PRINCIPLES OF APPLIED CLINICAL CHEMISTRY

CHEMICAL BACKGROUND AND MEDICAL APPLICATIONS

Volume 3

Plasma Proteins in Nutrition and Transport

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Volume 1 • *Maintenance of Fluid and Electrolyte Balance*

Volume 2 • *The Erythrocyte: Chemical Composition and Metabolism*

Volume 3 • *Plasma Proteins in Nutrition and Transport*

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Volume 3

Plasma Proteins in Nutrition and Transport

SAMUEL NATELSON, Ph.D.
University of Tennessee
Department of Environmental Practice
College of Veterinary Medicine
Knoxville, Tennessee

and

ETHAN A. NATELSON, M.D.
University of Texas Medical School and
St. Joseph Hospital
Houston, Texas

PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

Natelson, Samuel.
Principles of applied clinical chemistry.

Includes bibliographical references and indexes.
CONTENTS: v. 1. Maintenance of fluid and electrolytc balance. – v. 2. The erythrocyte. – v. 3. Plasma proteins in nutrition and transport.
1. Chemistry, Clinical. 2. Metabolism. 3. Metabolism, Disorders of. I. Natelson, Ethan Allen, 1942– joint author. II. Title. [DNLM: 1. Chemistry, Clinical. QY90 N273p]
RB27.N38 616.07'56 75-4798
ISBN 978-1-4684-3586-3 ISBN 978-1-4684-3584-9 (eBook)
DOI 10.1007/978-1-4684-3584-9

A Division of Plenum Publishing Corporation
227 West 17th Street, New York, N.Y. 10011

Preface

This book, the third volume in the series, continues to explore the application of chemistry to our understanding of the functioning of the human in health and disease.

It is the objective of the authors to continue to present, in this and subsequent volumes, the biochemical aspects of clinical chemistry, and to indicate how this knowledge applies to the diagnosis of disease and the treatment of the patient. For this purpose, the literature is reviewed carefully and the findings of the different study groups are integrated, to present an overall view of the present status of the various fields. The text is written with the intent to serve in the training of clinical chemists, clinical pathologists, and medical students in clinical biochemistry. It is also intended to serve as a reference text for the practicing physician who desires a more rational approach to the use of the clinical chemistry laboratory, as an aid in understanding (1) the chemical changes in disease and (2) the logical use of the laboratory data in the treatment of the patient.

This volume is concerned with the plasma proteins and their significance in normal human metabolism. The immunoglobulins are not included in this study since, along with complement and clotting factors, they form an integrated system concerned with defense against invading organisms. These will be discussed in Volume 4 of this series.

A historical introduction (Chapter 1) is followed by a general presentation of the composition and properties of proteins (Chapter 2). The classification of the plasma proteins and the various techniques used in protein fractionation are reviewed briefly in Chapter 3.

This discussion of the individual plasma proteins follows their natural grouping, in accordance with their function. Major stress is placed on the normal function of the protein in health and disease. For example, albumin is discussed as a source of nutrition and means of transport of important elements and metabolic intermediates to the tissues (Chapter 4). This is the pattern set for the discussion of the other plasma proteins (Chapters 5 and 6). In accordance with this approach, transferrin, ceruloplasmin, haptoglobin, and hemopexin are considered related in their transport of iron, copper, and the iron-bearing porphyrins (Chapters 7–10). This leads naturally to a discussion of iron and copper metabolism.

In a similar manner the function of prealbumin as a carrier of thyroxin- and retinol-binding proteins is discussed along with thyroxin-binding globulin (Chapters 11 and 12). Gc globulin and vitamin D transport are linked in Chapter 13. The steroid-hormone-binding globulins (Chapter 14) and transcobalamins (Chapter 15) are discussed, stressing their significance in disease.

The glycoproteins and the lipoproteins (Chapters 5 and 6) are each assigned about one-sixth of the total pages in the text. The glycoproteins' mode of formation is indicated, and diseases that result due to errors in their catabolism, such as fucosidosis and mannosidosis, are explained. The chemistry of the proteoglycans is related to certain genetic defects in their metabolism, such as the mucopolysaccharide storage diseases (e.g., Hurler's and related syndromes).

The structure, function, and metabolism of the lipoproteins are explored in detail in Chapter 6, correlated with a discussion of the genetic and acquired lipoproteinemias, which in turn are related to the common disease states associated with the lipemias.

For the student of clinical chemistry, certain technical details are explained in the Appendix, which include an explanation of the convention in naming the steroids and an elaboration of the biosynthesis of cholesterol and the structures of the phospholipids. A brief explanation of the significance of the parameters used in the text in characterizing the proteins is also given.

The text is illustrated with a substantial number of halftones and drawings, demonstrating the principles being explored. By far, most of these are from the authors' own experiences. The more than 2000 references cited have been carefully chosen so that they represent reviews of various subjects. The text stands supported by these references

and selected readings, which are recommended to the reader for additional study.

Although it is not the intention of this volume to dwell on the analytical procedures used for assaying the various proteins, a brief summary of the principles behind the techniques used is given at the end of each chapter, to be used as an aid in evaluating the laboratory findings in the various diseases.

The authors wish to thank Dr. George Mozes of the library staff of Michael Reese Hospital and Ethel D. Natelson for researching the reference material. Of special value were the efforts of Dr. Stephen E. Natelson of the Fort Sanders Presbyterian Hospital of Knoxville in proofreading the manuscript and contributing to its medical significance.

Most of this text was written while the senior author was still at the Michael Reese hospital, and the authors are grateful to the library and secretarial staff at Michael Reese Hospital for their valuable assistance.

Samuel Natelson
Ethan Allen Natelson

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Sec. I

Nature of the Plasma Proteins

1

Introduction

1.1 *HISTORICAL BACKGROUND*

The nature of *protoplasm* was a subject of interest to all biological investigators after the discovery of the microscope. In the sense it was used in the 17th and 18th centuries, it referred to the viscous sol in the cell in which the organelles were suspended. The similarity of this material to the whites of eggs was noted early and some, such as Fourcroy at the end of the 18th century, referred to protoplasm as *albuminoid substances* or literally, substances resembling the white of an egg. Berzelius is quoted as saying that serum albumin is the same as egg white and that the leukocytes are only globular forms of albumin.

Liebig and Mulder, in 1840, analyzed the whites of eggs and the protoplasm of animal tissues and plasma and came to the conclusion that these albuminoid substances, which they now called *proteins*, had a constant composition as far as carbon, nitrogen, and sulfur was concerned.[1] According to Denis, all proteins were therefore the same, and fibrin was only coagulated albumin.[2,3]

The invention of a precise method for nitrogen assay by Dumas showed that all of these albuminoid substances were not the same, and by 1860 classification of these variously called *albumins* (*Eiweissstoffe* in German), *proteins*, or *proteids* by different authors, took note of these differences.

In 1841 and subsequently,[4,5] it was noted that dilution of serum with water, after acidification with dilute acid, resulted in the precipitation of some of the protein. Since acidification of milk precipitated casein from which cheese was made, the precipitate (globulins) was

called *casein*, whereas the dissolved protein was called *albumin*. This was probably the first advance in the fractionation of plasma proteins.

Denis, in studying the solvent effect of salts on blood proteins, noted that if higher concentrations of $MgSO_4$ or Na_2SO_4 were used, some of the proteins would precipitate. He introduced the term *globulin* for these precipitates.[6] He stated that he derived the term from Berzelius, who observed the precipitation by salts of the *globules* of blood. Berzelius had noted that hemoglobin crystallizes when laked blood is dried. He called these crystals *haematocrystalline* and referred to them as *globulins* since they were derived from one of the globules (erythrocytes) of blood.

The confusion at that point in time is obvious today since these experimenters did not distinguish, in many cases, between blood, plasma, and serum, and some even considered the cells to be suspended proteins. Schmidt in 1862 then applied the term *globulin* to the proteins precipitated from acidified plasma, and pointed out that this precipitate (which included fibrinogen and fibrin) was distinct from the albumin in solution.[7] The systematic fractionation of proteins had now begun on a rational basis by many experimenters, including the physiological chemists (clinical biochemists) Hoppe-Seyler and Weyl.[8,9]

What appeared to be a minor improvement in protein fractionation turned out to be a major advance, when $MgSO_4$ and Na_2SO_4 were replaced by $(NH_4)_2SO_4$ by Mehu.[10] Hofmeister then succeeded in 1889 in crystallizing egg albumin after precipitation with half-saturated $(NH_4)_2SO_4$.[11] Five years later, Gürber crystallized serum albumin by a similar procedure.[12] *The significance of these achievements can be appreciated only if one bears in mind that at that time no compound was considered homogeneous or isolated unless it was crystallized.* Thus it was clearly shown that albumin was different from the globulins and that proteins were organic compounds which could be purified and isolated. This concept has persisted until the present time. Notably, Sumner and Northrup were awarded the Nobel prize in chemistry for obtaining the proteins (enzymes) urease, pepsin, and trypsin in crystalline form.[13,14]

The development of antitoxins by Behring and subsequently Roux at the end of the 19th century, motivated the interest of clinical biochemists to study means for isolating these materials from the antiserums being developed. It was quickly shown that the antibodies were in the globulin fraction.[15,16] Further studies showed that, after the antibodies were precipitated by 0.28–0.33% saturated ammonium

sulfate more globulin remained in solution. This could be precipitated by 0.34–0.46% saturated ammonium sulfate. The fraction precipitated by the lower concentration of ammonium sulfate was then called the *normal globulins* or *euglobulins*, whereas that requiring higher concentrations of salt was called *false globulins* or *pseudoglobulins*. By electrophoresis it can be shown that the euglobulins are mainly γ globulins ($\sim 70\%$), the pseudoglobulins being a mixture of the others.[17]

At the turn of the century, interest developed in the applications of electrochemistry to protein solutions, originating in the studies on ion mobilities or *transference numbers* by the colloid chemists. As early as 1892, it had been shown that hemoglobin would move in an electrical field.[18] Subsequently it was shown that albumin and globulins were *amphoteric* and would move to the anode or cathode, depending upon the acidity or basicity of the solution.[19] The concept of an *isoelectric point* at which they did not move at all was then developed. The transport of proteins in an electrical field was referred to as *cataphoresis* at that time. Michaelis, using Sorensen's newly developed concept of pH, studied this problem in detail, and introduced the term *electrophoresis* for the process.[20] His value of 4.7 for the isoelectric point (pI) of albumin is still accepted.

Attempts to unravel the composition of proteins by hydrolysis were begun as early as 1875. However, the preparations used were impure and the methods of hydrolysis with $Ba(OH)_2$ destroyed much of the protein.[21] Drechsler and his student Emil Fischer systematically studied the composition of proteins by acid hydrolysis and developed the concept of the peptide bond and that proteins are polymers of amino acids. By 1900 the concept of a polypeptide had been firmly established.[22] With Abderhalden, Fischer showed that the enzymes pepsin and trypsin also hydrolyzed proteins to amino acids.[23]

It was found early that proteins insoluble in water were soluble in weak salt solutions. It was also shown that if the concentration of salts was increased, the proteins would precipitate. Hofmeister showed that salts of a certain anion (e.g., NaCl, Ca_2Cl, $AlCl_3$) could be arranged in a fixed cation order, reflecting their ability to affect the solubility of proteins. Others extended this idea to different anions (NaCl, Na_2SO_4, Na_3PO_4). These lists are known as Hofmeister's series.[24,25] It was shown that the efficiency in precipitating proteins went up dramatically with the degree of hydration and charge on the anion or cation.[25] (See Table 1.1.)

TABLE 1.1

Relative Effectiveness in Protein Precipitation of Various Ions, Lyotropic[a] or Hofmeister Series[b]

Cations:	Ba	Sr	Ca	Mg
Anions:	NO_3	Br	Cl	SO_4

[a] This term is used because the degree to which the ions are hydrated correlates with the effectiveness in protein precipitation.

[b] Efficiency in protein precipitation increasing from left to right.

Independent of Hofmeister's studies, Lewis and Randall developed the concept of ionic strength (μ) which is equal to half the sum of the concentration of the ions, c, multiplied by their valence squared (V^2). Thus, $\mu = \frac{1}{2} \sum cV^2$.[26] The ionic strength of a 0.1 molar solution of Na_2SO_4 would be 0.1 + 0.1 + 0.4 divided by 2, or 0.3. This partly explains the dramatic increase in effect on the solubility of proteins by an increase in valence as shown in the Hofmeister series.

The application of these ideas on the effect of salts on the solubility of proteins was used by Cohn in fractionating the plasma proteins during World War II on a large scale.[27,28] His technique of varying the concentration of alcohol and salts in fractionating serum proteins lends itself to large-scale production, and variations of his procedure are still utilized at this writing.

In order to obtain the physical and chemical characteristics of a protein, a homogeneous preparation is necessary and some criteria for purity needed to be developed. For this purpose, attempts were made to arrive at the molecular weights of the proteins. The molecular weights of proteins had been studied by various techniques since the middle of the 19th century. At first, the proteins were analyzed for sulfur, and assuming one sulfur atom per molecule, a maximum value could be estimated. This was followed by methods for measuring a colligative property such as freezing point, vapor pressure, and especially osmotic pressure. Using the latter techniques, estimates of 70,000 were obtained for serum albumin.[29] This value is close to the actual value of about 66,300.

A major advance in the measurement of the molecular weights and

homogeneity of the proteins was the introduction of the ultracentrifuge by Svedberg. The power of this tool was demonstrated when it was shown that hemoglobin was homogeneous, with a molecular weight of 66,800, showing that each molecule had four iron atoms. Thus the method of equivalents and the ultracentrifuge complemented each other.[30] Today, almost every protein isolated is studied in the ultracentrifuge and its sedimentation constant in Svedberg units is determined as a necessary part of its identification.

Alongside of the development of the ultracentrifuge, the mobility studies of proteins in an electrical field continued, resulting in the development of the Tiselius apparatus for fractionating serum proteins.[31] The discovery that this procedure could be used for the diagnosis of certain conditions such as multiple myelomas hastened the development of an improved apparatus and the use of media other than the cumbersome electrophoresis in the absence of a supporting medium. The use of paper, cellulose acetate, starch, agar, acrylamide gel, and a host of other substances and varied techniques followed. These will be discussed later in this text.

An important contribution of Tiselius was the replacement of the terms *euglobulin* and *pseudoglobulin fractions* with the *electrophoretically obtained fractions* and the development of the concept of α, β, and γ globulins. He also showed that the antibodies were present mainly in the γ globulins.[31]

It was pointed out above that the development of the antitoxins and serum therapy created interest in the development of methods of protein fractionation. The development of column chromatography in the last 25 years, using various media which separate proteins by charge or molecular weight, coupled with chemical and immunochemical techniques for identification, have made protein isolation in pure forms almost a routine procedure. The substances most commonly used are Sephadex, cellulose, acrylamide, and their derivatives. Salt precipitation followed by dialysis is still widely used for preliminary purification.

The introduction of automatic amino acid analyzers, sequencers, and synthesizing apparatus for polypeptides and proteins has dramatically shortened the time between the discovery of a polypeptide or protein and elucidation of its structure. Examination of the quaternary structure of the proteins by X-ray diffraction is a major subject of study today.

Simultaneously with the development of protein chemistry, development in the understanding of the mechanism of immunization was achieved. Landsteiner showed that by condensing closely related aniline derivatives (e.g., *o*-, *m*-, and *p*-toludines) with albumins, and immunizing animals with this material, antibodies were formed. With this method closely related proteins could be distinguished. He called the attached group *haptens*. Thus he established immunochemical techniques as a method of identifying a particular chemical by binding it to a protein.[32] Quantitative procedures were then developed by Heidelberger and Kabat for measuring the amount of antigen or antibody by precipitin techniques.[33] These procedures were the basis for the radial immunoassays used in the routine clinical laboratory today.

An outgrowth of the application of immunochemical techniques to the study of protein chemistry was the development of the various procedures for immunoelectrophoretic identification of proteins.[34] Variations of the original technique of Grabar and Williams are used for identification of the nature of an apparent aberrant fraction obtained by electrophoresis of serum. In addition, this technique has been applied to the study of clotting defects, such as Factor 8 deficiency. Numerous other applications to the identification of proteins and polypeptides have been used for the diagnosis of disease by routine clinical analysis.

Immunochemistry played a major role in the resolution of the differences between the γ globulins IgM, IgG, IgA, and IgD. These techniques led to the discovery of IgE and its relationship to the *reagin* of allergy.

It is now apparent that Berzelius was not far afield when, in the 1820s, he related the "globules" of blood to the plasma proteins. In order to discuss the clinical biochemistry of the immunoglobulins we need to consider the leukocytes from which they originate. To discuss the chemistry of the proteins making up the clotting factors, the platelet must be considered. We cannot present a clear picture of the function of the various complement components without invoking the macrophage, lymphocyte, and neutrophil. The chemistry of IgE is meaningless without considering the chemistry of the basophil and mast cell. For this reason, the chemistry of the immunoglobulins will be considered in a subsequent volume together with the proteins of the complement system, clotting factors, components of the inflammation reaction, and the chemistry of the leukocytes.

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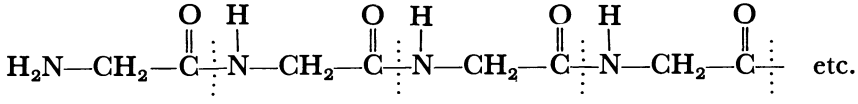
2

Protein Composition and Properties

2.1 *CHEMICAL COMPOSITION OF PROTEINS*

Before discussing the plasma proteins, it is advantageous to first review the fundamental chemistry of the proteins in general.

Proteins, on hydrolysis with acid, alkali, or enzymes, can be broken down to amino acids of which glycine is a simple example. The amino acids contain an amino group and a carboxyl group. Since carboxyl groups split out water with amino groups to form amides, glycine can be made to polymerize. In this case we would have the following linear polymer (the dotted lines separate the glycyl radicals):



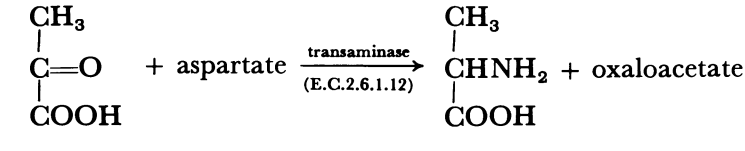
Polyglycine

Notice that an amino group appears finally at one end and a carboxyl group at the other. The carboxyl group may occur in the form of the amide.

If the chain consists of only two amino acids it is called a dipeptide. If three or more amino acids are linked, then we have a tri-, tetra-, etc. peptide. If the chain becomes longer, we call it a polypeptide. Chains with molecular weights above 10,000 are called proteins, although the line of demarcation between polypeptide and protein is ill defined and depends mainly on the point of view of the experimenter.

There are 18 amino acids commonly found in proteins, and two more that also occur not infrequently. Of these, eight are called the

amino acids *essential* for humans. An essential amino acid is one which cannot be synthesized in the human. Actually, it is the chain which cannot be synthesized. For example, alanine is not an essential amino acid because it can be synthesized from pyruvic acid derived from glucose.



Pyruvic Acid Alanine

The keto group can be converted to an amino group by transamination from aspartic acid. Similarly, aspartic and glutamic acids can be synthesized in the body from oxaloacetate and ketoglutaric acids, respectively, both derived from the citric acid cycle. However, *there is no endogenous source in the body for phenylpyruvic acid.* Therefore phenylalanine is an essential amino acid. If phenylpyruvic acid is supplied, however, from outside sources, then phenylalanine can be made. If phenylalanine is available, it can be oxidized to tyrosine and thus *tyrosine is considered to be a partially essential amino acid since it is essential in phenylalanine deficiency.*

Proteins comprise a mixed polymer, containing all of the common amino acids plus ammonia, and can reach molecular weights in excess of 60,000. These chains can then be cross-linked, often by —S—S— bonding, as in the immunoglobulins, and by other types of linkages, so that molecular weights in excess of several million can be obtained.

Since the amino acids have the amino group in the α position, they can be considered as derivatives of glycine. A typical polypeptide is shown in Figure 2.1.

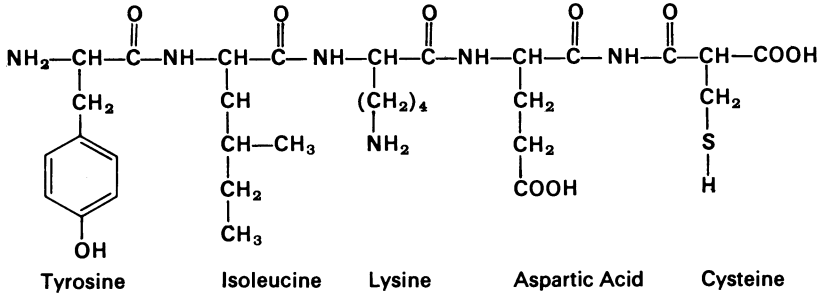


FIGURE 2.1 A typical polypeptide.

Note that the chain (called the *backbone* of the polypeptide) is the same as for polyglycine, and only the groups attached vary. Thus we can have strongly acidic groups (aspartic acid), weakly acidic groups (tyrosine, cysteine), neutral groups (isoleucine), and basic groups (lysine) attached to the polyglycine chain.

A protein comprising a preponderance of basic groups would be basic in nature, such as the histones. On the other hand, a preponderance of acidic groups would result in an acidic protein. The composition of specific proteins and their structure may be obtained from the numerous monographs on this subject (see Section 2.5).

The structures of the most common amino acids are given in Table 2.1. They are arranged to show the group which would be suspended from the polyglycine backbone of the protein. In polypeptides, the first three letters of the amino acid are used as an abbreviation. When, with dicarboxylic acids, the free carboxyl group occurs as an amide, "n" is substituted for the last letter (e.g., Asn for the amide of Asp).

Amphoteric Nature of The Amino Acids

Since the amino acids have carboxyl and amino groups, they are both basic and acidic. Thus they are *amphoteric* having both positive and negative charges. The amino acid can thus exist in three forms depending upon the pH of the solution:

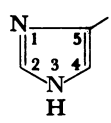
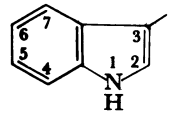
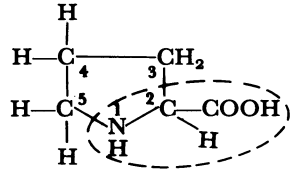
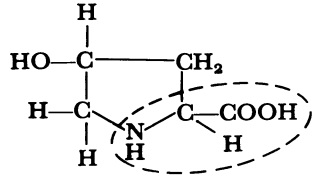
$H_2N—CH_2—COO^-$	$H_3\overset{+}{N}—CH_2—COO^-$	$H_3\overset{+}{N}—CH_2—COOH$
Negative Charge (alkaline solution)	Zwitterion (isoelectric point)	Positive Charge (acid solution)

The word *zwitter* in German means hybrid, which explains the term zwitterion for the form with both positive and negative charges. Actually, careful measurements have shown that even at the isoelectric point most of the amino acids are in the acidic or basic form, and very little exists as the zwitterion. At the isoelectric point some amino acid can also exist uncharged.

The importance of the amphoteric nature of the amino acids is due to the fact that they can react with either anions or cations. When polymerized, the proteins formed are also amphoteric, and move to the

TABLE 2.1

Twenty Most Common Amino Acids Obtained on Hydrolysis of Proteins or Polypeptides, Separating, for Emphasis, the Groups Which Make Up the Backbone of the Protein Chain (Polyglycine) from the Groups Hanging from the Chain

Amino Acid	Formula
Glycine	$H - - - - CH(NH_2) \cdot COOH$
Alanine	$CH_3 - - - - CH(NH_2) \cdot COOH$
Valine[a]	$(CH_3)_2CH - - - - CH(NH_2) \cdot COOH$
Leucine[a]	$(CH_3)_2—CH_2—CH - - - - CH(NH_2) \cdot COOH$
Isoleucine[a]	$CH_3—CH_2—CH(CH_3) - - - - CH(NH_2) \cdot COOH$
Cysteine	$HS—CH_2 - - - - CH(NH_2) \cdot COOH$
Methionine[a]	$CH_3S—CH_2—CH_2 - - - - CH(NH_2) \cdot COOH$
Serine	$HO—CH_2 - - - - CH(NH_2) \cdot COOH$
Threonine[a]	$CH_3—CH(OH) - - - - CH(NH_2) \cdot COOH$
Aspartic acid	$HOOC—CH_2 - - - - CH(NH_2) \cdot COOH$
Glutamic acid	$HOOC—CH_2—CH_2 - - - - CH(NH_2) \cdot COOH$
Arginine[b]	$H_2N—C(:NH)NH(CH_2)_3 - - - - CH(NH_2) \cdot COOH$
Lysine[a]	$H_2N(CH_2)_4 - - - - CH(NH_2) \cdot COOH$
Hydroxylysine[d]	$H_2N—CH_2—CHOH—CH_2—CH_2 - - - - CH(NH_2) \cdot COOH$
Phenylalanine[a]	$C_6H_5—CH_2 - - - - CH(NH_2) \cdot COOH$
Tyrosine[c]	$HO—C_6H_5—CH_2 - - - - CH(NH_2) \cdot COOH$
Histidine[b]	
Tryptophane[a]	
Proline[d]	
Hydroxyproline[d]	

footnotes opposite

anode or cathode on electrophoresis, depending upon the pH of the buffer.

2.2 *PRIMARY, SECONDARY, TERTIARY, AND QUATERNARY STRUCTURE*

As can be seen from Table 2.1, the polypeptide chain is attached to radicals of varying charge ranging from plus to minus. For this reason, as the chain gets longer and can curl up, it does so because of the attraction of one portion of the chain for another. In this way the molecule can be curled to form a helix or a random coil, ending finally in a globular form. This can be seen from Figure 2.2 which also appeared in Volume 2, p. 386, of this series.

Polypeptide chains can be bound to other similar or different chains. A most common link occurs by oxidation of cysteine residues on the chains to form —S—S— linkages between the chains. In this case the chains can be separated by reduction, to regenerate the —SH radical. For example, in the immunoglobulins four chains are bound by —S—S— linkages.

In the case of hemoglobin, four polypeptide chains are bound to each other by amino acid radicals capable of forming hydrogen bonds, semipolar bonds, by electrostatic attraction or salt formation. Examples of such bonding of different chains are lysine and aspartate, tyrosine and lysine, cysteine and lysine, threonine and proline, and others. For this reason, dissociation of the chains of hemoglobin (Hb) takes place in concentrated solutions of urea, as shown by molecular weight estimation of Hb with the ultracentrifuge (see Section 9.1.3, Volume 2).

The *primary* structure of a protein refers to the sequence of the amino acids in the polypeptide chains starting from the amino end of the chain.

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[a] The eight "essential" amino acids in the human.

[b] In rats, synthesis of arginine and histidine are too slow and therefore they are essential to this animal.

[c] Can be made from phenylalanine in the human. In phenylketonuria this cannot be done and tyrosine becomes an essential amino acid.

[d] Hydroxylysine and hydroxyproline are present in collagen. In *Marfan's syndrome*, a collagen disease, they appear in the urine. In proline and hydroxyproline the portion of the molecule incorporated in the chain is encircled with a dotted line.

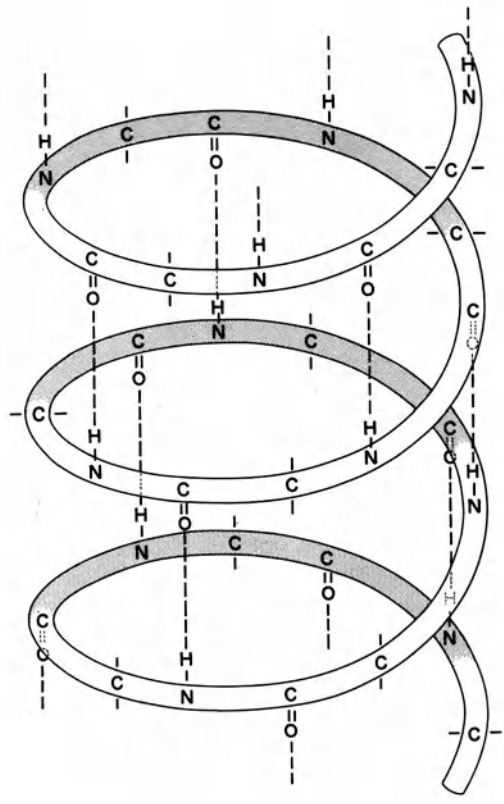

FIGURE 2.2 Schematic representation of the helix structure of the globins. Hydrogen bonding maintains the rigid structure. There are 3.6 amino acids per turn, on the average, hydrogen bonding occurring between an NH group and the C=O group four residues back.

The *secondary* structure refers to the organization into stable helices and connecting amino acids.

The *tertiary* structure is the manner in which the polypeptide chains are folded into a three-dimensional figure. The tertiary structure of myoglobin is shown in Figure 9.2, Volume 2 of this series.

The *quaternary* structure, refers to the manner in which the polypeptide chains, comprising the protein, are linked to each other. In the case of hemoglobin, this refers to two pairs linked together for a total of four polypeptides. On the other hand, albumin and myoglobin are

single polypeptide chains, and there would not be a quaternary structure to consider.

2.3 *PROTEINS IN SOLUTION*

Technically speaking, a protein does not dissolve in water. Instead, it imbibes water and swells until the boundaries of the protein molecule are the same as the boundaries of the solvent. For this reason, when reconstituting lyophilized serum, it is necessary to agitate the lyophilized serum with water for at least one half hour in order to complete the swelling process.

A solution is a molecular dispersion of one phase in another. It has certain characteristic properties. For example, it does not show the *Tyndall effect.* That is, when illuminated at right angles to the viewer, it does not show a cloudy beam of light. A protein "solution" does not meet these criteria.

Dispersions of one phase in another, such as proteins in water, emulsions, stable clay suspensions, etc., are referred to as *colloidal suspensoids or emulsoids.* The term *suspensoid* is reserved by some colloid chemists to refer to colloids whose charge only maintains them in suspension by electrostatic repulsion. Gold sols, such as those obtained when performing the colloidal gold test with spinal fluid, are typical suspensoids. Where the particle remains suspended in a solvent because of its charge, plus a water envelope surrounding each particle, the sol is referred to as an *emulsoid.* The terms suspensoid and emulsoid should not be confused with the terms suspension or emulsion. A suspension is a dispersion, such as sand in water which settles out. The word *emulsion* is reserved for stable dispersions of oils in water. Notice the use of the general term *sol* for colloidal dispersions of all types.

A protein "solution" would be classified as a colloidal emulsoid because the molecules are charged, and because each molecule holds an envelope of water around it. This whole system is called a *micelle.* This term was first introduced by Nägeli. Each molecule of protein remains suspended not only by repulsion of like changes but also by random bombardment of water molecules. When dehydrated, several protein molecules share one envelope of water. This phenomenon is called *coacervation.* Finally, if the charge of the protein is neutralized, it

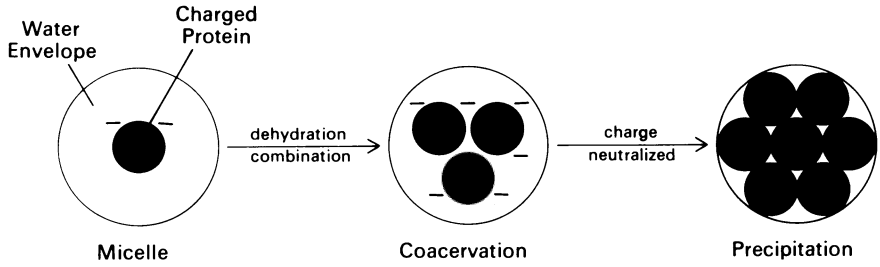

FIGURE 2.3 Schematic representation of the nature of the protein micelle and the steps in its precipitation.

will precipitate (coagulate). This is illustrated schematically in Figure 2.3.

Since the amino acids which comprise the protein are amphoteric, the protein is amphoteric and has $—COO^-$ and $—NH_3^+$ groups on its surface. Thus it can adsorb anions and cations depending upon the pH of the solution. At alkaline pH, the negative charges predominate and the proteins move to the anode. At acid pH, they move to the cathode. At some intermediate pH they do not move in either direction, since the charges are balanced. This is the *isoelectric point* of the protein (pI), and is one of its characteristics. Since this point depends upon the salts adsorbed, it is often not a true indicator of the acidic or basic nature of the protein. It is often noted that, as the concentration of buffer is decreased, the isoelectric point shifts toward a pH at which the charges on the protein, because of the —COOH and $—NH_2$ radicals on the surface, are balanced. This may even result in a change of direction of movement in an electrophoretic field at different buffer concentrations. *For this reason, the mobility of a protein in an electrophoretic field needs to be defined, not only in terms of the nature of the buffer, since different anions are adsorbed differently, but also in terms of the buffer concentration.*

In precipitating proteins with Bloor's reagent (alcohol–ether, 3:1), the water envelope is removed, causing the plasma proteins to precipitate. On addition of water they will dissolve instantly since they still retain their charge. If a salt solution such as ammonium sulfate is added, the charge is neutralized causing precipitation. In most cases, when precipitating plasma proteins for the purpose of their isolation, both the charge and water envelope are removed. If the protein is to be removed as an impurity, it is often *denatured* so as to ensure its elimination.

If a protein is heated so that chemical reactions can take place between groups of adjacent molecules, then the protein will harden and separate as in boiled milk. The protein is then said to be *denatured.* The reactions which take place include the oxidation of —SH groups to form —S—S— groups, the splitting out of water from a carboxyl group, as from aspartic acid, and an amino group, as from lysine, to form an amide ($RCOOH + H_2NR \rightarrow R \cdot C{:}O \cdot NH \cdot R$), ester formation, and condensation with the phenolic groups of tyrosine. Thus the polypeptide chains are bound tightly together to form a plastic material like hair or nails. The denatured protein then becomes insoluble in water and precipitates. *Denaturation* also refers to an irreversible unfolding of a polypeptide chain or partial fragmentation of the chain so that its activity or characteristics are changed. Dissociation of intact polypeptide chains which make up a protein will often result in denaturation. Catalase is an example of a protein which loses activity when the intact chains are separated. Recombination of the chains restores catalase activity.

Protein precipitants commonly used are trichloroacetic, perchloric, tungstic acid, phosphotungstic, phosphomolybdic, and metaphosphoric acids. Bases such as zinc and cadmium hydroxide are also employed. Heavy metal salts such as those of uranium and lead have served in the past. In each of the above cases, the proteins are at least partially denatured. Where the proteins are to be recovered, milder agents such as Na_2SO_4, $(NH_4)_2SO_4$, dilute alcohol, or acetone solutions are useful.

For the proteins to be acted upon by the enzymes of the intestinal tract, it is best that they be precipitated and denatured. Removal of the water envelope and charge is necessary so that the protein can bind to the hydrolytic enzyme. Thus the hydrochloric acid in the stomach denatures proteins so that they can be attacked by pepsin, which further denatures them for processing in the small intestine.

Proteins in Gels

If the molecule of the protein is long and rigid, it can be attracted to another molecule, aligning oppositely charged groups against each other. Hydrogen bonding is also present since the hydrogen of an —OH group is attracted also to a —C=O group, for example. In this way the molecules will line up, all oriented in the same direction. Gelatin

is a protein of this nature. For this reason gelatin sols will form a gel readily.

If a gelatin gel is vigorously shaken, so that the molecules become disoriented, then the gel liquefies. On standing, it will gel again. This phenomenon is called *thixotropy*. With the molecules all oriented in the same direction, they are gradually pulled together, squeezing out the liquid which was held in the gel by capillary forces. Thus the gel will contract, get harder and water will float above it. This phenomenon is called *syneresis*; these phenomena are illustrated in Figure 2.4.

The events shown in Figure 2.4 take place when plasma clots. The fibrinogen molecules are ellipsoids which move at random through the solution. When acted upon by thrombin, short polypeptides (fibrinopeptides) are split from the molecules, causing the ellipsoids to adhere to each other, generating the long fibrin molecules. Clotting then proceeds as in Figure 2.4. *In fact, clotting is gel formation.* As the clot ages, syneresis takes place, squeezing out the serum from the clot. This can be seen with clotted blood, as it stands. This process is aided by an *actomyosin system* (*thrombosthenin*), derived from the platelets, which contracts and

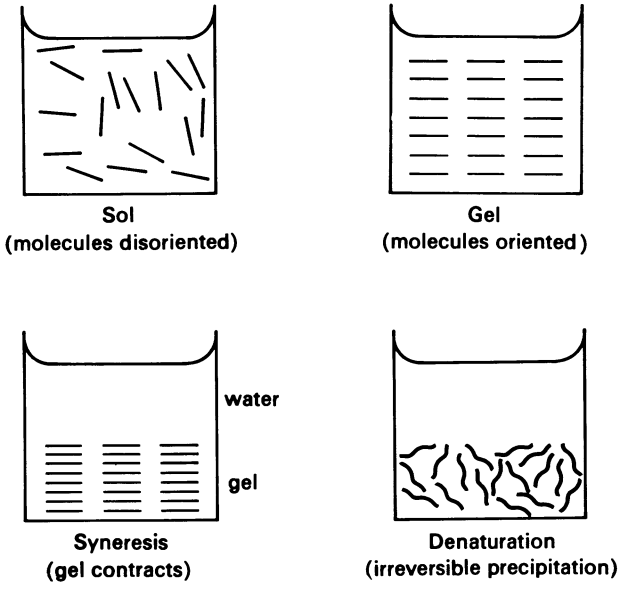

FIGURE 2.4 Schematic representation of the changes in molecular orientation which result in gel formation and denaturation.

accelerates the pulling together of the fibrinogen molecules, resulting in *syneresis*.

In the following pages the plasma proteins are discussed in greater detail with emphasis being placed on their properties and function in health and disease.

2.4 *RECAPITULATION*

Polypeptides are composed of amino acids, polymerized so as to form a polyglycine chain to which are attached a variety of acidic, basic, and neutral radicals. By means of hydrogen bonding and secondary valences, the polypeptides fold into a spiral, or form a random coil. Polypeptide chains may bind to one another by secondary valence bonds, or —S—S— linkages, to form high-molecular-weight proteins. Some proteins, like albumin and myoglobin, comprise a single chain.

Protein solutions in water are colloidal sols. The proteins are held in suspension by their charge and a water envelope. This system is then called a micelle (microcell). On the surface of the proteins are a plurality of both positive and negative charges which react with both positive and negative ions in the solution in which they are dissolved. Thus their properties in the electrical field (electrophoresis) are partially a function of adsorbed ions and thus depend on the nature of the buffer and pH at which the experiment is carried out. For this reason, it is not surprising to find that resolution in a borate buffer, for example, is different from that in a phosphate or barbiturate buffer.

Proteins whose molecules are elongated will tend to be aligned in solution to form a rigid gel. The molecules then are drawn together, squeezing out the water which now floats on top of the gel. Clotting of blood is an example of this phenomenon.

When proteins are caused to unfold, or when polypeptide chains, which comprise a protein molecule, are caused to dissociate from each other, the protein loses its characteristics and is said to be *denatured*. If heated or acted upon by enzymes so as to cause cross-linking of the polypeptide chains, the proteins become irreversibly denatured. Examples of such proteins are hair and fingernails.

Precipitation with solvents such as alcohols, acetone, and ether removes the water envelope from the protein molecules and they precipitate but are usually not denatured. Precipitation with salts such

as $(NH_4)_2SO_4$, NaCl, or $MgSO_4$ will also often not denature the proteins. However, precipitation with trichloroacetic and tungstic acid or heavy metals often will denature the proteins.

The *primary structure* of a protein describes the sequence of amino acids in the polypeptides which comprise the protein. The *secondary structure* describes the way the polypeptides are folded or coiled. The *tertiary structure* describes the three-dimensional figure formed from the proteins. The *quaternary structure* describes the way the polypeptides comprising the protein are bound to each other.

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3

Plasma Protein Survey

When blood is drawn, allowed to clot, and centrifuged, the supernatant obtained is called *serum*. Serum, in addition to other components, contains all the proteins of the blood other than those involved in the clotting mechanism (mainly fibrinogen) and the cell proteins. If the blood is mixed with an anticoagulant, such as heparin, or a calcium complexing agent, such as sodium fluoride or ethylene diamine tetraacetate, and then centrifuged, the supernatant obtained is called *plasma* and contains all the proteins of serum, plus *fibrinogen* and other *clotting factors*. The proteins of the plasma may be divided into three main categories: *albumin*, the *clotting factors* (including *fibrinogen*), and the *globulins*. The globulins include the proteins of the complement system, the immunoglobulins, and numerous other proteins with special functions (Table 3.1).

In discussing the plasma proteins, the numerous proteins and polypeptides which are carried by the blood and are not manufactured by the *liver* or *reticuloendothelial* system will not be included at this time. For this reason, the enzymes, many of which are discussed in Volume 2 of this series, and the polypeptide hormones are not considered in this section. The components of the clotting and complement systems are also not included, except for C3 which occurs in high concentration in the serum, since they will be considered in a succeeding volume. Antithrombin III is considered part of the clotting system and is not included. Fragments of proteins, such as the heavy and light chains of the immunoglobulins are also not considered here. Proteins which appear in significant quantities only in disease states are also not listed in Table 3.1. This includes carcinoembryonic antigen (CEA) and α-fetoprotein.

TABLE 3.1

Major Plasma Proteins[a]

Protein	Molecular weight	Concentration, mg/100 ml	Electrophoretic[b] mobility	Biological function
Prealbumins				
Thyroxine binding (TBPA)	55,000	10–40	7.6	Thyroxine transport
Retinol binding (RBP)	21,000	3–6		Vitamin A transport
Albumin	66,300	3500–5500	5.92	Maintain osmotic pressure, transport of bilirubin, free fatty acids, anions, and cations, cell nutrition
α_1 Globulins				
α_1 Acid glycoprotein (α_1S)	40,000	55–140	5.7	Unknown, inactivates progesterone
α_1 Antitrypsin (α_1AT)	54,000	200–400	5.42	Antiserine-type protease
α_1 Glycoprotein (9.5S, α_1M)	308,000	3–8	α_1	Unknown
α_1 Glycoprotein B (α_1B)	50,000	15–30	α_1	Unknown
α_1 Glycoprotein T (α_1T)	60,000	5–12	α_1	Unknown, tryptophane poor
α_1 Antichymotrypsin (α_1X)	68,000	30–60	α_1	Chymotrypsin inhibitor
α_1 Lipoproteins, high density (HDL)	28,000	254–387	α_1	Lipid transport
α_2 Globulins				
G_c Globulin (Gc)	51,000	40–70	α_2	Vitamin D transport
Ceruloplasmin (Cp)	134,000	15–60	4.6	Copper transport, peroxidase activity
α_2 Glycoprotein, histidine rich (HRG)	58,000	5–15	α_2	Unknown
Zn-α_2-glycoprotein (Znα_2)	41,000	2–15	4.2	Unknown, binds Zn^{2+}
α_2 HS-glycoprotein (α_2HS)	49,000	40–85	4.2	Unknown, binds Ba^{2+}
α_2 Macroglobulin (α_2M)	725,000	150–420	4.2	Inhibitor of thrombin, trypsin and pepsin
Transcortin (TC)	49,500	< 7	α_2	Cortisol transport

Haptoglobins (Hp)				
Type 1-1	100,000	100–220	4.1	Binds hemoglobin, prevents loss of iron
Type 2-1	200,000	160–300	α_2	
Type 2-2	400,000	120–260	α_2	
α_2 Lipoproteins (VLDL)	250,000	150–230	Pre-β	Lipid transport
Thyroxine-binding protein (TBG)	58,000	1–2	α_2	Thyroxine transport
β Globulins				
Hemopexin (Hpx)	57,000	50–100	3.1	Binds heme
Transferrin (Tf)	76,500	200–320	3.1	Iron transport
β Lipoproteins (LDL)	250,000	280–440	3.1	Lipid transport
C4 Complement component (C4)	206,000	40–80	β_1	Complement system
β_2 Microglobulin ($\beta_2\mu$)	11,818	Trace	β_2	Common portion of the HL-A transplantation antigen
β_2 Glycoprotein I (β_2 I)	40,000	15–30	1.6	Unknown
β_2 Glycoprotein II (GGG)	63,000	12–30	β_2	C3 activator (activates properidin)
β_2 Glycoprotein III (β_2 III)	35,000	5–15	β_2	Unknown
C-Reactive protein (CRP)	118,000	1	β_2	Opsonin, motivates phagocytosis in inflammatory disease
C3 Complement component (C3)	180,000	55–180	β_2	Complement system
Fibrinogen (ϕ, Fib.)	341,000	200–600	2.1	Blood clotting
γ Globulins				
Immunoglobulin M (IgM)	950,000	60–250	2.1	Antibodies, early response
Immunoglobulin E (IgE)	190,000	0.06	2.1	Reagin of the allergy system
Immunoglobulin A (IgA)	160,000	90–450	2.1	Tissue antibodies
Immunoglobulin D (IgD)	160,000	15	1.9	Cell surface and plasma antibodies
Immunoglobulin G (IgG)	160,000	800–1800	1.2	Antibodies, long range

[a] Does not include clotting factor, complement factors, or enzymes except fibrinogen, C3 and C4 of complement which occur in substantial concentration.

[b] Tiselius moving boundary electrophoresis in Tiselius units ($cm^2\ V^{-1}\ sec^{-1} \times 10^5$, at 0°C, pH 8.6, and ionic strength 0.15).

It is apparent, that any listing of the "plasma proteins," as distinct from other proteins found in the plasma, for reasons of practicality, needs to be arbitrary. Others (e.g., certain enzymes) have been described in Volume 2. Some (e.g., the immunoglobulins, clotting factors, and complement) will be presented in Volume 4 of this series. Other proteins, with special functions, will be discussed in subsequent volumes.

3.1 *CLASSIFICATION*

The plasma proteins are listed in Table 3.1. All have a significant half-life of at least a week and are considered constant components of plasma. Concentration, as can be seen from Table 3.1, ranges from 0.06 mg/100 ml for IgE to 4000 mg/100 ml for albumin. Molecular weights range from 12,000 for β_2 microglobulin to almost one million for IgM.

In the classification of the plasma proteins, there is no scheme which serves the purpose perfectly. We have arbitrarily, for convenience divided the proteins into a few major classes. First, the proteins which primarily carry nutrients to the cells. These include albumin and the lipoproteins. Second, the transport proteins which carry other substances, whose prime function is to supply some chemical, necessary for normal cell metabolism, in small quantities. This includes transferrin, ceruloplasmin, the proteins which transport vitamins A and D, transcobalamines, and those concerned with steroid hormone transport, as examples. The term glycoprotein applies to almost all proteins, and these are discussed as an example of the mechanism of protein synthesis. We classify the proteins of the complement, clotting, and immunoglobulins as proteins concerned with defense against disease.

The *lipoproteins*, HDL, LDL, and VLDL share the functions of carrying various lipids and are a natural group for discussion. These, along with albumin, are in fact *transport proteins* since they serve to bind and transport metabolities. Other transport proteins listed in Table 3.1, which function to transport a specific biologically important compound, are proteins carrying hydrocortisone (TC, transcortin), thyroxin (TBPA, thyroxin binding prealbumin, and TBG, thyroxin binding globulin), vitamin A (RBP, retinol binding protein), copper (Cp, ceruloplasmin), iron (Tf, transferrin), heme (Hpx, hemopexin), and hemoglobin (Hp, haptoglobin). To these should be added vitamin B_{12} (transcobalamins),

discussed in Volume 2 of this series, and Gc globulins, which transport vitamin D.

Almost all proteins carry small amounts of bound carbohydrate in their structure. However, others carry substantial quantities of carbohydrate of from at least 10 to as high as 40%. These precipitate from serum when diluted 160-fold by 95% ethanol (e.g., 25 μl serum plus 4 ml ethanol). The precipitate is then referred to as containing the *glycoproteins*. The glycoproteins, which carry in excess of 30% of carbohydrate, are often referred to as *mucoproteins*. These will not precipitate with 7% perchloric acid, but will precipitate with 5% phosphotungstic acid.

Among the glycoproteins are the haptoglobins, hemopexin, and at least ten others, in the prealbumin, α_1, and α_2 electrophoretic fractions. Those in higher concentration are listed in Table 3.1. In this volume, the glycoproteins other than those with special function, such as the complement and clotting factors, which are all glycoproteins, will be discussed as a group.

Other proteins, which do not lend themselves to ready classification in the above groups will be discussed separately at appropriate places in the text. For example, proteolytic enzyme inhibitors, such as the antitrypsin and α_2 macroglobulin will be discussed in Volume 4 of this series together with the clotting factors and antithrombin.

3.2 *PROTEIN FRACTIONATION*

Fractionation of the plasma proteins has been studied by four techniques: salt and alcohol or acetone fractionation, ultracentrifugation, electrophoresis on various media, and column chromatography with Sephadex or DEAE-cellulose. Of special use, where prothrombin and related substances are concerned, is the technique of concentration by total adsorption on a substance such as magnesium hydroxide, barium sulfate, barium stearate, tantalum oxide, or Celite followed by elution with a strong salt solution such as sodium citrate.

Identification and assay of the plasma protein, present in lower concentration, is now commonly done by adsorption to an antibody which itself is adsorbed to or combined with an insoluble support. If the antibody is attached to an inert support in a column, the protein may

be separated in one pass. It can then be washed and eluted, producing a homogeneous preparation. This is known as *affinity chromatography.*

3.2.1 *The Serum Electrophoretic Pattern*

Studies of the movement of ions and colloids in electrical fields in solution go back to the middle of the 19th century. At that time, Hittorf studied the relative rates of appearance of anions and cations at the electrodes.[1] The term *transference numbers* was applied as a measure of the relative mobility of the ions.[2] The transference number of an ion is the fraction obtained by dividing the current carried by that ion by the total current carried by both ions.

The equipment used for measuring ionic mobilities by the moving boundary method resembled very closely the apparatus used by Tiselius[6] for the same purpose with serum (Figure 3.1).

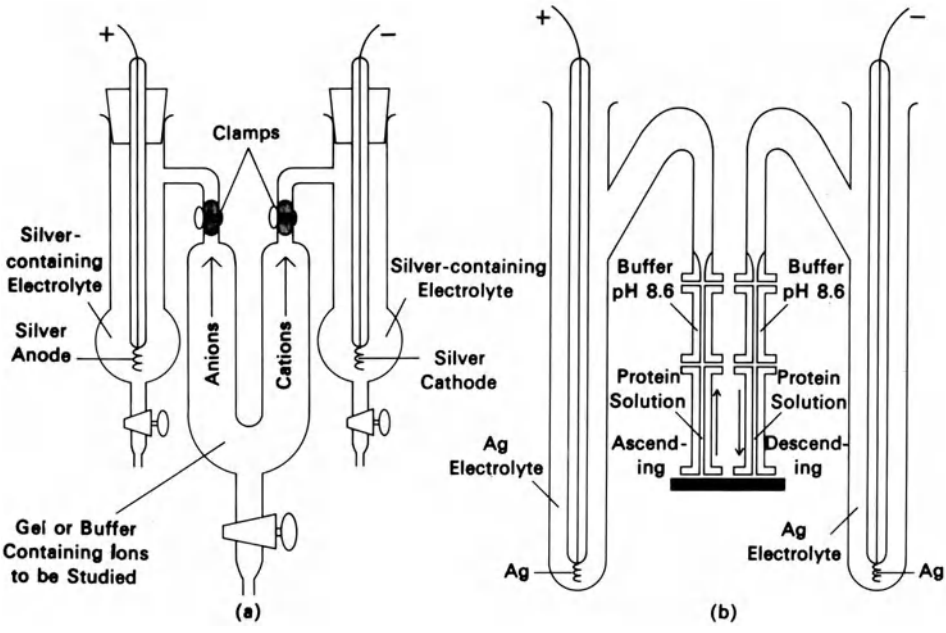

FIGURE 3.1 Comparison of the (a) Findlay apparatus (1909) for measuring transference numbers with the (b) Tiselius apparatus (1934).

3.2.2 *Moving-Boundary Electrophoresis*

Cataphoresis is the study of glues, gelatin, hemoglobin, and other colloids by the moving boundary procedure. Cataphoresis is considered a special form of *electroendosmosis.*[3] In electroendosmosis, a voltage applied in the presence of an electrolyte at both ends of a glass capillary causes the liquid to move towards the cathode. The water is positively charged relative to the wall of the capillary. In this procedure, the liquid moves and the solid phase (glass) remains stationary, whereas in cataphoresis, the solid phase (e.g., glue, gelatin, gum mastic) moves and the liquid is stationary (see Figure 3.2). Thus the two procedures were considered equivalent.[4]

Michaelis introduced the term *electrophoresis*, since the proteins being studied moved to the anode or cathode, depending upon the pH of the solution. The movement of proteins in an electrical field continued to be the subject of study, and in the late 1920s and early 1930s Longsworth, at the Rockefeller Institute in New York, developed an improved apparatus for this type of study.[5] Tiselius, coming from Svedberg's laboratory, joined this group and further improved the Longsworth apparatus.[6] He finally published his findings on the electrophoresis of the plasma proteins by the moving boundary method in 1937.[6a] Figure 3.1 compares the Tiselius apparatus with the Findlay apparatus used for measuring transference numbers.[7] In the Findlay apparatus, applying the two clamps permits removal of the contents of the compartment containing the material being studied. In the Tiselius apparatus, this is accomplished by sliding the section desired over to one side. The flanges on the Lucite section keep the liquid from flowing. The temperature in this apparatus is kept at 4°C.

With the Tiselius apparatus, the location of the proteins is measured by a change in refractive index using a cylindrical lens (Schlieren lens). Alternatively, the solution can be scanned with ultraviolet light. The importance of this contribution was that it clearly divided the proteins into four groups, albumin and the α, β, and γ globulins. Subsequently the protein trailing the albumin fraction was resolved and labeled the α_1 fraction.[8] Thus we have α_1 and α_2 fractions. Figure 3.3 shows the electrophoretic pattern as obtained with the moving boundary procedure. Electrophoretic mobility is still measured by the moving boundary method and is given in units of $cm^2\ V^{-1}\ sec^{-1}$ at 0°C at

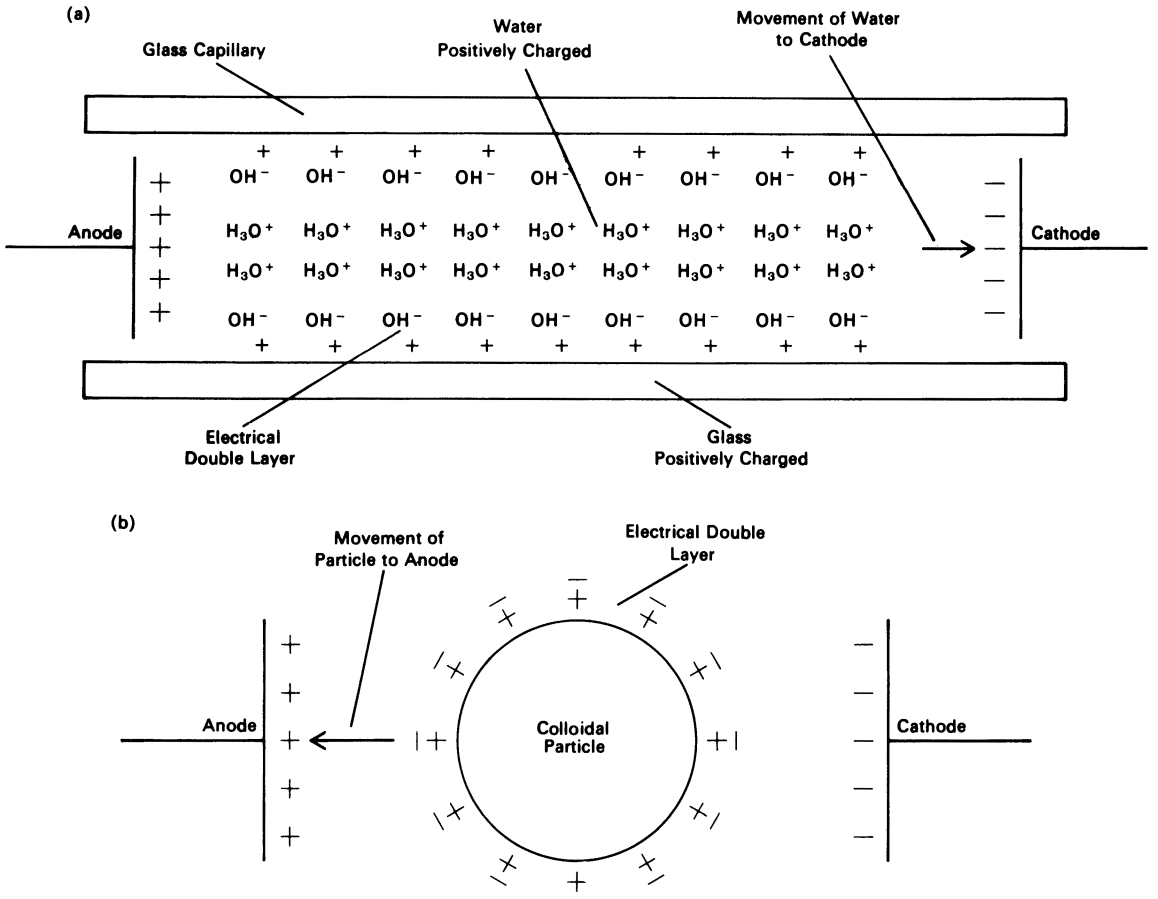

FIGURE 3.2 Comparison of electroendosmosis (a) and cataphoresis (b). In electroendosmosis, water moves relative to solid phase. In cataphoresis, solid phase moves relative to water.

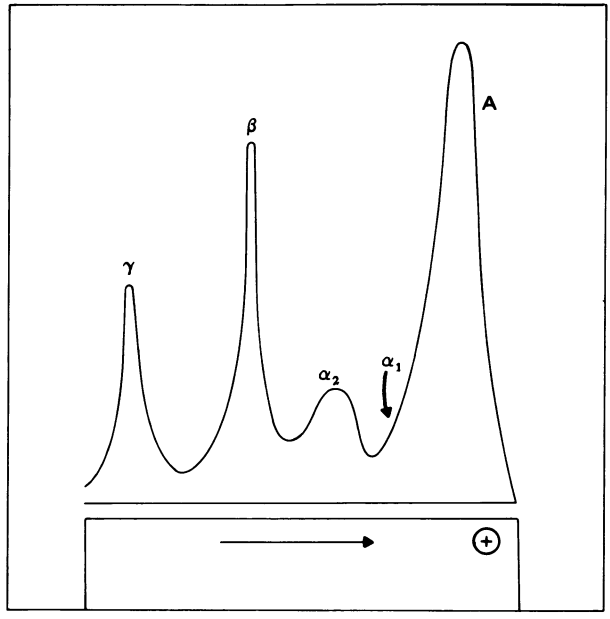

FIGURE 3.3 Tracing from a Schlieren diagram of the electrophoretic pattern obtained in the descending limb of the Tiselius apparatus.

pH 8.6. Since this unit is very small, it is usually multiplied by 10^5 and referred to as the Tiselius unit.

3.2.3 *Zone Electrophoresis*

Zone electrophoresis refers to the separation of the proteins into bands on a supporting medium, as distinct from the moving boundary procedure.

By the 1920s electrophoresis was being studied on supporting media such as paper. Attention was paid especially to the movement of ions in an electrical field by several investigators.[9] Electrophoresis on paper was extended soon to plasma and serum by Tiselius and others.[10] *The discovery that paper electrophoresis could be used in the routine diagnosis of disease, especially multiple myeloma, lipoid nephrosis, agammaglobulinemia and others, caused its wide adoption by clinical laboratories in the 1950s.*[11] Figure 3.4 illustrates the electrophoretic patterns obtained with paper in the normal adult and in various diseases.

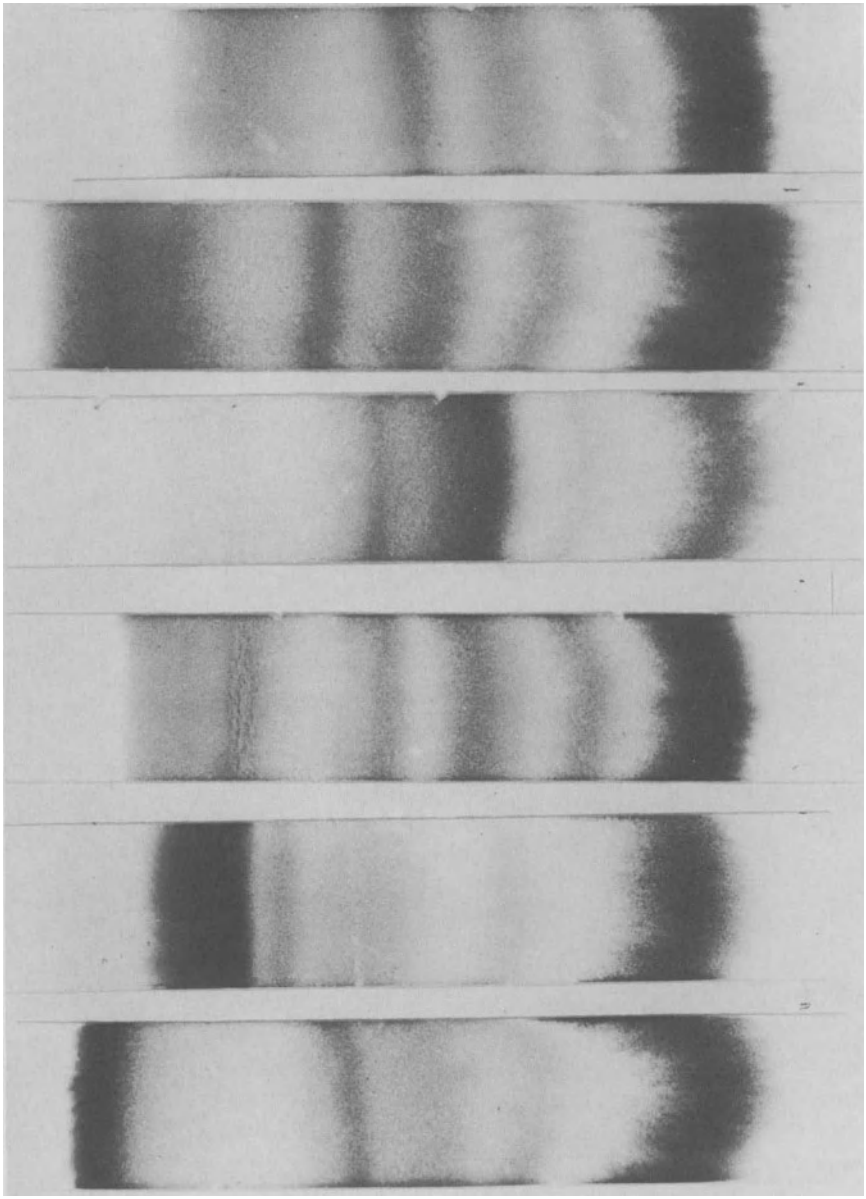

FIGURE 3.4 Paper electrophoretic patterns obtained with normal adult serum and in certain disease states (Whatman, 3mm chromatography strips, pH 8.6, barbiturate buffer, 0.05 M, 16 hr, 5 V/cm). In descending order from top to bottom: normal adult, viral hepatitis (polyclonal), lipoid nephrosis, leukemia myelogenous, myeloma, (IgM), myeloma (IgG).

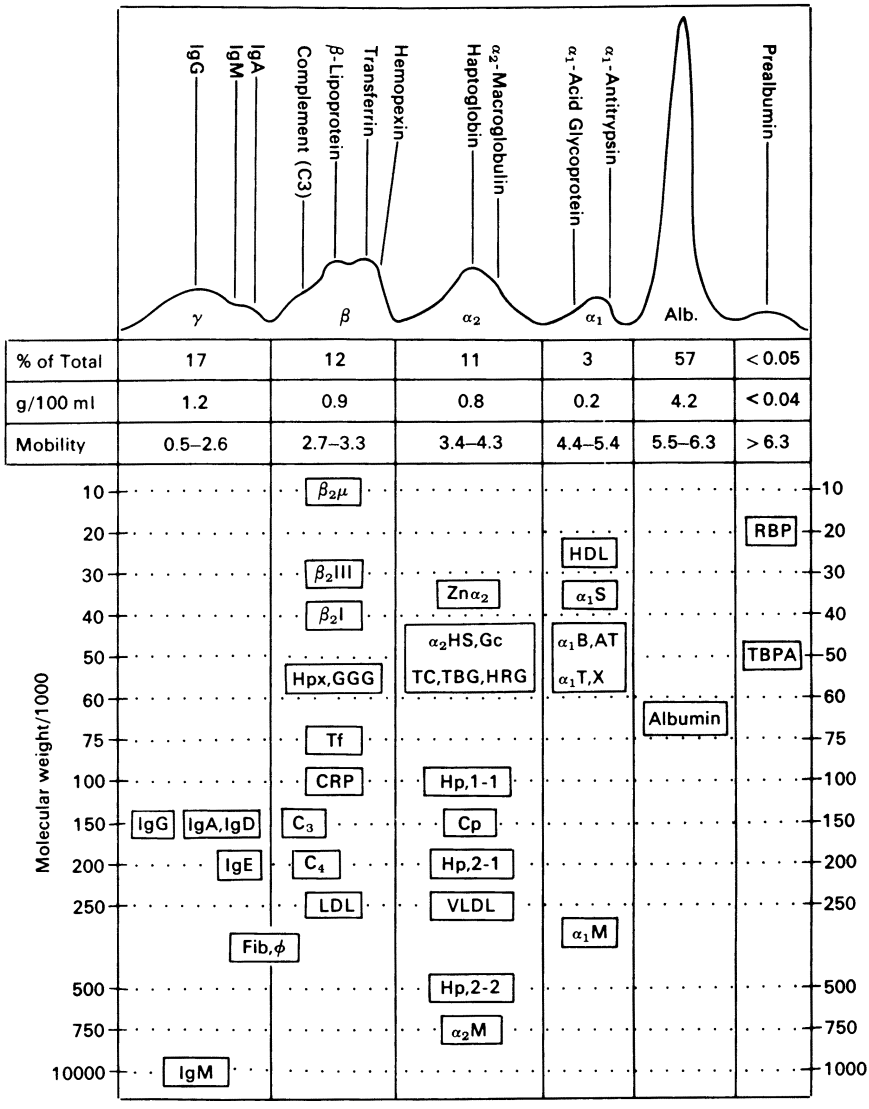

FIGURE 3.5 Serum electrophoretic pattern obtained with cellulose acetate at pH 8.6. The various proteins of Table 3.1 are placed according to their position in the electrophoretic field and their approximate molecular weight. A logarithmic plot of molecular weights is used. Mobility is that observed with the Tiselius moving-boundary system.

The problem with paper was that it required 16 hrs before adequate resolution of the fractions was obtained. The hydroxyl groups on the cellulose, and probably some carboxyl groups, caused trailing and reduced the resolution of the patterns. To reduce trailing and speed up the process the paper was acetylated. In other words, *cellulose acetate* was prepared. This dissolves in organic solvents and can then be spread on a glass surface to form a thin layer of porous cellulose acetate, on evaporation. Somewhat improved resolution was obtained with this medium, which is now widely used in the clinical laboratory.[12] In Figure 3.5 an electrophoretic tracing obtained with cellulose acetate is arranged so as to locate the different proteins listed in Table 3.1.

Separation of all major plasma proteins was not achieved with cellulose acetate and other media were explored so as to obtain greater resolution. *Starch block* was used successfully in resolving hemoglobins, and led to the isolation of hemoglobin A_2.[13]

Fine resolution was not achieved until the introduction of *starch gel*.[14] This medium led to the separation of numerous globulin fractions particularly haptoglobins, hemopexin, and Gc globulins. However, it is a difficult procedure, requiring a cold room and special care in preparing the starch gel. A marked improvement was obtained when *acrylamide gel* was used resulting in the resolution of the plasma proteins to at least 20 to 25 fractions.[15] Figure 3.6 illustrates electrophoresis with acrylamide gel. At least 15 bands are noted with this system.

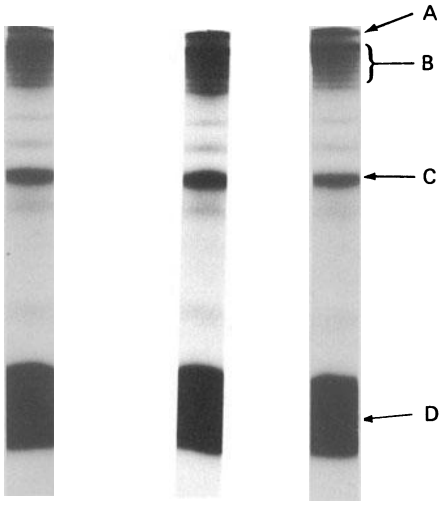

FIGURE 3.6 Resolution obtained with acrylamide gel. A are the gamma globulins, B contains the haptoglobins. C is the transferrin band, and D is albumin.

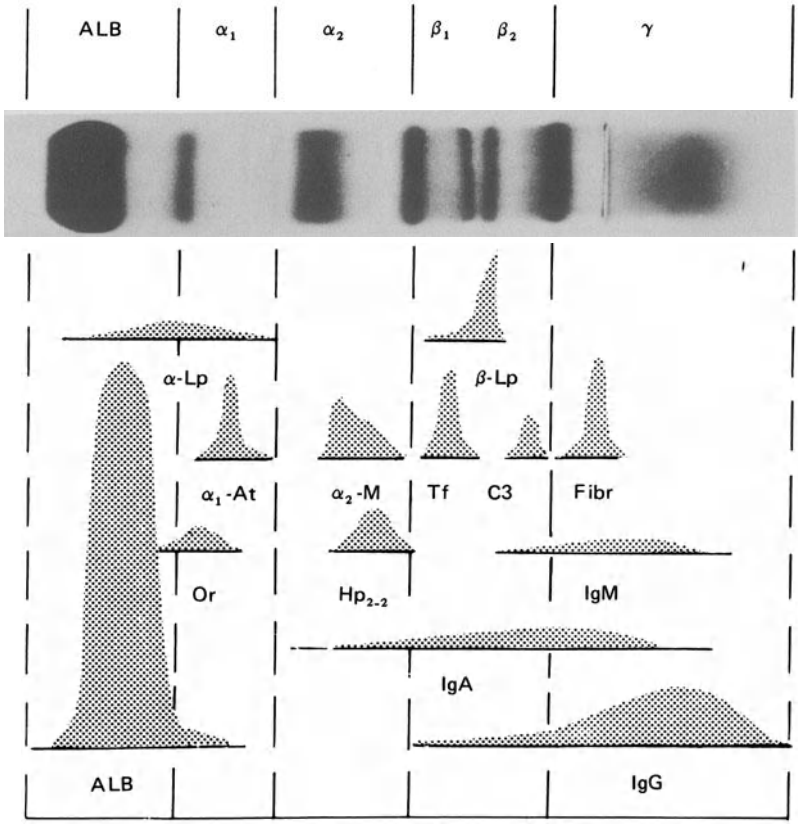

FIGURE 3.7a Electrophoresis of normal human serum on agar. Courtesy of C. B. Laurell, Dept. of Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden.

From the end of the 19th century, the movement in an electrical field of colloids, cells, proteins like hemoglobin, and various gums were studied, in a film of *agar*, on a microscopic slide. With the microscope, the Tyndall effect was employed to observe the moving boundary.[9]

Improvements in the quality of agar available resulted in highly purified *agarose*. This was developed originally for immunochemical precipitin studies. It proved to be an excellent medium for the resolution of the lipoproteins by electrophoresis.[16] It also permitted the development of various ways of combining the resolution of proteins by electrophoresis and the specificity of antibodies for specific proteins. Figure

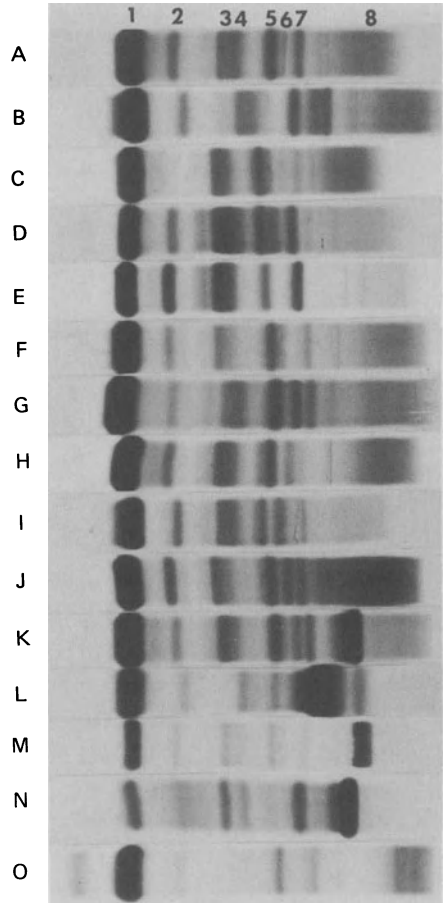

FIGURE 3.7b Electrophoretic patterns on purified agarose, comparing the normal with pathological sera. The numbers represent the major fractions: 1, albumin; 2, alpha-1-antitrypsin; 3, alpha-2-macroglobulin; 4, haptoglobin; 5, transferrin; 6, beta-lipoprotein; 7, C3 complement; and 8, gamma globulin. The letters represent: A, normal; B, hyperbilirubinemia with bilirubin-conjugated albumin; C, alpha-1-antitrypsin deficiency; D, nephrotic syndrome; E, protein losing enteropathy; F, haptoglobin deficiency due to hemolytic anemia; G, hyperbetalipoproteinemia; H, deficiency of C3 complement; I, hypogammaglobulinemia; J, hepatic cirrhosis; K, IgG multiple myeloma; L, IgA multiple myeloma; M, IgM multiple myeloma; N, Bence-Jones proteinuria with two monoclonal zones in gamma region; O, cerebrospinal fluid in multiple sclerosis. (Courtesy of Tsieh Sun, *Ann. Clin. Lab. Sci.* **8**:221 (1978).)

3.7a is an electrophoretic pattern of human plasma on purified agar. Note the resolution of the β lipoprotein from C3 and transferrin. Figure 3.7b shows the resolution of the plasma proteins with purified agar, in various disease states.

In *immunoelectrophoresis*, the proteins are resolved by electrophoresis. Antibodies placed in a trough alongside the electrophoretic path diffuse into the gel and delineate the various proteins.[17] An example of this type of pattern is shown in Figure 3.8.

For quantitative purposes, antibody to a specific protein may be distributed in the gel, and the serum subjected to electrophoresis. The

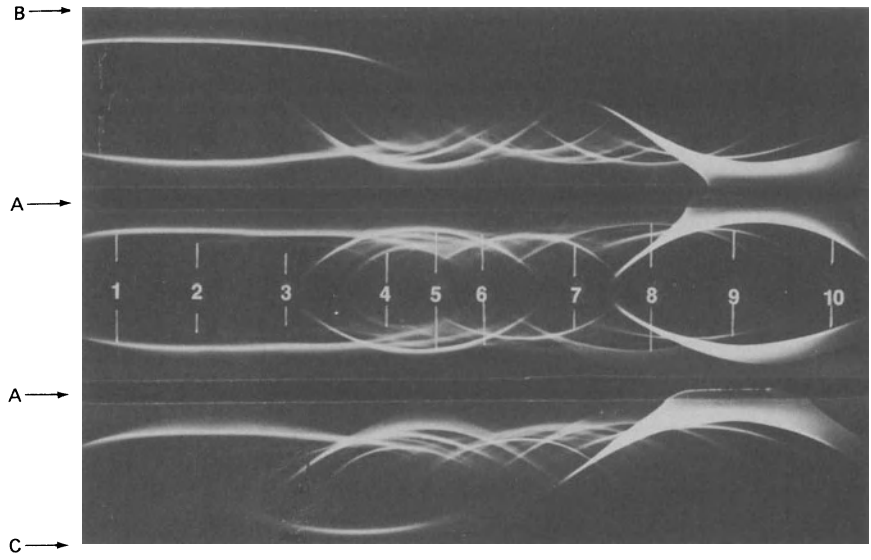

FIGURE 3.8 The resolution of proteins obtained with immunoelectrophoresis from serum of patients with viral hepatitis. The arcs labeled are (1) IgG; (2) IgM; (3) IgA; (4) hemopexin; (5) transferrin; (6) α_2 macroglobulin; (7) Haptoglobin; (8) Gc globulin; (9) α_1 acid glycoprotein; (10) albumin.
The antisera in the troughs are (A) anti-whole human serum (B) anti-IgG, and (C) anti-IgA. Note the markedly elevated IgG and IgA. Photographic procedure described by Century, B., Vorkink, W. B., and Natelson, S., *Clin. Chem.* **20**:1446 (1974).

higher the concentration, the further the protein will move and still form a precipitating arc. This technique has been referred to as *rocket electrophoresis.*[18] One routine application is the detection of clotting factor 8 in plasma. Figure 3.9 illustrates rocket electrophoresis. Alternatively, two or more antibodies are used in the gel. Since the proteins have different mobility they are easily resolved (Figure 3.10).

A variation of rocket electrophoresis is referred to as *crossed immunoelectrophoresis.*[19] Agarose gel electrophoresis is performed in the usual way in the absence of antibody. The agarose strip containing the pattern is inserted into a plate containing antibodies to human serum. Electrophoresis is then conducted at right angles to the original direction. Thus the various proteins move into the gel containing the antibody, forming rocketlike figures (Figure 3.11).

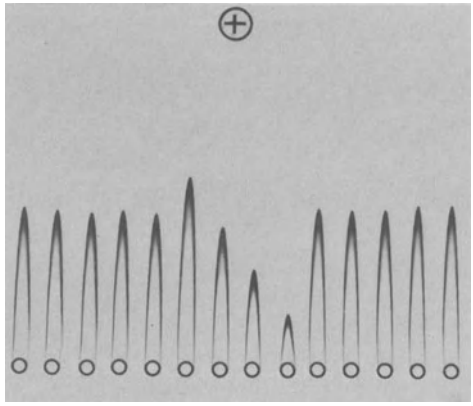

FIGURE 3.9 Illustration of the patterns obtained with rocket electrophoresis. The gel contains antibody to human albumin. The four peaks in the center show a typical standard curve. The others show the reproducibility of the procedure. The serum is deposited in the well and the electric voltage applied. (Courtesy of Dako-Immunoglobulins, Ltd., Copenhagen, Denmark.)

