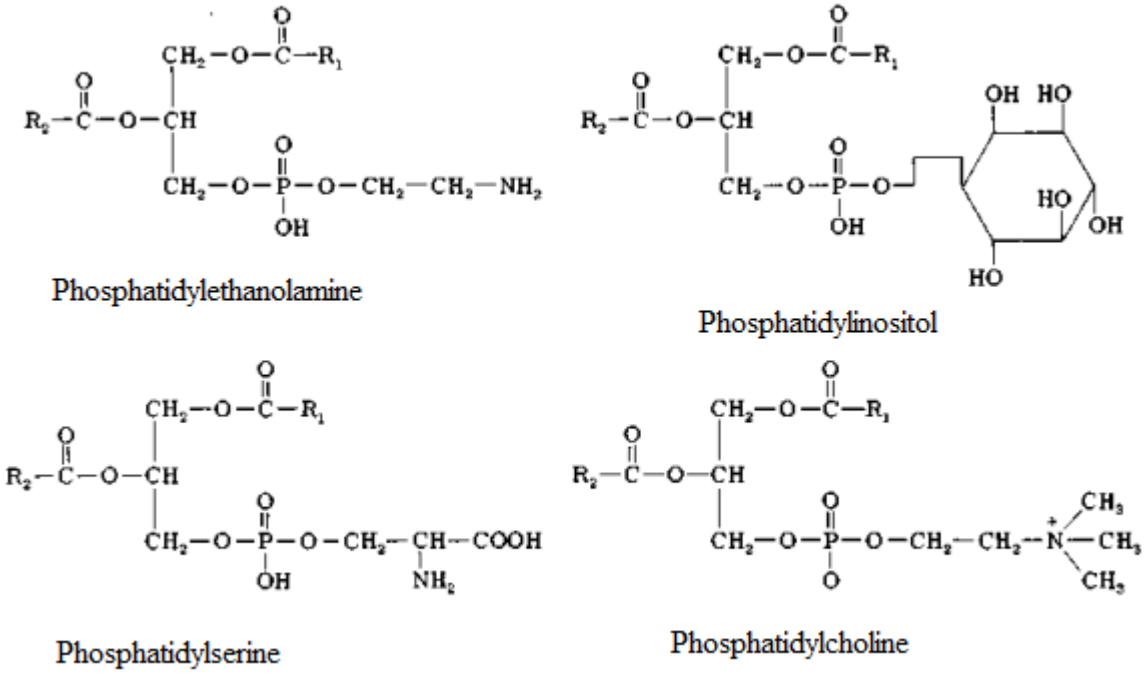


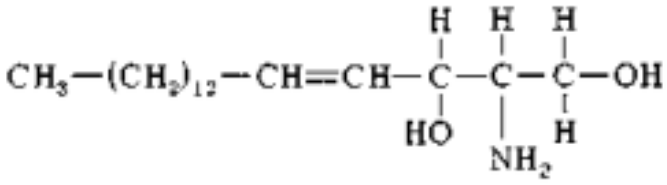
Fig. III.1 Structural formulas of common phospholipids.

Not all phospholipids contain two 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 that differ from ester bonds to the alcohol group of glycerol. Plasmalogens contain a monovinyl ether as well as a monofatty

acyl group. On hydrolysis, this α, β-unsaturated ether yields a long-chain aldehyde, which accounts for Feulgen's 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.

a. 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 tissue 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 of an amino group linked with monosaccharides, hexosamines, and neuraminic acid. For example, a crystalline ganglioside containing equimolar amounts of sphingosine stearic acid, glucose, galactose, *N*-acetylgalactosamine, and A-acetylneuraminic acid has been isolated from bovine brain. 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. The occurrence of some of these inborn errors of metabolism has also been noted in animals. Undoubtedly more animal diseases will be reported in the future.

3 CHEMISTRY OF SOME LIPIDS

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

3.1 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 animals. 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 and, even then, is likely absorbed to protein. Usually, when the fatty acid content of a tissue or diet is referred to the fatty acids ate 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 arc steam distillable. The metabolic differences between the short chain VFA and the long chain FFA will be discussed later.

The Geneva systems 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 monounsaturated 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 ‘‘-enoic” to designate the appropriate number of unsaturated bonds.

Two systems are 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-l. 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. Their essential function appears to be related to their presence as an integral part of cell membranes and as substrates for prostaglandins, thromboxane and leukotriene synthesis.

3.2 Chemical Reactions of Fatty Acids

Only selected chemical reactions of physiological and clinical importance will be discussed.

3.2.1 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. Solubility was inversely related to the length of the carbon chain at all temperatures caproic acid being the most soluble, about 1 g per 100 g water, and stearic acid being the least. 0.00018 – 0.0005 g per 100 g water depending on the temperature. The VFA, acetic, proprionic, and butyric acids are miscible with water in all proportions and 3.7 g of valeric acid is soluble in 100 g of water. 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 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.

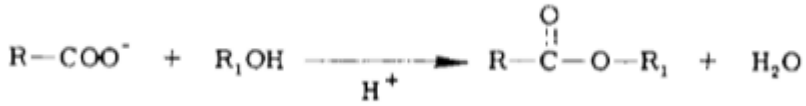
The sodium and potassium salts of fatty acids are soluble in water and insoluble in organic solvents. Thus, extraction of lipid samples with lipid solvents after saponification, but before acidification, separates the non-saponifiable lipids, mainly steroids and hydrocarbons from the saponifiable ones. 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 on the organic solvent used. For example, 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 epithelial cells of most mammalian species.

3.2.2 Formation of Salts

Free fatty acids 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.

3.2.3 Formation of Fatty Acid Esters

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



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.

3.2.4 Hydrogenation and Halogenation

Unsaturated fatty acids can be converted to saturated fatty acids by the addition of 1 mol of hydrogen at the site of each double bond. 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 mol of a halogen is added at each unsaturated bond. 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.

3.2.5 Halogenation is pertinent to clinical medicine.

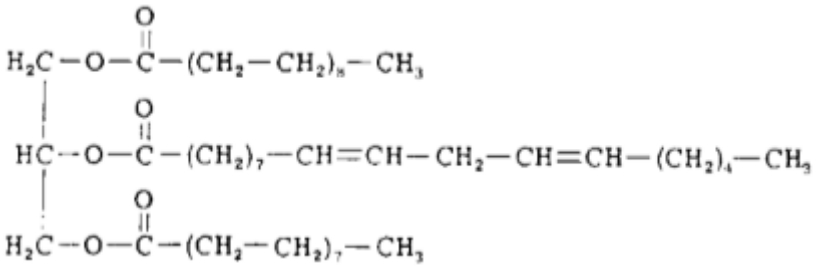
Fatty acids in glycerides used in absorption studies are often labeled with radioactive iodine, usually ^{125}I, by means of halogenation. Lipids labeled by this means are cheaper, more easily handled, and more easily assayed than comparable ^{14}C-labeled compounds. The specific tests using these compounds are discussed in Chapter 16. Whether fatty acids labeled with

radioactive iodine are handled by the organism precisely as are natural fatty acids is open to question.

3.3 Chemistry of Neutral Acylglycerols

Of the neutral acylglycerols, triacylglycerols are by far the most common in nature. Triacylglycerols 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-phosphate, 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 is discussed later.

The term "simple glycerides" refers to triacylglycerols 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 αinstead of *sn-1* and *sn*-3. Thus, the following triglyceride could be referred to as β-linoleo-α-stearopalmtin or as sn-1-stearyl-2-linoleolyl-3-palmityl triacylglycerol:



The fatty acids comprising the triacylglycerol 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 *sn*-2 position of the naturally occurring triacylglycerols.

An exception to this finding are the triacylglycerols of the domestic pig: in this species, palmitic acid is consistently found in the *sn-2* position.

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 alcoholic solution of a strong alkali results in formation of salts of fatty acids along with glycerol.

3.4 Chemistry of Steroids

The complicated chemical structure of steroids is covered only briefly to illustrate how the same basic structure can be varied to allow a wide variety of physiological roles. 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. III.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 also 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 at various sites; and (6) these side groups can be oriented in either the a or 0 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 on the type of group, and its orientation, at C-3, C-11, C-17.

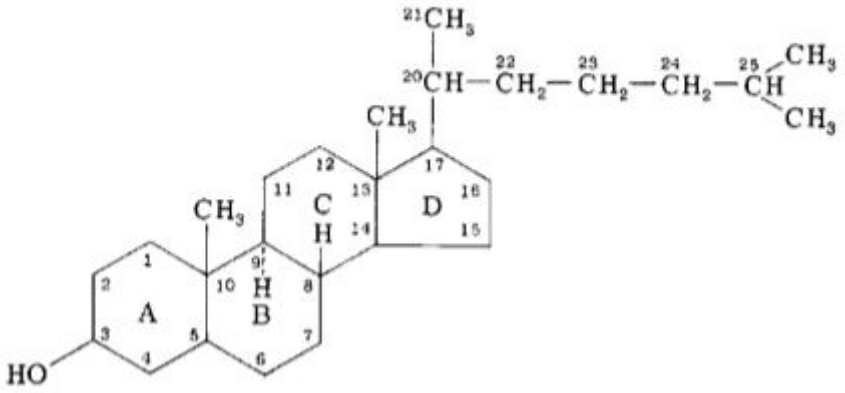


Fig. III.2 Structural formula of cholesterol showing numbering of carbon atoms and designation of rings.

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 occurring steroid, is implicated in vascular disease, and has diagnostic importance e.g. in hypothyroidism. Cholesterol has the basic steroid skeleton with a branched side chain at C-17, a secondary,

3-oriented alcohol at C-3, and a double bond between C-5 and C-6 (Fig. III.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 and plasma. The significance of the esters and reactions catalyzing their formation are discussed later.

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 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 have been used for the detection of cholesterol. Since these colorimetric methods are not specific for cholesterol, current assays are based, mainly, on enzymatic reactions.

4 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. The generalizations developed in studies with rodents and humans should be applied with caution to herbivores.

The major portion of the lipid ingested is in the form of triacylglycerols, but some cholesterol, cholesterol ester, and phospholipid are also present in many 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, rats, and humans. This lipase may be of physiological importance, particularly in the newborn, when the secretion of pancreatic lipase is low.

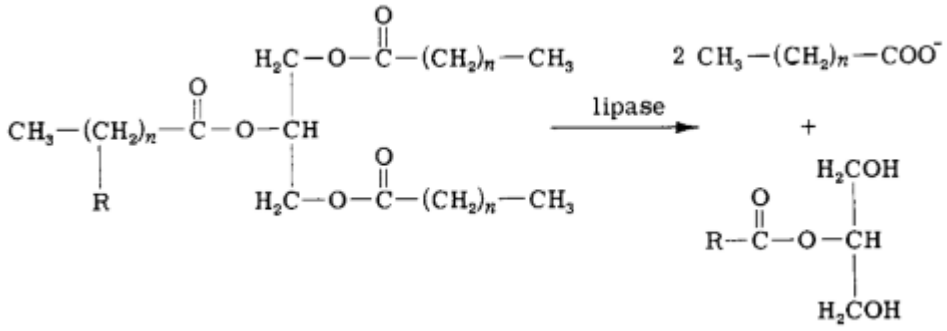
The manner in which lipids are absorbed was uncertain for many years. The basic concept that triacylglycerols are only partially hydrolyzed to monoacylglycerols (MG) and FFA.

Elaborating on the theory, 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. 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.

4.1 Digestion of Lipids

4.1.1 Hydrolysis of Triacylglycerol to MG and FFA

Pancreatic lipase acts specifically on the α-ester bond of the triacylglycerol, thereby releasing MG and FFA. Any further hydrolysis takes place only after isomerization of the remaining fatty acid moiety of the MG to the A 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. 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 substrate concentration.

Pancreatic lipase hydrolyzes all triacylglycerols with long chain fatty acid moieties at about the same rate, if they are adequately and equally emulsified. 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 triglycerides 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.

4.2 Formation of a Micelle Suitable for Absorption

Although bile salts aid in the formation of emulsions prior to hydrolysis of triacylglycerol. 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. Such aggregates are referred to as micelles. Lipid molecules are readily trapped and concentrated in the micelle. 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. Such MG are the most common ones formed by the action of pancreatic lipase because the naturally occurring triacylglycerols commonly have an unsaturated fatty acid at the β position. These mixed micelles arc probably the form in which lipid is absorbed by the mucosal cell. Small amounts of di- and triacylglycerol may also enter the micelles. 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.

Although the formation of micelles by bile salts aids in the absorption of lipid, it is dispensable for triacylglycerol absorption.

Thirty to forty percent of the ingested triacylglycerol is absorbed in the absence of bile in dogs and rats. 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 triacylglycerol 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.

4.2 Entry into the Mucosal Cell

There was 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. The droplets are not quantitatively related to lipid absorption and, therefore, represent an event taking place after actual absorption. After absorption, the MG and FFA are again combined to form triacylglycerols. This step appears to be the rate-limiting step in lipid absorption. Triacylglycerol synthesis can proceed via two pathways:

1) the glyceride-glycerol of the monoglyceride is utilized as the backbone;

2) a new glyceride backbone is formed by acylation of *sn*-glicerol 3-phosphate. The fatty acids are incorporated after being "activated" to -Acyl-CoA derivatives. The phosphatidic acid formed in pathway (2) is dephosphorylated and the resulting diglyceride acylated to form the final product.

The lipase of the mucosal cells may be of significance as a control point for determining which pathway of triacylglycerol synthesis predominates and may thereby provide a means of balancing available glyceride-glycerol backbone with available FFA. In contrast to pancreatic lipase, mucosal lipase is specific for MG. 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 re-esterified.

The enzymes of the mucosal cell responsible for activation and re-esterification of the FFA prefer fatty acid with a carbon chain of 12 carbons or more. Therefore, there can be partitioning of fatty acids in the mucosal cell, 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 triacylglycerol with medium and short chain fatty acids is more rapid than that of triacylglycerol containing long chain fatty acids because of more rapid hydrolysis, and the resulting FFA escapes the rate- limiting step of re-esterification to triacylglycerol. This observation is of clinical importance in treatment of any lipid malabsorption syndrome, such as short bowel syndrome, biliary obstruction, lymphatic obstruction, and, as mentioned, pancreatic insufficiency.

4.3.1 Steroid Absorption

The cholesterol resulting from the hydrolysis of 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. The cholesterol is then re-esterified and transferred to the lymph as part of the chylomicron.

4.3.2 Phospholipid Absorption

Phospholipids in the diet are hydrolyzed to phosphoglyceride and FFA in the lumen of the small intestine and absorbed in this form. Subsequently, the phosphoglyceride is re-esterified in the mucosal cell to form various diacyl derivatives.

4.4 Formation of the Chylomicron

Within the mucosal cell, the resynthesized triacyiglycerol, 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 on protein synthesis in the mucosal cell.

The chylomicron apparently leaves the mucosal cell by reverse pinocytosis. 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.

5 FATE OF DIETARY LIPIDS

5.1 Chylomicron Metabolism

Chylomicrons are rapidly removed from the circulation and their contents utilized by adipose tissue, cardiac muscle, liver and probably lung. The kinetics and the mechanism of chylomicron utilization have been extensively investigated. Triacylglycerol is the major component of chylomicrons, undergoes hydrolysis to glycerol and its constituent fatty acids. These components may be utilized for synthesis of new triacylglycerols and phospholipids or oxidized to CO_2. Whether the initial degradation takes place on the external plasma membrane, or intracellularly 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 triacylglycerol is catalyzed by lipoprotein lipase. The inactive bound form of this enzyme is activated in the plasma by heparin. Because the hydrolysis of triacylglycerol 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. A congenital deficiency of lipoprotein lipase resulting in hyperlipemia of exogenous origin has been reported in a puppy. Certain tissues contain lipoprotein lipase activity as well. Adipose tissue lipase is responsive to diet, increasing during feeding and decreasing during fasting. During lactation, this activity decreases, whereas the lipase associated with mammary gland increased.

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 re-esterified and stored as triglyceride; in liver, a portion may be oxidized, but another portion is re-esterified and released back into the plasma in the form of a very low density lipoprotein.

5.2 Medium and Short Chain Fatty Acids

The medium and short chain fatty acids are absorbed and transported to the liver via the portal system. The major portion is oxidized in the liver and does not enter the peripheral circulation. The fate of the large quantities of VFA absorbed into the portal system in ruminants varies with each compound.

5.3 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. The

output of cholesterol is also variable, increasing when the intake increases and decreasing when the intake is lessened. Cholesterol is excreted in the form of bile acids and as free cholesterol and its derivatives in bile.

6 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 triacylglycerol is most important. Cholesterol and cholesterol ester are also important quantitatively in specific species (e.g., the chicken) and important for clinical and diagnostic 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.

6.1 Free Fatty Acids

Only a small portion of the FFA are free in the plasma: most are bound to albumin. 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. 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. The concentration of FFA in most species in the post absorptive stage is 300-600 μmol per liter of plasma. The level of FFA increases from three to four folds during prolonged fasting or chronic nutritional stress.

6.2 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 ultracentrifugation, the classes have been named by 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 (triacylglycerol and cholesterol ester) and a surface made up of the lipoprotein apoprotein in association with cholesterol and phospholipid. The structural basis of the lipid-protein complex is not clear, but the bonding is not primarily covalent. 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 studies of lipoproteins in domestic animals have made it clear that species variations exist, and more recently, that even within species, in this case equines, significant differences occur studies in the cow indicate that such investigations 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.

6.2.1 Methods of Isolation of Plasma Lipoproteins

The two most widely used techniques for isolation of plasma lipoproteins are electrophoresis and ultracentrifugation. The correspondence between the fractions isolated by each of these methods is clear, at least in humans. The electrophoretic method of separation is the same as that for other plasma proteins except that lipid-specific stains can be 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.

Separation of the plasma lipoproteins by ultracentrifugation is based on differences in density, mainly arising from the lipid component of the lipoproteins. This method separates the lipoproteins into an almost limitless number of subgroups. By this means, the lipoproteins are currently divided into six subfractions: HDL, LDL, IDL, VLDL, chylomicrons, and chylomicron remnants. As seen in Fig. III.3, these divisions are arbitrary.

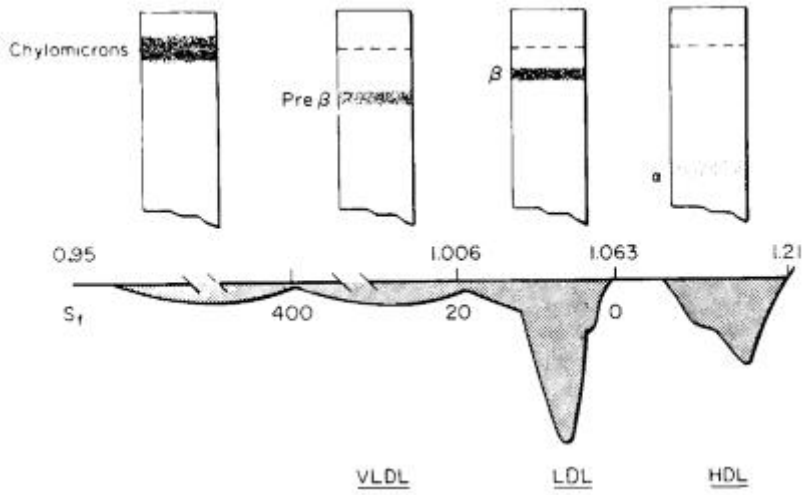


Fig. III.3 Electrophoretic and ultracentrifugal patterns of plasma lipoproteins of humans.

The relative densities are expressed as Svedberg units (S) based on the flotation rate of the lipoprotein in a density gradient. IDL (intermediate density lipoprotein, d = 1.006-1.019 *g/ml* has been designated recently as a specific class because it has been identified as a functionally important lipolytic intermediate between VLDL and LDL (Fig. III.4).

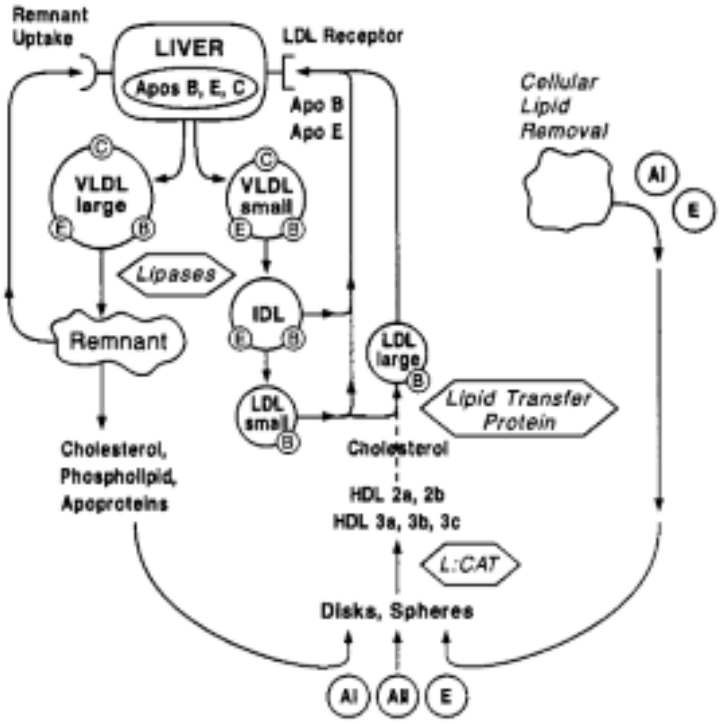


Fig. III.4 Dynamics of cholesterol transport. Large circles, lipoproteins; small circles, apolipoproteins; hexagons, enzymes and carrier proteins.

The final class, chylomicron remnants, is made up of particles derived from chylomicrons following partial removal of triacylglycerol by the action of lipoprotein lipase. Thus, these particles are characterized by being richer in cholesterol and denser than chylomicrons. The detailed relationship between the electrophoretic and ultracentrifugal separations is shown in Fig. III.3. The type and quantity of lipid found in each group are given in Table III.1.