A variation of the electrophoretic procedure in gels is the system of *isoelectric focusing*. In this procedure an ampholyte is dissolved in the gel (e.g., acrylamide gel) so as to create a pH gradient when the electrical field is applied. The protein that is sought moves to an area where it is at the pH of its isoelectric point and remains there for isolation. This has been used for the purification of certain specific proteins such as the clotting factors and hemoglobins.[20]

If sodium *dodecyl sulfate* (SDS) is added to serum before electrophoresis is carried out, the protein surfaces begin to resemble one

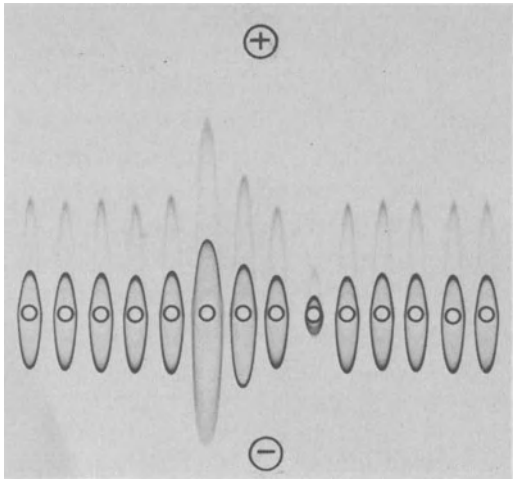

FIGURE 3.10 Double-rocket electrophoresis. Simultaneous quantitation of IgG and IgA. The gel contains antibodies to the heavy chain of IgG and IgA. The standard curve comprises the three center patterns. The dark, cigar-shaped patterns represent the precipitated IgG. The lighter patterns are the IgA precipitates. (Courtesy of Dakopatts Co., Denmark.)

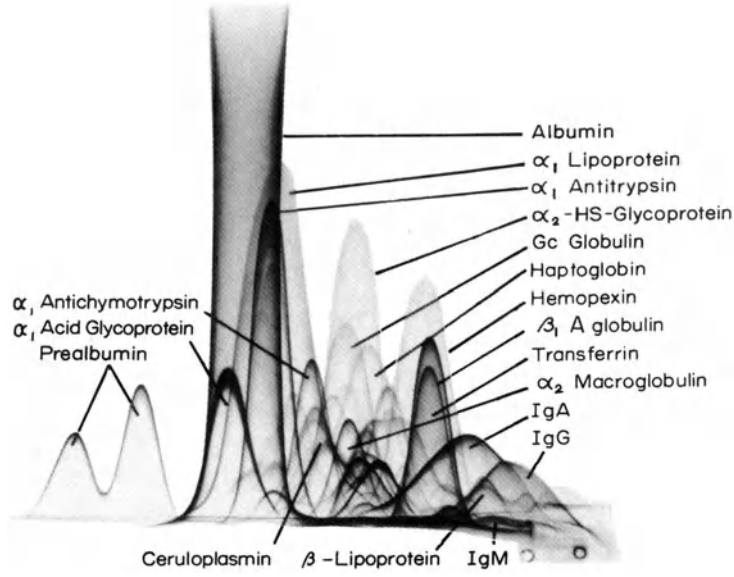

FIGURE 3.11 Two-dimensional electrophoresis of normal human serum (2 μl) plus 1 μl prealbumin as an internal standard in a gel to which rabbit antihuman serum has been added after the first electrophoretic run. Over 40 peaks are seen, some are identified. (Courtesy of Biorad Corp, Richmond, Calif.)

another. This is due to the fact that the fatty chain is held by the protein, leaving the sulfate residue exposed. The proteins then differ only in molecular weight and not in surface charge as far as the separation medium is concerned.[21] This is referred to as *SDS electrophoresis*, and with the help of suitable standards it can be used to obtain a rough estimate of the molecular weight of a particular protein. Figure 3.12 is an SDS electrophoretic pattern of human serum on acrylamide gel.

3.2.4 *The Ultracentrifuge*

The development of the ultracentrifuge by Svedberg in the 1920s and 1930s permitted the characterization of colloids and then proteins by their sedimentation rate.[22] This is a function not only of the molecular weight but also the specific volume and frictional ratio. The

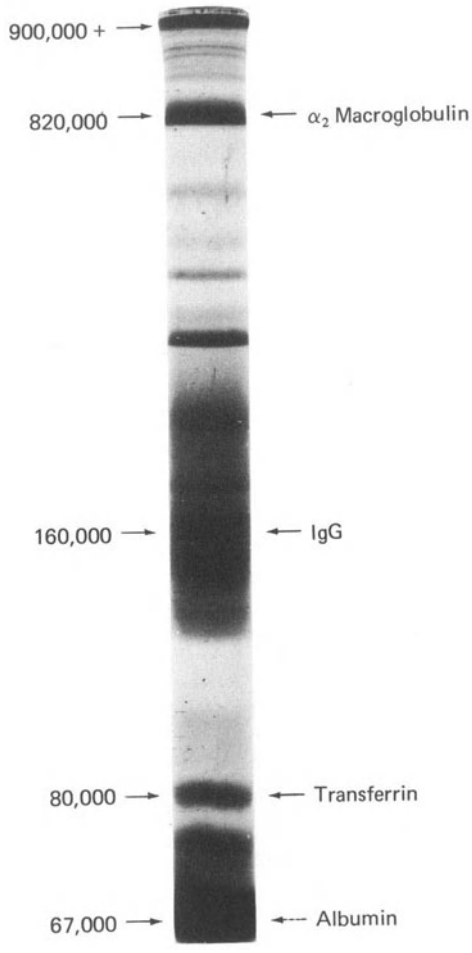

FIGURE 3.12 Pattern obtained in acrylamide gel with human serum after adding sodium dodecyl sulfate to the serum. (Courtesy of Amadeo Pesce, University of Cincinnati.)

frictional ratio is a measure of the distortion from a sphere that the protein exhibits. Thus a high frictional ratio means an elongated molecule. (See Appendix, page 538.)

The ultracentrifuge was first applied to serum in 1935.[23,24] Results were disappointing and all that could be said was that there were at least two fractions in normal serum, one of which was albumin. However, an "X-protein" was noted whose sedimentation coefficient varied with the density of the solution. This was identified in 1950 as the low-density *lipoprotein.*[25] Thereafter numerous laboratories purchased analytical ultracentrifuges for characterizing various types of lipemia

and studied their relationship to atherosclerosis. This technique has been replaced largely by electrophoresis and other procedures for characterizing the lipoproteins. However, for various other uses, including fractionation of cells into their components and isolation of various proteins, the ultracentrifuge has wide application.

In succeeding chapters each of the major components of the plasma proteins will be discussed in detail.

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Sec. II

Plasma Protein Metabolism

4

Albumin in Nutrition and Transport

As pointed out in Section 1.1, albumin literally means egg white. A distinctive characteristic of albumin, noted early, is the fact that it is coagulated by heat. This was observed when eggs were boiled. When milk was boiled, the film of coagulate, formed at the surface, was recognized as albumin, as distinct from casein which does not coagulate with heat. *It was also recognized that urine from some patients formed a coagulate with heat.* To distinguish this coagulate from other precipitates which form, the urine is heated after adding a few drops of acetic acid. The presence of albumin in the urine as an indication of kidney disease has been noted from the middle of the 19th century.

Albumin possesses no enzymatic nor hormonal properties, although it does inhibit or enhance the activity of various enzymes. It serves three major functions in the body: *First it is of nutritional value* serving as a source of amino acids and protein in cellular metabolism.[1] *Second, it is involved in maintaining the osmotic pressure of the blood.* Albumin accounts for 75% of the colloid osmotic pressure of plasma.[2] *A significant decrease of albumin level in the blood is usually accompanied by edema of the tissues.* It is also responsible, to some extent, for the osmotic gradient observed in the kidney (see Volume 1, p. 298). This is intimately related to normal kidney function.[3] *The third major function is as a carrier or solubilizer for certain substances, such as fatty acids, bilirubin, and certain anions and cations.* In this regard it is a major means of transport of these substances.

4.1 *PHYSICAL AND CHEMICAL PROPERTIES OF ALBUMIN*

Both bovine and human albumin were isolated in a high degree of purity toward the end of the 19th century.[4,5] In crystalline form,

1
Glu Ser Lys His Ala Asp
7
Val
Ala His Arg Phe Lys Asp Leu Gly Glu Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr
15
20
30
Leu
Asp Ala Val Cys Thr Lys Ala Phe Glu Thr Val Glu Asn Val Leu Lys Val His Glu Ile Asp Phe Pro Cys Glu Gln
50
35
40
Glu
57
Ser
Ala Glu Asn Cys Asp Lys Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu Arg Glu Thr
60
70
80
83
Glu Lys Ala Cys Cys Asp Ala Met Glx Gly Tyr
91
85
Gln
Pro
96
Glu Arg Asn Glu Cys Phe Leu
100
103
Gln His
Asp Val Glu Pro Val Leu Arg Pro Leu Asp Pro Asx Asp Asp Lys
120
110
140
Val
Met
Cys Thr Ala Phe His Asp Asp Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
130
Arg His
Ala Glx Cys Cys Glx Thr Phe Ala Ala Lys Tyr Lys Thr Ala Phe Leu Glu Pro Ala Tyr Phe Tyr Pro
170
160
150
Ala
Asp
Lys Ala Ala Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg
180
Asp
186
Leu Arg Gln Lys Ala Ser Ser Ala Lys Gly Glu
190
Arg
198
Cys Ala Ser Leu Gln Lys Phe Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg
200
210
220
Phe
Gly His Cys Cys Glu Thr His Val Lys Thr Leu Asp Thr Val Leu Lys Ser Val Glu Ala Phe Glu Ala Lys Pro
240
230
Asp
Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glx Asx Glx Asx Thr Ile Ser
250
260
272
Ser
Lys
Cys Pro Glu Lys Cys Glu Lys Leu
280
Leu
Leu Glu Lys Ser His Cys Ile Ala
290
Glu
1
2
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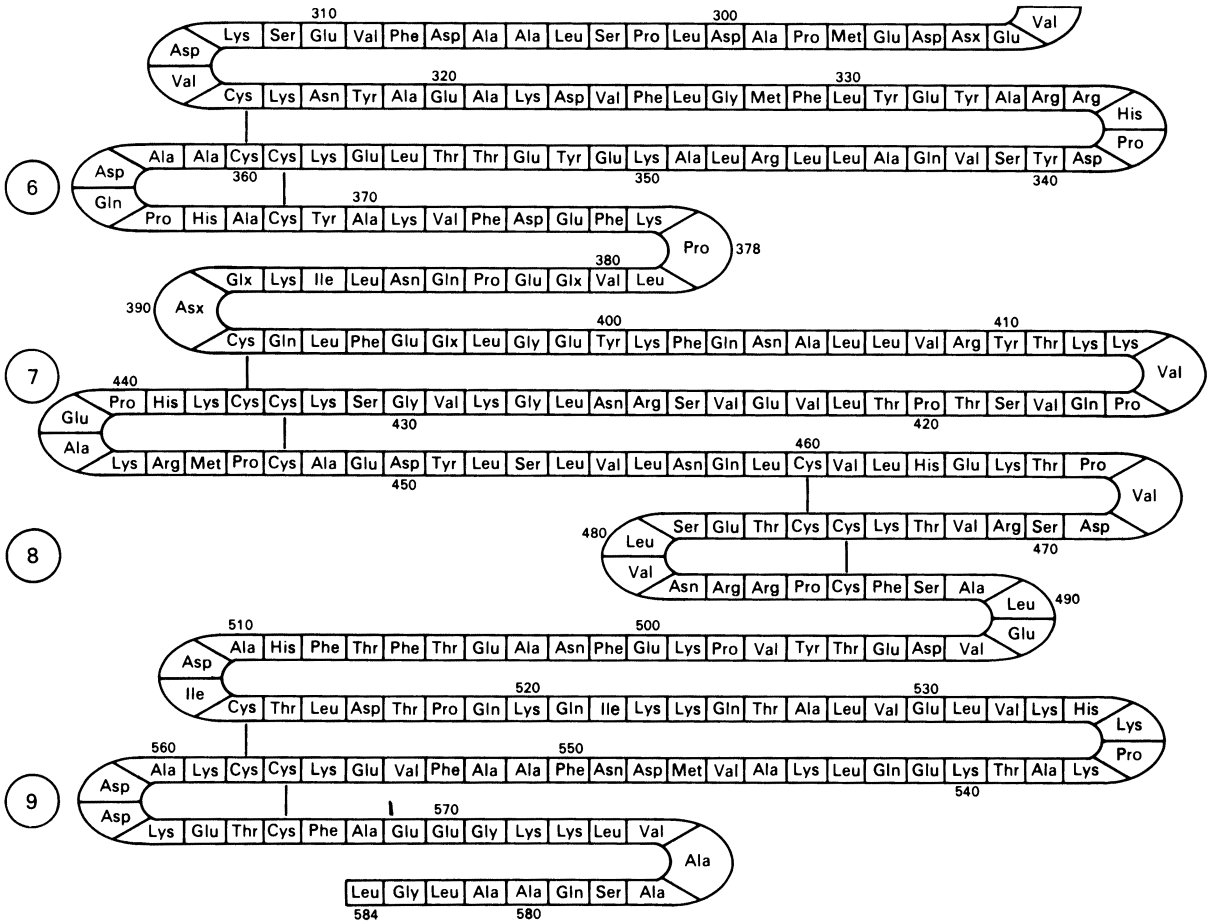

FIGURE 4.1 Amino acid sequence of human albumin. Numbers at the left indicate coil number. Adapted from Behrens *et al.*, *Fed. Proc.* **34**:591 (1975).

albumin is usually obtained as the oleate, palmitate, or stearate.[6] Free albumin is difficult to crystallize.

The fatty acids cannot be removed by ion exchange except at low pH. At pH 8.6 on paper or other media, serum albumin gives rise to a single band. At pH 5.1, the albumin complex with fatty acids separates from the free albumin, resulting in the formation of a double band.[7] Albumin may also combine with itself to form a dimer. For this reason a faster settling band is often observed on ultracentrifugation.[8]

Albumin is composed of one polypeptide chain, coiled to form a globular molecule.[9,10] This is shown in Figure 4.1. There are 16 disulfide bonds (—S—S—) from cysteine to cysteine residue, as indicated in Figure 4.1. This explains the stability of the molecule. From the structure in Figure 4.1, a molecular weight of 66,248 is calculated. This is similar to values obtained from the sedimentation constant, diffusion, and other procedures. The molecule shows a sedimentation constant at 20°C of 4.6×10^{-13} and 6.5×10^{-13} Svedberg units for the monomer and dimer, respectively.[11] Its diffusion constant is 6.1×10^{-7} units.[12] Its partial specific volume is 0.733. This is the reciprocal of its specific gravity. The frictional ratio (f/f_0) is 1.28, confirming a prolate ellipsoid of 140 × 40 Å in size.[13] Its electrophoretic mobility, in Tiselius units, is 5.9 toward the anode at pH 8.6 and ionic strength 0.15. The nitrogen content of albumin is 16.46%. Traditionally, a value of 16 or 16.25% has been used as the nitrogen content of albumin.

Albumin absorbs light at 279 nm mainly because of its tryptophan content. The absorbance at 279 nm for a 0.1% solution ($A^{1g/liter}_{279\,nm}$) is 0.531. Its isoelectric point at ionic strength of 0.15 is 4.7. Its isoionic point extrapolated to ionic strength zero is 5.2.

Approximately 48% of the molecule is arranged as the α helix and approximately 15% is in the β pleated sheet form. The rest is wound to make up the globular molecule.

Albumin will also fluoresce, the excitation wavelength being at 279 and the emission wavelength at 343 nm. This is due mainly to its tryptophan content.

4.2 *MICROHETEROGENEITY OF SERUM ALBUMIN*

At acid pH 2–5 the albumin molecule expands, as indicated by ultracentrifugal, diffusion, and other measurements. The molecule becomes more elongated without change in molecular weight, the helices tending to open up so that the interior parts become more

accessible.[14,15] The molecule becomes more soluble in ethanol, indicating that hydrophobic parts have been exposed. There are thus fewer sites for binding dodecyl sulfate. In addition, approximately 50 more carboxyl residues become exposed.

The fact that more than one form of albumin exists at acid pH can be shown by an inhomogeneous band on gel electrophoresis. The term *microheterogeneity* was applied to indicate this and to distinguish it from the tendency of albumin to polymerize (macroheterogeneity) and the various genetic variations of albumin found in different individuals (see Section 4.4).[16]

If electrophoresis of pure albumin is carried out between pH 3.5 and 4.5, two bands will be observed. The more rapidly moving peak is called the *F form* (faster) and the slower moving peak is called the *N form* (normal). The amount of each formed depends upon the pH. The data obtained could be represented by the equation, $N + 3\,H^+ \rightarrow F$, more of F forming as the pH is lowered.[17] These observations, along with data obtained of the effect of 8 M urea on albumin solutions, which has a similar effect, suggest that the F form is a result of the unfolding of the N form of the albumin molecule, which is the stable form above pH 4.0.[18] This unfolding has been demonstrated with the electron microscope.[19] The change from N to F form is rather abrupt and takes place at pH 3.5.

At alkaline pH 9, electrophoresis also shows that some isomerization begins to take place. This process is accelerated by heating to 65°C or exposure to 2 M urea solutions. At pH 11.4 the molecule unfolds to expose 11 of the tyrosine residues. This is similar to its behavior in acid solution.[20–22]

Another process causing microheterogeneity of albumin at alkaline pH, results from the *intramolecular exchange from a thiol linkage to a disulfide linkage*. This can be demonstrated to occur between albumin and cystine and/or vasopressin, thus binding these compounds tightly to albumin. The reaction takes place at pH 8 or higher. It has been suggested that on electrophoresis at pH 8.6 this is an additional cause for obtaining a band of albumin instead of a sharp peak.[23,24]

4.3 *NORMAL DISTRIBUTION AND ORIGIN*

The normal concentration of albumin in male human blood serum is 42 ± 3.5 g/liter. Albumin comprises 58 ± 4% of the total serum proteins.[25] At birth, the percentage of albumin is substantially higher

67 ± 5%, but the amount, in absolute terms, is lower. The reason for this is that the total protein in the adult is 70 ± 4 g/liter, whereas in the infant it is only 53 ± 3 g/liter. Thus the total albumin in the serum of the newborn is 35 ± 3 g/liter.[26]

In recent years, the introduction of dye-binding techniques for albumin estimation, without the use of suitable corrections for the presence of neonatal jaundice, hemolysis, and turbidity in the blood, has resulted in the publication of erroneously high values for albumin and total protein in the newborn. The values listed above are based on extensive studies, using specific procedures by the authors and others.[27,28]

Serum albumin levels are lower in women than in men by approximately 9%. Thus, in the female, the mean total protein is 66 ± 4 g/liter. The serum albumin concentration is at 38 ± 4 g/liter.

Serum albumin levels decrease with age, and in adults over 65 years of age albumin levels will be approximately 10% lower than at 30 years of age. The serum albumin level is responsive to diet, and in populations where beef is plentiful and is the main source of protein in the diet, albumin levels are higher.[29]

Only 40% of the albumin in the body is retained in the circulation. Approximately 50% leaves the circulation per hour and is recycled by way of the thoracic duct. Albumin is present in all tissues, including the skin. All of the albumin is synthesized in the liver by the hepatocytes. However, at any one time less than 2% of the body pool is in the liver. *About 50% of the energy expended by the liver is for the synthesis of albumin.*[30,31]

The sequence of events in albumin synthesis has been arrived at mainly by the use of [^{14}C]-labeled arginine, following its incorporation into albumin. After *messenger RNA (mRNA) synthesis*, directed by DNA in the nucleus, albumin synthesis proceeds in the cytoplasm under the direction of transfer RNA (tRNA) also synthesized in the nucleus. The *ribosomal RNA (rRNA)*, which reads the message from mRNA is synthesized in the *nucleolus*. A specific activated amino acid residue is carried by tRNA to the site of the growing peptide chain, which has been coded by mRNA.[32,32a]

Ribosomal proteins are synthesized in the cytoplasm and move to the nucleolus so as to combine with RNA to form the ribosomes. There are two RNA molecules made in the nucleolus, a lighter RNA of 18S, the other a heavy RNA (40S). These, together with the proteins make up a single ribosome with two subunits of 40 and 60S, respectively. The

ribosome now returns to the plasma. The 60S subunit is held in the center of the endoplasmic reticulum, whereas the 40S subunit holds the mRNA, thus forming an mRNA-40S-60S complex. As each specific amino acyl tRNA adds its amino acid to the growing peptide, the peptide moves to the next codon on the mRNA.

The *codon* for each amino acid comprises three nucleotides. Since there are 584 amino acids in albumin (Figure 4.1), 1752 nucleotides are required to code for albumin. Each ribosome is separated from the next one on the mRNA by 90 nucleotides (30 codons). Thus we need

TABLE 4.1

Albumin Content of Various Human Body Fluids[36–50]

Fluid	Albumin, g/liter	Total protein, g/liter	Albumin (electrophoretic), (%)
Serum			
adult	40 ± 4	69 ± 5	58 ± 2
newborn	34 ± 2	52 ± 3	64 ± 4
Urine	0.30 ± 0.2	0.50 ± 0.25	40 ± 3
Cerebrospinal	0.16 ± 0.3	0.23 ± 0.6	67 ± 5
Sweat, eccrine[a]	0.20 ± 0.2	0.65 ± 0.3	30 ± 3
Lymph			
thoracic duct	21 ± 2	33 ± 3	63 ± 5
liver	33 ± 4	50 ± 5	60 ± 4
Tears[a]	0.61 ± 0.3	3.60 ± 2	17 ± 2
Peritoneal cavity	20.0 ± 2	30.0 ± 5	65 ± 5
Pleural cavity	16.0 ± 3	26.0 ± 4	63 ± 5
Amniotic (at term)	1.14 ± 0.3	1.9 ± 0.5	60 ± 4
20–24 weeks	1.53 ± 0.5	2.36 ± 0.6	65 ± 5
6–16 weeks	1.80 ± 0.6	2.77 ± 0.7	65 ± 5
Milk[a]			
mature	1.0 ± 0.2	11.0 ± 2	10 ± 1.0
colostrum (at birth)	5.0 ± 1.0	17 ± 3	30 ± 3
Gastric juice	0.54 ± 0.1	3.0 ± 0.5	18 ± 3
Bile			
main duct	4.2 ± 0.4	7.5 ± 0.4	56 ± 4
gall bladder	14.6 ± 1.5	26.1 ± 3	56 ± 4
Synovial	1.8 ± 0.3	2.9 ± 0.4	62 ± 3

[a] Only a small fraction of the protein which moves to the albumin zone on electrophoresis tests as serum albumin, immunochemically. In human mature milk, it represents only 10% of the total protein. In tears and sweat the percentage of serum albumin in the "albumin" fraction is less than 1%.

TABLE 4.2

Distribution of Albumin (in Grams) in Various Tissues of the Adult[36,49,50]

Tissue	Grams	Tissue	Grams
Plasma	124 ± 15	Cardiac muscle	46 ± 5
Liver	3.5 ± 1	Spleen	6.2 ± 1.5
Kidney	3.5 ± 0.8	Skin	55 ± 6
Brain	2.4 ± 0.5	Total[a]	310 ± 15

[a] Of the total albumin, approximately 124 g is in the plasma and 186 g is in the interstitial fluid spaces and tissues.

approximately 20 ribosomes (20 × 90 = 1800) to form the albumin molecule. Actually a proalbumin is synthesized. This loses five or six amino acids and is then secreted directly into the plasma as albumin by way of the *Golgi apparatus.*[33]

The liver makes approximately 14–15 g of albumin daily. *Additional albumin can be synthesized, since two-thirds of the hepatocytes are in the resting stage at any one time.*[34]

Newly formed albumin is added directly to plasma. From there, equilibrium is established with the lymph and other tissues.[35]

Albumin is present in all secretions and excretions of the body and is normally present in urine, tears, bile, perspiration, gastric juice, amniotic fluid, muscle, eye, and other tissues. Some figures for the concentration of albumin in various fluids of the body are given in Table 4.1. The values in the table were obtained mainly by electrophoretic techniques. For this reason, in some cases, the "albumin" represents a mixture of proteins, only part of which will react with human serum albumin antibodies. This is especially true for tears, sweat, and human milk. In the others listed, the bulk of the albumin is essentially serum albumin. Table 4.2 lists the concentration of albumin in plasma and plasma-free tissues.

4.4 *ALBUMIN FUNCTION IN THE HEALTHY HUMAN*

The functions of albumin have been listed at the beginning of this chapter. They may be summarized as *nutritive*, to maintain the *osmotic pressure* in the various body fluids, and for *transport.* Albumin as a transport protein will be presented first.

4.4.1 *Albumin as a Transport Protein*

Albumin binds both anions and cations. When isolated from blood it appears pinkish because of its strong complex with hematin.[51] It also binds the steroids such as estradiol, progesterone, cortisol, corticosterone, aldosterone, and testosterone.[52–55] It also binds prostaglandins[56] and urates.[57] In these cases it maintains these materials in solution and transports them to their site of action.

Unconjugated bilirubin is insoluble in water and is retained in solution by binding to albumin.[58] The bilirubin is bound to the same sites to which dyes such as HABA [2(4′-hydroxyazobenzene benzoic acid)], phenol red, and numerous others are bound. Advantage is taken of this fact in determining *residual bilirubin binding capacity* in the newborn. In this case, HABA is added to the serum and the amount of dye bound to albumin is determined as a measure of the reserve binding capacity of albumin for bilirubin.[59]

Normally the residual binding capacity in the blood of the newborn is of the order of 25%. In cord blood the normal level is 72–97% since bilirubin levels are very low in cord blood, in the absence of disease.

Salicylate, sulfa drugs, and numerous others, including Warfarin, will also bind tightly to albumin.[60–62] In this respect, albumin aids in the neutralization of these drugs and in their transport to sites where they may be eliminated. Albumin will also bind the catechol amines.

A major function of albumin is the binding of the fatty acids, oleic, palmitic, linoleic, stearic, and some phospholipids.[7,63,64] *In this regard albumin is the major transport protein for free fatty acids, not the lipoproteins.* The sites for fatty acid binding are hydrophobic clefts into which the aliphatic chain is inserted. Thus albumin is of major significance in fatty acid metabolism. *In analbuminemia (see Section 4.5.2) the major symptoms are those due to ineffective lipid metabolism.*

The site where various substances bind on albumin has been the subject of intensive study. Cu^{2+} and Ni^{+} ions bind at the amino group in position 1 of Figure 4.1. A chelate compound is formed between this amino group, the cation, the two peptide nitrogens which follow, and the 1-nitrogen of the imidazole of histidine at position 3.[65] Dog albumin has a tyrosine residue at position 3. As a result it does not bind copper as well as human albumin. Human albumin has a major role in copper transport (see Section 5.4). This is a possible explanation of the fact that the dog is more sensitive to copper poisoning than man. Other bivalent

ions such as Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} are bound less strongly and at several locations on the albumin molecule.[66,67] Other proteins in plasma bind these ions more tightly. *For this reason, except for copper and possibly zinc, albumin is not considered an important transport means for most cations.*

The only amino acid which seems to have an affinity for albumin is the L form of tryptophan. This property has been used to separate L from D-tryptophan.[68] The former binds to albumin near the histidine residue at position 146 at the tip of loop 3 (see Figure 4.1). Pyridoxal phosphate binds at the tip of loop 4 (Lys, 233).[69] The sites for binding of fatty acids are in the loop region 7–8 and less at loops 6–7. Bilirubin binds to an area with histidyl, tyrosyl, and arginyl residues. It has been proposed that it binds to these amino acids in loop 3.[70,71]

Although there are specific proteins in the plasma for the transport of the various steroids, anions, and cations, albumin acts to help transport any overflow when these are overloaded. Its major transport function is for the transport of fatty acids and bilirubin. In the case of bilirubin it serves a protective function in jaundice because of the toxicity of bilirubin to neurological tissue. This is of particular significance in the newborn (see Volume 2, p. 358).

4.4.2 *Albumin in Nutrition and Osmotic Regulation*

Albumin is synthesized in the liver. *The most important factor regulating albumin synthesis is the state of nutrition of the individual.*

Starvation causes a rapid decrease in the rate of albumin synthesis. However, the capacity to synthesize albumin persists even after long periods of fasting. Refeeding results in a prompt increase in the rate of albumin synthesis. Apparently, the mechanism for the synthesis of albumin, including mRNA, remains essentially intact during starvation.[72]

On a protein-deficient diet, where carbohydrates and fats are fed in adequate amounts, there is a rapid shrinkage of the nucleolus and a decrease in rRNA production. Feeding the essential amino acids results in a restoration of the rate of albumin synthesis.[73] Most dramatic is the effect of tryptophan. *In vivo* and *in vitro* studies indicate that tryptophan is necessary for polysomal aggregation and continued protein synthesis by subcellular systems.[74,75]

A major factor in the immediate control of albumin synthesis is the colloidal osmotic pressure in the hepatocyte environment. *When the plasma colloid osmotic pressure is raised by administration of albumin, globulins, or even high-molecular-weight dextran, albumin synthesis is depressed.*[76] If the colloid osmotic pressure around the cell is decreased, then albumin synthesis is stimulated. In these experiments, the animal is subjected to plasmapheresis. As the plasma is removed, albumin from the albumin pool around the cells (see Table 4.2) is removed and moves into the plasma. This reduces the albumin concentration around the cells and stimulates albumin synthesis.[77] Furthermore, if liver cells are prepared from nephrotic animals with low plasma-albumin levels, the rate of albumin synthesis is substantially higher than that in normal hepatocytes.[78]

4.4.2.1 *Hormonal Effects on Albumin Synthesis*

For optimum protein synthesis, in general, it would be expected that normal functioning of the pituitary and its target glands, the thyroid and adrenal is necessary. However, the thyroid seems to have a specific action in connection with albumin synthesis.

Thyroidectomy results in a rapid decrease of mRNA and rRNA synthesis with a resultant decrease in albumin synthesis. Administration of *triiodothyronine* rapidly corrects this defect.[79] Triiodothyronine thus has been shown to be directly involved in the control of rRNA and mRNA synthesis and the binding of rRNA to the endoplasmic reticulum. *Control of ribosomal production is thus an important function of the thyroid hormone.*

Administration of *cortisone* results in an increase in the size of the liver and an increase in the rate of formation of rRNA, tRNA, and mRNA. This is associated with a marked increase in the amino acid pool in the liver and albumin synthesis.[80] Associated with this effect is a negative nitrogen balance. This has been interpreted as the result of a general antianabolic effect of cortisone.[81] On cortisone administration, the total albumin in the body remains constant, while a shift of albumin from the tissues into the plasma takes place resulting in elevated plasma albumin levels. This has been noted also with Cushing's syndrome.[82]

Insulin increases albumin synthesis by making available energy in the form of ATP from increased glucose metabolism.[83] *Testosterone* has

an effect similar to the thyroid hormone. The *growth hormone* also serves to stimulate albumin synthesis. Triiodiothyronine has an additive effect with both testosterone and the growth hormone.[84]

4.4.2.2 *Albumin in Cell Nutrition*

The half-life of albumin, as determined by [^{131}I]-albumin studies, is 17 ± 5 days. Thus albumin is constantly being synthesized and consumed. Very little of this is lost as albumin in the urine, sweat, and from other excretions. *The liver synthesizes 14–17 g of albumin daily and almost all of this is consumed by the cells of the various tissues as a balanced amino acid source to synthesize other proteins for their special function.* The albumin readily leaves the vascular system and approaches the cell wall. There it is engulfed by *pinocytosis* and hydrolyzed and reformed into the various proteins. In the process of pinocytosis, invaginations are formed in the surface of the cell which close to form fluid-filled vacuoles. This has been observed with the electron microscope.[85] (See Figure 7.3.) Since the adult human consumes about 50 g of protein daily, it is apparent that approximately one-third of this nourishes the cells as albumin which has been formed in the liver.

Presenting the cells with a formed protein rather than amino acids, facilitates protein production. Hydrolysis of albumin generates about 50,000 cal/mol. This is also the amount of energy required to build the protein. Thus albumin carries to the cell not only a balanced set of amino acids but also much of the energy required to build it into another protein. When only amino acids are supplied, the cell needs to generate the 50,000 cal/mol and requires that all the amino acids be present in the proper proportion at the same time. Thus, supplying the cells with albumin is an efficient way of supplying the raw material for protein production.

There is a pool of 310 g albumin throughout the body of which 130 g is in the plasma. Partly for this reason, man is able to go without food for weeks and still carry on the necessary protein synthesis for survival. See Table 4.2.

4.5 *ALBUMIN VARIANTS*

Albumin's evolutionary history is traced back to the period of its first appearance in the teleost fish.[86] It is absent from the elasmobranch and cyclostome.[87] In tadpoles, its level is less than 0.6 g/liter which

increases during metamorphosis to 8 g/liter.[88] It appears that the development of high levels of albumin corresponds with the problem of maintaining a high osmotic pressure and retaining water as the animals moved to dry land.

Studies on the nature of the different albumins in various animals indicates that its mutation rate is about twice as fast as that of hemoglobin.[89] This has been used as support for the "molecular clock" concept of mutation rates.

Genetic variations in the nature of albumin found in the blood may be divided into three groups: First, where an amino acid substitution has taken place in the albumin chain. This is detected electrophoretically in the heterozygote by the appearance of two bands, one being normal albumin, the other being the variant. This is called *bisalbuminemia.* A second form of abnormality is the appearance of a postalbumin band on staining of the electrophoretogram obtained with acrylamide or starch gel or cellulose acetate. This is a result of the formation of a *dimer of albumin.* In this case the dimer represents only 10–15% as compared to normal albumin. Immunologically it behaves like normal albumin. A third type of abnormality of genetic origin is an almost complete absence of albumin in the serum. This is called *analbuminemia.*

4.5.1 *Bisalbuminemia*

In 1955, it was noted by Scheurlen that the albumin band, obtained on Tiselius electrophoresis at pH 8.6 of serum from a 23-year-old diabetic, split into two bands.[90] The double albumin peak appeared to be unrelated to his disease. A few years later, several investigators observed similar phenomena.[91,91a,92] By studying kindred, it was apparent that in these cases one of the peaks represented normal albumin and the other a variant. Since the peaks were of approximately equal height it became apparent that these individuals were heterozygotes. The term *bisalbuminemia* has been applied to this phenomenon. The bisalbuminemia is also observed in the urine of these individuals.

In these early studies, one set of investigators labeled the faster peak A_1 and the slower A_2. The others labeled the faster peak A and the slower B. They were dealing with the same type of variant. This variant has been observed in at least 100 individuals in central

Europe.[93] The two albumins differ by the substitution of lysine for a dicarboxylic acid, resulting in the slow-moving (B or A_2) variant. Immunologically the two components behave as though they are identical. A similar substitution of lysine for glutamic acid occurs in Albumin Oliphant.[94]

Since albumin synthesis seems to be about equal for both normal and abnormal forms, it has been proposed that these genes are co-dominant for the synthesis of albumin.[95] A case of bisalbuminemia, observed in the laboratory of the senior author, is somewhat different. Figure 4.2 shows a pattern obtained on a serum sample obtained from an apparently healthy blood donor. The abnormal albumin (the more rapid peak) occurs in approximately one-fourth the concentration of normal albumin. This would suggest a slower rate of synthesis or a more unstable product.

Since these early studies, double albumin peaks have been observed in at least 25 different families. Some show albumin peaks with higher mobilities than the normal.[96] For example, in Albumin Naskapi, a high-mobility albumin occurs with a gene frequency of 0.138 in these North American Indians.[97] It is called Albumin Naskapi after that Indian tribe.

The location of some of the variants reported are shown in Figure 4.3. The fastest component, called *Gent*, was reported by Wieme.[98] A very slow albumin, Albumin Mexico, was first reported by Melartin.[99]

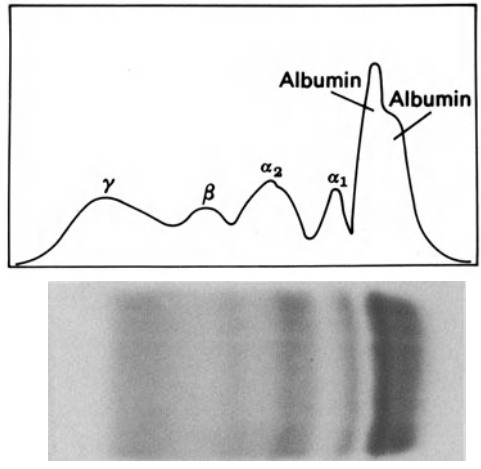

FIGURE 4.2 **Bisalbuminemia in an adult. Tracing and original pattern on cellulose acetate; pH 8.6, barbiturate buffer.**

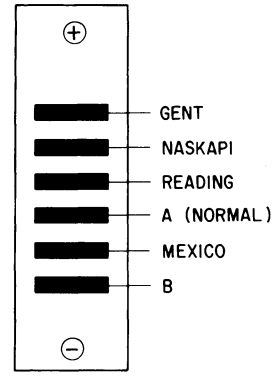

FIGURE 4.3 Schematic representation of the relative mobilities of various albumins at pH 8.6.

The abnormal albumins have been called *paralbumins*, and bisalbuminemia has been referred to as the *monomeric variants* to distinguish them from the dimeric variants (see below). Paralbumins are also found in ovarian cysts.

The fact that albumin travels in the electrophoretic field with what appears to be a normal mobility does not necessarily signify that it is normal albumin or is homogeneous. In a heterozygote, two different albumins may arrive at the same location. The albumin is then said to be *polymorphic*. The albumins can be readily distinguished by various properties such as their ability to bind various components such as thyroxine[100,101] and others.[102]

Dimeric Albumin Variants

In 1959, a genetic albumin variant was reported where 90% of the albumin was normal (A) albumin but there was a trailing band comprising the other 10%. This was soon identified as a dimer of albumin.[103] Other cases were reported subsequently where as high as 25% of the albumin was present in the dimeric form.[104,105]

It soon became apparent that these abnormal albumins were not different in nature from those observed in bisalbuminemia. The substitution of one amino acid for another merely produced a variant which tended to polymerize.[106] It has already been pointed out that even normal albumin will form dimers (macroheterogeneity; see Section

4.2). In addition, some of the monomeric variants tend to denature or polymerize on standing. This is true for Albumin Paris.[107] The number of families reported with dimeric albumin variants is less than 10, at this writing, and it is thus a rather rare condition. The presence of a dimeric form of albumin has not been shown to be related to any disease process.

4.5.2 *Analbuminemia*

In 1954, an electrophoretic pattern obtained from a 31-year-old woman was shown to contain no albumin.[108] Total protein was 4.8 g/100 ml. Her brother's serum also contained no albumin with a total protein of 5.4 g/100 ml. The father and mother showed serum albumins of 3.3 and 3.1 g/100 ml, respectively. A study of 220 relatives showed no defect. With an immunochemical technique it was found that the brother's serum contained 1.6 mg albumin/100 ml.[109]

Since this case was reported, approximately ten others have been presented in the literature. In each case a minute amount ranging up to 290 mg/100 ml of albumin has been reported.[110–113]

The cause of the defect is a markedly reduced rate of albumin synthesis. The half-life of albumin normally present in serum is 15 days. In these patients the half-life of albumin ranges from normal to 115 days.[112,113] Thus the defect is not due to rapid destruction of albumin. Analbuminemia is inherited as an autosomal recessive.

The clinical symptoms in these individuals are mild. With no albumin the total protein would be expected to be 3 g/100 ml. However, it is approximately 5 g/100 ml in the patient with analbuminemia; and therefore, synthesis of other proteins is increased as a form of compensation. Edema, although persistent, is mild. There is some hypotension and an elevation of the lipoproteins and γ globulins. The colloid osmotic pressure of the serum is about one-half the normal. The sedimentation rate is elevated. The absence of albumin to bind fatty acids requires that other proteins serve the purpose. This is not entirely compensated for since these patients show reduced rates of fatty acid metabolism and elevated serum triglycerides and cholesterol.[113]

Overall, the patient with analbuminemia seems to thrive, and it is apparent that albumin is not essential for life.

4.6 *SERUM ALBUMIN CHANGES IN DISEASE*

In disease, the ratio of albumin to globulin level, referred to as the A/G ratio, almost invariably falls. This is due to the fact that in disease the liver is usually involved in some way, and albumin synthesis is reduced. At the same time the level of γ globulins usually rises and this is often accompanied by an increase in the α_2 and β globulin levels. Table 4.3 summarizes the data obtained by various experimenters for some of the conditions where the A/G ratio is lowered significantly.

Note that in multiple myeloma, with a huge increase in the γ globulin level, the total protein is elevated and the albumin level is reduced. In the nephrotic syndrome, serum albumin levels are often

TABLE 4.3

Mean Serum Albumin, Globulin, and Total Protein Levels and A/G Ratios Reported in Disease in Humans by Salt Fractionation, as Compared to the Normal[114–119]

Condition	Cases	Albumin, g/liter	Globulin, g/liter	A/G	Total protein, g/liter
Adults[a]	340[a]	44	26	1.7	70
Newborns[a]	142[a]	38	16	2.4	54
Severe starvation	2	8.0	28	0.3	36
Kwashiakor	37	14	30	0.5	44
Lipoid nephrosis	21	14	31	0.5	45
Cholera	16	23	36	0.6	59
Meningitis	16	23	38	0.6	61
Smallpox	10	24	35	0.7	59
Hepatic cirrhosis	103	25	41	0.6	66
Tuberculosis (advanced)	42	28	47	0.6	75
Obstructive jaundice with biliary cirrhosis	41	29	42	0.7	71
Viral hepatitis	82	31	39	0.8	70
Lupus erythematosis	15	31	38	0.8	69
Tetanus	15	29	46	0.6	75
Sarcoidosis	11	31	48	0.6	79
Multiple myeloma	37	29	69	0.4	98
Venereal lymphogranuloma	59	32	56	0.6	88

[a] From the senior author's laboratory after lipid extraction and by the biuret procedure.

reduced to less than half the normal value, whereas the major increase in serum protein is in the α_2 and β region of the electrophoretic pattern.[119]

In malnutrition, such as in kwashiokor, the albumin level is markedly reduced.[120] Patients who are admitted into a hospital after a prolonged illness (see Table 4.3) also show low albumin levels usually because of malnutrition.[121] These patients, unless treated, are poor surgical risks since after surgery the albumin is needed for repair and proliferation of the tissues and for minimizing edema, especially at the sites of repair.[103]

Malnutrition with hypoalbuminemia will also result from food idiosyncracies, especially in children. This includes the so-called *gluten intolerance* or *grain dyspepsia* in breast-fed infants. The use of hypoallergic milks, and other measures designed to avoid the intake of the food which is not tolerated, readily corrects these conditions.[122]

In the nephrotic syndrome, and especially lipoid nephrosis, loss of albumin to the urine is huge. Hypoalbuminemia in these cases, is the result of inability of the liver to keep up with the rate of loss of albumin through the kidneys.[123]

In major infections such as cholera the fall in albumin results not only from some losses through the kidney but also because of the toxic effect of the disease on albumin synthesis. There is, in these conditions, also a rise in the rate of catabolism of all proteins, resulting in a negative nitrogen balance.

Surgical and accidental trauma, including burns, also will often result in impaired albumin synthesis and an increased rate of catabolism. It has been suggested that this is the result of stress since injection of adrenaline in guinea pigs results also in decreased albumin synthesis and increased catabolism.[124–126] However, hydrocortisone, cortisone, or prednisone administered in high dosage results in an increase of serum albumin levels by as much as 30%.[127]

In the presence of a gammopathy such as in multiple myeloma, or macroglobulinemia, and in the presence of malignancies, where huge amounts of γ globulin components are being synthesized, albumin levels will drop significantly (Table 4.3). This is partly due to the demand for amino acids for the synthesis of the antibodies. A major factor is lack of appetite with inanition in these patients so that they are usually suffering from malnutrition. In these patients the nitrogen pool is lowered and also diverted to synthesizing proteins other than albumin.

4.7 *I.V. ALBUMIN THERAPY*

Human albumin preparations are used as plasma expanders in traumatic or surgical shock or shock due to burns.[128] Their high stability permits their storage for extended periods of time. They withstand heating at 60°C for 10 hr, which removes any adsorbed hepatitis virus.

In recent years, with the widespread use of hyperalimentation, albumin preparations, are used by some in place of amino acid hydrolyzates as a nitrogen source.[129] Albumin is utilized more efficiently as a source for protein synthesis. It also permits the use of smaller volumes. *Normal Serum Albumin* (*Human*) is supplied in 50-ml quantities of a 25% solution. It is a viscous preparation and diluting it presents some problems. This preparation and *Plasma Protein Fraction* (*Human*) are also supplied in 250-ml quantities of a 5% solution. The significance of these terms is explained in Section 4.7.1. In Section 4.3, the nutritive properties of albumin were discussed and in practice it appears that, where available, human albumin has significant application in patient management.

Preparation of Purified Albumin

The source for albumin is outdated blood bank blood. In addition, substantial amounts are obtained by bleeding a donor, separating the plasma, and returning his red cells (plasmapheresis). Human placentas have also been used as a source for albumin. They have the disadvantage that they contain a high concentration of a heat-stable alkaline phosphatase.[130]

Albumin was first purified by exhaustive dialysis against water. Under these conditions the globulins precipitate and the albumin remains dissolved. In fact, only the euglobulins were precipitated in this procedure. Half-saturated ammonium sulfate (2.1 mol/liter), 25% sodium sulfite, or saturated sodium sulfate (25%, 1.8 mol/liter) precipitate the globulins. These techniques have been used to determine the A/G ratio. Reducing the pH to 4.4 and increasing the $(NH_4)_2SO_4$ concentration then will precipitate crystalline albumin. The presence of fatty acids such as oleate or palmitate favors crystallization.

For large-scale production, the method of E. J. Cohn developed during World War II is still being used.[131] The albumin is prepared from "Fraction V," after globulins are removed by precipitation, at pH 4.8 with 40% ethanol at an ionic strength of 0.11 at −5°C. The product is 96% pure. After heating at 60°C for 10 hr to destroy any hepatitis virus, the product is sold as Normal Serum Albumin (Human) for intravenous use. Simplified variations of this procedure are used by several pharmaceutical manufacturers to produce an albumin of 83% purity which is sold as *Plasma Protein Fraction (Human)* or PPF. This is approved by the U.S. Food and Drug Administration for intravenous use.

When albumin is precipitated with trichloroacetic acid, it readily redissolves in organic solvents such as 80% ethanol. This is not true for the globulins. Advantage is taken of this phenomenon to rapidly separate albumin in fairly pure form or to determine the albumin/globulin ratio in serum.[132]

Addition of Hg^+ ion to albumin results in the reaction of all free —SH groups with mercury; two-thirds of the albumin molecules react. The product can be crystallized. The mercury is removed by dialysis against cysteine to form *mercaptoalbumin* which now contains 1.0 sulfhydryl group per molecule, as compared to 0.64 sulfhydryl group in albumin prepared by other procedures.[133]

Albumin can be purified by electrophoresis, isoelectric focusing, or column chromatography. With column chromatography, albumin is bound to DEAE-cellulose more tightly than the globulins and can then be eluted by salts and at a lower pH.[134]

In recent years, affinity chromatography has been applied to the preparation of pure albumin.[135] Antibodies to albumin, prepared by immunizing a rabbit or goat against human albumin, or a fatty acid such as palmitate, or bilirubin, or a dye which binds albumin are linked to a solid support such as dextrans, cellulose, agarose, or resins. The diluted serum is passed through a column prepared from these materials and only albumin binds to the column. Elution is usually done with a solution of high salt concentration.[136]

Albumin as prepared contains substantial amounts of fatty acids tightly bound. Thus even crystalline albumin is a mixture of albumin and albumin bound to fatty acids. These can be removed by polar solvents without denaturing the albumin. Albumin free of fatty acids is available commercially (Sigma Chemical Co., St. Louis, Mo.).

4.8 *SERUM ALBUMIN ASSAY*

Albumin assay in human serum takes two different approaches. In the classical procedure the globulins are precipitated by sodium sulfate (25%) and the albumin remaining in the supernatant is digested with sulfuric acid to form NH_3 which is determined by distillation, aeration or diffusion, and titration (*Kjeldahl procedure*).[137] Calculation traditionally assumes 16.25% nitrogen in the molecule. The value should be corrected to 16.46%. Nonprotein nitrogen is also determined after total protein precipitation and this value is subtracted from the total nitrogen eetermined on the albumin solution. If total serum protein is sought, the sulfuric acid is added directly to the serum for digestion. Catalysts such as Se, $CuSO_4$, or H_2O_2 are added to accelerate the reaction, and K_2SO_4 is added to raise the boiling point of the mixture. The Kjeldahl procedure is still the reference procedure for protein estimation.

Currently, assay for albumin and total protein with color reagents is employed widely, especially where the concentration of protein is low, or for microanalysis. *The Folin-Ciocalteu* reagent (phosphotungstic acid) is used to detect tyrosine and calculation is made by a suitable factor.[138] *The Sakaguchi reagent* for arginine has been used in the same way.[139] A most widely used reagent for total protein and albumin estimation is biuret.[140] Alkaline copper solutions form a pink-colored complex with the biuret linkage. In this procedure, precipitation of the globulins with 25% sodium sulfite is done in the presence of ether to remove lipids and bilirubin interference. For total protein, the proteins may be precipitated with Bloor's reagent (ethanol–ether, 3:1), the precipitate being then redissolved in water and biuret added.[26] Where extractants such as ether or Bloor's reagent are not used, interference from bilirubin, slight hemolysis, and lipemia is experienced.[140]

In automated systems, advantage is taken of the fact that certain dyes will bind selectively to albumin with a shift of the wavelength of maximum absorption. Globulins adsorb some of the dye but substantially lesser amounts. HABA [2(4′-hydroxyazobenzene)-benzoic acid] has been widely used.[141] Bromcresol green (3,3′,5,5′-tetrabromo-*m*-cresolsulfonphthalein) is also used and is claimed to suffer less from bilirubin interference.[142]

Albumin is also estimated roughly from the electrophoretic pattern on cellulose acetate after total protein has been estimated by biuret.

For ultramicroprocedures, the albumin may be estimated by

immunochemical techniques, particularly by radial immunodiffusion.[143]

4.9 *RECAPITULATION*

Human albumin comprises a single polypeptide of 584 residues coiled to form an ellipsoid. It accounts for 75% of the colloid osmotic pressure of serum. A major function of albumin is to supply the cells of the body with a raw material for protein synthesis. Albumin serves to bind the free fatty acids of the serum and is a major means for their transport.

Approximately 15 g albumin is synthesized daily in the liver. In plasma it represents approximately 58% of the total protein and is present normally in a concentration of 40 g/liter. At any one time, approximately 50% of the total albumin is present throughout the body outside of the vascular system. It is present in all secretions and excretions of the body.

A major function of albumin is to transport bilirubin to the spleen for eventual removal. It thus acts as a buffer against the neurotoxic effects of circulating bilirubin in the various forms of jaundice. This is of great significance in neonatal jaundice. Albumin also serves to bind drugs and toxic substances so they can be transported safely for excretion.

On electrophoresis at pH 8.6, albumin has the highest mobility of the plasma proteins with the exception of a small amount of prealbumin. Variants occur and are most apparent when they change the position of albumin in the electrophoretic field. Most variants result from the substitution of one amino acid for another in the albumin polypeptide chain. The appearance of two peaks of approximately equal concentration in the heterozygote is referred to as *bisalbuminemia.*

In some patients, amino acid substitution results in a tendency for albumin to form a dimer. This is noted as a band trailing the albumin peak. It usually is not more than 15% of the total albumin. These are called *dimeric variants.*

A rare condition occurs where the patient has practically no albumin visible on electrophoresis of his serum. This is referred to as *analbuminemia.* These patients have elevated globulin levels which compensates in some measure for the lack of albumin. They survive to adult-

hood and thus albumin, in the human, is apparently not essential for life.

In disease, in general, albumin levels are depressed. This may result from the toxic effect of the disease on the liver as in cholera. It may also result from excessive losses in the urine as in the nephrotic syndrome. When the liver is engaged in the synthesis of huge amounts of gamma globulins, as in multiple myeloma, the albumin level falls because of the diversion of the amino acids to antibody synthesis. Where tumor invades the liver, this is aggravated by reduced ability of the liver to synthesize albumin. With malnutrition, the albumin level falls because of lack of adequate nitrogen intake. This is associated with edema resulting from the decreased colloid osmotic pressure of the blood.

In all of the conditions cited above, the ratio of albumin to globulin (A/G ratio) falls. An A/G ratio of less than one is said to be *inverted* and an indication of a disease process. Purified albumin has been administered I.V. as a plasma expander in shock on a wide scale, especially during World War II. In recent years I.V. albumin administration has assumed a major role in supplying protein nitrogen in hyperalimentation. This has resulted in a marked expansion in the production of purified human albumin, mostly from outdated blood and some from plasmapheresis of donors, where the erythrocytes are returned to the donor.

Albumin assay is performed after the albumin is separated by salt precipitation, selective dye binding to albumin, and estimation from the electrophoretic pattern. For minute amounts of albumin, immunochemical procedures are employed.

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5

Glycoproteins and Proteoglycans

A glycoprotein is defined as a protein or polypeptide to which a carbohydrate is attached by a covalent bond. These conjugated proteins are of major biological importance, comprising enzymes, hormones, antibodies, membranes, and the ground substance of every cell. They include not only most of the soluble globulins of the plasma, but insoluble proteins of connective tissue and the lubricant secretions of the various organs of the bodies, the mucoproteins.

Some proteins of the body, such as albumin, retinol-binding protein, thyroxine-binding prealbumin, and hemoglobin contain no carbohydrate. Others, such as the blood group substances, may contain as much as 80% carbohydrate. The proteins of the complement system and hemostasis are glycoproteins. Haptoglobin and ceruloplasmin contain about 20 and 10% carbohydrate, respectively.

Study of the composition of the plasma glycoproteins, especially those which occur in low concentrations, was hampered by the fact that they were heat labile, turning black on heating because of the browning reaction. When heated, the aldehyde group of the carbohydrate portion of the molecule reacts with an amino group of the protein to form a Schiff base, which then polymerizes, turning brown or black. Furthermore, microtechniques were needed for investigating the composition of proteins which occurred in minute amounts in the serum. In the last 25 years, the aminoacid and carbohydrate sequences have been elucidated for many of the glycoproteins.

5.1 *ISOLATION OF THE PLASMA GLYCOPROTEINS*

When 95% ethanol is added to plasma or serum in a ratio of 100:1, some of the proteins of the serum will precipitate. On examina-

TABLE 5.1
Normal Distribution Obtained for the Glycoprotein Fractions of Serum by Electrophoresis[a]

Prealbumin and albumin, %	Globulins, %			
	α_1	α_2	β	γ
2–9	18–22	25–35	22–27	13–18

[a] Results are in percentage of total carbohydrate.

tion, these are found to contain substantial amounts of carbohydrate. These alcohol precipitable, carbohydrate-carrying proteins are the major glycoproteins.[1,2]

If 0.75 *N* perchloric acid is added to serum in the ratio of 8:1, then most of the proteins in the serum will be precipitated. A more soluble carbohydrate-containing fraction remains in solution and may be precipitated with phosphotungstic acid. This latter mixture of proteins is often referred to as the *mucoprotein fraction.* It comprises mainly the α_1-*acid glycoprotein* which travels with the α_1 globulin fraction on electrophoresis.[3]

If serum is spotted on paper, cellulose acetate or some other medium and subjected to electrophoresis at pH 8.6, one may stain for the protein fractions, namely albumin, α_1, α_2, β, and the γ globulins. If one stains instead with the *periodic acid–Schiff reagent* (PAS), the carbohydrates are revealed[4,5]. Carbohydrate occurs in all the protein fractions but mainly in the α and β globulin regions. The distribution of these carbohydrate-containing fractions may be seen in Table 5.1.

Six monosaccharides comprise the main components of the polysaccharide linked with plasma proteins. These are the hexoses, galactose and mannose, the hexosamines, glucosamine and galactosamine, and fucose and the sialic acids. Xylose is also found occasionally.[6] The concentration of these components of serum proteins are listed in Table 5.2. Adding the mean values of Table 5.2, one obtains

TABLE 5.2
Concentration of Monosaccharides in Plasma Proteins, mg/100 ml of Serum

Hexoses	Hexosamines	Sialic acid	Fucose
121 ± 2.1	83 ± 4.9	60 ± 3.7	8.9 ± 0.6

TABLE 5.3

Distribution of Monosaccharides Among the Various Electrophoretic Fractions of Human Serum, g monosaccharide/100 g Protein

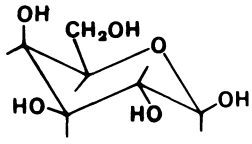
Protein	Hexoses	Hexosamine	Sialic acid	Fucose
Albumin	0.20	0.06	0.10	0.01
α_1 Globulin	7.5	6.3	4.1	0.55
α_2 Globulin	5.9	4.2	3.0	0.40
β Globulin	2.9	1.9	1.5	0.20
γ Globulin	1.9	1.5	1.2	0.30
Total	1.6	1.1	0.87	0.11

a total of 273 mg/100 ml of protein-bound carbohydrate. This value increases by a factor of two or three in the inflammatory and neoplastic diseases, the increase being in all of the components.[7] The distribution of these monosaccharides among the various protein fractions of the serum is given in Table 5.3.

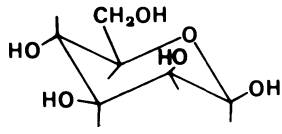
Separation of the Carbohydrate Moiety

The sugars found in the glycoproteins are galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, mannose, *N*-acetylmannosamine, *N*-acetylneuraminic acid, fucose, xylose, and in the cell wall of bacteria, *N*-acetylmuramic acid. The structures of these sugars are given in Figures 5.1 and 5.2. None of the plasma glycoproteins have been shown to contain glucose, except for Clq, a component of the complement system. However, the tissue (e.g., collagen) and membrane glycoproteins do contain glucose.[8] The carbohydrate units of glycoproteins are first isolated as glycopeptides with a minimum number of amino acids attached after extensive enzymic proteolytic digestion.[9] From a study of these glycopeptides, the size, number, and composition of these carbohydrate units can be estimated. The point of attachment to the polypeptide then can be determined.

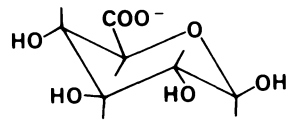
The carbohydrate units range in size from a molecular weight of approximately 3500 (as in fetuin) to single monosaccharide residues of molecular weight 162 (as in the collagens). There may be only a single carbohydrate unit per molecule as in ribonuclease, or there may be as many as 800, as in the ovine submaxillary glycoprotein. Since



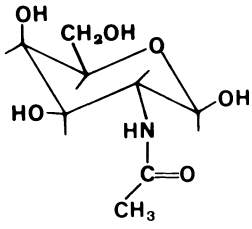
β-D-Galactose (Gal)



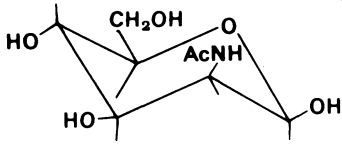
β-D-Mannose (Man)



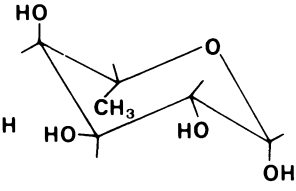
β-D-Glucuronate (GlcUA)



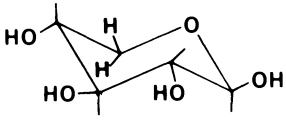
β-*N*-Acetyl-D-Galactosamine (GalNAc)



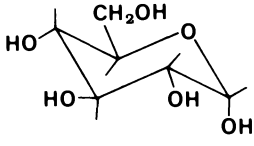
β-*N*-Acetyl-D-Mannosamine (ManNAc)



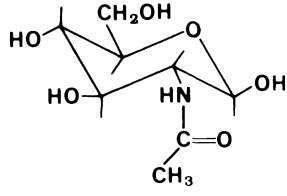
α-L-Fucose (Fuc)



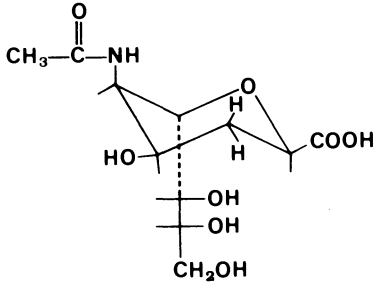
β-D-Xylose (Xyl)



α-D-Glucose (Glc)



β-*N*-Acetyl Glucosamine (GlcNAc)



N-Acetyl Neuraminic Acid (NANA)

FIGURE 5.1 Conformation of the sugars commonly found in the carbohydrate portion of the plasma glycoprotein molecules, with commonly used abbreviations (see Appendix, Volume 2, pp. 535–541).

glycoproteins vary so much in molecular weight, from 14,500 for ribonuclease to 1×10^6 for the ovine submaxillary glycoprotein, the average spacing of the carbohydrate units along the peptide chain (that is, the number of amino acids per carbohydrate unit) is a better

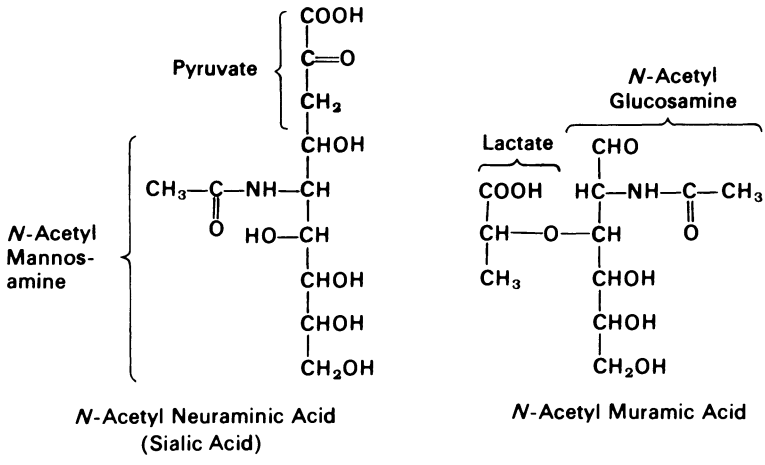


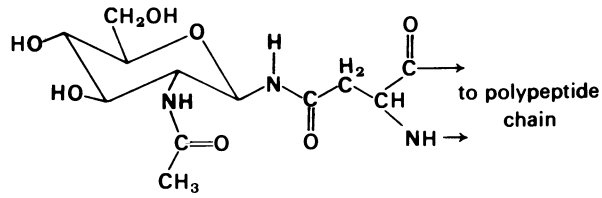
FIGURE 5.2 Comparison of the structure of *N*-acetyl neuraminic acid and *N*-acetyl muramic acid. Neuraminic acid is derived from *D*-mannosamine and pyruvic acid; *N*-acetyl muramic acid is D-glucosamine connected by ether linkage with lactic acid. In sialic acid the acetyl group may be replaced with the glycyl residue.

index of the extent of carbohydration of the protein than the number of units per molecule. This spacing of carbohydrate units may be seen to vary considerably, with as few as six amino acid residues per unit in the ovine submaxillary glycoprotein, to as many as 779 amino acid residues per carbohydrate unit in the IgG immunoglobulins.

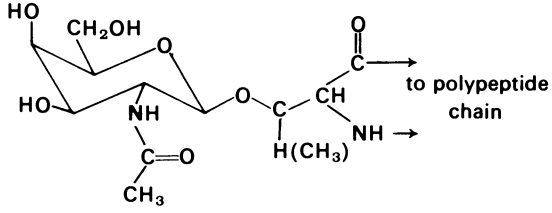
It may be noted, that most glycoproteins contain only one type of carbohydrate unit. A few, however, like thyroglobulin and the glomerular basement membrane, contain more than one distinct type.

5.2 *THE NATURE OF THE CARBOHYDRATE PROTEIN LINKAGE*

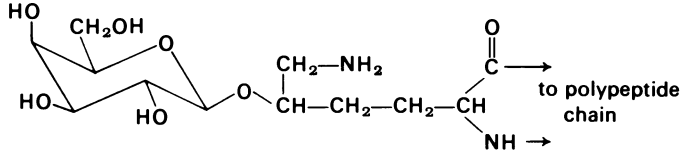
The covalent attachment of the carbohydrate units of glycoproteins has been uniformly shown to involve carbon 1 of the most internal sugar residue, and a functional group on an amino acid in the peptide chain. The three distinct types of glycopeptide bonds have been shown to occur in protein from animal tissues (see Figure 5.3). *The first glycopeptide bond to be ascertained was between carbon 1 of N-acetyl-glucosamine and the amide nitrogen of asparagine. This represents a glyco-*



β-D-*N*-Acetyl-Glucosaminyl-Asparagine Linkage



β-D-*N*-Acetyl-Galactosaminyl-Serine Linkage
(threonine linkage, CH_3 for H)



β-D-Galactosyl-Hydroxylysine Linkage

FIGURE 5.3 Linkages of the carbohydrate portion of the glycoprotein molecules in the polypeptide chain.

sylamine type linkage.[10] It was originally described in ovalbumin,[11] and has subsequently been found in a large variety of glycoproteins from diverse sources, including IgG immunoglobulin,[12] ribonuclease,[13] deoxyribonuclease,[14] α_1-acid glycoprotein,[11] fetuin,[13] thyroglobulin,[14] and ovomucoid.[15] It is the most common type of linkage of carbohydrate to protein in the plasma proteins.

A second glycopeptide linkage involves an O-glycosidic bond to serine or threonine. It is easily split by very mild alkali. The sugar component of this type of linkage is often *N*-acetylgalactosamine. The *N*-acetylgalactosamine is involved in the linkage of various mucins.[16] The molecular weight of these proteins is generally on the order of several millions. In these glycoproteins, the number of oligosaccharide residues per total amino acid residue is very high and solutions of these proteins

are very viscous. In the mucins, serine and threonine account for up to 40–50% of amino acid residues present, and a large proportion of these are substituted by carbohydrate groups. The sugar involved in this linkage is galactose or xylose, or more often *N*-acetylgalactosamine, usually in a β linkage[17] (see Figure 5.3). This type of linkage occurs in the immunoglobulins.

A third type of glycoprotein linkage occurs in basement membranes[18] *and collagens.*[19] *It involves an O-glycosidic linkage between a galactose residue and the hydroxyl group of hydroxy lysine.* This linkage is an unusual one, for it involves a glycosidic substitution on to a hydroxyl group which at physiological pH values is positively charged. Contrary to the *O*-glycosides of serine and threonine, this glycopeptide bond is very stable to alkaline hydrolysis permitting the isolation of the carbohydrate unit attached to hydroxylysine in very high yield. This linkage occurs in the Clq component of the complement system. It occurs commonly in collagen.

Studies of a number of glycoproteins such as ovalbumin, various ribonucleases, the immunoglobulins, and others, have revealed that where an oligosaccharide is attached to the amide of aspartate through *N*-acetylglucosamine, the sequence is always Asn-X-Thr- (or Ser-), where X is any amino acid with the exception of proline and the aromatic amino acids.[19–26] For example, in IgM, X can be alanine,

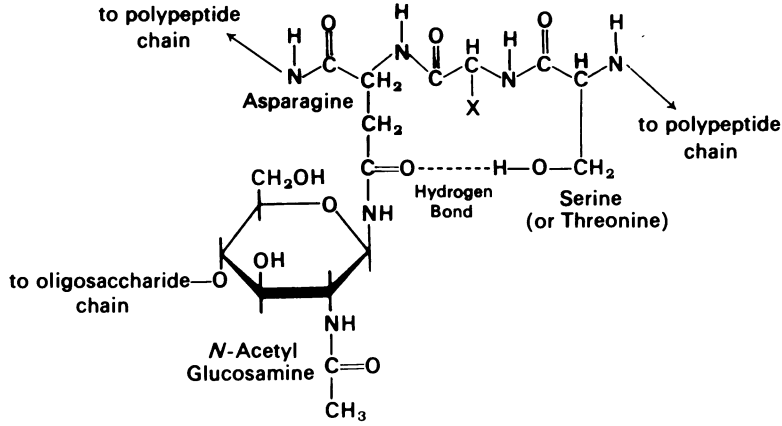


FIGURE 5.4 Proposed reason for occurrence of glycoside attachment at sequence —Asn-X-Ser(Thr)-. Hydrogen bond loosens hydrogen on amide, permitting substitution of glycoside residue.

asparagine, glycine, isoleucine, or leucine. For α_1 acid glycoprotein, X occurs as lysine, threonine, or alanine. In ceruloplasmin, X can be valine or leucine. In transferrin, X occurs as valine and lysine. These few examples suggest that this is more than a coincidence. It has been suggested that, in order for the *N*-acetylglucosamine to be attached, the hydroxyl group of the serine or threonine residue needs to form a hydrogen bond with the carboxyl group of the asparagine (Figure 5.4). This serves to activate the hydrogen on the amide and permit substitution of the glycosidic linkage.[27]

5.3 *STUDIES OF THE STRUCTURE OF THE HETEROSACCHARIDES*

In order to comprehend the function of the oligosaccharides attached to the polypeptides in the glycoproteins, certain facts need to be explored. These may be listed as follows:

1. The monosaccharide units which make up the oligosaccharides need to be identified.
2. The nature of the carbohydrate–peptide linkage needs to be ascertained.
3. The monosaccharide sequences and branching need to be unraveled.
4. The sites on the polypeptide chain where the oligosaccharides are attached need to be located.

Elucidation of the structure of the heterosaccharides attached to the plasma proteins is a complex problem. Examination of Figure 5.1 reveals that, after attachment of the monosaccharide to the polypeptide chain, there remain four or five hydroxyl groups to which a second monosaccharide may be attached. Thus, the sequence of a simple disaccharide, GlcNAc—Gal, may signify at least four isomers. If another monosaccharide is added, it can then be bound to GlcNAc or Gal, in any of seven different locations. The linkage of the hydroxyl groups in the 1 position can also be α or β. Thus an oligosaccharide comprising only a few monosaccharides can give rise to a huge number of possible structures.

Another problem which presents itself is the fact that synthesis of

the heterosaccharide side chains attached to polypeptides is relatively inaccurate, when compared to the precision with which the polypeptides are synthesized. The polypeptides are synthesized on a template which guides their formation. No such template exists for the addition of the monosaccharides. These sugars are added one by one by a mechanism which is presented in Section 5.4. If a monosaccharide is in short supply, it may be omitted. This gives rise to glycoproteins prepared in the same individual which may vary slightly. This phenomenon is called *microheterogeneity*, and accounts for some of the heterogeneity observed when isolating glycoproteins such as ceruloplasmin, the haptoglobins, and others.

In spite of the complexity of this field of study, substantial progress has been made in elucidating the structure of the polysaccharide side chains of a number of proteins. The term used by the experimenters in this field for these side chains is *heterosaccharide* to indicate a structure made up of different sugars. *Oligosaccharide* derives from the Greek *oligos*, meaning little or a few, and saccharide, meaning sugar. Thus the term oligosaccharide denotes a lesser number of monosaccharides forming a chain or matrix. *Polysaccharide* is reserved for the large complex carbohydrate structures, usually made up of a single monosaccharide such as glycogen and cellulose (glucose), inulin (fructose), mannan (mannose), galactan (agar, galactose), and others.

A major means for unraveling the sequence and linkages of the glycoproteins is the use of specific carbohydrases, all of which occur in the lysosomes of cells. These include neuramidinases, the α and β galactosidases, fucosidases, glucosidases, hexoseaminidases, and xylosidases and finally β-aspartylglucosylamine: amidohydrolase to release the sugar from the aspartylamide of the polypeptide chain. This is outlined schematically in Figure 5.5.[26]

In the normal individual, ingested glycoproteins are dismantled with the use of the enzymes shown in Figure 5.5. Genetic deficiency of any of these enzymes results in the accumulation of glycoproteins, glycolipids, and polysaccharides, resulting in a number of diseases, including the lipidoses, Hurler's disease, and related syndromes. In addition, where sulfate is attached to the carbohydrate, as in collagen, sulfatases are required for digestion. These sulfatases also serve in the elucidation of the complex carbohydrate structures.

Digestion of certain proteins is incomplete, unless the carbohydrate is first removed. Thus, where carbohydrate forms a high percentage of

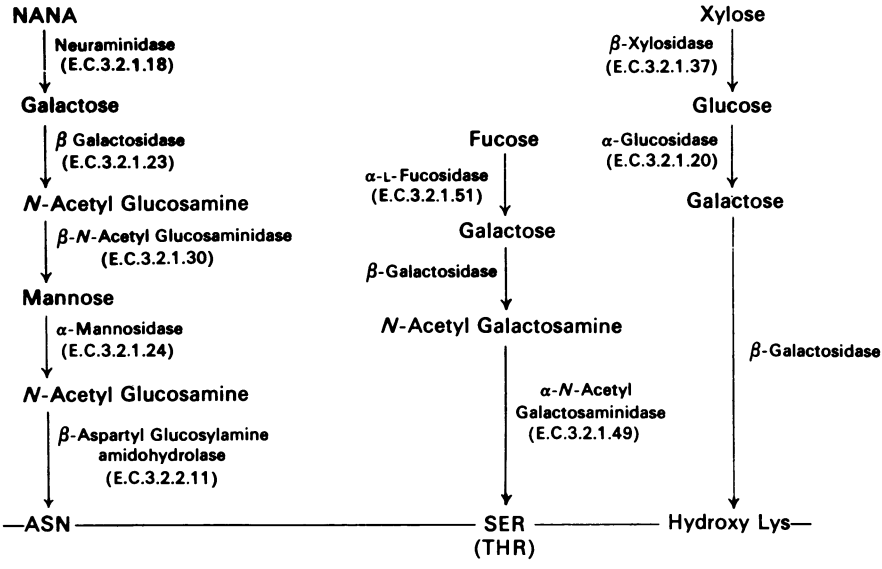


FIGURE 5.5 Representation of the lysosomal enzymes employed in studies of heterosaccharide structure and in digestion of glycoproteins.

the molecule, it serves to protect the polypeptide chain from proteolytic attack.

5.4 *HETEROSACCHARIDE SYNTHESIS*

Under the electron microscope, the endoplasmic reticulum of the cell appears as an extensive network of tubules, vesicles, and lamellae (variant of *laminae*) which in Latin means thin flat layers or leaves. It comprises a complex of membranes of extensive surface, which are composed of 60–70% protein and 30–40% phospholipid by weight. During homogenization of cells the endoplasmic reticulum breaks up, forming closed vesicles called *microsomes*. Many of these vesicles carry ribosomes on their outer surface. The cytochromes b_5 and P450-NADPH enzyme systems are located in the endoplasmic reticulum as well as a substantial number of other enzymes, *especially those involved in the synthesis of the carbohydrate moiety of the glycoproteins.*

Some of the endoplasmic reticulum of the cell appears "rough" because of the accumulation of ribosomes on its surface. It is here that

the polypeptides are assembled.[27–29] A message derived from RNA translation (mRNA) results in the synthesis of the polypeptides on the ribosomes bound to the endoplasmic reticulum (see Figure 5.6). It will be noted that protein synthesis also takes place on ribosomes bound to the nuclear membrane, which is, in effect, part of the endoplasmic reticulum.

The purpose of the embedding of the ribosomes in the endoplasmic reticulum is so that the formed peptide chain can pass into the cisternal space beneath. This isolates the newly formed molecule for further processing. In the cisternal compartment, some modification takes place, such as disulfide bond formation and hydroxylation of lysine and proline by the b_5 and P-450 systems. This is required for collagen synthesis and the glycosylation steps, which are the topic of discussion in this section.

The polypeptide moves along the smooth surface of the cisterna with energy supplied from ATP and encounters areas where specific transferases are located for sequentially glycosylating the particular glycoprotein being synthesized by the cell. This is shown schematically in Figures 5.6 and 5.7.

Most commonly, as far as the plasma glycoproteins are concerned, *N*-acetylglucosamine is the first monosaccharide to be attached to the amide nitrogen of an asparagine of the polypeptide chain (Figure 5.4).[30–32] In some cases, as indicated in Figure 5.5, attachment is to the hydroxyl appendage of serine or threonine. In this case, it is not

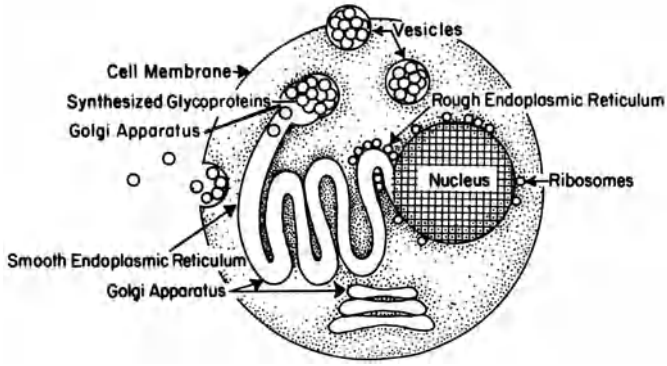


FIGURE 5.6 Schematic representation of the function of the endoplasmic reticulum and Golgi apparatus in the synthesis of glycoproteins, formation of vesicles, and discharge of the glycoproteins into the blood stream.

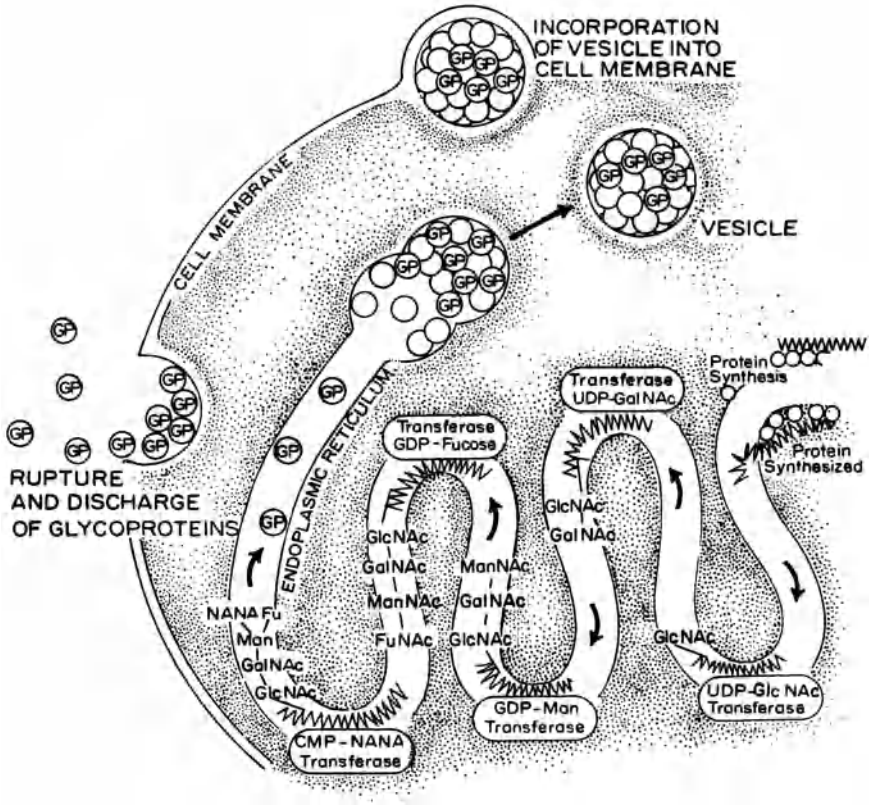


FIGURE 5.7 The role of the various transferases and activated monosaccharides in glycoprotein synthesis. In this hypothetical case, the sequence should be, NANA (Fu)-Man-GalNAc-GlcNAc-.

unusual for the *N*-acetylgalactosamine to be the first sugar attached. In special proteins, such as collagen, the attachment to hydroxylysine also occurs. The innermost monosaccharide is then often galactose.