Table III.1

Lipid Composition of Serum Lipoproteins of Human Beings

Density class (g/cm^3)	Electrophoretic migration	Percent lipid composition			
		TG	PL	CE	Chol
<1.006	Pre-β	63-75	16-25	13-16	4-6
1.006-1.063	ß	15	26	46	13
1.063-1.21	α	8	48	40	4

6.2.2 Formation of Lipoproteins

Chylomicrons are synthesized in the intestinal cells and released into the lymph and blood streams as one means of transporting absorbed triacylglycerol to various tissues in the body. The action of lipoprotein lipase, which is attached to the surface of endothelial cells, makes the fatty acids and glycerol available for uptake and further metabolism the nascent VLDL particles are synthesized in the liver for transport of endogenously formed triacylglycerols to peripheral tissues. Apolipoproteins B, C and E are associated with VLDL. IDL and LDL are generated from VLDL within the plasma by the action of lipoprotein lipase and, thereby, are rendered smaller and with relatively more cholesterol than VLDL. LDL are the lipoprotein the end products of VLDL catabolism and, hence, are the major carriers of plasma cholesterol. The importance of this class of lipoproteins in the development of atherosclerosis is mentioned later. During the processing of VLDL to IDL and LDL, specific apolipoproteins are lost so that IDL contain apoproteins B and E. LDL are characterized by their content of almost exclusively apoproteins B.

HDL are apparently formed in several peripheral tissues as well as in liver and plasma. These smallest of the lipoproteins are now conceived as the transporters of cholesterol in the form of cholesterol ester from peripheral tissues to the liver for excretion. The esterification of cholesterol is carried out by lecithin-cholesterol acyltransferase (LCAT; phosphatidylcholine-sterol acyltransferase), an enzyme in plasma. This enzyme is activated by apoproteins A-I, but the significance of this activation in the overall role of LCAT activity is unknown. This so-called reverse cholesterol transport has been suggested as an important factor in the risk of vascular disease. The apolipoproteins participating in this process include apoproteins E, apoproteins A-I, and apoproteins A-II.

The apolipoproteins facilitate lipid transfer between tissues by acting to maintain the structure of the lipid particle, as was

originally thought, but they do much more as well. Certain apolipoproteins by their affinity for specific receptors on cells act to regulate the distribution of lipid among tissues. Apo B-I00 and E mediate the interaction of LDL with its receptors on the cell. The role of these receptors in regulating plasma levels of LDL, and cholesterol, was the subject of the recent Nobel Prize in Medicine and was reviewed in detail at that time. As mentioned above in connection with LCAT, certain apolipoproteins act also as cofactors for enzymes involved in lipid transport and metabolism. The lipoprotein lipase associated with the vascular endothelial cells requires the presence of apolipoproteins C-II to carry out efficient hydrolysis of triacylglycerol in chylomicrons and VLDL. For a complete review apolipoprotein structure and function.

The site of synthesis of the apoproteins is less clear: liver is apparently the major site of apoproteins E synthesis, but it is becoming clear that other cell types also synthesize this cholesterol carrier. Apoprotein B the triglyceride carrier associated with VLDL and chylomicrons, is synthesized *de novo* in both liver (B-100) and intestine (B-48). The sites of synthesis of the A apoproteins is the liver and the intestine. The site of synthesis of C apoproteins has been studied less, but owing to the low synthesis in the intestine, liver would be the primary site of origin.

6.2.3 Interrelationships among Lipoproteins

The HDL and LDL 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 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-3-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" from those panicles with triglycerides arising from digestion and absorption. Conceptually, this scheme is still valid, but great advances have been made in the isolation and characterization, including amino acid sequence in some cases, of the apoproteins of lipoproteins. Using the nomenclature introduced by, 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 acyl-transferase (LCAT) in plasma and apoprotein C-II for lipoprotein lipase in endothelial cells lining the capillaries of many tissues. These studies have provided evidence for interrelationships between apoproteins. There is a dynamic interaction among the lipoproteins that regulates the transport, deposition, and tissue utilization of their components.

When the triglyceride carriers VLDL and chylomicrons enter the plasma, their major protein is apoprotein B (Fig. III.4). As they enter the plasma, they receive their complement of apoprotein C and apoprotein E. The addition of apoprotein C alters the electrophoretic properties of VLDL and, more important metabolically, provides one of the activators of lipoprotein lipase that act to remodel the particles for transport and effective tissue uptake. 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 apoprotein C returns to HDL, to be utilized again by nascent VLDL. Apoprotein B remains with the remnant particle and IDL and eventually can be recovered with the LDL fraction. The ultimate fate of LDL is removal by the liver. In those species in which LDL levels are low, for example, cow and rat, presumably, formation of cholesterol-(apoprotein E)-rieh HDL and hepatic

removal is very efficient. In other species, notably man, in which LDL is high, this portion of the dynamic process is not clear.

Human fibroblasts have a specific receptor for LDL. Binding of LDL to this receptor initiates a series of reactions resulting in adsorptive endocytosis of the LDL particle. 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, 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, apoprotein A is now 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. The activity of LCAT is important in maintaining the proper complement of cholesterol esters among the various lipoproteins. Because some of the cholesterol taken up by HDL and converted to cholesterol ester may come from peripheral cells, the level and complement of lipid in HDL particles (i.e. their ability to accept tissue cholesterol) may be factors in controlling excess cholesterol deposition in peripheral tissues. In this regard, the possible importance of HDL in the normal hypercholesterolemia of lactating cows is discussed later.

6.3 Dynamics of Lipid Transport

The source, mobilization, transport, and disposition of endogenous lipid are 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. As with dietary lipid, most of this lipid is triacylglycerol.

Adipose tissue lipid is mobilized in the form of FFA and glycerol is concomitantly released. Thus, hydrolysis of the glyceride ester 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. 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. Increased lipolysis does not ensure release of FFA from the tissue. If a source of *sn*-glycerol 3-phosphate is available, the fatty acid can be re-esterified 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-phosphate. The glycerol released during lipolysis cannot be reutilized for esterification because adipose tissue lacks the enzyme glycerokinase, which catalyzes the formation of glycerol 3-phosphate from glycerol. In summary, two factors enhance the mobilization of FFA: increased lipolysis and/or decreased esterification (the reverse reaction).

The FFA released from adipose tissue cannot diffuse into the plasma unless albumin is available to solubilize them. 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: for example, 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 oxidation purposes.

Free fatty acids can be disposed of in the liver in a variety of ways:

1. The FFA may be oxidized completely to CO_2 and H_2O. If there is a block in the utilization of Acetyl-CoA derived from 3-oxidation, complete oxidation cannot occur and the ketone bodies 3-hydroxy-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. The fate of triacylglycerol 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 diagrammatically in Fig. III.4.

6.4 Factors Affecting Fat Mobilization

6.4.1 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. The catecholamines probably do not act during the normal FFA release in the postabsorptive 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. Several peptides, including ACTH, TSH, MSH and vasopressin, have lipolytic actions. The action of a given peptide is limited to certain species. In each case, the site of action is triglyceride lipase and involves cyclic AMP. Another peptide, glucagon, acts similarly on adipose tissue.

Growth hormone also stimulates lipolysis but by a different mechanism. This hormone stimulates synthesis of the enzyme adenylate cyclase, which in turn catalyzes production of cyclic AMP. 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. 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. Furthermore, increased glucose uptake by adipose tissue in the presence of insulin tends to enhance esterification of FFA, thus decreasing their release. Adipose tissue has been described as being exquisitely sensitive to insulin. 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. These compounds are synthesized in mammalian tissues from the essential fatty acids. Adipose tissue in the presence of prostaglandins 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.

6.4.2 Neural Factors

Electrical stimulation of the autonomic fibers of adipose tissue results in the release of FFA into the circulation. This reaction is presumably due to the release of norepinephrine from the sympathetic fibers in the neural network. 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

exercis. In this respect, lipolysis by adipose tissue and glycogenolysis by the liver are comparable responses to emergency situations and are elicited by similar mechanisms.

6.5 Abnormalities of Plasma Lipoproteins

There have been a few studies in domestic animals to clearly establish that abnormalities and deficiencies described in man occur with significantly regularity in another species. The extensive studies in man provide an experimental model for conditions that are being observed in domestic animals. 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.

6.5.1 Inherited Lipoprotein Deficiencies

Two disorders of genetically determined origin have been described; the complete lack of apolipoprotein, abetalipoproteinemia. and a lack of normal HDL in Tangier disease. There appears to be normal apoprotein synthesis in this disease, but the catabolism of apoprotein A is abnormal and rapid, resulting in low levels.

a. Abetalipoproteinemia. The lack of, 6-lipoprotein is manifest in infancy by retarded growth, steatorrhea, and abdominal distension. The acanthocytosis accompanying this syndrome. Later, the very low plasma cholesterol levels led to the demonstration of the absence of lipoproteins. 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 lipoprotein. The

concentrations of cholesterol, phospholipid, and glycerides in plasma are the lowest recorded in any human disease. The lack of lipoprotein results in an inability to transport glyceride, either as VLDL or as chylomicrons. 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.

When patients are fed diets high in glycerides, chylomicron do not appear in the plasma. When fed high carbohydrate diets, which normally result in the release of endogenous lipid into the plasma, patients show no increase in the plasma glyceride concentration, nor is the presence of pre β-lipoprotein observed. These patients could digest and absorb MCT, but this diet could not overcome the lack of essential fatty acids. As 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. Glyceride would accumulate due to the lack of the protein complex to transport it to other tissues.

b. Lipoprotein Deficiency is lack of 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. As in abetalipoproteinemia the levels of plasma cholesterol and phospholipid are below normal. Glyceride concentrations in the plasma are high to 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 abetalipoproteinemia. 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 band and no distinct preβ band.

6.4.2 Hyperlipoproteinemias

In contrast to the hypolipoproteinemias hyperlipoproteinemias 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 is a familial occurrence. Before the advent of lipoprotein analysis, many of these conditions in both categories were referred to hypercholesterolemia and hyperglyceridemia. These names are accurate, specific terms that are clinically valid only in the absence of lipoprotein analysis.

On the basis of lipoprotein patterns and plasma lipid analyses, divided hyperlipoproteinemias into five categories. It is recommended for consistency that, initially, the findings of comparative studies in domestic animals be similarly categorized. Classes of lipoproteins that have been identified in dogs, in cattle and in pigs are chylomicron. VLDL, LDL, HDL1 and HDL2. Canine VLDL, LDL, and HDL2 correspond to human VLDL, LDL and HDL. Canine HDL has HDL mobility but is similar in size and density to LDL. The methods for sampling and analysis of the plasma have been described in considerable detail. As immunoassay methodologies, have become available for each apoprotein, the determination of their concentrations in specific disease states and in hyperlipoproteinemias added greatly to our understanding of lipid transport and deposition.

Table III.2, adapted from Fredrickson, 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 Table III.2. 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 and in idiopathic hyperchylomicronemia form in a dog.

Table III.2

Diseases Associated with the Hyperlipoproteinemias of Human Beings

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

In severe diabetes mellitus 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 Type I. The plasma usually remains turbid, indicating the presence of VLDL also. On analysis, total triglycerides (TG) (chylomicrons) and cholesterol (VLDL) are elevated. Diabetic hyperlipemia appears to be caused by impaired lipolysis of chylomicrons secondary to a deficiency of lipoprotein lipase rather than to overproduction of VLDL.

Type II is characterized by an increase in LDL. In this type of hyperlipoproteinemia, the plasma cholesterol is markedly increased in the absence of a similar increase in the glyceride level. Type II occurs basically in human beings, the most common syndrome being the formation of xanthomas and atheromas. This syndrome is now referred to as familial hypercholesterolemia and has been related specifically to mutations in the LDL receptor. A lipoprotein pattern typical of Type III is commonly associated with hypothyroidism and obstructive liver disease, although Type IV is also seen. Type III varies from Type II only in that the plasma glycerides are also markedly elevated. Specific immunoassay of apoprotein E has revealed abnormally high plasma levels of this protein in Type III.

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 III.2 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 VLDL and chylomicron areas in samples where this occurs.

In human beings, Type IV seems to have 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 VLDL as well. It is not well distinguished from Type IV and may be a combination of abnormalities such as Types I and IV.

The incidence of hypercholesterolemia in certain families makes it clear that genetic factors other than mutations in the LDL receptor contribute to the signs associated with premature atherosclerosis. Evidence is accumulating that mutations in the genes coding for apolipoproteins are factors in this phenomenon. Apoprotein B the predominant protein in LDL has been shown to be polymorphic. The discovery of polymorphism in apoprotein B in pigs allowed documentation of an apparent relationship between mutant apoprotein B hypercholesterolemia and early onset of atherosclerosis. The LDL in pigs with mutant apoprotein B is catabolized abnormally, and the hypercholesterolemia is a result of this defect. It is predictable; both in domestic animals and humans, that polymorphism among apolipoproteins will be found to be related to an increasing number of genetically linked abnormalities in lipid metabolism.

6.5 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 VLDL. 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. Ethionine CCL, puromycin and orotic acid all appear to cause fatty livers by blocking synthesis of protein, particularly the apolipoproteins. Similarly, choline deficiency results in fatty liver owing to the lack of synthesis of phospholipid a necessary component of lipoprotein complex. 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 or administration of cortisone. The ultimate cause of the fatty liver and subsequent hypertriglyceridemia in these studies is presumably mobilization of FFA derived from adipose tissue. 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.

7 BIOSYNTHESIS OF LIPIDS

The pathways for the synthesis of phospholipids and sphingolipids 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.

7.1 Fatty Acid Synthesis

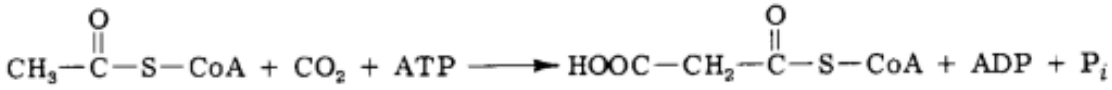
7.1.1 Tissues Involved in Fatty Acid Synthesis

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. 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.

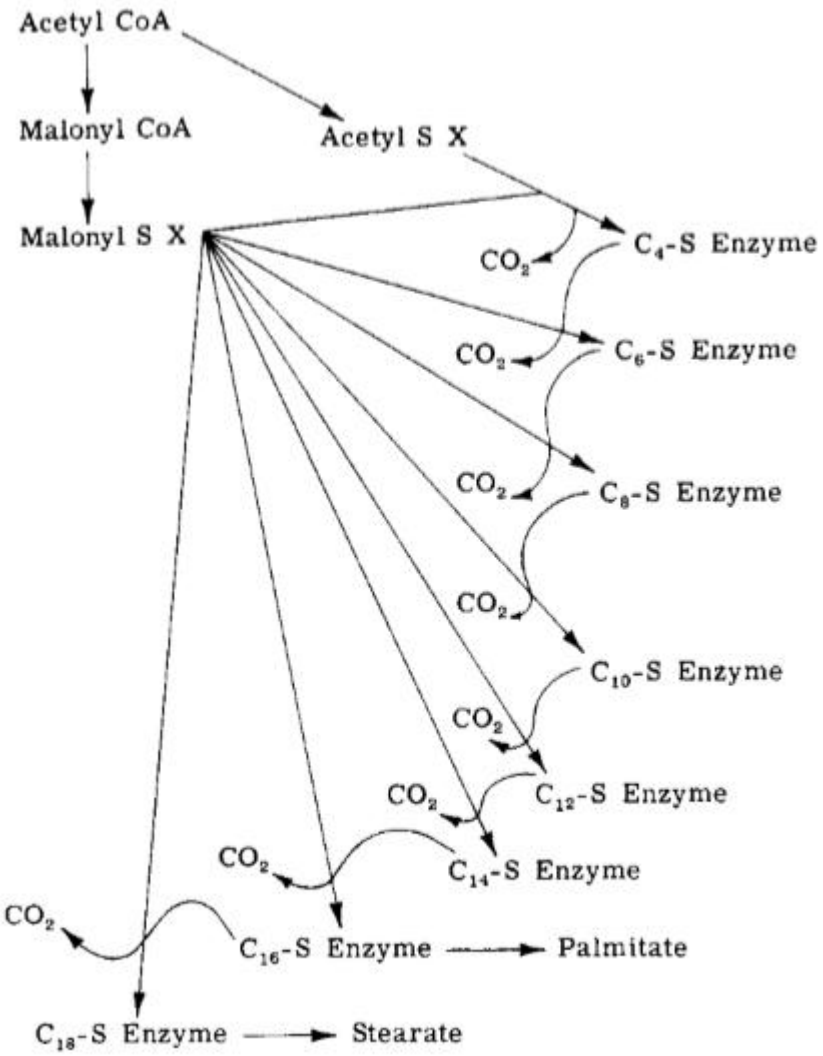
7.1.2 Pathway of Fatty Acid Synthesis

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. The first step is the formation of Malonyl-CoA by the addition of an active CO, to acetyl- CoA. This carboxylation is catalyzed by acetyl-CoA carboxylase, a biotin enzyme, and requires ATP:



In step 2 catalyzed by a multienzyme complex called fatty-acid synthase, one acetyl-CoA is coupled stepwise with several malonyl-CoA molecules, depending on 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. Studies in bacteria and in plants have revealed that the substrates participate not as CoA derivatives but as an acyl derivative of 4-phosphopantetheine that is covalently bound to serine of the peptides. This peptide is referred to as acyl carrier protein (ACP).

Fatty-acid synthase has been isolated in pure form from pigeon liver, rat liver, and mammary gland of lactating rats and cows. In all cases, it has been impossible to isolate a peptide comparable to ACP of lower forms:



But the multienzyme complex does contain 4-phosphopantetheine. The liver and mammary gland synthases 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. The complete set of reactions taking place on the multienzyme complex is shown schematically.

7.1.3 Cellular Compartmentalization and Fatty Acid Synthesis

In all mammalian *tissues* examined, the *enzymes* 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 base been suggested. Investigations indicated that, in mammary gland, citrate acts as the acetyl carrier. In this scheme, 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.

7.1.4 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. All of these are changes that would tend to maintain homeostasis. Fasting reduces lipogenesis in both liver and adipose tissue, whereas ingestion of large quantities of carbohydrate markedly increases lipogenesis in both tissues. 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. 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.

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. The degree of depression of hepatic lipogenesis is related to the linoleate, or at least to

polyunsaturated fatty acids, content of the diet and not to the total lipid content. Long-term feeding of diets high in lipid content appear 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.

It is now clear how dietary and hormonal changes influence the enzymatic activity of liver and adipose tissue. Several intracellular mediators of the dietary response have been demonstrated. Most of these are compounds known to alter the activity of Acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis. Citrate, and palmitylcarmtine have been shown to activate this enzyme. Palmityl-CoA has been shown to inhibit both the carboxylase. These activities have been used to explain the extent of lipogenesis seen in various nutritional states. Furthermore, the ratio of palmitylcarmtine to palmityl-CoA in the cell has been used to explain lipogenic responses. Hormonal control of lipogenesis by glucagon and adrenaline, which both inhibit, is due to phosphorylation and, thereby, inactivation of acetyl-CoA carboxylase by the cAMP-dependent protein kinase. Dietary modulations appear to also be mediated in part by the phosphorylation state of acetyl-CoA carboxylase.

7.2 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 is 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.

Phosphatidic acid is the first intermediate on the pathway unique to glyceride synthesis. The acylation of glycerol-3-P takes place in two steps, with the *sn-1* position being filled first and *sn-2* filled sequentially to produce phosphatidic acid. There is positioned specificity as well: saturated fatty acids tend to fill the t position and unsaturated fatty acids, the 2 position.

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-xn-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 dear. 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 diphosphidiacylglycerol, the precursor of cardiolipin phosphatidylglycerol, and phosphatidylinositol. The alternate path is dephosphorylation in the reaction catalyzed by phosphatidate phosphatase to yield 1,2-diacyl-sn-glycerol. Reaction of this diglyceride with cytidine derivatives of choline and ethanolamine results in (he 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. 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.

7.3 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 bite acids and steroid hormones is covered elsewhere in this volume. The pathway of cholesterol biosynthesis and its control are briefly discussed here.

7.3.1 Pathway of Cholesterol Synthesis

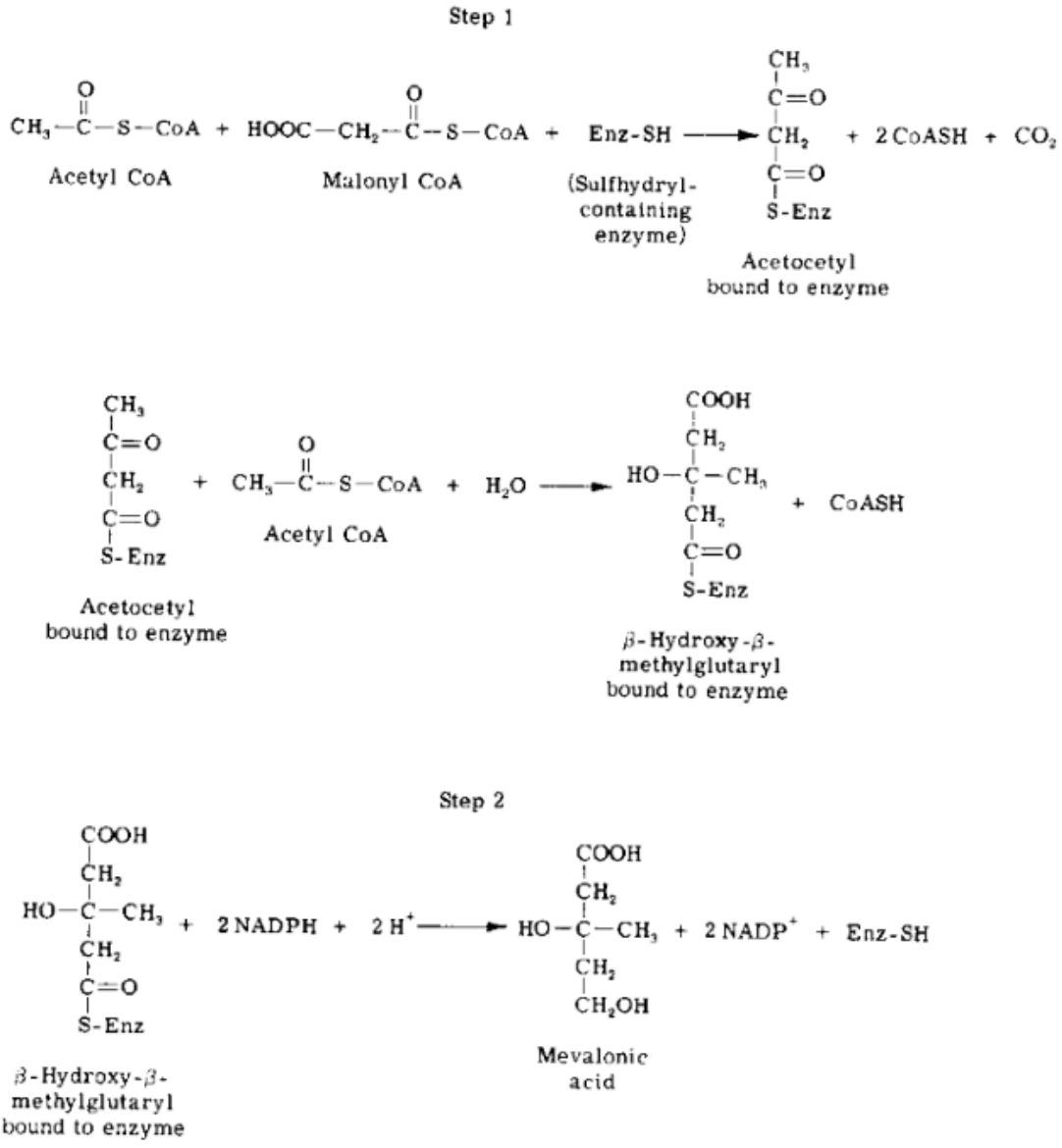
Cholesterol can be synthesized by many tissues of the body, but liver is the primary endogenous site in most animals. Cholesterol is synthesized from acetate in discrete stages. Step 1 involves the conversion of acetyl-CoA to 3-hydroxy-3-methylglutaryl-Co A (HMG-CoA). These reactions can take place either in mitochondria, where they are catalyzed by the same enzymes that form acetoacetate, or extramitochondrially with the reactants bound to an enzyme complex. The next step, the conversion of HMG-CoA to meyalonic acid, is the rate-limiting step of cholesterol synthesis and the site of dietary control. Next, meyalonic acid is converted to squalene, which subsequently is cyclized to lanosterol. Last, lanosterol is converted to cholesterol.

7.3.2 Regulation of Cholesterol Synthesis

The extent of cholesterol synthesis by the liver is inversely proportional to the cholesterol content of the diet. 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, this feedback control has little effect on the total body

cholesterol synthesis. 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 chat responds to dietary control. This reaction is the first one that is unique to cholesterol synthesis and is virtually irreversible. Many drugs designed to reduce cholesterol synthesis and. thereby, circulating levels of cholesterol act on this enzyme. It is also because of the regulatory importance of this enzyme in cholesterol synthesis that measurement of plasma mevalonate has been suggested as a gauge of hypercholesterolemia.



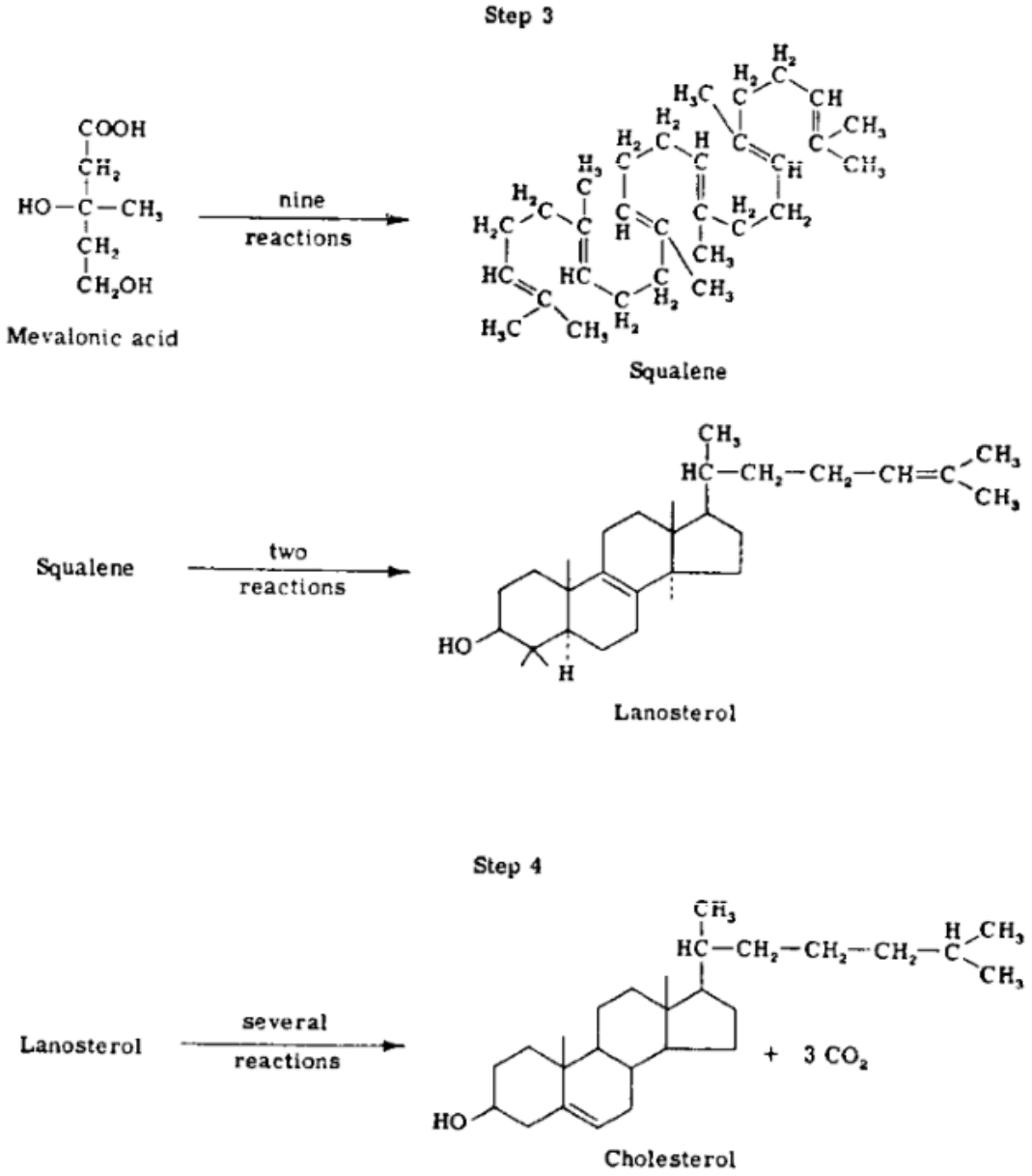


Fig. III.5 Synthesis of cholesterol.

7.3.3 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 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 trans esterifies the fatty acid of lecithin to cholesterol, resulting in the formation of lysolecithin and cholesterol ester. 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. These patients have other plasma protein deficiencies which appear attributable to the absence of LCAT.

8 OXIDATION OF FATTY ACIDS

The spiral path of long chain fatty acids to acetyl- CoA during oxidation within the mitochondrion 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 the rate-limiting step in fatty acid oxidation. The acyl-CoA derivative in the cytosol can arise from activation of FFA entering the cell 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. The enzyme catalyzing this reaction appears to be a part of the inner mitochondrial membrane. The activity of die 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. The intracellular effectors of this key enzyme are known.

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. 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. 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, a rate-limiting step in gluconeogenesis. Ketogenesis would result from the excessive production of acetyl-CoA in a situation in which the availability of oxaloacetic acid is limited or from saturation of the citrate-oxaloacetate condensing reaction with acetyl-CoA derived from FFA. The Acetyl-CoA-carnitine transferase, suggested previously as a means of transferring acetyl units out of the mitochondria, would act according to this hypothesis as an intramitochondrial shuttle for the two acetyl-CoA pools.

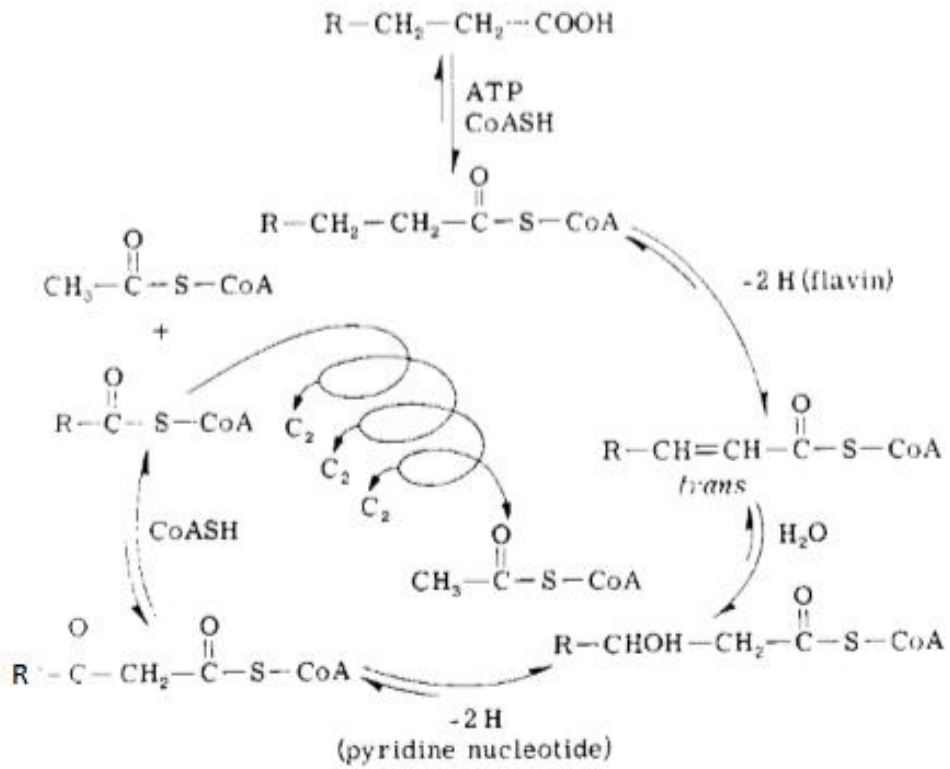


Fig. III.6 β-Oxidation of long chain fatty acids to acetyl-CoA.

9 SPECIAL ASPECTS OF LIPID METABOLISMIN DOMESTIC ANIMALS

9.1 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) special features of lipogenesis, and (5) the use of the concentration of FFA in the plasma as a metabolic indicator.

9.1.1 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. 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. Therefore, the early studies by Leal and co-workers are of more than comparative interest.

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.

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. The intestinal mucosa of lambs readily incorporates fatty acids into triglyceride and, to isolated a small extent, cholesterol ester. In contrast, studies with rats revealed incorporation of fatty acids in both triglyceride and cholesterol ester. Differences between the two species were also noted in the

fatty acids most readily esterified and the position of glycerol preferentially esterified with a given fatty acid.

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. 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. 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. Because of the ruminant's dependence on 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. As might be expected, the fatty acid composition of the milk of cows fed protected tallow was modified to reflect the composition of dietary lipid.

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

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. A major portion of the propionate is utilized for gluconeogenesis. It now seems clear that butyrate does not contribute carbon directly to glucose production but may increase glycogenolysis in the liver of lambs,

presumably by activation of phosphorylase. In other ruminants, the sole effect of butyrate on hepatic glucose output is via increased glyconeogenesis. 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, a key enzyme in gluconeogenesis.

9.1.2 Lipoprotein Complement and Lipid Transport

As mentioned, cows normally develop hypercholesterolemia during lactation. This hypercholesterolemia is accompanied by an increase in the α-lipoproteins. These alipoproteins contain both HDL and LDL. the latter accounting for about 50 % of the increase in serum lipids occurring during lactation. The fraction with a buoyant density of LDL is HDL with a higher content of core lipid.

Suggested three possible explanations for the high HDL levels in the plasma of lactating cows: (1) adaptation to lactation by increasing the apo C reservoir. (2) increased VLDL utilization by mammary gland, and (31 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 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. Suggested that, if VLDL catabolism and transesterification were coupled, more cholesterol ester could be packaged into a single lipoprotein, yielding some 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.

If either the second or third mechanism is substantiated, it could have practical significance. In the second mechanism, the degree of the increased HDL would be an indication of the capacity of mammary gland to form milk fat. The level of 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.

9.1.3Transfer 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. 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. The plasma concentration of FFA was unchanged across the gland. Earlier work in both the lactating cow and the goat had indicated that the mammary gland utilizes neutral lipids from the plasma. Studies with labeled fatty acids administered in the diet or as isolated chylomicrons verified that plasma lipid is a precursor of milk lipid. Plasma lipids contribute 35-75 % of the fatty acid in milk depending on the nutritional state of the animal. Linoleate infused postruminaly 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. 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.

Similarly, labeled butyrate is incorporated into milk fat of lactating cows. There is evidence that the ruminant mammary gland forms large quantities of oleate from plasma stearate.

Suggested that the depression in milk fat concentration observed after administration of glucose to cows (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 appears to be open to question. The depression to changes in the availability of propionate and acetate from the rumen and to enzymatic changes in adipose tissue and mammary.

Milk fat during lactation depends on the intestinal absorption of lipids, lipid levels in plasma. 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. 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, 44% of milk fat can be of direct dietary origin and implied that increased bioavailability 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.

9.1.4 Lipogenesis

The glucose carbon is not incorporated into milk fat. Availability of acetate and butyrate as precursors of fatty acids

sufficed to explain this finding. As the role of citrate lyase in fatty acid synthesis became clear, it was suggested that glucose carbon failed as a precursor of fatty acid due to low citrate cleavage enzyme activity in ruminant tissues. 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 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. The activity of citrate cleavage enzyme is known to be very sensitive to the dietary and hormonal changes. 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 as it is in monogastrics being fed high fat, low carbohydrate diets.

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. Longer term carbohydrate loading studies will be required before the low activities of citrate cleavage and malic enzymes can be attributed solely to an adaptive response to the nutrients absorbed from the alimentary

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 and thereby spare glucose for its indispensable roles, i.e. the central nervous system, lactose production, and support of the fetus. Gluconeogenesis, mainly from propionate, is essential for survival of the ruminant. The magnitude of the hepatic glucose formation is difficult to measure,

but in the ruminant, the glucose 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.

The ruminant, then is more dependent on gluconeogenesis than the monogastric animal, and 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 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. 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 livers 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 gluconeogenesis. In ruminant ketosis, the utilization of ketone bodies equals or exceeds that in normal animal up to certain limits. As in

nutritionally stressed monogastric animals, the major precursor of ketone bodies in the ruminant is FFA, but formation of β-hydroxybutyrate in the ruminal epithelium contributes a portion, as it does in the normal ruminant.

9.1.5 Significance of Plasma FFA in the Ruminant

The plasma FFA level is a sensitive clinical index of fat mobilization. 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. In contrast, measurements in pregnant sheep indicated that the level of FFA correlated well with the severity of ketonemia. Although the discrepancy may be due to species differences.

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. 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, we have found levels similar to those reported 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.

Scientists suggested the use of the plasma concentration of FFA instead of body weight changes as an index to estimate maintenance. 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. 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. Application of such an index, however, might aid in the prevention of ovine pregnancy toxemia and bovine ketosis.

9.2 Equine Lipemia

Lipemia in the equine has been observed accompanying maxillary myositis and equine infectious anemia. 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. Mature, non- lactating 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 in pregnant ponies and pony mares that had just foaled. This metabolic disorder is particularly prevalent and most serious in pregnant animals. It is accompanied by fatty infiltration of the liver, heart, and kidneys.

Since FFA are increased, a metabolic stress 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. Conceptually, the circulating lipid would be in the form of VLDL and this has been confirmed. This is also consistent with the observations that lipemia is more serious in obese ponies.

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 gauge fat mobilization and the response to metabolic stress.

9.3 Canine Hyperlipemia

Lipemias in dogs are of relatively frequent occurrence. Hyperlipemia in a puppy suspected deficient in lipoprotein lipase. The lipoproteins of dogs characterized in association with

atherogenic and non-atherogenic 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 HDL2 level (α_1). In hypothyroidism, lipoprotein values varied but the hypercholesterolemias were associated with increased HDLl (a,i levels. Diabetic dogs also had hypercholesterolemia in association with increased HDLl (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.

9.4 Hypercholesterolemia and Atherosclerosis in Pigs

Application of new techniques from molecular biology and immunology for detection of polymorphism in genes and gene products has proved to be a powerful tool for tracing subtle genetic contributions to multifactorial disease syndromes such as cardiovascular disease and cancer. Using polyclonal antibodies to specific forms of apo B, had demonstrated polymorphism in this protein in cattle, sheep, chicken, swine, rabbit, monkey, mink, and fish.

The syndrome in pigs is accompanied by hypercholesterolemia and early development of atherosclerotic lesions in coronary arteries. In contrast to the hypercholesterolemia described in humans and rabbits, the mutant pigs have normal LDL receptor activity. In a subsequent publication, the hypercholesterolemia was documented to be due to abnormal catabolism of LDL in the pigs with mutant apo B. The authors suggest that the root of the hypercholesterolemia in the mutant pigs is defective binding of LDL to the normal receptor. The reduced catabolism specifically involves a more buoyant species of LDL. How this is related to a mutation of apo B or to

the abnormal lipid composition of the mutant LDL is not clear. What is clear is that the risk of atherosclerosis is greatly increased in pigs carrying the mutation in apo B. The potential of studies of this type in clarifying relationships between genetic background and the risk of diseases with a suspected genetic component, in veterinary as well as in human medicine, cannot be overestimated.

Test questions for Chapter III

1. Clinical value of determination of lipids metabolism indicators (cholesterol, β-lipoproteins, phospholipids, ketone bodies) in different biological material.
2. Diversity of lipoproteins and their clinical significance.
3. Clinical value of TBA-active products content research.
4. Clinical value of steatorea.

CHAPTER IV
SERUM PROTEINS AND DYSPROTEINAEMIA

1 INTRODUCTION

Reference to the nitrogenous compounds of blood plasma includes all those organic and inorganic nitrogen-containing compounds of blood. These include organic macromolecular compounds such as the proteins and nucleic acids, smaller molecular compounds such as glutathione, urea, and creatinine and inorganic compounds such as nitrate. The nonprotein nitrogen (NPN) compounds are those grouped together as that fraction of the N-containing compounds of plasma that are not removed by the common protein-precipitating agents such as trichloracetic acid (TCA). The principal components of the NPN fraction are urea (50 %) and amino acids (25 %) which total about 35,7 mmol N/liter (50 mg N/dl) in plasma in contrast to protein nitrogen which totals more than 714 mmol N/liter (1 g N/dl) in plasma (or 6,25 g protein/dl). This chapter deals primarily with this later, significant fraction, which is classed collectively as the plasma or serum proteins. Emphasis is placed on the diagnostic and interpretive aspects. Readers are referred to the many excellent texts on biochemistry for the details of protein structure, metabolism, and function.

2 CLASSIFICATION OF PROTEINS

1.2 Structural Classification

Proteins are most frequently classified according to their form and composition:

1. Primary structure is the amino acid structure of a single polypeptide chain.
2. Secondary structure refers to the helical structure of the chain (e.g., α-helix).
3. Tertiary structure is the folding of the helices into different shapes: globular (myoglobin), linear (fibrinogen), etc.

Note that these are monomers.

4. Quaternary structure refers to the combination of two or more monomers: dimers (creatine kinase), tetramers (hemoglobin), etc.

2.2 Chemical Classification

Proteins are also classed according to their chemical composition:

1. Simple proteins contain the basic elements of the amino acids: carbon, hydrogen, oxygen, nitrogen. and sulfur
2. Conjugated proteins are those bound to elements, prosthetic groups or to other compounds:
   a. Metalloproteins. – ferritin (iron)
   b. Phosphoproteins – casein (phosphate)
   c. Lipoproteins – high density lipoproteins (triglyceride, cholesterol, cholesterol ester)
   d. Glycoproteins – glycohemoglobin (hemoglobin A_{IG})
   e. Nucleoproteins – ribosomal proteins

2.3 Physical Classification

Proteins have also been classified by their physical behavior:

1. Water and salt solubility
2. Density
3. Molecular weight

3 METABOLISM OF PROTEINS

3.1 General

The intake of nitrogenous compounds and the maintenance of nitrogen balance in animals essentially revolves about the intake and metabolism of the amino acids of the ingested proteins. Little free amino acids or ammonia are present in the diet. In the adult, excretory losses are balanced by intake so that

a nitrogen equilibrium is maintained. Major routes of loss are lactation, illness with cell breakdown, and urinary or gut losses, all of which can result in a negative balance unless nitrogen intake is increased. Positive nitrogen balance occurs during pregnancy, growth, and recovery from disease.

3.2 Synthesis of Proteins

The fundamental units of protein structure in nature are the 20 natural amino acids. The essential amino acids of this group are those not synthesized by animals and therefore must be supplied in the diet (Table I). The non- essential amino acids are those that are synthesized through transamination reactions from the carbon skeletons shown in the figures of Chapter 3. 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 catalyzed by an enzyme familiarly known as glutamic-pyruvic transaminase (GPT) or now as alanine aminotransferase (ALT).

The α-amino acids are linked together by peptide bonds by the protein-synthesizing mechanisms within cells on the rough endoplasmic reticulum (RER). The intricacies of the process by which genetic information for replication 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, in the nucleus, a precise sequence of nucleotides of DNA (chromosomal material) forms a template upon which a complementary messenger RNA (mRNA) is replicated (transcription). This transcription is catalyzed by DNA-directed RNA polymerases. The newly formed mRNAs now move to the cytoplasm to become bound with the ribosomes. Meanwhile, transfer RNAs (tRNA) or triplet nucleotides have become bound to their specific amino acids also in the cytoplasm. The tRNA (the anticodon) with its amino acid next moves to its complementary

triplet segment (the codon) of the mRNA. Succeeding amino acids are brought to the mRNA and sequential peptide linkages are formed until a terminator sequence on the mRNA is reached, at which time the completed polypeptide chain is released.