The transferase always acts by exchange of the monosaccharide bound to a nucleotide, such as in uridine diphosphoglucosamine (UDP-GlcNAc), for a hydrogen on the —CO:NH_2 or —OH group of the polypeptide, for example:

$$\text{UDP—GlcNAc} + \text{Prot—}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{—NH}_2 \xrightarrow{\text{transferase}} \text{UDP} + \text{Prot—}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{—}\overset{\overset{\text{H}}{|}}{\text{N}}\text{—GlcNAc}$$

$$\text{UDPGal} + \text{Prot—OH} \xrightarrow{\text{transferase}} \text{UDP} + \text{Prot—Gal}$$

The monosaccharide bound to the nucleotide acts as a substrate for a particular transferase and, in this form, is said to be *activated.* The nucleotide to which the monosaccharide is attached is not the same in every case. For example, for glucose, galactose, xylose, arabinose, and their *N*-acetylamino derivatives, uridine diphosphate is bound to the sugar. For mannose and fucose, guanosine diphosphate is the nucleotide portion of the activated complex. For the sialic acids (*N*-acetyl-, glycyl-, or glycolylneuraminic acids), cytidine monophosphate forms the activating complex. The structures of some of these activated substrates are shown in Figure 5.8.

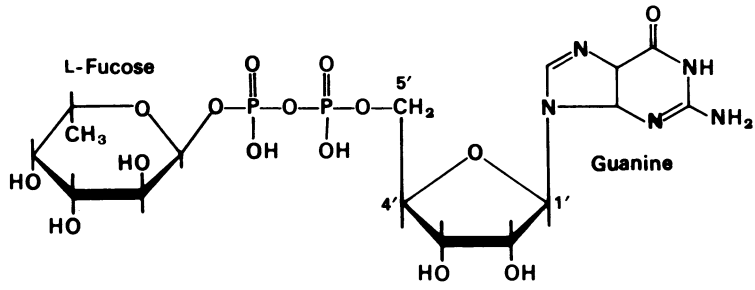
All monosaccharides found in the glycoproteins are derived from glucose. Figure 5.9 shows this relationship. In this figure, the activated forms of the monosaccharides used in the synthesis of the plasma glycoproteins are placed in a box. A total of ten are shown; however, the list is incomplete. For example, iduronate occurs as a component of some heterosaccharides.

Referring to Figure 5.7, after the first monosaccharide is added, the polypeptide moves along to the next station at which a second sugar is added. Branching may occur, as indicated. After NANA is added, no other sugar is added except that a second or third molecule of NANA may be attached to NANA. After fucose is added to a chain, it indicates the end of that chain or branch. It is not unusual for the same monosaccharide to be repeated in a chain. For example, as many as six mannose residues commonly occur in sequence in some glycoproteins.

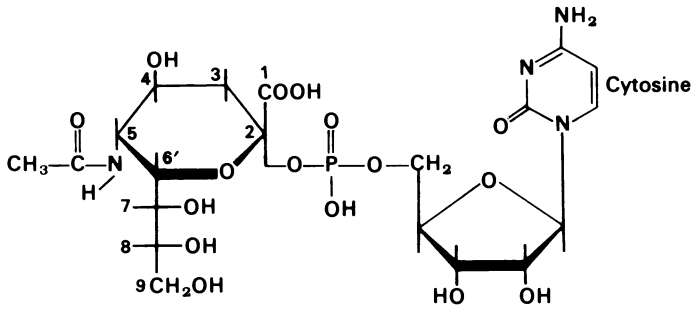
After the glycoprotein is synthesized, it may be sulfonated by a sulfotransferase using 3′-phosphoadenylylsulfate (PAPS) as the sulfate donor (see Volume 1, pp. 179–183).

When the glycoprotein is finally formed, it is pushed along the cisternae to a dead end, where the protein is concentrated in an enlarged balloon-like space. An approximately spherical portion, engorged with glycoprotein, breaks off to form a vesicle loaded with glycoprotein. This moves to the surface of the cell and fuses with the cell membrane. The vesicle then breaks open, expelling the contents of the vesicle into the blood stream.

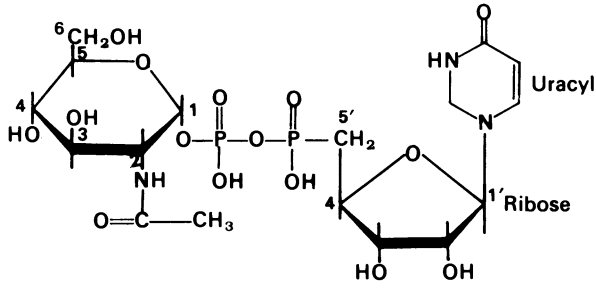
The endoplasmic reticulum and bulbar end resemble in appearance a deflated toy balloon. This shape permits the structures to be stacked one on top of each other. The complex so formed was first noted by Golgi and is therefore referred to as the *Golgi apparatus.*[33–37]



Guanosine-5′-diphospho-L-Fucose
(GDP-L-Fuc)

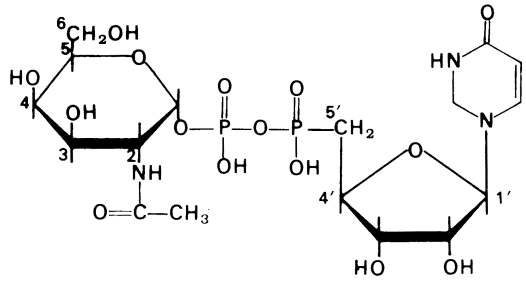


Cytidine-Monophospho-*N*-Acetyl Neuraminic Acid
(CMP—NANA)

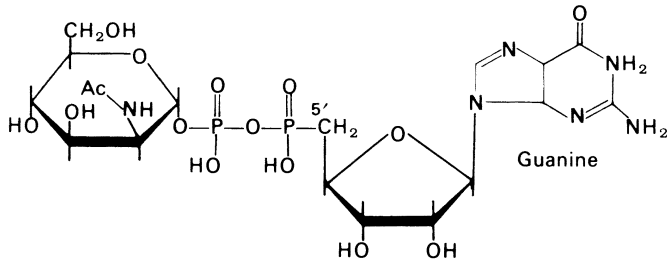


Uridine-5′-Diphospho-Acetyl Glucosamine
(UDP-GlcNAc)

FIGURE 5.8 Activated forms of the various monosaccharides employed in the synthesis of the heterosaccharides. UDP-Gal and UDP-Glc are not shown. They have structures similar to that of UDP-Glc-NAc and UDO-Gal-NAc, respectively, with the *N*-acetyl group replaced by an —OH group. (See Volume 2, p. 120.)



Uridine-5′-Diphospho-Acetyl Galactosamine
(UDP-GalNAc)



Guanosine-5′-Diphospho-Acetyl Mannosamine
(GDP-ManNAc)

FIGURE 5.8 (*continued*)

Figure 5.10 is a reproduction of an electron micrograph showing the structure and stacking of the Golgi complex.

Dolichol and Retinol in Glycoprotein Synthesis

From various sources, such as milk, hen oviduct, mammary gland, submaxillary gland, and other tissues rich in glycoprotein, oligosaccharides were isolated attached to a nucleotide. Typical examples are UDP ← GlcNAc ← Gal; UDP ← GlcNAc ← Gal ← NANA; UDP ← GlcNAc ← Gal ← Fuc; UDP ← GlcNAc ← sulfate; and others. From this the concept derived that an oligosaccharide attached to a single nucleotide could be transferred as a whole. This would be a more efficient system, since it could take place at one location on the endoplasmic reticulum.

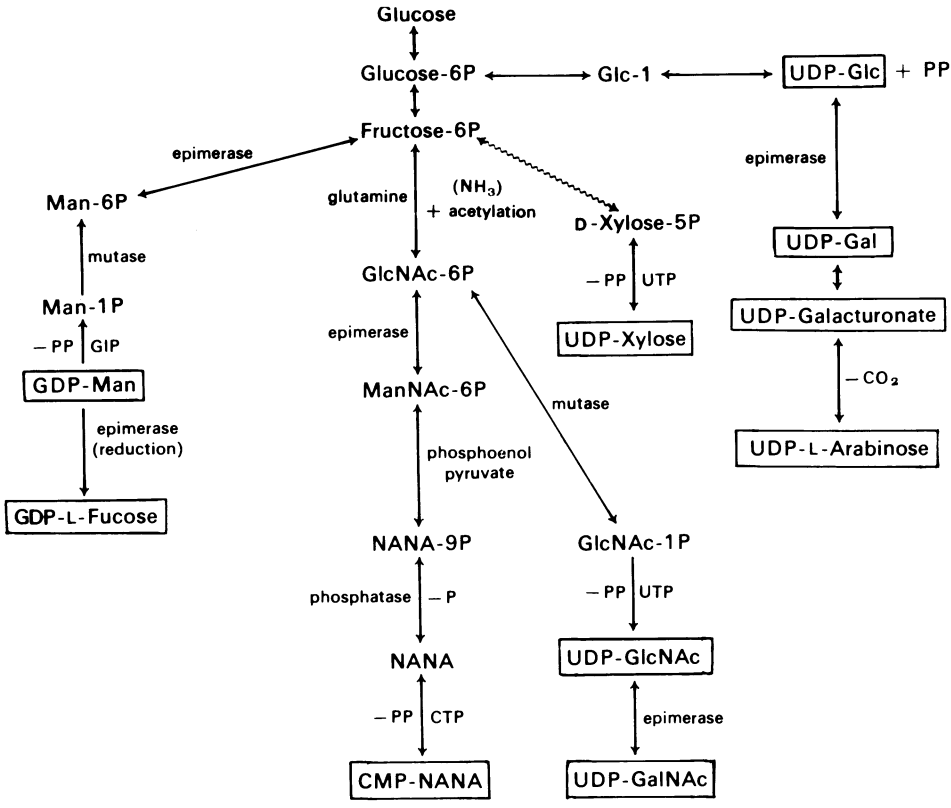


FIGURE 5.9 Formation of the activated monosaccharides from glucose. The activated sugars which take part in glycoprotein synthesis are underlined. See Volume 2, pp. 114–125 and 157 for the relationship between glucose, fructose, and UDP-glucose. All sugars are in the D-form except where indicated as L. A wavy arrow indicates a complex pathway.

Earlier it was pointed out that the endoplasmic reticulum was 30–40% phospholipid. Thus it would be reasonable to assume that a lipid anchored at a site could serve to collect the monosaccharides before transferring them to the newly synthesized protein. This seems to be the case for many of the glycoproteins synthesized. The lipids involved in the transport have been identified as *dolichol* and *retinol* (*vitamin A*).[38–46] See Figure 5.11.

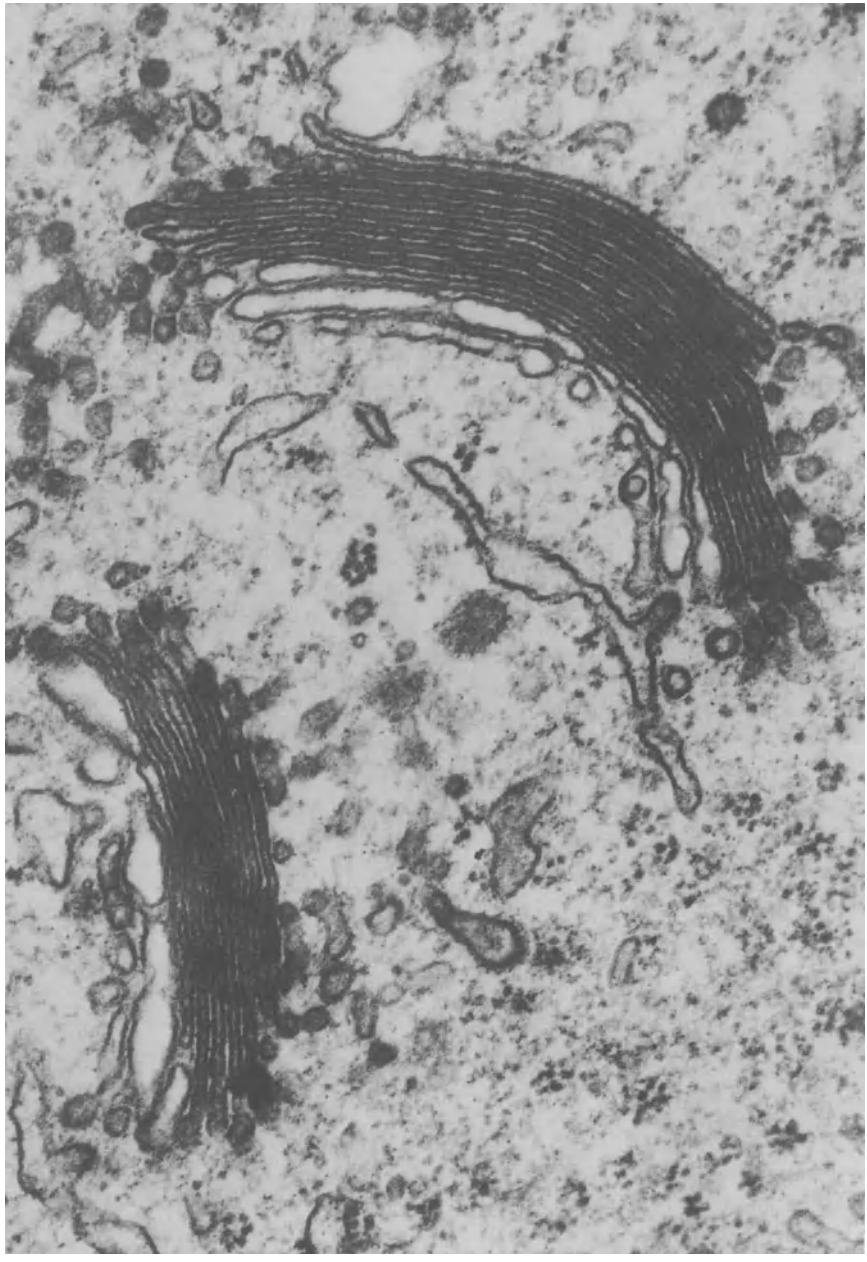
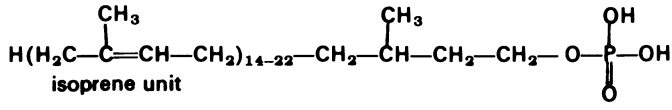
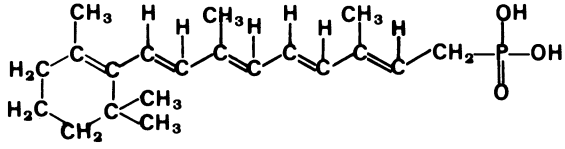


FIGURE 5.10 Electron micrograph showing the stacking of the Golgi apparatus. Scale: 1 in. = 0.37 μm. Courtesy of M. Dauwalder, Cell Research Institute, University of Texas at Austin.



Dolichol Phosphate



Retinol Phosphate
(vitamin A phosphate)

FIGURE 5.11 Structures of dolichol and retinol monophosphate. Both are polymers of isoprene. Dolichol is of variable composition, comprising 15–22 isoprene units. Retinol (vitamin A) contains only 4 isoprene units.

Each of the monosaccharide donors (Figure 5.8) donates its sugar to the dolichol by means of its transferase. These transferases are highly specific for nucleotide and sugar structure and are tightly bound to the "rough" and smooth endoplasmic reticulum. Retinol can substitute for dolichol.[47a,b] Thus, the glycosides are formed in a hydrophobic environment. This system is used to attach the first carbohydrate core chain to the newly synthesized protein. The system outlined in Figure 5.7 seems to add the side chains. The reaction taking place may be typified as follows, using UDPG (uridine diphosphoglucose) as an example:

$$\text{UDPG} + \text{Dol—P} \xleftrightarrow{\text{transferase}} \text{Dol—P}_2\text{—Glc} + \text{UMP}$$

$$\text{Dol—P}_2\text{—Glc} + \text{protein} \xrightarrow{\text{transferase}} \text{Protein—Glc} + \text{Dol—P}_2$$

$$\text{Dol—P}_2 \xrightarrow[\text{hydrolase}]{\text{—P}} \text{Dol—P}$$

A typical buildup of a core oligosaccharide is shown in Figure 5.12. This structure, transferred to the newly formed protein, serves as a base for addition of other sugars and possible sulfation before being released into the blood stream or some other secretion, such as mucus.

It has been known for some time that vitamin A was involved in some way with the formation of connective tissue. For example, a fetus could not retain its implantation in the uterus without vitamin A. The

$$\text{UDP}\leftarrow\text{GlcNAC} + \text{Dol-P} \rightarrow \boxed{\text{Dol-P}_2\leftarrow\text{GlcNAc}} + \text{UMP}$$

$$\text{GDP}\leftarrow\text{Man} + \boxed{\text{Dol-P}_2\leftarrow\text{GlcNAc}} \rightarrow \boxed{\text{Dol-P}_2\leftarrow\text{GlcNAc}\leftarrow\text{Man}} + \text{GDP}$$

$$\text{UDP}\leftarrow\text{Glc} + \boxed{\text{Dol-P}_2\leftarrow\text{GlcNAc}\leftarrow\text{Man}} \rightarrow$$

$$\boxed{\text{Dol-P}_2\leftarrow\text{GlcNac}\leftarrow\text{Man}\leftarrow\text{Glc}} + \text{UDP}$$

$$\text{Protein} + \boxed{\text{Dol-P}_2\leftarrow\text{GlcNAc}\leftarrow\text{Man}\leftarrow\text{Glc}} \rightarrow$$

$$\boxed{\text{Protein}\leftarrow\text{GlcNAc}\leftarrow\text{Man}\leftarrow\text{Glc}} + \text{Dol-P}_2$$

$$\text{Dol-P}_2 \xrightarrow{-\text{Pi}} \text{Dol-P}$$

FIGURE 5.12 Representation of the role of dolichol monophosphate in core glycosyl synthesis and transfer to form a glycoprotein. Each of the steps can be repeated; thus the final product may contain repeating units of GlcNAc and especially mannose.

mechanism discussed gives a role to vitamin A in glycoprotein synthesis, and thus the synthesis of ground substance and connective tissue. Its relationship to dolichol is not clear at present. Retinoic acid (oxidized retinol) relieves most symptoms of vitamin A deficiency. Reduction of retinoic acid to retinol has not been demonstrated in animal tissue. These problems need clarification.

Dolichol is synthesized from farnesyl pyrophosphate (Figure 5.13 and Appendix). Thus deficiencies in mevalonate synthesis or any

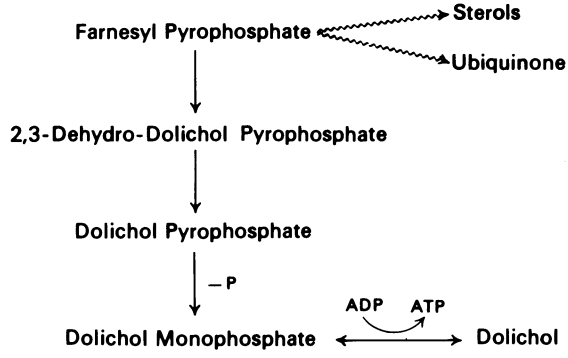


FIGURE 5.13 Synthesis of dolichol monophosphate in human tissue.

of the intermediate steps leading to dolichol synthesis may account for symptoms noted in certain diseases.

5.5 *GLYCOPROTEIN HETEROSACCHARIDE STRUCTURE*

The mechanism of heterosaccharide synthesis proposes that an oligosaccharide is built up on dolichol, which is then transferred to the newly synthesized protein. This is then added to as indicated in Figures 5.6 and 5.7. For this reason, it would be expected that the core structure of the oligosaccharides of the glycoproteins would resemble each other, and this is actually the case. A structure proposed for an oligosaccharide unit attached to dolichol is shown in Figure 5.14.[45,47]

One of the first glycoprotein oligosaccharide sequences determined was that for transferrin. Transferrin has two oligosaccharides which are identical. Their approximate structure is shown in Figure 5.15. In spite of extensive work on this problem, unresolved discrepancies still exist as to the structural detail.[48–50]

Another group of plasma proteins, where substantial progress has been made, is in the oligosaccharide structure of the immunoglobulins. This problem is complicated by the fact that all of the oligosaccharide chains are not identical. For example, IgE has four oligosaccharides per heavy chain, and IgM has five per heavy chain.[51–53]

An oligosaccharide, common to the immunoglobulins IgG, IgA, and IgE, is shown in Figure 5.16. This resembles that for transferrin, except for the attachment of the fucose residue. In these immunoglobulins, a high-mannose-containing oligosaccharide is also found.

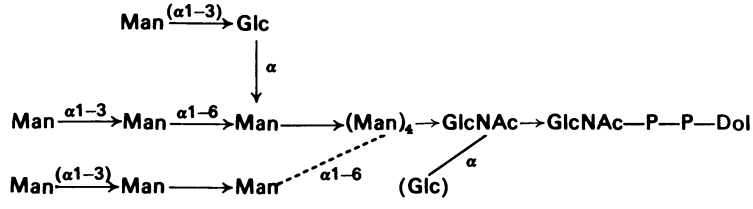


FIGURE 5.14 Provisional structure proposed for a dolichol-P-P-oligosaccharide.

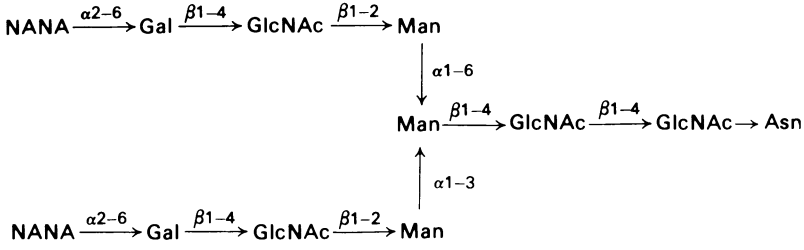


FIGURE 5.15 Structure of the heterosaccharides of transferrin. The transferrin molecule has two such identical structures.

Two of these structures are shown in Figure 5.16. A disaccharide isolated from human A erythrocytes is also shown in this figure.

These high-mannose-containing oligosaccharides resemble the single oligosaccharide attached to hen ovalbumin (Figure 5.17).[54]

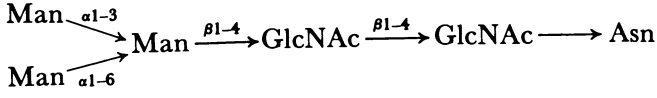
Human albumin does not contain a carbohydrate residue. One similar to that of the hen albumin oligosaccharide has been found in thyroglobulin.[55] Thyroglobulin also contains a unique oligosaccharide comprising a repeating glucuronate–galactosamine disaccharide attached to the peptide chain through a galactosyl xylosyl–serine linkage.[56] This structure also occurs in the proteoglycans (see Figure 5.42).

A glycopeptide has been isolated from human urine comprising glucose, fucose, and threonine. This is unusual in the fucose–threonine linkage, as follows[57]:

$$\text{Glc} \xrightarrow{\beta 1\text{–}3} \text{Fuc} \longrightarrow \text{Thr}$$

This is probably a fragment of an unidentified glycoprotein and suggests a normal type of linkage to the polypeptide chain.

In almost all of the glycoproteins found in substantial quantities in the plasma where attachment to asparagine occurs it is now apparent that an oligosaccharide core structure seems to be present. This may be represented by



Another example of such a structural type is that found in α protease inhibitor (antitrypsin). This is seen in Figure 5.18. This

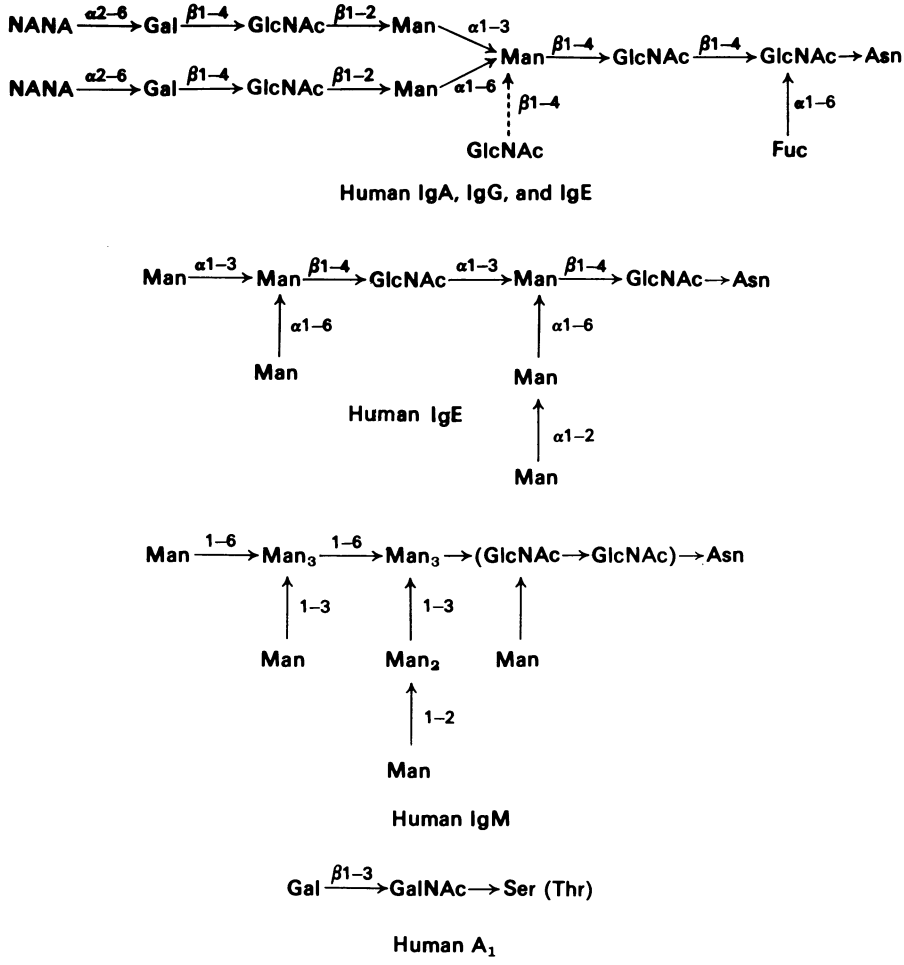


FIGURE 5.16 Some of the oligosaccharides of the immunoglobulins. The structure with the broken line attached to GlcNAc is found in IgE.

molecule has four oligosaccharides, two of which have a trisaccharide (NANA-Gal-GlcNAc) attached and two without.[58]

5.5.1 *Heterosaccharide Structure of the Blood Group Glycoproteins*

The major carrier of the oligosaccharides on the erythrocyte responsible for the immunological characteristics of blood groups is glycophorin (Volume 2, pp. 37–39, 51–55). This molecule is 60%

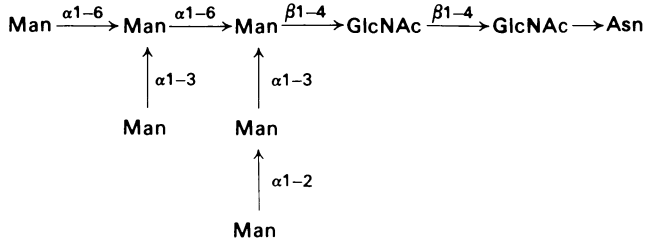
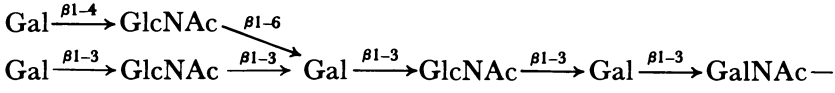


FIGURE 5.17 The glycopeptide of hen ovalbumin. Ovalbumin (mol. wt. 45,000) has only one oligosaccharide per molecule.

carbohydrate, of which sialic acid (NANA) represents more than 40%. The polypeptide chain traverses the cell wall. The carbohydrate-containing portion extends outside the cell (see Figure 5.19).[59] Variations in the structures of the oligosaccharides of glycophorin are responsible for the determination of some of the blood groups.

Glycoproteins having the blood group specificities are present in the secretions of the body such as saliva gastric juice and ovarian cyst fluid. These are attached to the protein at the hydroxyl group of a serine or threonine residue in the polypeptide chain. The blood group specificity is determined by the nature and linkage of the monosaccharides at the nonreducing end of the heterosaccharide matrix. The core, not involved in determining specificity, seems to be the same in all blood groups. It probably has the following structure:



To this oligosaccharide are attached fucose, galactose, and *N*-acetylgalactosamine, to form the determinants of the ABH and Lewis specificities as shown in Figure 5.20.[60–66]

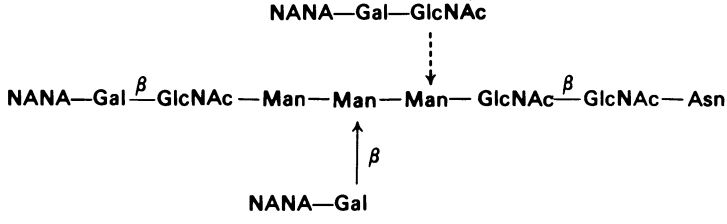


FIGURE 5.18 Oligosaccharides found in α-protease inhibitor (antitrypsin). There are four oligosaccharides per molecule, two with the NANA→Gal→GlcNAc, and two without.

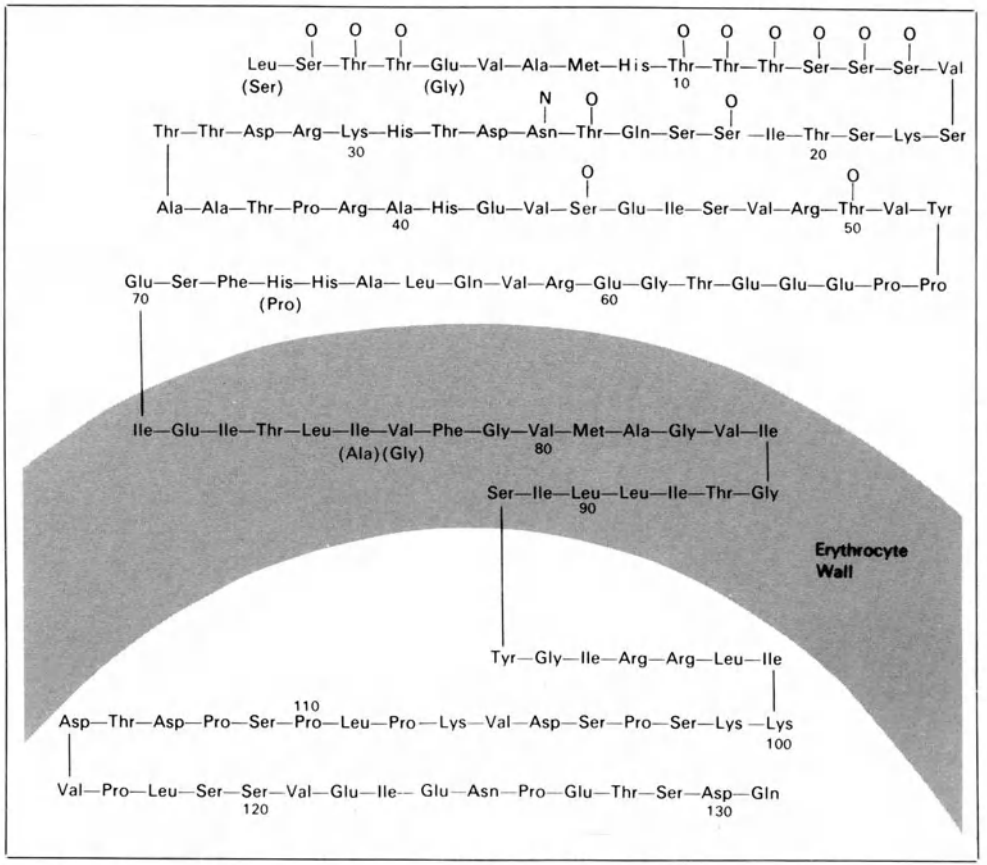


FIGURE 5.19 The arrangement of glycophorin on the erythrocyte wall. See Volume 1, p. 39. The symbol "O" represents the ether linkage of the oligosaccharide to serine or threonine, and the "N" represents the linkage to asparagine.

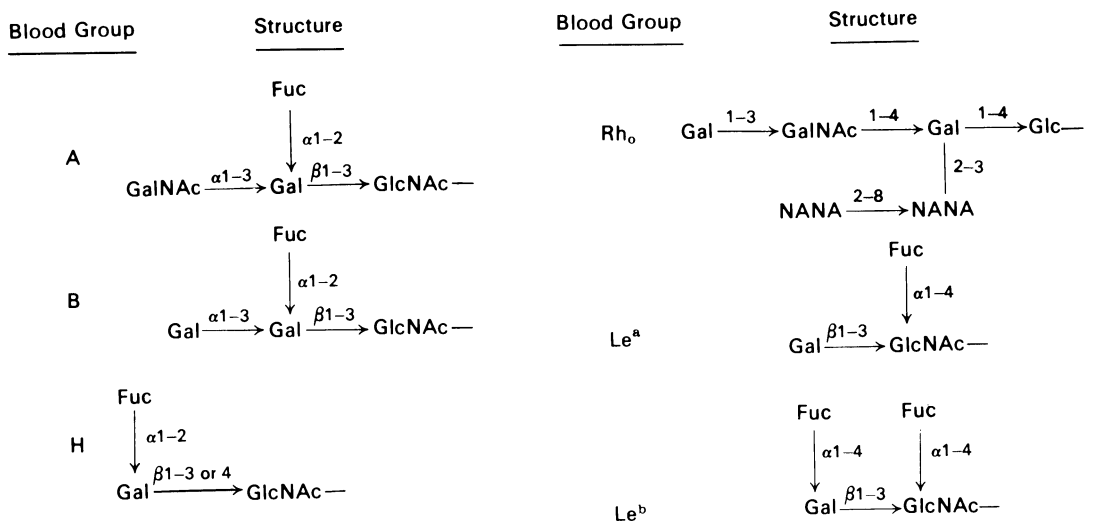


FIGURE 5.20 Structures determining A, B, H, Rh_0, and Lewis specificities of the glycoproteins.

The Lewis blood group system is primarily one of saliva and serum antigens rather than red cell antigens.[67] The red cells acquire their Lewis phenotype simply by adsorbing Lewis substances from the serum.[68] Since some individuals are nonsecretors (about 20%) they will not possess these antigens unless obtained by transfusion from some donor who is a secretor. Although rare, severe hemolysis during transfusion has been reported with Lewis incompatibility between donor and recipient.

The immunodominant sugar for each specificity is fucose for H, GalNAc for A, Gal for B, Fuc for Le^a, and two fucoses for Le^b. The ability to form the structures shown in Figure 5.20 is controlled by the action of genes at four independent loci, ABO, Lele, Hh, and Sese. *These genes are responsible for the synthesis of the specific transferases for the formation of the structures shown in the figure.* Thus the *A* gene produces a GalNAc-transferase, the *B* gene a Gal-transferase and the *H* and *Le* genes produce two different Fuc transferases. These enzymes have been identified in the milk, submaxillary glands, and gastric mucosa of mothers carrying the particular blood type.

The reactions mediated by the specific transferases during the formation of the erythrocyte are shown in Figure 5.21. Thus, the

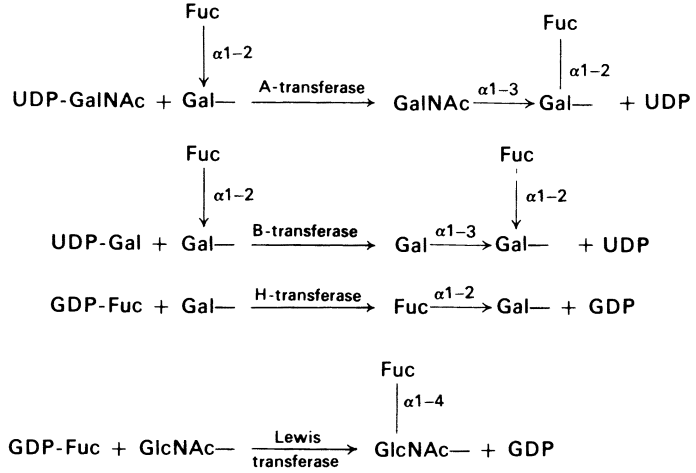


FIGURE 5.21 The mechanisms for the synthesis of the various blood group structures.

inheritance of blood groups, is the inheritance of the ability to synthesize certain glycosyl transferases and the relative activity of these transferases. The study of the structure of the numerous blood groups has developed into a study of the heterosaccharide structures and the enzymes responsible for their synthesis.

By the action of a suitable specific carbohydrase, types A and B can be converted to type H.[62] If erythrocyte type A is acted upon by an α-*N*-acetylgalactose-aminidase, and type B by an α-galactosidase, then both types of erythrocytes can by converted to O(H) by the use of suitable enzymes. This suggests the practicability of creating a large central erythrocyte bank of all type O blood, obtained naturally or by the action of enzymes on types A and B. Alternatively, by the use of a suitable transferase, a sugar can be added to an H antigen to make the A or B antigen.[64]

It is of interest that patients with type A blood lack the specific α-hexosaminidase and patients with B blood lack the α galactosidase. *Thus, patients with types A and B blood may be considered as patients with genetic deficiencies.*

A similar situation, as with the ABH system, exists for the MN system. For example, M specificity is readily transformed to N by incubation at pH 2 and 56°C.[65,66] This treatment removes one sialic acid, exposing the N-active terminal structure. This has another terminal sialic residue resistant to hydrolysis (Figure 5.22).

A composite structure indicating the location of the specific blood group antigens on the oligosaccharide is shown in Figure 5.23.[62] It can be readily seen from the figure, that it is possible to have one GalNAc on one leg of the structure and Gal on the other to make the AB blood type of the erythrocyte.

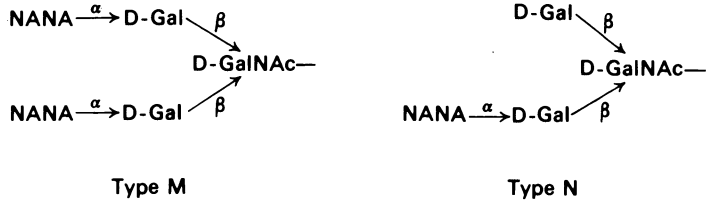


FIGURE 5.22 Proposed terminal immunoreactive structure for the MN system. Removal of one sialic residue yields the N terminal group. β-Galactosidase inactivates both types.

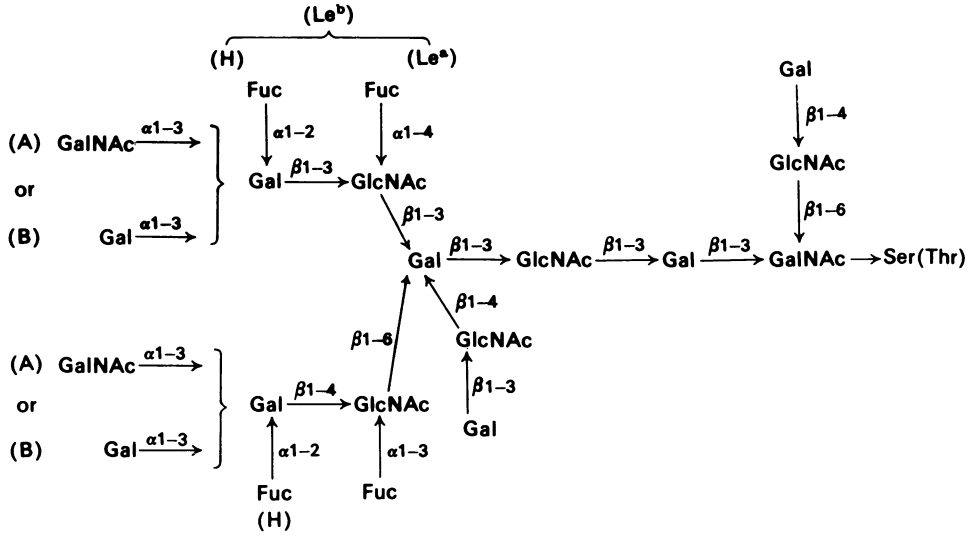


FIGURE 5.23 Composite structure of the blood-group-specific antigens on the erythrocyte.

5.5.2 *Mucin Heterosaccharides*

The mucins function as lubricants and thickening agents. In this group of glycoproteins the oligosaccharide chains are linked mainly to the hydroxyl group of serine and/or threonine of the peptide chain. The simplest structure found in most submaxillary mucins is one in which sialic acid is attached to the hydroxyl radical at C-6 of Gal-NAc. Typical oligosaccharides structures found in submaxillary mucin are shown in Figure 5.24.[69–72]

Four major groups of glycoproteins are identified by histochemical means: neutral, sialylated glycoproteins sensitive to neuraminidase, sialylated glycoproteins resistant to hydrolysis, acid glycoproteins (sulfated), and proteoglycans. These can all be located histochemically in the secretory granular cells.[73] In inflammation, the secretory cells of the airway increase in number, swell the tissue, and may block the airway. In chronic bronchitis, a higher percentage of sulfated glycoprotein is found and there is an increase in the neuraminidase-resistant glycoprotein fraction.

The blood group oligosaccharide structures are found in the mucus of the individual provided he is a secretor. For unexplained reasons,

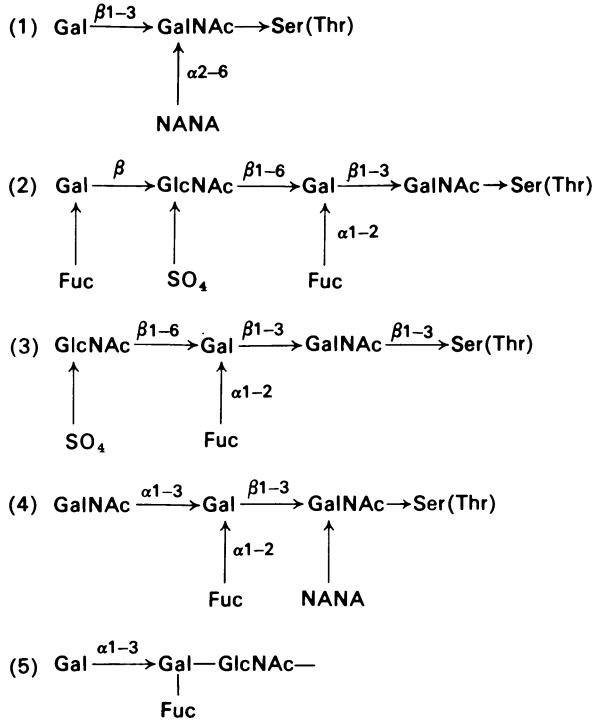


FIGURE 5.24 Some oligosaccharides found in submaxillary mucin. Number 4 has blood group A immunoactivity and 5 has B immunoactivity.

some humans do not secrete their blood type oligosaccharide structures and are referred to as *nonsecretors. It is from these structures and from oligosaccharides of human milk that much of the structure of blood group oligosaccharides has been determined.*[74,75]

5.5.3 *Collagen Heterosaccharides*

Substantial amounts of the heterosaccharides of collagen and the basement membranes are attached to the protein at the hydroxyl group of hydroxy lysine (Hyl). An oligosaccharide core common to these glycoproteins is Glc $\xrightarrow{\beta 1-2}$ Gal → Hyl (see Figure 5.25).[76] A characteristic sequence occurs around the point of attachment to the polypeptide. This is —Gly—X—Hyl—Gly—Y—Arg—, where X is alanine, serine, leucine, or phenylalanine and Y is glutamate,

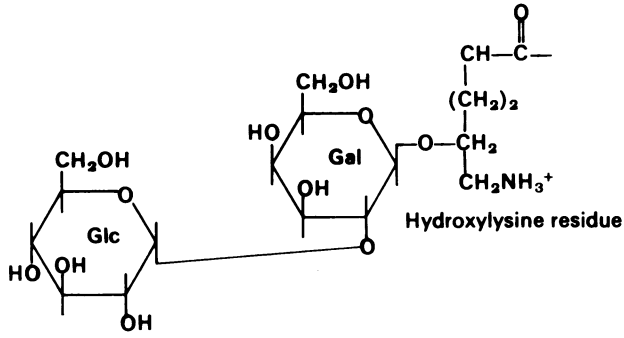


FIGURE 5.25 Core structures of the oligosaccharides of collagen and basement membranes.

histidine, or isoleucine.[77] Associated with collagen are the *proteoglycans*. These carry the acidic glycosaminoglycans as their carbohydrate moiety. They are discussed in Section 5.11.

Certain microorganisms carry the sequence, Glc $\xrightarrow{\beta 1-2}$ Gal $\xrightarrow{\beta}$, in their carbohydrate capsule. This is also a terminal sequence in the heterosaccharide of the basement membrane. High titer antibodies, produced in response to infection with these organisms, are antibodies against the glomerulus basement membrane. This plays a significant role in the pathogenesis of some forms of glomerulonephritis.[78] *These are autoimmune diseases, where circulating antibodies to the basement membrane serve to destroy it.*

5.6 *FUNCTION OF THE OLIGOSACCHARIDES OF GLYCOPROTEINS*

Most proteins have some oligosaccharide attached to the polypeptide chain. This includes most of the plasma proteins, enzymes, structural proteins, membrane proteins, hormones, antibodies, and others. A notable exception is human albumin. However, there are certain characteristics unique to certain glycoproteins, where the functional property is in the heterosaccharide structure. In these cases, the protein may be considered the carrier of a group of heterosaccharides. This is the case in the proteoglycans.

For the structural proteins, such as collagen and the basement membranes, *the function is that of a base for the construction of a form.* In this

regard, cellulose (as in wood) may be considered a typical example. The basement and other membranes and ground substances may be compared to cellophane. The use of sulfate for crosslinking and binding provides for a rigid structure, as in collagen, or a flexible container, such as the aorta or skin.

A second function is the property of the oligosaccharides *to increase viscosity or as "thickening" agents.*[74,75] Commercial methylcellulose serves such a purpose. In the human, the mucus is used as a lubricant and to retain moisture in the airways in the face of exchange of large volumes of air. This property is impaired in cystic fibrosis. In the plasma, the oligosaccharides maintain blood viscosity so as to retain the cells in suspension. In addition, the sialic acids are the major source of the charge on the cells.[79] This prevents pooling of the cells and the plugging of the fine capillaries. The sedimentation rate is a measure of the viscosity of the plasma.

A major function of the oligosaccharides is to provide a "fingerprint" so that the immunochemical system can distinguish those cells or particles which are foreign and those which are native to the particular human. If the sialic acid end groups of the plasma proteins are removed by neuraminidase exposing a galactose residue they are phagocytized in the liver.[79] The insulin receptor in the fat cells is a glycoprotein. If treated with neuraminidase and then galactosidase, it loses its ability to bind insulin.[80]

A most important set of oligosaccharides are those controlled by the histocompatibility gene complex which determines the carbohydrate configuration on the surface of cells. Rejection or acceptance of organ transplants depends upon whether the lymphocytes recognize the cells as foreign or native. A structure has been proposed for the nature of the type of heterosaccharide as would be found in cells, such as the leukocytes used for histocompatibility matching of patient and donor for transplant (see Figure 5.26).[81]

The implications of these studies are of major importance. *Development of techniques for determining oligosaccharide structure from a tissue sample (e.g., leukocytes) would permit matching of donor to recipient on a rational chemical basis.*

Another function attributed to the oligosaccharide portion of a plasma protein is that of *increasing solubility.* The sialic acid provides a charge distribution to maintain the colloidal state of the protein. In the case of a membrane, the charge on the sialic acid can be used to create a barrier and selectively permit only certain ions to pass the

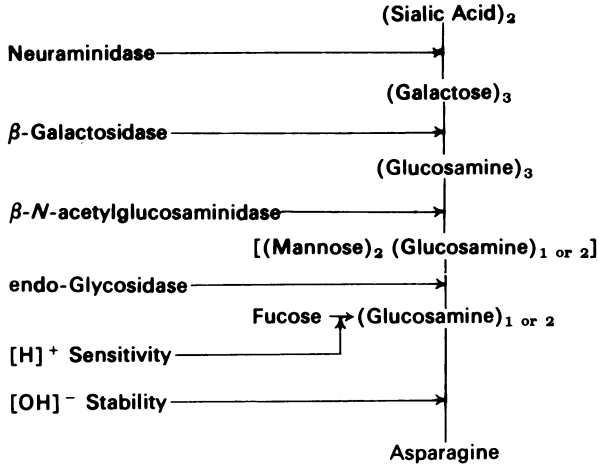


FIGURE 5.26 Proposed model of the structure of the H-2 oligosaccharides of the mouse. On the left are the enzymatic or chemical treatment which has been used in determining the structure.

membrane.[79] This is the chemical basis for the *blood brain* barrier which protects the nervous system against toxic substances.

On the other hand, for certain functions, the oligosaccharide portion of the protein seems to be superfluous and serves no definite function. For example, pancreatic ribonuclease exists in two forms, one with carbohydrate and one without.[82] Both have the same activity and amino acid sequence. *Homozygotes of the rare En(a)-erythrocyte type lack glycophorin in their erythrocytes.*[83] As a result, they develop antibodies to all blood types. The En blood group was discovered in England in 1965 in a patient whose serum agglutinated all erythrocytes tested. This patient had been transfused before, and had developed antibodies to A, B, and H cells. The name En was chosen to indicate the word *envelope* signifying the abnormal cell wall. En(a) was used for the second patient discovered in Finland with this abnormality.[84] None of these patients show any abnormalities except when sensitized by blood transfusion.

Although glycosyl transferases are found mainly on the endoplasmic reticulum, they do occur on the outer surface of cells. When this is the case, *the glycosyl transferase will bind its specific sugar located on the outer wall of a second cell or structure, thus binding to the second cell. Transfer of the sugar can initiate a biologically significant reaction.* It is this mechanism

which has been proposed for the adhesion of platelets to collagen fibrils, adhesion between gametes, glycosylation of adjacent cells in tissue culture (a signal for stopping cell growth), and other processes.[85,86]

A large glycoprotein, probably related to glycophorin, exists on the surface of cells such as fibroblasts and myoblasts. This has been named *large external transformation sensitive glycoprotein*, or simply LETS protein or LETS glycoprotein. *LETS glycoprotein serves to stimulate the cells to adhere to tissue and other cells.* Removal of LETS protein has a mitogenic effect. This results from the action of proteases and mitogens, like cytochalasin B. This has aroused substantial interest because of its significance in the proliferation and metastasis of tumors.[86a]

Regardless of the mechanism, polysaccharides on the surface of cells are of major importance in the recognition and interaction between circulating cells, such as between lymphocytes and the macrophage.

5.7 *ACUTE-PHASE REACTANTS*

The term *acute-phase reactants* (AP-reactants) refers to protein components of the plasma whose concentration is increased significantly in the acute phase of inflammatory processes.[87–89] These proteins are all glycoproteins whose carbohydrate moiety is in relatively high concentration and they are synthesized in the liver parenchymal cells. They are thus *trauma-inducible liver-produced plasma glycoproteins.* Table 5.4 lists the proteins commonly considered as the AP-reactants. The immunoglobulins which also contain carbohydrate are normally not considered acute phase reactants.

In addition to the proteins listed in Table 5.4, there are a substantial number of glycoproteins which satisfy the definition of an acute-phase reactant but are present only in small amounts in normal serum. From Table 5.4 it can be seen that a group of proteins is listed with diverse functions and with little relationship to each other. The reason for this is the fact that these proteins were found in high concentration with inflammatory disease, and it was assumed that they were part of this process.

With recent developments, it is possible to explain the reason

TABLE 5.4

Typical Acute-Phase Proteins

Protein	Concentration in normal plasma, mg/100 ml	Carbohydrates, %	Molecular weight	Isoelectric point, pI
α_1 Acid glycoprotein	75–100	41.4	44,000	2.7
α_1 Antiprotease (antitrypsin)	210–287	12.4	45,000	~3
Ceruloplasmin	27–63	8.0	160,000	4.4
C-reactive protein	Trace	—	138.000	β mobility
Fibrinogen	200–600	2.5	341,000	5.8
Haptoglobin (1-1)	30–190	19.3	85,000	4.1
α_2 Macroglobulin (plasmin and trypsin inhibitor)	110–310	8.0	725,000	5.4

for the increase of the concentration of these proteins. For example, in severe infection there is substantial hemolysis and a need for increased haptoglobin synthesis to dispose of the released hemoglobin (Section 9.11). For the same reason, ceruloplasmin concentration is increased in inflammatory processes (Section 8.7). Ceruloplasmin is also a glycoprotein containing 8% carbohydrate, the structure of whose oligosaccharide portion has been explored.[88] This would be expected as a defense mechanism against excessive bleeding. The clotting and inflammation reactions are part of the same system in defense against invasion by foreign bodies. The α_1 protease inhibitor (α_1 antitrypsin) and α_2 macroglobulin are also part of the clotting and inflammation mechanism and will be considered in Volume 4 of this series. Serum concentrations of α_1 antiprotease and α_2 macroglobulin are also increased significantly with inflammation. C-reactive protein and α_1 acid glycoprotein will now be discussed.

5.7.1 *C-Reactive Protein*

The cell walls of pneumococci are protected by an outer coat of polysaccharide. In pneumonia, antibodies to this polysaccharide are prepared in the human in defense against the invading organism. In

1930 it was noted that "acute phase" serum from a patient who had pneumonia formed a copious precipitate when a solution of an antiserum to the polysaccharide, prepared from pneumococci, was added.[90] The polysaccharide preparation is referred to as *C polysaccharide.* For this reason, the component of serum reacting with this polysaccharide, is called *C-reactive protein (CRP).*

Since the 1930s, CRP has been estimated in the serum of patients with a wide variety of inflammatory diseases, such as tumors, abscesses, peritonitis, acute infectious hepatitis, viral infections, autoimmune diseases, and especially in rheumatic fever and the acute phase of rheumatoid arthritis.[91–97]

CRP acts as an activator of the complement system.[98] In this regard, it resembles the immunoglobulins. CRP also affects the response of the platelets to inflammation.[99] It would therefore be expected that CRP is a member of the family of immunoglobulins. At this writing, no substantial evidence has been adduced that this is the case. The reason for this is the extremely low concentration normally present in serum. It is only recently that CRP has been detected in normal serum,[100] even though sensitive immunochemical techniques have been available for several decades. Electrophoretically, it travels with the β globulins and has a molecular weight of about 138,000.

When the erythrocyte sedimentation rate (ESR) is increased, it is recommended that the CRP test be done, particularly in exploring cases of rheumatoid arthritis.[101] In cardiovascular disease, elevated CRP levels are often found in myocardial infarction but not in angina pectoris or arteriosclerotic heart disease.

The test for CRP is easy to perform. The serum from the patient and the antiserum (Burroughs Wellcome Co., Research Triangle Park, North Carolina) are taken up in a capillary and the height of the precipitate is noted.[102]

5.7.2 α_1 *Acid Glycoprotein*

In 1948 it was shown that a 7% solution of perchloric acid did not precipitate all of the proteins. A highly soluble group of proteins remained. These could be precipitated by phosphotungstic acid.[103] The soluble fraction contained about 10% of the protein-bound carbohydrate of the serum. It was then variously referred to as *seromucoid,*

orosomucoid, or *mucoprotein-1* (*MP-1*). Most of this fraction was shown to consist of a single glycoprotein, moving with the α_1 plasma protein fraction. If borate is added to starch gel, this glycoprotein shows the highest electrophoretic mobility since it has a high binding capacity for borate. Because of its high negative charge, even at pH 4.5, it was considered an acid and labeled α_1 *acid glycoprotein.* It can be prepared in large quantities from commercially available Cohn fraction V supernatant, from the process for preparing human albumin, and globulins.[104]

α_1 Acid glycoprotein is about 45% carbohydrate. Its molecular weight is 40,000 and it contains 14 residues of sialic acid, 34 residues of neutral hexoses, 31 residues of *N*-acetylglusosamine, and two residues of fucose. The hexoses are galactose and mannose.[105]

The sialic acid residues are located terminally and linked to the heterosaccharide groups. Six of them are readily cleaved by neuraminidase. However, the others are buried in the molecule and are somewhat resistant to hydrolysis.[106,107] A structure for a typical heterosaccharide attached to the polypeptide chain is shown in Figure 5.27.[108] This structure is similar to that proposed for the glycoprotein found in fetal calves blood (fetuin).[109] There are five such carbohydrate units attached to the polypeptide chain.[110]

The structure of the polypeptide chain of α_1 acid glycoprotein is shown in Figure 5.28. There is substantial heterogeneity in the composition of this protein from different individuals and some of the substitutions are shown in the figure.[111,112]

There is considerable homology between the sequences of α_1 acid glycoprotein, haptoglobin, and the immunoglobulins. It is logical to assume that these proteins evolved from a single precursor in the

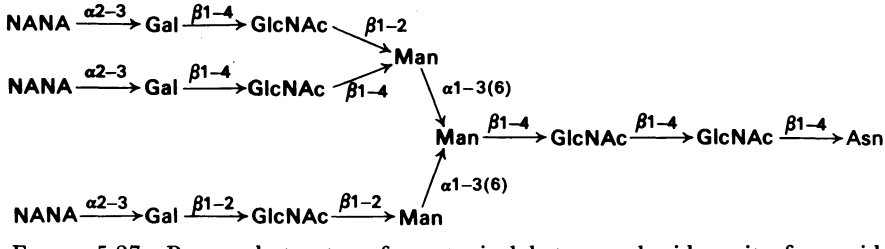


FIGURE 5.27 Proposed structure for a typical heterosaccharide unit of α_1 acid glycoprotein.

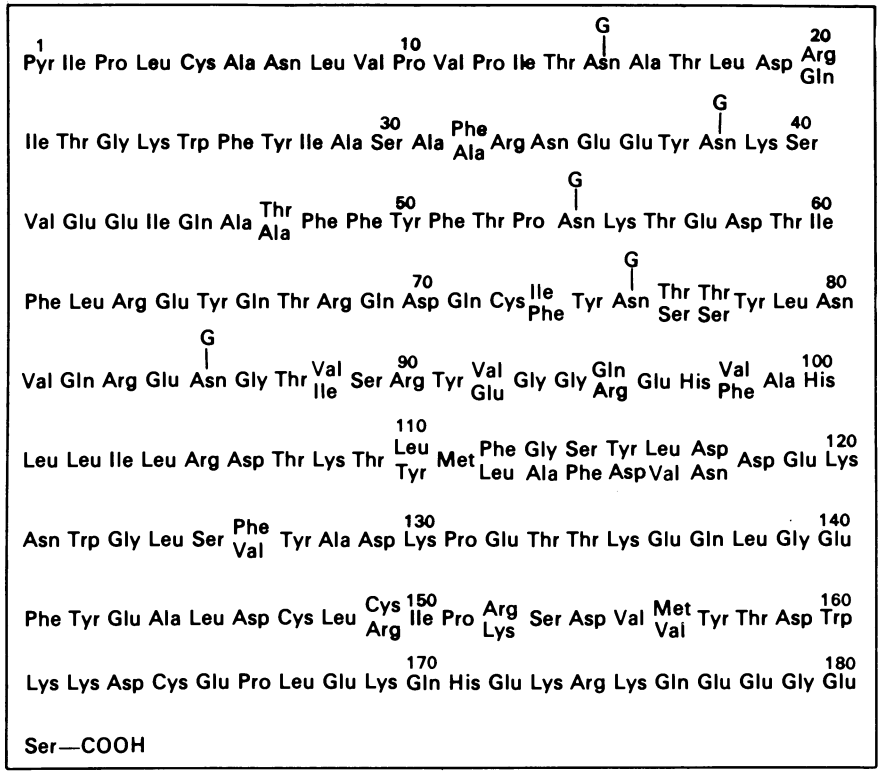


FIGURE 5.28 The amino acid sequence of α_1 acid glycoprotein showing some of the substitutions. Pyr stands for pyrollidone carboxylic acid which is the anhydride of glutamic acid. G represents carbohydrate.

distant past. However neither haptoglobin nor α_1 acid glycoprotein has any of the properties of the immunoglobulins in neutralizing invading organisms.

Biological Role of α_1 Acid Glycoprotein

Increased concentration of α_1 acid glycoproteins is the major factor in the increased concentration of glycoproteins in serum with inflammation.[113] The concentration of this glycoprotein is also increased in various unrelated conditions and diseases, such as during wound healing, pregnancy, rheumatoid arthritis, cancer, pneumonia, and numerous others.[113–115] The common denominator in all of these conditions is cell proliferation. *Tissues with a high cell proliferation*

rate release a factor which stimulates α_1 acid glycoprotein synthesis.[116] The significance of these observations is still not clear.

Normal human platelets carry substantial amounts of α_1 acid glycoprotein tightly bound to their membrane.[117] Platelets adhere to collagen. Dipyridamol inhibits binding of platelets to collagen. This property can be restored if α_1 acid glycoprotein is added to the system.[118] Since the clotting and inflammation are part of one overall mechanism, it would be logical for α_1 acid glycoprotein to be secreted for the purpose of increasing platelet adhesion to collagen when faced with an inflammatory reaction.

When α_1 acid glycoprotein is added to a solution of collagen, striated fibers are formed containing about 50% α_1 acid glycoprotein and 50% collagen. Under the microscope, the fibers show regularly spaced dense repeating zones 1200 Å apart. These fibers resemble, in appearance, the more closely spaced fibers (640 Å) seen in bone cartilage sections or produced when chondroitin sulfate is added to soluble collagen. The more widely spaced fibers contain 6 to 8 molecules of α_1 acid glycoprotein for every collagen molecule.[119]

The more widely spaced fibers of the type formed from α_1 acid glycoprotein and collagen are seen in normal Descemet's membrane.[120] They are also seen in certain tumors of the nervous system.[121,122] They are known to contain collagen and a glycoprotein. α_1 Acid glycoprotein is therefore implicated in the formation of certain membranes and fibers in combination with soluble collagen. *The crosslinking of collagen with α_1 acid glycoprotein seems to serve the formation of a mesh for the purpose of preparing a membrane or semirigid structure.*

5.7.3 *Low-Concentration Acute-Phase Glycoproteins*

From time to time, reports have appeared on the recognition of new glycoproteins, present in increased quantities in inflammatory conditions or disease. A number of these, like α_1 fetoglobulin and CEA have become of diagnostic importance. Some of these will be presented here with whatever data are available at present.

5.7.3.1. *Pregnancy-Associated Glycoprotein*

Although all of the acute-phase reactants are elevated in pregnancy, certain glycoproteins are associated only with pregnancy. They are generally referred to as *pregnancy-associated glycoproteins, or acute-phase*

proteins of pregnancy.[123,124] There are at least four such proteins. These include the pregnancy-specific β_1 glycoprotein,[123] pregnancy-associated plasma protein A (PAPP-A), placental lactogen, and chorionic gonadotrophin. Others which have been found to be elevated in pregnancy, such as steroid-binding β globulin (Section 14.8) and pregnancy-associated α_2 glycoprotein (α_2 PAG) are normal components of serum.

Pregnancy-specific β_1-glycoprotein was prepared from placenta.[126] An antiserum was prepared from this material and various sera tested. Only sera from pregnant women were positive in this test. The plasma protein travels with the β_1 globulin fraction on electrophoresis.

Pregnancy-associated α_2-glycoprotein is normally present in all sera at a concentration of about 1–2 mg/100 ml. This protein was noted first in 1959 and has been reported repeatedly as a new protein by numerous investigators. It has been called, Xh protein, Xm factor, pregnancy zone protein, α_2 pregnoglobulin, new α_2 macroglobulin, and others. The term pregnancy-associated α_2 glycoprotein (α_2 PAG) seems to be generally accepted at present. Some still call this protein *pregnancy-associated α_2 macroglobulin.*[127]

Human α_2PAG contains 10–11% carbohydrate and has a molecular weight of about 359,000.[128] It is not produced by the fetus since it cannot be detected in cord blood. The serum concentration of this protein rises sharply on estrogen administration. It has no significant binding properties for any of the steroid hormones. However, α_2 PAG is also elevated in cancer, rheumatoid diseases, and other inflammatory conditions.[125–128]

Although the concentration of α_2PAG is about 1–2 mg/100 ml in normal serum, it rises to 50–200 mg/100 ml in normal pregnancies.[128] *If α_2PAG levels do not rise significantly in the 9th to 12th week of the pregnancy, then it is claimed that there is 16 times the chance for spontaneous abortion.*

One function of α_2PAG seems to be as an immunosuppressive agent, to prevent the rejection of the fetoplacental unit. α_2PAG suppresses phytohemagglutinin-induced lymphocyte transformation and also the mixed leukocyte reaction.[129,130] It is synthesized by human leukocytes.[129]

5.7.3.2 *Zinc-α_2 Glycoprotein*

A glycoprotein found in normal serum in 1961, at a concentration of 20–200 mg/liter, binds zinc.[131,132] It is found in the α_2 region on

electrophoresis, and for this reason is called *Zn-α_2-glycoprotein* (*Zn-α_2-GP*). Its molecular weight is 41,000 and its sedimentation constant is 3.2. Its electrophoretic mobility is 4.2 and the isoelectric point is at pH 3.8.

In the urine, the concentration of Zn-α_2-glycoprotein is about 1 mg/liter.[134] The mean concentration found in amniotic fluid is about 9.8 mg/liter and is proportional to the protein content of the amniotic fluid.[133] Zn-α_2-GP is found in almost all the fluids of the body including sweat and saliva. With renal pathology, Zn-α_2-GP is lost in the urine because of its relatively low molecular weight.[132–134] In newborns, Zn-α_2-GP concentration in serum is lower than in adults by a factor of about one-half.

The function of Zn-α_2-GP is unknown. Zinc binds also to albumin and this is probably its major means of transport. Zinc is a required coenzyme for carbonic anhydrase and a number of enzymes in normal metabolism.[135–137] It is essential for the formation of normal mucin.[138] However, no relationship between zinc metabolism and Zn-α_2-GP has been established.

5.7.3.3 *Seromucoid Proteins*

In studies with perchloric acid precipitation of serum proteins it has been pointed out that a substantial amount of glycoprotein remains in solution. These proteins with high carbohydrate content have been referred to as *seromucoids* or *mucoproteins*. α_1 Acid glycoprotein is a major component of this fraction. These proteins are of relatively low molecular weight, and appear in Cohn fraction VI. Zn-α_2 Glycoprotein is another member of this group. A number of others have also been identified.

Numerous proteins which have been identified, such as members of the clotting and complement systems, enzymes such as cholinesterase and lysozyme have, what might be called, seromucoid or mucoprotein properties since they have low sedimentation rates and high carbohydrate content. These will be considered elsewhere. Here are presented those seromucoids whose function has not been identified at the present writing.

A protein, with a sedimentation coefficient of 2.9, is known as *β_2 glycoprotein I*. This protein has been crystallized.[139] It has the electrophoretic mobility of α_1 globulin and its molecular weight is about 48,000.[140] The protein occurs in serum at a concentration of

15–30 mg/100 ml.[141] There are two other proteins associated with β_2 glycoprotein I, and these are designated β_2 glycoprotein II and III. β_2 Glycoprotein II has been identified as C3 activator in the complement system. β_2 Glycoprotein III has a molecular weight of about 35,000 and occurs in plasma at a concentration of 5–15 mg/100 ml. No function has been ascribed to β_2 glycoproteins I or III.

If serum is diluted 1:2 with a pH 6.0 buffer and shaken with carboxymethylcellulose (CM cellulose), four proteins will be found to adhere. These include C1q of the complement system, lysozyme, and two glycoproteins designated *3.8S-α_2 globulin* and *9.5S-α_1 globulin.*[142] The 9.5S-α_1 globulin crystallizes readily. The designations 3.8S-α_2 and 9.5S-α_1 signify the sedimentation coefficient and electrophoretic mobility of these proteins. Because of their affinity for carboxymethylcellulose, these two proteins have also been designated CM-I and CM-III. C1q is referred to as CM-II, and lysozyme as CM-IV. The function of these proteins is unknown. They bind CM cellulose, heparin, and bivalent ions such as Ca^{2+}. They may be of significance in the clotting mechanism.

The *3.8S-α_2 globulin* has been found to be rich in histidine, with a molecular weight of about 58,500. The molecule is made up of two identical subunits, held together by ion binding. It is present in serum of adults at 9.2 $\pm$ 4.5 mg/100 ml and in newborns at 3.5 $\pm$ 0.9 mg/100 ml. The protein binds heparin and can be displaced by protamine. It is referred to as the *histidine-rich glycoprotein.*[143]

Another mucoprotein with high carbohydrate content (30%) is *8S-α_3 glycoprotein.* This protein contains about 9% of sialic acid. Its sedimentation coefficient is 7.87 and its molecular weight is about 220,000. It is present in serum at a concentration of 3.1–4.5 mg/100 ml.[144] It has a high solubility in perchloric acid and trichloroacetic acid because of its high carbohydrate content. The protein can be dissociated to lower-molecular-weight polypeptides by disulfide reducing agents.

Recently, a leucine-rich seromucoid of sedimentation constant 3.1 has been isolated. It has α_2 mobility and a molecular weight of about 49,000. It carries about 23% carbohydrate. It is present in normal serum at a concentration of about 2.1 mg/100 ml. Its leucine content is almost 17%. One out of every five amino acids is leucine. It is designated as *leucine-rich, 3.1S-α_2 glycoprotein.*[145]

Related to Zn-α_2 glycoprotein, a protein which precipitates with Zn^{2+}, is a protein which is readily precipitable by Ba^{2+}. This is an

α_2 protein with a sedimentation rate of 3.3, a molecular weight of about 49,000, and an electrophoretic mobility of 4.2. It is present in normal serum in a concentration of 40–85 mg/100 ml. The function of this protein is unknown. It is usually referred to as α_2 *HS-glycoprotein* or *Ba-*α_2 *glycoprotein.*[146]

Another seromucoid with a sedimentation rate of 3.3 occurs in the α_1 region on electrophoresis and is distinguished by the fact that it contains very little tryptophan. It is present in serum at a concentration of 5–12 mg/100 ml. It is designated α_1 *T-glycoprotein.*[147]

A mucoprotein with a sedimentation rate of 3.8, which is readily precipitated with protein precipitants, is designated α_1*B-glycoprotein.* Its molecular weight is about 50,000 and it is found in the α_1 region on electrophoresis. Its concentration in normal serum is 15–30 mg/100 ml.[148]

Two seromucoids present in very low concentration in serum have been used in the diagnosis of certain types of tumors. One is *α fetoglobulin*[149] and the other is *carcinoembryonic antigen (CEA).*[150]

α Fetoglobulin, which is present in fetal serum, also appears in the serum of patients with hepatomas. For this reason, it is of great interest in the diagnosis of these types of tumors. Its characteristics are listed in Table 5.5.[149]

Carcinoembryonic antigen has a molecular weight of 180,000 and is 49.9% carbohydrate. Thus it is a seromucoid. It moves with the α globulins on electrophoresis and has a sedimentation constant of 7. Sequential measurement of CEA provides an early warning of the recurrence and metastasis of large bowel cancer. The upper limit of normal for CEA is usually taken as 15 μg/liter. Values above 30 μg/liter suggest a high probability of an underlying cancer.[151]

The properties of the seromucoids present in serum in low concentration are listed in Table 5.5. All of these are most probably of biological significance. This area of knowledge needs to be investigated more thoroughly.

5.8 *GLYCOPROTEIN CATABOLISM*

In vitro, glycoproteins can be degraded by hydrolyzing the polypeptide chains with proteases, and then attacking the polysaccharide structure, as indicated in Figure 5.26. However, *in vivo*, the problem is

TABLE 5.5

Seromucoids Present in Small Amounts in Serum and of Unknown Function

Designation[a]	Alternative designation	Molecular weight	Sedimentation rate	Electrophoretic mobility	Concentration, mg/liter	Carbohydrate, %
α_1 B-GP	Easily precipitable	50,000	3.8	α_1	15–30	13.3
α_1 T-GP	Tryptophan poor	60,000	3.3	α_1	5–12	13.7
α_1 F	α_1 Fetoglobulin	64,000	4.5	α_1	<0.01	7.4
9.5S α_1	α_1 Macroglobulin	308,000	9.5	α_1	3–8	—
C.E.A.	Carcinoembryonic antigen	180,000	7.0	α_1	<0.015	49.9
3.1S α_2	Leucine rich	49,600	3.1	α_2	~ 2.1	23
3.8S α_2	Histidine rich	58,500	3.8	α_2	5–15	14
Zn-α_2	Zn-α_2 GP	41,000	3.2	α_2	2–15	18.2
α_2 HS-GP	Ba-α_2 GP	49,000	3.3	α_2	40–85	13.4
α_2 PAG	Pregnancy-associated macroglobulin	359,000	—	α_2	1–2	12
8S α_3	8-S-α_3 GP	220,000	7.87	α_3	3.1–4.5	30
PS β_1-GP	Pregnancy-specific GP	120,000	4.6	β_1	—	—
β_2 I	β_2 Mucoid	48,000	2.9	γ (1.6)	15–30	17.1
β_2 III	β_3 Mucoid	35,000	—	—	5–15	—

[a] GP stands for glycoprotein.

more complicated. Some sialic acid and fucose residues usually surround the glycoprotein with a protective coat which resists the action of proteolytic enzymes. These need to be removed before catabolism can proceed. Others are removed subsequently.

An *endo-β-N-glycosaminidase* then cleaves the polysaccharide from the polypeptide between the two *N*-acetylglucosamines (GlcNAc) which are attached to the asparagine chain. This yields a polysaccharide and a disaccharide (Fuc—GlcNAc—) still attached to the polypeptide. This is shown in Figure 5.29.[152] This enzyme is an "endo-" enzyme since it attacks the molecule from *within* the chain.

If fucose is attached to the *N*-acetylglucosamine, as it is in some glycoproteins, the peptide is then cleaved by a fucosidase. The GlcNAc is finally cleaved from the polypeptide by an *N-aspartylglucosaminidase.*

From Figure 5.29, it can be seen that hydrolysis proceeds sequentially removing each monosaccharide by an enzyme specific for that linkage. The sugars are then reutilized for glycoprotein synthesis or metabolized to yield energy for metabolism. Intermediates of varying carbohydrate composition also appear normally in the urine.

From Figure 5.29 it can be seen that, other than the proteolytic enzymes, at least seven enzymes are involved in the process of dismantling the heterosaccharides. These enzymes are located in the lysosomes. However, they can be released within and outside the cell for appropriate use. For example, when culturing fibroblasts from patients, these enzymes are released from the cells into the culture medium.

Any one of these enzymes can be deficient in a particular patient, giving rise to a variety of genetic disorders. The sphingolipidoses with carbohydrase deficiencies have been discussed in Volume 2, pp. 75–109. In addition, deficiency of mannosidase and fucosidase have been referred to in Volume 2, pp. 51–52. See also pp. 119 and 121 of this volume.

5.8.1 *Abnormal Glycoprotein Catabolism*

Deficiencies of almost every enzyme listed in Figure 5.29 have been reported in humans. The nature of the disease is usually discovered by finding substantial amounts of heterosaccharides in the urine. The composition of the heterosaccharide usually indicates which enzyme system is defective. From these data and direct enzymic analysis of

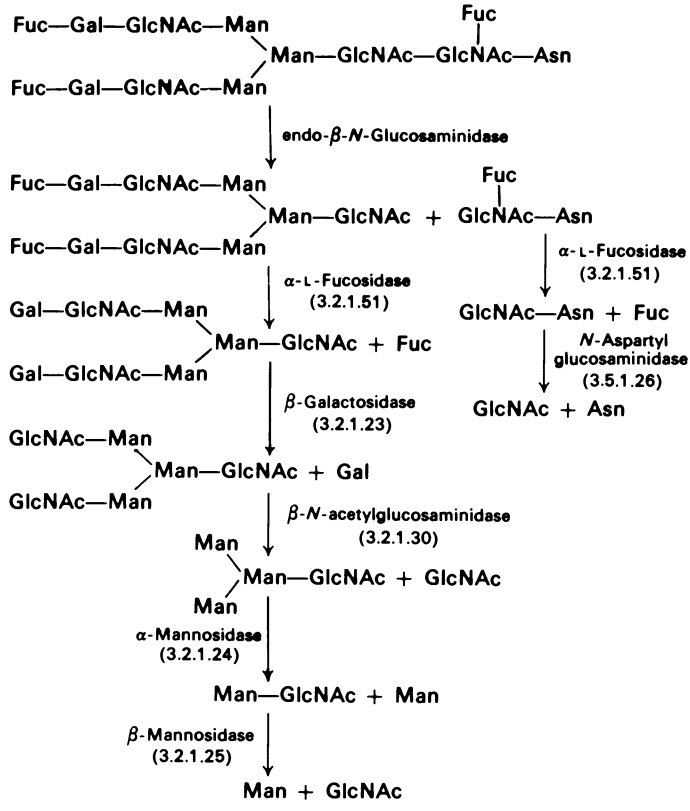


FIGURE 5.29 Steps in the catabolism of a typical heterosaccharide structure. The number in parentheses under each enzyme is its E.C. designation.

biopsy tissue the diagnosis can be made. In some cases, fibroblast culture permits the determination of the nature of the disease. This can be ascertained by adding the enzyme to the culture medium. If a specific abnormality exists, this will correct the deficiency.[152,153]

5.8.2 *Mannosidosis*

A patient was studied in 1967 with symptoms which resembled, to some extent, those of Hurler's syndrome. These symptoms included psychomotor retardation, slight gargoylelike facies, vacuolized lymphocytes, and recurrent infections.[154] This condition was identified as an *α mannosidase deficiency*. In this condition, tissue α mannosidase is reduced substantially. The disease, now called *mannosidosis*, had also been

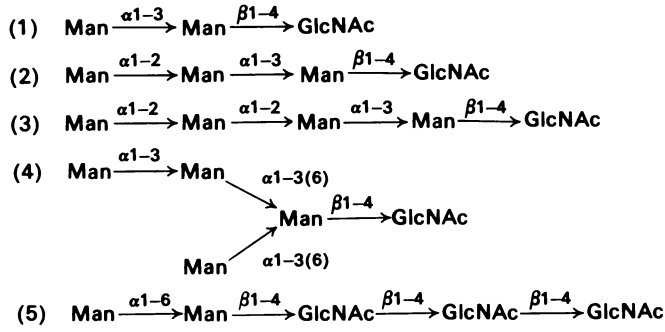


FIGURE 5.30 Polysaccharides found in human (Nos. 1–4) and bovine (No. 5) urine with mannosidosis.

observed in Angus cattle since at least the 1950s, but its etiology was not ascertained until 1972.[155] Polysaccharides found in human brain and other tissue and in urine from patients with mannosidosis are shown in Figure 5.30.[156] A sequence of three *N*-acetylglucosamines in succession, as observed in cattle, has not been identified in human glycoproteins.

Three different mannosidases have been isolated from human liver; A and B have their pH optima at acid pH (about 4.5) and C has its optimum pH at about 7.0. Forms A and B differ only in sialic acid content.[157] These enzymes are α mannosidases and do not hydrolyze the inner mannose linkages which are β linkages (Figure 5.30). *The deficiency in humans and cattle is an α mannosidase deficiency of all three mannosidases.*

The diagnosis of mannosidosis can be made by assay for the enzyme from serum, leukocytes, or biopsy material or by determination of the trisaccharide, $\text{Man} \xrightarrow{\alpha} \text{Man} \xrightarrow{\beta} \text{GlcNAc}$, in the urine. For this purpose the trisaccharide is isolated by gel filtration on a column. The aldehyde group is reduced with sodium borohydride and the resultant alditol is methylated. The methylated trisaccharide can be determined by gas chromatography.[158] Normally, this trisaccharide is not detected in urine. With mannosidosis values of 200–800 mg/liter are found.

The enzymatic method is most practical for routine use. The reaction can be carried out with leucocytes or serum and heterozygotes can be detected by determining their ratio to *N*-acetyl-β-glucosaminidase normally present.[159]

5.8.3 *Fucosidosis*

Fucosidosis, as a deficiency of α-L-fucosidase, was first discovered in 1966.[160–162] The clinical manifestations include progressive motor and mental deterioration, coarseness of facial features, cardiomegaly, hepatomegaly, and skeletal abnormalities including short stature. The tissues show vacuolated cytoplasm of epithelial and stromal cells suggestive of a lysosomal storage disease. The homozygote is mentally retarded and may develop grand mal seizures. The course is progressive so that by about 10 years of age the patients require complete nursing care being severely mentally retarded. The disease is transmitted as an autosomal recessive.

On electrophoresis in gels, leucocyte α-L-fucosidase shows an isoenzyme pattern resembling that for lactate dehydrogenase, with five or six major bands.[163] Fractionation on DEAE cellulose of kidney fucosidase yields two enzymes, one with an optimum pH at 6.5 and the other at pH 4.4. The pH 6.5 enzyme has a high molecular weight and is heat labile, whereas the enzyme with pH maximum at 4.4 is heat stable. When serum is incubated at 70°C for 20 min, normal serum retains 65% of its α fucosidase activity. No measurable activity is observed in the heat-treated serum of patients with fucosidosis.[164,165] Thus the deficiency is a deficiency in a specific enzyme.

With fucosidosis, fucose-containing polysaccharides are found

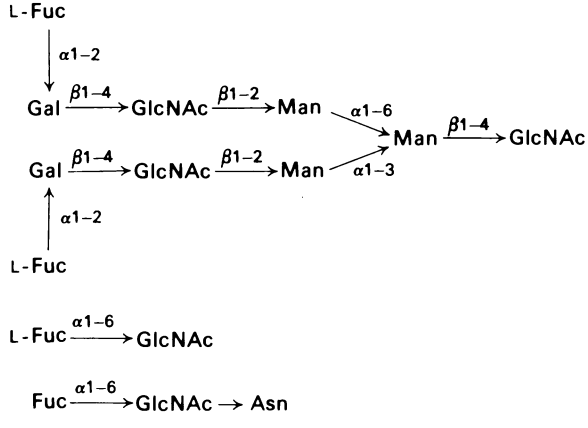


FIGURE 5.31 Polysaccharide fragments found in urine and tissues of patients with fucosidosis.

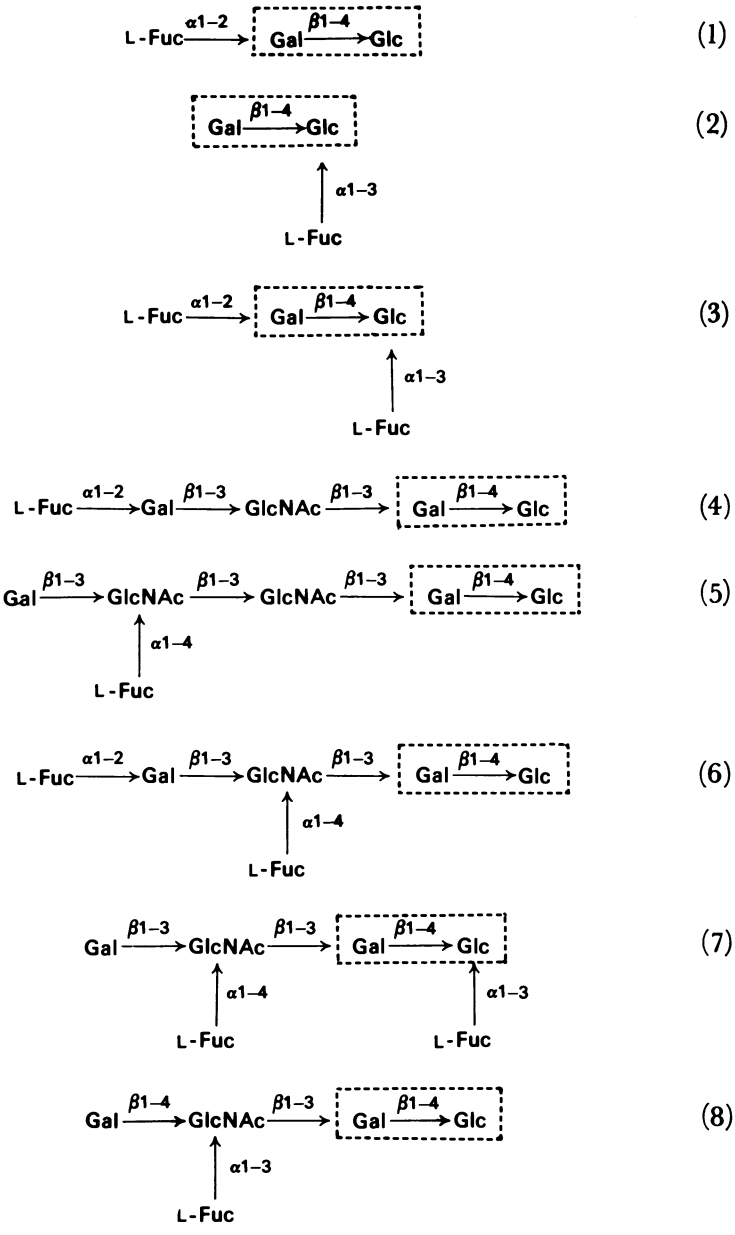


FIGURE 5.32 Some heterosaccharides containing fucose which are found in human milk, probably as lactose precursors. The fucoses are all of the L type, with the α linkage. The lactose portion of each molecule is enclosed in the dotted lines.

deposited in various tissues in the body especially in the lysosomes. This accounts for the cardiomegaly and hepatomegaly.

The disaccharide fragment, L-Fuc $\xrightarrow{\alpha 1-6}$ GlcNAc, is found in brain and urine, along with other polysaccharide fragments containing fucose,[160] see Figure 5.31.

The disease is variable, some of the patients living to adulthood with less severe symptoms. The reason for this is partially the variability of the polysaccharide chains. For example, a patient secreting Le^b-type blood group antigen will secrete more fucose-containing polysaccharides than one with the Le^a antigen (Figure 5.23). On the other hand the nonsecretor is at an advantage with this disease, since he does not release these fucose-containing heterosaccharides into the body fluids and secretions. The diet is important. Milk contains substantial amounts of fucose-containing polysaccharides. Other foods contain less. These polysaccharides cannot be metabolized by the infant with mannosidosis and some deposit in the lysosomes (Figure 5.32).

From Figure 5.32 it can be seen that all these polysaccharides contain lactose (Gal $\xrightarrow{\beta 1-4}$ Glc). *This seems to indicate that lactose, a predominant component of human milk (5%) derives from a higher polysaccharide containing fucose.*

5.9 *GLYCOSAMINOGLYCANS: THE MUCOPOLYSACCHARIDES*

The *mucopolysaccharides* (*glycosaminoglycans*) comprise a family of polymeric disaccharides with *N*-acetylchondrosine as a repeating unit and about one sulfate group per repeating disaccharide. *Chondrosine* is a disaccharide made up of a uronic acid, such as glucuronic or iduronic, linked to *N*-acetylgalactosamine. The chondroitin sulfates (see Figure 5.33) have the general formula

$$\left(\text{glucuronate} \xrightarrow{\beta 1-3} \underset{\displaystyle |}{\overset{\displaystyle SO_3H}{}}\text{GalNAc}\right)_n$$

When glucuronic acid (GlcUA) is replaced by its epimer at the 5′ position, it becomes iduronic acid (IdUA). In this case the polymer is

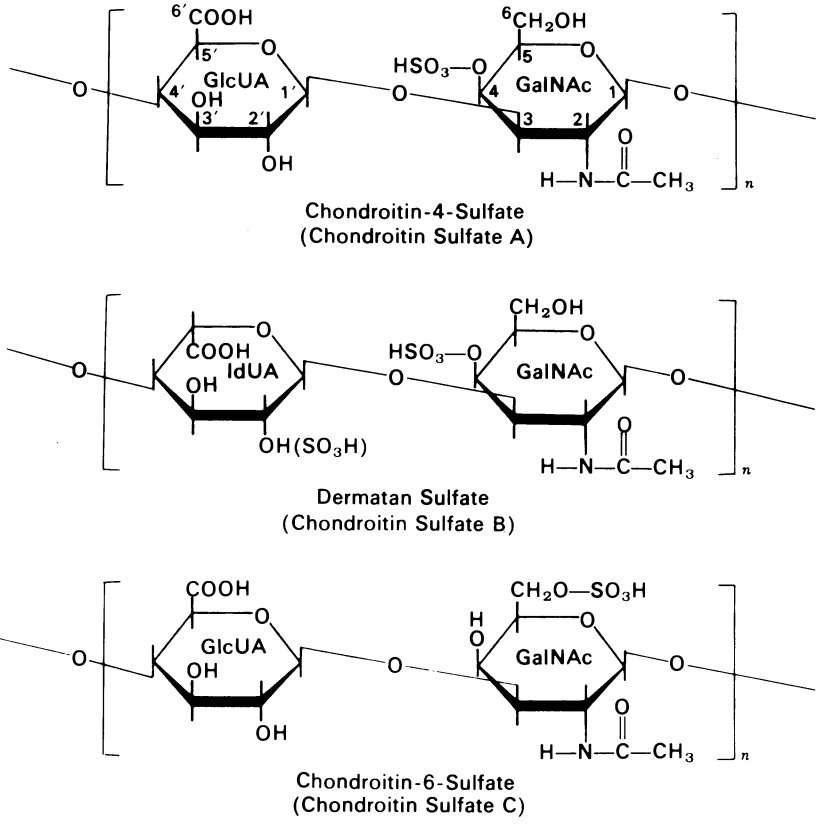


FIGURE 5.33 Structure of the repeating units of the various chondroitin sulfates. In dermatan sulfate, iduronic acid (IdUA) replaces glucuronic acid (GlcUA).

referred to as *dermatan sulfate*. The structure then becomes:

$$\left(\text{L-Iduronate} \xrightarrow{\alpha 1\text{–}3} \begin{matrix} \text{SO}_3\text{H} \\ | \\ \text{GalNAc} \end{matrix}\right)_n$$

Chondroitin sulfate acts to cross-link collagen, creating a flexible linkage between the tough protein filaments of cartilage. This serves as a resilient matrix for calcification to form bones and teeth and for the formation of structures such as the nasal septum. The sulfate is on the 4 or 6 position of the galactosamine. When in the 4 position, we have

chondroitin sulfate A. When in the 6 position, we have chondroitin sulfate C.

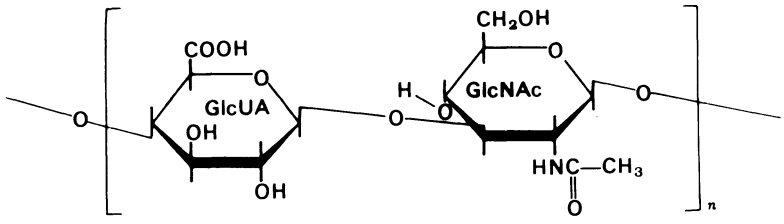
Dermatan sulfate, also called chondroitin sulfate B, is present in soft connective tissue and is the main cross-linking substance for collagen in skin, arterial walls, and cartilage. The sulfate is in the 4 position on the GalNAc portion of the disaccharide repeating unit. *Cross-linking of collagen with dermatan sulfate produces a softer and more flexible structure suitable for construction of tissues where more flexibility is required.*

In addition to the structural mucopolysaccharides, a number of others serve varied functions. *Hyaluronic acid*, a sulfate-free polysaccharide, related to dermatan sulfate in structure, is made up of repeating units of a disaccharide, GlcUA $\xrightarrow{\beta 1-3}$ GlcNAc. Its molecular weight ranges from 50,000 to as high as 8 million.[168–170] Hyaluronic acid is a thickening agent and lubricant. It is present in serum, synovial fluid, vitreous humor, umbilical cord (Wharton's jelly), and sperm fluid, and coats the walls of the gastrointestinal tract, where it protects against bacterial invasion and digestion by the gastrointestinal enzymes.

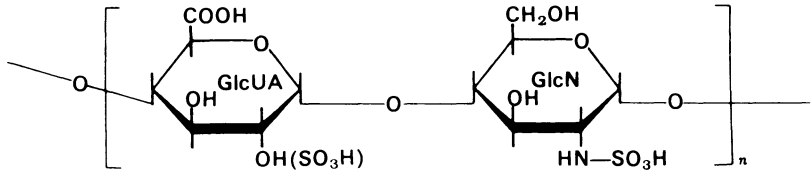
Pathogenic bacteria secrete enzymes called *hyaluronidases* (E.C. 3.2.1.35 and 3.2.1.36), which hydrolyze hyaluronic acid and thus gain access to the tissues. Sperm can release hyaluronidases, which along with acid phosphatase of semen dissolve the wall of the ovum permitting one of the sperm to penetrate the egg. The structure of hyaluronic acid is shown in Figure 5.34. Hyaluridase is also known as the "spreading factor." It can be assayed by its effect in lowering the viscosity of a solution of hyaluronic acid. Its activity accounts for the increased sedimentation rate of erythrocytes in rheumatoid disease.

Heparin, originally isolated from liver as its name indicates, is a major anticoagulant in the human protecting against thrombosis. It is present in liver, lung, and mast cells in relatively high concentration. It is composed of repeating units of a disaccharide having the structure GlcUA $\xrightarrow{\alpha 1-4}$ GlcN (see Figure 5.34).[171,172] Its molecular weight, after removing the sulfate esters, ranges from 600 to 20,000, depending upon the source and method of isolation. It occurs sulfonated on the nitrogen in the 2 position of glucosamine. Some sulfate ester also occurs at position 6.

Related to heparin is *heparitin sulfate* (*heparan sulfate*).[173] It is a variable structure (microheterogeneity), composed mainly of repeating units of a disaccharide similar to that of heparin, but where D-glucuronic acid (GlcUA) is replaced by its epimer, L-iduronic acid (IdUA)



Hyaluronic Acid



Heparin
(Heparinic Acid)

FIGURE 5.34 Comparison of the structures of heparin and hyaluronic acid. Heparin carries sulfate on the nitrogen, and often on the 2′ position of the glucuronate and 3 position of the glucosamine moiety.

(IdUA $\xrightarrow{\alpha 1-4}$ GlcNAc). In addition, disaccharides with D-glucuronic acid, also occur in the polymer, resulting in heterogeneity of the structure. As distinct from heparin, some of the glucosamine is acetylated. A typical sequence would be:

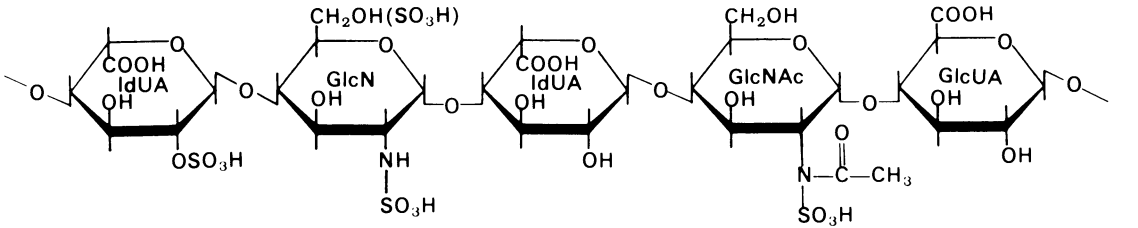
$$\xrightarrow{\alpha 1-4} \text{IdUA} \xrightarrow{\alpha 1-4} \text{GlcN} \xrightarrow{\alpha 1-4} \text{IdUA} \xrightarrow{\alpha 1-4} \text{GlcNAc} \xrightarrow{\alpha 1-4} \text{GlcUA} \xrightarrow{\beta 1-4}$$

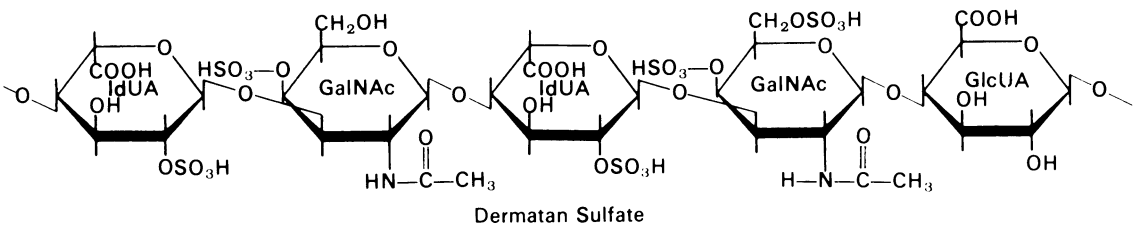
This structure is shown in Figure 5.35.

Heparitin sulfate occurs normally in liver, lung, spleen, and other organs, especially of the reticuloendothelial system, and its formation is related to heparin synthesis. It has no anticoagulant activity, however.

The structure of *dermatan sulfate* as indicated in Figure 5.33 is a simplification. When isolated, it contains a variable amount of glucuronic acid, which is integrated into the polymer chain. A typical structure is shown in Figure 5.35 and in simplified form can be represented as,

$$\xrightarrow{\beta 1-4} \text{IdUA} \xrightarrow{\alpha 1-3} \text{GalNAc} \xrightarrow{\beta 1-4} \text{IdUA} \xrightarrow{\alpha 1-3} \text{GalNAc} \xrightarrow{\beta 1-4} \text{GlcUA} \xrightarrow{\beta 1-3}$$





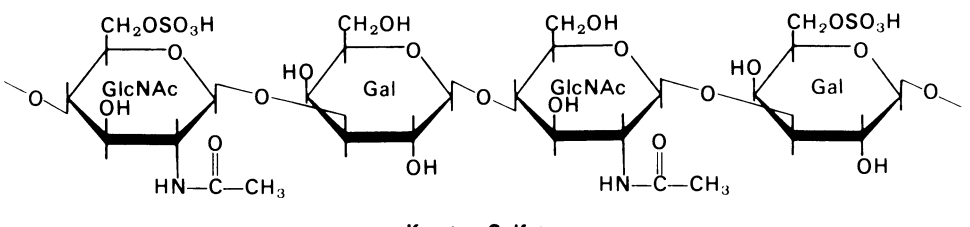


FIGURE 5.35 Structures of heparan sulfate, dermatan sulfate, and keratan sulfate.

A mucopolysaccharide occurs in the costal cartilage and cornea of the eye and other tissues, which is referred to as *keratosulfate or keratan sulfate.* It is composed of equimolar amounts of sulfate, *N*-acetylglucosamine, and galactose. The structure of keratan sulfate is variable and may contain mannose, L-fucose, and sialic acid, as isolated from various sources.[174–176] A representative sequence is elaborated in Figure 5.35. This may be represented as follows:

$$\xrightarrow{\beta 1\text{-}4} \underset{\displaystyle SO_3H}{\overset{|}{\text{GlcNAc}}} \xrightarrow{\beta 1\text{-}3} \text{Gal} \xrightarrow{\beta 1\text{-}4} \text{GalNAc} \xrightarrow{\beta 1\text{-}3} \underset{\displaystyle SO_3H}{\overset{|}{\text{Gal}}} \xrightarrow{\beta 1\text{-}4}$$

Catabolism of the Mucopolysaccharides (Glycosaminoglycans)

Most likely, the glycosaminoglycans are synthesized initially as *glycoproteins* as described in Section 5.4. This is supported by the finding of fragments of heparan sulfate in the urine which contain serine and the monosaccharides normally associated with a glycoside linkage in the glycoproteins, GlcNAc, Gal, and xylose.[175,177] They are then cleaved from the protein by various endoglycosidases.[178] The catabolism of the major heparan, dermatan, or keratan sulfate moiety then proceeds sequentially from the nonreducing end.[179]

It should be pointed out that glycosaminoglycans form complexes with polypeptides and it is possibly that they are transported in this form to sites of cartilage and ground substance synthesis, where they form complexes with collagen to build a structure such as cartilage, a blood vessel or a membrane.

Those studying glycosoaminoglycan catabolism have assigned sequential numbers to the various enzymes, approximately in the order in which they act on heparan, dermatan, and keratan sulfates. These are listed in Table 5.6 in the order in which they act on the glycosaminoglycans.

Figures 5.36 and 5.37 indicate the steps in the degradation of typical mucopolysaccharides.

In the *mucopolysaccharide (glycosaminoglycan) storage diseases* one or more of these steps cannot take place at the normal rate. This results in accumulation of the glycosaminoglycans in the lysosomes. The lysosomes become engorged and swell, resulting in the swelling of the liver,

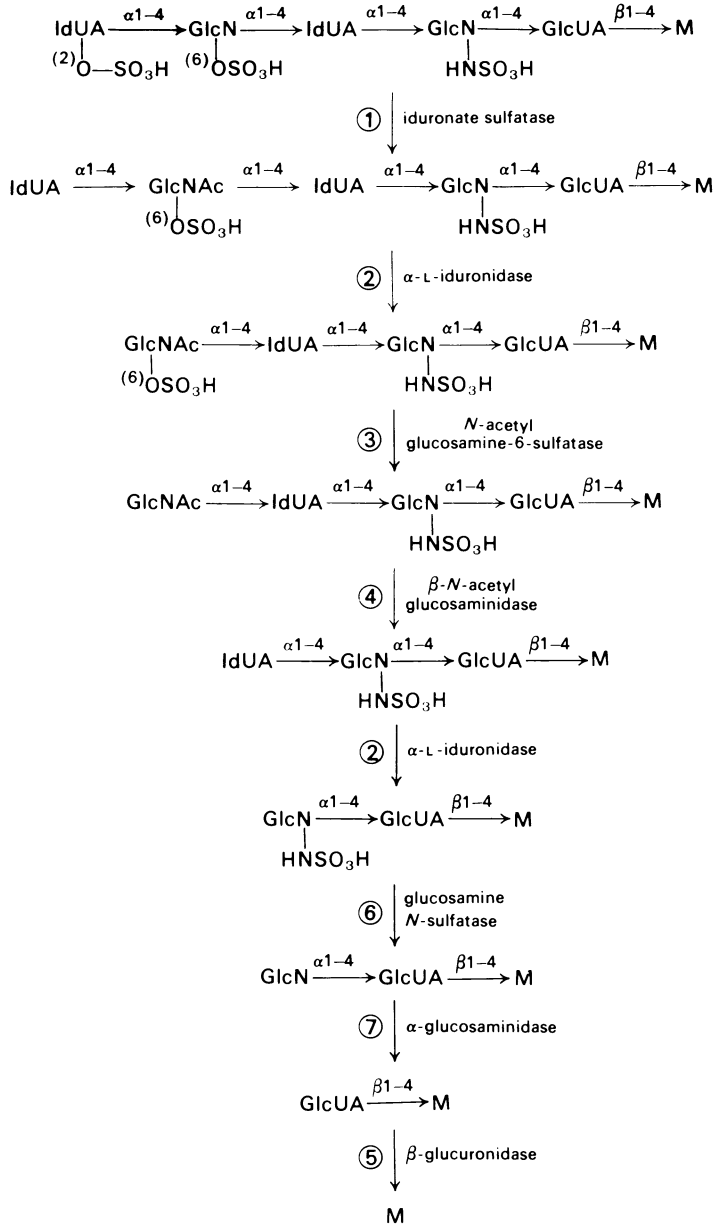


FIGURE 5.36 Steps in the catabolism of heparan sulfate. Heparan and dermatan sulfates have the same uronic acid components, but differ in that the amino sugar is galactosamine in dermatan sulfate, with β linkages. M represents the repeating units of the mucopolysaccharide. The circled numbers refer to the enzyme numbers in Table 5.6.

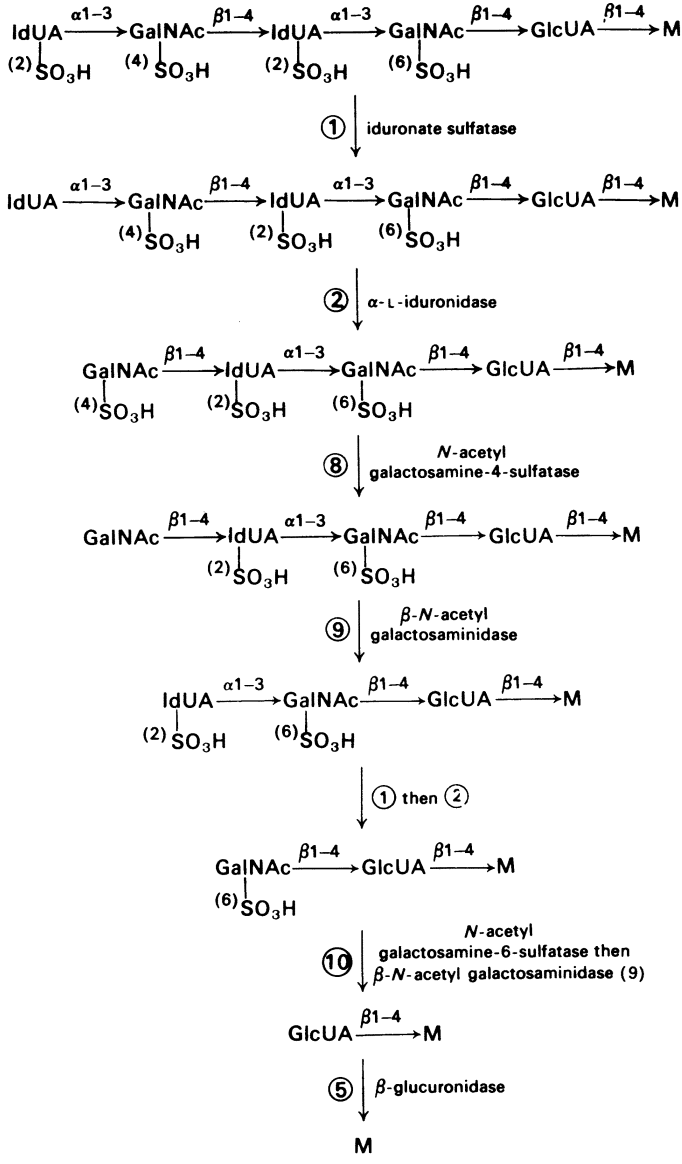


FIGURE 5.37 Steps in the catabolism of dermatan sulfate. M represents the continuation of the mucopolysaccharide chain. The circled numbers refer to the enzyme numbers in Table 5.6. The numbers in parentheses refer to the position of attachment on the sugar.

TABLE 5.6

Enzymes Responsible for the Catabolism of the Glycosaminoglycans in Approximate Order of Their Action on the Substrate

Sequence number[a]	Alternative designation	E.C. designation	Substrate
1	L-Iduronate sulfatase	—	L-Iduronate-2-sulfate (heparan and keratan sulfates)
2	α-L-Iduronidase	3.2.1.76	Iduronate α1-4 linkage (heparan and keratan sulfates)
3	*N*-acetylglucosamine-6-sulfate sulfatase (aryl-sulfatase B)	3.1.6.1[b]	*N*-Acetylglucosamine-6-sulfate
4	β-*N*-acetyl-α-glucosaminidase	3.2.1.50	α1 Linkage of *N*-acetylglucosamine
5	β-Glucuronidase	3.2.1.31	β-Glucuronides
6	(Heparan *N*-sulfatase) Sulfamidase (*N*-sulfatase)	—	α-Glucosamine-*N*-sulfate
7	α-Glucosaminidase	—	α-Glucosamine, 1–4 linkage
8	*N*-acetylgalactosamine-4-sulfatase	3.1.6.1[b]	*N*-Acetylgalactosamine-4-sulfate
9	β-*N*-Acetylgalactosaminidase	—	*N*-Acetylgalactosamine-β1–4 linkage
10	*N*-Acetylgalactosamine 6-SO_4-sulfatase	3.1.6.1[b]	*N*-Acetylgalactosamine-6-sulfate

[a] Sequence numbers of McKusick *et al.*[180]

[b] The same number is assigned to a group of aryl sulfatases.

heart, spleen, and other tissues. In addition, the mechanism for laying the foundation for the mineralization of bones and teeth is compromised, and a general dysostosis (defect in the ossification of cartilage) develops in severe cases called *dysostosis multiplex*. Figure 5.38 is an electron micrograph of the appearance of the lysosomes in a lysosomal storage disease.

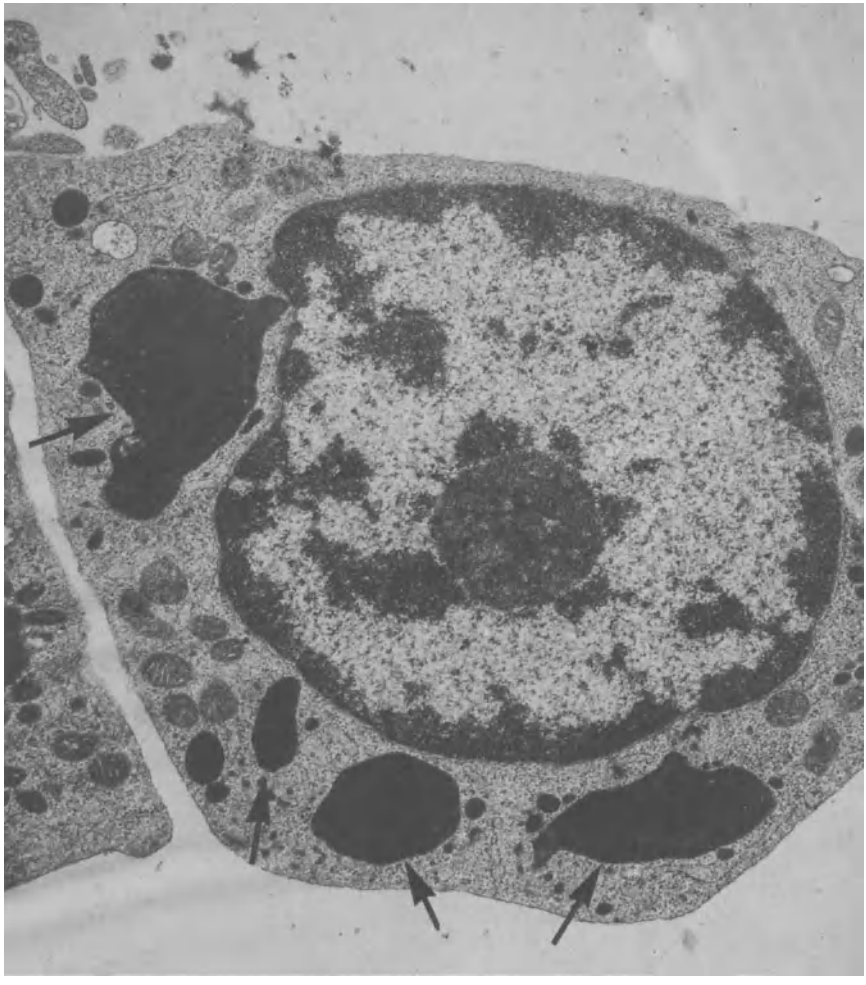


FIGURE 5.38 Electron micrograph showing enlarged lysosomes (arrows) due to storage of huge amounts of glycolipids in a granulocyte. Courtesy of Joseph Dardano, NASA Research Laboratories, Houston (× 13000).

5.10 *GENETIC GLYCOSAMINOGLYCAN STORAGE DISEASES*

The first clear descriptions of patients with genetic glycosaminoglycan storage diseases were those of Hunter in 1917 and Hurler in 1919.[181] Subsequently, these syndromes were referred to as *gargoylism*, from the facial appearance of the patients, *dysostsis multiplex*, from the skeletal deformities, and *lipochondrodystrophy*, on the assumption that the storage material was lipid in nature. In 1952, it was demonstrated by metachromatic staining of tissue, that the stored material was composed of mucopolysaccharides. The name *mucopolysaccharidosis* was then used[182] for Hurler's, Hunter's, and related syndromes.

In 1957, large quantities of heparan and dermatan sulfate were isolated from the urine of a patient with Hurler's syndrome.[179] Subsequently, this finding was confirmed by others. It was soon found that patients with related symptoms also excreted mucopolysaccharides, but of different composition. It then became possible to classify the diseases biochemically, first by the nature of the polysaccharides accumulating, and subsequently by the enzymatic defect which caused certain glycosoaminoglycans to deposit in the tissues and be excreted in the urine.[180] The classification is based on the enzymes listed in Table 5.6 and referred to in Figures 5.36 and 5.37. Table 5.7 classifies the mucopolysaccharidoses and identifies the genetic defect.

5.10.1 *Hurler's Syndrome (Type I H)*

Hurler's syndrome is designated MPS-1, the MPS standing for mucopolysaccharidosis. The MPS distinguishes these type numbers from those used for the glycogenoses, lipidoses, and others. We will dispense with this designation except where its deletion would result in confusion.

Hurler's syndrome (Type I H) is a progressive disease resulting in death at about age 10. The infants appear to be normal at birth. Subsequently they progressively deteriorate mentally and physically. Dwarfing becomes evident at about 2–3 years of age, at which point growth ceases, the cornea becomes cloudy, and the facies become coarse taking on the appearance of the gargoyle on medieval castles. Joints become stiff and claw hands develop. These patients also become

TABLE 5.7

Classification of the Mucopolysaccharidoses

Type	Syndrome	Genetic deficiency	Major urinary mucopolysaccharide	Symptoms
I H	Hurler's	α-L-Iduronidase	Dermatan sulfate, heparan sulfate	Mental retardation, skeletal deformities with gargoylism, corneal clouding, death before age 10
I S	Scheie's[a]	α-L-Iduronidase	Dermatan sulfate, heparan sulfate	Similar to Hurler's but much milder; normal intelligence, probably normal life span, mild skeletal changes, especially at the joints, corneal opacity
I H/S	Hurler/Scheie	α-L-Iduronidase	Dermatan sulfate, heparan sulfate	Symptoms intermediate that of Hurler's and Scheie's
II	Hunter's (severe or mild)	Iduronate sulfatase	Dermatan sulfate, heparan sulfate	Similar to Hurlers I, but less severe, moderate mental retardation, no corneal opacity, inherited as an X-linked recessive, death usually at about 15 years; in mild form patients survive to 30–60 years and are of fair intelligence

III A	Sanfillipo A	Heparan *N*-sulfatase	Heparan sulfate	Severe mental retardation in homozygote, very little to no corneal opacity
III B	Sanfillipo B	*N*-acetyl-α-D-glucosaminidase	Heparan sulfate	Symptoms similar to that of Sanfillipo A, but different etiology
IV	Morquio's	*N*-Acetylhexosamine-6-sulfatase	Keratan sulfate	Dwarfism, skeletal deformity, osteoporosis, defect in epiphyses of vertebral bodies resulting in shortening of the neck, no mental retardation, occasional corneal opacity
V	Scheie's		Now classified as type I S	
VI	Maroteaux–Lamy	*N*-acetylgalactosamine-4-sulfatase	Dermatan sulfate	Severe skeletal deformity and corneal opacity. No mental retardation, valvular heart disease. White cell inclusions. Normal intelligence. Survives to age 20. Variants can be mild or intermediate in symptoms
VII	β-Glucuronidase deficiency	β Glucuronidase	Dermatan sulfate, heparan sulfate	Hepatosplenomegaly, white cell inclusions, dysostosis multiplex, mental retardation

[a] A variant of Hurler's syndrome; formerly Type V.

deaf. Involvement of the heart valves and lung occur with stertorous breathing. Skeletal abnormalities develop with widening of the medial end of the clavicle. The sella turcica is enlarged, the optic chiasm being elongated, creating the J-shaped or ω sella.[179–182]

The disease is inherited as an autosomal recessive. It is estimated that there is an incidence of 1 out of 100,000 births. About 1 in 150 are carriers for the disease. Death usually results from pneumonia or heart disease.[183]

The basic defect, as indicated in Table 5.7, is a *deficiency of* α-L-*iduronidase*, enzyme No. 2 in Table 5.6, and Figures 5.36 and 5.37. It is therefore apparent that except for some removal of sulfate, *dermatan sulfate and heparan sulfate cannot be metabolized.* These patients possess adequate amounts of glucuronidase. For this reason, chondroitin sulfates A and C and keratan sulfate can be metabolized (see Figures 5.33 and 5.35).

Diagnosis can be first made by metachromatic staining by the urine of a paper impregnated with alcian or toluidine blue. Cetyl pyridinium salts will precipitate the mucopolysaccharides from urine. Albumin acidified with dilute acid will also precipitate the mucopolysaccharides.[184–186]

A most practical method for identifying some of the glycosaminoglycans is by agarose and acrylamide gel electrophoresis. Since the sulfated mucopolysaccharides are highly charged, they move fairly rapidly at pH 9.0 and separate. Chondroitin sulfates A, B, and C are available commercially. Heparan sulfate is readily prepared from beef lung. Electrophoresis can be carried out before and after the action of chondroitinases, which are available commercially. By these techniques, it is practicable to distinguish Hurler's from Hunter's syndrome or Sanfilippo A and B.[187]

Although it is practicable to demonstrate the accumulation of [^{35}S]-mucopolysaccharides by cultured fibroblasts and the correction of this defect by adding α-L-iduronidase,[188] a much simpler approach is to determine α-L-iduronidase in the leukocytes of the patient.[189–192] The use of α-L-iduronidase in cultured fibroblasts of amniotic fluid is useful in detecting Hurler's syndrome prenatally.[188] The heterozygote can be detected since the α-L-iduronidase concentration of leukocytes is about half that of the normal.

There is no treatment for Hurler's syndrome which will affect the course of the disease process. Infusions of α-L-iduronidase have been

tried in several patients. The urinary excretion of mucopolysaccharides decreased but the effect was transient and did not influence the progressive course of the disease significantly.[194] This needs to be explored further.

5.10.2 *Scheie's Syndrome (Type I S)*

A syndrome was discovered in 1962 which resembled Hurler's disease, except that it was observed in adults.[195] Enzyme studies revealed a deficiency of α-L-iduronidase.[196] Since the disease is a milder form of Hurler's disease, it was expected that some α-L-iduronidase activity was present. This could not be confirmed with cultured fibroblasts.[197] In tissue culture studies, Scheie cells will not correct the Hurler cell deficiency.

The patient with Scheie's syndrome shows severe clouding of the cornea, deformity of the bone structure, and involvement of the aortic valve. Intelligence is normal. Patients with Scheie's syndrome include college graduates and professionals. Deafness is common among these patients.

The disease is usually associated with corneal clouding and pigmentary degeneration of the retina and glaucoma leading to visual disability. Facies are coarse, with the broad mouth characteristic of Hurler's disease. Stiff joints develop as the patient matures with claw hand and other skeletal deformities and the development of carpal tunnel syndrome. Patients complain of stiff, painful legs which interferes with walking. Feet are misshapen. Stature can be normal, although short stature is usually reported in these patients.

Scheie's disease is inherited as an autosomal recessive. A woman with Scheie's disease is reported to have given birth to a normal son.[195] The frequency of Scheie's syndrome is estimated at 1 in 500,000 births.[198]

It is postulated that Hurler's and Scheie's syndrome represent homozygosity for two different alleles at the structural locus for α-L-iduronidase synthesis. This concept is supported by a phenotype with α iduronidase deficiency intermediate Hurler's and Scheie's syndrome. This is designated *Type I H/S mucopolysaccharidosis.*

Symptoms in patients with the hybrid Hurler–Scheie's syndrome, are intermediate in severity to the two primary syndromes. These

patients are short with dysostosis multiplex, mental retardation, and severe involvement of the heart and liver. The cornea is cloudy. Stiff joints and claw hand occur in these patients. Valvular heart lesions are a constant finding. There is a tendency to a receding chin. There is marked destruction in the sella turcica region and cribiform plate, with the development of an arachnoid cyst. Patients tend to be blind with degenerative optic nerve changes. Spinal fluid block at the level of C2 and C5 occurs.

Patients with the Hurler–Scheie's syndrome have been known to survive to their 20s and some females have become pregnant. On autopsy, mucopolysaccharide storage in connective tissue is observed throughout the body. The dura is thickened and contains infiltrates of foamy macrophages loaded with mucopolysaccharides.

The mucopolysaccharides found in the urine in Scheie's disease are the same as in Hurler's syndrome. In the less severe Scheie's disease with normal intelligence, eye surgery for glaucoma and corneal transplantation have been successful.[199]

The laboratory tests for Hurler's and Scheie's syndrome do not distinguish between these conditions. This can only be done clinically. However, the absence of α-L-iduronidase in the leucocytes of a patient who has gone beyond puberty is diagnostic of Scheie's or the compound disease, depending upon the clinical behavior of the patient.[200]

5.10.3 *Hunter's Syndrome (Type II)*

Hunter's syndrome is a disease of glycosaminoglycan accumulation in the lysosomes, as is Hurler's, but differs in its cause. It is a much milder disease and is distinct from Hurler's since there is no clouding of the cornea. The prominent features of this condition are stiff joints, dwarfing, coarse facial features, progressive deafness, mental deterioration, and progressive loss of vision due to papilledema and retinitis pigmentosa. In the severe form of the disease, patients live to about 15 years of age. In these cases hydrocephalus develops. The autonomic nervous control system is defective and diarrhea is a common problem in these patients.[201]

In the mild form of Hunter's syndrome the patients show rosy cheeks (plethoric appearance), the voice is hoarse, and heart disease is chronic caused by valvular, myocardial, and ischemic factors. Death

usually results from a heart attack. Degenerative arthritis and lung disease with airway obstruction is seen in the adults. With the mild form of Hunter's disease, patients have survived to beyond 60 years of age. Some show normal intelligence and minimum skeletal symptoms.[202]

The defect in the Hunter's syndrome is a deficiency of the enzyme L-*iduronate sulfatase* (see Figures 5.36 and 5.37 and Table 5.6. The first step in the catabolism of dermatan sulfate and heparan sulfate is blocked. As a result, these glycosaminoglycans accumulate in all tissues of the body, and are excreted in huge quantities in the urine. Keratan sulfate and chondroitins A and C are metabolized normally (Figures 5.33 and 5.35) since they do not contain the iduronate-2-sulfate residue. Since sulfates have a tendency to hydrolyze nonenzymatically to some extent, some metabolism of dermatan and heparan sulfate is possible. This may be a factor in reducing the severity of the disease in Hunter's as compared to Hurler's syndrome. An ameliorating factor is that with the sulfate attached, the glycosaminoglycans are more soluble. Thus, in Hurler's syndrome the material deposited in the lysosomes is more insoluble and accumulates more rapidly, resulting in more severe symptoms.

Hunter's syndrome is readily noted as a suspected mucopolysaccharidosis with the screening tests for mucopolysaccharides in the urine, as described under Hurler's disease.[184–186] Electrophoresis is useful, since the more highly sulfated dermatan and heparan sulfates have an increased mobility over the lesser sulfated mucopolysaccharides.[187] If Hunter's disease is suspected, a direct test for iduronate sulfatase is practicable in serum and leukocytes.[199,203,204]

Confirmation of Hunter's disease can be made by adding the normal cells to cultured fibroblasts from the patient to see whether this corrects the accumulation of [^{35}S]-mucopolysaccharides. Cloning can reveal the Hunter cells in heterozygotes. Occasionally the heterozygote cells become spontaneously enriched with Hunter cells during cell culture.[205] The use of cell cultures is still the preferred technique for prenatal determination of Hunter's syndrome in the homo- and heterozygote.[193]

In contrast to Hurler's disease, which is inherited as an autosomal recessive, Hunter's syndrome is inherited as an X-linked recessive. Mutations at the same X-chromosome locus probably account for the variability of the severity of this disease.

The Lyon hypothesis (see Volume 2, pp. 217, 218, 473) designed to explain mosaicism in erythrocytes, proposes that only one X chromo-

some is functional in each cell. There would therefore be two populations of cells in the Hunter heterozygote (trait) whether the maternal (Hunter) or paternal (normal) X-chromosome is active. This hypothesis is used to explain the mosaicism seen in cell cultures when the cells obtained from heterozygotes are stained with metachromatic stains to reveal the deposited mucopolysaccharides.

Attempts have been made to treat the Hunter's syndrome with plasma and lymphocyte transfusions to supply iduronate sulfatase.[194,206,207] Results have been only minimally effective. *An interesting approach is the use of a skin graft from a histocompatible sibling.*[208] This was effective in significantly decreasing heparan and dermatan sulfate excretion in the urine.

5.10.4 *Sanfilippo Syndromes A and B (Type III)*

Sanfilippo syndrome was first reported (1961) as a variant of Hurler's disease.[209] This case was subsequently shown to have the Type B form of the disease. Two years later the condition was described more fully and recognized as a disease different from Hurler's or Hunter's.[210]

The clinical course is severe. Progressive mental retardation is usually not marked until about 5–6 years of age. Subsequently, the patients lose the power of speech and survive only to about age 20. There is no clouding of the cornea, hepatosplenomegaly is slight, and the bone changes are less severe than in Hurler's or Hunter's disease. Dwarfing is moderate. The calvaria tends to be unusually dense and dorsolumbar vertebral bodies show ovoid dysplasia. Hirsutism is common. Mucopolysaccharide concentration in urine is not very high and may be negative. Cardiac valvular defects develop and in one case the mitral valve was replaced in a child 3 years old.[211]

Both types of Sanfillipo syndrome are inherited as autosomal recessives. The A type of the disease is more common by at least a factor of two.[212]

The defect in Sanfillipo A is the absence of the sulfatase which hydrolyzes the sulfate from the nitrogen in heparan.[213] This is listed as enzyme No. 3 in Figure 5.36 and Table 5.6. This enzyme is not required for the hydrolysis of dermatan sulfate (Figure 5.37) and dermatan sulfate is not found in the urine of these patients in increased amounts. *This*

distinguishes Sanfillipo from Hurler's and Hunter's disease. This enzyme is sometimes referred to as *heparan-N-sulfatase* and *heparin sulfamidase* since it acts on these substrates.

The enzyme deficiency of the B form of Sanfillipo disease is unrelated to that of the A form. In this case there is a deficiency of β-N-acetyl-α-glucosaminidase. This is listed as enzyme No. 4 in Table 5.6 and Figure 5.36. This enzyme is required to hydrolyze heparan sulfate (Figure 5.36) but not dermatan sulfate (Figure 5.37). Thus in both Sanfillipo A and B only heparan sulfate is found in increased amounts in the urine. Addition of *N*-acetyl-α-D-glucosaminidase to a culture medium of fibroblasts from patients with Sanfillipo B disease corrects the defect.[214]

In a case of Sanfillipo B disease, the presence of an inactive form of the *N*-acetyl-α-glucosaminidase was shown by immunochemical techniques.[215]

Urinary excretion of heparan sulfate with the clinical symptoms described, is evidence for pursuing the concept that the patient has the Sanfillipo syndrome. Serum or leukocyte assay for *N*-acetyl-α-glucosaminidase is effective in the identification of the Sanfillipo homo- and heterozygote.[216] The differences in structure of the heparan in the A and B syndrome has been used to differentiate these two conditions.[217,218] Prenatal diagnosis using cultured fibroblasts is also a practical procedure.[219]

5.10.5 *Morquio's Syndrome (Type IV)*

This syndrome is not uncommon among the French Canadians of Eastern Quebec Province.[220] It bears the name of Morquio who first described the syndrome in 1929. The disease is characterized by short limbs, out of proportion to the size of the torso. The neck is contracted so the head rests almost directly on the shoulders. As our understanding of the mucopolysaccharidoses developed, it became apparent that Morquio's syndrome was a mucopolysaccharidosis. Inclusions in leukocytes and brain cells, excessive secretion of keratan sulfate but not heparan or dermatan sulfate, coarse facies, cardiorespiratory insufficiency, prominence of the lower ribs noticed at 12–18 months, all pointed to a glycosaminoglycan storage disease.[221–224]

With Morquio syndrome, growth stops after age 6–7. In severe cases, dental enamel is abnormally thin.[223] Clouding of the cornea is

mild and deafness is an invariable finding. The joints tend to be excessively loose and instability at the wrist tends to incapacitate the patient. A constant feature of the syndrome is absence or severe hypoplasia of the odontoid process of the second cervical vertebra. Atlantoaxial subluxation is a major problem. Aortic regurgitation develops in some patients.

By the time these patients reach their teens, keratan sulfate excretion in the urine is reduced so that it is almost undetectable. Although knock-knees is a serious problem in the severe form of the disease, some of these patients with mild symptoms have straight legs.[222]

Patients with the severe form of Morquio's syndrome do not survive much beyond 20–30 years of age. Patients with the milder forms have lived beyond 60 years of age.

Figure 5.33 illustrates the fact that chondroitin sulfate contains sulfate ester on *N*-acetylgalactose at both the 4 and 6 position. Dermatan sulfate contains sulfate only in the 4 position. The sulfate in keratan sulfate is in the 6 position on *N*-acetylglucosamine and galactose (Figure 5.35). When fibroblasts cultured from a Morquio patient were incubated with chondroitin sulfate preparations, only sulfate at the 4 position could be hydrolyzed. Thus, the defect in Morquio's syndrome seems to be in deficiency of *N*-acetylgalactosyl-6-sulfate sulfatase.[225] Keratan sulfate, however, has the sulfate on galactose and *N*-acetylglucosamine and not on *N*-acetylgalactosamine (Figure 5.35). It is probable that the enzyme specificity is for sulfate on the 6 position and the *N*-acetyl group and the configuration of the monosaccharide does not interfere in its action as long as it is in the D-form.[226]

At present, the diagnosis of Morquio's syndrome depends upon the clinical appearance and behavior of the patient and the finding of keratan sulfate in the urine.

No effective treatment for Morquio's syndrome has been developed.

A syndrome which combines the features of Morquio's and Sanfillippo syndromes has been reported in a patient. *This 5-year-old male had an N-acetylglucosamine-6-sulfate sulfatase deficiency.*[227a] This is enzyme No. 3 in Table 5.6 and Figure 5.36. The patient was of short stature, with excessive coarse hair, hepatomegaly, mild dysostosis multiplex, and hypoplasia of the odontoid. The cornea was clear. Both keratan and heparan sulfate were found in the urine. Circulating

lymphocytes, stained with toluidine blue, showed ring-shaped deposits under the cell membrane.

Unlike Morquio's syndrome, cultured fibroblasts accumulated [^{35}S]-mucopolysaccharides. Both heparan and keratan sulfates contain sulfated *N*-acetylglucosamine, which is the substrate for the missing enzyme. Both parents showed partial deficiency of the enzyme. The syndrome is therefore inherited as an autosomal recessive.

5.10.6 *Maroteaux–Lamy Syndrome (Type VI)*

In 1963, a report appeared describing a patient with dysostosis multiplex, but differing from Hurler's syndrome in that the urine contained chondroitin sulfate (Figure 5.33). The syndrome is named after the authors.[227] In Type VI mucopolysaccharidosis the bone abnormalities become obvious at about age 2 with genu valgum (knees twisted outward), lumbar kyphosis (hunchback), contracture of the fingers, and anterior sternal protrusion.[228] There is severe restriction of movement of the joints. The corneas are cloudy and cardiac abnormalities, seen in Hurler's syndrome, are present. Hydrocephalus, requiring a shunt, is common, and there are neurological complications resulting from atlantoaxial subluxation (partial dislocation of the first vertebra).[229] The joints, especially the head of the femurs, are severely affected. Patients with the severe form of the disease rarely survive past their 20's.

Milder cases occur where the stiffness at the joints are minimal, stature is short, and corneas are cloudy. However, these patients are able to work at various occupations.[230] Cases intermediate the mild and severe form also occur. These patients resemble Scheie's syndrome patients but are shorter in stature. They may be of superior intelligence. However, there are substantial changes at their joints and hips.[231]

Even in the mild form of Maroteaux–Lamy syndrome, there is myelopathy because of compression of the cervical region of the spinal cord by thickened dura. Neurological deterioration proceeds slowly but progressively. Aortic stenosis also occurs.[229,231]

Some of the patients with a mild form of Maroteaux–Lamy syndrome have become pregnant. Severe neurological deterioration then occurs, especially in the last trimester.[229]

The defect in the Maroteaux–Lamy syndrome is the inability to hydrolyze the sulfate which occurs at the 4 position of N-acetylgalactosamine of dermatan

sulfate (*Figure 5.35*). *N*-Acetylgalactosamine-4-sulfatase and arylsulfatase B have been shown to be the same enzyme.[232] This distinguishes this disease from metachromatic leukodystrophy, where the defect is in arylsulfatase A (see Volume 2, p. 95).[233] The defect is in enzyme No. 8 of Table 5.6 and Figure 5.37.[236]

Lysosomal inclusions in the Kupffer cells and abnormal metachromatic staining in the leukocytes is severe in Type VI disease.[234,235] Inclusions are also seen in the cornea, conjuctiva, and skin.

Urinary excretion of dermatan sulfate exclusively suggests the Maroteaux–Lamy syndrome. This can be detected by acrylamide gel electrophoresis before and after treatment of the urine with arylsulfatase B. The determination of arysulfatase B activity in the leukocytes is the simplest specific test for Type VI mucopolysaccharidosis.[233–235] Prenatal diagnosis is also practicable with the use of synthetic substrates.[234]

5.10.7 *Sly Syndrome: β-Glucuronidase Deficiency (Type VII)*

In 1973, the first case of β-glucuronidase deficiency was reported.[237] This is listed as enzyme No. 5 in Figures 5.36 and 5.37 and Table 5.6. This patient was a black male, 7 weeks of age when first examined. He showed umbilical hernia, hepatosplenomegaly, metatarsus adductus, and the facies of the mucopolysaccharidoses patients. Circulating and bone marrow granulocytes showed metachromatic staining granules. Subsequently, he developed anterior chest deformity and bilateral inguinal hernias (common in the mucopolysaccharidoses) which were repaired. Between 2 and 3 years of age mental deterioration became apparent. Mucopolysaccharide levels in urine were only slightly elevated.

Several other patients have been reported subsequently. There are, however, substantial differences in the clinical course and biochemical findings. In the first patient, the cornea was clear and the patient was mentally retarded. Others showed severe corneal clouding and some showed normal intelligence.[238,239] All the patients which have been reported (about ten) showed the coarse facies and cardiac involvement. Several have shown the lung anomalies with repeated episodes of pneumonia common to the mucopolysaccharidoses.

β-Glucuronidase deficiency is inherited as an autosomal recessive, and the locus for the gene for β-glucuronidase synthesis has been found on chromosome No. 7.[240]

The deficiency of β-glucuronidase can be shown in the fibroblasts and leukocytes.[238,239] From Figures 5.36 and 5.37 it can be seen that the block occurs in enzyme No. 5. The disease is of varied expression. In some patients, only dermatan sulfate is found in the urine. In others, heparan sulfate is found. In some, both heparan and dermatan sulfate are found. In still others, only lower-molecular-weight polysaccharide fragments appear in the urine.[238]

Total deficiency of β-glucuronidase would be incompatible with life because β-glucuronidase is utilized for many purposes in the human. The fact that these patients have survived to term, indicates that they have varying amounts of glucuronidase activity. On immunoassay of tissue extracts and serum from these patients, cross-reacting material with antibodies for β-glucuronidase has been demonstrated.[241] Thus variable mutations in a structural gene for the enzyme is the probable defect in these cases.

The diagnosis of β-glucuronidase deficiency is readily made by measuring the β-glucuronidase concentration in fibroblasts, leukocytes, or serum using synthetic substrates available commercially.[242] Technology for demonstrating this condition prenatally is available.

5.10.8 *Glycosaminoglycan Lysosomal Storage Diseases*

The mucopolysaccharidoses are only one group of conditions where deposits of glycosaminoglycans are found in the lysosomes. The sphingolipidoses (discussed in detail in Volume 2, Chapter 3), are another example of this condition. They are grouped together for convenience since they concern the metabolism of globoside and related glycolipids. These are also deficiencies of some lysosomal carbohydrases.

In generalized gangliosidoses and Tay–Sachs disease, the gangliosides G_{M1} and G_{M2} accumulate because of a β-galactosidase and hexosaminidase deficiency, respectively, as follows:

$$\text{ceramide} \xrightarrow{\beta} \text{Glc} \xrightarrow{\beta} \underset{\displaystyle \text{NANA}}{\text{Gal}} \xrightarrow{\beta} \text{GalNAc} \xrightarrow{\beta} \text{Gal} \qquad (G_{M1})$$

$$\text{ceramide} \xrightarrow{\beta} \text{Glc} \xrightarrow{\beta} \underset{\displaystyle \text{NANA}}{\text{Gal}} \rightarrow \text{GalNAc} \qquad (G_{M2}, \text{Tay–Sachs})$$

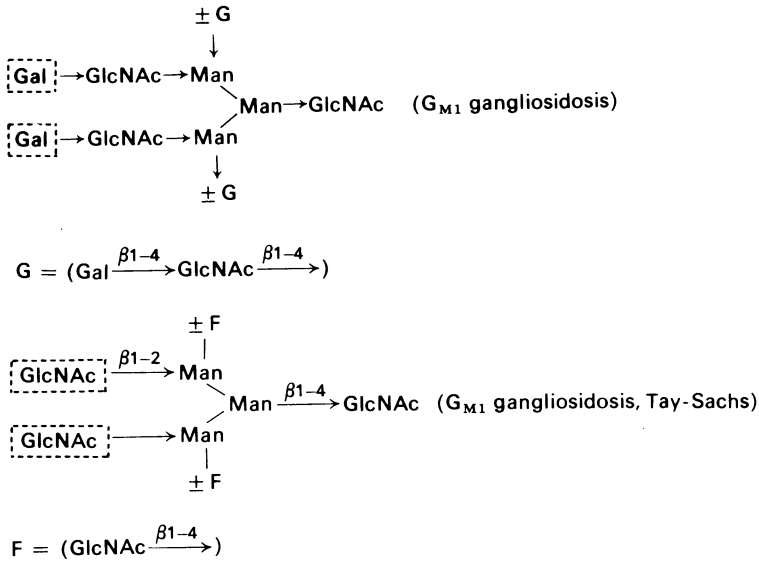


FIGURE 5.39 Polysaccharides found stored in the liver lysosomes of patients with generalized gangliosidosis and Tay–Sachs disease. The enzyme defect is emphasized by placing the monosaccharide which cannot be removed in a dotted box.

However, in addition to being unable to degrade the glycolipids, these patients also accumulate polysaccharides; their structure is shown in Figure 5.39.[242–244]

From Figure 5.39 it can be readily seen that these carbohydrates derive from a glycoprotein by endoglucosaminidase action (see Figure 5.29). *Thus the gangliosidoses and mucopolysacchasidoses are closely related to fucosidosis and mannosidosis.* They are all manifestations of defects in glycoprotein metabolism which results in storage of heterosaccharide complexes in the lysosomes. Thus they are all *lysosomal storage diseases.* Another example of this generalization is the condition which results from the inability to hydrolyze the last *N*-acetylglucosamine from the asparagine residue attached to a glycoprotein polypeptide chain (see Figure 5.29). This results in a severe lysosomal storage disease, resembling the most severe cases of Hurler's syndrome. *This disease is called aspartylglucosaminuria.*

5.10.8.1 *Aspartylglucosaminuria*

In Figure 5.29 it is pointed out that an endo-β-*N*-glucosaminidase cleaves most of the heterosaccharide chain from its attachment to the

polypeptide. This usually leaves one or two monosaccharides still attached to the polypeptide chain. In Figure 5.29, fucose is then removed leaving only *N*-acetylglucosamine attached to an asparagine residue. To cleave this last sugar from the polypeptide requires *N*-aspartylglucosaminidase (E.C. 3.5.1.26). A deficiency of this enzyme was first demonstrated in two mentally retarded siblings who had excreted large quantities of *N*-acetylglucosaminylasparagine (GlcNAC-Asn) in the urine.[245] Subsequently, this disorder was diagnosed in Finland[246,247] and the United States.[248] It is referred to as *aspartylglucosaminuria* or *AGU*. AGU, in Finland, occurs in 1 of 26,000 live births.

Symptoms of AGU resemble those of Hurler's disease. Features are coarse, and there is the typical visceromegaly and swollen lysosomes, vacuolated lymphocytes, impaired speech and motor clumsiness. Connective tissue lesions include dysostosis multiplex, umbilical hernia, and hypermobile joints. The cardiac symptoms of Hurler's and Hunter's disease are present with mitral insufficiency.

The major tissue change, as observed with the electron microscope, is enlargement of the lysosomes. Kupffer cell and kidney lysosomes are also involved.[245–249] Fibroblasts from skin biopsy also show deposits resembling the mucopolysaccharidoses. The cytoplasm of the neuronal cells from the cerebral cortex, cerebellum, and thalamic regions are filled with membrane bound vacuoles of electron-lucent granular material and electron-dense membranous or granular material with occasional lipid droplets.[248] The variety of lysosomal changes seen in aspartylglucosaminuria exceeds that seen in the mucopolysaccharidoses discussed above.

Reduced amounts of the enzyme *N*-acetylglucosaminyl-aspartamidohydrolase [2-acetamido-1-(β-L-aspartamido)-1,2-dideoxy-β-D-glucose aspartamidohydrolase, E.C. 3.5.1.26] have been demonstrated in leukocytes, seminal fluid, brain, liver, and spleen.[249–251] Measurement of the enzyme in cultured fibroblasts permits detection of heterozygotes and the condition can be detected prenatally.[252] The urine contains substantial amounts of glycoasparagines other than the simple GlcNAc-Asn compound. These are shown in Figure 5.40.[253]

5.10.8.2 *The Mucolipidoses (ML): Neuraminidase Deficiency*

Certain lysosomal storage diseases which result in the deposition of mucopolysaccharides in the lysosomes have been designated *muco-*

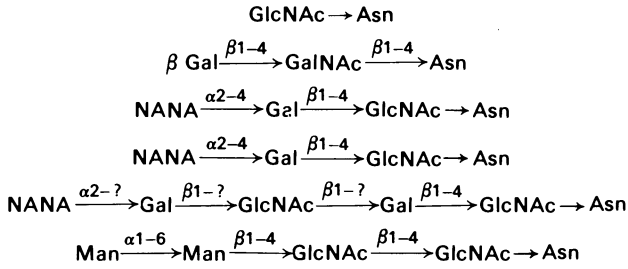


FIGURE 5.40 Glycoasparagine containing heterosaccharides from the urine of patients with aspartylglucosaminuria.

lipidoses. These conditions are distinct from the sphingolipidoses and related diseases in that the urine contains glycosaminoglycans but not lipid–carbohydrate complexes. In this group of diseases, there are at least four syndromes which resemble each other. These are labeled ML-I, ML-II, ML-III, and ML-IV. All are characterized by increased excretion of heterosaccharides containing high concentrations of sialic acids. Lysosomal deposits are also rich in sialic acid. *All show deficiency of α-N-neuraminidase, at least in some tissue.* Clinically, all these diseases resemble Hurler's syndrome of various degree of severity.[254,255a]

In ML-I, the condition is generalized. Abnormal storage of glycoprotein and/or glycolipids occurs in neuronal, mesenchymal, and visceral tissue.[256,257] The only enzyme defect is in lysosomal α-*N*-acetylneuraminidase, determined in cultured fibroblasts. Clinically, the disease resembles a severe type of Hurler's syndrome, with skeletal dysplasia, neurodegeneration with progressive ataxia, impaired speech and mental retardation. Death usually occurs before the end of childhood. These patients show the cherry red spot seen in Tay–Sach's disease. The urine contains numerous different heterosaccharide fragments containing sialic acid. Typical structures are shown in Figure 5.41.

In contrast to ML-I, ML-II and ML-III show enzyme defects not only in α-*N*-neuroaminidase but in other hydrolases as well. In addition, the disease is limited to certain tissues and lysosomal deposits are found only in the fibroblast cultures.

Clinically, mucolipidoses II and III resemble severe and milder forms of Hurler's disease. Mucolipidoses II, also called inclusion cell (I-cell) disease, is the more severe condition with severe mental retardation, hepatomegaly, cardiomegaly, recurrent upper respiratory

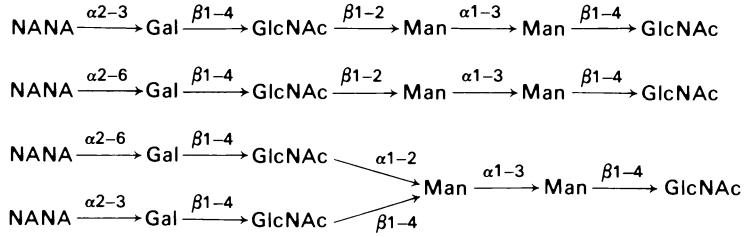


FIGURE 5.41 Typical sequences of heterosaccharides found in the urine of patients with the mucolipidoses. NANA represents α-*N*-acetylneuraminic acid.

infections, and umbilical hernia. All these conditions are progressive. Corneas remain clear. Most of these patients die before 6 years of age.[256,257] *Mucolipidosis IV is distinguished from the others by the fact that these patients develop cloudy corneas.*[255b] In other respects it resembles ML-I.

Mucolipidosis III is the most common of the conditions. Patients with mucolipidosis III can survive to adulthood. They develop the coarse facies and skeletal changes typical of Hurler's syndrome, with stiff joints, claw hands, severe changes in the lung structure, and the murmur of aortic regurgitation.[258] They are mentally retarded, and are usually assigned to the ungraded class in school. Cases have been reported where patients with mucolipidosis III have raised large families.[258,259]

Both conditions are inherited as autosomal recessives. The genetic relationship between mucolipidoses II and III is unknown, but it is apparent that they are variants of the same disease.[258–260]

A remarkable characteristic of the mucolipidoses is the marked elevation of lysosomal hydrolases in the serum. This includes almost all the hydrolases involved in carbohydrate metabolism including, α-L-iduronase, iduronate sulfatase, β glucuronidase, *N*-acetyl-β-hexosaminidase, aryl-sulfatase A, β galactosidase, α mannidase, and α-L-fucosidase. It is as though the lysosomes were disintegrating and dumping their contents into the body fluids. For this reason some have proposed a defect in the cell wall of the lysosomes as the cause of the disease. This has not been confirmed.[258,260]

In contrast to their high concentration in body fluids, the concentration of the hydrolases in connective tissue and fibroblasts is very low. As a result, mucopolysaccharides and glycolipids accumulate in fibro-

blast cultures. This can be demonstrated in tissue culture.[261] The fibroblasts of these patients tend to crack up on freezing because of an apparent defect in the cell membrane.

Sialyl polysaccharide derivatives are present in high concentration in the urine of patients with the mucolipidoses (seven to eight times the normal).[262] In Hurler's syndrome, sialoglucides are not elevated to more than two to three times the normal. In addition, deficiency in neuraminidase in fibroblasts and leukocytes has been reported in the mucolipidoses.[263,264] Sialic acid levels are also increased three- to fourfold in cultured fibroblasts from patients with ML-II syndrome.[263] Furthermore, the lysosomal hydrolases excreted by ML-II fibroblasts have an abnormal electrophoretic mobility as compared to the normal which is corrected with neuraminidase. *This has suggested that the mucolipidoses are primarily a neuraminidase deficiency in connective tissue.* The hydrolases elaborated contain excessive amounts of sialic acid, which normally would be removed. This increases their solubility and tendency to move out of the cells into the surrounding circulating fluids. The low concentration of hydrolases remaining in the lysosomes then causes polysaccharides to accumulate in the lysosomes of the connective tissue since they cannot be metabolized.

Diagnosis of the mucolipidoses is made by clinical findings of symptoms resembling Hurler's disease without excessive amounts of the mucopolysaccharides in the urine. The disease is confirmed by measuring *N*-acetyl-β-hexosaminidase and arylsulfatase A levels in serum. These should be elevated to about three times the normal. Prenatal diagnosis has been made by measuring the elevated enzyme levels in the amniotic fluid. The serum electrophoretic isoenzyme patterns of β hexosaminidase are abnormal.[265] Sialic acid levels measured in urine are helpful.

5.11 *PROTEOGLYCANS*

For centuries, bone and cartilage extracts were made for nutritional purposes (jellies) and glues. Interest was shown only in the collagen component of cartilage. Toward the end of the 19th century, attention was directed to acid-insoluble organic substances and it was discovered that these could be extracted from cartilage with dilute alkali. They

were, therefore, acids. In addition, they contained a high percentage of carbohydrate. Since they contained ester sulfates these compounds were labeled *chondroitin sulfates*, from the Greek *chondros* meaning cartilage.[266–268]

For the next 50 years, intensive studies were made on the structure of the chondroitin sulfates (now also called mucopolysaccharides, since related substances were found in mucins). During all this time, very little attention was paid to the polypeptides to which the mucopolysaccharides were attached. It was tacitly assumed that the mucopolysaccharides were synthesized and attached to a polypeptide to form a proteoglycan. The polysaccharide portion of the proteoglycan was then sulfated and subsequently split from the proteoglycan. The sulfated polysaccharide then reacted with collagen to form a polar bond.[269]

This assumption was supported by the observation that chondroitin sulfates coprecipitated with collagen to form insoluble striated fibers seen in cartilage, with the striated fibers 640 Å apart. For this reason, chondroitin sulfates were always purified by treatment with proteolytic enzymes to remove any adhering polypeptides (impurities). In spite of this treatment, it was always difficult to remove the serine residue clinging to the chondroitin sulfate.[270,271]

A new approach was taken in the 1950s when attempts were made to isolate the chondroitin sulfate–polypeptide complex intact by extracting with water or dilute saline.[270] These studies culminated in 1968 with the isolation of the chondroitin sulfate polypeptide. This was done by extraction with 4 *N* guanidine sulfate which serves to break the hydrogen bonds binding protein–chondroitin complexes to each other.[272–274]

The compounds isolated then came to be called *protein polysaccharides*, to distinguish them from the glycoproteins. Unlike the glycoproteins, they were sulfated and had been oxidized to form uronic acids. It was established soon that these mucopolysaccharides existed in covalent linkage with the polypeptides.[273,274]

In 1970, a review of this field appeared entitled "Protein Polysaccharides of Cartilage."[266] At that time, the generic name glycosaminoglycans was introduced and used internationally for what had been called the *chondroitins* or *mucopolysaccharides*. Only 3 years later, the term *protein polysaccharides*, which emphasizes the carbohydrate moiety, was replaced by the term *proteoglycans*, which stresses their existence as proteins.[267]

The average proteoglycan molecule, also called proteoglycan subunit (PGS), has a protein core of a molecular weight of about 200,000 and length of about 340 nm. To this core are attached about 100 chains of chondroitin and 50 chains of keratan sulfate; 90% of the proteoglycan subunit is carbohydrate and only 10% polypeptide. The molecular weight of the keratan sulfate is about 4000–8000 and of the chondroitins about 20,000. The total molecular weight is about 2.5 million.[275–277]

The repeating dipeptides of the glycosaminoglycan in cartilage are attached by a series of monosaccharides to serine of the polypeptide chain.[275–279] This series comprises xylose attached to serine, followed by galactose, another galactose, galacturonic acid, and then the repeating unit, glucuronate → *N*-acetylgalactosamine, of chondroitin sulfate. The sulfate esters are at the 4 or 6 position. For dermatan sulfate, the repeating unit is iduronate—*N*-acetylgalactosamine (Figure 5.33). Thus, the sequence for the core polysaccharide structure is

repeating disaccharide → GlcUA → Gal → Gal → xyl →
serine → (polypeptide)

This structure is elaborated in Figure 5.42. In cartilage, keratan sulfate is linked to the serine or threonine residues of the core protein by *N*-acetylgalactosamine.[277]

5.11.1 *Proteoglycan Aggregates*

It had been known for some time that articular cartilage contained substantial amounts of chondroitin sulfates and hyaluronic acid and lesser amounts of keratan sulfate. Studies with the electron microscope coupled with biochemical studies, showed how these three substances were bound together to form the structure of cartilage (Figure 5.43).[280,281]

The proteoglycan aggregates consist of proteoglycan subunits arising laterally at fairly regular intervals (20–30 nm) from the opposite sides of an elongated filamentous structure made up of hyaluronic acid, which ranges in length from 400 to 4000 nm.

The fragment of the proteoglycan adjacent to the hyaluronic acid binding site contains the keratan sulfate. The chondroitin sulfate chains

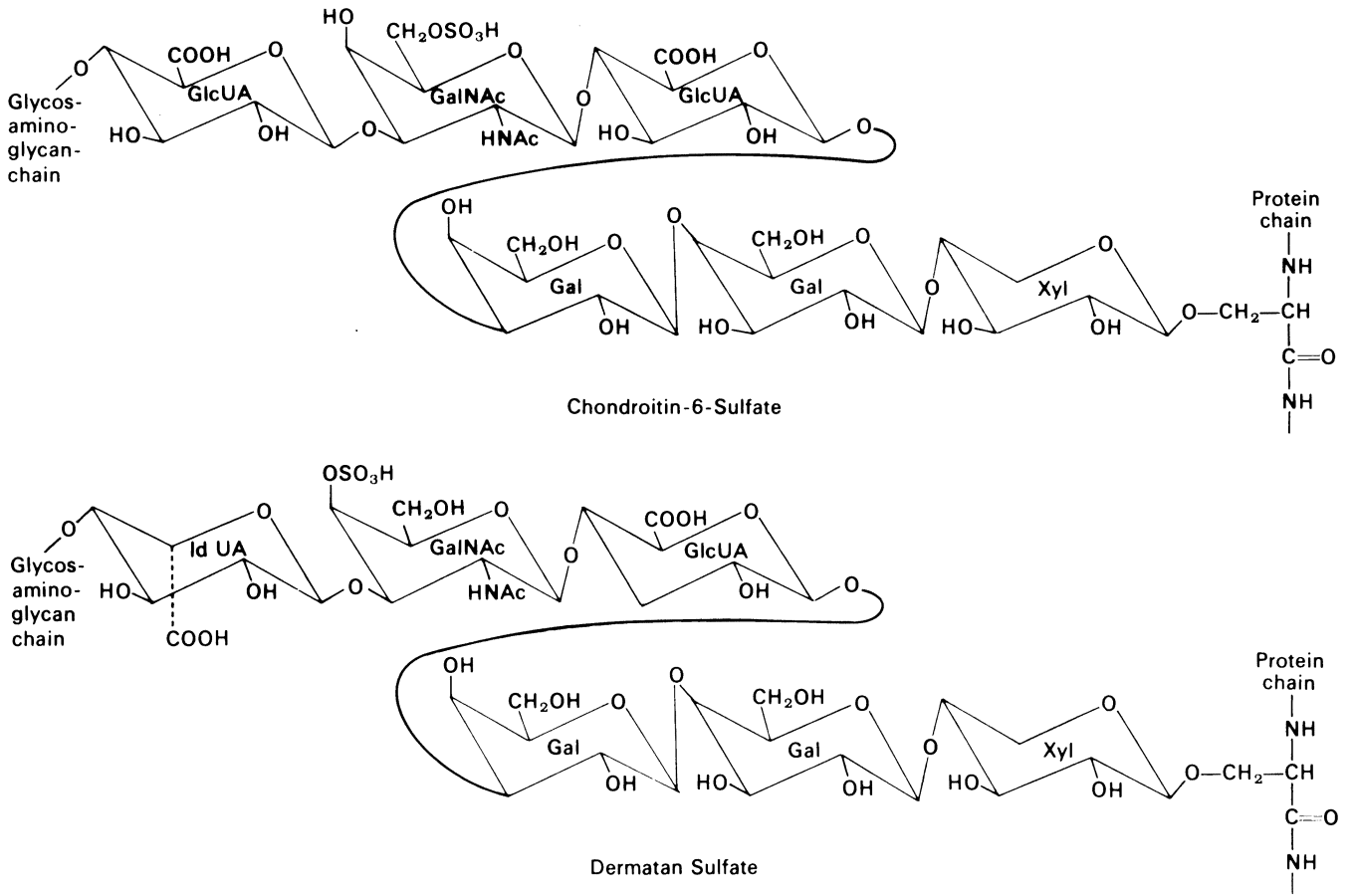


FIGURE 5.42 Linkage of repeating disaccharide units of chondroitin and dermatan sulfates to the polypeptide to form the proteoglycans.