Table IV.1

Natural Amino Acids

Essential amino acids		
Histidine	Lysine	Threonine
Isoleucine	Methionine	Tryptophan
Leucine	Phenylalanine	Valine
Nonessential amino acids		
Alanine	Cysteine	Proline
Arginine	Glycine	Serine
Asparagine	Glutamate	Tyrosine
Aspartate	Glutamine	

This is the molecular basis of the inheritance of protein structure as represented by DNA transcription and RNA translation into a protein identical in structure to its progenitor. Many replicates of the protein molecule can be synthesized on a single mRNA. When a cell divides, the genetic information is again 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., plasma cells in multiple myeloma, and excessively produce a single discreet species of protein, immunoglobulin M.

3.3 Catabolism of Proteins

3.3.1 Turnover of Proteins

Tissue and plasma proteins are constantly being degraded to their constituent amino acids, and the amino acids in turn are

sources of energy as well as sources of carbohydrate and fat carbon. Generally, amino acid carbons, after conversion to fat (lipogenesis) or to carbohydrate intermediates or glucose (gluconeogenesis) become sources of energy after their oxidation to CO_2 and H_2O. Carnivores derive as much as 40-50 % of their energy requirements from amino acids whereas omnivores and herbivores range from less than 10 % to about 20 %.

The rate of degradation of the plasma proteins is expressed as their turnover, fractional clearance, or half-time. Clearance rates may range from a few hours (some enzyme proteins) to as long as 160 days for hemoglobin in cow red cells. The rate of clearance of most plasma protein ranges between 1 and 3 weeks.

3.3.2 The Urea Cycle

Proteins, as the major source of nitrogen, are hydrolyzed in the gut to their constituent amino acids and absorbed by the intestinal mucosal cells. Gut bacteria may also degrade the amino acids so that ammonia itself may be absorbed. This is an important consideration in the management of liver disease. The amino acids and ammonia are transported to the liver via the porta) circulation and then on to other protein- metabolizing tissues.

About 75 % of the amino acids or ammonia are transported across the liver cell membrane to be taken up by the liver cell and on into the mitochondrion. In the mitochondrion, a number of mechanisms of deamination to generate ammonium ion are present: glutamate dehydrogenase, glutaminase. Also in the mitochondrion, ammonium ion from any source, bicarbonate.and ornithine form citrulline. Citrulline moves to the cytoplasm where arginine and then ornithine is formed with the release of urea. This cyclical process is known as the Krebs-Henseleit or urea cycle (Fig. IV.1). The compartmentalization between mitochondrion and cytosol coordinates the deamination reactions

within the mitochondrion with the aspartate and TCA cycle within the cytosol. Urea excretion by the kidney tubules is the major route of nitrogen excretion. *and most others routes,* e g., uric acid, *nucleic* acids, are relatively minor.

All animals are quite intolerant of ammonia in their cell or body fluids, and if is the ammonia form which is highly toxic. Fortunately, at the pH of blood 99% of the ammonia is in the form of ammonium ion:

$$NH_3 + H^+ \rightarrow NH_4^+$$

It is also the NH_3 form which readily traverses cell membranes where it is converted to the NH_4^+ form. Ammonia is particularly toxic to brain cells where it is thought to reduce their metabolic activity by reducing Krebs cycle activity. Glutamate is often increased and α-ketoglutarate decreased in the presence of increased ammonia. Also, ammonia itself may be toxic by decreasing neurotransmitters. Ammonia is clearly associated with hepatic encephalopathy of humans, dogs, and horses, but the mechanism is still unknown. It is often found in portasystemic shunts of young dogs.

Disposal of ammonia by the kidney tubules is a second important route of nitrogen excretion. In the tubules glutamine is deaminated to glutamate and ammonia by glutaminase. Ammonia in the tubular lumen is converted to ammonium by binding to H^+ and is therefore an equally important mechanism for removal of excess H^+ and the maintenance of acid-base balance.

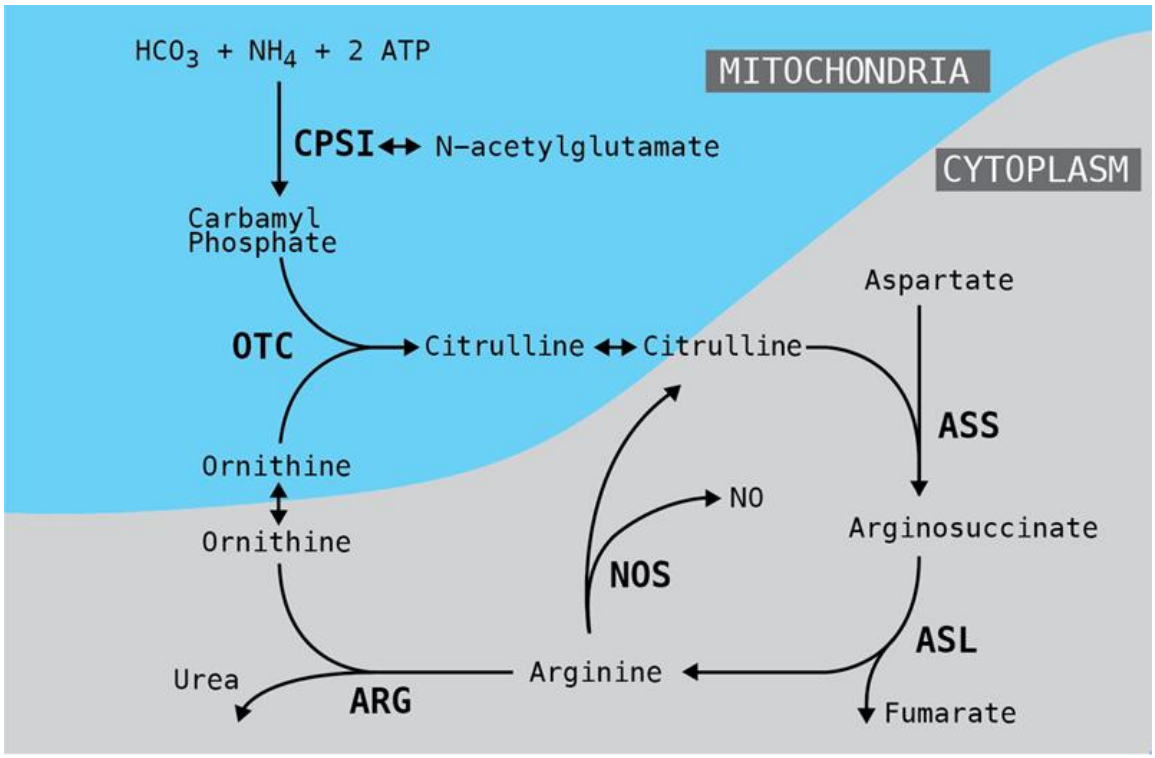


Fig. IV.1 The Krebs-Henseleit or urea cycle. Note the partitioning of (the reactions of the cycle between the cytosolic and mitochondrial compartments.

4. PLASMA PROTEINS

4.1 Sites of Synthesis

The major site of synthesis of the plasma proteins is the liver, with the second major site being the immune system consisting of the reticuloendothelial (RE) tissues, lymphoid and plasma cells. Structural, functional, and enzyme proteins which are synthesized in all body cells and tissues are present in plasma in minor quantities as a result of cell turnover. In general, plasma contains about 5-7 % (50-70 g/liter or 5-7 g/dl) protein. If hemoglobin is included, whole blood is composed of about 20 % or more of protein.

4.2 Functions

The functions of proteins in the body are innumerable. Proteins form the basis of structure of cells, organs, and tissues,

they maintain colloid osmotic pressure, they are catalysts (enzymes) in biochemical reactions, they are buffers to maintain acid-base balance, they are regulators (hormones) and they function in blood coagulation, in body defenses (antibodies), are nutritive, and are transport and carrier compounds for most of the constituents of plasma. The biological activity of proteins and polypeptides for these various functions are dependent on 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.

4.3 Factors Influencing Plasma Proteins

4.3.1 Age

At birth, plasma proteins of most animals are quite low due to the minimal quantities of immunoglobulins and low albumin. As the newborn animal ingests colostrum a rapid rise in the immunoglobulins occurs as a result of the absorbed maternal immunoglobulins. As the maternal antibodies decline due to normal turnover the neonatal animal rapidly gains immunocompetence and begins to synthesize its own immunoglobulins. On reaching young adulthood, normal adult levels of the albumins and globulins are reached.

With increasing age, the plasma proteins are seen to increase above the normal adult levels as a result of a small decrease in albumin and a progressive increase in the globulins.

4.3.2 Hormones, Pregnancy, and Lactation

During gestation, the total plasma protein decreases due to albumin decrease even though there is a slight increase in the globulins. Near term, there is a sharp rise in the α-globulins and with a corresponding rise in total plasma proteins. In lactation, the total plasma proteins again decrease due to an albumin decrease.

Some hormones (testosterone, estrogens, growth hormones) effect an increase in total plasma proteins owing their anabolic effects. Others (thyroxine, cortisol) tend to decrease the total plasma proteins because of their catabolic effects.

4.4 Handling and identification of proteins

The process of protein denaturation is the net effect of the alteration of the biological, chemical, and physical properties of the protein by disruption of its structure. It is, therefore, of great importance that proteins be handled in a way to prevent this structural change. Heat, ultraviolet light, surfactant detergents, and chemicals all have some effect on protein structure. If this effect is significant, the protein is denatured, its biological 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.

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 use of cellulose acetate electrophoresis to separate the various protein fractions is a commonly used first step in the investigation of protein dyscrasias. The agarose gel or high-resolution protein electrophoresis is now also used as a first step. Serum protein electrophoresis has evolved into an extremely useful technique because aberrations are observed in so many disease states. There are only a few diseases, however, for which the electrophoretic pattern can he considered pathognomonic.

5 METHODOLOGY

5.1 Total Serum Protein

There are a number of methods for the determination of total protein in serum or other body fluids. Classically, the N in the protein has been determined by the Kjeldal 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.

5.1.1 Chemical Methods

Biuret. The biuret 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 on the formation of the blue Peptide-Cu complex in alkaline solution.

The method is highly accurate for the range of protein likely to be formed in serum (10-100 g/liter: 1-10 g/dl), but it is not sensitive 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.

Phenol-Folin-Ciocalteau. The phenol-Folin-Ciocalteau method is extremely sensitive and is the method of choice for the dilute solutions such as the CSF. It is based on the reaction of the phenolic groups of tryptophan and tyrosine of proteins with the reagent to form a blue color. Automated as well as manual techniques are available.

Bicinchoninic Acid. A relatively new modification of the Lowry method which uses bicinchoninic acid (BCA) is more sensitive than the original and may eventually replace it as the method of choice for dilute solutions.

Precipitation Methods. Depending on their charge, proteins can be brought to their isoelectric point, where they will precipitate, by the addition of either anions or cations. Anionic precipitants such as trichloroacetic acid (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 total protein, e.g., sulfosalicylic acid method for urine protein.

5.1.2 Physical Methods

Refractometry. Proteins in solution cause a change in the 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 ns rapidity and simplicity, it is well suited for the office or the emergency clinical laboratory. Owing to its dependence on the transmission of light, it is important that the method be used only for clear, non-turbid and non-lipemic sera and fluids. A moderate degree of hemolysis or icterus does not interfere. Calibration of the refractometer should be frequently checked because this has been found to be a frequent source of error. 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.

Fibrinogen is a large protein of 340.000 relative molecular mass *(M_r)* units which constitutes about 5 % of the total proteins of plasma. It is most simply and rapidly estimated by the heat-precipitation-refractometer method 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 the 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 precipitated 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 or as the thrombin time.

5.2 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 on the fractionation method. In its simplest form, if either the albumin or globulin were determined, the other can be obtained by subtraction from the total serum protein. Ultimately, the accuracy of any of the chemical methods is based on comparisons with protein electrophoresis, the current standard of reference for serum protein fractionations.

5.2.1 Salt Fractionation

Salts added to proteins in solution dehydrate the proteins, causing them to precipitate and the solution to become turbid. 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 to include the zinc sulfate, sodium sulfate, and ammonium sulfate turbidity tests. These are turbidimetric 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 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 and foal where field tests for the detection of suckling have been evaluated using $ZnSO_4$ turbidity. The test was found to be a satisfactory method for the assessment of colostral immunoglobulin transfer to newborn foals when compared to electrophoresis and better than other rapid tests such as the sodium sulfate precipitation test. Another precipitation test for detection of hypogammaglobulinemia in calves is the glutaraldehyde coagulation test which also appears to have practical value in identifying hypogammaglobulinemic calves.

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 binds so poorly to the albumins of domestic animals that the method was unacceptable. The HABA dye method has now been replaced by 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 less outside the normal limits and is usually unacceptable at very low levels. The Coomassie

blue dye binding method for albumin is another innovation which is sensitive at low concentrations of proteins but suffers from variability.

Colorimetric Determination of Globulin. The reaction of glyoxylic acid with globulins forms a purple-colored complex which is proportional to the amount of globulin. The test has not gained wide acceptance in clinical biochemistry because of its variability. It has been adapted to some unitized blood chemistry systems, but it must be used with caution since its applicability to animal sera has not been established.

5.3 Electrophoretic Fractionation of Serum Proteins

The electrophoretic technique is the current standard of reference for the fractionation of the serum proteins in clinical biochemistry. The marked advances in technology of the past decade have made this previously elaborate technique into 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 as 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 (SPE) which basically differ only in the type of support media used. The cellulose acetate (CA) method is to be recommended for its relative simplicity and accuracy as, the test of choice. Its principle and the results of its use are described more fully as representative of the electrophoretic fractionation.

Principle. The principle of the electrophoretic separation of the serum proteins is based on the migration of charged protein particles in an electric field. The direction and rate of migration of the particles are based on the type of charge (+ or −) on the protein, the size of the protein, the intensity of the electric field,

and the support medium through which the protein particles are induced to migrate. It follows that it is of importance that the support medium, pH, buffer, and the electric current be described to allow comparison of SPE

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 cellulose acetate (CA), although methods using many other support media such as agar gel, agarose gel, starch gel, or polyacrylamide gel are available. Many of the latter yield greater separability of the serum proteins than does CA but in the clinical laboratory CA remains the most useful method. It should be recalled that the globulins are comprised of a myriad of separate protein moieties enzymes, carriers, antibodies, clotting factors and that a specific serum protein can be identified only by additional special techniques.

6 NORMAL SERUM PROTEINS

There are well over 200 plasma 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 of a species are synthesized under genetic control, it would be expected that variations in proteins would occur between individuals and between species. These variations are reflected in the species differences of the normal serum protein electrophoretic (SPE) patterns. Thus, in ruminants such as the cow, the normal SPE pattern exhibits an albumin, one α, one β. and one γ fraction. Table II lists some of the important serum proteins with their principal functions and conditions of alteration. Since there are significant differences in fractionation depending on the method, reference is being made here only to cellulose acetate electrophoresis. Normal values for total serum protein and its fractions are given elsewhere in this volume.

6.1 Prealbumin

Prealbumin is the most rapidly migrating fraction in human serum, is usually not visualized, may not exist in any domestic animal, but does exist in some birds. A protein with the characteristics of prealbumin, however migrates in the α_2 globulin region of the SPE of dogs. The only known function of prealbumin is thyroxine binding and transport.

6.2 Albumin

6.2.1 Structure

Albumin is the most prominent of the serum proteins on SPE. In animals, it constitutes between 35 and 50 % of the total serum proteins, in contrast to humans 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 homogeneous fraction discernible on SPE. It is often described as the only discrete protein species which can be detected by SPE. However, other methods such as starch gel 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. It is consistently observed in chronic liver disease in horses. A thyroxine-binding function of this postalbumin fraction has been observed in horses.

6.2.2 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 clearance (Tab. IV.2). There appears to be a direct correlation between albumin turn-significant hypoalbuminemic edemas occur only in the larger animals.

Table IV.2

Albumin Turnover in Animals

Species	Days
Mouse	1.90
Rat	2.50
Guinea pig	*2* 80
Rabbit	5.70
Pig	8.20
Dog	8.20
Sheep	14.28
Human	15.00
Baboon	16.00
Cow	16.50
Horse	19.40

This would suggest that edema develops in large animals because of slow replacement of albumin. Hypoalbuminemic edemas occur in small animals usually in the presence of posterior vena cava hypertension.

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 in the aqueous plasma. Binding to albumin also prevents the loss of plasma chemical constituents through the kidneys. The binding of unconjugated bilirubin or of fatty acids by albumin are examples of this function.

6.3 Globulins

6.3.1 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 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, 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 and the pre-β-lipoproteins (very low density lipoproteins, VLDL), which migrate in the α_2 position. The β-lipoprotein (low density lipoprotein, LDL), so named because it migrates in the β3 region on paper, also migrates in the α_2 region on CA. These 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, ceruloplasmin, and amyloid A (SAA) are also diagnostically important acute phase proteins (Tab. IV.3).

Table IV.3

Acute Phase Proteins: Markers of Acute Inflammatory Disease

Positive acute phase proteins	Negative acute phase proteins
α_1 -Globulins	Prealbumin
α_1-Antitrypsin	Albumin
α_1-Acid glycoprotein	Transferrin
α_2-Globulins	
α_2-Macroglobulin	
Ceruloplasmin	
Haptoglobin	
β-Globulins	
Fibrinogen	
Complement. C3, C4	
Protein C	
C-Reactive Protein	
Ferritin	
Amyloid A	

6.3.2 β-Globulins

β-Globulins trail the α_2 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 (C3, C4), hemopexin, transferrin, ferritin, and C-reactive protein (CRP). Fibrinogen slightly trails the β_2 and is another important acute phase protein. CRP and SAA are now considered the most diagnostically important of the acute phase proteins in humans and a recent study indicates it may be equally valuable in dogs. Some immunoglobulins, IgM and IgA, 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.

6.3.3**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 animal's IgA, IgM, and IgE are found primarily in the γ_1 region while IgG is found primarily in the γ_2 region. The specific identification and quantitation of the immunoglobulins require the use of sophisticated immunochemical techniques. Immunochemical assays are widely used in protein research and are now also widely used in the clinical laboratory. 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 the immunochemical tests are of two types: complete antigens which induce formation of specific antibodies and incomplete antigens (haptens) which, though they react with antibodies, do not elicit an immune response. Complete antigens, usually of molecular weight greater than 5000, are proteins, glycoproteins, complex carbohydrates, or nucleic acids that are recognized as foreign by the host and elicit an immunologic response from the host. Haptens are low molecular weight compounds which, if coupled to a larger molecular weight compound such as a protein, elicit an antibody response. Most antibodies used in radioimmunoassay (RIA) have been produced in response to haptens coupled to albumin, e.g., T4 antibody for T4 RIA.

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

The lymphocytic cell line is now known to play the central role in the immune system. There are two subpopulations, the B lymphocytes (bursa) and the T lymphocytes (thymus), which can be identified by special immunologic 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 blood and in 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 (IgG, IgA, IgM, and IgA), cats, and horses. 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 protein.

A specific plasma cell population of defined genetic origin, a clone, produces a specific immunoglobulin. Uncontrolled growth of a single B-cell clone (malignancy) results in the overproduction of a single chemical species of immunoglobulin which appears as a sharp ‘'monoclonal” spike or gammopathy on an electrophoretogram. A group of clones, each of a different genetic origin, can also overproduce a heterogeneous mix of immunoglobulins which appears as a diffuse or broad hyperglobulinemic region on the electrophoretogram and is described as a ‘‘polyclonal” gammopathy.

b. Immunoglobulins. The immunoglobulins are glycoproteins whose basic structure is a monomer made up 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. The structure of the H chain governs the class of immunoglobulin and is named by corresponding Greek letters:

γ = G, м = M, α = A, ε = E, δ = D.

The structure of the L chain is either kappa *(k)* or lambda (λ)

and denotes type. Structural variations in the variable regions 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). and IgG_4 and two subclasses of IgA (IgA_1 and IgA_2).

IgG, IgD, and IgE 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. IgD has been reported only in humans, where its function is unknown. IgE is involved in allergic and anaphylactic reactions and is present in dogs. IgA is a dimer of two basic units joined together by a secretory piece. IgA is found in the secretions of the respiratory, genitourinary, and gastrointestinal tracts. IgM is a cyclic pentamer of five basic units which forms 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.

7 INTERPRETATION OF SERUM PROTEIN PROFILES

The determination of serum proteins and their SPE profiles have evolved into important diagnostic aids in clinical biochemistry. This has occurred even though a specific diagnosis can seldom be made with SPE. Abnormal serum protein profiles can be identified with types of disease processes with some accuracy. SPE 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-to-globulin ratio (A:G).

Serum protein electrophoresis provides the percentage of albumin and classes of globulins and. knowing the total serum protein, 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 the absolute values. While there is merit in the suggestion, the A:G value retains usefulness in interpretation of automated chemistry data. Changes in A:G are often the first signal of a protein abnormality. The Appendixes give normal values for total serum protein and its fractions in domestic animals and birds.

7.1 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, therefore even minor changes in the SPE profile can be of significance and warrant close scrutiny.

7.1.2. Influence of Age and Development

In the fetus, the concentrations of total protein and albumin progressively increase with little change in globulins and an absence of γ. However, γ-globulin was detected in 25 % of bovine fetal serum samples, which was attributed to transplacental transfer. After nursing and up to 24 hours postpartum, baby pigs had large amounts of γ-globulin, which progressively decreased to 5 % of the total serum protein by 4 weeks of age. In the calf, precolostral serum normally contains no γ-globulin, but, within a few hours after ingestion of colostrum, γ-globulin appears in serum and absorption continues for up to 48 hours after birth, after which gut permeability ceases. In colostrum-deprived calves, immunoglobulin increases only minimally. In the developing foal from birch to 12 months of age progressive increases in albumin, globulins, and total proteins are seen.

In all animals, there is a general increase in total protein, a decrease in albumin and an increase in globulins with advancing

age, and in the very old the total proteins again decline. Similar changes due to age have also been 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 the SPE.

7.2.2 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 (DES) given to male calves and to chickens 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 because of its catabolic effect. 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. Thus, in general, hormonal effects of serum proteins are slight even though their effects on weight gain or body *composition may* be quite marked.

7.2.3 Pregnancy and Lactation

Generally, during the course of gestation albumins decrease and the globulins increase. In ewes, albumin decreases to a minimum at midgestation and returns to near normal at term. Globulins and total serum protein progressively decrease throughout gestation. 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. 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 pregnancy also occur.