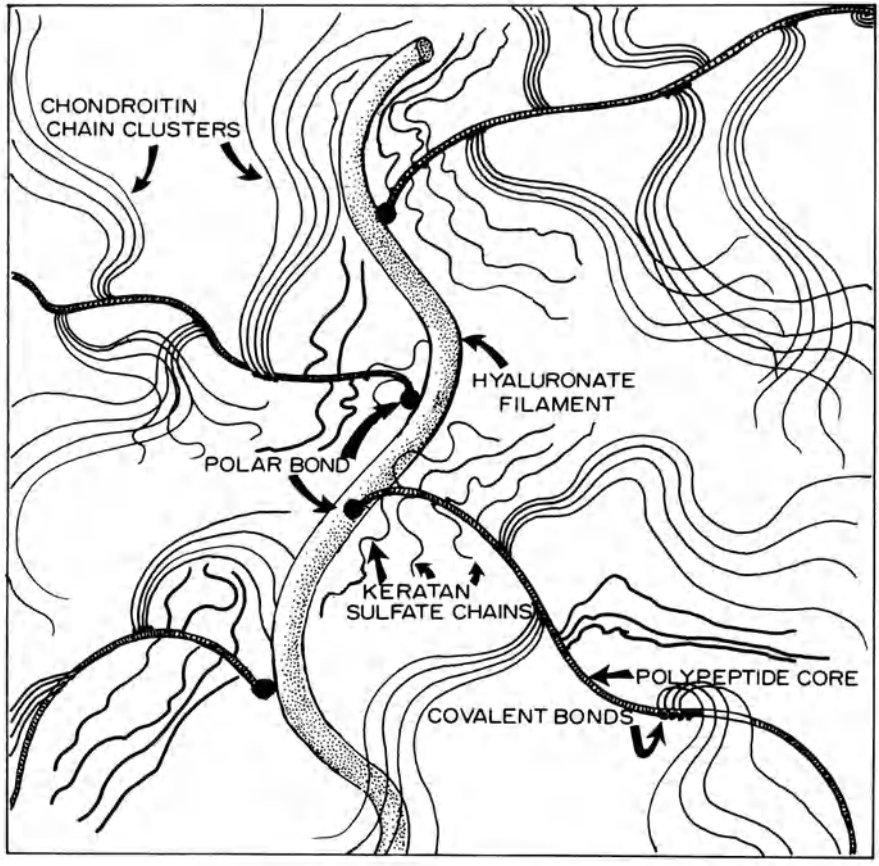


FIGURE 5.43 Schematic illustration of the structure of a proteoglycan aggregate. The polypeptide core is attached to a central hyaluronate filament. Attached to the polypeptide core are clusters of chondroitin sulfate chains. As the filament is approached, single short strands of keratan sulfate extend from the polypeptide chains. See Figure 5.44.

are further out, on the polypeptide chain. A schematic representation of this arrangement is shown in Figure 5.43. Figure 5.44 is an electron micrograph of a proteoglycan aggregate.

In cartilage, the core polypeptide and chondroitin chains of the monomer structure shown in Figure 5.43 tend to polymerize, the suspended chains entangling each other into a semi-rigid structure. This can be disentangled with 4 *N* guanidine salt solution at pH 7.4, which breaks the hydrogen bonding and solubilizes the monomers.

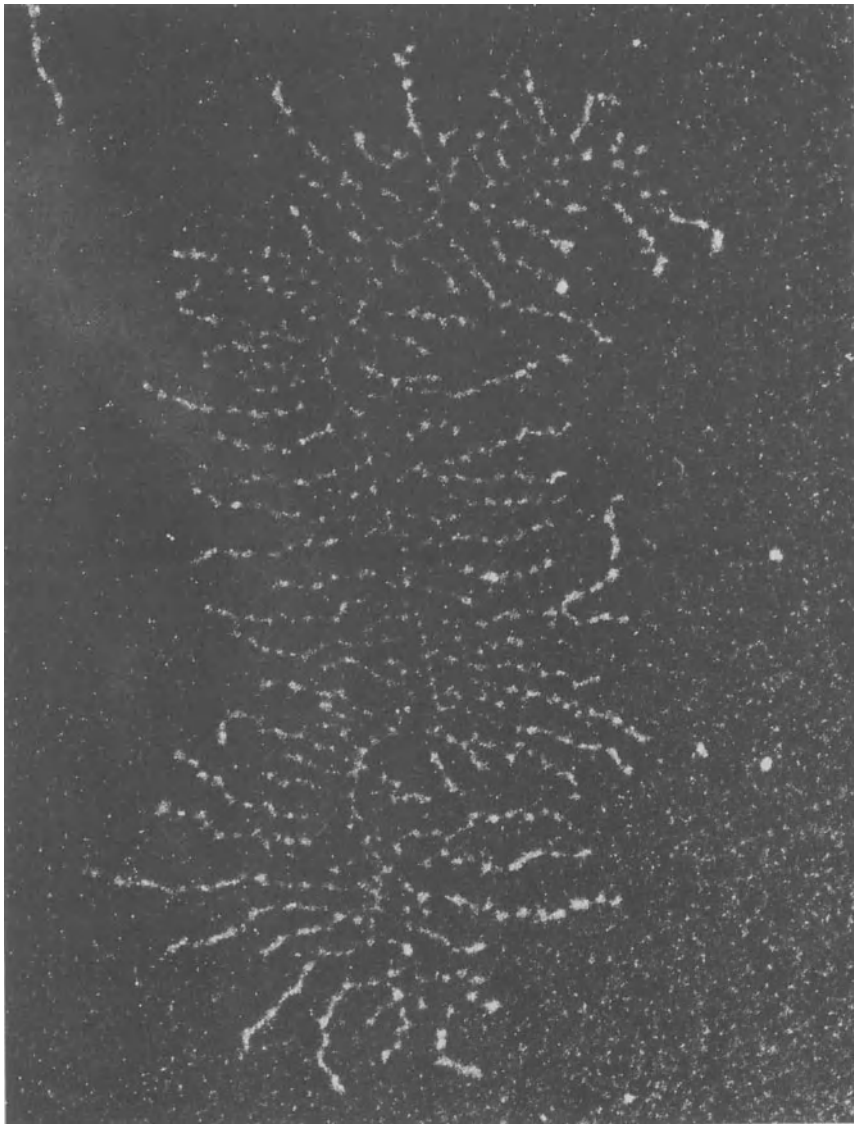


FIGURE 5.44 Proteoglycan–proteohyaluronate aggregate from bovine articular cartilage with 77 proteoglycan subunits arising from a filamentous backbone (hyaluronate), 1700 nm in length. Electron, dark-field, micrograph of proteoglycan-cytochrome-*c* complex. (Courtesy of Lawrence Rosenberg, Montefiore Hospital and Medical Center, Bronx, New York.)

In the cornea, the keratan sulfate is bound to the core polypeptide, not to serine, but through N-acetylglucosamine to asparagine, as in the glycoproteins of plasma (see Figures 5.3 and 5.4).[282–284] Cartilage keratan sulfate is bound to serine or threonine of the core polypeptide through *N*-acetylgalactosamine, as mentioned above.

Heparan sulfate, which occurs in many tissues of the body, is also a macromolecular complex attached to a common protein core, as determined in brain proteoheparan sulfate.[285] The heparan sulfate in the brain is a polymer of a disaccharide of glucuronate and glucosamine sulfates, sulfated at the nitrogen and at the 6 position of the glucosamine. Thus, it is also a proteoglycan. The polypeptide core contains serine and glycine in equimolar proportions.[285]

The proteoglycan structure of the neutral hyaluronate filament, to which the hyaluronic acid proteoglycans are attached, is still uncertain in 1979. The hyaluronate filament seems to be a single unbranched polymer attached to a protein differing from the other branched proteoglycans attached to it (Figure 5.43).[286,287]

5.11.2 *Proteoglycan Synthesis*

Almost every cell of the body has the capacity to synthesize proteoglycans. The mechanism of synthesis is similar to that described for the glycoproteins (see Section 5.4). The core protein is synthesized, and carbohydrates are then attached.[278,279,288] Retinol and dolichol are involved, and vitamin A deficiency results in impaired synthesis of the proteoglycans and disintegration of the cartilage.

The monosaccharides are transferred to the growing polysaccharide chain by transferases, as for the glycoproteins. Xylosyl transferase, galactosyl transferase I, galactosyl transferase II, and glucuronosyl transferase I serve to build the core polysaccharide chain. For chondroitin sulfate, this is followed by the action of *N*-acetylgalactosaminyl transferase I and glucuronosyl transferase II alternating to build the repeating units of chondroitin sulfate.

As the polysaccharide units are added to the growing chain, the molecules are sulfonated by "active sulfate." For this, 3′-phosphoadenylylsulfate (PAPS), and PAPS-transferase, are utilized (see Volume 1, pp. 180–182). In addition, *sulfation factor* (*somatomedin*) is required. Somatomedin is derived from the liver by action on the growth hormone. *If viable costal cartilage is added to a tissue culture medium containing sulfate,*

the sulfate will not be incorporated unless somatomedin is present. Growth hormone deficiency, as in the pituitary dwarf, results in defective sulfation of chondroitin and other aminoglycosoglycans, retarding the development of cartilage and thus the skeleton. A similar effect results from severe cirrhosis of the liver, under which condition somatomedin cannot be formed. It has been proposed that degenerative arthritis is partly a somatomedin deficiency resulting from a liver degenerative process or decrease in secretion of growth hormone.[289]

In the embryo, along with the synthesis of proteokeratans and proteochondroitins, there is simultaneous synthesis of hyaluronic acid and collagen so as to form the cartilage structure. Hyaluronidases appear at certain stages of embryo development. This permits break down of hyaluronate and remolding of the cartilage as the embryo develops. Circulatory hyaluronate removal is also necessary for cell differentiation and tissue construction.[290]

Proteoglycans synthesized by chondrocytes can be extruded so as to form cartilage (see Figures 5.6 and 5.7). On the other hand, proteoglycans and the glycosaminoglycans can be incorporated into cells such as fibroblasts by pinocytosis.[291]

The structure of the glycosaminoglycans and proteoglycan molecules vary, even when being synthesized by a single cell, because of the lack of precision in building the carbohydrate chains. This results in the heterogeneity of the synthesized proteoglycans and glycosaminoglycans as has been observed with the glycoproteins.[292–294]

Proteoglycan fragments which are formed with tissue destruction, as in injury or disease, need to be metabolized. For this purpose, carbohydrases are present, which serve to disintegrate and solubilize the carbohydrate fragments. Hyaluronidase is one such example. Various cathepsins present in liver and other tissues serve to hydrolyze the polypeptide core.[295] Healthy viable cartilage is protected from the cathepsins, since the intricate entanglement of chondroitin sulfate chains acts as a barrier to its action.

5.11.3 *Function of Proteoglycans in Health and Disease*

In different tissues, the type of glycosaminoglycan being synthesized varies also and the proportion in which their proteoglycans form a structure by complexing with collagen and elastin.

There are three major types of cartilage: fibrous, elastic, and hyaline. Fibrous cartilage is made up of parallel bundles of collagen fibers. Elastic cartilages are networks of fibers containing collagen and elastin. Hyaline cartilage is made up mostly of proteoglycans. In hyaline cartilage, only after removal of the proteoglycans by trypsin and alkali can the collagen fibrils be seen.

Cartilage may be easily deformable as skin or tendon, or it may have the hardness of bone cartilage. With advancing age, costal cartilage increases in stiffness. Cartilage, such as that of the vertebral disks, becomes dehydrated as the patient ages. This does not reflect changes in the osmotic properties of the proteoglycans but changes in their state of cross-linking and polymerization.[296]

Recent developments in our understanding of the structure of collagen has stimulated greater interest in the study of the biochemical changes which take place in cartilage in disease processes such as osteoarthritis. Although the subject is too novel for its applications to be apparent, questions are raised here which can be studied in the light of our recently acquired knowledge of the chemistry of the proteoglycans.

Joint cartilage can be damaged by trauma, inflammation, and mechanical or biochemical factors. A similar sequence of events can be produced by any of these factors. Osteoarthritis which results is therefore a heterogeneous disease. The earliest lesions seen in arthritis are on the surface of the cartilage. Alterations in the deeper zones with the distintegration of cartilage-forming clefts is progressive. The final phase is a failure of the reparative process, resulting in disintegration of the matrix, cell death, and total loss of cartilage integrity.[297] As the disease progresses, the rate of polysaccharide and DNA synthesis decreases. The cartilage which is synthesized shows formation of immature proteoglycan monomers, as indicated by alterations in the percentages of keratan and chondroitins being synthesized.[298,299] Cathepsins and other hydrolases are then released from the lysosomes which attack the partially degraded proteoglycans.

Where the disease is complicated by bacterial infection and inflammation, hyaluronidases are secreted by the bacteria which serve to disintegrate the filaments on which the proteoglycans are attached leading to disintegration of the tissue.

In rheumatic fever and rheumatoid arthritis, the inflammation affects not only the joints but also the proteoglycans of which the heart valves are formed, resulting in the typical heart defects observed with

these diseases. This has been repeatedly referred to in discussing the various mucopolysaccharidoses.

In peridontal disease, the cartilage supporting the teeth depolymerizes and disintegrates causing loosening and loss of the teeth. This occurs from trauma, as with malocclusion where excessive pressure is exerted at a particular point. More commonly it occurs with aging. The cause of this could possibly be connected with the decrease in liver function with age and decreased conversion of growth hormone (somatotropic hormone, STH) to the sulfation factor, somatomedin.

Major interest has been shown over the past 60 years in the role of cartilage in promoting mineralization of tissue and bone and teeth formation. If fibers are made by mixing collagen and chondroitin sulfate, both prepared from nasal septum, calcification will take place if placed in a calcifying solution (i.e., a solution containing Ca^{2+} and phosphate at the concentrations of normal serum). However, calcification does not take place in nasal septum *in vivo*. What factors guide the mineralization process?

Proteoglycans bind cations such as Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}, and others. These in turn cause adherence of anions such as phosphate, chloride, citrate, and sulfate. As a result, 90% of the NaCl in the body is found in the cartilage and connective tissue. This serves as a huge reservoir for NaCl, and is of major importance for animals who can only make periodic trips to salt licks since it is distant from their food supply. It is also probable that mineralization of bones and teeth starts with Ca^{2+} binding which then binds phosphate to form the hydroxy apatite structure.

Not only in the rheumatic diseases, but in other conditions including multiple epiphesial dysplasia[300] and cancer, such as in malignant glial cells,[301] abnormal proteoglycans are synthesized.

Another disease which deserves further study in connection with the proteoglycans is *cystic fibrosis*. The end group, on the nonreducing end of keratan sulfate is fucose. Thus in fucosidosis, keratan sulfate appears in the urine.[302] In cystic fibrosis, increased amounts of fucose have been reported in the urine.[303] This relationship needs to be explored.

The significance of the problems raised above are now being explored in the light of our recent understanding of the biochemistry of the proteoglycans.[304]

5.12 *RECAPITULATION*

Glycoproteins are proteins in which carbohydrate is attached by a covalent bond. *Proteoglycans* are acidic glycoproteins where some of the monosaccharides have been oxidized to uronic acids; they occur often as sulfate derivatives giving them a strongly acidic character.

Almost all the plasma proteins are glycoproteins. A notable exception is albumin. However, albumin of the hen's egg is a glycoprotein.

Glycoproteins are synthesized on the endoplasmic reticulum. A sequence of polysaccharides first accumulates on dolichol or retinol (Vitamin A). This core is transferred to a newly synthesized polypeptide in the Golgi apparatus and additional monosaccharides are added sequentially. Finally, sialic acid and fucose are added. In the case of the proteoglycans, sulfation proceeds as the carbohydrate chain is being constructed. The monosaccharides and sulfate are all added to the growing polysaccharide by transferases, specific for each monosaccharide or sulfate added.

The attachment of the heterosaccharide to the polypeptide chain is to the nitrogen of asparagine or to the hydroxyl group of serine, threonine, or hydroxylysine. The core monosaccharides attached to each type of linkage are characteristic. To asparagine, *N*-acetylglucosamine residues followed by mannose are attached most often. The mannose is then attached to two or three chains to form branches. *N*-acetylgalactosamine is usually the monosaccharide attached to serine or threonine. In the proteoglycans, xylose is attached to the hydroxyl group, followed by two galactoses and then a glucuronate residue. To this is attached repeating units of disaccharides. The repeating units for chondroitin sulfate are glucuronate-*N*-acetylgalactosamine sulfate. For dermatan sulfate, the repeating unit is sulfonated L-iduronate-GalNAc. For keratan, the repeating unit is sulfonated *N*-acetylglucosaminegalactose.

Heparin occurs in various tissues bound to a protein and is derived from a proteoglycan called heparan sulfate. The repeating unit in heparan sulfate is glucuronate-glucosamine. Hyaluronic acid has a similar sequence with the glucosamine acetylated.

In inflammation, the glycoproteins of the plasma such as α_1-acid glycoprotein, C-reactive protein, haptoglobin, ceruloplasmin, and those of the complement and clotting system are elevated. These are

called the acute-phase reactants or acute-phase proteins. They are all involved with different functions in the mechanism of defense against invading foreign bodies. Certain glycoproteins are associated with pregnancy. These are also acute-phase reactants.

There are at least 30 glycoproteins in plasma occurring in low concentration associated mainly with the α electrophoretic fractions of the serum. They tend to have a high percentage of carbohydrate and do not precipitate with dilute perchloric acid. They are referred to as seromucoids. The term *mucoprotein* is usually used to indicate a glycoprotein of high polysaccharide content, like those found in the mucus.

Glycoproteins and proteoglycans are degraded by lysosomal hydrolases, specific for each linkage in the heterosaccharide chain. Absence of any one of these results in accumulation of polysaccharides in the lysosomes. This results in swelling of the lysosomes and thus of the tissue. Accumulation of carbohydrate occurs in brain interfering with its function. This results in mental retardation. This occurs also with the sphingolipidoses and related diseases discussed in Volume 2 of this series.

When mannose or fucose cannot be hydrolyzed from the heterosaccharide, mannose- or fucose-containing polysaccharides appear in the urine. These diseases are called *mannosidosis* and *fucosidosis*, respectively. If the *N*-acetylglucosamine cannot be hydrolyzed from the aspartyl linkage of the polypeptide chain, the disease is called *aspartylglucosaminuria.*

A group of diseases occurs where the heterosaccharide chains, attached to the polypeptide of the proteoglycans, cannot be catabolized because of a lysosomal hydrolase defect. Hurler's and Hunter's syndrome are the prototypes for these diseases. They are referred to as the *mucopolysaccharidoses* after the nature of the polysaccharide found in the urine and deposited in the lysosomes. The mucopolysaccharides found in the urine and tissues in these diseases are dermatan and keratan sulfates. They are characterized by skeletal changes and characteristics of the lysosomal storage diseases.

Another group of lysosomal storage diseases are the *mucolipidoses.* In these conditions, the enzymes of the lysosomes are released into the plasma and are therefore in low concentration in the cells. The disease is limited to the cells producing cartilage, connective tissue, and skin. Some evidence has been adduced to suggest a neuraminidase deficiency in these patients.

In inflammatory disease, the proteoglycan aggregates which make up collagen are depolymerized. This exposes them to the action of cathepsins and hyaluronidase which serves to cause the cartilage to disintegrate.

A major function of the glycoproteins is for the purpose of distinguishing, by the defense mechanisms of the body, which cells are native and which are the invaders. For this purpose, heterosaccharide complexes are attached to polypeptides on the cell surface, unique for the individual. These are the histocompatability antigens. Similarly, the erythrocytes have glycoproteins bound to the cell wall which are characteristic of the various blood groups.

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6

Lipoproteins in Nutrition and Transport

6.1 *PLASMA LIPIDS: COMPOSITION*

Before discussing the lipoproteins, it is necessary to identify the nature of the lipids being transported by these proteins.

The word *lipid* is an all inclusive, general term, referring to compounds containing carbon, hydrogen, and oxygen, where the carbon content is high relative to the oxygen. This distinguishes them from carbohydrates, where the oxygen content is high. Nitrogen, phosphorus, and other elements may be present, but in lower amounts. This distinguishes the lipids from the proteins. In general, lipids are soluble in organic solvents and poorly soluble or almost insoluble in water. Thus, they are said to be *hydrophobic*, meaning that they "fear water" or tend to be immiscible with water, or *lipophilic*, meaning they "like" to be dispersed in fatty solvents.

When plasma is extracted with a nonpolar solvent, such as ether or carbon tetrachloride, less than 16% of its cholesterol content will be extracted.[1] The reason for this is that the lipids are bound to certain proteins to form the *lipoproteins*. In order to free the lipids from the lipoproteins, it is necessary to add a polar solvent to break the bonds holding the lipids to the protein. Methanol or ethanol serves this purpose. Mixtures of ether and ethanol 1:3 (Bloor's reagent),[2] ethanol and acetone 1:1 (Schoenheimer–Sperry reagent),[3] and methanol and chloroform or toluene 1:1 (Folch's reagents)[4] have all been used to extract the lipids from plasma.

Plasma lipids include mainly *cholesterol*, *cholesteryl esters*, *phospholipids*, *glycerides*, and *free fatty acids*. The distribution of these substances

TABLE 6.1

Serum Lipid Levels in the Normal Healthy Adult, Ages 18–45[5,6]

Lipid	Range, mg/100 ml	Geometric mean, mg/100 ml
Glycerides	52–141	86
Total cholesterol[a]	160–230	200
Free cholesterol (unesterified)	40–55	45
Esterified cholesterol	120–175	159
Cholesteryl esters[b]	207–294	275
Phospholipids[c]	160–250	185
Free fatty acids[d]	7–25	15
Total lipids[e]	500–790	620

[a] Values for true cholesterol level. Values obtained with nonspecific methodology are much higher.

[b] Calculated from the values for esterified cholesterol by adding the weight of oleate.

[c] The total phosphorus minus the inorganic phosphorus multiplied by 25.

[d] Values for freshly drawn serum; rises markedly on aging of serum.

[e] The sum of the weight of the cholesteryl esters and free cholesterol, plus the glycerides, phospholipids, free fatty acids, and a small amount of others not listed in this table (see text).

in plasma is shown in Table 6.1. It will be noted in Table 6.1 that four values for cholesterol are given: the total, free, and esterified cholesterol refer to the weight of cholesterol in the fractions. The value for *cholesterol esters* includes the weight of the fatty acid which esterifies the cholesterol.

The *total lipid levels* in plasma increase progressively from birth, when they are 300–500 mg/100 ml, to adulthood, when they range from approximately 400 to 800 mg/100 ml. This is a progressive increase in all of the components. For example, in individuals who are not obese, total cholesterol levels rise from 130–160 mg/100 ml at birth to 180–230 mg/100 ml at 40 years of age and remain approximately constant until 65–70 years of age, when they start to decrease together with the total lipid levels.[7,8]

In addition to the substances listed in Table 6.1, other lipids occur in smaller but biologically significant amounts in the plasma.

These include the plasmalogens, the steroid hormones, the prostaglandins, and the sphingolipids. The sphingolipids are discussed in Volume 2 of this series, pp. 75–110.

6.1.1 *Serum Cholesterol*

Cholesterol and its esters normally comprise about one-third of the total lipid in the plasma. The structure of cholesterol is given in the Appendix. Approximately three-fourths of the cholesterol is esterified, mainly as the oleate, linoleate, or palmitate. The level of cholesterol varies with age, ranging from low values on the order of 120 mg/100 ml to 150 mg/100 ml before puberty, and then rising to 180–210 mg/100 ml at age 20. Levels continue to rise so that at age 40, values range from 190 to 230 mg/100 ml. After age 60, levels begin to fall in healthy individuals and fall to levels on the order of 120–180 mg/100 ml at old age.[9–11] These values are lower than usually reported with less specific methods used with some instrumentation.

Serum cholesterol levels are markedly affected by diet, and weight reduction by dieting will cause a drop in plasma cholesterol levels. For example, cholesterol levels have risen in the United States from a range of 180–220 mg/100 ml in 1930 to 200–240 mg/100 ml in 1980. This has been directly related to a higher caloric intake because of general prosperity and public assistance programs for the indigent. Studies made on various populations of the world have shown that when an individual is on a mere subsistence diet, serum cholesterol levels can be as low as 80 mg/100 ml, as was observed in the African Bantu.[8] Among healthy overweight Americans, cholesterol levels of 240–300 mg/100 ml are not uncommon. For serum cholesterol levels in other countries, see Table 6.13.

The serum cholesterol in man may arise from two sources. With the diet, man ingests approximately 400 mg of cholesterol per day.[12] The liver, which is the major organ where cholesterol synthesis occurs, produces 700 mg per day in the normal adult. It must be pointed out that almost all tissues, including muscle, skin, kidneys, and others, synthesize some cholesterol. Thus a total of 1.1 g of cholesterol are being generated every 24 hr. This gives rise to approximately 500 mg of bile salts daily. About 600 mg of cholesterol are excreted daily,

primarily as coprosterol in the stool. A major portion of the bile salts and neutral steroids synthesized thus appear in the stool.[13] Substantial amounts of cholesterol are converted to the steroid hormones and eventually excreted, conjugated with sulfate or glucuronide, in the urine.

6.1.2 *Plasma Free Fatty Acids*

The structure and nomenclature of the fatty acids found in biological tissue are given in Volume 2, pp. 541–546. The most common fatty acids found in human tissue are straight-chain, even-numbered fatty acids. These may be saturated or unsaturated. They are listed in Table 6.2.

On electrophoresis, the free fatty acids of the plasma travel with albumin. There are three distinct binding sites on albumin for the fatty acids: the first binds two fatty acids, the second five, and the third twenty, per mole of albumin. The function of this complex is to transport the fatty acids to the sites where they are to be metabolized.[14,15] For this reason, albumin is often used as an additive to maintain fatty acids in solution in *in vitro* experiments.

TABLE 6.2

Mean Approximate Fatty Acid Composition[a] of the Lipid Components of Plasma Found in 25 Healthy Adults[10]

	Percent of total			
Fatty acid[b]	Free acid	Glycerides	Phospholipids	Cholesteryl esters
Myristic, 14:0	1.8–2.4	1–2	< 1	< 1
Palmitic, 16:0	30–32	24–28	28–31	12–14
Palmitoleic, 16:1	5–6	5–6	1.5–2.5	4–5
Stearic, 18:0	13.5–18	4–6	15–17	1.5–2.2
Oleic, 18:1	30–35	43–48	14–17	20–23
Linoleic, 18:2	9–11	12–18	22–25	50–55
Eicosatrienoic, 18:3	1–2	< 1	2–4	< 1
Arachidonic, 20:4	< 1	< 1	8–12	5–7

[a] Only the most abundant fatty acids are listed.

[b] The first number represents the number of carbons, the second (after the colon) the number of double bonds (see Volume 2, pp. 541–543).

The free fatty acid level in serum does not exceed 25 mg/100 ml in the normal individual. Because of the presence of lipases in normal serum, these levels may be found to be as high as 60 mg/100 ml, if the serum is allowed to stand at room temperature overnight. This is due mainly to hydrolysis of the glycerides in the plasma on standing.[5,6]

It can be seen from Table 6.2 that the most abundant free fatty acid in the human plasma is oleic, which comprises about 33% of the total. Next in abundance is palmitic acid, at the 25% level. Linolenic makes up approximately 10–20% of the free fatty acids, different values being reported by different investigators. The higher value is probably correct because of the tendency to obtain low values on analysis, owing to the ease with which linolenic acid is destroyed during processing. Other acids occur in small amounts, the most abundant of this group being palmitoleic (3%) and arachidonic (1.5–5%). *It is noteworthy that in both human plasma and cerumen (ear wax) the odd-numbered carbon (C-15) saturated acid, probably derived by elongation of leucine and isoleucine chains, has been consistently reported to appear in the gas chromatogram of the free fatty acids.*[14,16] Typical distribution of the free fatty acids is listed in Table 6.3.

Hormonal Control of Plasma Free Fatty Acid (FFA) Levels

The free fatty acids of plasma are in dynamic exchange with the adipose tissue. This is regulated by certain hormones. For example, insulin administration is followed by a rapid fall in plasma FFA

TABLE 6.3

Plasma Free Fatty Acid Distribution Observed with Methyl Esters by Gas Chromatography[14]

Fatty acid[a]	Percentage	Fatty acid	Percentage
12:0 (Lauric)	1.0	(Linoleic) 18:2	20.0
14:0	1.5	18:3	1.5
14:1 (Myristic)	1.0	20:0	1.0
15:0	5.2	20:3	1.5
16:0 (Palmitic)	25.0	(Arachidonic) 20:4	3.5
16:1 (Palmitoleic)	3.1	24:0	0.3
18:0 (Stearic)	2.0	24:1	0.3
18:1 (Oleic)	33.1		

[a] The first number represents the number of carbons, the second (after the colon) the number of double bonds (see Volume 2, pp. 541–543).

levels, whereas adrenaline causes a marked rise of plasma-free fatty acid levels.[17–21] Glucose metabolism is stimulated by insulin. This generates glycerol which esterifies the fatty acids and causes them to deposit as triglycerides. Insulin also has the property of inhibiting the *hormone-sensitive triglyceride lipase* (*HSTL*), or simply *hormone-sensitive lipase* (*HSL*), of the tissues which on activation hydrolyzes the triglycerides. Thus the fatty acids are prevented from leaving the fat depots by insulin. Prolactin, in large doses, has an effect similar to insulin.

Although insulin causes a lowering of the free fatty acid levels of plasma, other hormones, such as ACTH, TSH (thyroid-stimulating hormone), both α and β melanocyte-stimulating hormone (MSH), growth hormone, vasopressin, adrenalin, noradrenalin, and glucagon have the opposite effect, causing a rise in plasma FFA levels.[22] These substances all stimulate the activation of the *hormone-sensitive triglyceride lipase* (*HSTL*) directly or indirectly.[23] It is claimed by some that the effect is more an interference in the esterification of the FFA, which results in the increased plasma FFA levels. For a discussion of the lipases in the human, see the Appendix.

The prostaglandins inhibit the fat-mobilizing action of noradrenaline, corticotrophin, glucagon, and the thyroid-stimulating hormone.[24,25] Noradrenalin and corticotrophin activate adenyl cyclase in adipose tissue, causing the formation of cyclic AMP. Cyclic AMP activates the lipolytic enzyme.[19,21] It appears that the prostaglandins, especially PGE_1, inhibit the action of adenyl cyclase and thus the synthesis of cyclic AMP.[24,25] In support of this concept, cyclic AMP-induced lipolysis is not inhibited by PGE_1. These observations are summarized in Figure 6.1, HSTL signifying the active form of the hormone-sensitive triglyceride lipase.

The methylated xanthines, such as caffeine and theophylline block the action of the phosphodiesterase which hydrolyzes cyclic AMP.[26] This prevents cyclic AMP destruction, causing an elevation of cyclic AMP. As a result, the level of hormone-sensitive triglyceride lipase (HSTL) increases, resulting in hydrolysis of the deposited triglyceride in the adipose tissue. Thus, the drinking of coffee or tea causes a rise in the FFA level of the plasma.

Hormone-sensitive triglyceride lipase hydrolyzes the triglyceride to the diglyceride. The diglyceridases and monoglyceridases normally present in adipose tissue are not responsive to hormonal action. *Thus it is felt that the hydrolysis by HSTL of the triglyceride to the diglyceride is the*

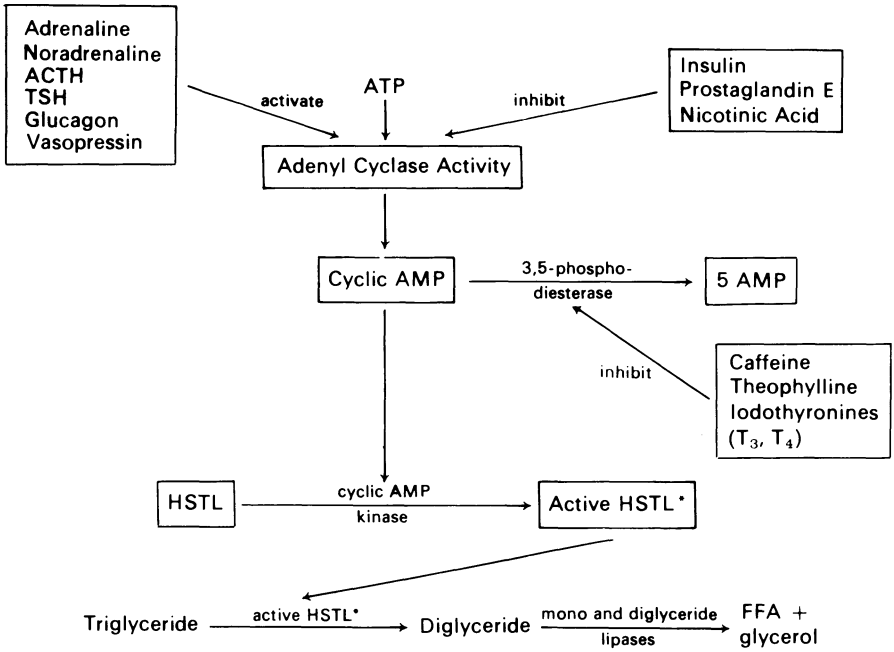


FIGURE 6.1 Schematic representation of a proposed mechanism for control of plasma level of the free fatty acids (FFA) by control of HSTL activity. HSTL* signifies the activated form of the hormone-sensitive-triglyceride-lipase.

step under hormonal control.[23] The thyronines (T_3 and T_4) act in many different ways in counteracting fat deposition. Not only do they stimulate formation of cyclic AMP but they also interfere in its hydrolysis by phosphate diesterase. Thus, intake of tetra- or triodothyronine results in an increased plasma FFA level.[27]

6.1.3 *Neutral Plasma Glycerides*

The term *glycerides* refers to esters and ethers of glycerol. This includes the *glycerophospholipids, mono-, di-, and triacyl glycerides,* and *plasmalogens.* The glycerides other than the phospholipids are called the *neutral glycerides.* The neutral glycerides are found in the β, pre-β, and chylomicron regions on the electrophoretic pattern.

On hydrolysis, the glycerides yield mainly oleic, palmitic, and linolenic acid. These three acids account for 80–94% of the fatty

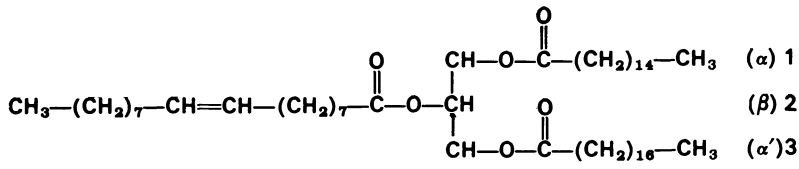


FIGURE 6.2 Structure of a typical mixed, neutral triglyceride.

acids combined with glycerol. The neutral glycerides may contain only one kind of fatty acid. Triolein and tristearin contain only oleic acid and stearic acids, respectively. Or they may be mixed. A mixed triglyceride is labeled as shown in Figure 6.2.

When referring to the neutral glycerides, the expression *triglycerides* is used most often, even though mono- and diglycerides are also present.

The plasma triglyceride level varies widely, ranging from about 50–150 mg/100 ml when fasting, to several thousands about 1 hr after a fatty meal. In the latter case the plasma is turbid.[28,29]

6.1.4 *Phospholipids*

The phospholipids are derivatives of phosphatidic acid (Figure 6.3). Phosphatidic acid forms phosphate esters with a number of compounds having an hydroxyl group, such as ethanolamine, choline, inositol, glycerine, and serine. To this list must be added the plasmalogens, where a hemiacetal is formed at the 1 position of glycerol which is then dehydrated to form a vinyl ether in place of an ester. Reduction of the vinyl group results in phospholipids with ethers at position 1. These are also not uncommon. The spingomyelins, which are also phospholipids, are discussed in detail in Volume 2 of this series, pp. 96–98. Table 6.4 lists the types of fatty acids found in the phospholipids. Note that the fatty acids in position 2 are mainly unsaturated, except for phosphatidyl inositol.

Phospholipids are usually estimated in serum by assaying for total and inorganic phosphorus and subtracting the value for inorganic phosphorus from the total. The resultant number is multiplied by 25. This factor is obtained from the percentage of phosphorus in lecithin.

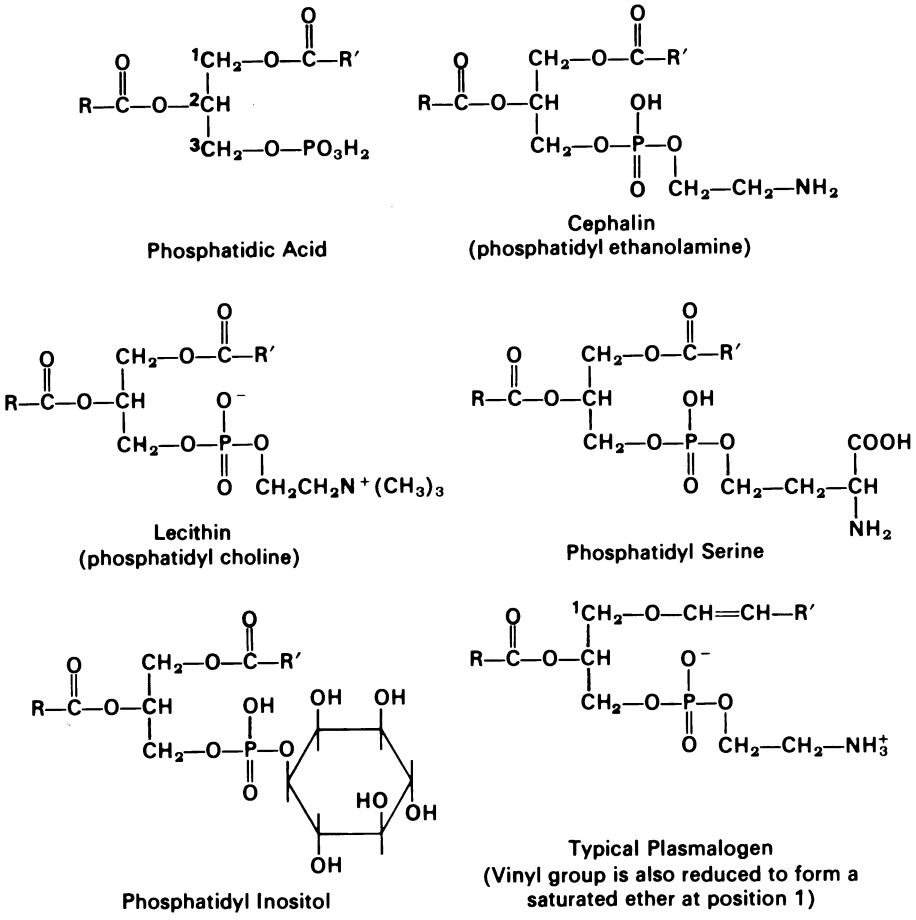


FIGURE 6.3 Typical phospholipids. R′ at position 1 are usually saturated and R at position 2 are usually unsaturated chains.

TABLE 6.4

Distribution of Fatty Acids in the Plasma Phospholipids

Position	Diacyl[a]	Plasmalogens	Glycerol ether[b]	Phosphatidyl inositol[c]
1	95% saturates	90% saturates	80% saturates	80% saturates
2	95% unsaturates	99% unsaturates	99% unsaturates	80% saturates

[a] Includes lecithins, cephalins, and phosphatidyl serines.

[b] Unsaturates in the 1 position are mainly oleate.

[c] Saturates are mainly stearate.

For example, total phosphorus = 12 mg/100 ml, inorganic phosphorus = 3 mg/100 ml; the difference, 9, multiplied by 25, equals 225 mg phospholipid per 100 ml serum. In serum from healthy adults, phospholipid values range from 150 to 290 mg/100 ml.[30–32]

Phospholipid Functions

As background for the discussion of the role of the lipoproteins in health and disease, the functions of the phospholipids in the human are summarized here.

1. *Phospholipids are major constituents of the cell membranes in the body.* In the erythrocyte, a bimolecular layer of phospholipid is formed with the lipid portions within the membrane and the polar portion (e.g., phosphocholine) on both sides of the membrane. When experimental films of phospholipids are made, they can be demonstrated to take this form.[33] This is shown in Volume 2, Figure 2.8, p. 47. (See also Figure 6.14.)

2. *Lecithins and cephalins serve as emulsifying agents to maintain the blood lipids in homogeneous form.* In lipoid nephrosis, for example, blood lipid levels may reach as high as 4000 mg/100 ml. However, if the phospholipid level is proportionately high, the plasma will remain clear. In other patients, with mild elevation of plasma lipid levels, the lipids will separate and rise to the surface if the plasma is allowed to stand. In these cases, it is found that the phospholipid level is not elevated in proportion to the total lipid concentration. (See Section 6.5.4 under hyperlipoproteinemias, and Figure 6.19, Type I.)

Lecithin serves a similar purpose in egg yolk. Lecithin is also used commercially as a natural emulsifying agent, and is added to various products especially chocolate. With this function, the phospholipid level, and the character of the phospholipids in plasma are undoubtedly related in some way to cholesterol deposition in the blood vessels and atherosclerosis.

3. *In blood coagulation, factor 3 of the platelets is a phospholipid.* This lipid is of the "cephalin" type where the amino group of ethanolamine is not methylated. Phosphatidyl serine seems also to serve this purpose. However, if pure synthetic compounds are used they are not as effective as natural "phospholipid." Mixtures of synthetic lecithin, cephalin, and phosphatidic acid have been claimed to be as effective as the natural lipid extract of the platelet.[34]

4. *Lecithin serves as a source for the esterification of cholesterol.* The enzyme lecithin–cholesterol acyltransferase (LCAT) occurs in human plasma. This enzyme mediates the transfer of the fatty acid from the β position of lecithin (mainly oleic or linoleic) to cholesterol to form cholesterol esters and lysolecithin.[35]

$$\text{lecithin} + \text{cholesterol} \xrightarrow[\text{(E.C. 2.3.1.43)}]{\text{lecithin–cholesterol acyltransferase}} \text{lysolecithin} + \text{cholesterol ester}$$

When blood is first drawn, the percentage of cholesterol is about 25%. As it stands, because of the presence of LCAT, and lecithin normally present in serum, it drops to 18%. Thus in analyzing for free cholesterol one needs to use fresh serum.[5,6]

5. *Phospholipids serve to form micelles with the fats in the intestine.*[36] This is a major factor in facilitating fat absorption.

6.2 *LIPOPROTEINS*

The first reports on the appearance of distinct lipoproteins in serum appeared in 1929.[37,38] In the late 1940s, motivated by studies on atherosclerosis, it was shown with the ultracentrifuge, that the plasma lipids were bound in a stable union to certain proteins.[39] At that time, this observation was considered remarkable since the mechanism for that type of binding was not apparent. These proteins were designated *lipoproteins*. It soon became apparent that the plasma lipoproteins served to transport cholesterol, glycerides, phospholipids, and fatty acids in the plasma, from sites of absorption and synthesis, to sites of utilization or storage.

At that time, the analytical ultracentrifuge had become available commercially, and extensive studies were being carried out measuring the sedimentation rate of various proteins as an aid in the study of their molecular weight, shape, and composition (see Appendix). In the presence of salts, these lipoproteins had a tendency to float, rather than sediment. By judicious selection of salt concentration, it was possible to separate them from each other and from sedimenting plasma proteins. A flotation rate (S_f), by means of which they could be characterized, was then defined.[40,41] The flotation Svedberg unit is 1×10^{-13} cm sec^{-1} dyne^{-1} g^{-1}, in an NaCl solution of 1.063 density at 26°C.

This concentration of NaCl was chosen so that the lipoproteins would not sediment, or would move upward. There is one exception: Albumin binds fatty acids, as has been presented in Section 4.4.1. On centrifugation in the ultracentrifuge in an NaCl solution of 1.063 g/ml density, this complex sediments. This results from the fact that free fatty acids make up only a small percentage of the albumin–fatty acid complex. There are only about 15 mg/100 ml of free fatty acids in serum as compared to 7 g/100 ml of albumin.[5,6]

Albumin has a density substantially higher than 1.063 and thus sediments in the centrifugal field. Most often, the albumin-free fatty acid complex, referred to as the *very-high-density lipoprotein (VHDL)*, is not considered a lipoprotein by those working in the field.[42]

6.2.1 *The Major Lipoprotein Groups*

On the basis of the density determination by ultracentrifugation, the lipoproteins have been divided into four classes (excluding VHDL). These are called *high-density (HDL)*, *low-density (LDL)*, *very-low-density (VLDL)*, *and chylomicrons*, as determined by their flotation rate (S_f) in the centrifugal field.[43] On electrophoresis, the chylomicrons stay at the origin, the high-density lipoproteins (HDL) spread over the α_1 and α_2 globulins, the low-density lipoproteins (LDL) move with the β globulins, and the very-low-density lipoproteins (VLDL) migrate just ahead of the β globulins, or in the pre-β region (see Figure 6.4). An additional class of lipoproteins, between the VLDL and LDL, is named

TABLE 6.5

Classification of the Lipoproteins by Sedimentation, Compared to Their Location on the Electrophoretic Pattern

Class	Density, g/ml	Flotation rate, S_f units	Electrophoretic location
Chylomicrons	0.95	> 400	Origin
VLDL	0.95–1.006	20–400	Pre-β
IDL	1.006–1.019	12–20	β
LDL	1.019–1.063	0–12	β
HDL	1.063–1.21	—	α
VHDL	> 1.21	Sediments	Albumin

[a] At a salt solution density of 1.063 HDL stays suspended and does not move upward.

intermediate-density lipoprotein (*IDL*). This class has also been referred to as *remnants*. It represents the lipoproteins of density 1.006–1.019 g/liter. This group of proteins moves with the β globulins on electrophoresis.[44] As mentioned above, the very-high-density lipoprotein (VHDL) is the albumin–fatty acid complex and is found in the albumin band on electrophoresis. Table 6.5 summarizes this classification scheme.

Some have recommended the subfractionation of the high-density lipoproteins into three groups, namely, HDL_1, HDL_2, and HDL_3. The HDL_2 group contains 60% and HDL_3 40% of protein. The HDL_1

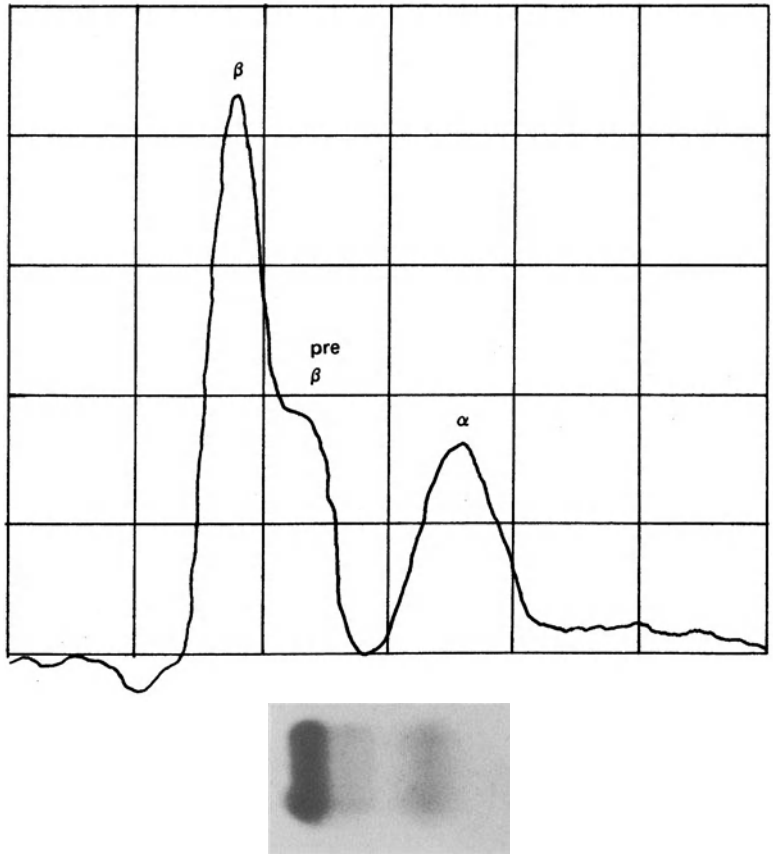


FIGURE 6.4 Electrophoresis and tracing of normal serum on agarose, stained with the lipid stain. The total lipid concentrations in this serum was 660 mg/100 ml. Total cholesterol was 210 mg/100 ml with 26% unesterified. Phospholipids were 212 mg/100 ml.

group is referred to as *sinking prebeta.* It is also designated *lipoprotein (a), Lp(a).*[45] It varies in density from 1.050 to 1.120 g/ml and contains about 27% protein. Of this about 15% is albumin, 65% apoB (see below), and 20% a protein called apolipoprotein (a) or apoLp(a). It moves with the prebeta fraction on electrophoresis. Lp(a) has been shown immunochemically to be present in about 75% of the population. When present, Lp(a) is found at a level of about 15–50 mg/100 ml.[46,47]

6.2.2 *Composition of the Lipoprotein Classes*

The major lipids associated with the lipoproteins are cholesterol and its esters, neutral triglycerides, and phospholipids. Some sphingolipids are also present, but in lesser amounts. For the chemical structure of the lipids see Appendix. The density of the neutral triglycerides is only about 85% that of water, and it is expected that they would comprise most of the chylomicrons and very-low-density lipoproteins. On the other hand, phospholipids are esters of glycerin which contain only two fatty acids instead of three, as in the triglycerides. In addition, they contain the relative heavy phosphate group. For this reason their density is significantly higher than that of the triglycerides or the cholesteryl esters. The phospholipids contribute, therefore, to the high density of the high-density lipoproteins (HDL). This is shown in Figure 6.5.

Figure 6.5 is an ultraviolet photograph of the lipoproteins of normal serum, as they float toward the surface, in the analytical ultracentrifuge.[43] The U.V. absorbance of albumin (VHDL) is higher than that of the solvent and the albumin is sedimentating. The peak is therefore shown reversed. It can be seen from Figure 6.5 that there is a rapid increase in flotation rate, as the percentage of lipids in the lipoprotein complex increases. The flotation rate is directly related to the lipid content of the lipoprotein and thus inversely to the protein content. Figure 6.5 also shows qualitatively the lipid content and composition of the lipids in the various lipoproteins. In the figure, the phospholipids are associated with the lipoprotein complexes of higher density, whereas the triglycerides are associated with the lighter fractions. Cholesterol and cholesterol esters are concentrated mainly in the LDL fraction. However, substantial amounts of cholesterol are present in the HDL fraction. As will be seen subsequently, *this distribution of cholesterol is of important clinical significance.*

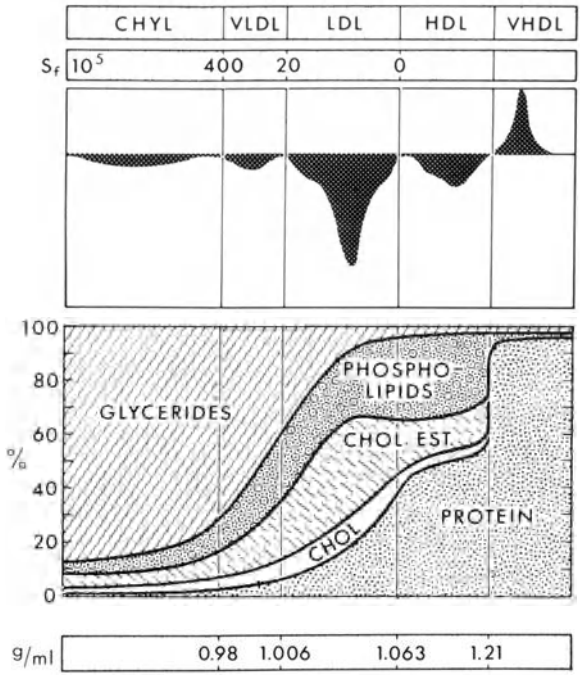


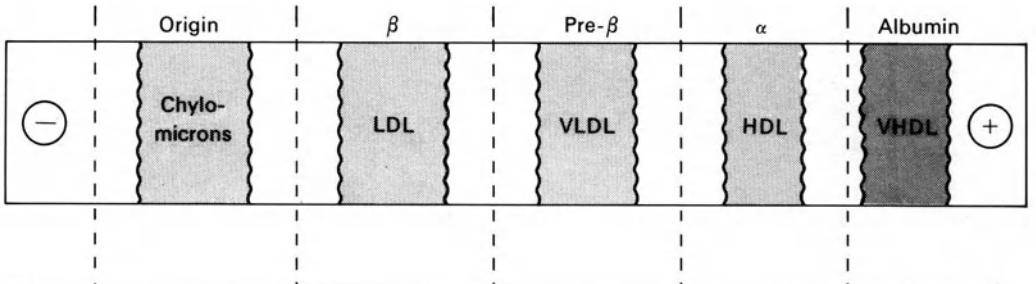
FIGURE 6.5 Schematic representation of the separation of the lipoproteins in the centrifugal field and their composition. The top portion is the U.V. diagram obtained by the centrifugation of normal human serum. The figures on the bottom (g/ml) represent the density of various fractions. The scan was obtained from normal human serum (after Scanu[43]).

From Figure 6.5 it is also apparent that only a very small amount of protein is present in the chylomicron fraction.

In Figure 6.6 the data of Figure 6.5 are correlated with the location of the various lipoprotein groups on the electrophoretic pattern. The lipoproteins do not take the same relative positions in the ultracentrifuge as they do by electrophoresis.

The four major lipoprotein classes of plasma have the following properties. Each class comprises different sizes, increasing with a decrease in density (Table 6.6).

1. *High-density lipoprotein, HDL, α_1 lipoprotein,* is isolated in the density range 1.063–1.21 g/ml. It includes two populations of particles, one set of diameter 7–10 nm, and another of diameter 4–7 nm. The lipid content is 45–55%, with phospholipid and cholesteryl ester as major components.



Density	< 0.98	1.006–1.063	0.98–1.006	1.063–1.21	> 1.21
Cholesterol Free Ester	 + +	 ++++ ++++	 ++ ++	 +++ +++	 —
Triglycerides	++++	++	+++	+	—
Free fatty acids	—	—	—	++	++++
Phospholipids	+	+++	++	++++	—
Contribution to turbidity	++++	—	+	—	—
Svedberg flotation units (S)	400+	0–20	20–400	—	Sediments

FIGURE 6.6 Correlation of the lipoprotein electrophoretic fraction with the lipoprotein classes derived from ultracentrifugal studies.

2. *Low-density lipoprotein, LDL, β lipoprotein,* is isolated in the density range 1.019–1.063 g/ml. The particle diameter is 20–25 nm. There are at least two subclasses. A more abundant, denser variety is referred to as intermediate-density lipoprotein, IDL. In the less dense variety (78% lipid) cholesteryl ester is the major component with smaller amounts of phospholipid, unesterified cholesterol, and neutral triglycerides.

3. *Very-low-density lipoprotein, VLDL, pre-β lipoprotein,* includes a wide range of triacylglycerol-rich particles, 30–80 nm in diameter; they contain about 90% of lipids. The density range is from 1.006–1.063 g/ml.

4. *Chylomicrons,* comprise larger particles (80–600 nm) with 80–90% of triacylglycerol, mainly of dietary origin, in contrast with that of VLDL, which is of mixed, largely endogenous origin. The protein content is only 1 to 2.5%. Density is less than 0.98 g/ml.

In preparing the apolipoproteins, the same reagents are used as for plasma lipid assay, such as Bloor's reagent (ethanol–diethyl ether, 3:1) or Folch's reagent (chloroform–methanol), 1:1) to remove the lipid from the lipoproteins to form the apolipoproteins[2–4] (see Section 6.2.3).

6.2.3 *The Apolipoproteins*

It was recognized early that there were differences in the nature of the proteins in the lipoprotein groups. The protein present in the β fraction (LDL) was called β lipoprotein and then B lipoprotein. The protein in the α fraction (HDL) was called the α lipoprotein or A lipoprotein.[48] The protein in the pre-β fraction, on electrophoresis (VLDL) was designated as, C lipoprotein. Numerous other designations were proposed as it became apparent that there were more than three apolipoproteins.[49,50] In 1971, a system was proposed using the designations A, B, and C to indicate the location on the electrophoretic pattern and Roman numerals to designate a specific apolipoprotein.[47] This designation is in general use today.[51,52]

With this system, two lipoproteins, apoA-I and apoA-II were found in the α (HDL) fraction, one apoB protein in the β (LDL) fraction, and three lipoproteins, apoC-I, apoC-II and apoC-III, in the pre-β (VLDL) fraction. It was also noted that small amounts of these proteins occurred as minor components in other fractions. In addition,

a small amount of a protein called the *thin-line protein* (see below) was designated D. This is also designated A-III, since it is found associated with A-II. Another protein shown to be present in the pre-β (VLDL) fraction was found to be rich in arginine and was first designated the *arginine-rich apolipoprotein.* It is now designated, apoE lipoprotein. The location of the various apolipoproteins are shown in Table 6.6, along with data on the size and composition of the particles of the lipoprotein groups.

6.2.3.1 *Apolipoprotein A (apoA)*

Chromatography of apo HDL on Sephadex or DEAE-cellulose yields two polypeptides which bind lipids, apoA-I and apoA-II.[53] These constitute more than 90% of the protein of the HDL fraction. Of this, about 65% is apoA-I and 25% apoA-II. The rest are listed as minor fractions in Table 6.6.

Human apoA-I is a single-chain polypeptide of 243 or 245 amino acid residues, as reported in two different studies (Figure 6.7).[54–57] The complete amino acid sequence of human apoA-I reported in these studies differs in about 23 amino acid residues. The molecular weight is about 27,000. There is no carbohydrate in apoA-I. ApoA-I exists in two polymorphic forms, apoA-I_1 and apoA-I_2. They have the same amino acid composition and probably represent two different conformations of the same molecule.

ApoA-I has a tendency to associate and is fractionated in the presence of urea to prevent polymerization.[58] Under physiological conditions about 55% of the molecule is in the form of the α helix, 8% as the β structure, and 37% disordered.[59] The helices contain the *amphipathic* structure so as to bind the lipids. The word, *amphipathic* was coined to designate helices with hydrophobic and hydrophilic surfaces on opposite sides of the helix so as to bind the lipids. The word derives from *amphi*, which means around or surrounded, and *path.* Thus the helix is surrounded by the lipid, binding to hydrophobic and hydrophilic surfaces. Binding is apparently mainly to the hydrophobic areas.[60]

Human apoA-I enhances the activity of lecithin–cholesterol acyl transferase (LCAT). This is apparently related to its property of binding lecithin.[61]

TABLE 6.6

The Characteristics of the Plasma Lipoprotein Fractions

Lipoproteins, mean, mg/100 ml	Mobility	Molecular[a] weight	Size, Å	Apolipoproteins		Polypeptide, %	Carbohydrate, %	Cholesterol, %		Phospholipid, %	Triglycerides, %	Free fatty acid, %	Total lipid, %
				Major	Minor			Free	Ester				
HDL M[b] 228 F[c] 249	α	200,000	75–100	apoA-I apoA-II	apoC-I apoC-II apoC-III apoD apoE	31.5	1.5	7	21.5	29	8	1.5	67
LDL M 458 F 535	β	2.1–4.6 million	215–220	apoB	—	19.2	1.8	8	38	22	10	1	79
VLDL M 156 F 148	Pre-β	5–10 million	300–800	apoC-I apoC-II apoC-III apoB apoE	apoA-I apoA-II apoD	7.2	1.7	7	15	18	50	1	91
Chylomicrons	Origin	1–10 billion	750–10,000	apoC-I apoC-II apoC-III apoB	apoA-I apoA-II	1.4	0.3	2	6	7	83	0.3	98

[a] Approximate values for lipid–lipoprotein complex.
[b] Males.
[c] Females.

NH_2—Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Asp Leu Ala Thr Val Tyr Val Asp-
1 10 20

Val Leu Lys Asp Ser Gly Arg Asp Tyr Val Ser Gln Phe Gln Gly Ser Ala Leu Gly Lys-
30 40

Gln Leu Asn Leu Lys Leu Leu Asp Asn Trp Asp Ser Val Thr Ser Thr Phe Ser Lys Leu-
50 60

Arg Glu Gln Leu Gly Pro Val Thr Gln Glu Phe Trp Asp Asn Leu Glu Lys Glu Thr Glu-
70 80

Gly Leu Arg Gln Glu Met Ser Lys Asp Leu Glu Glu Val Lys Ala Lys Val Gln Pro Tyr-
90 100

Leu Asp Asp Phe Gln Lys Lys Trp Gln Gln Gln Met Glu Leu Tyr Arg Gln Lys Val Glu-
110 120

Pro Leu Arg Ala Glu Leu Gln Glu Gly Ala Arg Gln Lys Leu His Glu Leu Gln Glu Lys-
130 140

Leu Ser Pro Leu Gly Gln Gln Met Arg Asp Arg Ala Arg Ala His Val Asp Ala Leu Arg-
150 160

Thr His Leu Ala Pro Tyr Ser Asp Glu Leu Arg Gln Arg Leu Ala Ala Arg Leu Glu Ala-
170 180

Leu Lys Glu Asn Gly Gly Ala Arg Leu Ala Glu Tyr His Ala Lys Ala Thr Glu His Leu-
190 200

Ser Thr Leu Ser Glu Lys Ala Lys Pro Ala Leu Glu Asp Leu Arg Gln Gly Leu Leu Pro-
210 220

Val Leu Glu Ser Phe Lys Val Ser Phe Leu Ser Ala Leu Glu Glu Tyr Thr Lys Lys Leu-
230 240

Asn Thr Gln—COOH
243

FIGURE 6.7 The amino acid sequence of human apolipoprotein A-I.

Human apoA-II consists of a polypeptide chain of 77 residues, which occurs mainly in the form of a dimer. The molecular weight of the dimer is 34,800.[62] The monomer has very little ordered structure. The dimer, however, has a globular form. The monomer and dimer are in equilibrium with each other with an association constant of 2.3×10^4 $(\text{mol/liter})^{-1}$ at body temperature. The association is between hydrophobic portions of the chain, and it has been suggested that lipids compete with this association for the same sites.[63,64] Thus lipid–polypeptide or polypeptide–polypeptide linkages occur. Dimerization promotes the formation of α helices, and it is probably in this form, and in the form of higher polymers, that it functions in binding the lipids. Like apoA-I, apoA-II has an affinity for phospholipids and cholesterol and functions in lipid transport. The amino acid sequence of the apoA-II chain is shown in Figure 6.8.

PCA Ala Lys Glu Pro Cys Val Glu Ser Leu Val Ser Gln Tyr Phe Gln Thr Val Thr Asp-
5 10 15 20

Tyr Gly Lys Asp Leu Met Glu Lys Val Lys Ser Pro Glu Leu Gln Ala Gln Ala Lys Ser-
25 30 35 40

Tyr Phe Glu Lys Ser Lys Glu Gln Leu Thr Pro Leu Ile Lys Lys Ala Gly Thr Glu Leu-
45 50 55 60

Val Asn Phe Leu Ser Tyr Phe Val Glu Leu Gly Thr Gln Pro Ala Thr Gln—COOH
65 70 75 77

FIGURE 6.8 The amino acid sequence of human apolipoprotein A-II. PCA stands for pyrrolidone carboxylic acid (cyclized glutamate).

Although there are no extended hydrophobic or hydrophilic sequences, in apoA-I and apoA-II, as can be seen from Figures 6.7 and 6.8, there are polar and nonpolar residues exposed on the outside of the helices permitting binding to the phospholipids. Typical nonpolar residues are phenylalanine, leucine, isoleucine, and alanine. The polar residues include aspartate, glutamate, lysine, serine, tyrosine, and tryptophan.

6.2.3.2 *Apolipoprotein B (apoB)*

The major protein of the low-density lipoprotein, LDL, β-globulin lipoprotein fraction, is designated apolipoprotein B or apoB. It comprises about 20–25% of the LDL fraction.[65] The purified apolipoprotein is only slightly soluble in water and polymerizes readily. Molecular weights have been reported, ranging from as low as 8000 to 270,000.[66] It is claimed that the higher estimates are due to aggregation of a relatively low-molecular-weight monomer. However, several investigators have maintained that the molecular weight of 250,000 is the correct one.[67] Sequencing of this protein is impracticable until this problem is resolved. With acrylamide gel electrophoresis, apoB remains at the origin, as would be expected from its high molecular weight.

Appreciable amounts of apoB are found in other lipoprotein fractions, and 40% of the protein of VLDL and 20% of the protein in the chylomicrons is apoB.[68,69]

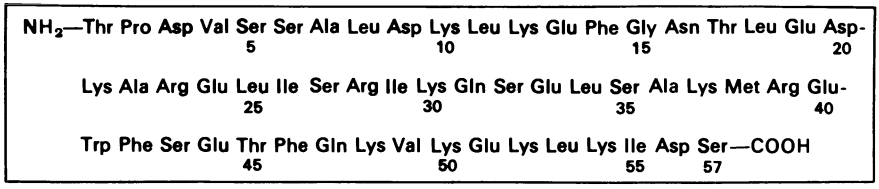


FIGURE 6.9 Amino acid sequence of apoC-I.

6.2.3.3 *Apolipoprotein C (apoC)*

Other than apoB, there are three lipoproteins found as major proteins in VLDL (pre-β fraction) which are labeled C. These are now designated C-I, C-II, and C-III. ApoC-I comprises about 5–10% of the protein of VLDL. Some of this protein is also found in significant quantities in the chylomicrons and HDL.

ApoC-I is a low-molecular-weight polypeptide which tends to associate. The monomer has a molecular weight of 6630 and has been shown to contain 57 amino acid residues (Figure 6.9).[70,71] In contrast to apoA-I and apoA-II, a large conformational change takes place when apoC-I self-associates or combines with lipids. *ApoC-I is an activator of lecithin–cholesterol acyltransferase (LCAT).*[72] These authors indicate that apoC-I does not activate lipoprotein lipase as had been reported by others.

The amino acid sequence of apoC-I is shown in Figure 6.9. The complete synthesis of apoC-I has been reported, using the solid-state synthetic approach.[73]

ApoC-II contains 79 amino acids and has a molecular weight of 9110.[74] Its amino acid sequence is shown in Figure 6.10. About 23% of the molecule is in the form of the α helix.[75] Like apoC-I, it contains

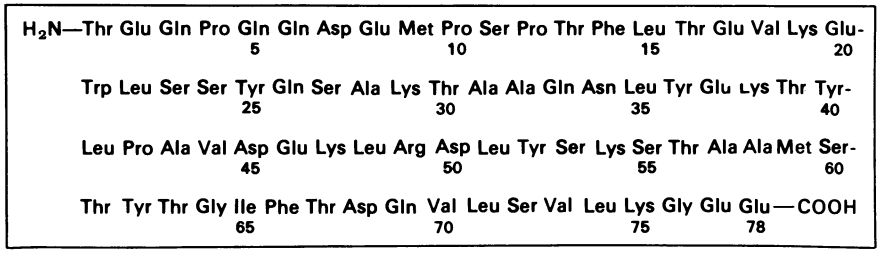


FIGURE 6.10 Amino acid sequence of apoC-II.

TABLE 6.7

Approximate Apolipoprotein Distribution in the Lipoprotein Groups in Percent

Apoprotein	Chylomicrons	VLDL	LDL	HDL
A-I	—	Trace	—	65–70
A-II	—	—	—	20–25
B	5–20	40	95	Trace
C-I	10–15	13	Trace	1–3
C-II	10–20	13	Trace	1–3
C-III	40–50	34	Trace	5–10

no carbohydrate and is found associated with apoC-I and apoC-III, not only in VLDL but also in the chylomicrons and HDL in small amounts. In VLDL it comprises about 10% of the protein fraction.

Heparin releases a lipoprotein lipase into the plasma. This lipase has also been designated *clearing factor*, since it will resolve lipemia (see Appendix). *ApoC-II activates this lipoprotein lipase.*[76]

ApoC-III is the major apoC protein in VLDL, comprising about 30% of the total protein. Thus VLDL lipoprotein is made up of about 40% apoB and 60% of the apoC proteins. ApoC-III also comprises about 45% of the protein in the chylomicrons and about 5–10% of the proteins of HDL.[77] The distribution of the proteins in the various lipid fractions is summarized in Table 6.7.

An oligosaccharide comprising galactosamine, galactose, and up to two residues of sialic acid is attached to the hydroxyl group of threonine at amino acid residue 74. This can be seen in Figure 6.11 which shows the amino acid sequence of apoC-III.[78] Three forms of apoC-III

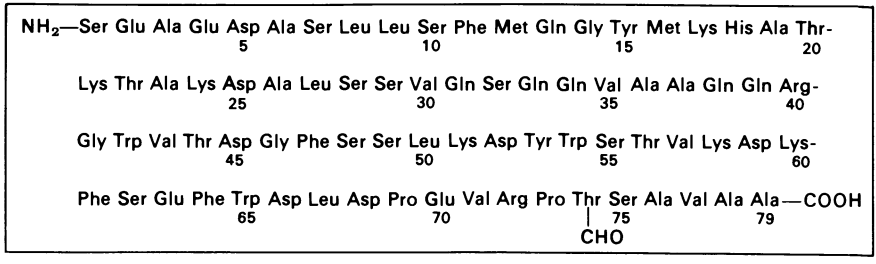


FIGURE 6.11 Amino acid sequence of apolipoprotein C-III. An oligosaccharide is attached at the Thr 74.

have been isolated: (1) without sialic acid, (2) with 1 mole of sialic acid, and (3) with two moles of sialic acid.[79,80] ApoC-III aggregates to form dimers and trimers. This is probably related to its affinity for the plasma lipids.

ApoC-III has no activating effect on the various lipases, and seems to have a nonspecific inhibitory effect on lipases in general.[81,82]

6.2.3.4 *Apolipoprotein D (apoD)*

Apolipoprotein D (apoD) was originally referred to as the *thin-line protein*, since it formed a thin precipitin line near the antigen well, using the Ouchterlony technique with anti-HDL.[51] The protein occurs in small quantities in HDL, LDL, and VLDL. This protein has also been designated apoA-III, since it was found first in HDL and its properties resemble these of apoA-I.[83]

ApoD is a glycoprotein with a molecular weight of 22,700, including 18% of carbohydrate. It contains all of the common amino acids, and both the amino and carboxyl ends are blocked. ApoD has been shown to be a powerful activator of lecithin–cholesterol acyltransferase (LCAT).[84–86] Thus, apoA-I and apoD are involved in the esterification of cholesterol and are coenzymes for this reaction. It has also been suggested that apoD is a specific carrier for lysolecithin, formed from the reaction of LCAT on HDL to transfer the unsaturated fatty acid (linoleic) to cholesterol. Figure 6.12 illustrates the action of lecithin–cholesterol acyltransferase (LCAT).

6.2.3.5 *Apolipoprotein E (apoE)*

In 1969, a protein was isolated from VLDL of normal individuals which was distinct from the other lipoproteins in that it was rich in arginine. It was referred to as the *arginine-rich* lipoprotein.[87] This protein comprises about 17% of the protein in VLDL. It is also present in HDL. Its molecular weight is estimated to be from 33,000 to 39,000, and it has been shown to have a high α helical content. Subsequently, the arginine-rich protein was given the designation apoE.[88,89]

There are three polymorphic forms shown to occur, apoE-I, apoE-II, and apoE-III. They are distinguished by their isoelectric points of 5.5, 5.6, and 5.75, respectively. *Lipoprotein X is present in high*

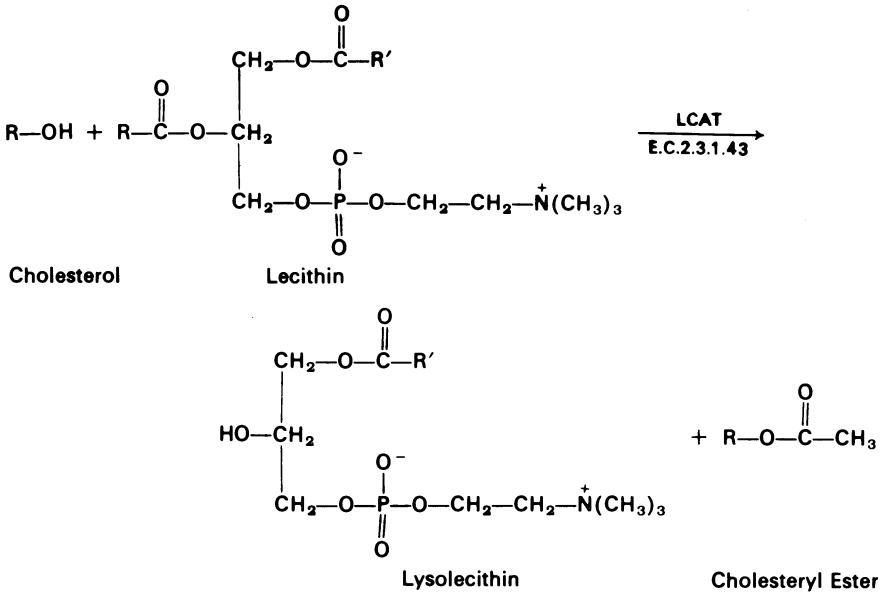


FIGURE 6.12 The action of lecithin–cholesterol acyltransferase (LCAT) in esterifying cholesterol and generating lysolecithin. The fatty acid transferred is usually unsaturated such as oleic and linoleic.

concentration in obstructive liver disease. It contains the three forms of apoE in its composition[90,91] (see Figure 6.16).

6.2.3.6 *Minor Apolipoproteins*

Other apolipoproteins are present in the lipoproteins, which have not been well characterized and whose function has not been elucidated. An example is apolipoprotein (a) which has been known for some time.[92]

In Figure 6.5, it will be noted that there is a shoulder on the LDL peak, on the side next to HDL. This has been designated by some as part of HDL and is referred to as HDL_1, whereas the two differently sized particles in HDL, referred to in Section 6.2.1, are called HDL_2 and HDL_3. The HDL_1 group, commonly referred to as lipoprotein (a) or Lp(a), contains 15% of albumin, 65% of apoB, and an unidentified protein, apo Lp(a). It is isolated in the density range of 1.05–1.09 on centrifugation and moves with the pre-β fraction on electrophoresis.[93] On electrophoresis, Lp(a) moves somewhat more slowly than

VLDL. Since it contains albumin, it sediments at the density used to separate VLDL and has been referred to as *sinking prebeta.*

In the normal adult, the amount of lipoprotein(a) is small (50 mg/liter when negative, and 300 mg/liter when positive). Lp(a) can be detected immunochemically, and about 75% of the population are found to be positive.[95,97] Its function is unknown. According to one study, the concentration of Lp(a) does not relate significantly to coronary artery disease.[95] However, many have claimed that patients whose serum contain Lp(a) are at higher risk for ischemic heart disease.[93,94] Its presence or absence from a particular serum and its concentration seems to be determined by polygenic inheritance.[95,96]

The serum of patients with obstructive jaundice contains a lipoprotein which moves to the cathode instead of the anode when subject to electrophoresis in gels. This lipoprotein has been designated *lipoprotein X* (LpX).[99,100] LpX contains only 6% protein. Free cholesterol comprises 25% of the molecule and phospholipids about 65%. The apoprotein contains about 40% albumin, the remainder being mostly the apoC proteins, with small amounts of apoA-I, apoD, and apoE.[101] ApoC-I and apoD are apparently on the surface of the lipoprotein X particle, since antibodies to these proteins react with lipoprotein X. The antigenic sites of albumin, apoC-II, and apoC-III, on the other hand, are deep in the molecule, and do not react unless lipid is first removed[102] (see Figure 6.16).

Under the electron microscope, lipoprotein X particles are globular and 300–700 Å in diameter. They tend to form stacks of disklike structures (rouleaux formation) similar to that seen with erythrocytes in certain diseases.[100]

Lipoprotein X is observed in patients with either acquired lecithin–cholesterol acytransferase (LCAT) deficiency, such as in alcoholic cirrhosis of the liver, or in the serum of patients with a genetic LCAT deficiency.[98] In obstructive jaundice bile salts are absorbed and appear in the serum. They strongly inhibit LCAT activity, resulting in a high percentage of free cholesterol and LpX formation.

Lipoprotein X has been resolved into two components, LpX_1 and LpX_2, with LpX_1 having the higher phospholipid-to-protein ratio. Both are rigid structures because of their high free cholesterol content.[103]

A protein occurs in bile which differs somewhat from LpX in protein-to-lipid ratio, and electrophoretic migration rate. However, if

albumin or serum is added to this protein, LpX is formed. Conversely, if bile salts are added to LpX, a particle with the migration rate of the bile protein is formed. This suggests that LpX originates from bile which is reabsorbed in an obstruction, and thus appears in the serum.[104]

6.3 *LIPOPROTEIN FORMATION*

The liver and intestine are the major sites of plasma lipoprotein synthesis.[105,106] The liver synthesizes only a single class of LpB particles (VLDL). The intestine secretes two classes of LpB particles, namely, chylomicrons and VLDL. Chylomicrons and VLDL are essentially triglyceride-laden LpB particles.

Apo-LpB is synthesized in the parenchymal cells of the liver, moves to the Golgi apparatus, where the carbohydrate moiety is attached, and is finally laden with phospholipids, cholesterol, and triglycerides and then secreted and picked up by the blood stream.[104] Initially, only LpB is present. After release from the site of origin, the nascent particle picks up other lipids, apoC, and apoE, finally forming the VLDL particle. This is actually a secondary particle. Thus lipoprotein fractions in plasma are in dynamic exchange with each other and with the free circulating lipids in the plasma.

VLDL is the precursor of LDL. The fate of apo-LpB has been followed in various ways, such as by amino acid and radioiodine labeling.[105–108] It seems that there is a stepwise delipidation of VLDL, so that the same particle will have progressively a VLDL, IDL, and finally an LDL composition. At first, LpC-I, C-II, C-III, and E become associated with VLDL, as it circulates in the plasma. Subsequently, as delipidation proceeds, affinity for LpC and LpE decreases, and when LDL reaches its final form, it has lost most of its LpC and LpE (Figure 6.13).