7.2.4 Nutritional influences

The plasma 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, 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, chickens and dogs. In humans, 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. Immunoglobulins are affected only following severe protein restriction, but the effects are reversible on protein repletion.

7.2.5 Stress and Fluid Loss

Temperature stress, either febrile or cold, is associated with nitrogen loss, increased adrenal activity, and increased protein turnover, resulting in decrease in total serum protein, decrease in albumin, and often an increase in $\alpha._2$-globulin. Similar findings are observed in crushing injuries, bone *fractures, and* extensive surgery. Tissue repair calls on protein reserves, and subsequent protein metabolism results in decreased albumin and increased α_2-globulin. In the inflammatory process, fluids and proteins move into the 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 hypoprotememia. Conversely, dehydration leads to hemoconcentration through reduction in fluid volume and consequently hyperproteinemia. During splenic contraction in the horse, a large mass of erythrocytes moves into the circulation with little or no change in serum protein levels.

7.2 The Dysproteinemias

The best method for the overall evaluation of protein status is through SPE. The SPE profile and evaluation of the absolute values provides an excellent basis for presumptive diagnoses and for additional studies of the patient. The derivation of A: G from chemistry panels or from the SPE can serve as a useful basis for interpretation.

Several classifications of SPE profiles have been proposed all based on disease. Table IV.4 gives a classification of SPE based on the A.G ratio and the nature of the profile.

Table IV.4

Classification of the Dysproteinemias Based on the Albumin-to-Globulin Ratio and the Serum Protein Electrophoretic Profile

A. Normal A: G-normal SPE profile
   1. Hyperproteinemia: dehydration
   2. Hypoproteinemia
      a. Overhydration
      b. Acute blood loss
      c. External plasma loss: extravasation from burns, abrasions, exudative lesions, exudative dermatopathies. external parasites; gastrointestinal disease, diarrhea
      d. Internal plasma loss: gastrointestinal disease, internal parasites

B. Decreased A: G – abnormal SPE profile
   1. Decreased albumin
      a. Selective loss of albumin: glomerulonephritis, nephrosis, nephrotic syndrome, gastrointestinal disease, internal parasites
      b. Decreased synthesis of albumin: chronic liver disease, malnutrition, chronic inflammatory disease
   2. Increased globulins
      a. Increased α_1-globulin
         I. Acute inflammatory disease: α_1-antitrypsin, α_1-acid

glycoprotein (orosomucoid, seromucoid)

b. Increased α_2-globulin

I. Acute inflammatory disease; α_2-macroglobulin, ceruloplasmin, haptoglobin

II. Severe active hepatitis: α_2-macroglobulin

III. Acute nephritis: α_2-macroglobulin

IV. Nephrotic syndrome: α_2-macroglobulin, α_2-lipoprotein (VLDL)

c. Increased β-globulin

I.Acute hepatitis: transferrin, hemopexin

II.Nephrotic syndrome. β_2-lipoprotein (LDL), transferrin

III.Suppurative dermatopathies: IgM. C3

d. β-*γ* Bridging

I. Chronic active hepatitis: IgA. IgM

e. Increased γ-globulin (broad increases) – polyclonal gammopathies: IgG. IgM, IgA

I. Chronic inflammatory disease, infectious disease, collagen disease

II. Chronic hepatitis

III. Hepatic abscess

IV. Suppurative disease: feline infectious dermatitis, suppurative dermatitis, tuberculosis

V. Immune-mediated disease: autoimmune hemolytic anemia, autoimmune thrombocytopenia, Aleutian disease of mink, equine infectious anemia, systemic lupus erythematosus, autoimmune polyarthritis, autoimmune glomerulonephritis, autoimmune dermatitis, allergies

VI. Tumors of the reticuloendothelial system (RES): lymphosarcoma

f. Increased γ-globulin (sharp increases) – monoclonal gammopathies: IgG, IgM, IgA

I. Tumors of the reticuloendothelial system (RES): lymphosarcoma

II. Plasma cell dyscrasias: multiple myeloma,

Aleutian disease of mink

III. Macroglobulinemia

IV. Canine ehrlichiosis Benign

C. Increased A: G – abnormal profile

1. Increased albumin: does not occur except in dehydration
2. Decreased globulins

c. Precolostral neonate

d. Combined immunodeficiency of Arabian foals

e. Aglobulinemia

7.2.1Normal A:G – Normal Profile

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

b. Hypoproteinemia. Overhydration through vigorous fluid therapy or excess water intake is 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.

7.2.2 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. Owing to its small size and osmotic sensitivity to fluid movements, albumin is selectively lost in renal disease gut disease and in intestinal parasitism. The hypoalbuminemia of intestinal parasitism is aggravated by increased albumin catabolism. Furthermore, owing 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. A-Globulins. α_1-Globulin increases are not of great diagnostic significance in animals, whereas α_2-globulin increases are a common finding. Many acute phase proteins migrate in the α_1- and α_2-globulin regions (Table IV.3). A rise in α_2-macroglobultn is commonly seen in acute inflammatory disease. This may or may not be accompanied by increases in ceruloplasmin or haptoglobin. In the nephrotic syndrome, α_2-globulins increase due in part to the α_2-macroglobulin and the lipoproteins.

II. β-Globulins. Increases in β-globulins alone are infrequent and found in association only with active liver disease, suppurative dermatopathies and in the nephrotic syndrome. Transferrin appears to be the major component which rises in active liver disease together with hemopexin and complement. IgM 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 the IgM and complement increases in the /3 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 are sharp monoclonal increases of multiple myeloma.

III. β-γ Bridging. The phenomenon of β-γ bridging is almost pathognomonic of chronic active hepatitis. 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-Globulin (Broad Increase): Polyclonal

Gammopathy. The diffuse or broad increases in the γ-globulins which characterize polyclonal gammopathies are due to the heterogeneity of the clones of plasma cells which produce a heterogenous mix of immunoglobulins. Any one or all of the immunoglobulins IgM, IgG, or 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. In these cases, the polyclonal increase is more marked and the hypoalbuminemia more severe than in chronic inflammatory disease. This phenomenon may 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 be immune processes directed against "self" autoimmune disease, or against external antigenic stimuli. In either case, a multiple immunologic 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 erythematosus (SLE). SLE is a multifaceted disease in the dog often found in association with autoimmune hemolytic anemia (AIHA), thrombocytopenia (AITP), glomerulonephritis (GN), and rheumatoid polyarthritis (RA|. The basic defect in SLE is the LE factor, an anti-DNA antibody, and the widespread dissemination of the DNA-anti DNA com-of albumin, (D, E) newborn and neonatal calves, and (F) immunodeficiency in a foal plex

throughout highly vascular structures. The LE cell is a granulocyte which has phagocytosed the DNA- antiDNA complex.

AIHA is characterized by acute erythrocyte destruction, accelerated bone marrow response to the anemia, and the presence of autoantibodies against the patient’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 proteins or prosthetic groups are in some way altered or mutated to become a "foreign" antigen which the body's immune mechanism now does not recognize as "self." Antierythrocyle antibodies are formed that coat the erythrocytes, and. in the presence of complement. hemolysis, fragmentation, or phagocytosis occurs. Diagnosis of AIHA is made by demonstrating a positive direct Coombs test.

Autoimmune thrombocytopenia (AITP) is another form of autoimmune disease in which the platelets either have 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. Definitive diagnosis is made by use of the PF-3 test.

Rheumatoid arthritis (RA) is characterized by the development of rheumatoid factor (RF). 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 hyperglobulinemic peaks can occur anywhere between the β_1 and γ_2 regions and range from very broad, diffuse peaks to very sharp, monoclonal spikes. 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-Globulin (Sharp Increases): Monoclonal Gammopathy. The monoclonal forms are characterized by sharp

"spikes" of immunoglobulin frequently but not limited to the γ region. A useful interpretive guideline is to visually compare the sharpness of these 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 macroglobulinemia with hyperviscosity and IgM monoclonal spikes has been reported in dogs. In multiple myelomas, Bence-Jones proteins (light chains) are detected in approximately 50% of the cases in humans and in dogs. 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, dog, cat, and rabbit. Aleutian Disease (AD) of mink, a valuable model of immunologic disease, is also characterized by plasma cell infiltration, hypoalbuminemia, hyperproteinemia, and hyper-gamma-globinemia, frequently with a monoclonal spike. Light chain disease, in which the M component was identified as L chains (Bence-Jones proteins) in the plasma and urine, has been observed in dogs. An IgA monoclonal gammopathy in a dog without Bence-Jones proteinuria or plasma cell infiltration has also been observed.

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 recent case of lymphocytic leukemia in a dog. A biclonal gammopathy has been observed in a dog with a combined myeloma and cutaneous lymphoma. Monoclonal gammopathies have also been observed in canine amyloidosis and are frequently seen in canine ehrlichiosis in association with large numbers of reactive plasma cells in the bone marrow.

Generally, the clinical characteristics of the monoclonal gammopathies are referable 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. Not all monoclonal gammopathies are pathologic, however, because it is becoming increasingly apparent that many are benign. In consequence, care must be exercised in the final evaluation of the monoclonal gammopathy.

7.2.3 Increased A:G – Abnormal Profile

a. Increased Albumin. True overproduction of albumin 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. Hypogammaglobulinemia occurs in about 15 % of foals less than 2 weeks of age and is attributed to failure of colostral transfer even though most foals had nursed. 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. A selective IgM deficiency has been reported.

Test questions for Chapter IV

1. Clinical value of the research of serum total protein concentration in blood and research proteingram.
2. Clinical value of the research of nitrogen compounds in the blood and urine.
3. Clinical value of the proteins content research of "acute phase".
4. Qualitative reaction on protein in the urine.
5. Diagnostic value of proteinuria.
6. What indicators characterize the coagulation system of the blood?
7. What classes of immunoglobulins and their functions do you know?
8. Features of colostral immunity formation in newborn calves.

CHAPTER V
CLINICAL ENZYMOLOGY

1 INTRODUCTION

Clinical enzymology has made great advances since the introduction of serum alkaline phosphatase as a diagnostic aid in 1927 by King and Armstrong. No longer is it confined to the measurement of serum enzyme activity as markers of cell integrity. Advancement in bioengineering and computer technology have linked enzymology, immunology, and molecular genetics to produce new clinical diagnostic procedures. Qualitative and quantitative non-radioisotopic immunodiagnostics with enzyme-linked monoclonal antibodies enhance the specificity and sensitivity of assays for metabolites, hormones, and infectious agents. Immunocytochemistry has advanced the specificity and sensitivity of light and electron microscopic histology and cytology. Endonucleases combined with DNA probes enable the diagnostician to detect the fingerprints of prepatent genetic defects and infectious agents before or without the aid of endogenous antibody development. Although enzymes appear to have only a passive part in these forms of biotechnology, understanding their fundamental action is essential to clinical diagnostics and basic research.

This chapter is intended to be an introduction to enzymes as primary clinical diagnostic aids and their secondary role in other diagnostic techniques. Additional information on clinical enzymology can be located in the serial publications Veterinary Clinical Pathology and Clinical Chemistry, the respective publications of the American Society of Veterinary Clinical Pathology and the American Association of Clinical Chemistry. The latter organization has a Division of Animal Clinical Chemistry. Advances in Clinical Chemistry and Methods in Enzymology are other serials containing information on clinical enzymology. Organizations which help standardize enzymology

arc the International Union of Biochemistry's Nomenclature Committee (EC) and the Expert Panel of the International Federation of Clinical Chemistry (IFCC).

2 BASIC ENZYMOLOGY

2.1 Enzyme Nomenclature

Enzyme nomenclature is based on the reaction enzymes catalyze. In general, the suffix “-ase" identifies an enzyme. The International Union of Biochemistry (IUB) established a commission on enzyme nomenclature to systematize, categorize, and catalogue enzymes. The system is numerical and based on the type of reaction catalyzed; a systematic name, a recommended name, 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 systematic name for LDH is S-lactate: NAD oxidoreductase, its recommended name is L-lactate dehydrogenase, and its EC number is 1.1.1.27. Abbreviations, recommended name, and EC number of sonie enzymes are given in Table V.1 and can also be obtained from the IUB listings.

2.2 Units of Enzyme Activity

The concentration of an enzyme is expressed directly as mass or indirectly as activity. Currently the most accepted expression of enzyme concentration is the rate of the reaction catalyzed, activity. It would be more precise to express the concentration as moles of enzyme per unit volume. The concentrations of most enzymes in scrum and tissues, however,

are usually too low to be measured as mass by current state-of-the-art enzymology.

For example, ALT occurs in plasma at less than 1 mg/liter, whereas the total plasma protein concentration is about 70 g/liter. The proportion of enzyme protein to total protein would be less than 1 in 7,000, and the purification, isolation, and determination of concentration would not be feasible for clinical chemistry.

Table V.1

Enzymes Used in Veterinary Medicine

Abbreviation	Recommended name	EC number
ALT (GPT)	Alanine aminotransferase	2.6.1.2
AP	Alkaline phosphatase	3.1.3.1
Amyl	γ-Amylase	3.2.1.1
ARC	Arginase	3.5.1.1
AST (GOT)	Aspartate aminotransferase	2.6.1.1
ChE	Cholinesterase	3.1.1.8
CK (CPK)	Creatinekinase	2.7.3.2
GGT	γ-Glutamyl transferase	2.3.2.2
GPx	Glutathione peroxidase	1.11.1.9
LDH	L-Lactate dehydrogenase	1.1.1.27
LIP	Lipase (triacylglycerol lipase)	3.1.1.3
OCT	Ornithine earbamoyltransferase	2.1.3.3
SDH	Sorbitol dehydrogenase	1.1.1.14
PK	Pyruvate kinase	2.7.1.40
TK	Transketolase	2.2.1.1
-	Trypsin	3.4.21.4

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 mmol of a substrate or the production of 1 mmol of product per minute under specified conditions of time, temperature, pH, and substrate concentrations. The unit specifies only the amount of substrate and time. The volume, temperature,

and substrate in which the unit is expressed vary with assay procedures used 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 must 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 actual units.

The IUB and the IUPAC have recommended a new unit to express enzyme activity, the katal (1 kat = 1 mol/second), which is consistent with the Systeme International. One U/liter is equal to 16 67 nkat/liter. How readily the kat will be accepted in clinical chemistry remains to be seen. At present the U remains the more commonly accepted expression of enzyme activity.

2.3 Kinetics

Enzymes catalyze reactions by complexing with the substrates to lower the energy of activation of the reaction without changing the equilibrium constant. The reaction will take place independent of the enzyme, but at a much slower velocity. There are four ways in which enzymes can catalyze reactions: general acid- base catalysis, covalent catalysis, approximation of reactant catalysis, and induction of strain in the substrate or enzyme. When the product is formed the enzyme- product complex disassociates.

Enzymes are characterized on the basis of their affinity for their specific substrate and cofactors, and the effects of inhibitors, activators, pH, and temperature on the reaction. The optimum affinity for the substrate is expressed as the Michaelis constant *(K_m)* which is the concentration of the substrate at one half the maximum velocity (V_{max}) of the reaction. It is determined by plotting the change in activity (Michaelis plot or the Lineweaver-Burke plot) as a function of changing substrate concentration

while the enzyme concentration is held constant. The lower the numerical value of the K_m the greater the affinity and specificity of the enzyme for the substrate. Given the V_{max} and number of binding sites for an enzyme, it is possible to determine how many moles of product are produced (turnover time, K_i) per second. Advanced recording systems permit a direct measurement of the enzyme's affinity for its substrate.

Calculation of the K_m for two-substrate reactions, such as for the dehydrogenases, requires a complex approach. The concentration of the primary substrate is varied while the enzyme and secondary substrate, the cofactor, such as NADH, is supplied in sufficient concentration that they do not limit the velocity of the reaction. Once the K_m for the primary substrate is determined, it is held constant at its V_{max} and the concentration of the cofactor is varied.

The affinity of the inhibitor and its mechanism of binding can be characterized by plotting (Lineweaver- Burke plot) the effect of the inhibitor on the substrates V_{max} and K_m. The lower the numerical value of the inhibitor constant (K_i) the greater the affinity of the inhibitor for the enzyme.

2.4 Enzyme Structure

An active enzyme, the holoenzyme, consists of a complex of proteins with various nonprotein modifications. The protein portion is made up of peptides known as protimers. One or more such protimers may make up the protein, apoenzyme, portion of the holoenzyme. The apoenzyme may undergo posttranslational glycosylation, acylation, phosphorylation, deamidation, sulfhydryl oxidation or aggregation within the confines of the sinus of the rough endoplastic reticulum. Posttranslational modification of a single protimer results in the formation of a monomer. Monomers can combine to form dimers, tetramers. and oligomeres to make up the conformation of the holoenzyme. The three- dimensional configuration of the enzyme gives it its specific catalytic action. A

change in the enzyme’s specific conformation results in reduction or loss of its specific activity.

No specific amino acid sequence characterizes a protein as an enzyme. Among some enzymes there are similarities in the amino acid sequences of their catalytic site. The serine proteases are a group of enzymes which have serine residue common to their catalytic site. There has been speculation that this similarity reflects the phylogenic development and essential nature of the enzyme.

Modification of the enzyme's environment, or matrix, such as temperature, pH, protein, or urea concentration, results in an alteration to its configuration. Therefore, the matrix in which an enzyme is evaluated determines its conformation and, in turn, its catalytic specificity and kinetic characteristics.

Enzymes secreted by cells for extracellular function are frequently synthesized and secreted as inactive proenzymes or zymogens. They are given the suffix "-ogen" or the prefix "pro-" to denote their inactive form. They are activated by proteases which cleave the zymogen into the holoenzyme and a remnant peptide. Many plasma zymogens are the serine proteases of coagulation and complement systems, which are themselves proteases which produce a cascading effect in their respective pathways.

2.5 Isoenzymes

Isoenzymes are multiple forms of enzymes found in the same species, with the same catalytic specificity, whose apoenzymes’ structures differ and therefore, have separate genomes. Previously, the IUB definition of the isoenzyme did not require derivation from separate genes, only that they be located in different tissues and have different physical features independent of being posttranslational modifications. The development of monoclonal antibodies, amino acid sequencing, and DNA hybridization increased the specificity of the definition of an isoenzyme. The IUB recommends that the term “multiple forms

of the enzyme" should be used as a broad term covering all isoforms of proteins catalyzing the same reaction and occurring in the same species. The term isoenzyme should apply only to those isoforms of an enzyme which arise from different genes and not those which differ only as the result of posttranslational modifications, such as glycosylation, acylation, phosphorylation, deamidation, sulfhydryl oxidation, or aggregation. As an example, the human hepatic, bone, and renal alkaline phosphatases (AP) have similar catalytic characteristics and etectrophoretic mobility when stripped of their sialic acid residues, but differ from intestinal AP and placental AP. Therefore, it appears that human placental, intestinal, and the three isoforms of tissue nonspecific APs are derived from three separate genes. By the IUB definition, then, there are only three, not the formerly defined five AP isoenzymes. This recommended definition does not yet speak to the possibility that the same gene for the apoenzyme may be found in more than one loci.

Although the IUB recommendation that the term isoenzyme be confined to enzymes derived from separate genes was made in 1977, it has not received wide acceptance in clinical biochemistry. For the sake of continuity and clarity, the term isoenzyme is used in this chapter to define both the isoforms and the parent isoenzyme.

Allelozymes are isoenzymes derived from the same loci, but with differing or an allelic genome. When there is a high frequency of different allelozymes for loci, it is described as being polymorphic. Human placental AP has a great deal of allelozyme polymorphism, but other species placental AP contains very little. Allelozymes are well characterized in only a few nonhuman species. The X-linked phosphoglycerate kinase of mice and glucose-6-phosphate dehydrogenase (G-6-PD) in the hare are allelozymes, and the artificially produced mosaicism of crossbred domestic cats with the Geffroy cat X-linked G-6-PD results from allelozyme polymorphism. These X-linked allelozymes in the

mouse and cat were used to demonstrate the poly- or monoclonal origin of neoplasia.

Hybrid isoenzymes are isoenzymes that result from the aggregation of two or more protimers. Two types of protimers are aggregated to make up the tetramers of the five LDH isoenzymes. Of the live, only LDH-I and LDH-5 are made up of all of the same protimer. The LDH-2, LDH-3. and LDH-4 isoenzymes are hybrids of the various combinations of the two protimers.

Isoenzymes can be characterized by zone and column etectrophoresis, kinetically with different substrates, inhibitors, and activators, and immunologically with antibodies against the isoenzyme. Zone electrophoresis as a function of total enzyme activity has been the most common clinical procedure used to quantify isoenzymes.

Isoenzymes have become a valuable means of phenotyping the genetic pool of both plants and animals. Quantification of isoenzymes has added to the specificity and sensitivity of clinical enzymology in human medicine, but less so in veterinary medicine. The reason for this difference is that in human medicine LDH and CK isoenzymes have a high level of efficaciousness as diagnostic and prognostic aids in the very frequently encountered disorder, cardiac infarction. However, cardiac infarction is an uncommon disease of animals.

2.6 Substrate

Although enzymes are specific for the reaction they catalyze, they may not be specific for the substrate. An enzyme’s affinity for one substrate may not be the same as that for another. This absence of substrate specificity *in vitro* has led to confusion when comparing data from one or more laboratories where the substrates used differ, but the unit of expression is the international unit. The confusion which can result from the lack of substrate specificity of an enzyme can be illustrated with AP. For a number of years, synthetic substrates have been used to assay

serum AP with units of activity expressed as an eponym unit. i.e. Bodansky and King-Armstrong unit. With the advent of the international unit, investigators converted the eponym unit to U by a conversion factor. This conversion led to confusion because the assays were not standardized and the user of the conversion factor fell to recognize the difference among assays. The most appropriate method of comparing values among reports of various laboratories is on the basis of the magnitude of change, rather than numerical units of activity.

The specificity of some enzymes is for general groups on the substrate, such as hexokinase (HK) which catalyzes the phosphorylation of D-glucose. D-fructose, and D-mannose at the same rate. For other enzymes, such as L-lactate dehydrogenase (LDH) (EC 1.1.1.27), used to measure blood lactate, there is a strict substrate requirement. LDH catalyzes the oxidation of L(+) – lactate and only poorly its mirror image, D(−) – lactate. This characteristic of LDH is of particular importance in veterinary medicine when measuring plasma lactate concentration in herbivores with lactate acidosis as the result of grain overload. Lactate acidosis is a disease in which the patient absorbs large amounts of D (−) – lactate synthesized by the rumen bacteria from grains. Use of a LDH to assay for D (−) – lactate underestimates total plasma lactate concentration.

Measurement of the maximal amount of enzyme activity 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 the case of LDH, 99 % of the substrate is converted to product. However, K_{eq} = 1, as in the ALT reaction, only 50 % of substrate is converted to product at equilibrium and the reaction appears to stop. If the reaction is reversible, the product may be reconverted to substrate as quickly as product is formed. This can be overcome by converting the product to a second irreversible byproduct. This

process is called ‘‘trapping.'' In the reverse LDH reaction, lactate is converted to pyruvate by LDH. In order to drive the reaction in the direction of pyruvate, hydrazine is used to "trap” pyruvate as irreversible pyruvate-hydrazine product. This technique permits a greater amount of LDH activity to be determined with a much smaller amount of substrate than would have been possible without the pyruvate being "trapped.”

Substrate exhaustion is frequently the technical reason for erroneous reporting of low activity in samples with a large amount of activity. This phenomenon is the result of an unusually large amount of enzyme activity consuming the substrate before the first reading is taken for either an end point or kinetic assay. This is guarded against by setting minimum and maximum limits of absorption for the first reading. Should that limit be exceeded the sample should be diluted with an appropriate diluent and the assay rerun.

In many reactions, the substrate or product is not readily measured, but a cofactor can be measured. Cofactors which can be measured conveniently and accurately are NADH and NADPH. They have peak absorption at 340 nm and an absorption coefficient (formerly the extinction coefficient) of 6.22×10^3 *M*/liter. Since the rate of appearance or disappearance of NADH is stoichiometrically related to substrate consumption, it is used to express the activity of enzyme in many oxidation-reduction reactions.

In some reactions, no cofactor is required, nor can the substrate or the product be readily measured. In such cases, the primary reaction can be coupled to a second reaction, which utilizes a product of the primary reaction and generates a measurable cofactor or product. An example of a coupled reaction in which three reactions are linked is that of the creatinekinase (CK) assay:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose 6-phosphate} + \text{ADP}$$

$$\text{Glucose 6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-PD}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

In a coupled assay, the substrate of the specific reaction, cofactors, and secondary enzymes are in unlimited quantities. The only variable is the amount of enzyme, CK. in the sample analyzed. The rate of generation of NADPH is then stoichiometrically proportional to the amount of ADP consumed.

Synthetic substrates are available for many proteolytic enzymes which have products or substrates not easily quantified. Those of hemostasis, trypsin, plasmin, cathepsin, and kinasis are a few with diagnostic value. The synthetic substrates are frequently chromogens conjugated to the natural substrate. When the reaction takes place, the chromogen is freed or modified to a form which can be spectrophotometrically measured and its absorption coefficient used to calculate the rate of the reaction.

The study of hemostasis has been revolutionized by the development of synthetic substrates for the proteolytic enzymes of the coagulation pathway. Previously, assay of one coagulation enzyme required linkage of a large portion of the pathway to form a fibrin clot. Now a single enzyme can be assayed independent of the remainder of the enzymes of the pathway. Additional developments can be expected in similar proteolytic pathways such as the complement cascade.

2.7 Cofactors

Cofactors are small molecules, loosely or tightly associated to enzymes as cyclic secondary bisubstrates. They are substrates common to a number of enzymes, where they are specific for the type of reaction catalyzed. Because they are common to a number of enzymes, they are secondary to the enzyme’s primary specific substrate. All of the water-soluble vitamins except C. are

substrates for cofactors which have no stable storage forms. The inability to synthesize and store these vitamins and the cofactors lose arrangement with a broad range of enzyme reactions make water-soluble vitamins pivotal points in homeostasis (Table V.2). An example of their importance is niacin, vitamin $B_{3,}$ the substrate for the NAD and NADP cofactors essential to the electron transportation of respiration. About 25 % of the body's enzymes have metals as cofactors and are called metalloenzymes. Some are further complexed with vitamin-derived cofactors, such as the metalloflavinproteins of oxidation-reduction enzymes. A loosely bound cofactor must be supplied in the enzyme assay in optimum amounts. When the cofactor is tightly bound, however, the addition of cofactor makes no difference to the assay unless the patient is deficient in the factor.

Table V.2

Vitamin Cofactors

Vitamin	Product	Enzym and reactions
Niacin	Nicotinamide of NAD and NADP	Dehydrogenase
Thiamine (B_1)	Thiamine pyrophosphate	Transketolase. pyruvate dehydrogenase
Riboflavin (B_2)	Flavin mono- and dinucleotides	Clutathione reductase
Pyridoxine (B_6)	Pyridoxal phosphate	Aminotransferases
Biotin (vitamin H)		De-, trans-, and carboxylases
Folic acid	Folates	Single-carbon transfer unit
B_{12}	Cobalamins	Isomerization and methylations

Enzyme assays can be used to detect dietary mineral deficiencies. Deficiency in dietary selenium and copper is

detectable by measuring their respective metalloenzymes, red blood cell (RBC) glutathione peroxidase and superoxide dismutase. Cofactor activation enzyme assays are used to detect vitamin deficiencies. Activation assays are performed by adding the factor under consideration to the assay of an enzyme requiring it, to demonstrate a difference in the rate of the reaction in a sample from affected versus the index or normal animals. An activation assay is used in the study of polioencephalomalacia, an idiopathic disease of cattle and sheep in which there is cerebrocortical necrosis and low red blood cell transketolase activity. The low activity is retated to the deficiency of thiamine cofactor, thiamine pyrophosphate (TPP). The magnitude of activation of a TPP-requiring enzyme, RBC transketolase, is measured before and after adding TPP to the assay. The magnitude of change that results from the addition of TPP to a patient’s sample is compared to that observed in the control sample. The greater the activation the greater the thiamine deficiency.

2.8 Inhibitors and Activators

Inhibitors or activators modify the rate of a reaction in which they are not substrates. They are generally small molecules and ions, and can even be enzymes. Their action may be reversible or irreversible. If covalently bound, they are irreversible. Reversible inhibition and activation is by electrostatic, hydrogen, and van der Waals bonds. The action may be competitive with the substrate at (he enzyme's catalytic site, or noncompetitive at a site elsewhere on the enzyme, an allosteric site.

Feedback inhibition of enzymes by the end products of a pathway are well in established in physiological control mechanisms as well as *in vitro* enzyme assays. Some inhibitors, essential to the homeostasis of the body, occur naturally in plasma. The acute phase reactants of plasma, α_l-antichymotrypsin, α_1-antitrypsin, inter-α-trypsin inhibitor,

antithrombin III, and antiplasmin, inhibit the proteolytic enzymes of digestion and coagulation which form from their respective zymogens leaked into plasma from their parent organ.

Inhibitors and activators are used as therapeutic agents as well as to control enzymatic reactions in the laboratory. Heparin is a commonly used enzyme inhibitor. It activates plasma antithrombin, the inhibitor of thrombin and other serine proteases of the coagulation pathway. Without heparin, antithrombin has only a small amount of inhibitory activity, and without antithrombin, heparin has no anticoagulation activity. Other inhibitors are lead, which inhibits enzymes of the porphyrin synthetic pathway, and the organic phosphate insecticides, inhibitors of cholinesterase.

Activators increase the rate of the enzyme reaction by promoting the active state of the substrate or the enzyme. Unlike cofactors, they don't enter into the reaction as bisubstrates. Some are themselves proteases, such as those which activate the plasma and pancreatic zymogens. Metal ions activate enzymes by stabilizing the conformation of the active site.

When divalent metal ions are essential to the enzyme, plasma containing EDTA will chelate the ion and inactivate the enzyme. Some metal ions play an integral role in the structure of the enzyme and are not readily exchanged with the surrounding matrix. Creatine kinases and all other kinases require Mg^{2+} for phosphate transfer. The more loosely associated activators are added to the assay to obtain optimum enzyme activity.

The substrates of allosteric enzymes serve as self-activators. The binding of 1 mole of substrate results in a conformational change which enhances the binding of additional substrate. Allosteric enzymes are polymers with two or more reactive sites. This allosteric activation produces a sigmoidal curve, similar to that of the O_2 saturation curve of hemoglobin, and is inconsistent with the Michalis-Menten model.

2.9 Temperature Effects

Changes in the temperature of incubation alter the rate of the reaction and dénaturation of the enzyme. The temperatures at which enzyme assays are conducted are usually either 37, 30, 25, or 22 °C. The higher the temperature, the more rapid the rate of the reaction, and the greater the rate of heat denaturation of the enzyme. Thirty degrees Celsius is the recommended temperature for reference assays. With computerized recording systems, it is possible, however, to carry out assays in a very short period of time, at a higher temperature, with little or no dénaturation. Many clinical laboratories prefer to use a 37 ^{o}C incubation, and. unlike the case of the international unit, a standard temperature of incubation has not been widely accepted for clinical enzymology.

The rate at which temperatures change the velocity of a reaction is fairly constant for the enzyme, provided the incubation period is not so long that heat dénaturation becomes a factor. Temperature conversion factors are available for approximate conversions to be used in comparative studies. When the activity is converted, it should be reported as converted and the method of conversion also reported. A reaction conducted at 37 °C is approximately twice as fast as one carried out at 25 °C.

2.10 pH Effects

The optimal pH of an enzyme assay is generally a very narrow range, and the type and concentration of buffer used can be critical. Any modification of pH changes the configuration of the enzyme, which may change the reaction characteristics of the enzyme. When the pH used in the assay is in the range of the *pK,* of the substrate, a change in pH can result in a change of the concentration of the disassociated and the non-dissociated form of the substrate. If the enzyme were specific for one form, the effect of the pH change could be the same as changing the substrate concentration.

The type of buffer used should be one that has a pK, within one pH unit of the optimum pH of the assay. Some buffers chelate metal ions and would be inappropriate for an assay of a metal-requiring enzyme. The product of some reactions is ionized and will require buffering. Therefore, it is essential that the molarity of the buffer be capable of maintaining the optimum pH throughout the assay period for as high a concentration of product as anticipated.

3 CLINICAL LABORATORY ENZYMOLOGY

3.1 Development of a Clinical Enzyme Assay

An enzyme, to be valuable as a clinical diagnostic aid, must be economically and readily assayable and must reasonably reflect pathological change in a specific tissue, organ, or group of organs. Even when meeting these requirements, an enzyme assay may not become commonly used in the clinical laboratory. An example of assay ability is the case of the three liver- specific enzymes, arginase (ARG). sorbitol dehydrogenase (SDH); L-iditol dehydrogenase and alanine aminotransferase (ALT). Serum ARG and SDH are liver specific enzymes in many animals and are specific, sensitive markers of hepatocellular damage. Serum ALT is also liver specific in many species but is used in favor of the former two enzymes because the assay for ALT is simpler than that for ARG. Sorbitol dehydrogenase is unstable in some species, and, furthermore, a large body of clinical background data for ALT was available before ARG and SDH assays were developed.

Each cell of an organ has a specific function and enzymes unique to that function. Disruption of the integrity of a cell by hypoxia allows soluble cytosolic enzymes to escape into the surrounding fluid. Once in these extracellular fluids, i.e., plasma, cerebral spinal fluid (CSF), urine, or milk, their activity is measured as an index of the cell's integrity. Cells need not be

necrotic to release their enzymes. Transitory hypoxia causes the cell membrane to temporarily lose its integrity, permitting enzymes to leak into the extracellular space. This reversible loss of integrity is observed microscopically as hydropic degeneration. Partial blood stasis results in hypoxia of hepatocytes which is evident as increased serum ALT activity in the absence of frank hepatocellular necrosis.

An increase in the loss of membrane integrity and necrosis of the cell are not the only way the activity of cell enzymes change in extracellular fluid spaces. The amount of an enzyme's activity in serum changes in proportion to the intracellular specific activity of the enzyme, without a change in total cell mass or cell integrity. This form of change is commonly observed in dogs treated with glucocorticoids. Normal dog liver contains little or no intestinal alkaline phosphatase isoenzyme (IAP) activity and none appears in serum. When a dog is treated with corticosteroids, synthesis of IAP in the liver is induced and the enzyme is observed in serum. Reduction of an enzyme's intracellular specific activity results in a decrease in its activity in serum. Serum ALT decreases in cyclosporine-treated dogs and rats to half the pretreatment value as the result of a decrease in ALT's specific activity in the liver.

A change in the total amount of tissue also results in a change in a serum enzyme's activity without induction or loss of integrity of the cell. As an example, the amount of serum creatine kinase (CK) increases in direct proportion to skeletal muscle mass.

High tissue specific activity of an enzyme does not mean the enzyme will be of diagnostic value. The range of activity within the reference population, anatomical location of the cell, the total mass of the tissue or the cell type, and the rate of catabolism in plasma are all variables to be considered when determining the value of an enzyme as a diagnostic aid. A wide range in an enzyme's activity within the reference population, such as serum

AP in ruminants, makes it difficult to determine what constitutes the abnormal value in an individual patient. However, the same enzyme measured sequentially in the same individual may be sufficiently constant to detect sequential changes within the same individual.

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 AP activity increases in bone and liver disorders, and. to assist in identifying the source of the increase, ALT or γ-glutamyltransferase (GGT) may be assayed as part of a hepatic profile.

Another consideration is whether the activity of the enzyme is detectable after it has been released. These considerations limit the organ-specific enzymes as markers of the site, magnitude, and type of disease affecting the patient. Most cells have enzyme activity specific for the cell, but only a few are of diagnostic significance. For example, the respiratory, intestinal, and urinary tracts have high specific GGT activity, but damage to these tissues does not result in clinically significant changes of serum GGT activity. This appears to be because their major cell mass faces the lumen of the bowel or tubule and is separated from the extracellular plasma space of the body by the basement membrane. The cells of these three organs discharge their GGT outside the body to be washed away as urine, feces, and pulmonary transudates and do not accumulate in plasma as does hepatic GGT. This is not true, however, for all tissues with lumens facing outside the body. Pepsinogen of the stomach, the digestive enzymes of the pancreas, and GGT of the biliary ducts are normally found in serum and increase in disease states of the respective tissue. Therefore, high enzyme specific activity in a tissue does not in itself mean that the enzyme will be efficacious as a diagnostic aid. Its efficaciousness must be validated by appropriate studies of induced and spontaneous disease.

Plasma is the most commonly sampled body material for clinical enzymology, but urine, gastric, intestinal, and pulmonary fluids, blood cells, and fixed tissue all contain enzymes of clinical diagnostic value.

Clinical enzymology of non-plasma fluids present a problem in quantification not found in plasma. Homeostasis of plasma volume and solutes makes it possible to express enzyme activity per unit of serum volume as the reference point. In the non-plasma samples used in clinical enzymology, however, unit per volume is not appropriate because the solvent, water, widely fluctuates. Enzyme activity in tissue is expressed as unit per weight of tissue, protein, DNA, number of cells, or nuclei. In urine, enzyme activity is expressed per mass of creatinine, because creatinine is excreted in direct proportion to the body's skeletal muscle mass. In lung, gastric, and intestinal fluids, there is no good constant to use as a reference point, and enzyme activity is generally expressed per unit volume. Whichever reference point is used, it must be one that permits valid results among laboratories as well as within the laboratory reporting the results.

Histochemical enzymology and enzyme-linked immunocytochemistry serve to identify cell types in neoplasias and enzyme deficiencies. Histochemical procedures can be semi quantified only by stringent, simultaneously stained controls in which the tissue thickness and length of staining time are closely controlled.

3.2 Validation of an Assay

Cost of reagents, time constraints, and limitations of the detection systems used, i.e., automated photometry, are limits to consider when performing clinical chemistry assays. Optimum conditions of clinical enzyme assays are not always the optimum conditions for the maximum velocity of the reaction. The optimum assay procedure is generally the reference procedure and contains such stringent limitations that it may not be a valid

clinical laboratory assay. Because of the multiple of variables, time, temperature, substrate, cofactor concentration, and range of enzyme activity to be anticipated, a sophisticated strategy is necessary to establish optimum assay conditions for the clinical chemistry laboratory. Two methods are in current use: the response-surface co-optimization and the simple method. The optimization methods require holding all but one variable constant while the remaining one is varied. Even when these methods are applied, however, there can be considerable variation in procedures used.

To establish the validity of a new clinical laboratory assay, the accuracy of the reference and clinical procedures are compared. The accuracy of an assay refers to how closely the assay can measure the true value of the enzyme activity as determined by the reference method. This is desirable but not always possible with unstable enzymes. A commercially available assayed quality control serum can be used for stable enzymes. The new assay method can then be compared with the reference method at low, high, and medium levels of activity to be encountered in the laboratory. If the method is new only from the standpoint of the species, it can be compared over the anticipated range and sample conditions encountered in-house with the established procedure.

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 variation (CV). Precision is of particular importance when one is assaying enzyme activity because many enzymes are unstable and control serums with known amounts of activity may not be available for all enzymes.

3.3 Specimen Requirements

Many enzymes require metal ions for maximal activity, and plasma containing the metal-chelating anticoagulants EDTA, citrate, or oxalate are unsatisfactory for their assay. Heparinized plasma can be used in some procedures. If there is doubt, serum should be used.

Some enzymes in serum are very stable at room temperature. Serum AP activity increases when kept at room temperature for a few days (Table V.3). Refrigeration and freezing preserve many enzymes, but others deteriorate even when frozen. Stability of an enzyme's activity in one species does not mean it will be stable in a second. Horse serum SDH, but not that of cattle, sheep, or goat, is unstable at room temperature, refrigerated, and frozen. Urine GGT is more stable at room temperature than frozen. Freezing results in concentration of salts and enzymes and deaggregation of some weakly bonded protimers. When thawed, the protimers may randomly reaggregate into inactive or less active configurations. A list of some enzymes and their storage characteristics are found in Table V.3.

The glassware in which a specimen is obtained must be carefully cleaned. Detergents interfere with many assays, and only thorough rinsing will reduce the detergent residue to a point which will not inhibit the enzymes. Whenever possible, commercially available collection vials should be used.

Serum must be separated from the cellular element of blood as soon as possible as hypoglycemic blood cells leak LDH and AST into serum even before hemolysis is apparent. Finally, clinical history is essential to interpretations of laboratory results as many drugs are themselves the cause of changes in enzyme activity.

Table V.3

Enzyme Stability in Serum

Enzyme	Storage	Time (days)	Activity (ft)
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 (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
L-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		71
Sorbitol dehydrogenase (EC 1.1.1.14)	Species dependent	8	68

3.4 Assay of Enzymes in the Clinical Laboratory

Differences in performing clinical assays are inevitable. Constant improvement in technology requires changing the assay procedure, but the slightest modification of a procedure may change the stoichiometry of the assay. Modification of an enzyme assay for whatever reason requires revalidation to assure that zero-order kinetics are not altered. Kits commercially prepared for human medicine are commonly modified for veterinary medicine. This may seem to be a subtle change, but, in fact, it requires invalidation of the procedure. Use of a fresh versus frozen sample requires revalidation. Some species have unusually high amounts of basal activity in serum, such as amylase in dogs and AST in horses, and the limits of linearity are exceeded with kits prepared for human use. Normal equine and bovine sera have a great deal of background absorption in the 340-nm spectrum not observed in humans or many other species. This background requires a patient blank, which may not be required in other species, or setting higher limits of starting absorption.

Enzyme assays in clinical biochemistry are generally carried out in one of two ways. The first is an "end point'' or “fixed-time'' procedure analogous to a colorimetric assay. The sample is added to the reaction mixture, and. after an 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 is then determined.

A second procedure, the "kinetic” or "continuous-monitoring" 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 since the reaction rate can be visualized throughout the time of the assay. A constant recording device is useful for this purpose because the linearity of the reaction rate can be 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, when the substrate is exhausted the assay must be repeated; and (2) if an activator is present, it can be detected on the graph (Fig. V.1).

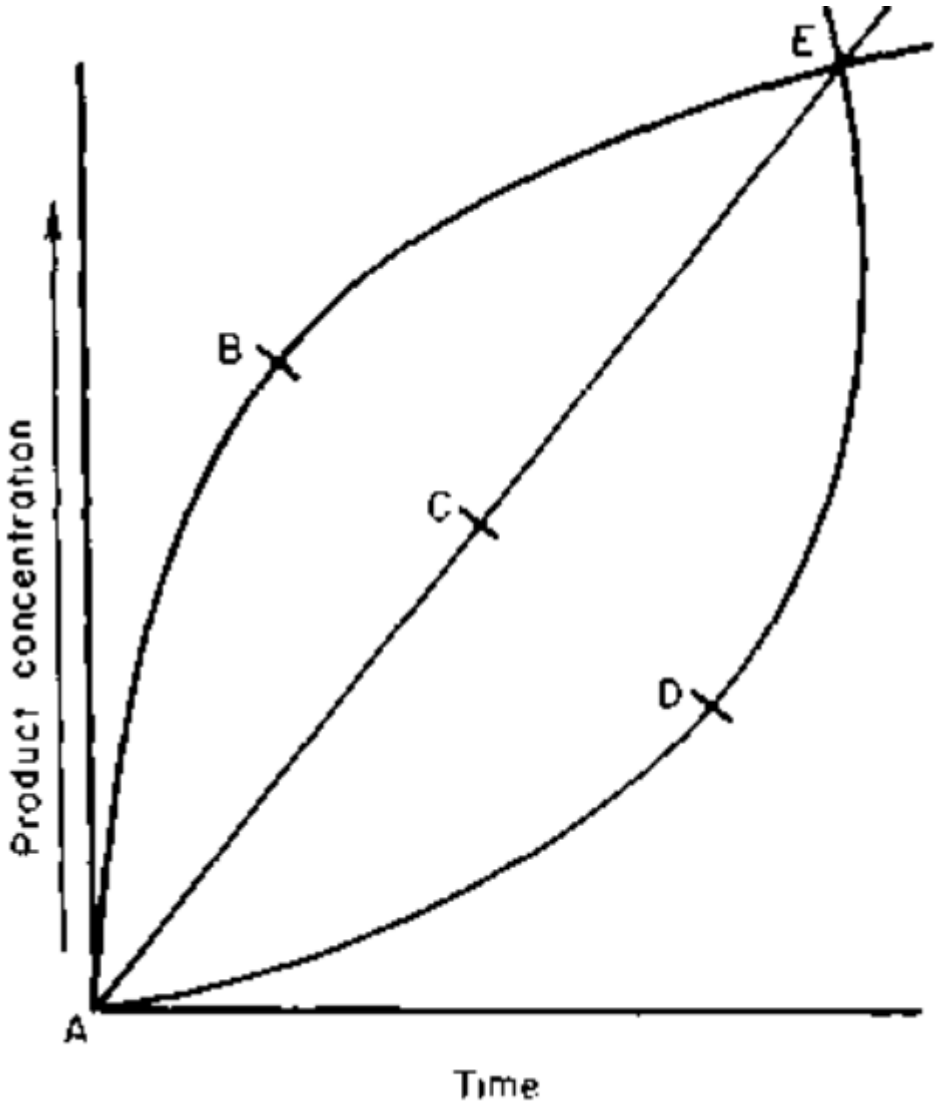


Fig. V.1. Illustration of potential hazards of using an end point enzyme assay. Line ACE is a zero-order reaction that permits accurate determination of enzyme activity for the entire reaction time. Curve ABE initially is a zero-order reaction of high rate followed by a reduction in rate, possibly caused by exhaustion of substrate. Assay at point E would be in error. Curve ADE has an initial lag phase which also would be erroneous.