The *lipoprotein lipase*, which mediates the delipidation of the original triglyceride-rich VLDL, is located on the capillary epithelium, in adipose tissue and muscle.[111] It is this enzyme which is released into the plasma after intravenous injection of heparin. This is the "clearing factor" or *postheparin lipolytic activity* (*PHLA*).[112] In addition to triglyceride lipase, lechithinase, monoglyceridase, and phospholipase are also released into the plasma at the same time.[113] A lipase of

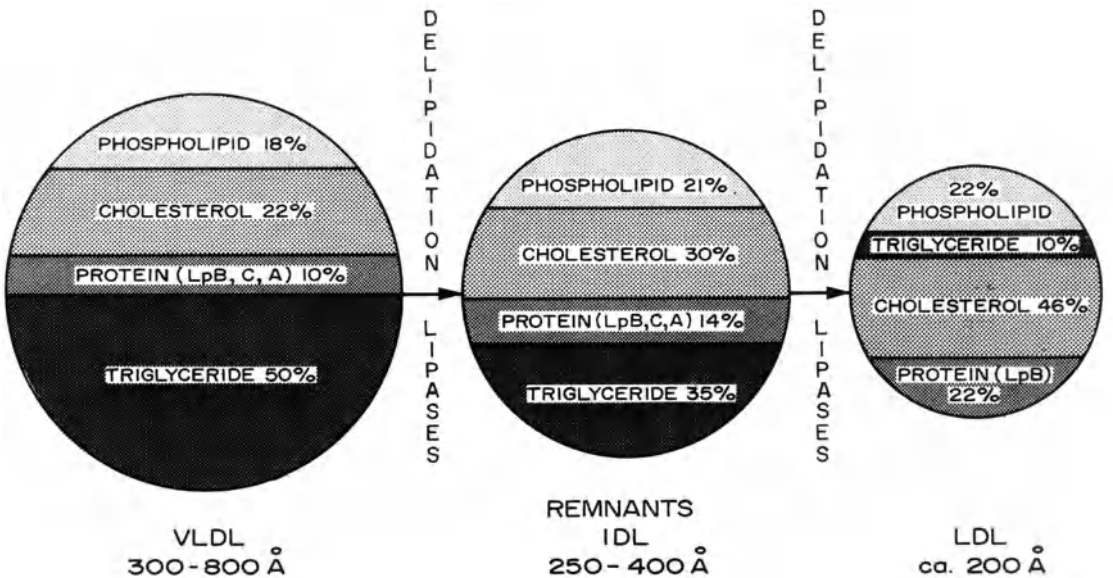


FIGURE 6.13 Approximate percentages of the various components of the lipoprotein particle as VLDL changes to IDL and then LDL. Note the increase in cholesterol protein and phospholipid concentration and decrease in the triglycerides as they are hydrolyzed by lipoprotein lipase. Lipoproteins C, E, and small amounts of A, first picked up by VLDL in the plasma, are lost as VLDL transforms to LDL.

hepatic origin, with similar amino acid composition but somewhat different specificity than lipoprotein lipase, is also released into the plasma at the same time.[114,115] After the actions of the lipoprotein lipase, the hepatic lipase acts to hydrolyze chylomicron remnants.

There seem to be two forms of lipoprotein lipase. One acts on chylomicrons and VLDL and is activated by apoC-I, and the other which is activated by apoC-II, is more effective on the smaller VLDL particles.

The absorptive cells of the intestinal epithelium are the source of the chylomicrons. The entire particle is synthesized within the intestinal cell. Extrusion of the chylomicron particle into the intestitial space takes place by reverse pinocytosis. The chylomicrons are then transported by intestinal lymphatics to the thoracic duct and thence to the systemic blood stream. The amount of chylomicron synthesis is a function of how much fat is to be absorbed.

As soon as the chylomicron reaches the blood stream it is attacked by the lipoprotein lipase and the other lipolytic enzymes and follows the course of VLDL catabolism.

The liver also synthesizes the apo-LpC proteins and secretes them, apparently, originally as components of HDL.[109,110,116,117] They are then transferred to the other lipoprotein particles. LpA is synthesized by both liver and intestine.[118,119] Thus the high-density lipoprotein (HDL) seems to be released into the plasma independently from VLDL. Exchange then takes place in the plasma to transfer protein and lipid moieties between the various particles. HDL has a half-life of 4.6 days in the human plasma.

The nascent HDL is disk shaped and rich in phosphatidylcholine and free cholesterol. The apoproteins A-I, A-II, and C, and the phosphatides seem to be involved in keeping the cholesterol suspended. The spherical shape of HDL develops in the plasma after the other components are taken up and after esterification of some of the cholesterol by LCAT.[121]

The major function of HDL seems to be in cholesterol transport. At the adrenal cortex, the free cholesterol is converted to the steroid hormones. This is stimulated by ACTH administration.[120]

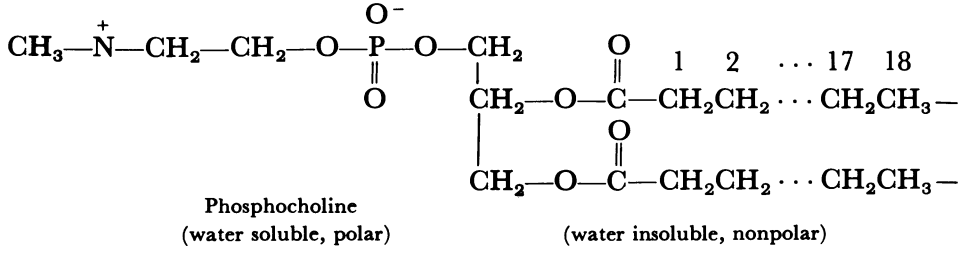
Lipoprotein E is also synthesized in the liver and intestine, and is released along with LpA and LpC in HDL.[118,121] Increased plasma LpE levels are observed when monkeys are put on a high cholesterol diet.[122] Thus, lipoproteins A, C, and E participate in the production of

a vehicle for cholesterol transport. These proteins are transferred to other particles only after the HDL reaches the blood stream.

In summary, chylomicrons, LDL, VLDL, and IDL are involved mainly in glyceride transport, HDL is a cholesterol carrier, and VHDL (albumin) carries the fatty acids. All are synthesized mainly in the liver and intestine.

Lipid Binding to the Apoproteins

Phospholipids are emulsifying agents. They have a polar and nonpolar part of the molecule as indicated in the following formula for lecithin:



The molecule can thus be represented by a polar, water-soluble (hydrophilic) head and a twin, nonpolar, water-insoluble (lyophilic) tail. When added to water, the phospholipids will take a position at the surface, with the head in the water and the tail in the air. If shaken to form small particles, little globules will form which remain suspended indefinitely. A double-molecular layer will form in which the heads of the phospholipid are in the water, and the tails oriented toward the center of the shell of the spherical particle. Since they all have the same charge they will repeal each other, and remain suspended. This can be seen in Figure 6.14 which shows a double layer of molecules. Actually, with ordinary hand shaking a series of concentric layers, with water in between, is formed. These can be broken up to form the type shown in Figure 6.14, prepared ultrasonically.[123,124]

On the other hand, if neutral lipid is present, then the lipophilic portion of the phospholipid will dissolve in the lipid, and the hydrophilic end will remain in the water. If shaken, an emulsion will form, comprising a dispersion of globular particles (see the structure of a

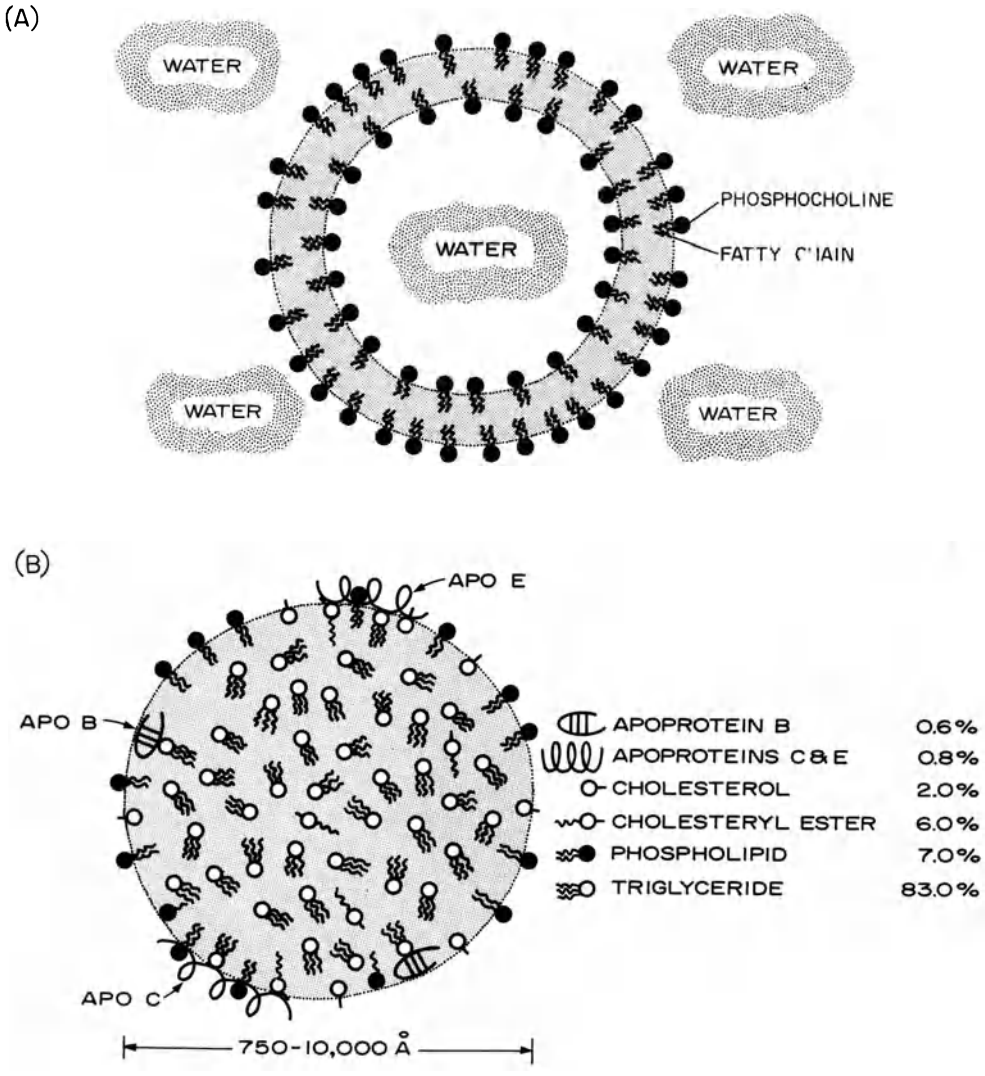


FIGURE 6.14 Comparison of a dispersion of phospholipid in water (A) to the chylomicron particle (B). When shaken by hand, the particle is made up of a series of concentric layers, interspersed by water each 20 Å in thickness. The particle shown forms by ultrasonification. In the chylomicron, the phospholipids form a monolayer, some of which is free and some bound to apoproteins C and E.

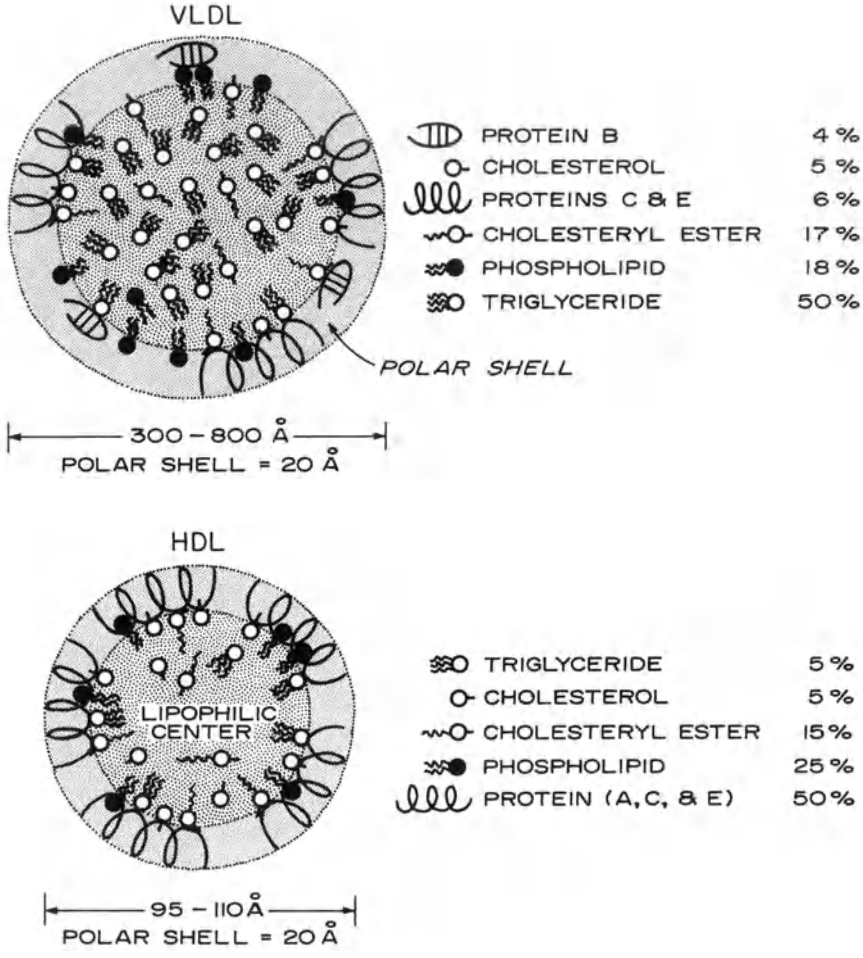


FIGURE 6.15 Comparison of proposed structure for HDL and VLDL particles. A polar shell (20–30 Å) formed by phospholipid and phospholipid protein complexes, holds the particle's spherical shape. Polar groups like the —OH groups of cholesterol are oriented so that the polar group is in the shell. The helix is oriented so that the hydrophilic amino acids are outside, and the lipophilic amino acids are inside, so as to bind cholesteryl esters and triglycerides on the underside (amphipathic). The choline phosphate portion of the phospholipids is bound to the upper part of the helix.

micelle, Figure 2.4). This can be seen in Figure 6.14, using the chylomicron as an example.

It will be noted that the particle is also stabilized by the presence of small amounts of protein which comprise less than 1.5% of the weight of the chylomicrons. The distribution of the proteins in the chylomicron is similar to that seen with VLDL. After a fatty meal, the plasma abounds in chylomicrons and the plasma appears turbid. By a process of delipidation, chylomicrons are gradually reduced in size and digested in a manner similar to that for VLDL. See Figure 6.13.

The structures of the VLDL, IDL, LDL, and HDL particles are more rigid than that of the chylomicrons. The higher concentration of protein permits stabilization of the 20Å outer shell. This can be seen in Figure 6.15. Apoprotein B can bind both polar and nonpolar groups, depending upon its orientation. This is especially true for the helical proteins A, C, and E with their amphipathic helices, polar on one side and nonpolar on the other. Thus one side binds the phosphoethanolamine derivatives of the phospholipids, whereas the other side can bind the tryglycerides and cholesterol esters.[125]

In Figure 6.15 it can be seen that HDL is 50% apoproteins A, C, and E. This makes for a rigid particle of more uniform size than VLDL. In VLDL, apoprotein B comprises about 40% of the protein. As the particle is delipidated to form LDL, the protein becomes almost exclusively B (see Figure 6.13). The outer shell of LDL is made up almost exclusively of phospholipids and apoprotein B, and cholesterol is distributed mainly throughout the inner core. From the above, it appears that HDL has a different history of formation from LDL and VLDL.

In lipoprotein X a somewhat different structure results, largely from the high free cholesterol content, and the presence of substantial amounts of albumin in the core. The core, instead of being nonpolar as in VLDL and HDL, is actually polar. The core is aqueous, and the lipoprotein X particles resembles the phospholipid–water vesicle of Figure 6.14. There are also some sphingolipids in the molecule. The albumin seems to be dissolved in the aqueous core where it serves to bind any free fatty acids and the fatty acid appendages of esterified cholesterol, triglycerides, and the sphingolipids. ApoC-I and apoD are on the surface as determined immunochemically. The apoC-II, C-III, and the albumin do not react immunochemically and are buried in the core.[126] This is represented schematically in Figure 6.16.

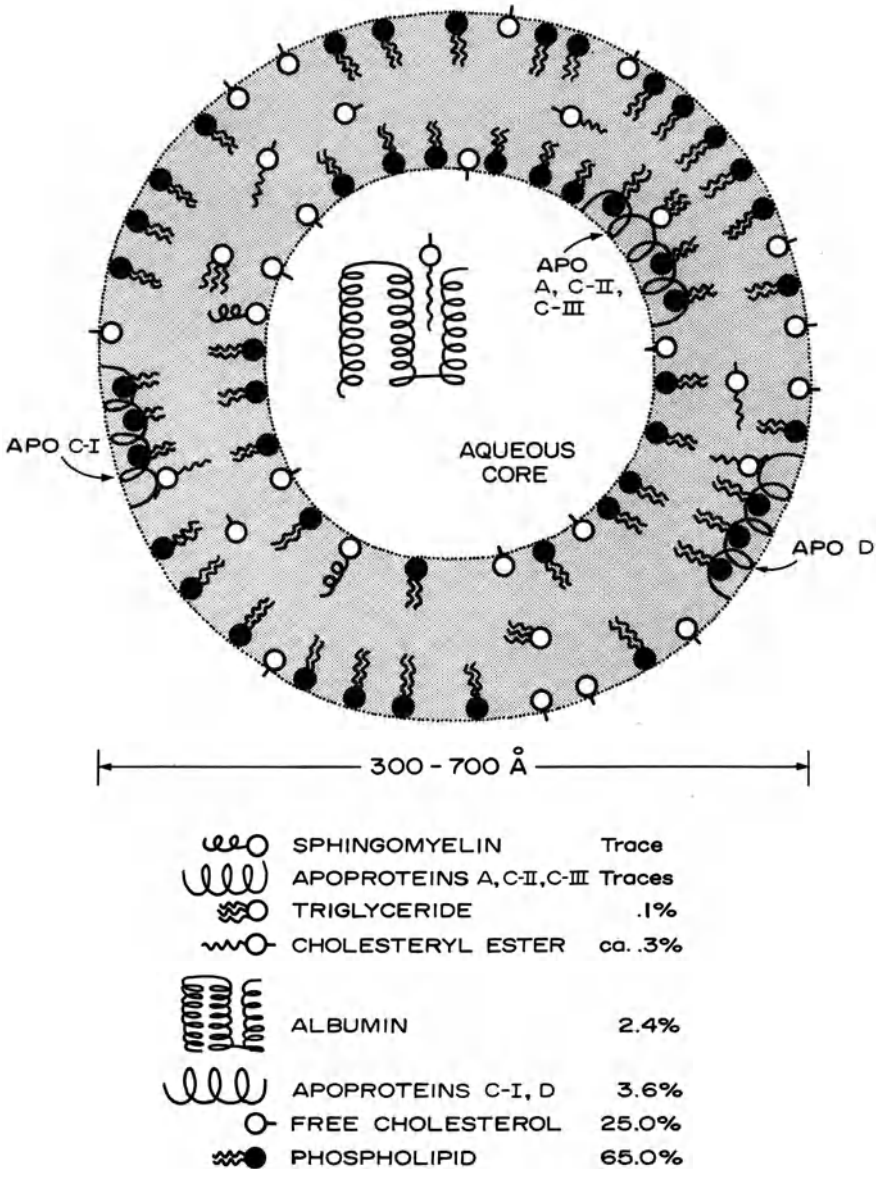


FIGURE 6.16 Schematic model of the structure of lipoprotein X showing the double layer. The albumin is dissolved in the aqueous core. ApoC-I and D are detectable immunochemically and are on the surface. ApoC-II, C-III, and albumin are not so detectable. Lipoproteins E are also present, but in low concentrations.

6.4 *NORMAL LIPOPROTEIN METABOLISM*

The concentration of the plasma lipoproteins is in a steady state, the rate of synthesis being responsive to the rate of degradation of the formed lipoproteins. In addition, the lipids like cholesterol, phospholipids, and triglycerides exchange rapidly between lipoprotein particles and membranes in the body.[127,128] This is also true for the A, C, D, and E polypeptides. For example, the cholesterol and phospholipids of the erythrocyte cell wall derive directly from the lipoproteins. The exchange of apoC proteins between VLDL and HDL can be readily demonstrated *in vitro*.[129]

The phospholipids, cholesterol, triglycerides, and proteins of the lipoprotein particles are transferred to the cell membranes, including the cell walls, mitochondria, microsomes, and others. In this manner they serve not only in the construction of the cell membrane but also as a means for transporting lipids for metabolism, to generate energy for cell metabolism. *Along with albumin, the lipoproteins have a major roll in supplying amino acids for protein synthesis and triglycerides as the major source of energy in the body.* The cholesterol of the lipoproteins is an important source for the synthesis of the steroid hormones and bile salts.

From the above, it is clear that the major function of the lipoproteins is in cell nutrition and in supplying raw materials for the synthetic functions of the cells. After performing their function, any remnants of the lipoproteins complexes are processed by the hepatocytes so as to recover residual lipids and amino acids.[130–133]

6.5 *ABNORMAL LIPOPROTEIN METABOLISM*

The fat stores in the body are designed to serve as the major raw material for energy and the production of certain substances such as the steroid hormones necessary for cellular metabolism. Any defect in the mechanism for the release of fat from these stores, or in the mechanism of depositing the lipids in the adipose tissue will have a major effect on the well-being of the individual. Of prime importance is the transport of these lipids from the intestine to the adipose tissue and finally to the site of utilization. In this function the lipoproteins are of major importance.

There are two distinct problems in lipid metabolism. One concerns the way fatty acids or steroids are metabolized, the other the metabolism of glycerol. The latter will not be dwelt on here, since it is a problem in carbohydrate metabolism. (See Volume 2, p. 114.)

Defects in lipid metabolism may be secondary to some other disease, such as lipoid nephrosis, diabetes mellitus, hypothyroidism liver disease, and others. On the other hand, the defect may be of genetic origin, specific to lipid transport or metabolism. The aberration may concern the manner in which fatty acids are synthesized, catabolized, or only the mechanism of transport. These various conditions will be presented here.

6.5.1 *Genetic Defects in the Plasma Lipoproteins*

Genetic defects in plasma lipoproteins can be classified into two major groups, those which result primarily from an increase in one or more of the lipoproteins (hyperlipoproteinemia) and those which result in decreased concentration of a particular lipoprotein fraction (lipoprotein deficiency). The two genetic defects which result in decreased synthesis of a particular lipoprotein are deficiency, or absence of apo-LpA and apo-LpB. The deficiency of apo-LpB was discovered first and has received the most attention. This will be presented first, therefore. Deficiency of apo-LpA and apo-LpB are referred to as *hypolipoproteinemias.*

6.5.2 *Hypo- and A-β-lipoproteinemia*

In 1950, a new syndrome was reported in an 18-year-old girl and subsequently in her younger brother.[134] The condition was marked by retinitis pigmentosa, ataxic neuropathology, and malabsorption of fat. Circulating erythrocytes were remarkable in that they resembled in appearance the mace used by knights in combat, in that they were crenated (having notches on the edge because of shrinkage), and had horns projecting from their surface. These erythrocytes were termed *acanthocytes*, the prefix, *acantho-* meaning hornlike (Figure 6.17). *An important observation was the fact that serum cholesterol levels were very low.*[135]

It was soon found that LDL, VLDL, and chylomicrons were

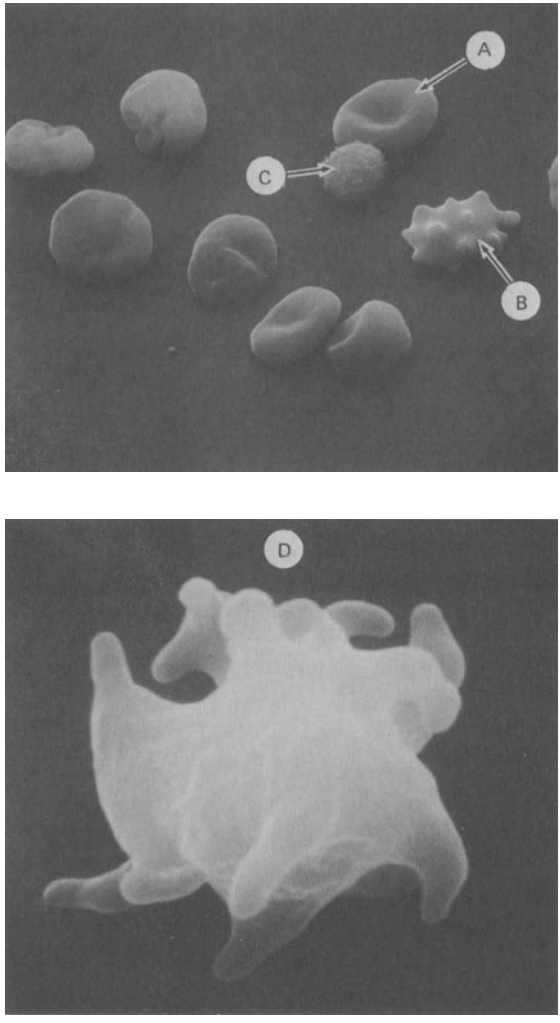


FIGURE 6.17 Types of erythrocytes seen in hypo- and a-β-lipoproteinemias. A. normal erythrocyte; B. echinocyte; C. lymphocyte; D. close-up of unusual spiculated erythrocyte. When the echinocyte takes a spherical form, it is referred to as an acanthrocyte (acanthocyte).

present in only minute amounts or entirely absent from the plasma of these patients, and the name *a-β-lipoproteinemia* was given to the disease.[136,138] By 1980, more than 40 patients with symptoms of a-β-lipoproteinemia had been reported.

In this condition, steatorrhea is noted in infancy, but by about the 5th year of life some tolerance to fat develops. The condition is distinguishable from celiac disease or cystic fibrosis in that the enzyme content and bile acids of the intestines are present in normal amounts. The cells lining the villi of these patients are loaded with lipids and they therefore appear foamy on staining. The lipid is taken into these cells but cannot seem to move to the interstitial spaces.[136]

In the severe form of this disease apo-LpB is completely absent. This condition is inherited as an autosomal recessive.

A variant of this disease, with milder symptoms, is called *familial hypo-β-lipoproteinemia.* In this condition, the homozygote has symptoms similar to that observed with a-β-lipoproteinemia. However, the heterozygote has normal levels of VLDL. LDL concentration however, is only one-tenth of that found in normals.[139] Hypo-β-lipoproteinemia differs from abetalipoproteinemia in that it is inherited as an autosomal dominant.[139–142]

The problem in both hypo- and a-β-lipoproteinemia is due to a decreased synthesis of lipoproteins containing apoB. In the absence of apoB, transport of lipids across the walls of the intestine is impaired. Decreased availability of fatty acids results in decreased synthesis of cholesterol and derived products. Decreased synthesis of sphingomyelin and other sphingolipids results in the neurological symptoms.

In these patients, HDL and thus LDL contains no apoC-III.[143–145] This contributes to decreased binding of lipids and the hypocholesterolemia.

Some patients with a-β-lipoproteinemia die in childhood. Others survive to maturity and adulthood. This is probably indicative of the fact that there are several variants of this disease with varying degrees of severity.

Investigation of the adult kindred of homozygotes with hypo-β-lipoproteinemia, who were obligatory heterozygotes, and showed reduced LDL levels, revealed that their life expectancy was higher by 9–12 years than normal controls. Since LDL cholesterol (LDL–C) concentration was low in these patients, the LDL-to-HDL cholesterol ratio was 1:1 instead 2.4:1 as in the normal control. These low LDL–C levels were apparently inherited as a dominant characteristic. Most of these patients were asymptomatic. A few showed some neuropathy.[144,145] Serum LCAT levels are high in this condition and most of the cholesterol is esterified in the plasma.[143] Thus familial low

LDL levels are antiatherogenic (see hyper-α-lipoproteinemia, Section 6.7).

6.5.3 *Hypo-α-lipoproteinemia, Tangier Disease*

In 1960, a lipid dyscrasia was discovered in patients living on Tangier Island off the coast of Virginia.[146] By 1980 about twenty-five such kindred had been uncovered. The symptoms in these patients resemble somewhat those observed in hypo-β-lipoproteinemia. A characteristic of the disease is the presence of enlarged, lobulated tonsils with a distinctive orange or yellowish gray color. This is due to deposits of cholesteryl esters. Cholesteryl esters deposit in lymph nodes and there is usually hepatosplenomegaly, foam cells in the marrow, and other tissue with mild neurologic symptoms. Most of these patients live to adulthood.

The disease is inherited as an autosomal recessive. Cases have been reported from various parts of the United States, Europe, and New Zealand.[146–150]

The neurological symptoms are present in most cases, although mild in some. These include general muscular weakness, sensory loss, paresthesia, diminished reflexes, and transient diplopia and ptosis. Fat deposits in the cornea have also been reported.[188]

The changes in plasma lipids in these patients is mainly in the high-density lipid fraction (HDL). The amount of HDL is reduced significantly. However, its composition is similar to that of the normal.[151] A small amount of a lipoprotein particle labeled HDL_T (T for Tangier disease) has been reported. This contains apoproteins other than A-I and A-II as demonstrated immunochemically.[152]

The genetic defect in Tangier disease is a decrease in the amount of apoA-I and apoA-II in the plasma. Two reasons for its absence have been proposed: One proposes a decrease in the rate of synthesis of A-I and A-II, the major decrease being in apoA-I. Normally the ratio of A-I to A-II is 3:1. In the patient with Tangier disease, this ratio drops to 1:12.[152] Others have presented evidence that the problem results from increased rates of catabolism of A-I and A-II.[153] The result is the same, decreased availability of A-I and A-II leads to decrease in plasma HDL levels and decreased cholesterol transport.

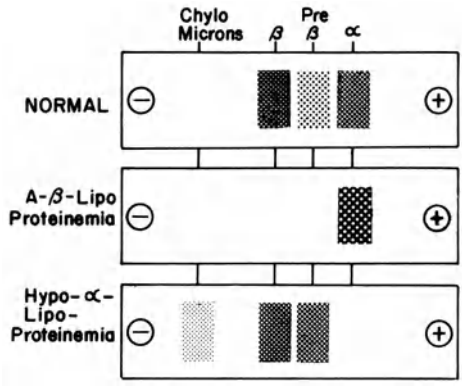


FIGURE 6.18 Comparison of plasma lipoprotein electrophoresis patterns in the abetalipoproteinemias contrasted with that in the normal and in Tangier disease.

Some amelioration of the condition in Tangier disease results from increase in chylomicron, VLDL, and LDL levels to compensate for the loss of HDL. This is illustrated in Figure 6.18. For this reason, the condition is mild, compared to hypo- or a-β-lipoproteinemia. Heterozygotes with Tangier disease show minimal or no adverse symptoms of the disease.

Lecithin–cholesterol acyltransferase activity (LCAT), in the plasma of patients with Tangier disease is normal, resulting in cholesteryl ester formation. These need to be transported by LDL and VLDL, since HDL is in short supply. Shortage of adequate transport means, for the amount of cholesterol esters formed, results in the excess being phagocytized by the white cells and cells of the reticuloendothelial system. This explains the presence of foam cells in these tissues. Since adequate means for transport is lacking, the cholesteryl esters accumulate in the liver, spleen, and lymph nodes, and other reticuloendothelial tissue. This accounts for the enlarged tonsils and hepatosplenomegaly. Lack of adequate amounts of cholesteryl ester and other lipid intermediates for the formation and metabolism of the central nervous system would cause the neurological symptoms.

6.5.4 *The Hyperlipoproteinemias*

Hyperlipemia associated with various diseases such as diabetes, lipoid nephrosis, celiac disease and the sphingolipidoses has been known for at least 100 years. Some of these conditions are acquired and some

are of genetic origin. The association of high cholesterol levels with obesity and cholesterol deposits in atherosclerosis focused attention on the hyperlipemias, as causative conditions leading to congestive heart failure.[154]

In 1967, in a series of articles, Fredrickson *et al.* proposed a system of classification of hyperlipemias of genetic origin.[155] The classification was based on the changes in the lipoprotein fractions in these diseases. Thus the classification was based on the relative amounts of chylomicrons, β, pre-β, and α fractions on electrophoresis using lipid stains. In addition, chemical analysis and the ultracentrifuge was used to evaluate the concentration of chylomicrons (origin), VLDL (pre-β), LDL (β), and HDL (α) in these patients (see Figures 6.5 and 6.6).

Five "types" were delineated, based on the lipoprotein distribution, correlated with plasma cholesterol and triglyceride concentration, and with other plasma parameters and symptoms. This classification was eventually revised by the World Health Organization.[156] The nature of these conditions, as clarified subsequently, will now be presented.

6.5.4.1 *Familial Fat-Induced Hyperlipemia (Type I)*

There are two genetic diseases where patients on a normal diet develop severe *hyperchylomicronemia*, Types I and V. However, these conditions are distinct from each other in the nature of the genetic lesion. *Type I disease results from a deficiency of postheparin lipoprotein lipase activity.* (*PHLA*). Thus, heparin administered to the patient with Type I disease has no effect in clearing the serum of chylomicrons. In contrast to this, the plasma of the patient with Type V disease clears up somewhat on administration of heparin. Postheparin lipoprotein lipase has been discussed in Section 6.2.3.3 and in the Appendix.

Administration of heparin releases at least two isoenzymes with lipase activity. One derives from the luminal surface of the capillary endothelium in muscle and adipose tissue, and the other from the liver. The combination of lipases (PHLA) are relatively nonspecific in their action, hydrolyzing triglycerides, diglycerides, monoglycerides, phospholipids, and fatty acid acyl CoA. In this manner, the chylomicrons are dismantled and the free fatty acids released, so that they can be metabolized. VLDL is also attacked, reduced to LDL, and finally metabolized.[157]

For full activity, the PHLA isoenzyme which is released from the capillary endothelium, needs to be activated by certain apolipoproteins. ApoC-I and apoC-II are activators for this enzyme (see Section 6.2.3.3). Absence of apoC-II has been reported in one patient.[158] This patient's condition resembled that of a patient with lipoprotein lipase deficiency. Addition of HDL containing C-II restored the lipoprotein lipase activity. This indicates that Type I disease can result from any of several factors. There may be a lipase deficiency, a lack of response to endogenous lipase releasing factors, or a defect in the lipase molecule.[159]

Although Type I hyperlipoproteinemia has been recognized as a genetic disease since 1939,[160] it was not until 1957 that it was recognized that the condition is due to a decrease in the concentration of lipases (PHLA) in the plasma.[161] *The deficiency of lipoprotein lipase in the tissues of patients with Type I disease has also been demonstrated.*[162] Type I hyperlipoproteinemia is a rather rare disease, only about 50 cases having been documented by 1980.

Patients with Type I disease will show symptoms of episodic abdominal pain. These may be severe enough to result in exploratory laparotomy. Some will show yellow to orange lipid deposits under the skin (eruptive xanthomas), hepatosplenomegaly, lipemia retinalis, and foam cells in bone marrow, spleen, and liver. Some will show arcus (fat deposits in the iris). Some will develop a pancreatitis. All of the patients show a creamy serum, in which the fat rises to the top when kept in the refrigerator for 48 hr, leaving a clear lower layer. Total lipids are as high as 4000 mg/100 ml, with cholesterol levels of over 1000 mg/100 ml. On a fat-free diet, the serum clears up and the symptoms recede. Usually the glucose tolerance curve is essentially normal.

After a fatty meal, these patients will develop eruptive xanthomas, severe abdominal pain, nausea, vomiting, and diarrhea. The severe lipemia will then persist for at least a week. This is the reason for referring to this disease as *fat-induced hyperlipemia.*

Patients will vary in their response to heparin. In some, heparin will release monoglyceride hydrolase but not triglyceride lipase into the plasma.[163] The Type I classification thus covers a relatively broad range of conditions, the common denominator being the PHLA deficiency.

Patients with severe lipemia will show rouleaux formation of their

TABLE 6.8

Classification of the Hyperlipoproteinemias

Type	Familial designation (frequency)	Lipoproteins	Chylomicrons (fasting)	Serum appearance (fasting)	Cholesterol mg/100 ml	Triglycerides, mg/100 ml	Total lipid, mg/100 ml	Post-heparin lypolysis	Response to glucose	Genetic lesion
I	Lipoprotein lipase deficiency; hyperchylomicronemia (rare)	Elevated chylomicrons	Very high	Creamy, separates on standing	>260 (or normal)	>1000	>1500	None or very low	Normal glucose tolerance	Post heparin lipoprotein lipase activity (PHLA) deficiency
II	Hypercholesterolemia; hyper-β-lipoproteinemia (common)	(a) Elevated β (LDL) (b) Elevated β and pre-β (LDL & VLDL)	Low to absent	Clear, light yellow	>260	(a) normal (b) 150–400	900–1200	Normal	Normal to sl. abnormal glucose tolerance	LDL receptor and internalization deficiency
III	Dys-β-lipoproteinemia; broad-β-disease; remnant disease (less common)	Elevated β fused to elevated pre-β (increased HDL and IDL)	Low to absent	Clear, occasionally cloudy	>260	>150	900–1200	Normal	Carbohydrate inducible, abnormal glucose tolerance	C-II activatable lipase deficiency and E-III deficiency
IV	Hypertriglyceridemia (endogenous); hyper-pre-β-lipoproteinemia (common)	Elevated pre-β (VLDL)	Low to absent	Clear, occasionally cloudy or milky	>260	>150	900–1600	Normal or slightly depressed	Carbohydrate inducible, abnormal glucose tolerance	Deficiency of apoC-II activator of lipoprotein lipase. Defective delipidation of VLDL from endogenous synthesis
V	Mixed hypertriglyceridemia; hyperchylomicronemia	Elevated chylomicrons; elevated pre-β	High	Cloudy to creamy	>260	>400	>1200	Normal or low	Fat and carbohydrate inducible, abnormal glucose tolerance	With Type IV, probably obesity of genetic origin, resulting in hyperinsulinism and fat intolerance

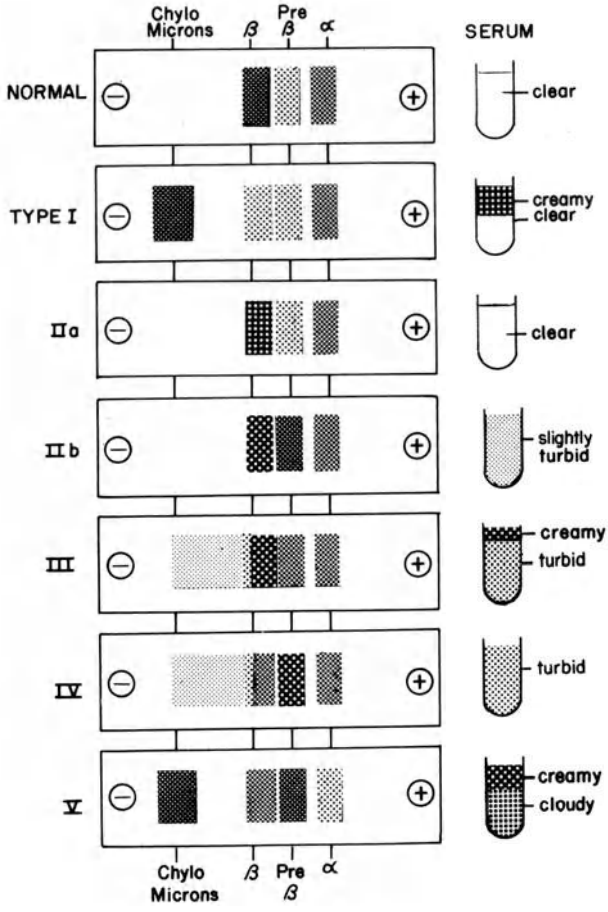


FIGURE 6.19 Comparison of the lipoprotein patterns obtained with agarose or cellulose acetate electrophoresis for the various familial hyperlipoproteinemias. The test tube shows the appearance of the serum after standing for 24 hr.

erythrocytes and a tendency to a prolonged bleeding. This has been attributed to the fact that heparin-activated lipase, deficient in these patients, is required for normal platelet function.[164]

Figure 6.19 compares the lipoprotein pattern of Type I disease with the other familial types. Table 6.8 lists some of the criteria for characterizing Type I disease.

6.5.4.2 *Familial Hyper-β-lipoproteinemia (Type II)*

This condition was described in the 19th century because of the obvious xanthomatous lesion seen in patients with no jaundice or other evidence of liver disease.[166] In subsequent papers it was referred to as xanthoma tendinosum, and when the high cholesterol level were noted, familial hypercholesterolemic xanthomatosis. For some time it was also confused with Type III disease and called xanthoma tuberosum (see Section 6.5.4.4). It is a relatively common condition occurring in about 0.2–9.5% of the population.[172]

By the middle of the 19th century, it was established that the nature of the disease, still called *xanthoma tuberosum multiplex*, was not a skin disease but a genetic form of hyperlipidemia.[167] In this condition, even the heterozygote will develop xanthomatous lesions, along with some hyperlipidemia. Discussion of this disease comprised a major portion of the classic text by Thannhauser, in 1950, summarizing the accumulated knowledge of the lipoidoses.[168] He stressed the hypercholesterolemia and the cholesterol in the xanthomatous deposits, as being characteristic of the disease.

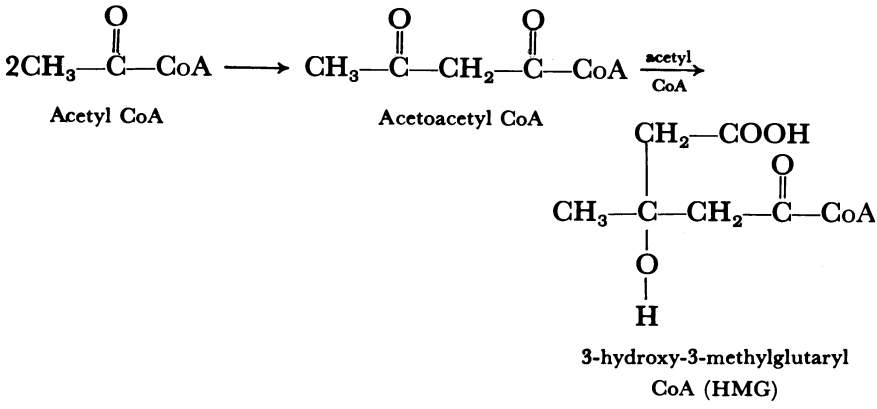
With the introduction of the ultracentrifuge into lipid studies, and the application of lipoprotein electrophoresis, the increases in the β-lipid fraction (LDL) was detected.[169,170] Hyper-β-lipoproteinemia is not an uncommon disease. In one study, over 600 such cases were discussed.[155] The condition has been reported from numerous countries including the Middle East. It is estimated that between 2 and 5 out of every 1000 Americans are heterozygous for this disease.[171]

Figure 6.19 demonstrates the nature of the defect in Type II hyperlipemia. A marked elevation of the β lipoprotein fraction LDL is shown in the figure. In these patients, cholesterol concentration is elevated, (300–600 mg/100 ml) in adults and 230–500 mg/100 ml in patients less than 20 years of age. Plasma triglyceride levels can be normal or somewhat elevated (50–500 mg/100 ml). Concentration of LDL cholesterol is greater than 210 mg/100 ml in adults and 170 mg/100 ml in children.[165] *On a low fat diet, plasma LDL and cholesterol levels remain elevated.* The chronically elevated LDL levels, after exclusion of the other hyperlipemia syndromes, defines the Type II hyperlipoproteinemia. In Type II disease, VLDL and triglycerides are generally elevated to about twice that of the normal.

The defect in the rate of synthesis of LDL is not the cause of the

disease, since this is within normal limits.[172] *The problem seems to be a decreased rate of LDL catabolism.*[173,174] In addition, there seems to be an increased rate of apolipoprotein B synthesis.[175]

The rate-limiting step in cholesterol synthesis is the reduction of 3-hydroxy-3-methyl glutaryl-CoA(HMG) by a reductase (see Appendix). In the synthesis of cholesterol, two moles of acetyl CoA condense to form acetoacetate. An additional mole of acetyl CoA is added to form 3-hydroxy-3-methylglutaryl CoA.



The reduction of HMG to form mevalonate and then an isoprene unit, which subsequently polymerizes to form cholesterol, is mediated by HMG-CoA reductase. *This reduction is inhibited by cholesterol which serves as feedback mechanism to prevent synthesis of excessive amounts of cholesterol.* On the other hand, in cell cultures, LDL stimulates HMG-CoA reductase activity and cholesterol synthesis.

From these observations, a mechanism is proposed to explain the slow rate of catabolism of LDL and increased plasma cholesterol levels in familial hypercholesterolemia (Type II hyperlipoproteinemia). The events in cellular LDL metabolism are visualized as follows[176]:

1. *A protein receptor exists at the surface of the cell which binds the LDL particle.* This has been demonstrated *in vitro* with fibroblasts.[177]

2. *The protein-LDL complex moves into the cell.* This phenomenon is referred to as *internalization.* The internalized protein-LDL vesicle is called an *endosome.* These endosomes fuse with the lysosomes.

3. *Hydrolysis follows to liberate free amino acids and small polypeptides from the proteins, at the same time splitting linolenic acid from the cholesterol*

esters. The linolenic acid and amino acids are metabolized, and the cholesterol moves to the cholesterol synthesis site in the cell.

4. *The liberated cholesterol suppresses the activity of HMG-CoA-reductase, thus slowing down cholesterol synthesis. It also activates* cellular, acyl-CoA–cholesterol acyltransferase, promoting the storage of incoming cholesterol as cholesterol oleate.[178] Plasma HDL cholesterol is predominantly esterified with linoleate. The reesterified cholesterol is stored as oleate or palmitoleate.

5. *The protein receptor is constantly being synthesized and consumed.* It has a half-life in fibroblasts of 25 hr. In this way the cell regulates the rate of LDL movement into the cell and the rate of LDL catabolism.

The cell receptors thus serve to control the rate of LDL catabolism and cholesterol synthesis. It is proposed that this mechanism is defective in Type II hyperlipidemia.

Fibroblasts from patients with Type II hyper-β-lipoproteinemia lack functional LDL receptors. Thus LDL does not bind to the surface of these cells.[179] Fibroblasts from the parents of these homozygotes, necessarily heterozygous for the disease, have 50% of the normal LDL binding capacity.[176]

Type II hyperlipoproteinemia classification includes patients with genetic differences. In some of these patients, who are apparently homozygotes, only partial suppression of HMG-CoA reductase in cultured fibroblasts takes place.[180]

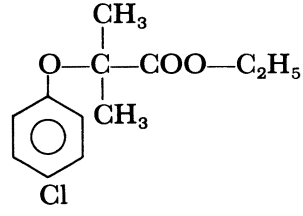
In addition to the defect in binding capacity for LDL and decreased rate of internalization, it is necessary to account for the increased rate of synthesis of apolipoprotein B, the presence of increased amounts of plasma IDL particles,[147] structural abnormalities seen in the cell membranes, and essentially normal total blood cholesterol in many of these patients.[154] Some have claimed that the major defect is an inability for the LDL of these patients to bind cholesterol esters securely because of a protein defect. For this reason, the cholesterol deposits in different tissues to produce the xanthomas.[181] Lipids are also deposited in the cornea and in the eyelids (xanthelasma).[188]

In the World Health Organization Bulletin devoted to the classification of the familial hyperlipoproteinemias, it was recognized that Type II included variants with significantly different symptoms. For this reason, Type II was reclassified into Type IIa and Type IIb. Both types show an LDL cholesterol concentration of 220 mg/100 ml or more. The major difference lies in the VLDL (pre-β) concentration.

In Type IIa the VLDL triglyceride concentration is below 1.25 mmoles/liter. In Type IIb the VLDL triglyceride concentration is greater than 1.25 mmoles/liter, resulting in a markedly increased pre-β, on lipoprotein electrophoresis. This can be seen in Figure 6.19.

Patients with Type II hyper-β-lipoproteinemia are at risk in developing atherosclerosis and cardiac pathology. Fortunately these patients respond to diets low in cholesterol containing lipids in the form of polyunsaturates, such as linoleic acid glycerides.[182] Plasma cholesterol levels can be reduced by as much as 30% with these regimens.[183] In acute cases, the resin *cholestyramine* is effective in preventing reabsorption of bile acids. Since bile acids derive from cholesterol, this serves to put a drain on plasma cholesterol.[184] See Section 6.9.

Clofibrate, the ethyl ester of 2-(4-chlorophenoxy)-2-methylpropanoic acid, has also been used by some to lower plasma cholesterol levels but has some objectional side effects.



Clofibrate

In severe cases with abdominal pain and pancreatitis, plasma exchange will ameliorate the condition and produce temporary relief.[185]

In view of the frequency of occurrence of Type II disease and since it can be detected in infancy, it has been recommended that screening for this condition should be carried out in the newborn.[186–189]

6.5.4.3 *Mutations Affecting LDL Metabolism*

In studies with cultured fibroblasts from humans with hypercholesterolemia, Type II disease, several mutations have been uncovered affecting the LDL pathway, both at the cellular level and the rate of secretion of plasma LDL.[175,178,190] One which has been discussed above is familial hypercholesterolemia (a dominant disorder) where

the mutant cells lack functional LDL receptors. In another, the number of receptors is markedly reduced. This is the more common form of Type II disease. A third mutation results in an inability to internalize the receptor–LDL complex once formed. In one study of 22 cases of Type II disease, 12 were receptor negative, 9 were receptor defective, and 1 showed the internalization defect.

Two other mutations of LDL metabolism have been known for some time. These are the so-called *Wolman syndrome* and *cholesterol ester storage disease.*[191] In the Wolman syndrome, a lysosomal acid lipase which hydrolyzes cholesteryl esters and triglycerides is absent. In the *cholesterol ester storage disease variant*, only about 1–5% of this lipase is present in the lysosomes. As a result, cholesteryl esters and triglycerides accumulate in nearly all the tissues of the body and death ensues within the first year of life.

A-β-lipoproteinemia and hyperlipoproteinemia are also mutations which affect LDL metabolism.

6.5.4.4 *Dys-β-lipoproteinemia, Broad-β Disease (Type III)*

This condition has also been referred to as *hyper-β or hyper-pre-β disease or mixed hyperlipemia* and also *idiopathic hyperlipoproteinemia.*[192–194] The disease is seen only in adults. Because of the confusion between this disease and other secondary lipemias it is difficult to estimate its frequency of occurrence. However, it is not an uncommon form of hyperlipemia. *The diagnostic features which define this disease are the presence of xanthomas and a floating β which moves as a pre-β on electrophoresis.*

In this condition, some of the LDL particles have an excessive amount of triglycerides causing them to float in the ultracentrifuge in a solution of density 1.006. These resemble VLDL in regard to their specific gravity. Since the particles move upward in the centrifugal field, they are referred to as *floating*, β particles. Electrophoretically they still move to the β region, since by composition and charge they are still LDL. The net result is a broad β peak, spanning the β and pre-β areas with trailing to the origin.[195,196] This can be seen in Figure 6.19.

The floating β particles are derived from VLDL and are referred to as *remnants* or *intermediate-density lipoproteins* (IDL). The disease is sometimes referred to as *remnant hyperlipoproteinemia.* It should be pointed out that some apparently healthy individuals have a double pre-β, one

moving slower than normal. The slower band is partially degraded VLDL and is still rich in triglycerides. This can usually be resolved on agarose. However, it can often give the appearance of a broad-β, the various bands, β, slow VLDL, and rapid VLDL all fusing to form one broad band.[197]

Patients with broad-β disease are usually first noted by the fact that they develop xanthomas in the skin over the joints, such as in elbows and knees. This is referred to as *xanthoma tuberosum multiplex*, and suggests Type III disease. This is somewhat distinct from Type II disease where the xanthomas occur mainly on the extensor tendons of the hands and feet and the Achilles tendon (xanthoma tendinosum).

In 1954, the floating β particle was found in these patients, with the ultracentrifuge.[192] These authors reported xanthomas in 23 patients, with floating β particles, none of whom were under 20 years of age. This condition was finally characterized as an entity distinct from other lipoproteinemias in 1968.[193]

Development of xanthomas on the surface of the joints, in the palm of the hands, and in the ankles is observed only after the patient has reached adulthood. The deposits are mainly cholesterol esters, with some carotene-like substance which gives the lesions the yellow-to-orange appearance. Plasma triglyceride and VLDL cholesterol levels are markedly elevated. On the other hand, LDL and HDL cholesterol levels are not elevated significantly. For example, in two patients with total cholesterol levels of 352 and 660 mg/100 ml, 236 and 545 mg/100 ml, respectively, were in the VLDL fraction. In the normal, most of the cholesterol ($>75\%$) is distributed between the LDL and HDL fractions, in a ratio of about 2.4 to 1. In the VLDL fraction, the cholesterol to glyceride ratio is also elevated.[194] The cholesterol to triglyceride ratio in VLDL in normal individuals is approximately from 16–30 to 1. In Type III disease the ratio ranges from 26–52 to 1. This ratio is also generally high in Type IIa disease (17–92 to 1) but not IIb (18–37 to 1).[195]

In 1973, the important observation was made that patients with familial dys-β-lipoproteinemia (Type III disease) had elevated apolipoprotein E levels.[196] However, subsequent studies showed that the LpE-III fraction was absent in these patients which may represent the genetic defect.[198] *In addition, the C-III activatable lipoprotein lipases are deficient in these patients.*[198a,199]

These observations have led to the conclusion that the major defect

in Type III disease is in the delipidation of VLDL to generate normal LDL. In the normal individual, a specific proportion of triglycerides, cholesterol, and apoprotein is maintained, as VLDL is delipidated. This serves to produce a stable configuration, maintaining the binding properties for cholesterol esters. Thus LDL can bring the cholesterol ester to sites of metabolism. In Type III disease, a particle (remnant IDL) is produced which is not LDL and therefore cannot bind properly to the cell. Deficiency of E-3 and a partial defect in lipase activity contributes to the high triglyceride levels in the abnormal LDL particle. Direct study of the delipidation process in patients with Type III disease has generally confirmed these concepts.[200]

Type III disease responds readily to diet, cholestyramine, or clofibrate therapy. The lesions redissolve and symptoms of the disease become minimal.[270] Patients with this condition are at high risk for cardiovascular disease and need to be maintained on a low cholesterol, polyunsaturated fat diet indefinitely.[263–266]

6.5.4.5 *Hyper-pre-β-lipoproteinemia (Type IV)*

This condition has also been called *familial endogenous hyperglyceridemia, carbohydrate induced hyperlipemia* and *familial hypertriglyceridemia.*

Hyper-pre-β-lipoproteinemia, as the name indicates, is the disease process which leads to increased VLDL or pre-β-lipoprotein in the plasma, without chylomicronemia, and which is of genetic origin. A distinctive feature of this disease is that the increased amounts of glycerides come from endogenous fatty acids which have been synthesized from carbohydrates taken in the diet. Thus the condition is therefore said to be *carbohydrate inducible.*[204] Although normal individuals also synthesize fats from carbohydrates, this is exaggerated in patients with Type IV disease. Since the source of the excess lipids comes from endogenous sources, Type IV hyperlipidemia has also been referred to as *endogenous hyperlipemia.* Type IV disease seems to be inherited as a dominant trait.

Type IV as well as Type V hyperlipemias represent groups of disorders and not single diseases. For example, in families of patients with Type III disease, individuals with Type IV disease are commonly found. The electrophoretic patterns of Type IV and Type V diseases are also simulated by a variety of acquired diseases. A patient with Type IV disease will show a Type V electrophoretic pattern after eating

a few fatty meals. Patients with both Types IV and V disease have elevated serum uric acid levels.

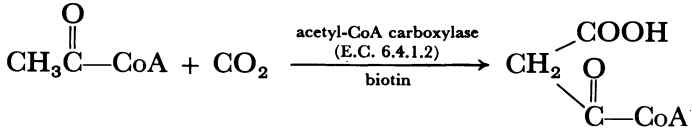
Patients with Type IV disease are generally obese. About 20% of these patients show eruptive xanthomas. Hepatomegaly is also present in about 20% of these patients. Total cholesterol levels are elevated and triglyceride levels are elevated moderately. Fasting serum will not be turbid unless the patient has had a fatty meal in the last 24 to 48 hr.

On electrophoresis of the fasting serum, an increased pre-β is noted, and chylomicrons may be absent or present in small amounts (see Figure 6.19). If *untreated, about 80% of these patients will develop ischemic heart disease.* The diagnosis is confirmed, if close relatives with Type IV disease can be found. Patients with Types I, III, or V disease, often exhibit a Type IV pattern. Thus a Type IV-like syndrome can be secondary to some other condition.

The exact cause of Type IV disease, *familial carbohydrate inducible hypertriglyceridemia*, is still not completely resolved. The symptoms which are prominent in this condition are obesity, high VLDL (pre-β) concentration derived from endogenous sources, and the funneling of ingested carbohydrate to triglyceride formation. All of these symptoms have led to the concept that this results from a tendency toward hyperinsulinism. The proponents of this idea base their conclusions on the following observations:

1. Glucose gives rise to pyruvate by the Emden–Meyerhof pathway (see Volume 2, p. 114). Pyruvate then enters the mitochondria to be oxidized to acetate.

2. Acetate moves into the cytoplasm, where, by a cluster of seven enzymes, fatty acids are synthesized. In order for this to take place, acetyl-CoA reacts with CO_2 to form malonyl-CoA. Palmitic acid is used as an example in the following equations.



Acetyl-CoA Malonyl-CoA

$$\text{Acetyl—CoA} + 7\text{Malonyl CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow$$
$$\text{CH}_2(\text{CH}_2)_{14}\text{—COOH} + 7\text{CO}_2 + 8\text{CoA} + 14\text{NADP}^+ + 6\text{H}_2\text{O}$$

Palmitatic acid

3. The oxidation of fatty acids (see Appendix) on the other hand, requires that the mitochondria take up the fatty acid for oxidation, eventually, to CO_2 and water, by the oxidative cycle. *Insulin prevents mitochondrial fatty acid uptake, whereas glucagon enhances fatty acid uptake and oxidation.*[203]

4. Insulin-resistant states, associated with hyperinsulinism, are associated with increased triglyceride and VLDL production.[204]

5. Patients with familial hypertriglyceridemia are usually obese. Obesity with high caloric intake tends to result in hyperinsulinism.[205]

6. In addition to obesity, insulin-resistant states are associated with estrogen therapy,[206] and prolonged glucosteroid therapy, which leads to a compensatory hyperinsulinism.[207] Both of these conditions lead to hyperglyceridemia and elevated VLDL levels. Estrogen therapy, in patients with familial hypertriglyceridemia, aggravates the condition and results in severe elevation of serum triglyceride levels which leads to pancreatitis.[208,209]

7. Hyperinsulinism has been demonstrated immunochemically in a majority of patients who have been diagnosed as having Type IV disease.[210,211]

All the facts listed above suggest that obesity common to patients with Type IV disease, and which seems to be of genetic origin in the familial form of the disease, results in hyperinsulinism. This would account for the rapid conversion of glucose to endogenous triglycerides. Since obesity may result from any of numerous genetic defects in metabolism, this classification includes a variety of inherited conditions. Some use the term *polygenic* to indicate the plurality of genetic causes.

About 100 g triglycerides per 24 hr are taken in daily by the adult in the American diet. In addition, depending upon the carbohydrate intake, up to about 30 g of triglycerides are generated daily endogenously from glucose. Normally, most of the carbohydrate does not take this metabolic pathway leading to lipogenesis. In Type IV hypertriglyceridemia, presumably because of hyperinsulinism, a higher percentage of carbohydrate is converted to triglycerides and transported as VLDL or pre-β. Practically none of it forms chylomicrons.

In Type V hypertriglyceridemia (see below) ingested triglycerides seem to be the major source of the triglycerides, and these result in a

persistent chylomicronemia. Serum of patients with Type IV hypertriglyceridemia do not show increased levels of chylomicrons unless a fatty meal has been ingested within the last 24–48 hr.

Patients with Type IV disease are at very high risk for ischemic heart disease. These patients benefit from a low caloric diet. It is important that their weight be controlled, correlated with a reduction in their cholesterol level. *Alcohol must be avoided since it also increases triglyceride synthesis leading to intrahepatic triglyceride accumulation in the liver.*[212] Alcohol seems to inhibit the fatty acids in the hepatocyte from entering the mitochondria to be oxidized.[194] Although serum cholesterol levels will decrease on a low caloric intake in these patients, somewhat elevated triglyceride levels will persist. If xanthomas have been formed, then clofibrate or cholestyramine therapy will serve to redissolve them by reducing plasma cholesterol levels.

6.5.4.6 *Familial Mixed Hypertriglyceridemia, Type V Disease*

This disease has also been called hyperchylomicronemia and *pre-β-mixed* or *combined hyperlipemia.* This condition is often called to the attention of the physician because of bouts of abdominal pain and pancreatitis.[213] It usually becomes manifest after puberty or in adulthood. The fasting serum is cloudy or creamy. On standing in the refrigerator for 24–48 hr, a creamy upper layer forms. The lower layer remains turbid (see Figure 6.19).

Total cholesterol level is usually greater than 400 mg/100 ml and the electrophoretic pattern shows a severe chylomicronemia, sometimes associated with an elevated pre-β (VLDL). β Lipoproteins (LDL) are also usually elevated.[214] The α-lipoprotein band (HDL) is somewhat less dense than normally seen. This is shown in Figure 6.19.

Since some of these patients have both elevated chylomicron levels and elevated pre-β levels, this disease is designated as a *Familial mixed or combined disease.* The diagnosis is confirmed if close relatives are found with Type V electrophoretic patterns.

The distinction between Type V and Type IV disease is made when a fatty meal will result in severely elevated chylomicron levels, which will persist for much longer periods of time than that observed in Type IV disease.

The tendency to ischemic heart disease is great with Type V disease but less than that observed with the Type IV condition. It

appears that chylomicronemia and higher triglyceride levels are not directly associated with the deposition of cholesterol in the vascular system (atherosclerosis). This is true for Type I disease also.

Conditions resulting from triglyceride deposits, on the other hand, are more common with Type V disease. For example, *lipemia retinalis* and *hepatosplenomegaly* are more common in Type V than in Type IV disease.[215]

Studies with kindred have indicated that Type V disease is inherited as an autosomal recessive. The fact that two parents with Type IV disease can have children with Type V disease suggests that both conditions are variant expressions of the same disease. For this reason, many of the suggestions for revision of the classification of dyslipoproteinemia lump Type IV and Type V into one group which is referred to as *familial hypertriglyceridemia*.[216]

Eruptive xanthomas will appear in patients with Type V disease when their plasma triglyceride level exceeds 1500 mg/100 ml, after a series of fatty meals. These will redissolve, when the patient is put on a low-caloric low-fat diet. Because of the higher triglyceride levels seen in Type V disease, eruptive xanthomas are more common in this condition than in Type IV disease. Uric acid levels are elevated in Type V disease, but they are also elevated in Type IV disease.[217]

About 75% of patients with familial Type V disease have abnormal glucose-tolerance curves.[211] A majority of them are hyperinsulinemic and, as in Type IV disease, this has been suggested as a basic genetic defect in these patients. Practically all of these patients tend to be obese, and it has been suggested that the hyperinsulinism is secondary to their high carbohydrate intake when not under control.

These patients are maintained by a low-caloric, low-fat, high-protein diet.[218] Some of these patients tend to have high plasma glucose levels. In that case tolbutamide or phenformin therapy is recommended, since insulin will tend to aggravate the hyperglyceridemia.[219,220] Table 6.8 summarizes the criteria used to identify a patient as a Type V hyperlipemia and compares this condition with other genetic defects.

6.5.4.7 *The Hyperlipoproteinemia Types: Summary*

Examination of the preceding pages and Table 6.8 indicates that the serum of patients with familial hyperlipoproteinemia are divided

into the following six types: I—increased chylomicrons; IIa—increased LDL; IIb—increased LDL and VLDL; III—increased IDL (remnants); IV—increased VLDL; and V—increased VLDL and chylomicrons.[221]

The metabolic lesions which have been identified, by 1980, are listed as follows:

1. Familial hyperchylomicronemia, Type I is a lipoprotein lipase deficiency.
2. Familial hypercholesterolemia, Type II, is an LDL receptor deficiency. This may be due to a total or partial loss of binding of LDL to the cell surface, or it may be a deficiency in bringing the LDL-binding protein into the cell for metabolism (internalization deficiency).
3. Familial dys-β-lipoproteinemia (Type III) is a deficiency of apolipoprotein C-II activatable lipases and apoprotein E-III, which are necessary for conversion of VLDL to LDL. As a result *delipidation of VLDL to LDL is defective*, resulting in the production of remnants (IDL) with decreased effectiveness in binding to cells and metabolism.
4. Familial hypertriglyceridemia (Type IV) seems to be a deficiency of apoC-II, which is a major activator for lipoprotein lipases. This results in elevated pre-β (VLDL) because of defective delipidation. These patients are at highest risk for ischemic heart disease.
5. *The relationship of carbohydrate metabolism to Type IV and Type V diseases is still not resolved.* It is apparent that several genetic diseases are being considered. The patient with fasting chylomicronemia (Type V), resolvable with heparin, is distinct from the Type IV patient with high pre-β and no chylomicronemia. Hyperinsulinism is often found in Types IV and V.

Familial lecithin–cholesterol acyltransferase deficiency needs to be classified with the hyperlipemias and this will now be presented.

6.5.5 *Familial Lecithin–Cholesterol Acyltransferase (LCAT) Deficiency*

In *familial lecithin–cholesterol acyltransferase (LCAT) deficiency*, esterification of plasma cholesterol cannot take place and, as a result, most of the cholesterol in the plasma is unesterified. Total cholesterol levels exceed 300 mg/100 ml with only about 5% being esterified. At the same time, plasma triglyceride and lecithin concentrations are increased, while plasma lysolecithin concentration is decreased.[222]

The most obvious clinical sign of this disease is the formation of arcus-like deposits of cholesterol in the periphery of the cornea.[223] These patients are somewhat anemic, with hemoglobin levels at about 10 g/100 ml with some target cells. Bone marrow aspirates may contain foam cells. Serum albumin levels are reduced and hyaline casts and protein appear in the urine.[223] Postheparin lipolytic activity is generally low normal to normal, and these patients usually show normal glucose-tolerance curves.[224]

With the high free cholesterol in the plasma, abnormal lipoprotein X (LpX) appears in the plasma of these patients (see Figure 6.16).[225] This is also the case in alcoholic cirrhosis when the percentage of free cholesterol in the plasma can reach 90% of the total.

The lecithin–cholesterol acyltransferase (LCAT) is located in the high-density lipoprotein (HDL).[226] In the normal patient, this enzyme is responsible for the exchange of oleate or linoleate from circulating lecithin to cholesterol. Thus it is responsible for esterifying the cholesterol carried in HDL (see Appendix).

$$\text{lecithin} + \text{cholesterol} \xrightarrow[\text{(E.C. 2.3.143)}]{\text{LCAT}} \text{cholesteryl ester} + \text{lysolecithin}$$

The esterified and free cholesterol in HDL is then exchanged with LDL, VLDL, and chylomicrons, and indirectly controls their cholesterol content.

In the absence of LCAT, the cholesterol is mostly free. This changes the electrophoretic mobility of HDL and VLDL. VLDL (pre-β) now moves to the β position and HDL to the α_2 position on electrophoresis. An electrophoretic pattern is then obtained with no pre-β and the HDL displaced somewhat from its normal position toward the cathode.[227]

The high plasma-free cholesterol concentration results in a high free cholesterol level in the erythrocyte membrane and target cells appear. If these cells are incubated with normal serum containing LCAT, they assume a normal appearance.[228] In obstructive jaundice, bile salts move into the plasma and inhibit LCAT. The erythrocytes of these patients then also take on the target cell appearance, which can be corrected by incubating these cells with LCAT.[229]

If blood from a normal individual is transfused into these patients, the phospholipid level drops and cholesterol esters rise, due to the effect of the LCAT administered.[230]

TABLE 6.9

Serum Lipid Concentration in Patients with Lecithin–Cholesterol Acyltransferase (LCAT) Deficiency Compared to the Normal

Lipid	LCAT deficiency, mg/100ml	Normal mg/100 ml
Free cholesterol	105–580	45–60
Esterified cholesterol	0–10	135–180
Triglycerides	100–570	50–150
Phospholipids, total	120–800	150–250
Lecithin, %	75–80	68–78
Lysolecithin, %	1.3–2.8	4–10
Sphingomyelin, %	13–20	12–22
Cephalin, %	3–4.5	3–6
Lecithin–cholesterol acyltransferase	None	43–47 μmole ml^{-1} hr^{-1}
Lipoprotein X	Present	Absent

The net effect of LCAT deficiency is a general rise in total lipids since its major function seems to be not only in esterification of cholesterol but also in dissolution of surface lipids in the chylomicrons.[231,232] The presense of LCAT stimulates transfer of apoA from HDL to VLDL and apoC from VLDL to HDL. This suggests that esterification of cholesterol, whose esters then bind to apoA, is involved in the transfer of the apolipoproteins between the various lipoprotein particles.

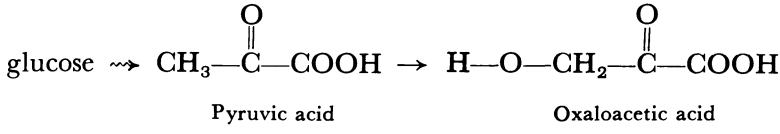
The concentration of the lipid fractions in the serum of patients with LCAT deficiency is summarized in Table 6.9.

6.6 *ACQUIRED HYPERLIPEMIAS*

In the normal individual, within 1 hr after a fatty meal, a lipemia (chylomicronemia) is observed in the serum. This is resolved within the next 2 hr and the serum becomes clear again. In certain disease states, such as diabetes, hypothyroidism, or liver disease, the lipemia will persist for prolonged periods of time. With certain conditions, such as the nephrotic syndrome, or subsequent to a viral hepatitis, the serum may be chronically lipemic. These conditions can be readily mistaken for a familial type of hyperlipemia. The term *phenocopies* has

been used to describe these conditions because of their resemblance to the phenotypes of the familial hyperlipemias.

Fatty acids are metabolized by conversion to acetate. This reacts with oxaloacetate to form citrate, which goes on to be oxidized by the oxidative cycle. Oxaloacetate is regenerated in the cycle, but some is lost on each pass. The oxaloacetate lost is replaced by oxaloacetate derived from pyruvate, which in turn is derived from glucose (see Appendix). Thus fat burns in the fire of the carbohydrates.



In diabetes, with the blockage of the effective utilization of acetate, fatty acid metabolism is blocked. *For this reason total lipids in the serum of the diabetic are elevated.* This results in increased levels of chylomicrons, VLDL, and LDL. In severe diabetes, the serum may be cloudy (see Figure 6.21). Cholesterol levels are also elevated in the diabetic. *The reason for this is the fact that cholesterol derives from acetate.* Acetoacetate may be converted to cholesterol in the cell stroma by a series of reactions beginning with the condensation of a third acetate molecule with acetoacetate, followed by a reduction to form *mevalonic acid* in the mitochondria. (See Appendix).

The blockage of acetate metabolism, due to a shortage of supplies of oxaloacetate and ATP needed to form acyl-CoA from fatty acids because of decreased glucose metabolism, results in accumulation of acetate and ketone bodies. This stimulates cholesterol synthesis. In the diabetic, the percentage of free cholesterol will be normal (about 25% of the total) since lecithin–cholesterol acyltransferase (LCAT) activity is usually normal (Figure 6.20).

In the discussion of Types IV and V diseases (familial hypertriglyceridemia) it was pointed out that hyperinsulinemia is a common finding in these obese patients. This has been shown to be the result of reduced numbers of binding sites for insulin on the adipocytes (fat cells) and circulating monocytes. *This is reversible by caloric restriction and weight loss.* When these patients are not on a low-caloric diet the numbers of insulin binding sites increases in proportion to their weight loss.[210,211,264] There is an inverse correlation between the degree of obesity and numbers of insulin binding sites. If the patients who have

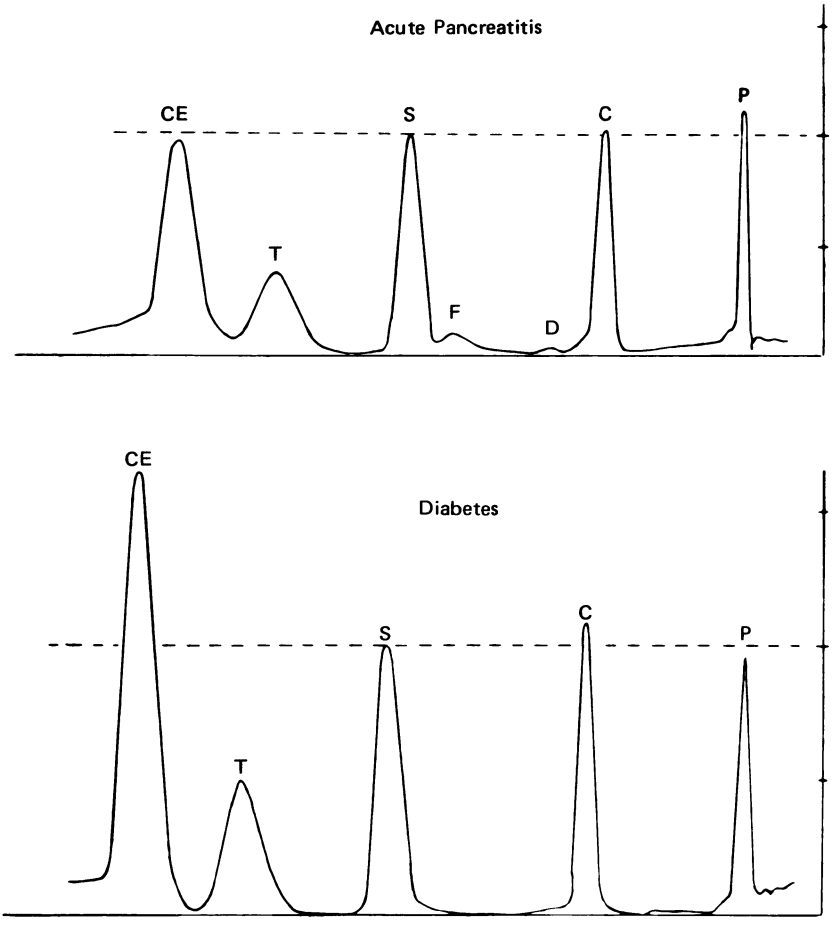


FIGURE 6.20 Densitometer tracing from a thin layer chromatogram, as in Figure 6.21, of a patient with pancreatitis compared with a mild diabetic. Compare the triglyceride levels with that of the normal in Figure 6.22.

lost weight are put back on a high carbohydrate diet, the number of insulin binding sites drop promptly. Obesity from any number of causes, including inactivity and overeating, results in increased numbers of insulin binding sites and hyperinsulinism as a mode of compensation for this defect. This in turn leads to decreased glucose and thus fat metabolism, maintaining the obesity.

In *hypothyroidism*, total lipid and cholesterol levels will be high (see Figure 6.21). There will be a tendency for the patient to be obese.

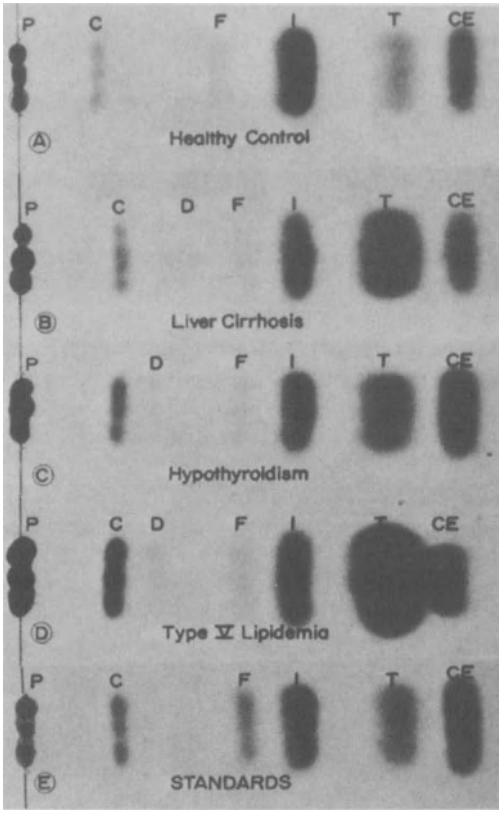


FIGURE 6.21 Thin-layer chromatogram obtained with serum extract from a healthy adult (A) as compared to those obtained from a patient with liver cirrhosis (B), hypothyroidism (C), Type V lipidemia (D), and a set of standards (E). The letters P,C,D,F,I,T, and CE represent phospholipids, free cholesterol, diglycerides, free fatty acids, internal standard, triacylglycerols (triglycerides), and cholesteryl esters, respectively. [Reproduced from *Clin. Chem.* **23**:835–841, (1977).]

The reason for this is that the oxidative cycle slows down in hypothyroidism. *In vitro*, the iodothyronines (thyroxine and triodothyronine) have very little effect on the oxidative cycle in physiological concentrations. It has been proposed that the rate of oxidation of fats is controlled by a balance between the hormones, adrenaline, and ACTH, which tend to block the cycle and the iodothyronines which unblock it.[233] Thus thyroxine increases the metabolic rate.

The net effect of the decrease in availability of the iodothyronines is to cause an elevation of plasma total lipids. As a result, acetate accumulates leading to the formation of excessive amounts of acetoacetate and then cholesterol. The high cholesterol and total lipids of hypothyroidism may simulate the Type IV, genetic hyperlipoproteinemia.

In hyperthyroidism, the total lipid and cholesterol level are often low. However, this is not always the case, even though the metabolic rate is increased. The reason for this is that quite frequently hyperthyroidism is accompanied by other diseases which interfere with normal lipid catabolism. Furthermore, these individuals tend to show anxiety and are sensitive to stress.

Acute stress will cause a rapid, temporary rise in plasma total lipids and cholesterol levels.[233,245] This has been attributed to the mobilization of fats from body stores by adrenaline and ACTH.

With *nephrosis*, the lactescence of serum was reported as early as 1813.[234] In 1980 the exact nature of this phenomenon has not been completely resolved.

Patients with *renal disease* have a high incidence of hyperlipidemia resulting in a high incidence of ischemic heart disease and other cardiovascular complications.[235] About 50% of the deaths of patients undergoing hemodialysis result from cardiovascular causes. Children affected by the nephrotic syndrome are at high risk for arteriosclerosis in childhood. It is not uncommon for serum total lipids to exceed 4000 mg/100 ml, and for cholesterol levels to exceed 1500 mg/100 ml in these children.[236,237] Phospholipids are elevated proportionally, so that in many cases, although the serum lipids are very high, they are dispersed and do not separate readily on standing.

The electrophoretic protein patterns of the nephrotic patient is very characteristic. Albumin and globulin concentrations are depressed and a marked elevation of the α_2 and β globulins are noted. This is due mainly to elevation of the plasma lipoproteins.[238] On the other hand, the electrophoretic pattern of urine proteins is complementary to that of the serum proteins. Albumin, and α_1 and γ globulins are prominent.[239] This has been suggested as an explanation for this condition. In the nephrotic syndrome, proteins, such as albumin and gamma globulin (e.g., IgG) are able to pass the glomerulus, and thus appear in the urine. The heavier lipoprotein, particles such as HDL, VLDL, LDL, and the chylomicrons cannot. This accounts for the complementary electrophoretic patterns of serum and urine in nephrosis, and stimulates further synthesis of all proteins in an attempt to maintain the colloid osmotic pressure. Thus all the plasma proteins are synthesized at a higher rate, but the lipoproteins remain behind resulting in the hyperlipoproteinemia.