Approximation of zero-order kinetics becomes limited when the rate of enzyme activity is very high. Reagents for an enzyme

generally have sufficient substrate concentration to maintain zero-order kinetics only for the time period of the assay or for magnitudes of enzyme activity 2 to 3 times greater than the activity normally occurring in the patient's serum. When performing an enzyme assay, it is imperative that the limitations of zero-order kinetics be maintained if high precision in quantification is required. In some clinical situations, a high level of precision is not required, in which case the results of the assay exceeding the limits of linearity can be reported as "greater than" the value established as the upper limit of linearity. Alternatively, the assay can be repeated on a diluted sample, or the size of the sample can be reduced and appropriate dilution corrections made.

Immunoassays for enzymes have been developed as isolation procedures improved. Monoclonal antibody technology allows for the production of a specific antibody against a protein in an impure sample. In turn the specific antibody can be used to isolate the pure enzyme for further study and use as an assay standard. Combining radio labeling with immunoassays has permitted the development of very specific, sensitive assays for inactive, active, and inactivated enzymes such as serum pepsinogen and trypsinogen. However, this high specificity and absence of cross-reactivity have disadvantages in veterinary clinical laboratory diagnostics. Without species, cross-reactivity the veterinary laboratory would have to maintain a number of species-specific immunoassays for the same enzyme. Immunoassays tend to be more expensive and complex in clinical laboratories and may never have the multiple species advantage of measurement of enzyme activity.

3.5 Enzyme Histochemistry and Cytochemistry

The identification of cell types on the basis of their enzyme content is a routine histochemical diagnostic procedure which is limited only by the availability of soluble substrates with insoluble

products. The linking of enzymes with monoclonal antibodies against cell-specific epitopes has increased the sensitivity and specificity of characterizing undifferentiated neoplastic cells and permits identification of cell structure and products by light and etectron microscopy.

3.6 Enzymuria

Renal diseases and nephrotoxicosis are reflected in changes in enzyme activity of urine, but not serum. The kidney is functionally compartmented along the length of the nephron, with the majority of its cells facing into the lumen of the tubule. When the integrity of these cells is lost, their contents are discharged into the tubule’s lumen. Plasma and tubule epithelium leak enzymes into urine, but only a few of the plasma enzymes in urine have been of diagnostic value. Pancreatic amylase in urine is of diagnostic value in active pancreatitis in humans, but not in dogs with the same disorder. To date, in veterinary medicine, enzymuria has been of diagnostic value only in renal disease, specifically in nephrotoxicities and renal transplantation rejection.

The nephron's functional organization of the proximal and distal tubules epithelium enzyme content differs. Attempts to localize the damage through ratios and patterns of enzymuria have met with limited success. Subcellular localization of damage inthemicrovillar versus intracellular space appears to be possible by measuring urinary AP and GGT as indicators of nephrotoxicosis and urinary N-acetylglucosamine (NAG) as an indicator of renal hypoxic loss of vascular integrity in transplant rejection. Much like the case of hepatic congestion, the increase in urinary enzyme activity as an indicator of nephrotoxicities is so sensitive that the renal lesion causing the hyperenzymuria is reversible and not readily evident by light microscopy or the results of renal function tests. To some investigators this sensitivity suggested that diagnostic enzymuria is not a valuable diagnostic aid; to others it reflects the great reserve capacity of

the kidney and the insensitivity of microscopy and renal function tests.

Urinary enzyme assays are not as easily performed as are serum enzyme assays. The point of reference is generally the activity per unit of creatinine clearance or more commonly per gram of creatinine. Urine is not as homogeneous as serum, anti the great ranges in pH, molarity, and variety of solutes found in urine differ considerably from those in serum. Some of the solutes may be inhibitors at one concentration but not at another. Therefore, as the concentrations of the solutes are changed by changes in the urine solvent, water, so might change the enzyme activity. The inhibitor may have to be eliminated by dialysis before an assay is performed, or, if the inhibitor is a constant, the specific gravity may have to be standardized before the assay is performed. Urea in high concentration disrupts weak hydrogen bonds; thus, enzymes dependent on aggregation by weak hydrogen bonds may be rendered inactive at one concentration of urea, but not at a lower concentration.

Very few validated urine enzyme assays ate commercially available. Therefore, before a serum enzyme assay kit is used for urine, it must be revalidated. The validation must include the following: high and low sensitivity, limits of linearity, presence of inhibitors or activators by column chromatography or dialysis, storage stability, reproducibility at various solute dilutions, capability of the buffers to maintain the reaction pH over the anticipated range of salt concentrations, and pH of the urine. Urine is a very poor environment for a molecule that depends on its conformation to function.

3.7 Quality Control

To maintain the quality of any enzyme assay, some form of control must be followed to assure that unpredicted variables are detected. Quality control (QC) of an assay is a part of the laboratory’s quality assurance program that is concerned with the

range of variables from errors in labeling of a sample to review of the patient's accumulated laboratory data. Potential unpredictable variables in the QC of the assay range 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 accuracy and precision of the assay.

Unlike many metabolites measured in clinical chemistry laboratories, some enzymes are insufficiently stable to lend themselves to a QC program. The quality control program consists of assaying the same sample with known activity, in the same range of activity, every time the assay is performed. When a large series of samples is assayed, as many as one in six of the samples in the series will be QC samples to assure within-run precision. In general, the closer the numerical value approaches the lower limit of sensitivity of the assay, the less the precision. It is essential to assay QC samples with values over the range of values to be expected. The precision of the assay for three criteria is determined statistically for the QC sera: within-run, within-day, and day-to-day. Ninety-five percent limits of confidence are the limits of reproducibility for the QC sample acceptable in the clinical laboratory. Thus, when the test value of control serum is within 2 SD of the mean of the control value, the assay of the unknown sample is accepted. With the acceptable level of precision set at 95 %, as many as l in 20 of the accepted values may be incorrect. As long as an error is to be accepted, it should be recalled that the more assays performed, the larger will be the number of incorrect values accepted. In a quality assurance program, the detection of these incorrect values becomes the function of the individual reviewing the appropriateness of the patient’s accumulated data.

In general, an assay procedure which QC’s CV is greater than 10 % has poor precision. This does not mean that the assay is inappropriate for clinical use. Its appropriateness will depend on

the magnitude of sensitivity needed by the clinician to interpret the results.

When the value for the QC sample is established, the QC serum is used each time an assay is performed, a record maintained, and the value plotted on a graph. The within-run and within-day precisions should be greater than the day-to-day precision, as day-to-day values reflect a greater number of variables, such as reagent decay and various operator idiosyncrasies. The graph will show trends or progressive changes in the value of the QC serum. The trends serve to detect the procedural errors before the variable becomes so great that the data are valuates.

Assayed and unassayed lyophilized QC serum with normal or abnormal activity can be purchased from commercial sources. Unassayed QC serum is less expensive and more appropriate in the individual laboratory where the methodology differs from the method of the commercial sources. Commercial programs, however, offer a number of benefits. The commercial system generally supplies a uniform QC serum over a long period of lime, keeping track of its rate of deterioration, which permits individual laboratories to compare their results with other laboratories using the same or a different procedure. This comparison is of particular value when evaluating infrequently performed procedures.

4 ENZYME-LINKED DIAGNOSTICS

4.1 Immobilized Enzymes

The immobilization of enzymes adsorbed to solid supports such as membranes, beads, and tubes permits physical separation of the enzyme from its substrate, buffer, and product. This separation allows for the conservation of the enzyme in diagnostics and industrial preparative methodology. Immobilized enzyme techniques have been applied with the potentiometric technology of ion-selective electrodes (ISE) for the direct assay of

glucose and urea in whole blood. This is performed by binding glucose oxidase to a membrane where it catalyzes the oxidation of glucose in the sample to form hydrogen peroxide. Hydrogen peroxide is oxidized on contact with a platinum electrode, and the electrical current produced is measured by a voltmeter. The current produced is proportional to the glucose concentration. This procedure has the potential for *in* vivo monitoring of blood metabolites.

4.2 Enzyme Immunodiagnostics

Conjugation of enzyme with antibody has been a major development in competitive protein binding assays. Enzyme immunoassays (ETA) have high sensitivity, low cost, reduced need for radioisotopes, and aid automation. The specificity of an assay is dictated by the specificity of the antibody, and when monoclonal antibodies are used assay specificity is very high. Although radioimmunoassay (RIA) is of equal specificity and of greater sensitivity than ETA. ETA is frequently the preferred method. The ETA reagents have relativity long shelf lives, require commonly available measuring methodology (photometry), and involve little or no government regulation. The catalytic activity of the assay enzyme amplifies the antigen-antibody complex and has the potential for measuring more than one analyte at a time in a single aliquot of sample.

Enzymes are the source of sensitivity of the ETA. An ETA for mouse IgG can detect 24,000 molecules of IgG/ml (6 μg/ml). By using radioactive substrate, the sensitivity of the ETA can be further enhanced. When [^{3}H] AMP is used with AP. A detection system capable of measuring 600 molecules/ml is produced.

Two basic ETA procedures arc in use to measure soluble antigens bound to solid phases. They differ fundamentally in the need to separate the enzyme-antibody complex from the enzyme-antibody-antigen complex before the enzyme activity is measured. The homogeneous or nonseparation enzyme

multiplied immunosorbent technique (EMIT) does not require a separation step. The unique feature of EMIT is that the enzyme is inactive or has limited activity when complexed with antigen. It becomes activated when the enzyme-antigen aggregates with antibody. Free antigen of the sample competes with the enzyme-labeled antigen for the antibody to produce an inverse relationship between the amount of enzyme activity and the unlabeled analyte of the sample. The EMIT procedure is limited by the kinetics of the enzyme-antigen-antibody complex and is currently restricted to analysis of small molecule analytes in toxicology, pharmacology and endocrinology.

The second ETA procedure is a heterogeneous, or separation. Enzyme-linked immunosorbent assay (ELISA). In contrast to the situation in the EMIT, the enzyme in the ELISA is always active. Before enzyme activity can be measured in an ELISA the unbound enzyme-antibody or enzyme-antigen complex must be separated from the bound complex. This separation step makes the ELISA a more complex procedure and more versatile than the EMIT. Immobilizing the ELISA complex on a solid phase simplifies the separation step to inverting the tube or plate over a sink ELISA is a popular method for qualitative detection systems in which color changes are detected with the unaided eye and little or no quantification is required; it is used to detect parvo virus in canine feces and lymphosarcoma virus in fetine blood.

Enzyme-linked immunosorbent histochemistry has provided a very specific diagnostic procedure. By using antibodies against various markers of cells, it has become possible to separate cell types, whose light and electron microscopic features are the same, on the basis of their membrane epitopes.

4.3 Molecular Genetics

Linking ETA with nucleotide hybridization has resulted in the development of diagnostic procedures designed to detect the nucleotide sequence of aberrant genes and latent infectious

diseases. Selective use of enzymes permits the insertion and extraction of specific sequences of nucleotides from both RNA and DNA. Given the amino acid sequence of a protein, the probable nucleotide sequence(s) of its gene can be synthesized and labeled with a radioisotope or biton. This labeled single strand of nucleotides, the probe, can be hybridized with native DNA or RNA of a given cell to probe for the presence of messenger RNA. Abnormal genes of the patient or infectious agent, or erroneous rearrangement of chromosomes.

Restriction endonucleases are a group of enzymes derived from bacteria that cleave DNA at specific sites to produce fragments of DNA. Combining restriction endonucleases with DNA probe hybridization to produce selective fragments of DNA has produced a new diagnostic procedure based on restriction fragment length polymorphism (RFLP). In veterinary medicine, RFLP technology and other nucleotide hybridization procedures are just beginning to be used to identify bacteria and viruses and to phenotype genetic diseases. In the future, this technology has the potential of selecting desirable as well as undesirable genetic traits in pretransplantation ova.

5 ENZYMES OF DIAGNOSTIC IMPORTANCE

5.1 Alkaline Phosphatase

Alkaline phosphatases (AP; EC 3.1.3.1) were the earliest serum enzymes to be recognized to have clinical significance when, in the 1920s, it was discovered that they increase in bone and liver diseases. Since then they have been the subject of more publications than any other enzyme. Alkaline phosphatases are a group of isoforms of nonspecific enzymes which hydrolyze many types of phosphate esters whose natural substrate or substrates are unknown. Because APs catalyze the dephosphorylation of ATP, are located in most cells, and have high specific activity in the brush borders of secretory epithelium and bone, their

activities are speculated to be a part of the ATP-dependent membrane "pumps" and calcification. In addition, because people with inherited AP deficiency secrete large amounts of ethanol-amine phosphate, APs are believed to be associated with membrane phospholipid synthesis.

The term “alkaline" refers to the optimal alkaline pH of this class of phosphatases *in vitro.* The optimum pH of 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. All APs studied are dimers composed of monomers with molecular weights of 40.000-7.000. They are Zn^{2+} metaloenzymes which are dependent on loosely bound Mg^{2+} for activity. Because of the dependency of APs on Mg^{2+} activation, assays of plasma containing EDTA must have Mg^{2+} added. The presence of identical active sites in bacteria and mammalian APs has demonstrated their evolutionary conservation, and their presence in nearly all cells speaks to their importance in the cell.

The two AP isoenzymes of nonhuman animals are the bone, kidney, placenta, and liver isoforms multiple forms of tissue-unspecific AP (UAP) and the intestinal and steroid-induced hepatic isoforms of AP (IAP). In people, placental AP (PAP) is a third isoenzyme. The isoforms are separated on the basis of their catalytic site differences, catalytic activity, inhibitors, etectrophoretic mobility before and after removal of sialic acid moieties, immunogenicity, and, in some cases, various amino acid sequences. Temperature inactivation is another means of characterization of the isoforms, but all APs are inactivated by heating at 60 °C for 10 minutes. In clinical biochemistry, cellulose acetate, agarose zone etectrophoresis, and inhibitors are the most common methods of determination, but polyacrylamide disk get etectrophoresis gives the best separation. Inhibitors used to characterize AP isoforms seldom do so completely. Each of these criteria has been used to identify APs under normal and abnormal conditions.

The isoforms of UAP are glycosylated homodimers with molecular weights of 140.000-170.000 and contain sialic acid moieties. Removal of sialic acid from the UAP isoforms results in the formation of a single form of AP. The isoforms of UAP have common immunochemical and heat inactivation properties and are inhibited by L-levamisole and L-homoarginine but not by L-phenylalanine. The UAP isoforms make up all of the serum AP. In young, growing animals bone UAP is the predominant form which decreases as maturation progresses and the epiphysis closes. Total serum AP activity is 2 or 3 times greater in puppies, foals (Fig. V.2), and kittens than it is in adults. In the mature animal, hepatic UAP dominates in serum. Cholestasis at any level results in an increase in serum hepatic UAP. In patients with cholestatic disorders, serum UAP is found in high molecular weight membrane fragments which also contain γ-glutamyltransferase (GGT), 5'-nucleotidase, and nucleotide pyrophosphatase. The disassociation of these aggregates is probably the reason total serum AP increases in activity when stored at any temperature. Renal UAP is not found in serum. Located in the brush borders of renal epithetium, UAP is lost into urine where it accompanies GOT in membrane fragments. Both are markers of nephrotoxicity.

Human placental AP (PAP) is a dimer or aggregates of a monomer of 58.000-64.000 daltons with a high degree of polymorphism. The placental AP of most other species, including cat, dog. cow, sheep, pig, rat, mouse, rabbit, hamster, and guinea pig, is an isoform of UAP. Although serum PAP is found in large quantities in sera of pregnant women, placental UAP was not found in the serum of pregnant mares or other species.

Calf IAP is a sialic acid-free glycoprotein homodimer of 160,000 daltons. It and its steroid-induced hepatic isoform are inhibited by L-phenylalanine but not by L-levamisole, and it is stable at 65 °C for 10 minutes. Hepatic IAP has an etectrophoretic mobility closer to that of hepatic UAP than IAP but when the sialic acid residues are removed from the hepatic IAP, it has the same

mobility as the AP derived from the intestine. Antibody against canine hepatic IAP reacts agalnst IAP but not hepatic UAP. Hepatic IAP and hepatic UAP are found in bile duct canaliculi epithelium.

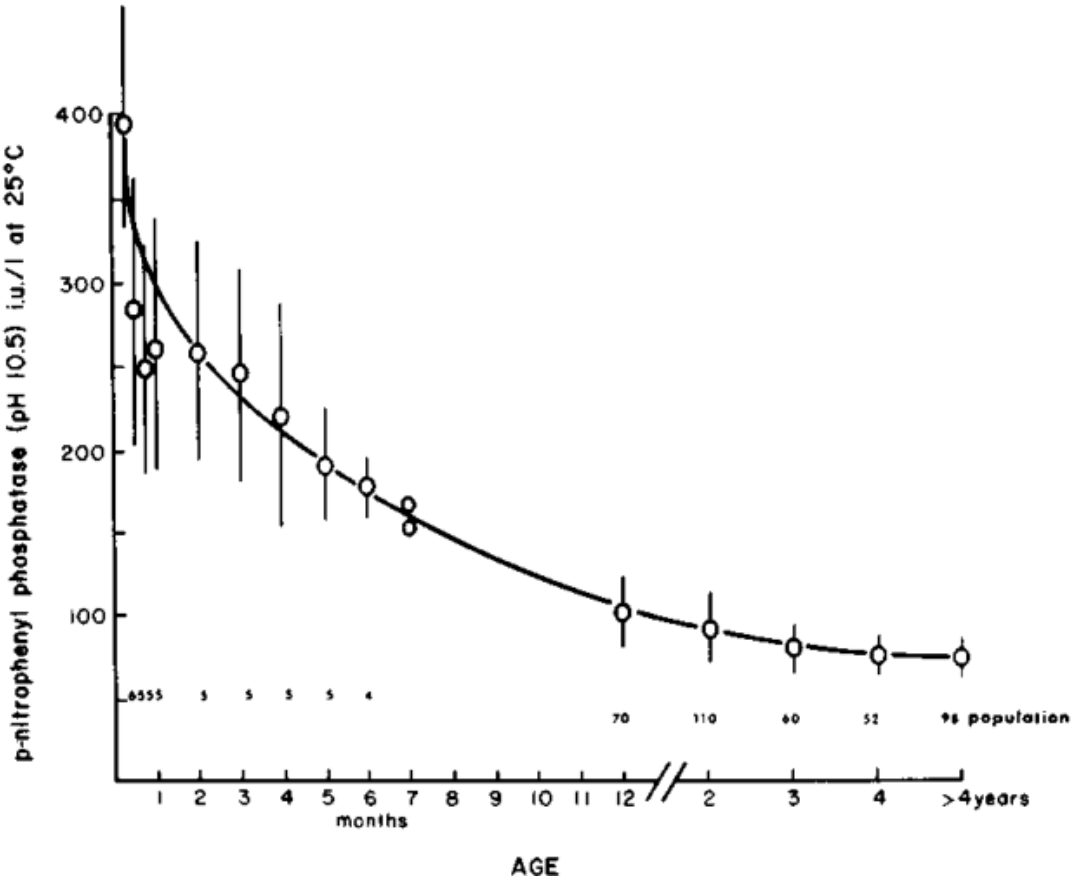


Fig. V.2 Mean and standard deviation of serum alkaline phosphatase activity in Thoroughbred horses in relation to age.

In the past, reports of serum IAP have been based on the separation of IAP from UAP on the basis of L-phenylalanine inhibition and electrophoresis. These techniques do not separate hepatic IAP from intestinal derived IAP. No intestinal or hepatic IAP activity is observed in the serum of a normal dog. But, in various diseases, hepatic IAP increases independent of treatment with pharmacological steroids. Experimentally induced mesenteric infarction and pancreatitis in dogs resulted in increased polyacrylamide get-separated serum IAP, but bile duct obstruction, laparotomy, duodenal perforation, or small bowel

obstruction did not. Although these observations suggest that the increased serum IAP was of intestinal origin, no attempt was made to separate the two forms of IAP. Serum IAP has been reported in serum of horses with and without gastrointestinal disorders, but the L-phenylanine inhibition assay did not separate the two forms of IAP. The value of total serum IAP activity as a diagnostic aid in gastrointestinal disorders remains unknown. If a clinical L-levamisole inhibition AP assay can be developed to separate hepatic IAP and hepatic UAP, then AP isoenzymes may become of value for the definitive diagnosis of hepatic disorders.

Total serum AP activity has diagnostic value in hepatic and bone diseases in dogs and cats. It is of little value in hepatic diseases of horses and ruminants because of the broad range of reference values against which the patients' values must be compared. The range of serum AP value in goats may be 10-fold with no evidence of hepatic damage. Values within the individual are fairly constant for sequential evaluation.