The elevation in lipoproteins is mainly in the VLDL and to a

lesser extent in LDL.[236,240–243] On the other hand, in lipoid nephrosis of childhood a substantial amount of the cholesterol is carried by an elevated level of HDL.[240]

Gout occurs commonly in connection with obesity. In England, gout was associated with affluence. A high percentage of gouty patients have elevated cholesterol and triglyceride levels. Conversely, a high percentage of patients with elevated cholesterol and triglyceride levels have high uric acid levels.[244] The mechanism proposed to explain this phenomenon is based on the fact that for each molecule of acetyl-CoA added to a fatty acid chain, two molecules of NADPH from the pentose phosphate shunt mechanism are oxidized. Resultant stimulation of the shunt mechanism results in increased levels of phosphoribose pyrophosphate (PRPP), which leads to purine and then urate formation. Ingestion of alcohol aggravates this phenomenon.

With *liver disease* and jaundice, increased concentration of plasma cholesterol and total lipids in the plasma are often noted. In obstructive jaundice, without substantial parenchymal damage, cholesterol levels will be elevated, and the percentage of free cholesterol will be increased proportionately. However, with extensive hepatocyte destruction and appearance of bile salts in the plasma, as in alcoholic cirrhosis, lecithin–cholesterol acyl, transferase (LCAT) is inhibited, and although total cholesterol levels are reduced, the percentage of free cholesterol can reach levels in the plasma as high as 90% of the total, simulating a genetic LCAT deficiency. In Figures 6.21 and 6.22, the thin layer chromatogram of the serum extract from a moderate case of alcoholic cirrhosis is contrasted with that from a case of hypothyroidism and a normal individual. The scans in Figure 6.22 brings this out more clearly.[245]

After *severe viral hepatitis*, a lipemic serum develops and can persist for several years in some patients. This can be cleared with heparin, but it turns cloudy again when heparin administration ceases. Using the thymol turbidity test (a rough estimate of total lipoprotein levels), 85% of blood donors who had a history of viral hepatitis were positive.[246] Unless the history of these patients is available, these cases may be misdiagnosed as genetic types of hyperlipoproteinemia, Types III, IV, or V.

One of the sequellae from some of the familial hyperlipoproteinemias is a *pancreatitis* and acute abdominal pain resulting therefrom. For this reason, pancreatitis from any cause will simulate a hyperlipo-

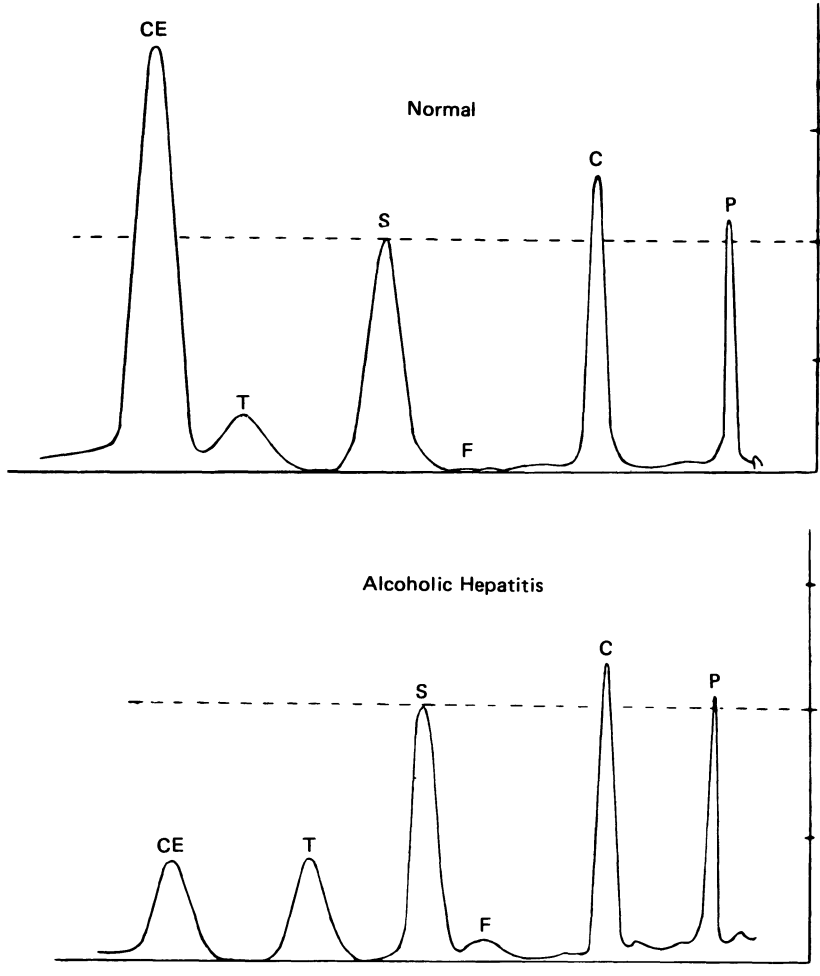


FIGURE 6.22 Comparison of the tracing from a thin-layer chromatogram, as in Figure 6.21, of the normal individual with a patient with alcoholic cirrhosis. The symbols are as in Figure 6.21.

proteinemia, especially since it is often accompanied by a hyperlipemia.

In the *myelomas*, elevated serum lipid concentration occurs not infrequently and this and other diseases involving the immunochemical system can also simulate familial hyperlipoproteinemia; this includes several of the autoimmune diseases. In *porphyria* (see Volume 2, pp.

TABLE 6.10

Clinical Manifestations of the Familial Hyperlipoproteinemias and Acquired Conditions Which Can Show Similar Symptoms and Lipoprotein Abnormalities

Type	Symptoms	Acquired condition
I	Noted below age 10; markedly elevated serum triglycerides and moderately elevated total cholesterol; eruptive xanthomas, hepatosplenomegaly, lipemia retinalis, abdominal pain, pancreatitis	Pancreatitis, insulin-dependent diabetes mellitus, dysglobulinemias, lupus erythematosus
IIa and IIb	In IIa, elevated cholesterol; in IIb, elevated cholesterol and triglyceride; xanthomas of skin or tendon, arcus cornealis, xanthelasma, ischemic heart disease	Nephrotic syndrome, hypothyroidism, hepatic obstruction, acute prophyria, autoimmune disease, gout, diet high in cholesterol
III	Occurs above age 20, elevated cholesterol and triglycerides, abnormal glucose-tolerance curve, hyperuricemia, planar and tuboeruptive xanthomas, obesity	Occasionally seen in the autoimmune diseases
IV	Mildly elevated cholesterol, elevated triglycerides, hyperuricemia, abnormal glucose-tolerance curve, obesity	Nephrotic syndrome, gout, insulin-dependent diabetes difficult to control, glycogen storage diseases, dysglobulinemias, alcoholism, pregnancy, pancreatitis, high carbohydrate diet
V	Occurs in adulthood, mildly elevated cholesterol, markedly elevated triglycerides, hyperuricemia, eruptive xanthomas, abdominal pain (pancreatitis), premature vascular disease	Insulin-dependent diabetes difficult to control, alcoholism, pancreatitis, autoimmune disease, dysglobulinemias, glycogen storage disease, nephrosis, myxedema

318–330) abdominal pain, associated with elevated plasma lipids has often been reported.

Some of the common diseases which produce the symptoms seen in the familial hyperlipoproteinemias are summarized in Table 6.10.

6.7 *HIGH-DENSITY LIPOPROTEIN; HYPER-α-LIPOPROTEINEMIA*

In 1951, a report appeared which indicated an inverse relationship between HDL concentration and cardiovascular disease.[253] This has since been substantiated in numerous studies, involving thousands of individuals and different ethnic groups.[247–252,254]

In one study, plasma HDL cholesterol (HDL–C) concentration was measured in large groups of individuals. Those with the highest values (> 95 percentile) were then selected, and familial studies were carried out. It was observed that the condition was inherited as an autosomal dominant.[255] The condition (not a disease) was referred to as *hyper-α-lipoproteinemia.* These individuals, from this and other studies, have been shown to be resistant to cardiovascular disease. The predominant protein in HDL is apoA-I.[256] This protein is elevated in these individuals, and it appears that this is an inherited trait.

Examination of Figure 6.5 and Table 6.6 reveals that about 46% of the plasma cholesterol is present in the LDL fraction and 28% in the HDL fraction. The remainder is distributed in the other fractions. These are mean figures for all age groups. At birth, the ratio of HDL–C to LDL–C is 1.2:1. In adults, an inversion occurs and the ratio becomes approximately 1:2.4.[257,258] In cord blood, HDL–C accounts for about one-half of the total plasma cholesterol.[259]

The predominant atherogenic component of plasma seems to be LDL–C, whereas the antiatherogenic component is HDL–C. An inverse correlation seems to exist in human plasma between these two components.[246] Determination of HDL cholesterol and total cholesterol may be used to provide a rough estimate of LDL cholesterol by difference. Triglycerides, even at high concentration, do not seem to put the individual at high risk for ischemic heart disease.

The larger low-density particles, LDL, VLDL, and the chylomicrons can be precipitated by a combination of a polyanion and salt (e.g., heparin–$MnCl_2$). Cholesterol, determined in the supernatant, gives the HDL–C. Comparison with the total cholesterol concentration allows calculation of the percentage of cholesterol in the HDL fraction. If the ratio of total cholesterol to HDL cholesterol is significantly greater than 4.4 in women and 5.0 in men, the patient is at risk for ischemic heart disease; this is shown in Table 6.11. Values of HDL–C

TABLE 6.11

Relationship Between Ratio of Total Cholesterol to HDL-Cholesterol and Risk for Coronary Heart Disease (CHD)[249]

	Ratio of total cholesterol to HDL-cholesterol	
Risk, % of normal	Men	Women
50	3.43	3.27
Normal	4.97	4.44
200	9.55	7.05
300	23.4	11.04

lower than 55 mg/100 ml in women and 45 mg/100 ml in men places the proband at increased risk for CHD (see Table 6.12).

Examination of Figure 6.5 indicates that the highest percentage of phospholipids occurs in the HDL fraction. This serves to retain cholesterol in solution, and to prevent deposition in the vascular bed. A high cholesterol level, with most of it in the HDL fraction does not, therefore, place the patient at the same disadvantage as a high cholesterol level in the LDL fraction. For this reason the expression *familial hypercholesterolemia* as a disease refers to increased cholesterol levels in the LDL and VLDL fractions only (Type II hyperlipoproteinemia).

TABLE 6.12

Correlation of the Incidence of Coronary Heart Disease (CHD) with the Concentration of HDL-Cholesterol in Men and Women[252]

HDL-C, mg/100 ml	Incidence/1000 men[a]	Incidence/1000 women[b]
> 75	0	4
65–74	25	13.9
55–64	59.7	39.7
45–54	51.0	49.5
35–44	104.5	54.5
25–34	100.0	164.2
< 25	176.5	—

[a] Standard risk, 77.1/100,000.
[b] Standard risk, 43.6/100,000.

The distribution of cholesterol between HDL and LDL in the parents is inherited by the offspring. Where parents are at high risk for ischemic heart disease, it would be advantageous to determine the ratio of total cholesterol to HDL–C in the neonate. Since ischemic heart disease does occur in children occasionally, this may be of value in guiding the diet fed to the infant.[260] *It must be kept in mind that an individual with a cholesterol value above 250 mg/100 ml is at three times the risk for cardiovascular disease than an individual with a cholesterol value of 194 mg/100 ml.*[261]

The data accumulated in the various statistical studies on the relationship of HDL and LDL to atherosclerosis have been obtained mainly on people who are about 50 years of age. The reason for this is that studies on younger people (e.g., 20 years of age) would require 30–40 years to complete. Total cholesterol values increase with age, and Tables 6.11 and 6.12 probably understate the risk for younger people.

Diabetics have a greater risk for CHD than that shown in Tables 6.11 and 6.12. This is also true for patients with gout, alcoholic cirrhosis, kidney disease, and others. Women at high risk for CHD increase that risk when taking contraceptive pills.

Moderation of diet and physical exercise serves to raise serum HDL–C levels.[269] Certain drugs such as nicotinic acid and clofibrate have similar effects. However, the use of drugs should be limited to the exceptionally severe case. See Sections 6.8 and 6.9.

6.8 *HYPERLIPEMIA AND NUTRITION*

There are two factors controlling the lipid composition of the plasma in the healthy individual. One is of genetic origin and the other is the diet. To illustrate this statement, let us examine the total cholesterol level in the serum of healthy individuals from different ethnic origins. Table 6.13 lists the serum cholesterol levels observed in adults in different countries.[262]

From the table, it can be seen that, regardless of the diet, whether affluent or poverty stricken, there is a lower and upper limit encompassing the values. Thus a hard-working, poor dirt farmer in Finland will still have a higher cholesterol than most sedentary, wealthy

TABLE 6.13

Fasting Serum Cholesterol Levels (mg/100 ml) Observed in Different Countries

Parameters	Japan	Greece	Italy	United States	Finland
Population mean	150	175	200	225	250
Lower limit (2.5 percentile)	75	100	125	150	175
Upper limit (97.5 percentile)	225	250	275	300	325
Mean difference from 150	0	+25	+50	+75	+100

Japanese, and equal to that of the mean of the Greeks. The most affluent country of all, with the highest percentage of fat in the diet, the United States, still shows lower cholesterol values than the Finns. Thus, inheritance is of major significance in the tendency toward high serum cholesterol levels.

Atherosclerotic coronary heart disease is a phenomenon of the affluent society. Diseases characterized by fatty deposits in the arteries are not a natural or necessary component of the aging process.[263–265] The ready availability of concentrated foods such as bread, pastries, ice cream, alcoholic beverages, sugar, and fatty meats, such as pork, frankfurters, and hamburgers, makes it practicable to ingest a very-high-caloric diet, rich in saturated fats, without distending the intestinal system. Since lipids are generated from any food, regardless of whether carbohydrate or protein, the dietary contribution to the total plasma cholesterol level is a sum of everything in the diet and not just the lipid component.[266]

Various factors have contributed to mass population obesity in the United States, associated with hyperlipemia and rising cholesterol and triglyceride levels. These include affluence, television addiction connected with inactivity and increased food consumption, massive consumption of alcoholic beverages, public assistance programs in the cities with emphasis on food supplementation, and "fast food" restaurants which serve concentrated low-bulk foods. These, coupled with nondietary factors which result in high blood pressure and/or hyperlipemia, as ensues from cigarette-smoking, addiction to caffeine-containing beverages, and from various medicinals which can be purchased without prescription, have made cardiovascular disease of epidemic proportions in the United States.[267,268]

From the discussion above, it is apparent that the approach to the obese, hyperlipemic patient requires first that a diagnosis be made to determine whether any of the genetic conditions of Table 6.8 or acquired diseases of Table 6.9 are present. This requires that the total cholesterol, triglycerides, and high-density cholesterol levels be determined as a minimum. Assuming that there is no congenital or acquired disease, then the patient's response to a low-caloric diet needs to be explored first.

Various diets have been proposed, most of which comprise a diet low in saturated fats and cholesterol and high in roughage or bulk, along with some increase of muscular activity.[268,269] The public has now been made aware of the problem, and various means for weight reduction have become part of the American culture. As a result, recent surveys have shown a significant decrease in the incidence of cardiovascular mortality.[265,266]

Where an acute problem persists, such as with xanthomatous deposits, certain drugs have been effective in correcting these conditions. These will now be discussed.

6.9 *HYPOLIPIDEMIC DRUGS*

The hypolipidemic drugs are effective for one of two reasons. Either they affect the rate of lipoprotein production or the rate of lipoprotein removal.[270]

Cholestyramine is the quaternary ammonium salt of a copolymer of styrene with 2% divinylbenzene. It is therefore an anionic exchange resin. It binds bile acids in the small intestine interfering with their reabsorption during their enterohepatic circulation. This increases bile acid production and cholesterol metabolism. Much of this is compensated for by increased cholesterol synthesis.[271]

An apparently more effective property of this drug is in its ability to reduce LDL concentration by increasing the rate of catabolism of its apoprotein moiety.[269] In support of this concept, cholestyramine has been observed to *increase* cholesterol levels when the hypercholesterolemia is due to increased VLDL or IDL and not LDL.[272,273]

Cholestyramine is the drug of choice for treatment of Type II hyperlipoproteinemia. It is especially effective in Type IIa, where LDL

alone is elevated. It is counterindicated in Types III, IV, and V where VLDL or IDL is in excess, since it will increase the production of these lipoproteins, and increase triglyceride levels.[270–274]

The usual dose of cholestyramine in adults is 16 g/day, administered in two to four doses. This is increased by 4–8 g every 2–3 weeks until a maximum of 32 g/day is reached.

The major side effect is constipation. This may be accompanied by nausea, vomiting, and abdominal distention. In addition, gastrointestinal obstruction has also been reported with elevated alkaline phosphatase levels.

Clofibrate (*ethyl p-chlorophenoxyisobutyrate*) inhibits cholesterol synthesis by inhibiting the reduction of 3-hydroxy-3-methylglutaryl CoA to mevalonate (see Section 6.5.4.2). It also has other useful effects, such as decreasing free fatty acid and triglyceride synthesis, increasing triglyceride metabolism, excretion of neutral and acid sterols in the stool, and efflux of cholesterol from tissues.[274,275]

The major action of clofibrate is the reduction of VLDL concentration. This is accomplished not only by inhibiting VLDL synthesis, but mainly by increasing the clearance rate of VLDL and IDL. VLDL and IDL levels drop when clofibrate is administered. This may or may not be accompanied by a fall in LDL levels.[276,277] In some cases, LDL levels may actually rise. It is effective in lowering triglyceride levels, when due to elevation of VLDL, as in Type IV disease.

Clofibrate is most effective in the treatment of patients with Types III, IV, and V hyperlipoproteinemias. Its usefulness in Type II disease is limited.

Clofibrate will potentiate the action of anticoagulant drugs, such as the dicumarols. After extensive studies on large groups of patients, it appears that clofibrate increases the rate of mortality and incidence of cancer in patients treated for extended periods of time.[278,279] In addition, it results in nephropathy, myopathy, and in obstruction of the hepatic ducts, resulting in liver damage.[314–318] For this reason, it is recommended that its use be discontinued, except for short periods of time under acute conditions.

Clofibrate (Atromid) is available in 500 mg tablets. A total dose of 2 g, in four doses is administered daily.

Nicotinic acid (as the amide, niacin), aside from its action as a vitamin, is a plasma-lipid-lowering agent, which has its effect by depressing VLDL synthesis and thus that of its products, IDL and

LDL.[256–258] Plasma triglyceride levels can be shown to be lowered by as much as 60%. Cholesterol levels are also reduced.[259] Some patients who fail to respond to diet and a cholestyramine regimen have been shown to respond to nicotinic acid.

A common side effect of nicotinic acid therapy is intense cutaneous flushing and pruritis. These symptoms usually disappear within a week after the patient is maintained on the drug. Nausea, vomiting, and diarrhea have also been reported. Prolonged therapy may cause hyperpigmentation, dry skin, and acanthosis nigricans. Hepatotoxicity, abnormal glucose-tolerance curve, glycosuria, and hyperuricemia which appear regress after the drug is discontinued.

Nicotinamide may increase the vasodilating and postural hypotensive effect of ganglioplegic antihypertensive agents.[280–282]

Evaluation by the Coronary Drug Project Research Group of nicotinic acid as a lipid-lowering agent, indicated that, although there was no effect on overall mortality from coronary heart disease, there was a significant decrease in the number of infarctions.[283]

Nicotinic acid is most useful where the increase in lipemia is due to an increase in VLDL and its products IDL and LDL. Thus it is best used in Types III, IV, and V disease, but it is also useful in Type II disease.

Because of its many side effects, nicotinic acid and its derivatives are used rather infrequently in controlling hyperlipemia and obesity.

Another approach to the treatment of hyperlipemia is to administer *β-sitosterol.* This plant sterol has the structure of cholesterol, except that an ethyl group is attached at carbon 24 in the side chain (see Appendix). It is unabsorbed, but binds to sites which normally bind cholesterol. Thus it interferes with cholesterol absorption. The drug is most effective in states characterized by LDL excess, as in Type II disease.[270]

Some patients, for unexplained reasons, will absorb plant sterols. This results in xanthomatous deposits made up of these sterols such as β-sitosterol. Unless balance studies are done on the amount of β-sitosterol appearing in the stool, this drug may serve to aggravate an existing condition. β-Sitosterol has a mild laxative effect, sometimes causing nausea and vomiting.

β-Sitosterol is available as a prepared solution. The dosage is 30 ml, one-half hour before meals and at bedtime.

Colestipol is an insoluble high-molecular-weight copolymer of tetraethylenepentamine and epichlorhydrin. Like cholestyramine, it serves to bind bile acids and carry them out with the stools.[284,285] It behaves in a manner similar to cholestyramine. It is administered as water-soluble beads, 4–5 g, three or four times per day.

p-Aminosalicylic Acid was used in the treatment of pulmonary tuberculosis. It was noted that the drug had a hypocholesterolemic effect. A preparation containing *p*-aminosalicylic acid with vitamin C (PAS-C) has been successful in lowering cholesterol in Types IIA and IIB patients.[286] In these patients it serves to reduce serum LDL levels. The drug has been used for many years in the treatment of tuberculosis and is generally well tolerated, although some gastrointestinal effects are noted. Alcohol intake is counter-indicated when taking this drug for the purpose of reducing cholesterol levels.

In adults, the dosage is 8–9 g/day. In children this is reduced to 5–6 g/day.

D-*Thyroxine* is the isomer of the normally physiologically active L-thyroxine. Thyroxine has been used in the past to reduce obesity and hyperlipemia in hypothyroid patients. For the euthyroid patient with hyperlipoproteinemia, the D form of thyroxine was introduced. D-Thyroxine lowers serum LDL and thus cholesterol levels. It seems to act by accelerating lipid and LDL metabolism, and has been applied in Type II, lipoproteinemias.[287]

D-Thyroxine has been shown to be cardiotoxic. For this reason, the Coronary Drug Project Report recommends discontinuance of its usage.[288] Other objectionable side effects are neutropenia, glycosuria, and glucose intolerance, and abnormal liver function.

Neomycin, the antibiotic from *Streptomyces gradiar* has a hypolipemic effect when administered orally. It seems to form insoluble complexes with the bile acids in the intestine.[289,290] Its action thus resembles that of cholestyramine. The major effect is in lowering plasma cholesterol levels with little or no change in glyceride levels.[285]

Although only about 3% of neomycin administered is absorbed, it has resulted in serious oto- and nephrotoxicity in some patients. For this reason it is not used generally. When given, it is administered in doses of 0.5–2 g/day.

In conclusion, all of the hypolipidemic drugs have objectionable side effects of varying degree. For this reason, it is expedient to attempt to control the hyperlipemia with a dietary regimen designed for the

particular patient. For acute treatment, such as the dissolution of xanthomas and reduction of severely elevated cholesterol and/or triglyceride levels, a drug should be selected only after the diagnosis has been made as to the nature of the problem in the particular patient.

6.10 *LIPID AND LIPOPROTEIN ASSAY*

Total lipids are extracted from serum with Bloor's or Folch's reagent as described in Section 6.1. After evaporation, the total lipids are estimated gravimetrically.[3,291,292] Total phospholipids are estimated by oxidizing the residue and estimating the phospholipids from the phosphate liberated (see Section 6.1.4).[293]

Total cholesterol is estimated enzymatically.[294,295] In this procedure the cholesterol esters are hydrolyzed by a lipase and the cholesterol oxidized by cholesterol oxidase which liberates peroxide. The peroxide, in the presence of a peroxidase, oxidizes a substrate to generate a color.

$$\text{cholesterol ester} \xrightarrow[\text{(E.C. 3.1.1.3)}]{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acid}$$

$$2\ \text{cholesterol} + O_2 \xrightarrow[\text{oxidase (E.C. 1.1.3.6)}]{\text{cholesterol}} \text{cholestenone} + H_2O_2$$

$$\text{substrate} + H_2O_2 \xrightarrow[\text{(E.C. 1.11.1.7)}]{\text{peroxidase}} \text{colored compound} + H_2O$$

Alternatively, cholesterol is assayed by the Lieberman–Burchard reaction. In this reaction, the cholesterol, dissolved in chloroform, reacts with a mixture of acetic anhydride and sulfuric acid, 15:1. A greenish-blue color develops which is read at 625 nm.[296]

For unesterified (free) cholesterol, the free cholesterol is precipitated as the digitonide and the color developed with the Lieberman–Burchard reagent.[296,297]

A more stable color is obtained with cholesterol using the Kiliani reagent. This comprises a 0.4% solution of anhydrous ferric chloride in a 2:1 mixture of glacial acetic acid and concentrated sulfuric acid. A purple color is obtained which is read at 560 nm.[298,299]

Triglycerides are estimated enzymatically. The triglycerides are hydrolyzed by a lipase, and the glycerol is phosphorylated with ATP. The ADP generated is estimated by converting it back to ATP with enolphosphate. The pyruvate generated, reduces NADH to generate NAD. The decrease in absorbance at 340 nm is read, to estimate the triglyceride content.[300,301]

$$\text{triglycerides} \xrightarrow[\text{(E.C. 3.1.1.3)}]{\text{lipase}} \text{fatty acids} + \text{glycerol}$$

$$\text{glycerol} + \text{ATP} \xrightarrow[\text{(E.C. 2.7.1.30)}]{\text{glycerol kinase}} \text{glycerylphosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[\text{(E.C. 2.7.1.40)}]{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} \xrightarrow[\text{dehydrogenase (E.C. 1.1.1.27)}]{\text{lactate}} \text{lactate} + \text{NAD}$$

Alternatively, the triglycerides are hydrolyzed by heating with alkali and the glycerol is estimated by oxidation to formaldehyde. The formaldehyde is estimated by its reaction with chromotropic acid.[302,303]

The lipoprotein fractions are estimated by electrophoresis on cellulose acetate or agarose. Paper electrophoresis does not produce results as good as those obtained with these media. After staining with a fat stain, such as oil red, fat red 7B, or Sudan black, the concentration of each fraction is evaluated by densitometry.[304,305]

Acrylamide gel electrophoresis separates the particles by size. Thus the order is HDL, LDL, VLDL, and chylomicrons.[305] With agarose or cellulose acetate the order is HDL, VLDL, LDL, and then chylomicrons. Partly because of this inversion, the agarose or cellulose acetate systems are used more frequently.

High density lipoprotein (HDL) is located by cellulose acetate or agarose electrophoresis, followed by densitometry. Its cholesterol content can then be estimated enzymatically *in situ* (Figure 6.23). For high-density lipoprotein cholesterol (HDL-C), advantage is taken of the fact that all of the lipoprotein fractions other than HDL can be precipitated by a divalent salt and polyanionic material. Most commonly heparin and Mn^{2+} are used.[306,307] Alternatively, Mg^{2+} with dextran sulfate has been recommended.[308] The cholesterol determined, usually enzymatically, on the supernatant is the HDL-cholesterol.[309] In healthy individuals a value of 460 ± 104 mg/liter is considered the normal range.

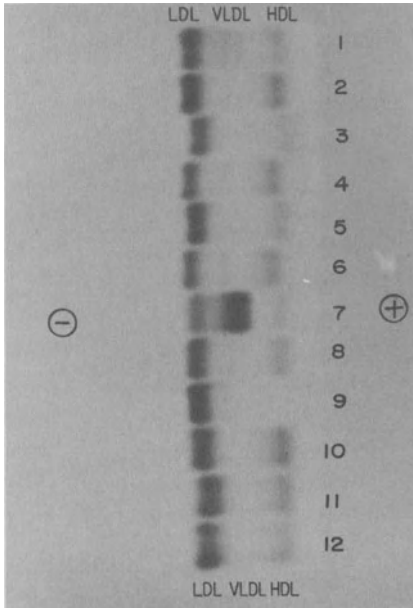


FIGURE 6.23 High-density lipoprotein cholesterol (HDL–C) estimation by electrophoresis, staining with cholesterol oxidase followed by a peroxidase to produce a color. The concentrations are estimated with the densitometer. Notice the high VLDL-cholesterol levels in No. 7, and very low HDL–C levels in Nos. 3, 7, and 9. (Courtesy of Helena Laboratories, Beaumont, Texas.)

Low-density lipoprotein (LDL) levels are important in uncovering hypo-β-lipoproteinemias. This is assayed by electrophoresis and densitometry. This condition, along with hyper-β-lipoproteinemia, can be detected by electrophoresis of cord blood.[309]

Increasing interest has developed in the assay of the apolipoproteins by radioimmunoassay. The focus has been on the assay of apoB apoA-I, A-II, and C-II. In normal individuals the concentration of these proteins, as determined immunochemically, are: apoB, 830–930; apoA-I, 1000–1300; apoA-II, 300–450; and apoC-II, 45–60 mg/liter.[310,311] These assays are of particular value in the diagnosis of the various hypo- and hyperlipoproteinemias.

Assay for lipoprotein X as an indicator of cholestasis has aroused increasing interest. This is readily assayed by electrophoresis because of its movement to the cathode, whereas all other lipoproteins move to the anode.[312,313] In the presence of high concentration of bile salts and other metabolites sometimes present in the plasma, lipoprotein X will change mobility and move to the anode. Thus a false negative will be obtained. Lipoprotein X is elevated when serum-free cholesterol levels are elevated along with alkaline phosphatase as in bile duct

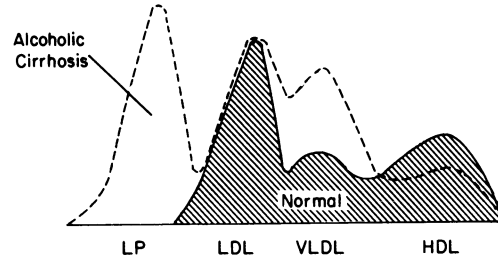


FIGURE 6.24 The location of lopoprotein X with cellulose acetate electrophoresis as compared to HDL, VLDL, and LDL in the normal individual.

obstruction. Figure 6.24 compares the scans of electrophoretic patterns for normal serum as compared to a serum containing LpX.

6.11 *RECAPITULATION*

The blood lipids, cholesterol, cholesteryl esters, triglycerides, and phospholipids are solubilized in the plasma by certain proteins. The protein lipid complexes are called lipoproteins. These can be separated into four major groups by electrophoresis or in the ultracentrifuge. These are referred to as the chylomicrons, low-density lipoprotein (LDL), very-low-density lypoprotein (VLDL), and high-density lipoprotein (HDL) in order of increasing density. In the electrophoretic field, the mobilities of these proteins, in order of increasing mobility to the anode, are the chylomicrons (origin), LDL (β globulins), VLDL (pre-β), and HDL (α globulins). In addition to these fractions, albumin carries the free fatty acids and has been referred to as the very-high-density lipoprotein (VHDL).

VLDL originates in the liver and small intestine from endogenous sources. It carries most of the triglycerides in fasting serum. It is rapidly digested into an intermediate form (IDL) and finally LDL. The LDL, which is about 50% cholesterol by weight, adsorbs to the parenchymal cells of the liver, is internalized and metabolized. Excess fatty acids, not needed for immediate use, are stored in the adipose cells as triglycerides. Under the influence of a hormone activated lipase, they can be released into the plasma as free fatty acids and glycerol, for metabolism by the various tissues.

The major protein of LDL is referred to as apoB. It is a high-molecular-weight protein which binds triglycerides and cholesteryl esters. HDL is composed of several helical low-molecular-weight

proteins referred to as apoA, apoC, apoD, and apoE. These helical proteins appear to be synthesized with HDL, move to the blood stream, and exchange with VLDL and the chylomicrons, so that VLDL and the chylomicrons also contain these helical proteins.

As VLDL is acted upon by lipases, it forms an intermediate lipoprotein (IDL) and finally LDL. LDL loses its affinity for the helical proteins, and contains almost exclusively apoB. These apoproteins are *amphipathic*, that is, they have affinity for the polar end of the phospholipid and nonpolar hydrocarbon chains of the fatty acids.

In patients with lecithin–cholesterol acyltransferase deficiency, cholesterol cannot be esterified and forms a lipoprotein complex in the bile, which then moves into the blood stream. This is referred to as lipoprotein X.

Lipid disorders occur because of either overproduction or faulty removal of one or more lipoproteins. These may result from increased intake of cholesterol and triglyceride in the diet, increased amounts of endogenous cholesterol or triglycerides released from the liver and small intestine as VLDL, defects in the clearing enzymes at catabolic sites, or abnormalities in the lipoproteins themselves.

A system has been devised which divides the familial disorders into different types. Type I refers to a defect in the release of lipase by heparin. This results in a severe chylomicronemia. Type II is a deficiency in the binding of LDL and results in a hypercholesterolemia. Type III derives from a deficiency of apoC-II activatable lipase, which interferes in the conversion of VLDL to LDL, resulting in the accumulation of VLDL and IDL (remnants) containing triglycerides and cholesterol. Type IV is a hyperglyceridemia resulting from an accumulation of VLDL from endogenous sources. Type V is also a hyperglyceridemia resulting mainly from an accumulation of chylomicrons and some VLDL from the diet.

Some suggest that Types I and V are variants of the same disease, Type I being the infantile or juvenile type, and Type V being the adult type of the disease, with only a partial postheparin lipase deficiency. Others consider Types IV and V as hyperglyceridemias which are variants of the same disease.

Diabetes, hyperinsulinism, hypothyroidism, gout, the nephrotic syndrome, and other diseases can simulate the various types of familial hyperlipemias. In any case, hypercholesterolemia and triglyceridemia, usually associated with obesity, place an individual at risk for cardio-

vascular disease. These conditions respond to diet and, in severe cases, to certain drugs which lower blood lipids.

Inheritance of certain lipoprotein patterns seems to result in a decrease in the incidence of atherosclerosis and heart disease. One such condition is familial elevated HDL (hyper-α-lipoproteinemia) with an elevated percentage of cholesterol in the HDL fraction. Another is familial hypo-β-lipoproteinemia with decreased concentration of LDL in the plasma. In both cases, a higher percentage of cholesterol in the plasma is carried by HDL. These conditions, as well as some of the familial diseases tending toward an increased risk for ischemic heart disease, can be detected in the neonate.

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Part B

Plasma Proteins in Transport of Intermediates, Minerals, and Hormones

Sec. III

Plasma Transport Proteins

7

Transferrin: Iron Metabolism

7.1 *TRANSPORT PROTEINS*

By their very nature all plasma proteins will tend to bind anions and cations and various types of organic compounds. Almost every type of bond, including primary and secondary valences and Van der Waals forces are employed for this purpose. Thus there are *polar bonds*, such as in salt formation with anions or cations, since proteins are amphoteric in nature. With *semipolar bonds chelate formation* occurs, especially when binding metal ions. *Hydrogen bonding* is common since the oxygen on the carbonyl group and the nitrogen of the amide both will bind protons. Insertion of hydrocarbon portions of a molecule into a hydrophobic pocket formed in a protein fold is common. This is used in treating proteins with dodecyl sulfate so as to provide a uniform electrical charge for all proteins, in acrylamide gel electrophoresis.

Binding forces may be weak, readily reversed, to very strong and irreversible with every shade between. The proteins may be phosphorylated to aid in binding such ions as magnesium, zinc, or calcium. In these cases, a chelate complex is usually formed with an adjacent amino acid residue suspended from the backbone of the protein.

The binding to proteins in the plasma serves several purposes. Some of these have been discussed in the chapter on albumin. Thus they may function as a carrier to detoxify and aid excretion of substances such as drugs or bilirubin. Binding may serve as a reservoir for storage of a particular ion or metabolite, such as with the transcobalamines or ferritin.

In this chapter, we are concerned mainly with the transport

TABLE 7.1

Selected Plasma Transport Proteins

Protein	Transported material	Relative electrophoretic mobility	Molecular weight	Concentration, mg/liter
Thyroxine-binding prealbumin (TBPA)	Thyroxine	Prealbumin	54,980	100–400
Retinol-binding protein (RBP)	Vitamin A	Prealbumin (α_1 fraction when free of TBPA)	21,000	30–60
Transcortin (TC)	Steroids	α_2	55,700	60–80
Thyroxine binding globulin (TBG)	Thyroxine	α_1	58,000	10–20
Ceruloplasmin (Cp)	Copper	α_2	151,000	150–600
Transferrin (Tf)	Iron	β	76,000	2000–3200
Transcobalamin II	B_{12}	Post α_2	53,900	25–35
Haptoglobin (Hp)	Hemoglobin	α_2	100,000 subunit (up to 4 subunits per molecule)	1000–2200
Hemopexin (Hpx)	Heme (hemin)	β	57,000	500–1000
Vitamin-D-binding protein (DBP), Gc globulin	Vitamin D and 25-hydroxy vitamin D	α_1	52,000	400–450

proteins in plasma which serve to carry the hormones and various metabolites from the site of production to the area of their utilization. Albumin will be excluded since it has already been dealt with. The lipoproteins and immunoglobulins will also be excluded. Here we will deal only with those listed in Table 7.1.

7.2 *TRANSFERRIN*

Plasma iron is bound to a protein called *transferrin* which has a molecular weight of approximately 76,500[1] and a sedimentation coefficient ($s_{20,w}$) of 5.3. Transferrin has been called various names such

as *siderophilin*[2] and β_1 *metal-binding globulin* since it also binds copper.[3] Transferrin occurs in the plasma at a concentration of 200–320 mg/100 ml.[4] Since it comprises a substantial portion of the β globulins it can readily be seen as the initial and highest β peak in the normal electrophoretic pattern (see Figure 3.5).

One molecule of transferrin binds, stoichiometrically, two molecules of ferric iron.[5] At the same time it binds two bicarbonate radicals. Each binding site is independent of the other. The transferrin concentration is somewhat in excess of 2.5 g/liter of plasma or 33 μmole/liter. This can bind 66 μmole iron per liter. The normal plasma iron concentration is 60–150 μg/100 ml, all of which is bound to transferrin. On the average, this represents about 19 μmole bound iron per liter. Thus the plasma is only about 30% saturated with respect to iron. If saturated with ferric ion, serum changes from a straw color to a pale pink, which is the color of iron-saturated transferrin, with an absorption maximum at 460–470 nm. The transferrin ferrous ion complex is colorless.

The existence of an iron deficiency state is often explored by measuring the *serum iron* and the *iron-binding capacity*. *The iron-binding test* is performed by first measuring the serum iron. An aliquot of the serum is then incubated with an excess of a ferrous salt. Residual ferrous salt is measured with the phenanthrolenes, as a measure of the amount of iron complexed to the transferrin.[6] The amount of iron originally present in the serum plus the amount adsorbed is the *iron-binding capacity*. Normally this value ranges from 250 to 410 μg Fe/100 ml. The iron-binding capacity is actually an approximate measure of the amount of transferrin present. Albumin and other proteins in the serum also bind small amounts of iron.

The adsorption of Fe^{3+} to transferrin changes the conformation of the molecule so that its electrophoretic mobility changes. This permits the separation of four forms of transferrin, depending upon the degree of saturation with Fe^{3+}.[7] This can be seen in Figure 7.1.

Transferrin has been isolated, crystallized, and substantial progress has been made in elucidating the sequencing of the amino acids in its makeup.[8] It comprises a single chain, which is longer than that for albumin.

Transferrin has two equal chains of carbohydrate attached, and the sequence of the sugars has been ascertained. They are attached to the molecule at asparagine residues.[9] The oligosaccharide has two molecules of *N*-acetylneuraminic acid (sialic acid, NANA), two

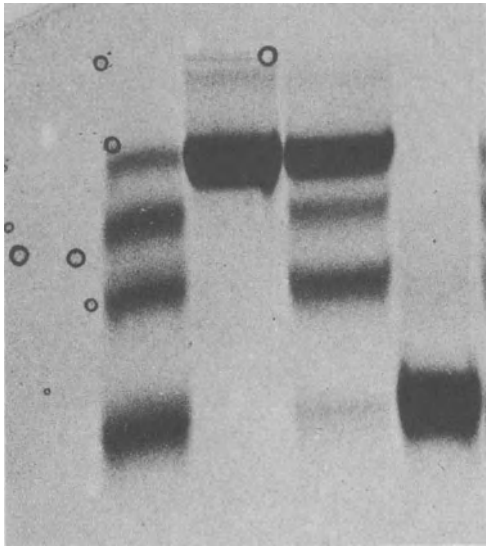


FIGURE 7.1 Electrophoresis of transferrin in acrylamide gel in 6 M urea showing the existence of four bands. From left to right the transferrin solutions show iron saturation of 60, 0, 30, and 100%. (Courtesy of D. L. Makey, V. A. Hosp., Minneapolis, Minn.)

molecules of galactose, four molecules of glucosamine (GlcNAc) and three molecules of mannose (9, 10). This is shown in Chapter 5, Figure 5.15, page 95.

Cerebrospinal fluid contains a protein that binds iron which moves in the β_2 region on electrophoresis. This is in the same area as the so called spinal fluid tau (τ) band. This is not identical with transferrin which migrates in the β_1 region. However, the two proteins react equally with transferrin antibody. This peculiarity was explained when transferrin could be given a β_2 mobility by hydrolyzing off four neuraminic acid residues per molecule with neuraminidase.[10] One fourth of the transferrin of the cerebrospinal fluid is synthesized locally.[11]

Multiple transferrin bands ranging from β_2 to β_1 mobility are found in cerebrospinal fluid as well as umbilical cord blood. These are not distinguishable immunologically. On treatment with neuraminidase, to remove sialic acid, they all assume β_2 mobility. On this basis it has been postulated that the protein is first synthesized, and the carbohydrate is added later.[12]

One of the most studied proteins is the iron-binding protein of eggwhite, *ovotransferrin.* This protein is most often referred to as *conalbumin,* and is synthesized in the oviduct of the hen. Studies on the iron-binding sites of ovotransferrin indicate that binding of Fe^{3+} displaces

three protons from the –OH group of three tyrosine residues and forms a chelate, which involves two histidine imidazole groups.[13] It also turns pink on saturation with iron.

An iron-binding protein, *lactoferrin* (*lactotransferrin*) also occurs in human milk and colostrum. It differs significantly in its amino acid content from human serum transferrin, mainly in the content and distribution of the basic amino acids. Its carbohydrate content differs in detail from serum transferrin and it is also different immunologically.[14]

Adsorption of iron to lactoferrin is the same as for transferrin, there being two distinct binding sites which bind one Fe^{3+} and one bicarbonate ion at each site.[15,15a] Copper also binds in a similar fashion. The absorption spectrum of lactoferrin saturated with iron is the same as that for transferrin. Lactoferrin has a molecular weight of 75,000 and a sedimentation rate of 4.93S. It also consists of a single polypeptide.

7.2.1 *Transferrin Variants*

Transferrin was originally labeled protein C in early experiments with starch electrophoresis.[16] It was noted that in certain Blacks, the C was replaced by a slower moving protein which was referred to as D. Both were soon identified as transferrins.[17] The variation in the nature of this protein was soon shown to be under genetic control, the variation being due to the substitution of one amino acid for another. Soon variants were found which traveled faster than the normal *transferrin C*. These were then referred to as transferrins B. Subscripts were then introduced and finally locations or names were used. At present, about 25 variants have been reported. Most of these are rare. Table 7.2 lists some of the variants in order of their mobility.[18]

In most cases, there is a single amino acid substitution. For example, in $D_{Chicago}$ arginine is substituted for histidine. In B_2, a glycine residue is replaced by glutamate. On the other hand, the genetic variant D, seems to have two substitutions, an exchange of glycine for aspartate and another glycine for asparagine.[19–21]

The gene frequency of most variants are very low. For example,

TABLE 7.2

Relative Mobility of Some Genetic Variants of Human Transferrins[19–23]

Transferrin designation	Source	Electrophoretic mobility to anode(%) relative to transferrin C[a]
B_{Lae}	New Guinea	+195
B_0	U.S. Caucasian	+165
B_{0-1}	Navajo	+112
$B_{Atalanti}$	Greek	+97
B_1		+97
$B_{Lambert}$	U.S. Caucasian	+90
B_{1-2}	Italo-African	+90
B_2	U.S. Caucasian	+70
B_3	Japanese	+63
B_{Austin}		+48
C	Normal	0
$D_{Adelaide}$	Australian Caucasian	−60
D_0	U.S. Black	−60
D_{Wigan}	English	−70
D_{0-1}	English	−75
$D_{Montreal}$	Canadian Caucasian	−88
$D_{Chicago}$	Chinese	−95
D_1	U.S. Black	−100
$D_{Ralford}$		−102
$D_{Finland}$	Finnish	−107
D_2	African Black	−160
$D_{Indonesia}$	Indonesian	−170
D_3	U.S. Black	−180

[a] Distance traveled between C and D_1 is arbitrarily called 100. Others are relative to this measure.

the gene frequency for B_2 is 0.005, for $D_{Chicago}$, 0.03, for D_1, 0.06. Others show even a lower gene frequency. Of interest is the fact that the gene frequency for Transferrin C is only 0.554 in the Australian natives of the western desert.[22,23]

Transferrin variants are readily located by saturating the serum with $^{59}Fe^{3+}$ and following with electrophoresis on acrylamide gel. The radioactivity is detected by scanning or by autoradiography. Usually the variant is observed in a heterozygote in which case TfC and the abnormal Tf will be detected.

7.2.2 *Transferrin Function*

The major function[62a] of transferrin is the transport of iron. Since it forms a tightly bound chelate with iron, it also serves to prevent the loss of iron into the urine. Bacteriostatic properties have been assigned to transferrin since the apoprotein will prevent bacterial growth when added to a culture medium by its sequestering of the iron.[34] Transferrin will inhibit enzyme activity where ferrous iron is required as a cofactor.[25] The high concentration of transferrin in eggs and milk contributes to their resistance to bacterial action. Patients with hypotransferrinemia may suffer from repeated infections. This in part could be related to the absence of bacteriostatic action of transferrin.[26,27b]

Since only about one-third of the circulating transferrin is required to bind the circulating iron, transferrin also acts as a buffer to prevent surges in the available ionic iron which would result in excessive iron deposition in the tissues.

Transferrin is synthesized in the parenchyma of the liver.[28] The half-life of transferrin in the plasma is about 8 days. The daily turnover is about 10–15 mg/kg of body weight.[29,30] By measuring the rate of transferrin production it has been calculated that it takes 2 min to synthesize a molecule of transferrin as compared to 1 min for albumin. The transferrin chain is longer, and also requires attachment of oligosaccharide.

7.2.3 *Transferrin and Iron Metabolism*

Absorption of ingested iron takes place mainly in the stomach and duodenum. Since there is danger of iron intoxication, due to the sporadic intake of substantial quantities of iron, especially from meats, the iron is promptly oxidized to the ferric state, and complexed with a protein present in the lining of the stomach and intestinal walls and in the reticuloendothelial system, called *ferritin*. Fe^{2+} which reaches the serum is adsorbed to transferrin and oxidized to Fe^{3+} with the aid of *ceruloplasmin*, the copper-containing protein in serum. The Fe^{3+}, complexed to the transferrin molecule, can then be transported to the tissues.[30a,31]

Ferritin is a complex of a protein called *apoferritin* and a variable amount of $(FeO, OH)_8 \cdot (FeOPO_3H_2)$. Apoferritin has a molecular weight of approximately 445,000.[32,33] The rate of Fe absorption is

regulated by the amount of iron already in storage throughout the body, and especially the amount within the cells of the gastrointestinal tract in the form of ferritin.[34–36] Transferrin of the plasma does not seem to affect Fe absorption. *Complete saturation of transferrin, of the plasma does not block Fe absorption from the gastrointestinal tract.* However, in iron deficiency, the amount of transferrin synthesized decreases.[30] Since the transferrin is not being utilized, however, its turnover rate is decreased, resulting in a net increase of serum transferrin levels (Table 7.5).

Even in the presence of anemia, or reduced plasma levels of Fe, if the tissue levels of ferritin are high, additional Fe will not be absorbed. Ferritin is 23% Fe, and the Fe is loosely bound so that it is in equilibrium with plasma Fe.[36] Some inorganic iron is also present in the cells as a colloidal aggregate called *hemosiderin*, which is 35% iron. Here iron is precipitated as the oxide, so that disassociation to Fe ions is very slight. Excessive deposits of hemosiderin in disease is called *hemosiderosis.*[37]

The ferritin of the tissues act as an iron store for eventual transfer to the bone marrow by means of transferrin in the plasma. There the iron is stored as ferritin by the RES (reticuloendothelial system) cells and used for hemoglobin production. Thirty percent of erythroblasts still contain ferritin which disappears as the cell matures to form an erythrocyte. The scheme of iron absorption and metabolism is shown in Figure 7.2.[38–41]

In Figure 7.2 one can see that iron is solubilized by digestion in the stomach of the protein-bearing iron compounds in the food, such as meat. The ferric ion is reduced to the more soluble ferrous iron and absorbed directly from the stomach or upper duodenal cells into the plasma where it adsorbs to transferrin. Inorganic iron also goes directly to the liver from the intestines, where it reacts with apoferritin to form ferritin, the iron being oxidized in the process. This acts as a storehouse for iron, for production of hemoglobin. As needed, the iron is released from the ferritin, reduced, and moves into the plasma where it is reoxidized to the ferric form, as it complexes to transferrin. This is catalyzed by ceruloplasmin, using oxygen from oxyhemoglobin as the oxidizing agent.[31] It is then transported to the bone marrow where it is utilized. Transferrin itself is also an oxidase, and can oxidize Fe^{2+} in the absence of ceruloplasmin.

The mature erythrocyte circulates until it becomes senescent. It is then phagocytized by the RES cells of the liver and spleen. Any hemoglobin attached to haptoglobin or heme complexed with hemopexin are

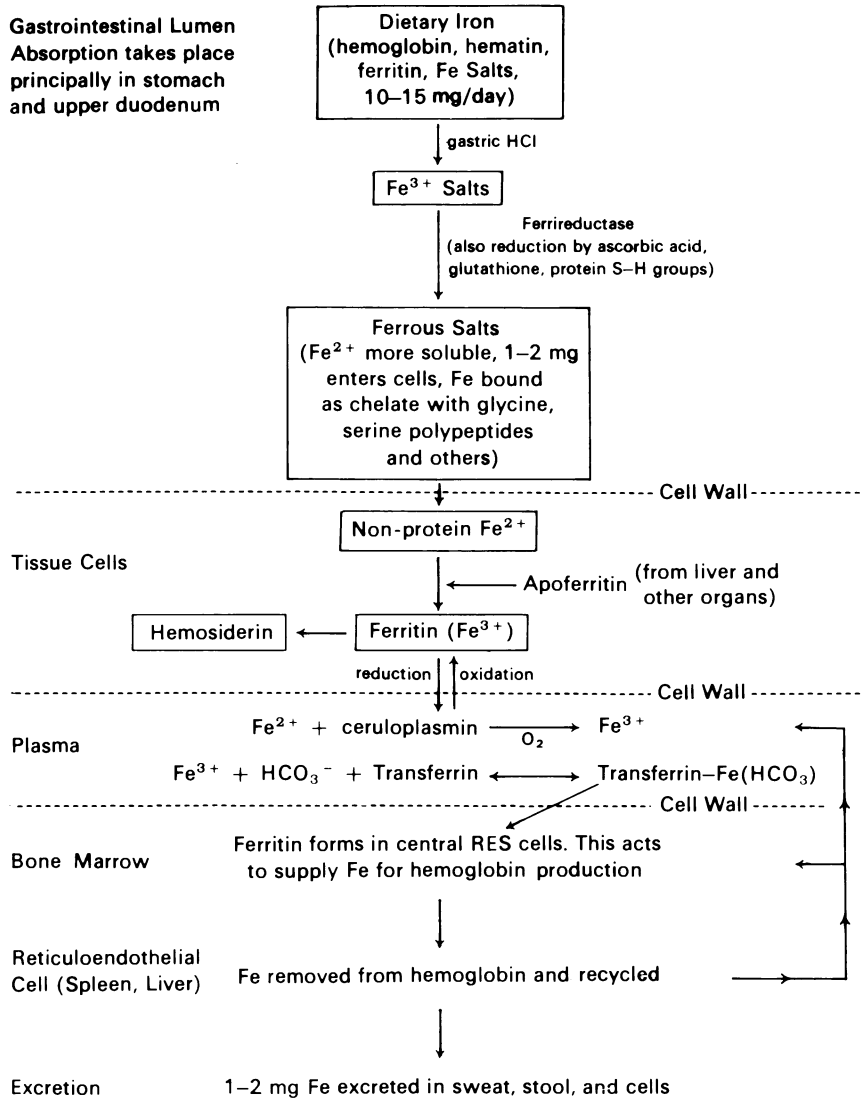


FIGURE 7.2 Schematic representation of iron metabolism.

also phagocytosed or enter the RES cells by pinocytosis. The red cells are disrupted and the iron is extracted and stored as ferritin in the RES cells. If excessive amounts are accumulated, the iron is transferred to the plasma transferrin from where it is carried to the parenchymal cells for storage.[41–43] See Figure 7.3.

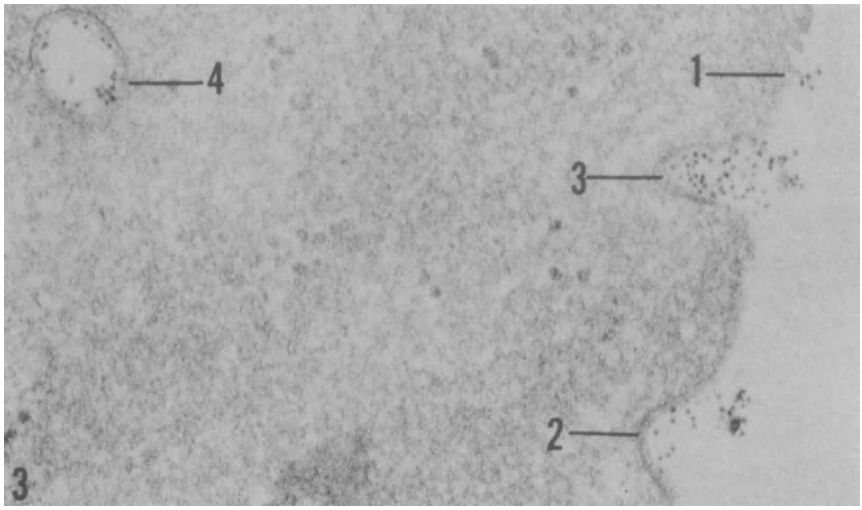


FIGURE 7.3 Electromicrograph showing pinocytosis of transferrin by the blood reticulocyte. The transferrin is bound to a transferrin antibody which in turn is bound to ferritin. Adsorption to the cell wall is shown at 1, invagination at 2 and 3 and inclusion within the cell in a vesicle at 4 (× 94,050). Reproduced at 94%. [Courtesy of Sullivan, A. L., Grasso, J. A., and Weintraub, L. R., *Blood* **47**:133 (1972).]

The daily turnover of hemoglobin iron is about 30 mg/24 hr. There are about 3–5 g iron present in the body. At any one time, only 3–4 mg are circulating. Thus the iron stored, mostly as ferritin, is 1000 times that circulating. All the circulating iron is bound to transferrin. Some evidence has been adduced to indicate that transferrin actually penetrates the reticuloendothelial cell when it releases its iron.[44.44a]

The mechanism of pinocytosis is shown in Figure 7.3. An anemia, with reticulocytosis, was induced in a rat with phenylhydrazine intoxication. Figure 7.3 shows one of these reticulocytes which has been incubated with transferrin for 5 min. The transferrin adsorbs to the cell. This cell is then incubated with a transferrin antibody to which ferritin has been conjugated, so that it can be visualized with the electron microscope. Such a mechanism is proposed for the movement of iron-bearing transferrin into the cells of the reticuloendothelial system.[44a]

7.3 *FERRITIN AND IRON STORAGE*

Ferritin is composed of 24 subunits each with a molecular weight of 18,500. These are readily dissociated from each other by acetic acid at 0°C or sodium dodecyl sulfate.[45,46] The apoferritin molecule is a hollow shell of internal diameter of 74 Å and external diameter of 122 Å. By X-ray diffraction it has been shown that there are six channels in the hollow molecule which permit flat molecules up to 5 Å across to enter.[47] The iron content of ferritin is 300–400 μg iron per mg protein which calculates to 2400–3200 iron atoms per molecule.

The amino acid composition and sequence has been developed for the individual subunits of ferritin and these vary with the tissue from which the ferritin originates.[48–51] Thus each ferritin is a unique gene product depending upon the tissue. When human liver, spleen, kidney, and heart ferritins are subject to isoelectric focusing, they are all found to be heterogeneous (*isoferritins*). Most tissues contain five to six isomers, some being common to several tissues. About 12 distinct isoferritins have been identified in human tissues.[52,53] Amino acid analysis indicates changes in the acidic amino acids in the subunits which results in differing electrophoretic mobility.[54] Like the lactic dehydrogenases, only two or possibly three different subunits are involved, the proportion being different in different isoferritins, resulting in the various isoferritin molecules. Heart and liver ferritin each contain two subunits species, H + HL and L + HL. The HL species is common to both tissues. H is more acidic than L and travels faster at alkaline pH, Apoferritin of the liver consists largely of L subunits.[55]

If iron is administered in large doses, the amount of apoferritin synthesized by the liver increases rapidly.[56,57] Several hypotheses have been proposed for the way the ferritin shell accumulates the iron. The most probable one seems to be that soluble ferrous ion penetrates into the channels where it is adsorbed to a catalytic site in which carboxyl groups and histidine residues serve to complex the iron. The channels are wider at the inside (12 Å) and narrower outside (9 Å). Adsorption in the channel stimulates the oxidation to the ferric form by molecular oxygen. This forms a microcrystalline product which moves into the center of the shell. Further iron is added to the crystal surface at a rate dependent on the number of available sites and the concentration of ferrous ion.

Mobilization of ferritin iron stores requires that the iron be reduced

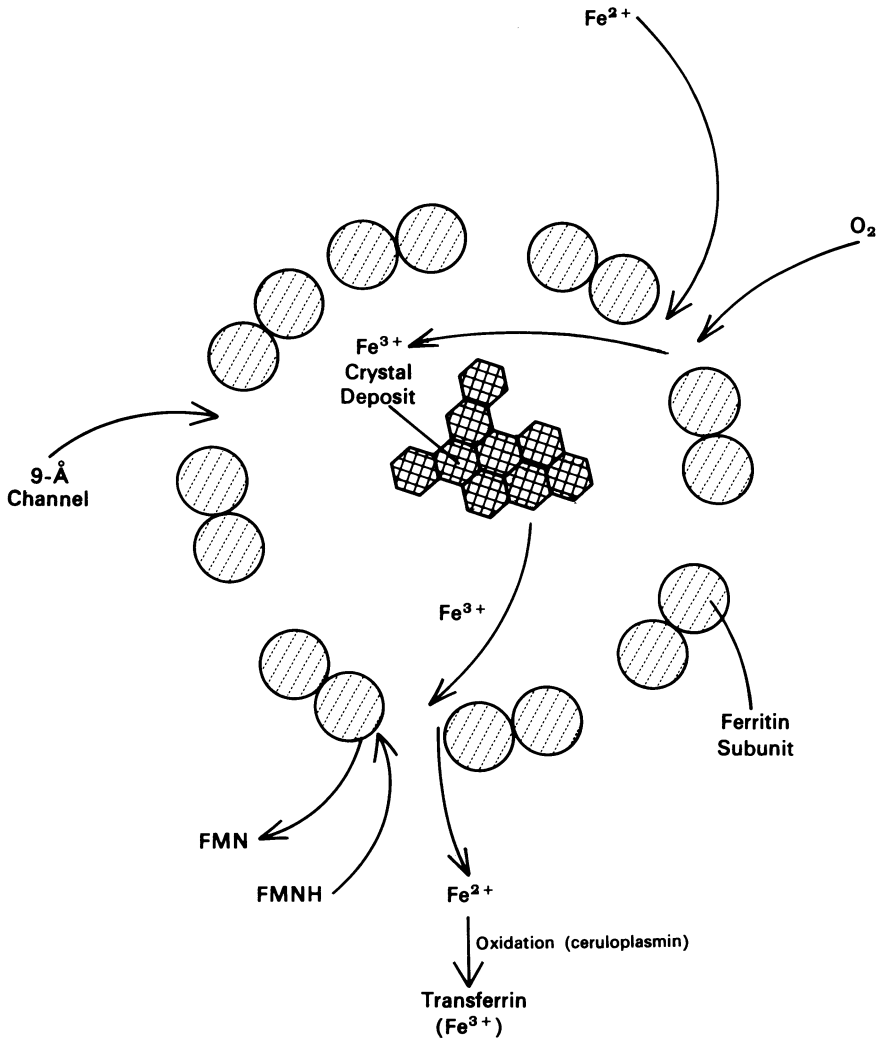
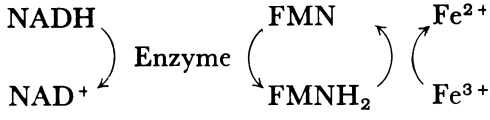


FIGURE 7.4 Schematic representation of the storing and release of iron from the ferritin molecule to plasma transferrin.

to the soluble ferrous form. This is done by reduction of the ferric iron by an NADH–$FMNH_2$ coupled oxidoreductase present in liver cells in the microsomal fraction.[58,59] Xanthine oxidase, which was purported to be implicated in the process,[58] is at present not considered of major importance.

In the process, NADH reduces FMN (Flavine mononucleotide) to

$FMNH_2$, enzymatically. The $FMNH_2$ binds to ferritin, reducing the iron. It is then released for recycling. This may be shown as follows:



Ferrireductase system

Figure 7.4 is a schematic drawing indicating the way iron is stored in the ferritin molecule.

Although ferritin exists in all tissues, substantially all iron is stored in the liver and reticuloendothelial system. The reticuloendothelial system ferritin derives most of its iron from senescent erythrocytes.[39] The liver parenchymal cell ferritin obtains most of its iron from transferrin. In the RE system, reduction of iron depends mainly on ascorbic acid. In the parenchymal cells, reduction is by the ferrireductase system.

In addition to storage of iron as ferritin, iron is also deposited in the cell stroma as granules surrounded by a protein film. These are called *hemosiderin granules*. The iron content of these granules is higher, and the nitrogen content correspondingly lower than that found in ferritin. It has been suggested that hemosiderin is derived from ferritin by partial digestion of the protein shell, releasing the micelles of iron which are then phagocytized.[60]

Normally, half the storage iron is in the form of ferritin. In the presence of ascorbate deficiency and iron overload, a shift takes place and a greater percentage is stored as hemosiderin.[60,61]

7.4 *ABERRANT IRON METABOLISM*

Aberrant iron metabolism may result in iron deficiency or excessive iron deposits in the body. The most common form of anemia in males and females, both in adults and children, is due to iron deficiency. Typically, iron deficiency is accompanied by a hypochromic and microcytic anemia in which the cells are pale and are variable in size and shape. This is seen in Figure 7.5. Bone marrow stains, with Prussian blue, show decreased amounts of iron in the bone marrow (Figure 7.6). Hypochromic anemia is not always consequent to reduced total body

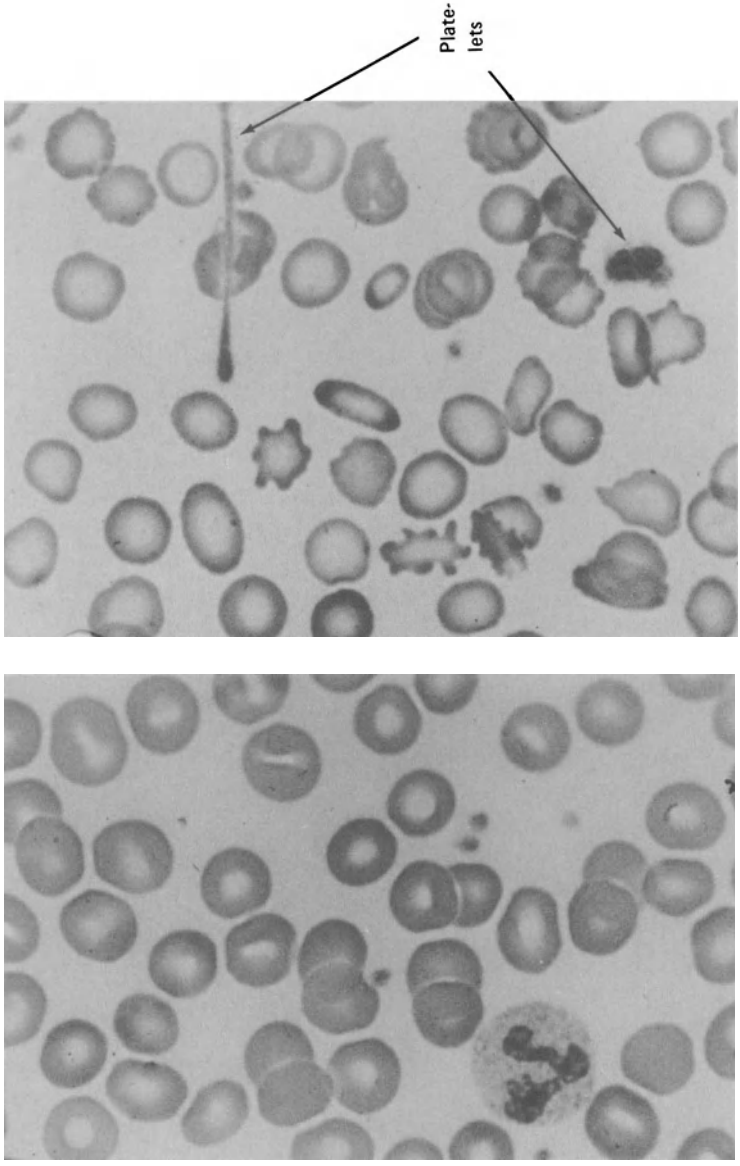


FIGURE 7.5 Anemia, due to iron deficiency (right), compared to the normal blood film (left). Note the hypochromia and poikilocytosis. Platelet appearance is also abnormal.

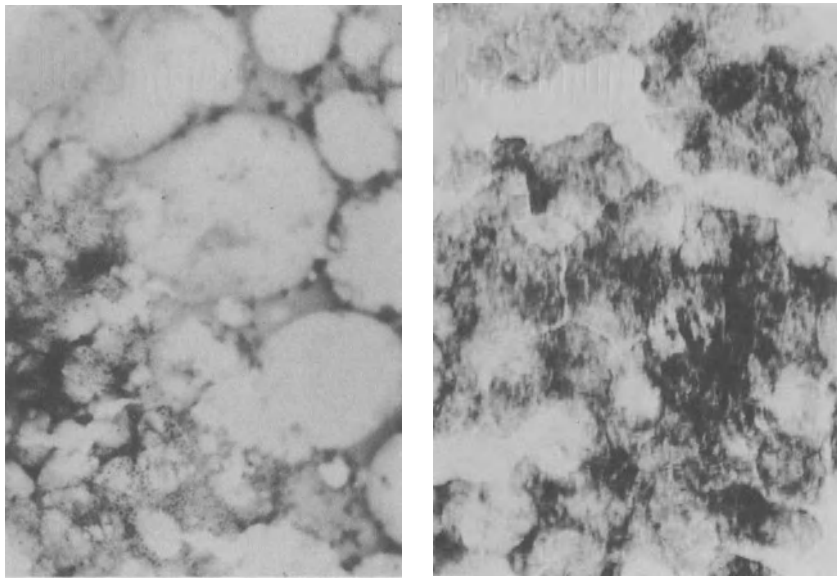


FIGURE 7.6 Comparison of bone marrow preparations, stained with Prussian blue, obtained from a patient with an iron deficiency anemia (left) and a normal adult (right).

iron stores but may result from any of at least four causes which may be summarized as follows:

1. Increased iron losses (usually blood loss) resulting in a negative iron balance.
2. Inadequate iron intake and/or general malnutrition.
3. Defective reutilization of iron released from senescent erythrocytes, resulting in loss of iron available to developing red cells.
4. Decreased amounts of, or absence of, transferrin or ferritin necessary for the transport and storage of iron.

These causes for hypochromic anemia due to iron deficiency will now be considered in the order in which they are listed.

7.4.1 *Increased Iron Losses due to Hemorrhage*

Losses of iron during menstruation are often not replaced before the next menstrual period. As a result *hypoferremia* (*low iron blood concentration*), is more common in women regardless of the country where the

studies have been made.[61] For example, in the United States, 12.6% of women are anemic as compared to 0.8% of men. About the same figures are noted in the more technically advanced Latin American countries such as Mexico and Venezuela. However, in countries where adequate nutrition presents a serious problem, such as in India, 35% of women show an anemia associated with iron deficiency while only 6% is observed in men.[62] Table 7.3 shows the distribution of iron in the body and a suggested daily allowance.

In males, the major cause of iron deficiency is gastrointestinal blood loss. This may be due to peptic ulcer disease, ulcerative colitis, alcoholic liver disease with esophageal varices, hiatal hernia, neoplasm, diverticulosis, or hemorrhoids.[63,64] Similar problems, of course, may also occur in women. In tropical countries, hook worm infestation is a common cause of gastrointestinal bleeding.[64a] In all affected persons, such bleeding may be potentiated by any underlying defect in the normal blood coagulation mechanism[65] or by habitual ingestion of gastrointestinal irritants such as aspirin.[67] Iron deficiency anemia has also been reported in individuals who donate their blood at regular intervals to blood banks.[66]

Certain rare disorders are associated with chronic bleeding and iron loss resulting in anemia. Among these are *paroxysmal nocturnal hemoglobinuria*, where excessive urinary iron loss may occur, and in *idiopathic pulmonary hemosiderosis* where repeated hemorrhages into

TABLE 7.3

Approximate Distribution of Iron in the Adult Male and Female and Daily Needs[62]

Location	Male (mg Fe)	Female (mg Fe)
Hemoglobin	2100	1750
Tissue (myoglobin)	350	300
Storage (ferritin)	1000	400
Total	3450	2450
Daily allowance[a]	15 ± 5	18 ± 5
Actually absorbed	0.9 (~6%)	1.3 (~7%)

[a] Males normally take in their allowance. Many women take in about 10 mg/day or about 56% of their allowance.

alveolar capillaries allows iron to be sequestered in pulmonary macrophages as hemosiderin.[68]

Chronic self-inflicted bleeding in mentally ill persons is not uncommon, especially in women. In these cases, the psychiatric problem may first be brought to light by the persistence of an unexplained iron deficiency anemia.[69,70]

7.4.2 *Inadequate Iron Intake and Nutrition*

The normal iron requirement is estimated as 5–10 mg/day for men and 7–20 mg for women.[71] This corresponds to about 6 mg Fe per 1000-calorie intake. In the United States, the male diet usually contains the minimum iron requirement. This is often not true for women and children in the first year of life. So called "enriched" bread contains, in addition to the vitamin B complex added, 2.7 mg Fe per 100 g flour.[72]

In many countries, diets do not contain adequate amounts of iron, especially that required by women. As a result, iron deficiency anemia in women is common. This is true in the United States, Latin America, Scotland, Norway, China, India, and Africa.[72]

Even if the diet contains adequate amounts of iron, an iron deficiency due to malabsorption may be acquired because of disease or some congenital defect. After a total or partial gastrectomy, iron deficiency is the most common cause of anemia.[73] Since absorption of iron occurs mainly in the proximal upper gastrointestinal tract, surgical procedures which interrupt the continuity or lessen the time food is exposed to the duodenum may impair iron absorption. Diseases associated with chronic steatorrhea also result in defective iron absorption and iron deficiency anemia.[74]

Cow's milk does not have adequate iron to supply the iron requirements of the infant (1.5 mg/kg/24 hr). Cow's milk contains approximately 75 μg/100 g milk. *On the other hand, human breast milk does contain adequate amounts of iron.* In Uganda, the practice was to feed infants exclusively on cow's milk. This resulted in an iron deficiency anemia called *Bahima disease.*[75,76]

At birth, the hematocrit of new borns is in excess of 50%. This serves as an iron store for the first 30 days of life. This is markedly decreased if the umbilical cord is clamped prematurely. Dietary requirements of infants fall to 1 mg/kg/24 hr at 1 year of age and

0.5 mg/k/24 hr at 18 months.[77] From there on the requirement rises to 4 mg/day at 2 years of age to 10 mg/day at puberty. Adolescents require 10–20 mg/day.[78] Premature infants usually have markedly elevated hematocrits (55–60%). However, at the end of 30 days the stored iron is used up rapidly and more iron is required than in the normal infant by a factor of almost two. This process of Fe metabolism is disrupted in certain infants when excessive amounts of iron need to be stored because of unexplained internal bleeding. This is accompanied by enteropathy resulting in decreased Fe absorption and protein loss.[79] In many of these cases administration of iron clears up the condition.

During pregnancy, the mother loses 680 mg iron, which is the equivalent of 1300 ml blood. In addition, the blood volume is expanded in pregnancy and this requires an additional 450 mg iron. The iron requirement of the pregnant woman then goes up by about 2.5 mg/day. If this additional requirement is not supplied, an iron deficiency anemia will result.[80]

7.4.3 *Defective Iron Reutilization*

Defective iron reutilization seems to accompany many chronic disorders such as rheumatoid arthritis, subacute bacterial endocarditis, chronic renal disease, and many malignant diseases such as Hodgkin's disease.[81] Such a condition also occurs in the absence of other apparent disease and is referred to as *primary defective iron reutilization.*

A distinctive feature of this condition is the combination of *a low plasma iron*, a *decreased iron-binding capacity* (TIBC), and *increased RES iron stores.* Associated with these conditions are a normochromic or mildly hypochromic anemia. Erythrocyte protoporphyrin and coproporphyrin are increased, as they are in iron deficiency. Plasma copper levels range from 81 to 147 μg/100 ml (mean = 114 μg/100 ml), in the normal. In a group of these patients, the mean serum iron-binding capacity was only 191 μg/100 ml with a range of 118 to 267 μg/100 ml.[82]

Ferrokinetic studies have shown that the degree of iron red cell reutilization correlates with the resulting anemia.[83] Table 7.4 describes the results of ferrokinetic studies in patients with various types of hematological disorders. Measurements were obtained after reinfusing 24 ml of autologous blood labeled with 10 μg ^{59}Fe as ferrous citrate. Red cell survival data were derived by labeling the erythrocytes with ^{51}Cr.

TABLE 7.4

Ferrokinetic Studies in Patients with Various Types of Hematological Disorders Compared to Healthy Adults

Condition	Hematocrit (%)	Serum Fe (μg/100 ml)	Fe-binding capacity (μg/100 ml)	Plasma Fe disappearance T/2 (min)	Plasma Fe turnover (μg/kg/day)	Abdominal ^{59}Fe 24 hr post entry (%)	RBC ^{59}Fe incorporated day 7 (%)	RBC turnover (μg/kg/day)	^{51}Cr red cell T/2 survival (day)
Normal	42–48	60–160	270–370	50–120	300–450	30–40	70–85	220–320	25–31
Pernicious anemia	16	100	280	30	1400	36	5	70	22
Aplastic anemia	15	210	270	310	290	78	2	10	27
Myeloid metaplasia	20	70	300	28	1070	62	15	160	21
Iron lack	18	15	430	13	200	29	80	160	18
Sideroblastic anemia	24	220	230	100	950	40	17	160	26
Polycythemia vera	60	35	370	25	860	30	75	660	26
Hemolytic anemia	20	90	340	30	1510	30	60	900	10
Hemochromatosis	45	210	220	150	600	52	50	300	28
Chronic disease	29	25	227	39	320	39	75	240	23

Iron deficiency anemia shows the rapid uptake of ^{59}Fe by the "hungry" erythroid elements of the marrow. Hence the extremely short plasma iron disappearance time. Red cell survival of the hypochromic and microcytic cells is usually shorter than normal. *Polycythemia vera* also represents an iron-deficient state as far as the marrow is concerned. In this illness, red cell production is only limited by the iron supply.

Hemolytic anemias demonstrate extremely shortened red cell survival which also results in an apparent reduction in iron incorporation at day 7 of the study. Actually, newly formed erythrocytes containing ^{59}Fe are hemolyzed before day 7, artifactually lowering the result. Erythropoiesis may also be ineffective in some of the hemolytic anemias such as sickle cell disease.

Patients with *hemochromatosis* are typically not anemic and have normal marrow function. Their extremely high serum iron competes with the ^{59}Fe and some of the latter is taken up by the liver. Thus, we find a mildly prolonged iron disappearance time and reduced incorporation of the isotope into newly forming erythrocytes. The red cell survival is normal.

The anemia of chronic disease is characterized by surprisingly normal ferrokinetic studies in view of the persistent anemia. The anemia results primarily from a defect in iron utilization with reticuloendothelial cells failing to release metabolized iron to developing red cell precursors. Iron in the form of ferrous citrate, given intravenously, is thus utilized more efficiently than iron derived from breakdown of erythrocyte hemoglobin. A mild reduction in erythrocyte survival contributes to the anemia which is usually not symptomatic.

The data in Table 7.4 show that *pernicious anemia* is similar to *sideroblastic anemia* in that both disorders are characterized by ineffective erythropoiesis. Thus, these marrows contain many active erythroid precursors which avidly incorporate radioactive iron. However, many of these cells break down in the marrow without returning the radioactive label to the peripheral blood in the form of new, labeled, red cells. Hence, we see a normal or even short plasma iron disappearance time (^{59}Fe leaving the blood for the marrow after injection) and increased iron turnover, but poor return of the label to the peripheral blood in the form of [^{59}Fe]-labeled red cells (reduced iron incorporation at day 7).

In *aplastic anemia* typical findings include the greatly prolonged

plasma iron disappearance time since there are few erythroid precursors in the marrow to utilize this element. The reduction in red cell iron incorporation at day 7 is striking.

Myeloid metaplasia is another disease characterized by ineffective erythropoiesis. Here, there is usually fibrosis in the bone marrow and extramedullary marrow tissue in both the liver and greatly enlarged spleen. Hence, total body scanning demonstrates a large percentage of the ^{59}Fe concentrated in the abdominal segment (liver and spleen) and often a shortened red cell survival as the result of excessive red cell breakdown in the spleen (hypersplenism).

A *chronic disease, such as malignancy,* causes a deficiency of transferrin in the plasma because of decreased protein synthesis taking place. A decrease in plasma transferrin causes the iron to accumulate on apoferritin within the histiocytes or precipitate as hemosiderin. This explains the low serum iron, low iron-binding capacity and increased iron stores indicative of inadequate iron reutilization seen in certain chronic diseases, such as neoplasm, rheumatoid arthritis, folic acid deficiency, and chronic blood loss. In these conditions, orally administered iron is absorbed, but does not affect the severity of the anemia.