Assays for APs 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. Conversion is of value for comparative studies only when conditions of the assay are taken into account. When converting, data derived from different methods, it is satisfactory within the normal range, but, when outside the range, the activities are not usually comparable.

5.2 Creatine Kinase

Creatine kinase isoenzymes (CK; EC 2.7.2.2) are the most organ-specific serum enzymes in clinical use. They catalyze the reversible phosphorylation of creatine by ATP to form creatine phosphate, the major storage form of high-energy phosphate required by muscle.

The four creatine kinase isoenzymes are dimers of two protimers with individual molecular weights of 40.000, one with muscle (M) and the other with brain (B) subunits. The isoenzymes are numbered CK_1, (CK- BB), CK_2, (CK-MB), and CK_3, (CK-MM). The CK_2 is the hybrid isoenzyme. A fourth variant form, CK-Mt, is found between the mitochondrial membranes and makes up about 15 % of the total cardiac CK activity. In the horse, rabbit, and dog, CK_3, is the dominant form in heart and skeletal muscle.

A number of conditions and compounds inhibit CKs. All are activated by Mg^{2+} but inhibited by an excess. They are unstable when stored at room, refrigerator or freezing temperatures owing to rapid oxidation of sulfhydryl groups at the active site. Thiol agents added to the assay reactivate the enzymes. In 1981 the U.S. Food and Drug Administration reported that, of 17 methods of CK isoenzyme separation studied, ion-exchange chromatography was the most useful. In human medicine, where CK isoenzyme quantification is in more common use than in veterinary medicine, specific monoclonal antibodies against the CK isoenzymes are used in separation. In veterinary medicine, separation of the CK isoenzymes has not been demonstrated to be of any greater diagnostic value than total serum CK activity.

Creatine kinases are found in many types of cells, but they have their highest specific activity in skeletal muscle. Although CK has a high specific activity in the brain, very little or none is found in normal cerebrospinal fluid (CSF) or serum. Increases in total 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. Increases in CK in CSF have been associated with a number of disorders in dogs, cats, cattle, and horses. Spinal fluid CK values should have significant diagnostic value in the detection of non-inflammatory central nervous system disorders and have been observed to increase in some diseases and neoplasia; however, more

information concerning the sensitivity of this diagnostic method is needed.

Very high total serum CK value consisting of CK_2, and CK_3, are observed in moderate amounts of muscle ischemia in patients in prolonged recumbency, convulsions, or shivering. These high values rapidly return to normal as the result of the enzyme's short serum half-life. The CKs are such sensitive indicators of muscle damage that, generally, only large increases in serum activity are of clinical significance. Nutritional myopathies arising from vitamin E and selenium deficiencies are diagnosed on the basis of increased total serum CK activity and can be confirmed by low selenium metalloenzyme glutathione peroxidase activity in the red blood cells.

5.3 Alanine Aminotransferase

Alanine aminotransferases (ALT: EC 2.6.1.2) (formerly glutamic pyruvic transaminase; GPT) catalyzes the reversible transamination of L-alanine and 2-oxoglutarate to pyruvate and glutamate in the cytoplasm of the cell. Pyridoxal 5'-phosphate is a cofactor tightly bound to ALT and many other aminotransferases. Some apoenzyme without the cofactor is always found in plasma, and in order to obtain maximum activity it is necessary to add pyridoxal 5'-phosphate to the assay. Treatment of rats and dogs with cephalosporins results in a decrease in tissue and consequently serum ALT and AST activity. The decreased activity *is* the result of a metabolite of the drug and can be partially relieved by the addition of pyridoxal phosphate to the assay. The assays for ALT and AST are frequently done with LDH to produce a measurable product. When high concentrations of substrates of LDH occur in the plasma of the patient, the lag phase is prolonged and interferes with the transaminase assay.

Although ALT activity is normally found in serum and spinal fluid, it is not found in urine. Its absence in urine is a reflection of

very low renal specific activity. It is relativity stable at room temperature, refrigerated, and frozen.

The greatest specific activity of ALT in primates, dogs, cats, rabbits, and rats is in the liver. It is a well-established, sensitive liver-specific indicator of damage. However, ALT in the tissues of pigs, horses, cattle, sheep, or goats is too low to be of diagnostic value. It is used as an indicator of hepatopathy in toxicological studies which use small laboratory rodents as well as dogs.

5.4 Aspartate Aminotransferase

Aspartate aminotransferases (AST; EC 2 6.1.1) glutamic-oxaloacetic transaminase**;** GOT, catalyzes the transamination of L-aspartate and 2-oxoglutarate to oxaloacetate and glutamate. They share many of the features of ALT such as puidoxal 5'-phosphate as a cofactor. The two isoenzymes of AS, one cytosolic and the other mitochondrial, have molecular weights of about 92.000, and multiple forms exist. The presence of AST in so many tissues makes their serum level a good marker of soft tissue damage but precludes its use as an organ-specific enzyme. With the introduction of CK as a highly specific marker of muscle damage, the value of AST assays has decreased. Because ASTs have a longer serum half-life than CK, it was suggested that its marked elevation could serve as an indication of muscle damage after CK has fallen. However, this has not found support in clinical medicine, and AST as a diagnostic aid has given way to more organ-specific serum enzymes.

The upper limit of normal equine serum AST activity is considerably greater than that observed in other species. Therefore, assay procedures established for other species are usually unsatisfactory for the detection of increases in equine serum AST activity. In the horse, when the activity is high, it is best to dilute the sample one-half to one-third or to reduce the volume of serum sample and correct for the change in volume.

Red blood cells contain a large amount of AST which leaks into plasma before hemolysis is seen.

5.5 Sorbitol Dehydrogenase

Sorbitol dehydrogenase (SDH; EC 1.1.1.14) (recommended name L-iditol dehydrogenase; IDH) catalyzes the reversible oxidation of D-sorbitol to D-fructose with the cofactor NAD. It has a molecular weight of about 95.000, is localized in the cytosol, and appears to be derived from a single gene. Activity in plasma is low in dog and horse plasma but appreciably greater in cattle, sheep, and goat serum. Aside from the testes, it is found in appreciable amounts only in hepatocytes. Therefore, an increase in plasma SDH is consistent with hepatocyte damage.

Early reports of the magnitude of instability of SDH distracted it from use in clinical diagnosis. Usage has demonstrated it to be stable in equine serum for only a day or two but much more stable in the sera of rodents, sheep, goats, and cattle. Quality control sera are not routinely analyzed for SDH, but an SDH kit with a poorly stable, lyophilized control is commercially available.

A preincubation period is required to permit the reduction of endogenous keto acids by the other endogenous dehydrogenases. The clinical assay is in the direction of sorbitol formation. A high fructose substrate concentration is required because product inhibition occurs when 10 % of the substrate is converted to sorbitol. The syruplike texture of highly concentrated fructose starting solution requires that the assay preparation be well mixed before the initial absorption measurements are recorded. A high starting absorbance of the assay and the inherently high background absorbance of equine and bovine serum at 340 nm make it necessary to use a patient blank. Heparinized plasma can be used, but serum is preferred. Metal-chelating anticoagulants, such as EDTA and oxalate, decrease SDH activity.

The enzyme is liver specific in all species of animals and hepatic injury appears to be the only source of increased SDH activity. Although SDH is liver specific in all species, previously established usage of ALT in dogs and cats has limited SDH as a diagnostic indicator of hepatocellular damage to horses, cattle, sheep, and goats.

5.6 Lactate Dehydrogenase

L-Lactate dehydrogenases (LDH; EC 1.1.1.27) catalyzes the reversible oxidation of pyruvate to L(+)- lactate with the cofactor NAD. The equilibrium favors lactate formation, but the preferred assay method is in the direction of pyruvate because pyruvate has an inhibitory effect on LDH. Lactate dehydrogenase isoenzymes are tetramers of two protimers with molecular weights of about 35,000. The two homomers are LDH-1 (heart; LDH-HH) and LDH-5 (liver and muscle; LDH-LL or LDH-MM). The hybrids are LDH-2 (LDH- H_1L_3), LDH-3 (LDH-H_2L_2), and LDH-4 (LDH-H_1L_3). Multiple forms of LDH isoenzymes have been reported, and a sixth form, LDH-X, has been reported in humans. Both electrophoretic and immunoinhibition methods are used to separate isoenzymes.

Tissues contain various amounts of the LDH isoenzymes, and serum isoenzyme profiles have been used to identify specific tissue damage by electrophoretic separation. There is as much as 150-fold greater LDH activity in the RBC than in plasma. Thus, minimal hemolysis appreciably increases plasma LDH. Anticoagulants such as EDTA and oxalates indirectly inhibit the enzyme; therefore, heparinized plasma or serum is the preferred sample.

Lactate dehydrogenase isoenzyme profiles were the first isoenzyme profiles used in clinical veterinary medicine in an attempt to detect specific organ damage. The instability of LDH-4 and LDH-5 when frozen requires that specimens for isoenzyme separation be refrigerated but not frozen. The introduction of

more highly organ-specific procedures has resulted in LDH no longer being in common use in veterinary medicine.

5.7 Cholinesterase

Serum cholinesterase (ChE) activity is composed of two distinct cholinesterases. The major substrate is acetylcholine, the neurotransmitter found at the myoneural junction. Acetylcholinesterase (AChE: EC 3.1.1.7) found at the myoneural junction is the true ChE and is essential in hydrolyzing acetylcholine so that the junction can be reestablished and prepared for additional signals. The myoneural junction AChE is also found in RBC, mouse, pig brain, and rat liver. Only a small amount of AChE is found in plasma. The ChE of plasma is a pseudocholinesterase, butyrylcholinesterase (ButChE: EC 3.1.1.8), which hydrolyzes butyrylcholine 4 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. Four aggregates of ChE's single monomer are found in serum with molecular weights ranging from 80,000 to 340,000.

There are a number of inhibitors of ChE. They include drugs as well as naturally occurring substrates. Currently, the most important inhibitors are organophosphate insecticides. Because of the high environmental background concentrations of organic phosphates in some areas, it is essential that reference values be established for the geographic area to be studied. 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 humans with acute infection, pulmonary infection, muscular dystrophy, chronic renal disease, and pregnancy as well as insecticide intoxication.

Serum is the specimen of choice, and small amounts of hemolysis do not interfere with the assay. The stability of ButChE is variable, activity lasting at least 6 hours at room temperature, many weeks at refrigeration, and months at – 4 ^{o}C.

5.8 Lipase

Serum pancreatic lipases (EC 3.1.1.3; triacylglycerol lipase) catalyze the hydrolysis of triglycerides preferentially at the 1 and 3 positions, releasing two fatty acids and a 2'-monoglyceride. There are five lipases in serum, but the majority of serum lipases are pancreatic. Human pancreatic lipase is a glycoprotein with a molecular weight of about 48.000. Bile salts and a cofactor, colipase, form a complex necessary for optimum catalytic activity. Lipase and colipase are of pancreatic origin. The enzyme is water soluble, but the substrate is not; therefore, the reaction takes place at the water-lipid interphase of the lipid micelle where a micelle-bile salt-colipase-enzyme complex forms. Colipase is cleared by the kidney, but lipase is not. This disproportionate loss of colipase results in less serum lipase assayed than is actually present, unless colipase is added. In acute pancreatitis and renal failure serum lipase may increase for different reasons. In pancreatitis, it is the result of increased total lipase and colipase activity, but in renal failure it may be the result of normal serum lipase in the face of retention of colipase. Albumin or calcium ions enhance lipase activity. The means by which they do this are 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. Serum lipase is stable at room temperature, refrigerated, and frozen. Formerly, lipase assays were titrimetric and were run overnight. More recently, sensitive but expensive kinetic assays containing colipase have become commercially available.

Pancreatic lipase is destroyed by heating at 56 ^{o}C for 3 hours. Lipoprotein lipase is normally inactive in serum unless

activated by heparin, in which case it can contribute an appreciable amount of activity; it is destroyed at 37 °C. All serum lipases may be assayed when measuring pancreatic lipase unless an appropriate method is selected to omit the other three nonpancreatic enzymes.

Normally, serum lipase activity is very low in dogs or cats, but in pancreatitis the activity increases significantly. Total serum lipase activity is the most pancreas- specific serum enzyme available for pancreatitis in the dog. However, the lipase assay is relativity expensive and more difficult to perform than serum amylase. Therefore, serum amylase is the more common enzyme assayed when pancreatitis is suspected. Although total serum lipase activity increases in renal failure, the magnitude of increase is seldom sufficient to cause a diagnostic problem. The serum lipase activity increased to varying magnitudes in dogs treated with prednisone or dexamethasone. In some, it increased sufficiently to possibly confuse a diagnosis of pancreatitis.

5.9 α-Amylase

α-Amylases (EC 3.2.1.1) are calcium-dependent metalloenzymes that randomly catalyze the hydrolysis of complex carbohydrates, e.g., glycogen, at α1-4 linkages. The products of this action are maltose and limit dextrins. The enzyme is a Ca^{2+} metalloenzyme which requires one of a number of activator ions such as Cl^- or Br^-. Therefore, EDTA and citrated plasma are inappropriate for an amylase

There are at least seven α-amylase isoenzymes in human serum and four in canine serum but none in canine salivary glands. Canine serum amylases can be separated into two peaks by get filtration. One peak was glucoamylase, the enzyme formerly called maltase and thought to be the major enzyme interfering with the saccharogenic serum amylase assay. All canine tissues except salivary glands and liver have α-amylase. Pancreas and duodenum have more than 6 times the activity

found in other tissues. The lack of tissue specificity of the isoenzyme profile and extremely high total pancreas-specific activity suggest that only total serum amylase activity is of diagnostic significance.

In humans, serum amylases have a molecular weight of 40,000-50,000, which are small enough to be cleared by the kidney into urine where they can be used as a diagnostic aid for pancreatitis. The same is not true in canines. In the dog, serum amylases are cleared by the kidney but cannot be assayed in urine. The reason for this difference may be that canine serum amylases are present in an inactive form in the urine matrix. Evidence of renal clearance of serum amylases is observed in dogs with renal failure and hyperamylasemia. This presents a diagnostic problem because many dogs with acute pancreatitis have prerenal azotemia. The problem can generally be resolved by observing the specific gravity of the urine, which in renal failure is low but in prerenal azotemia is high.

The serum amylase activity can be assayed by either a saccharogenic or an amyloclastic procedure. The saccharogenic procedure measures the rate of appearance of reducing substances from the starch substrate. The reducing substrates are maltose and glucose. Dog plasma contains glucoamylase as well as amylase, and thus the saccharogenic procedure incorporates the activities of both enzymes. Scrum amylase in dogs and other animals is considerably greater than that in human serum. The amount of glucoamylase in dog plasma varies and is not correlated with either amylase or pancreatic inflammation. Therefore, the saccharogenic procedure is not acceptable for assay of canine serum amylase.

Amyloclastic methods for serum amylase activity 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. Use of synthetic substrates in amyloclastic assays is currently the more

accepted method of assaying for canine serum amylases. The procedure uses a dye linked to a starch substrate. The dye is freed as the starch is hydrolyzed, and the rate of appearance of the soluble dye complex 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 more classic procedures and has given comparable results.

The commercial amyloclastic or dye-substrate procedures are designed for use in human sera. Normal total canine serum amylase activity is 5 to 6 times greater than that in human serum, and the kit's substrate concentration may limit the reaction when used for canine serum. These kits can be modified for use with dog serum by reducing the amount of serum or diluting it with saline.

5.10 γ-Glutamyltransferase

γ-Glutamyltransferase (GGT; 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 common acceptor, and synthetic substrates are often used. The number of isoforms of GGT, if any, is unclear. Current information suggests that there is a large amount of posttranslational modification of a single apoenzyme. GGT occurs as membrane-associated aggregates which makes it difficult to obtain not only its molecular weight but also a consistent specific activity in tissues. Dilution of saline-suspended kidney homogenates with either urine or serum results in an increase in activity, which is thought to be the result of disassociation of aggregates of enzyme and exposure of

additional active sites. Estimates of its molecular weight range from 90,000 to 350.000.

The physiological function of GGT is unknown, but it is speculated that the enzyme is associated with glutathione metabolism. With the exception of muscle, all cells have some cytosolic and membrane GGT activity. The greatest amount of cellular GGT is in the brush borders of renal and bile duct epithelia. Serum GGT is derived from liver and found in high molecular weight membrane fragments which also contain AP, 5'-nucleotidase, L-leccyl-β-naphthy-lamindase, and nucleotide pyrophosphatase, enzymes which have a common cell location as well. Renal GGT is the source of urinary GGT and. with urinary AP. is a marker of active nephrotoxicity. Basal serum GGT is very low in dogs and cats as compared to ruminants, but it is a sensitive and specific marker of cholestasis and bile duct proliferation in all species examined. Cow, ewe, and doe colostrum but not mare colostrum contains a large amount of GGT which, with colostral antibodies, is readily transferred across the neonate's intestinal wall into the plasma.

Serum GGT is stable at room temperature for 8 hours and frozen for as long as 40 weeks in humans. In horses, serum stored at -30 °C for 4 weeks lost 50 % of its activity but lost appreciably less at − 20 °C. Human and equine urinary GGT is relativity stable at room temperature and when refrigerated, but it is inactivated to varying degrees as the result of cryoconcentration of urea and enzyme at the time of freezing. Inactivation in urine can be prevented by dialysis or the addition of albumin or dimethyl sulfoxide to the urine before freezing. Urinary GGT concentration is relativity constant, when expressed as enzyme per gram of urinary creatinine, on a day-to-day basis in normal dogs and horses. The various statements concerning the stability of GGT in urine and serum require that investigators validate their individual methods of storage of samples in studies using GGT activity.

Cholestatic disorders of all species examined result in increased serum GGT activity. Prednisolone treatment of dogs induces hepatic GGT, as well as AP, and results in an increase in serum GGT values. The induction of GGT offers a unique feature in the selection and breeding of tolerance to naturally occurring toxins. The unique specificity and sensitivity of GGT as a marker of bile duct epithelial proliferation are most evident in the rapid rise in serum GGT, with no increase in the serum SDH. after a natural exposure of sheep to the hepatotoxin sporidesmin of facial eczema. Sporidesmin is a mitogen of bile ductal epithelium, and the increase in activity appears to be the result of an increased amount of bile duct epithelium. The absence of a rise in serum GGT in sheep fed on pastures containing sporidesmin is used to select strains of sheep inherently resistant to sporidesmin.

5.11 Trypsin

Trypsins (EC 3.4.21.4) are serine proteases which hydrolyze the peptide bonds formed by lysine or arginine with other amino acids they are secreted by the pancreas as the zymogen trypsinogen, which is converted to trypsin by intestinal enterokinase or trypsin itself. The trypsin isoenzymes, 1 and II, can be immunologically separated and have differing physical characteristics. They are activated by Ca^{2+} and Mg^{2+}. Natural irreversible inhibitors occur in soybeans, lima beans, and egg whites. Pancreatic trypsinogen and trypsin occur in plasma, where trypsin is inhibited by α_1-antitrypsin and α_2-macroglobulin. Trypsins have molecular weights between 23.000 and 26.000. They and their zymogens in plasma are stable at room temperature for several days and frozen for at least 5 years.

Fecal trypsin’s proteolytic activity is determined in feces and intestinal contents as a measure of pancreatic sufficiency. Their value is limited, however, by the destruction of trypsin as it passes through the intestinal tract and by interference with bacterial proteases.

Plasma trypsin, trypsinogen, and antitrypsin-trypsin complex occur in plasma in proportion to pancreatic mass. Immunoassays for trypsinlike material measure all three forms. This is a relativity new procedure, and an assay has been established for the dog. Serum trypsinlike immunoreactivity (TLI) decreases in canine exocrine pancreatic insufficiency. In humans, serum TLI increases in conditions which also result in increases in serum amylase, including renal failure.

5.12 Glutathione Peroxidase

Glutathione peroxidases (GPx; EC 1.11.1.9) are metalloenzymes containing four atoms of selenium per molecule of enzyme. They catalyze the oxidation of reduced glutathione by peroxide to form water and oxidized glutathione. Their assays are coupled to glutathione reductase to produce a detectable chromogen. There are two isoenzymes with glutathione peroxidase activity. One is the Se-dependent seleno-GPx and the other a Se-independent glutathione transferase. The seteno-GPx can use either hydrogen peroxide and organic hydroperoxides as substrate, but the glutathione transferase uses organic hydroperoxides and has low activity with hydrogen peroxide. Therefore, assays of tissues other than blood which use organic hydroperoxides, such as cumene hydroperoxide, should not be expected to demonstrate a correlation between blood and Se concentration.

Because of the high concentration of selenium in GPxs, there is good direct correlation between the amount of red blood cell GPx activity and the selenium concentration of other organs. The enzyme has good stability in whole red blood cells. There are, however, discrepancies concerning its stability in frozen hemolysates. The lack of prolonged stability has made quality control difficult for the assay of this enzyme. Expression of the amount of GPx activity has been based on blood volume. However, GPx is an intracellular enzyme, and the red blood cell

concentrations of blood can vary by as much as 50 % in some species. A more appropriate measure is U/mg hemoglobin or U/dl red cells.

Test questions for Chapter V

1. Localization of enzymes in different organelles of the cell.
2. Features of the structure and division of enzymes in animal body.
3. Application of enzymes for diagnostics and treatment. Give examples.
4. Application of enzymes as analytical reagent.
5. International SI units of enzyme activity determination.
6. The role of isoenzymes in the diagnosis of kidney disease.
7. The role of isoenzymes in the diagnosis of cardiovascular system.
8. Clinical and diagnostic value of isoenzymes in the pathology of the liver.
9. Clinical and diagnostic value of determination of: α-amylase, transaminases, alaninaminopeptidaze, alkaline and acid phosphatase activity.

APPENDIX I
SI UNITS

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

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 PREFICS

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 D

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

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) Karmen unit (KU) Reitman-Frankel unit (RFU) (0.001 OD[b]/min × ml)	0.48	U/liter
Glutamic pyruvic transaminase (GPT), (ALT)	Sigma-Frankel unit Karmen unit Wroblewski-LaDue unit (WLU) Reitman-Frankel unit (0.001 OD/min × ml)	0.48	U/liter
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 CORECTION 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 STORADGE 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.

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