For patients with *primary defective iron reutilization* without any other apparent complicating disease, testosterone, I.M., has been found to be effective.[83] Testosterone seems to act by increasing stem cell proliferation and iron mobilization from the RES. Corticoids have been used in a similar manner.[85]

The reduction in serum transferrin may reflect a generalized decrease in protein synthesis or may result from increased binding of transferrin by the histiocyte. Bone marrow examination in such patients typically reveals normal appearing red cell precursors with increased iron deposition within histiocytes.

Therapy in such patients is directed at the underlying chronic disease process (i.e., chemotherapy for certain neoplasms, antibiotics in chronic infections, etc.). The anemia is seldom severe enough to require transfusion with hematocrit values above 27%.

In patients with no apparent underlying chronic illness, primary defective iron utilization (primary refractory anemia) may reflect a preleukemic state. Therapy in such patients may involve androgens[83] or corticosteroids.[85] Transfusions may or may not be required, depending upon the severity of the anemia.

7.4.4 *Congenital Atransferrinemia*

Since 1961, when it was first reported in Germany, several reports have appeared describing patients with a hereditary deficiency of transferrin.[86–90] In several of these patients transferrin was not detectable immunochemically. The others had serum transferrin levels of 3–39 mg/100 ml. In the patients without transferrin, there was apparently a structural gene deletion. In the others, the rate of transferrin synthesis was defective.

All of the patients described had a marked hypochromic anemia from early infancy and required repeated blood transfusions. Serum iron was low and iron-binding capacity was less than 20% of normal. The *half-life of transferrin* in normal individuals is from 6.7 to 8.4 days. In those patients who had some transferrin, the half-life was normal. Normally, the half time of clearance of administered radioactive iron is 80–120 min. In these patients it was less than one-third these values. The administration of purified human transferrin to some of these patients at 3 to 6 months intervals resulted in a mild increase in reticulocyte count followed by an increase in blood hemoglobin concentration. In some kindred of these patients (6 and 9 years of age) transferrin levels (immunochemically) were as low as 12–14 mg/100 ml without anemia. It is most likely that in these patients some other protein had taken over the transferrin function.

7.4.5 *Iron Overload*

Iron overload[91–93] may occur in a variety of chronic anemias, especially when blood transfusion is required frequently as in aplastic anemia and thalassemia major. Inasmuch as virtually all iron entering the body is retained, iron overloading may also result from increased oral iron intake and/or increased iron absorption as in *primary hemochromatosis*. Clinical symptoms of organ failure may result from iron overload regardless of how the iron is introduced. In the case of iron overload from decay of senescent red cells, the initial accumulation may occur in the reticuloendothelial cells, but after a short time, parenchymal cells of vital organs such as the heart and liver also become saturated with iron.

7.4.6 *Hemochromatosis*

Hemochromatosis[94,95] is a hereditary disorder of iron metabolism resulting in excess deposition of iron in the tissues, especially the liver and pancreas. In severe forms, there is bronze pigmentation of the skin associated with cirrhosis and diabetes mellitus. There are also bone and joint changes.[96,97]

Hemochromatosis has been extensively studied and the defect appears to be due to the fact that of the iron absorbed in the intestine, only a small percentage is retained in the mucosal ferritin. Consequently, the transfer rate of this iron is high.[96,97] This leads to the conclusion that the ferritin system in the mucosa, which limits iron absorption, is defective. The net result is a marked increase of iron bound to transferrin and other plasma proteins. These unload their iron in the parenchymal cells of the liver and other tissues. Plasma transferrin levels are low, as they are in all conditions with excessive iron storage. *Plasma transferrin concentration promptly returns to normal if the excess iron is removed from the patient by repeated venesection, indicating that the low levels are secondary to the disease process.*[98]

Reticuloendothelial cells obtain most of their iron by phagocytosing senescent erythrocytes. With dietary iron overload, the iron deposits are equally distributed between the RE cells and parenchyma of the liver, spleen, and marrow. Deposits in other tissues are minimal. In idiopathic hemochromatosis, RE deposits are minimal, the hemosiderin being preferentially located mainly in the parenchymal cells of liver, heart, and endocrine glands. This has suggested that the disease is at least partly a result of the inability of the RE cells to store iron. In support of this idea, after injection of heat-damaged [^{59}Fe]-labeled erythrocytes in the normal individual, about two-thirds of the ^{59}Fe ingested by the RE cells is returned to the plasma in two hr. With hemochromatosis, *all* of the ^{59}Fe is returned in this period.[96,97]

Idiopathic hemochromatosis is inherited but the mode of inheritance has not yet been established unequivocally.[98,99]

7.4.7 *Iron-Loading Anemias*

Anemias associated with iron overload[100–105] may result from both hereditary and acquired conditions associated with ineffective erythropoiesis and chronic hemolysis. Many of these patients also have a

repeated requirement for blood transfusion. Common among these patients are those with sideroblastic anemia, myeloid metaplasia, preleukemic states, hemoglobinopathies, red cell enzyme defects, and aplastic states. Severe clinical problems with iron overload typically begin after transfusion of approximately 100 units of blood. This provides an increase in total body iron stores of about 25 g. In practice, it is not rare to encounter patients who have received from 100 to 300 units of blood and have massive iron overload.

An interesting example of an iron overloading anemia is *transferrin-immune complex disease.* This disorder is a result of the production of an autoantibody to transferrin. This produces a circulating immune complex to which the serum iron is bound very tightly. In one such case the serum iron level rose to 800 μg/100 ml, which is about eight times the normal.[106] The patient showed the bronzed skin due to hemosiderin deposits, cirrhosis of the liver, and diabetes mellitus, as observed in hemachromatosis.

Porphyria cutanea tarda is another defect of genetic origin which results in iron excess with the symptoms of hemochromatosis.[107]

7.4.8 *Laboratory Findings with Aberrant Iron Metabolism*

The laboratory findings in *iron deficiency anemia* are marked by variable reduction in hemoglobin concentration and hematocrit value. When the hemoglobin concentration falls below 10 gm/100 ml, hypochromic erythrocytes are usually obvious upon examination of the blood film. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) are reduced in both adults and infants.[108,109] See Volume 2, pp. 6–10 for normal values.

The chief finding on the blood film, shown in Figure 7.5, is the low concentration of hemoglobin in the red cells. Very small microcytes and a moderate number of poikilocytes are also usually present. Serum iron levels are low, and iron-binding capacity (TIBC), is somewhat elevated.[108] Of greater value is the percentage transferrin saturation, calculated from the iron-binding capacity (TIBC) and serum iron.

$$\% \text{ transferrin saturation} = \frac{\text{serum Fe } (\mu g/100 \text{ ml}) \times 100}{\text{TIBC } (\mu g/100 \text{ ml})}$$

The normal value is 20–45%. In iron deficiency anemia, values of the order of < 16% will be observed.

Plasma transferrin concentration can be measured directly by radioimmuno-assay techniques or by radial immunodiffusion.[110,111] Commercial kits are available for this purpose (Abbott Laboratories, North Chicago, Ill.). The normal concentration of transferrin is 2.5 g/liter ± 20%. The concentration found in patients is lower than that calculated from the less specific transferrin saturation values. The procedure is useful for investigating the cause of an unexplained anemia. Table 7.5 illustrates the fact that high transferrin levels occur in iron deficiency anemia, whereas in anemias due to most other discase processes these levels are low. Transferrin levels are between normal and somewhat elevated in pregnant women or women on birth control pills.[110,111]

Of importance is the *measurement of body iron stores.* This is done qualitatively by staining a bone marrow film or liver biopsy (see Figure 7.6) with Prussian blue and counting the blue hemosiderin particles. The stains are graded as to normal, below normal as observed in iron deficiency, and above normal as noted with iron overload.[112]

Alternatively, sideroblasts in marrow aspirations may be counted after staining with Prussian blue. *A sideroblast is a nucleated red cell (normoblast) containing stained iron granules.* The percentage of sideroblasts, as compared to the total normoblast population, is then estimated. Normally 30–50% of the normoblasts are sideroblasts. In iron deficiency

TABLE 7.5

Transferrin Levels in Serum of Patients with Various Types of Anemia Compared to Serum Iron Values[110–111]

Cause of anemia	Number of patients	Serum Fe (μg/liter)		Transferrin (g/liter)	
		Range	Mean	Range	Mean
Fe deficiency	16	180–500	330	3.10–5.10	3.63
Chronic illness	8	150–620	420	1.11–1.90	1.38
Cirrhosis	15	370–1440	730	1.30–1.60	1.17
Neoplasms	20	100–870	390	1.20–1.85	1.28
Pernicious anemia	1	2410	—	1.85	—
Sickle cell	1	1240	—	1.40	—
Normal[139]	25	600–1600	900	1.87–3.12	2.60

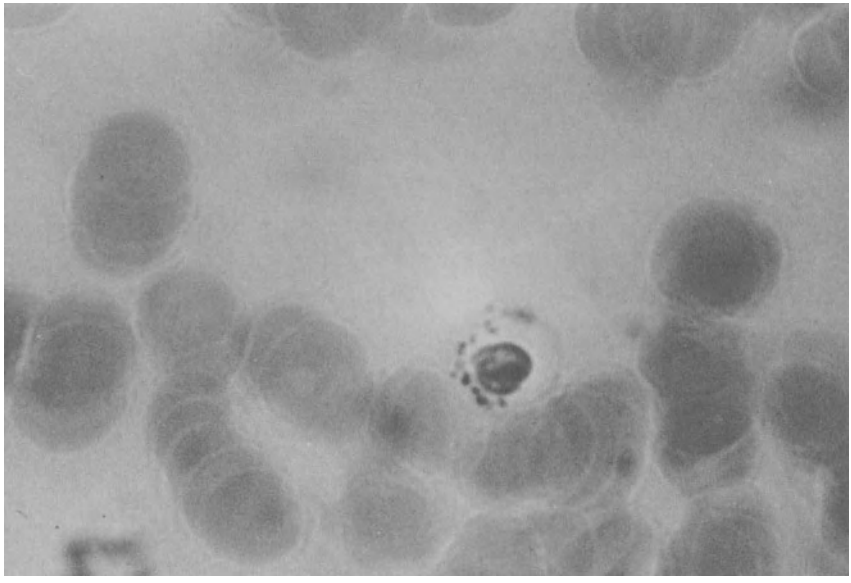


FIGURE 7.7 Iron deposits around the nucleus of a marrow cell in sideroblastic anemia. A ringed sideroblast.

a value of 2.5 ± 2% is often noted. In anemias secondary to other diseases, values of about 13 ± 6% will be found. Marrow sideroblast counts correlate well with the determination of transferrin saturation.

In sideroblastic anemia, iron deposits on the mitochondria throughout the cell. Excessive iron stores are often accompanied by the appearance of *ringed sideroblasts* where the stained granules form a ring around the nucleus of the cell[113] (see Figure 7.7).

A semiquantitative test for measuring iron stores is to administer a chelating agent, I.M., such as *deferoxamine* (*DF*) and measure the iron excretion in the urine. Excretion in the normal, after 500 mg of (DF), is about 0.7 mg (range 0.1 to 3.6 mg). In the iron-deficient patient, values are from 0 to 1.7 mg. In sideroblastic anemias, values from 8 to 16 mg were obtained.[114] This test is more useful in testing for iron overload rather than for an iron deficiency.

Most commonly, as a measure of iron stores, serum ferritin levels are measured by radioimmunoassay. The mean level, normally, is about 69 μg/liter in men and 35 μg/liter in women. In iron deficiency anemia levels are very low being less than 10 μg/liter. With iron overload ferritin levels are high, serum levels being over 1000 μg/liter.[115–119] With

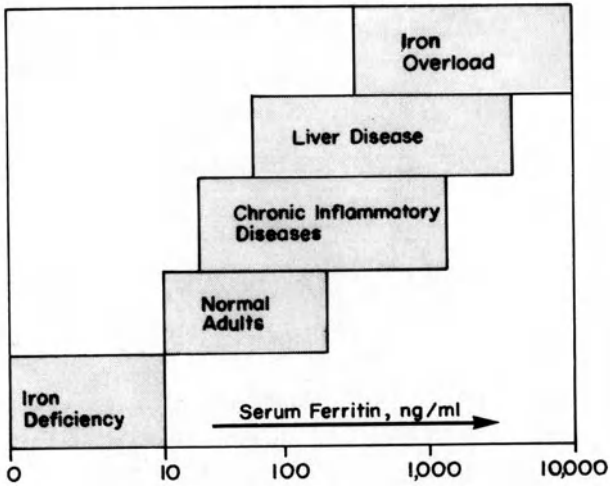


FIGURE 7.8 Serum ferritin levels found in health and disease.

certain tumors such as Hodgkin's disease serum ferritin levels are also markedly elevated[119a] (see Figure 7.8).

There is from 15 to 80 μg/100 ml of *protoporphyrin* in the normal erythrocyte. In iron deficiency anemia, *erythrocyte protoporphyrin* (FEP) is increased, by as much as five times.[120] In using this test, the absence of lead poisoning needs to be shown since FEP is markedly increased in lead poisoning also. It is also increased in any of the conditions resulting in defective heme synthesis.

Absorption of cobalt correlates closely to that of iron. Since radioactive cobalt was first readily available, the absorption and excretion in the urine was introduced as a measure of iron absorption. This is the *cobalt excretion test.* In the test 0.5 μCi of ^{57}Co, with 20 μmoles of nonradioactive cobalt, is administered orally. In the normal, 10 μmoles $\pm$ 5% is excreted during a 6-hr period. In iron deficiency values as high as 30% are excreted.[121] In certain conditions, where there is increased iron absorption, in the absence of an iron deficiency, such as idiopathic hemochromatosis, the cobalt excretion test also shows elevated values. With acute blood loss, hemolytic anemias, thalassemia, and sideroblastic anemia, elevated cobalt excretion is also noted. ^{55}Fe can be used for the same purpose. This test is not widely used except for research purposes.

For confirming iron deficiency or overload, the *chemical tests*

commonly employed are for serum iron, iron-binding capacity, ferritin, and transferrin.

7.5 *MANAGEMENT OF THE PATIENT WITH ABNORMAL IRON METABOLISM*

Treatment for an iron-deficiency anemia is best done by oral administration of ferrous sulfate. For adults, a dosage of 200 mg of iron are given in several daily doses. Ferrous gluconate and ferrous fumarate have been used also for this purpose. For children, a dosage of 1.5 to 2.0 mg of Fe^{2+} has been recommended.[122] As hemoglobin regeneration occurs, less iron will be absorbed, and absorption will drop to about 5% after 30 days. Therapy is continued for at least 6 months in order to replenish iron stores. Parenteral iron therapy is resorted to when the patient is uncooperative, is unable to tolerate oral iron compounds, cannot absorb iron from the gastrointestinal tract, or is losing blood at a rate too rapid to be corrected by oral intake, such as in hereditary hemorrhagic telangiectasia (proliferation of capillaries which tend to bleed under the skin and elsewhere). In these cases, iron-dextran complex is used.[84] This can be given intramuscularly or intravenously. In using this material, it is recommended that a small dose be first administered to see if the patient has any hypersensitivity to the drug.[123]

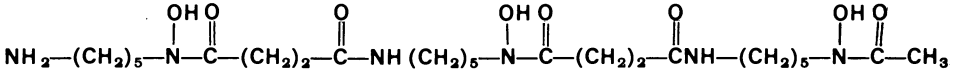
For patients with an iron overload there are two major procedures used for removing excessive iron. One is by bleeding and the other is by the use of chelate compounds.

When 500 ml of blood is removed from an individual, about 200–250 mg of iron are removed. An equal amount of iron is mobilized from iron stores. Advantage is taken of this fact to remove excessive iron stores resulting from iron overload. For idiopathic hemochromatosis, with a storage pool of 20–40 g, it is apparent that treatment needs to extend for more than 1 year. This is so because if one 500 ml unit is removed weekly, then it takes 4 weeks to remove 1 g or 1 year to remove 13 g. Under this regimen, erythrocyte production increases three to six times normal, and the hematocrit remains within 10% of its normal level, thus not interfering in the oxygen transport system.

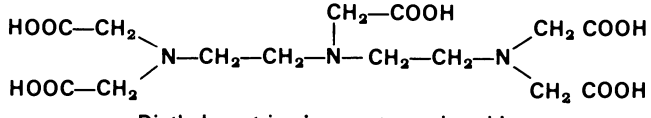
If an anemia develops before adequate amounts of stored iron have been removed, the phlebotomies can be interrupted until the anemia is

corrected before proceeding again with the treatment.[124] This technique has also been applied to the alcoholic cirrhotic patients with iron overload and in the iron loading anemias (98).

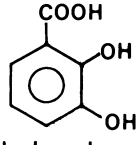
Where marrow erythropoietic reserves are limited, as in patients requiring blood transfusion, phlebotomy is impracticable. In these cases, a substance which chelates iron is administered[125] (see Volume 1, p. 142). A commonly used chelating agent derived from actinomyces, *Streptomyces pilosus*, is *deferoxamine* (*desferrioxamine B, DF*). The action of this drug is almost entirely localized to the hepatic parenchymal ferritin pool.[126,127] Also useful is the Ca salt of *diethylene triamine pentaacetic acid* (*pentetic acid, DTPA*). Although DF is metabolized in the body, DTPA is excreted unchanged into the urine within 24 hr. In contrast to DF, DTPA chelates extracellular iron only. The amount excreted in the urine is about the same as with DF because of the fact that iron stores in the cells are in dynamic equilibrium with extracellular iron.[128]



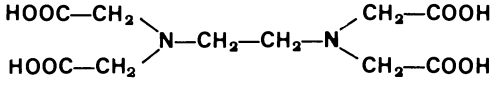
Deferoxamine
(Desferrioxamine B, DF)



Diethylene-triamine-pentaacetic acid
(DTPA)



2,3-Dihydroxy benzoic acid
(2,3 DHB)



Ethylene-diamine-tetraacetic acid
(EDTA)

FIGURE 7.9 Structure of some compounds used to chelate iron so as to remove it in iron overload.

Both DF and DTPA are ineffective when given orally. DTPA is usually given intravenously and DF, intramuscularly or subcutaneously although it can be given intravenously. Orally, 2,3-dihydroxybenzoic acid (pyrocatechuic acid; 2,3-DHB) causes increased excretion of iron in the stool. However, at the 100 mg/kg dosage level only 6.5 mg of iron per day are excreted.[129]

Chelating agents have been effectively used to remove excessive deposits of iron in patients with thalassemia major. In adults, 0.15–1 g of DF is given I.M. daily, often with the addition of 1–3 g DTPA added to any blood which is used to transfuse the patient, to correct an anemia. Usually, 500 mg of ascorbic acid is given at the same time to keep the iron in the ferrous state. With 20 mg/kg of DF daily, in combination with 500 mg ascorbic acid, combined urinary and fecal iron excretion is about 31 mg. With maximal doses of DF given by slow intravenous infusion, in combination with ascorbic acid, iron excretion in thalassemic patients with iron overload can reach 200 mg Fe/day.[130]

Figure 7.9 illustrates the structure of some of the commonly used chelate compounds employed to remove excessive deposits of iron, with iron overload. Note the resemblance of the DTPA structure to that of EDTA. Calcium EDTA has also been used to chelate iron but has more side effects and is less effective and specific for iron than calcium DTPA.

7.6 *ASSAY FOR TRANSFERRIN AND IRON*

Transferrin is best assayed by immunochemical techniques, especially by radioimmunodiffusion[131] or electroimmunodiffusion.[132] Values obtained by these procedures are generally lower by about 20% than those found by saturating the serum with iron and measuring the total iron adsorbed. This is so because proteins other than transferrin and other metabolites also adsorb iron.[133]

Low serum transferrin levels are found in many diseases which directly or indirectly result in decreased protein synthesis by the liver. These diseases include liver cirrhosis, malabsorption, kidney disease, and chronic infection.[134] Elevated serum transferrin levels are often found in iron deficiency anemias. In the last trimester of pregnancy, elevated transferrin levels have been reported.

Using immunochemical techniques, serum transferrin levels range from 2.6 to 3.1 g/liter with a mean value of 2.75 g/liter.

Total serum iron is readily determined by precipitation of proteins with trichloroacetic acid and adding KCNS to the supernatant to produce a red color for Fe^{3+}, with an absorption maximum at 480 nm. The color development is independent of pH.[135] The phenanthrolines and related compounds, such as bipyridyl, also yield pink to red colors, which are more sensitive but require reduction to the ferrous state. The amount of color developed is pH dependent.[136] Assay for the iron with the phenanthrolines does not require protein precipitation and for this reason finds application in determining iron-binding capacity.[137]

For total iron in serum, atomic absorption spectroscopy is readily applied.[138] With the X-ray spectrometer, the iron can be determined in blood or serum without destroying the specimen.[139]

Serum contains 0.9–1.6 mg Fe/liter. Whole blood of males contains 0.5 g Fe/liter. Whole blood of females, contains less (0.45 g Fe/liter) since the hematocrit of females is lower. The total body iron content is approximately 65 mg/kg of which 40 mg/kg is located in the circulating blood.

Serum ferritin levels are determined by immunochemical techniques exclusively.[140] Several companies supply kits for its determination. The mean ferritin level for males is 69 μg/liter and for females 35 μg/liter. Values differing $\pm 15\%$ from these values are considered in the normal range.[141,142]

7.7 *RECAPITULATION*

Transferrin comprises a single long-chain polypeptide of 670 amino acid residues and two oligosaccharide chains totaling 5000 in molecular weight. The weight of the whole molecule is thus approximately 81,000. There are about 2.5 g/liter of transferrin in human serum. Each transferrin molecule binds two ferric ions. Normally, transferrin is saturated to the extent of 30% in human serum.

Variants of transferrin with higher and lower mobility, on electrophoresis, are observed. However, with few exceptions the gene frequency is very low. Normal transferrin is referred to as transferrin C or TfC. The slower variants are referred to as D transferrins and the faster variants as B. There seems to be no biologically significant difference in the iron-binding properties of the different variants. Absence of

transferrin, atransferrinemia, has also been reported in a few sets of kindred.

Transferrin serves to carry ferric iron from body stores to various tissues where it can be incorporated into hemoglobin, myoglobin, and the cytochromes. In carrying out this function, transferrin enters the iron-bearing cells, probably by pinocytosis, and adsorbs ferrous ions. The ferrous ions are formed from ferric deposits in ferritin and as hemosiderin by reduction by an NADH–FAD reductase system or by ascorbic acid. Transferrin leaves the cell, and in contact with oxygen may act as an oxidase to oxidize the iron to the ferric state. Alternatively, ceruloplasmin of the plasma can act as an oxidase to oxidize the iron to the ferric state.

In the ferric state the transferrin iron complex is pinkish. It distributes the iron to sites where it is needed, such as to the bone marrow normoblasts for hemoglobin production or to the parenchymal cells of the liver for storage. The iron is precipitated in the ferric state within molecules called ferritin. In addition, some is precipitated in a more insoluble form as hemosiderin. Senescent erythrocytes are processed by the reticuloendothelial cells in the liver and spleen so as to recover the iron and deposit it as ferritin. Excess is picked up by transferrin and transported to the parenchymal cells of the liver for storage.

Ferritin is made up of 24 subunits. It is structured in such a manner as to store huge quantitites of iron. Stimulation by the presence of excess iron results in an increase in the amount of ferritin synthesized and a selective increase in the formation of certain subunit types. Oxidation by an NADH–FAD oxidoreductase system permits the oxidation of Fe^{2+} to Fe^{3+}, to be stored as small crystals in the hollow of the ferritin molecule.

RE cells acquire their iron mainly from senescent erythrocytes. Their ferritin storage capacity is relatively small and the turnover rate of iron stores in these cells is therefore rapid. Parenchymal cells acquire their iron mainly from plasma transferrin, circulating heme–hemopexin and hemoglobin–haptoglobin.

Iron release from ferritin is controlled by the extent of iron bound to transferrin and thus the body's need. Ascorbic acid facilitates release of iron from RE cells reducing the Fe^{3+} to the more soluble Fe^{2+} form.

Parenchymal liver cells have a ferrireductase system which is effective in the reduction of iron. The RE cells rely mainly on ascorbic acid for Fe^{3+} reduction.

Iron deficiency is the most common cause of anemia. This results from intake inadequate to match the nutritional requirement. Chronic excessive bleeding into the gastrointenstinal tract from any of numerous causes may result in an iron-deficiency anemia. Excessive bleeding at menstruation is a common cause in women. Iron deficiency is not uncommon in infants on cow's milk, which will not supply the infant's iron requirement. *Breast milk contains adequate amouts of iron for this purpose.* Atransferrinemia, a rare condition, also is associated with an iron-deficiency anemia.

Iron deficiency is usually treated with oral administration of ferrous salts. Where gastrointestinal problems exist, this can be given parenterally.

Disease processes which result in excessive erythrocyte destruction will result in iron deficiency anemias unless intake of iron is increased to match the losses. At the same time, these conditions will overload the RE system resulting in iron deposits in the RE cells and also the parenchymal cells of various tissues. These are referred to as the iron overload anemias. These include the aplastic, congenital (e.g., thalassemia, sickle cell), cirrhosis of the liver, and other conditions such as idiopathic hemochromatosis.

Severe iron overload is often treated by phlebotomy. Reversal of myocardiopathy, improvement in hepatic function, and increased life expectancy have been demonstrated with this procedure. In the less severe cases the use of chelating agents to solubilize and excrete the iron deposits is widely applied successfully.

For diagnostic purposes the chemical procedures used most frequently for the iron deficiency anemias and iron overload are the serum iron and iron-binding capacity levels. These are also a rough estimate of the transferrin level. The transferrin concentration is also readily measured by immunochemical techniques. Serum ferritin levels correlate well with the extent of iron stores and are assayed for this purpose.

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8

Ceruloplasmin: Copper Metabolism

8.1 *CERULOPLASMIN*

Ceruloplasmin (*ferroxidase I*) first described in 1948, is the blue copper-containing oxidase present in the α_2-globulin fraction of human serum (see Figure 3.5, Table 3.1). Close to 95% of the serum copper is located in this fraction.[1,2] The molecular weight of the major form of ceruloplasmin, referred to as ceruloplasmin I, is 134,000 ± 3000.[3] Consistent with its molecular weight, its sedimentation coefficient is 7.25*s* and diffusion coefficient 4.46×10^{-7} cm^2/sec. There are about 1065 amino acid residues in the molecule. The amino acid composition has been determined (Table 8.1).[3,4] Substantial progress has also been made in sequencing the molecule.[5,6]

Ceruloplasmin is a glycoprotein and the oligosaccharides attached to the polypeptide chains contain glucosamine, mannose, galactose, fucose, and sialic acid (see Table 8.2). There are apparently three oligosaccharide units attached to ceruloplasmin with 14–22 carbohydrate residues in each oligosaccharide.[7,8] When fractioned on a Sephadex column, the ceruloplasm fraction with the highest carbohydrate concentration is referred to as *ferroxidase I*. This comprises by far most of the ceruloplasmin in the serum.

Attached tightly to the ceruloplasmin molecule are six atoms of copper per mole of protein. In addition, variable amounts of copper (0.5–1 atom per molecule) can be adsorbed readily and are held loosely. This can be readily removed by passing a solution of ceruloplasmin over a chelating resin (Chelex). Thus six copper atoms are actually held as part of the ceruloplasmin molecule.[3]

TABLE 8.1

Amino Acid Composition of Human Ceruloplasmin

Amino acid	Number of residues	Amino acid	Number of residues
Aspartic acid	127.1	Isoleucine	53.2
Threonine	76.7	Leucine	70.4
Serine	62.0	Tyrosine	63.5
Glutamic acid	119.5	Phenylalanine	49.0
Proline	46.3	Histidine	39.4
Glycine	76.6	Lysine	65.7
Alanine	49.4	Ammonia	113.1
Half-cystine	15.0	Tryptophan	21.9
Methionine	26.5	Arginine	41.1
		Total	1065.2

Ceruloplasmin is synthesized by the liver and released into the plasma *with the copper attached.* Cupric ion added to plasma is not picked up by ceruloplasmin, but adsorbs to albumin. *Thus albumin binds any cupric ion which finds its way into the plasma.*[9] By first reducing the Cu^{2+} in ceruloplasmin to the Cu^{+} state, the copper can be stripped from ceruloplasmin to form apoceruloplasmin.[10,11] When Cu^{2+} is complexed with apoceruloplasmin, an absorption maximum at 610 nm is noted. This is responsible for its blue color. In addition, a maximum appears at 332 nm also due to copper since it is not observed with apoceruloplasmin.[11–13] Thus not all of the copper in ceruloplasmin is equivalent, nor related to the blue color.

TABLE 8.2

Carbohydrate Composition of Human Ceruloplasmin

Carbohydrate	Number of moles/mole protein
Glucosamine	19.2
Mannose	14.2
Galactose	12.3
Fucose	1.6
Sialic acid	8.6
Total	55.9

8.2 *CERULOPLASMIN COPPER*

Ceruloplasmin copper is classified into three groups. Type 1 is blue and detectable by electron paramagnetic resonance (epr). Type 2 does not give the blue color and is also detectable by epr.* Type 3 is not detectable by epr. *All the copper is apparently in the cupric form.* There are six copper atoms per molecule. Only five of these can be accounted for as to type. For this reason a fourth type of copper has been postulated.[3,14] To this should be added the fifth type (readily removable by passing a solution of ceruloplasmin over a chelating resin). The different types of copper are shown in Table 8.3. Note that there are two types of copper designated as Type 1. In one case, after reduction to the monovalent state, the copper is rapidly reoxidized. The second is more stable in the monovalent state.

Assuming that there are 6.5 copper atoms (at. wt. 63.6) per mole of ceruloplasmin, and a molecular weight of 134,000 for the protein, then there should be 0.309% copper per ceruloplasmin molecule. Values ranging from 0.275 to 0.340% have been reported by various experimenters.[3,15]

Several investigators distribute the six copper ions in ceruloplasmin with two in Type 1, as shown in Table 8.3, two in Type 2 and the other two as a pair of binuclear copper ions called *a cluster*, Type 3.[16] The absorption spectrum of ceruloplasmin contains four bands; three are due to Type 1 copper. These are at 610 nm, $\epsilon = 11{,}300\ \text{M}^{-1}\ \text{cm}^{-1}$; 459 nm, $\epsilon = 1{,}200\ \text{M}^{-1}\ \text{cm}^{-1}$; and 794 nm, $\epsilon = 2{,}200\ \text{M}^{-1}\ \text{cm}^{-1}$. The one due to Type 3 copper is at 332 nm, $\epsilon = 4{,}100\ \text{M}^{-1}\ \text{cm}^{-1}$.

* *Electron paramagnetic resonance* (*epr*), also called electron spin resonance (esr), detects an unpaired electron. A spinning electric charge (electron) generates a magnetic field. The electron, being a magnet can then take two alternative positions in a magnetic field. If the magnetic field is designated as H and the energy as E, then the difference in energy between the two orientations is given as $\Delta E = (h\gamma/2\pi)H$, where γ is the *gyromagnetic ratio* of the electron. This is analogous to nuclear magnetic resonance (nmr) where an unpaired proton (+charge) is being examined. However, γ for the free electron is 1000 times that for a proton, bringing the resonance frequency to the microwave region. The sample is placed in the resonant cavity of a microwave generator and the first derivative of the absorption curve is plotted against the change in the magnetic field. Since the unpaired electron imparts magnetic properties to the compound, the compound is *paramagnetic*. If electrons are paired, then magnetic fields cancel out and the electrons cannot be detected by epr. The compound is then *diamagnetic*.

TABLE 8.3

Nature of the Copper Bound to Ceruloplasmin[a]

Type	Number of copper atoms	Additional designation	EPR detectability	Comment
1	1	Fast-type Cu^{2+}	Detectable	Blue, reoxidized rapidly
1	1	Slow-type Cu^{2+}	Detectable	Blue, reoxidized slowly
2	1	Permanent type Cu^{2+}	Detectable	Not blue, binds anions
3	2	Type 3 Cu^{2+} (paired)	Not detectable	Spin-coupled pair of cupric ions, not blue
2	0–1	Cu^{2+} removable by resin chelate	Detectable	Not essential part of enzyme, variable in content
4[b]	1	Type 4 Cu^{2+} (possibly cuprous)	Not detectable	Necessary to account for six copper atoms per molecule

[a] From Ref. 3.

[b] Some classify this as a Type 2 copper.

Type 1 cupric ions react with nitrous oxide by charge transfer (see Figure 8.2), Type 2 does not. When reduced to the monovalent state, Type 3 cupric ion clusters react with nitrous oxide giving epr signals at $g = 2$ and $g = 4$. This is direct evidence of cupric–cupric interaction, the distance between the two copper atoms calculating to somewhat less than 6 Å.[16]

Copper forms chelates with mercaptoethanols (—SH compounds). For example, sodium diethyl dithiocarbonate, penicillamine, and other —SH compounds are used in forming chelates with copper for extraction and assay.[17]

Apoceruloplasmin contains four —SH groups which are occupied when ceruloplasmin is reconstituted by adding copper. This is best done by adding cuprous ion and oxidizing to the cupric state after it has been complexed with apoceruloplasmin.[11] In addition, it appears that histidine residues are also involved in the formation of the tightly bound copper chelates.[12] Figure 8.1 illustrates the formation of copper chelates with —SH compounds containing an amine group. Figure 8.2 compares a proposed structure of a copper-binding site in ceruloplasmin to produce the blue color, with that in blue copper sulfate.

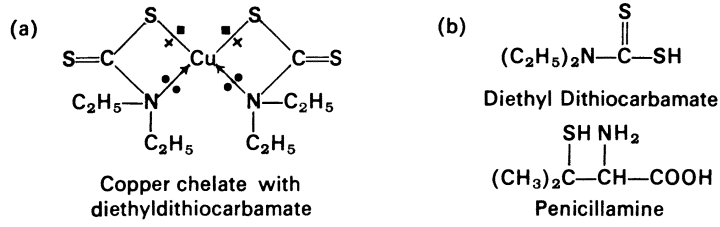


FIGURE 8.1 (a) Proposed structure of a chelate compound formed between molecules of diethyl dithiocarbamate and bivalent copper. The arrows represent semipolar bonds; both pairs of electrons being derived from the nitrogens. (b) Comparison of the structure of two compounds which chelate copper, diethyldithiocarbamate, and penicillamine.

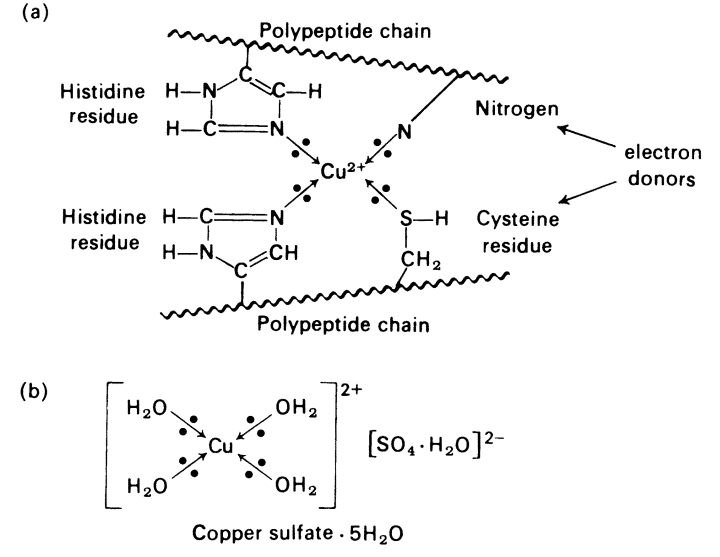


FIGURE 8.2 (a) The linkage of the Type 1 copper in ceruloplasmin to obtain the blue color. Two histidine and one cysteine residue supply two nitrogen and one sulfur atoms, each of which serve to donate a pair of electrons to form a semipolar bond with the copper. An additional two electrons, from some unidentified nitrogen compound, serve to complete the octet. This is a coordination compound and is blue. The copper ion carries two positive charges. (b) Cu $SO_4 \cdot 5H_2O$, as a typical coordination compound. Compare with the structure of the blue copper in ceruloplasmin above. One water is bound to the sulfate ion.

The blue color in ceruloplasmin is due to the formation of a *coordination compound* with copper.* A cysteine sulfur, and nitrogens from two histidine residues, are involved. To complete the structure, electrons from another nitrogen source are supplied in the structure proposed in Figure 8.2a.

It will be noted in Figure 8.2a, that two electrons from each of two histidine nitrogens and a sulfur from a cysteine residue each donate a pair of electrons. In addition, another nitrogen from some other residue donates a pair of electrons. This forms a stable octet. The electrons are relatively loosely bound and are responsible for the blue color.

In Figure 8.2b the structure of $CuSO_4 \cdot 5H_2O$ is compared to that of the blue copper in ceruloplasmin. Here also a stable octet is formed from pairs of electrons, in this case donated by the oxygen atoms of water. NH_3, CN^-, NO, and other groups can be substituted for the water to form numerous *coordination compounds.*

These types of linkages are of great importance in biological systems. *It is by this type of bond that enzymes and substrates are bound to metal ions. If the metal ion is a transition element, which readily changes its valence reversibly, such as copper, then it serves to guide the flow of electrons along the metabolic pathway.*

For similar reasons, the transition elements serve as catalysts in the heavy chemical industry. *Thus, both in living and nonliving systems the transition elements serve to activate the processes transpiring.*

One of the characteristics of the coordination compounds is that the ligands are held loosely so that the binding electrons absorb light readily in the visible region. They thus appear colored. Examples are

* Vanadium, chromium, manganese, iron, cobalt, nickel, copper, and zinc are *transition elements*, where low-energy 3*d* orbitals are being completed, while the outer two 4*s* electrons remain the same. Electron shift is possible from the 3*d* orbital to the 4*s* orbital (oxidation), or back to the 3*d* orbital (reduction). Another property of these elements is that their ions can form stable complexes with numerous molecules or radicals, which can donate a pair of electrons for the formation of a *coordinate bond.* These attached groups are referred to as *ligands.* H_2O, NH_3, CN^-, NO_2^-, SCN^-, —SH, >S=O and others, are examples of ligands. Such compounds are called *coordination compounds*, and their structures have been studied extensively. Common examples used in the laboratory are potassium ferrocyanide, $K_4Fe(CN)_6$, and sodium nitroprusside, $Na_2Fe(CN)_5NO \cdot 2H_2O$. In Figure 8.2, the compound $CuSO_4 \cdot 5H_2O$ is illustrated as an example, with H_2O as the ligand donating two electrons to form the coordination compound. NH_3 can substitute for the H_2O in this compound.

red hemoglobin, blue hemocyanin, hydrated or ammoniacal solutions of copper, and gems, such as rubies, emeralds, aquamarines, and numerous others.

The structure in Figure 8.1a is somewhat different. Here the copper has displaced two hydrogens to form two ionic bonds. Two of the five electrons of the nitrogens are available to move toward the copper ion. A stable system of eight electrons around the copper now forms. In contrast to the coordination compounds in Figure 8.2, these electrons are more tightly bound. This results in a nonpolar compound more soluble in organic solvents than water. The chelate of copper with diethyldithiocarbamate will form a brown precipitate and is extracted readily into organic solvents for assay. Compare this structure with that for the Ca-EDTA complex in Volume 1, p. 142.

8.3 *PREPARATION OF CERULOPLASMIN*

Ceruloplasmin has been prepared in large amounts from the Cohn plasma protein fraction IV–1. The procedure comprises fractionation of fraction IV–1 on DEAE-cellulose followed by precipitation with 25% alcohol at $-10°C$, followed by salt precipitation at pH 7.0.[18] Improvements use additional purification on Sephadex gel.[19] The purified product contains approximately 25 μg Cu/mg nitrogen. This calculates to about 95% of purity.

The motivation for the preparation of large quantities of ceruloplasmin was for possible use in the treatment of Wilson's disease (see below) so as to remove excess copper from the liver. For this the apoprotein is needed. The copper was then removed with *diethyldithiocarbamate*, a strong chelating agent for copper. The apoprotein then contained only about 3% of its original copper content.[10] This was not of value in Wilson's disease since it would not remove excess copper deposits, because apoceruloplasmin will not complex Cu^{2+} readily. The Cu^{2+} needs to be reduced first as indicated in Section 8.1.

8.4 *NATURE OF THE CERULOPLASMIN CHAIN*

Ceruloplasmin has been dissociated into its structural units by reductive cleavage with mercaptoethanol. On dissociation, three smaller

molecules were obtained as indicated by sodium dodecyl sulfate (SDS) electrophoresis. *p*-Hydroxymercuribenzoate and 6 M guanidine could be used, indicating a rupture of —S—S— linkages and hydrogen bonding.[20–22]

These units have been designated H, L, and H′, the H standing for *heavy* and the L standing for *light*. The respective molecular weights were, H = 69,000, H′ = 53,000, and L = 16,000. This has been interpreted as indicating a tetramer made up of a heavy and light chain. The intermediate chain (H′) would be the heavy chain (mol. wt. 53,000). The light chain (L) has a mol. wt. of 16,000. The H chain would represent a heavy and light chain combined (69,000).

By analogy with hemoglobin (Volume 2, p. 385), the chain structure would be $\alpha_2\beta_2$, where α is the heavy chain (53,000) and β the light chain (16,000). Dissociation could form the α, β, or $\alpha\beta$ chains as in hemoglobins. The total molecular weight would be 138,000, which is close to the accepted molecular weight of about 134,000.

8.5 *GENETIC VARIANTS*

The common form of ceruloplasmin is called CpB. This designation was chosen when forms of ceruloplasmin were discovered which traveled more rapidly (A forms) and more slowly (C forms) to the anode, with starch gel electrophoresis at pH 9.0.[23] Most of the variants have been found in American Blacks, although one variant CpThai has been found in a Thai. Other variants were found in blacks in Galveston (CpGal), Bridgeport (CpBpt), and in New Haven (CpNH).[24–26] Ceruloplasmins A, B, and NH are not uncommon among Negroes. Others have gene frequencies of less than 0.03%. The allele frequencies for these four types are summarized in Table 8.4.

In locating the ceruloplasmins on starch, agar, or acrylamide gel electrophoretic patterns, use is made of the fact that ceruloplasmin is an efficient oxidase for phenols and aniline derivatives. p-Phenylene diamine will oxidize to a dark brown in the air when it contacts ceruloplasmin and this and related compounds have served to locate the transferrin on the electrophoretic pattern. Figure 8.3 is a schematic representation of the location of the various ceruloplasmin variants on the electrophoretogram.[23–30]

TABLE 8.4

Ceruloplasmin Allele Gene Frequencies

Ethnic group	Studied	Cp^A	Cp^B	Cp^C	Cp^{NH}
Negroes					
America	1700	0.052	0.942	0.003	0.006
Haiti	323	0.12	0.874	0.002	0.012
Nigeria	520	0.15	0.837	0.003	0.115
West Africa	236	0.07	0.924	0.006	—
Angola	909	0.05	0.935	0.004	0.009
Mozambique	580	0.04	0.955	0.004	—
Bantu	302	0.04	0.960	—	0.005
India					
Hindu	212	0.016	0.982	0.002	—
Muslin	18	0.028	0.972	—	—
Mahato	213	0.014	0.986	—	—
Bombay	287	—	1.000	—	—
Bengal	978	0.012	0.986	0.002	—
Western Europe and U.S. (Caucasians)					
United States	334	0.006	0.994	—	—
Germany	224	0.013	0.985	0.002	—
Ireland	240	0.010	0.998	0.002	—
Iceland	106	0.004	0.996	—	—
Greece	210	0.036	0.960	0.004	—
Crete	155	0.068	0.932	—	—
Far East					
Korea	115	0.009	0.978	0.013	—
Australian aborigines	520	0.002	0.998	—	—
New Guinea	560	—	1.00	—	—
Near East					
Iran	198	0.003	0.990	0.007	—
Pakistan	96	0.005	0.995	—	—

Several cases of *aceruloplasminemia* were suspected. On careful examination, a very narrow band, moving more rapidly than ceruloplasmin A was detected. Thus, these patients have a very low concentration of ceruloplasmin oxidase activity. CpX is one such example.

The various ceruloplasmins cannot be distinguished immunochemically by the Ouchterlony technique and from analogy with hemoglobin probably result from a point mutation with, most likely, a single amino acid substitution.

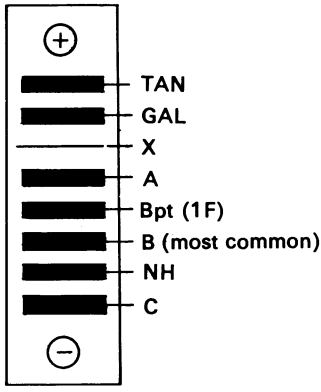


FIGURE 8.3 Relative mobilities of the various ceruloplasmin variants at pH 9.0. The 1F and Bridgeport variants have about the same mobility. The X variant is present in very low concentration or has low oxidase activity and is represented as a line.

Oxidase activity in the different forms does vary, especially with respect to the response to oxidase inhibitors such as cyanide or azide. For example, CpA is more susceptible to inhibition whereas CpBpt resists inhibition to these substances. This may be related to skin color since mainland Greeks show only half the frequency of CpA that the darker Cretans show. Also possibly related to this is the fact that the gene frequency of CpA in American Blacks is less than half that of the Haitians and only one-third that of Nigerians.[31]

8.6 *CERULOPLASMIN FUNCTION AND THE TISSUE OXIDASES*

Ceruloplasmin serves as the major source of copper for the tissue copper bearing oxidases. As a result, copper or ceruloplasmin deficiency will have a significant effect on hemopoiesis, cerebral function, the integrity of the cartilage, hair and connective tissue, oxidative metabolism, skin pigmentation, adrenaline formation, and protection of the cells against the accumulation of superoxide and peroxides. For these reasons, and in order to lay the groundwork for the discussion of the various effects of copper deficiency and overloading, it is necessary to present succinctly the nature of some of the copper-bearing enzymes involved.

Ceruloplasmin is the major oxidase in the serum. As such it is responsible for oxidizing the ferrous to ferric iron which is transported by transferrin

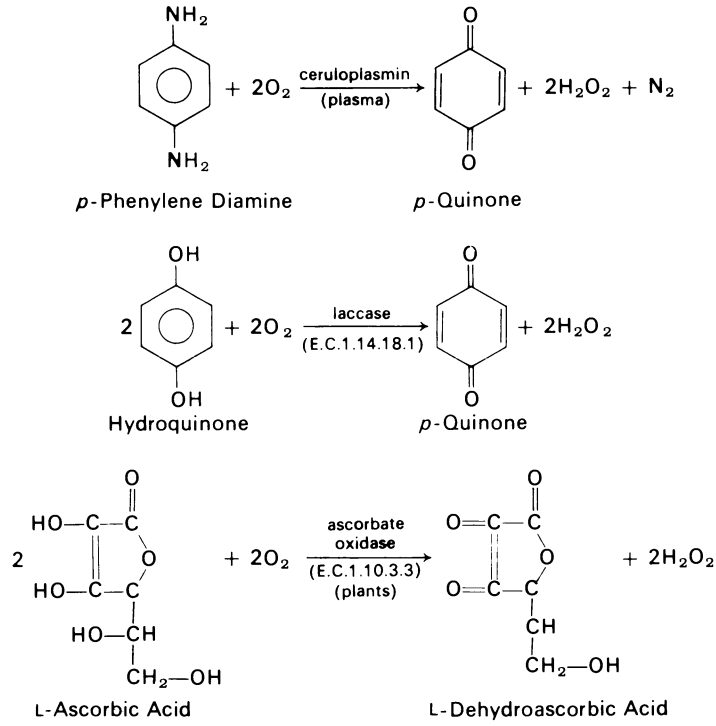


FIGURE 8.4 Comparison of the action of ceruloplasmin in the oxidation of aromatic compounds to form quinones with two plant enzymes. For ceruloplasmin or laccase (a tyrosinase) either *p*-phenylenediamine or hydroquinone may serve as substrates.

(see Section 7.2). In this function, it is several orders of magnitude more effective than the autooxidation of Fe^{2+} by transferrin, in the presence of oxygen and the absence of ceruloplasmin.[32] *Thus ceruloplasmin is an important participant in the process of hemopoiesis.*

In contrast to apotransferrin, which is an antioxidant, apoceruloplasmin has no antioxidant activity. The reason for this is that apotransferrin will adsorb circulating ferric or ferrous iron. On the other hand, apoceruloplasmin will not adsorb free Cu^{2+}. *Ceruloplasmin will however inhibit the autooxidation of unsaturated fatty acids,* such as linolenic acid, and in this regard has a significant function in maintaining the stability of the highly unsaturated fatty acids in serum.[33]

Ceruloplasmin is a member of a group of enzymes referred to as copper-bearing oxidases. Copper-containing oxidases catalyze the oxidation of

aromatic compounds such as *o*-dihydroxyphenol (catechol), *p*-phenylenediamine, or *p*-hydroxyaniline to quinones, liberating hydrogen peroxide in the process (Figure 8.4).

Some of the copper-containing oxidases require pyridoxal phosphate for their activity. These are members of a group of amine oxidases (also called diamine oxidase) given the number E.C. 1.4.3.4. The amine is oxidized to an aldehyde, generating hydrogen peroxide:

$$2H_2O + O_2 + RCH_2NH_2CH_2NH_2 \xrightarrow[\text{(E.C. 1.4.3.6)}]{\text{pyridoxal-containing amine oxidase}} RC(H)=O + H_2O_2 + NH_3$$

Specific copper-bearing oxidases of this type are found in all tissues and in plasma. For example, histaminase (diamine oxidase) protects against the effects of histamine and destroys the toxic amines cadaverine and putrescine generated by bacteria.[34,35]

Amine oxidases are also of significance in maintaining the structure of cartilage. Copper deficiency results in the destruction of cartilage, blood vessels, and connective tissue. Among those copper-bearing amine oxidases identified and located in human or animal tissue and plasma, are benzylamine oxidase, spermine oxidase, and lysyl oxidase all being found in connective tissue.[36,37]

Lysyl oxidase of connective tissue is usually prepared by extraction from the aorta. The enzyme serves to oxidize lysyl residues, suspended from a polypeptide, to an aldehyde. Three aldehyde residues then condense with a lysine residue to form a pyridine ring referred to as a *desmosine residue.*[38] The reactions are formulated in Figures 8.5a and 8.5b.

It is this cross-linking which serves to stabilize and strengthen the connective tissue. Since copper is supplied to lysyl oxidase by ceruloplasmin, *ceruloplasmin is also partly responsible for the maintenance of the integrity of connective tissue.*

Tyrosinase (*monophenol monoxygenase, E.C. 1.14.18.1*) is a copper-bearing enzyme which is responsible for the browning of an apple when it is cut and exposed to air. In the human, the enzyme is responsible for the formation of melanin, which is the dark pigment in the skin. The enzyme is a typical oxidase which carries out the reaction shown in Figure 8.6. *Ceruloplasmin thus has a role in pigmenting the skin and hair.*

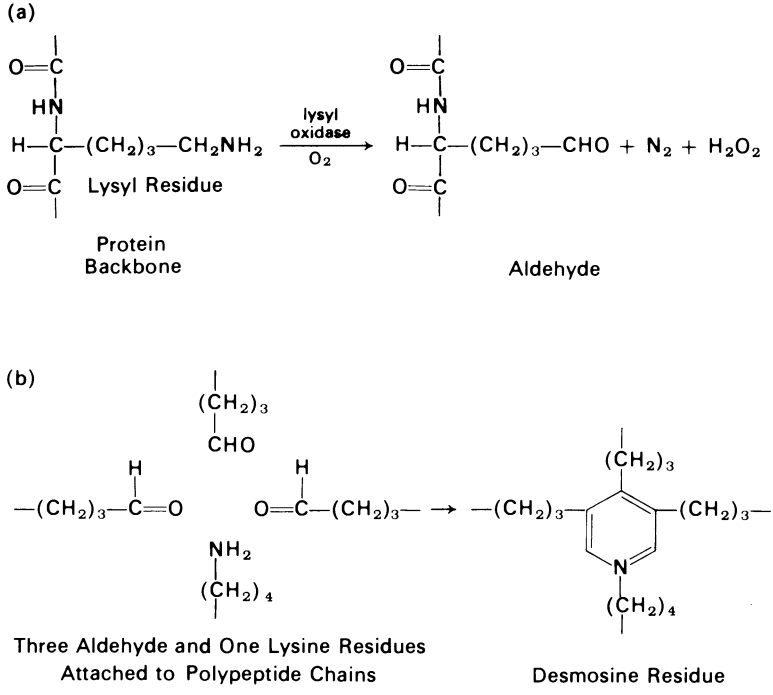


FIGURE 8.5 (a) Oxidation of the $-CH_2NH_2$ group from a lysyl residue attached to a polypeptide chain to form an aldehyde for cross-linking. (b) Condensation of three aldehydes suspended from polypeptide chains with one intact lysyl residue to form a pyridine ring which is now the center of a flexible network which serves to strengthen the connective tissue, such as in the aorta.

Cytochrome oxidase (*cytochrome c oxidase, E.C. 1.9.3.1*) is also a copper-bearing enzyme, and copper deficiency results in distortion and destruction of the mitochondria (see Volume 2, p. 490).

From the above, it is apparent that there must be some mechanism for transporting copper and incorporating it into the structure of these copper-bearing oxidases. Although Cu^{2+} is not readily exchangeable with ceruloplasmin, it has been demonstrated that in the presence of hydrogen donors which reduce Cu^{2+} to Cu^{+}, the copper is readily exchangeable. It has been demonstrated also, that ceruloplasmin donates copper to the oxidases.[39,40] Copper, which reaches the liver directly from the intestine or is transported to the liver by albumin, is incorporated into the ceruloplasmin as it is being synthesized. It is then released to the blood stream, where it is carried to the tissues. At the

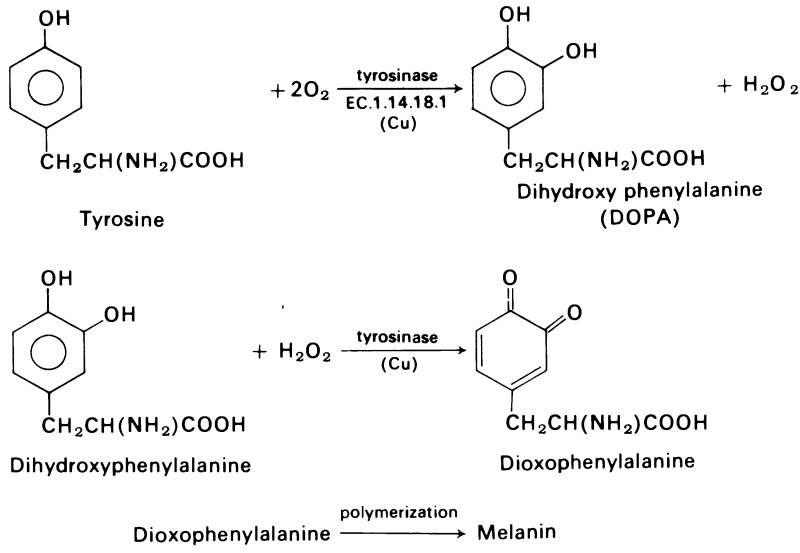


FIGURE 8.6 The action of tyrosinase on tyrosine to form melanin. The presence of dihydroxyphenylalanine or ascorbic acid stimulates this reaction. This enzyme is widely distributed in nature and has been called, phenolase, catechol oxidase, laccase, orthophenolase, and urushiol oxidase. Its official name is *monophenol monooxygenase*.

tissues, the ceruloplasmin probably enters the cell by pinocytosis, as for transferrin, and the Cu^{2+} is reduced to the Cu^{+} state. The copper then leaves the ceruloplasmin and is picked up by the various enzymes (e.g., cytochrome oxidase, amine oxidases) and reoxidized. The copper-depleted ceruloplasmin could then return to the liver, where it would be refitted with copper or be excreted or metabolized. *Regardless of the mechanism, ceruloplasmin serves to bring copper to the oxidases in the cells.* Figure 8.7 shows this ceruloplasmin function.

Livers of mammals, including humans, contain a low molecular weight (58 amino acid residues, approximately 6000) polypeptide to which most of the copper is bound.[41,42] This protein is similar in composition to a zinc and cadmium-binding protein called *metallothionein*, reported earlier.[43] In any case, this seems to be the major liver storage protein for copper, just as ferritin is the major storage protein for iron. This *copper thionein* probably acts as a reservoir for copper for the production of ceruloplasmin. It contains 2.5% copper.

An important enzyme required for normal nerve conduction is

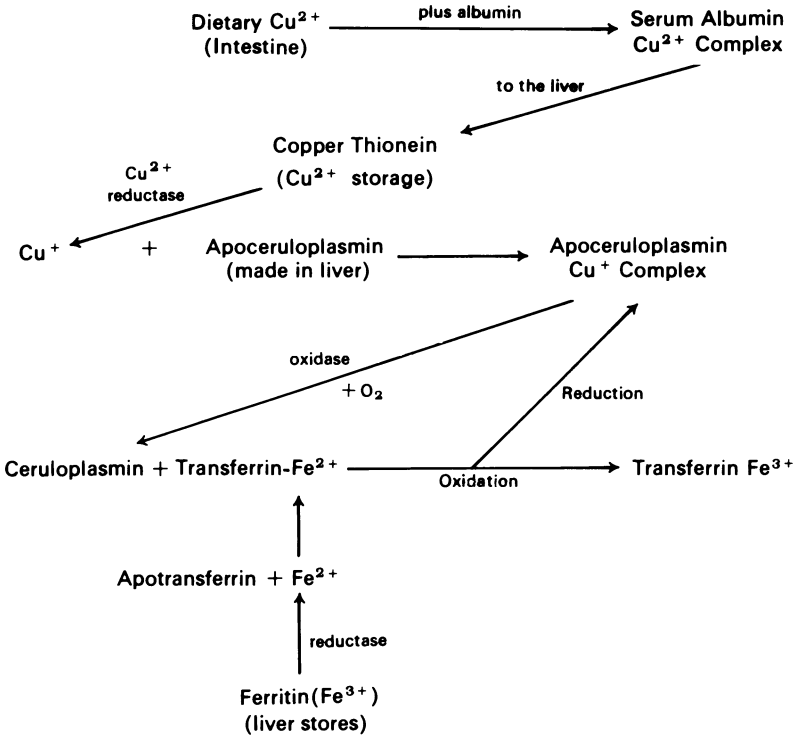


FIGURE 8.7 Movement of dietary Cu^{2+} to the liver to be stored as hepatocuprein. In the liver, some copper is reduced to monovalent copper forming a complex with apoceruloplasmin. Oxidation follows so as to form ceruloplasmin. Ceruloplasmin serves to oxidize the ferrous ion complexed to apotransferrin to form transferrin (Fe^{3+}).

dihydroxyphenylalanine-β-hydroxylase (*dopamine β-hydroxylase, DA-β hydroxylase*). This enzyme is a copper-bearing enzyme which mediates the oxidation of dihydroxyphenylethylamine to noradrenaline. The sequence of events in the synthesis of noradrenaline and thus adrenaline is shown in Figure 8.8. Tyrosine is oxidized by O_2 in the presence of tyrosinase so as to insert an additional hydroxyl group into the benzene ring (Figure 8.6). The dihydroxyphenylalanine formed is then decarboxylated and a hydroxyl group inserted into the side chain. *This reaction is mediated by dopamine β-hydroxylase.*[44] Ascorbate and O_2 is required for the reaction.

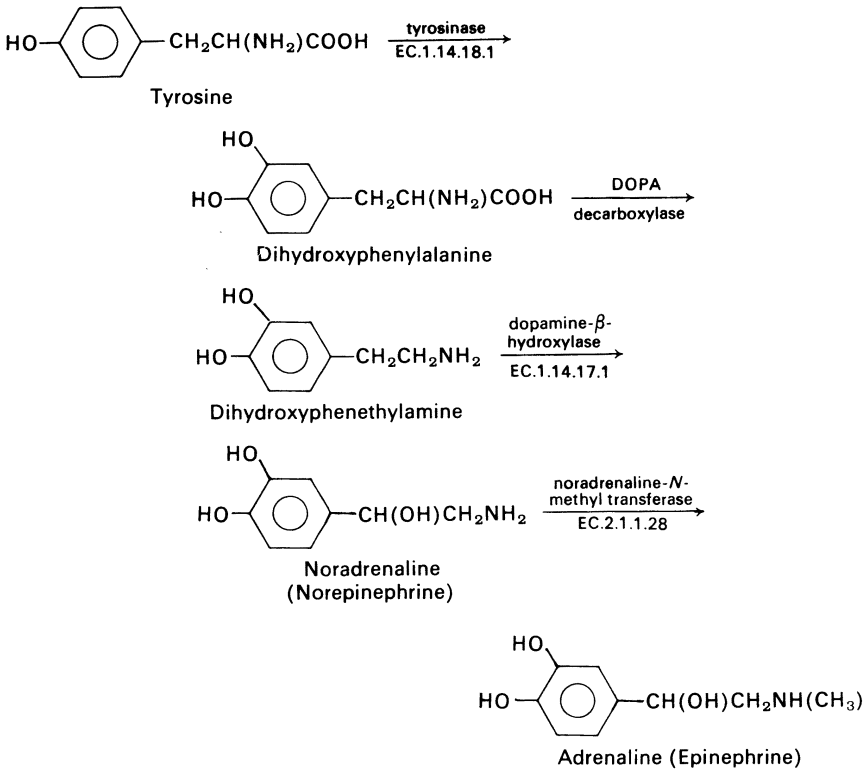


FIGURE 8.8 The role of the copper-carrying oxidases in adrenaline and noradrenaline production.

In 1938, Mann and Keilin prepared a crystalline copper-bearing protein from erythrocytes which they called *hemocuprein or erythrocuprein.*[45] Subsequently, a similar protein was isolated from liver, named *hepatocuprein*, and from brain, *cerebrocuprein.* This is referred to in Volume 2, p. 43. It was shown then that all three proteins from the brain, liver, and erythrocyte of the human are isoenzymes, having the same molecular weight and function.[46] *The cupreins have been shown to be superoxide dismutases.*[47] Ascorbate and O_2 is required for the reaction.

When certain oxidases, such as *xanthine oxidase*, act on their substrate in the presence of oxygen, then the *superoxide ion* is formed:

$$\text{xanthine} + O_2 + H_2O \xrightarrow[\text{(E.C. 1.2.3.2)}]{\text{xanthine oxidase}} \text{urate} + \text{superoxide}$$

Superoxide is oxygen, which has been only partially reduced with one hydrogen. If reduced with a second hydrogen, we have the *peroxide ion*:

$$H^0 \text{ (from donor)} + O_2 \longrightarrow \underset{\text{Superoxide ion}}{O_2^-} + H^+$$

$$H^0 + O_2^- \longrightarrow \underset{\text{Peroxide ion}}{O_2^{2-}} + H^+$$

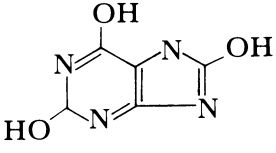
Superoxide is a powerful oxidizing agent and would destroy the tissues, if it were not for the presence of the cupreins. The cupreins cause a rearrangement of charges in such a way that superoxide is converted to hydrogen peroxide. The peroxidases, such as catalase and glutathione peroxidase in the erythrocyte and other tissues, then destroy the peroxide (see Volume 2, pages 262 and 468).

$$2O_2^- + 2H^+ \xrightarrow[\text{(E.C. 1.15.1.1)}]{\text{superoxide dismutase (erythrocuprein)}} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow[\text{(E.C. 1.11.1.6)}]{\text{catalase}} 2H_2O + O_2$$

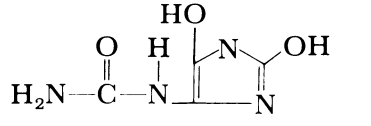
The copper contained in the erythrocyte and other tissue cupreins is carried to the tissue by ceruloplasmin. The red cell emerges into the blood stream with the erythrocuprein, containing the required amount of copper. *Thus ceruloplasmin must also be considered when examining the cell defenses against superoxide.*

Another copper-containing oxidase present in kidney, liver, and other tissues is *uricase* (*urate oxidase*). This mediates the oxidation of uric acid by oxygen to form allantoin and hydrogen peroxide. The allantoin is further oxidized to form unidentified products.



Uric acid

$$+ O_2 \xrightarrow[\text{uricase}]{\text{(E.C. 1.7.1.3)}}$$



$$+ CO_2 + H_2O_2$$

Allantoin

TABLE 8.5

Functions of Ceruloplasmin

Direct function	Significance
Oxidation of Fe^{2+} to Fe^{3+}	Required for hemopoiesis
Carries Cu to bile	Aids in excretion of excess copper taken in the diet
Oxidizes tyrosine	Required for melanin formation to produce skin pigment
Antioxidant for unsaturated fatty acids	Preserves highly unsaturated fatty acids esters in the plasma, such as of cholesterol, vitamin A, and triglycerides
Carrier of copper to the cells	Brings copper to sites where oxidases are being synthesized. Their function is outlined in Table 8.6

TABLE 8.6

Some Copper-Bearing Oxidases and Their Function in the Human

Oxidase	Function
Ceruloplasmin	Oxidizes Fe^{2+} to Fe^{3+}, oxidizes dihydroxyphenols and aromatic diamines to quinones
Cytochrome oxidase (a3) also cytochrome-c oxidase (E.C. 1.9.3.1.)	Completes the oxidation of hydrogen derived from organic substrates in the cytochrome oxidase system[a]
Amine oxidases (E.C. 1.4.3.6) benzylamine oxidase spermine oxidase diamine oxidase lysyl oxidase	Required for cross-linking cartilage and elastin for strength and flexibility; for detoxification of amines such as histamine; for intermediate formation by oxidizing amines in the metabolic chain
Tyrosine oxidase (E.C. 1.15.1.1) (tyrosinase)	For melanin formation and skin pigmentation; for catechol amine formation
Superoxide dismutase (cupreins)	For destruction of the superoxide radical in the protection of tissue from destruction
Dopamine-β-hydroxylase (E.C. 1.14.17.1)	For catechol amine formation (adrenaline and noradrenaline) and normal nerve function
Thiol oxidase (E.C. 1.8.3.2)	Oxidizes —SH to —S—S— linkages; important in cross-linking hair proteins; present in hair roots

[a] See Volume 2, p. 490.

In this section we have reviewed the function of ceruloplasmin *per se* and its indirect function of acting as a source of copper for the synthesis of numerous copper-bearing oxidases in the body. Table 8.5 summarizes the direct functions of ceruloplasmin. Table 8.6 summarizes the functions of the various copper-bearing oxidases whose copper is derived mainly from ceruloplasmin.

8.7 *CERULOPLASMIN AND COPPER IN DISEASE*

Ceruloplasmin occurs in normal serum at a concentration of 170–360 mg/liter with a mean at 265 mg/liter.[48,49] At birth, ceruloplasmin levels, when measured by immunochemical techniques, are approximately at the same concentration as found in adults.[48] However, its concentration is much lower when measured by oxidase activity, a mean value of 65 mg/liter being reported.[50] By the age of 2 years ceruloplasmin levels rise to somewhat above normal adult levels, ($\approx$425 mg/liter). Ceruloplasmin levels drop gradually for several years thereafter until they reach adult levels at puberty, depending upon the diet.

In cerebrospinal fluid, only 2.2 mg/liter of ceruloplasmin is found.[51] In tears the level is 33 mg/liter[52] and in amniotic fluid 26 mg/liter.[52a] Ceruloplasmin concentration is very high in liver bile (900 mg/liter), and even higher in gallbladder bile (1200 mg/liter).[53] Normal serum also contains some apoceruloplasmin ($\approx$33 mg/liter) which can be measured by radioimmunoassay.[54]

8.7.1 *Copper Deficiency and Ceruloplasmin*

The amount of circulating ceruloplasmin seems to be related to the availability of copper for its production. *In copper deficiency, due to decreased intake of copper, ceruloplasmin levels are low.*[55]

The amount of copper in the human diet far exceeds requirement. However, in *infants with diarrhea* or malabsorption, and a diet consisting almost exclusively of milk, copper deficiency associated with a low serum ceruloplasmin level has been reported.[56,57] *Patients on intravenous fluid feeding for extensive periods* have been reported to develop an

anemia associated with reduced serum copper and ceruloplasmin levels.[58] Copper deficiency due to copper deficient diets have been reported in sheep, cattle, pigs, fowl, and experimental animals such as rats.[59,60] These are always associated with a decreased serum ceruloplasmin concentration.

The symptoms of copper deficiency due to inadequate intake can be correlated clearly with the functions of copper and ceruloplasmin as indicated in Tables 8.5 and 8.6. *Anemia* develops because of ferroxidase deficiency and decreased cytochrome oxidase activity. A *neutropenia* is also observed. The skeletal system shows *osteoporosis*, and is readily fractured. This is associated with *a deficiency of lysyl oxidase and abnormal collagen with low desmosine residue concentration* (see Figures 8.5a and 8.5b).

The cardiovascular system becomes defective and in certain animals, a "falling sickness" is observed due to heart failure. This is associated with abnormal elastin, due to low desmosine content (lack of cross-linking). Lysyl oxidase and cytochrome oxidase levels are low.[61,62]

The most dramatic effect of copper deficiency in man is *mental deficiency associated with ataxia, hypogeusea (loss of taste), and demyelinization of nervous tissue.*[63] Cytochrome oxidase, and catechol amine levels are very low.[64]

The decrease in tyrosinase levels in the tissue results in a decrease in the melanin in the skin and color in the hair (achromotrichia).[65] The keratin in the hair is abnormal due to the deficiency of the copper-containing oxidase in the roots of the hair which cross-links proteins by the oxidation of —SH to —S—S— groups. The hair then takes on a kinky appearance. The skin also loses its tensile strength due to lysyl oxidase deficiency and defective cross-linking of elastin and collagen.[66]

A genetic condition occurs which demonstrates all the symptoms observed with copper deficiency in man and domestic and experimental animals. This is referred to as *Menkes' kinky and steely hair syndrome.*[67,68]

The name of the disease stems from the kinky (pili torti, twisted hair) and gray appearance of the hair due to a severe copper deficiency. The disease is X-linked and recessive. Progressive encephalopathy, metaphyseal abnormality, steely hair, micrognathus (small jaws), and abnormal elastic tissue in the arteries are observed in this disease. Death usually occurs before 3 years of age.[69] The incidence of the condition is one in every 35,000 live births.[70] Lysyl oxidase levels in tissues are low, as are blood and tissue copper levels. Cytochrome oxidase levels in tissue are low and there is a marked decrease in the synthesis of the

neural transmitter substances, dopamine, noradrenaline, and adrenaline.[71]

Although the ceruloplasmin levels are very low in patients with Menkes' syndrome, the apoceruloplasmin levels are about the same as those observed in the normal (33 mg/liter).[54] In this condition, the jejunal mucosa contains abundant copper but blood and tissue levels are very low. *This suggests a defect in jejunal mucosal transport.* The little copper absorbed is not enough to produce adequate amounts of ceruloplasmin and the result is a clear case of copper deficiency.

Administration of copper orally to patients with Menkes' syndrome is, as would be expected, ineffective. *Furthermore, administration of copper parenterally is also not effective.* Thus the exact nature of the disease needs clarification.

8.7.2 *Normal Copper Content in the Human*

The total body copper in a 70-kg man is between 12.6 and 18.8 mmoles (800–1200 mg).[56] The plasma concentration of copper is 12.7–25.4 μmol/liter (0.8–1.6 mg/liter). Of this copper, 90–95% is in the ceruloplasmin and the rest is adsorbed to albumin.[72,73] If packed erythrocytes are examined, the concentration is found to be somewhat less than half of that in plasma, 630 μg/liter of packed cells.[74] In the erythrocytes, practically all the copper is attached to erythrocuprein.

A normal diet contains 780–1100 μmol copper, while the daily requirement is only 40 μmol (2.5 mg). Only one-third of the copper ingested is absorbed, and thus about 300–400 μmol finds its way into the liver. This is still ten times the requirement.[56,75] Eighty percent of this is excreted in the bile complexed with bilirubin and other pigments and ceruloplasmin. Most of this finds its way to the stool. The other 20% is excreted in the urine. A 24-hr urine specimen from an adult contains 40–100 μg of copper (0.6–1.6 μmol/24 hr).

Gall bladder bile contains 1200 mg/liter of ceruloplasmin.[76] From 100–1000 ml of bile are secreted per 24 hr in the adult (see Volume 1, p. 257). Assuming a mean value of 500 ml, this would suggest a secretion of 600 mg of ceruloplasmin per 24 hr. Since ceruloplasmin is 0.3% copper, this represents 1.8 mg of copper or about 29 μmol of copper. The amount of copper excreted daily is about 10 times that quantity, and this must be excreted and complexed with other compounds. *On*

electrophoresis and examination of the bile fractions most of the copper is found complexed with the bile pigments including bilirubin.[77]

8.7.3 *Copper Overload*

Copper overload may result from ingestion of excessive amounts of copper in the diet. Ingestion of about 1 g of copper at a time results in nausea, gastritis (stomach cramps), and vomiting. If more than 10 g are taken, the patient will suffer from shock and extensive hemolysis and eventually, hepatic necrosis, nephropathy, coma, and death.[78] In India, suicide is not infrequently induced by ingestion of gram quantities of copper sulfate.[79] Acute copper poisoning has been reported when citrated and carbonated beverages have been allowed to stand in contact with copper vessels, pipes, or check valves.[80]

Copper intoxication is often detected after a preliminary diagnosis of liver disease due to the presence of jaundice and elevated levels of serum enzymes such as SGOT, SGPT, alkaline phosphatase, arginase, lactate dehydrogenase, and other abnormal "liver function tests." Hemolytic anemia resulting from excessive copper intake has been reported.[81] The diagnosis is made when serum, urine, and liver biopsy copper levels are obtained.

8.7.4 *Ceruloplasmin, Copper, and Liver Disease*

Copper is excreted mainly through the bile duct. For this reason, for normal copper excretion, an intact liver and bile duct system is necessary. In liver disease, some copper is retained, the amount depending upon the severity of the disease process. A defense mechanism then increases the rate of ceruloplasmin synthesis in order to complex as much copper as possible. This can be seen in Table 8.7 with diseases of the liver other than Wilson's disease, which will be discussed later.

The greatest elevation of serum ceruloplasmin concentration is seen in primary biliary cirrhosis where the mean is twice that found in the normal see Table 8.7.[82] This is also observed with *bile ducts obstruction* from any of several causes.[83] This follows from the fact that in these cases, copper excretion is interfered with. Associated with the rise in ceruloplasmin level is a rise in serum copper levels. This is to be expected

since the ceruloplasmin formed derives its copper from the plentiful supply in the liver. *Huge deposits of copper in the liver are seen in primary biliary cirrhosis, values approaching that seen in Wilson's disease.* Urinary copper is of questionable value since many of the patients with liver disease excrete amounts in the urine which are in the normal range. However, in some patients values to three times the normal are often obtained.[82–84]

In *acute hepatitis* elevated ceruloplasmin levels are noted. However, in *chronic hepatitis* these values decrease, and liver stores increase due to decreased protein synthesizing capacity. This is partly the case in *alcoholic cirrhosis*, the serum ceruloplasmin levels decreasing and liver copper increasing in the prolonged chronic form of the disease.[82–85]

In patients with primary biliary cirrhosis, copper retention and deposits are found, not only in the liver, but also in the spleen and renal cortex. In some, *Kayser–Fleischer rings* have been observed.[83] Kayser–Fleischer rings are golden-brown rings of pigment at the periphery of the corneas in Descemet's membrane. These were first observed in patient's with Wilson's disease (hepatolenticular degeneration) which is discussed below. See Figure 8.9.

8.7.5 *Wilson's Disease*

A group of rare diseases of genetic origin, characterized by increased deposits of copper in the liver and other organs, as in primary biliary cirrhosis but of greater severity, are grouped under the heading *Wilson's disease.* The reason for this is that Wilson published a review of this and related conditions he had observed in practice, and collected from the literature, going as far back as the 1860s.[86]

Wilson's disease is characterized by degenerative changes in the brain, particularly in the basal ganglia, and cirrhosis of the liver.[87]

In the basal ganglia of the brain, including the thalamus, are located lens-shaped (lentiform) nuclei. In Wilson's disease these degenerate, giving rise to the designation of Wilson's disease as *hepatolenticular degeneration*, since both the liver and brain are involved.[87,88]

Pathognostic signs of Wilson's disease are Kayser–Fleischer rings, seen in the cornea (see Figure 8.9), and large deposits of copper noted on liver biopsy. In the severe form of the disease, seen mainly in young adults, neurological signs are prominent such as spasticity, rigidity,

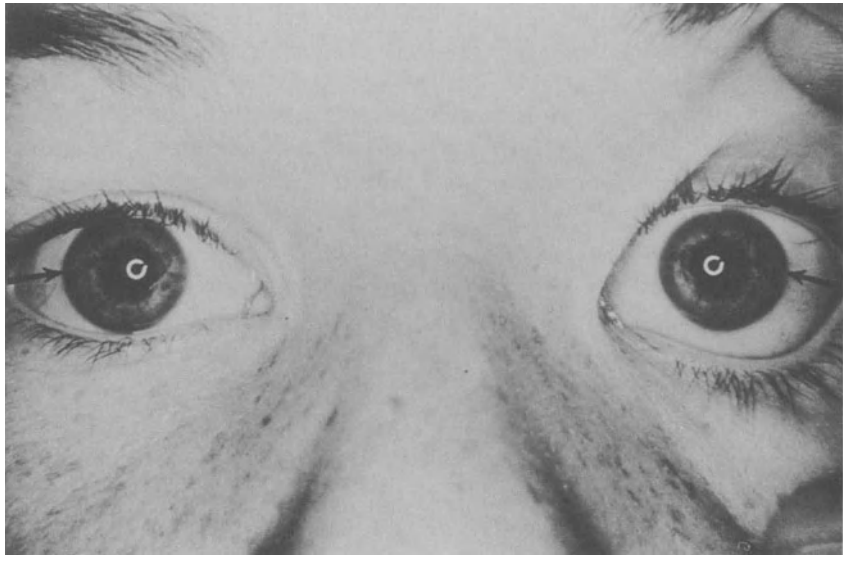


FIGURE 8.9 Kayser–Fleischer rings in the cornea of a patient with Wilsons disease. These are indicated by the arrows. The small bright ring in the center of the eye is a highlight.

dysarthria (impaired speech), and dysphagia (difficulty in swallowing). In many of these cases, liver disease is minimal. In some of these patients, a condition simulating that of multiple sclerosis called pseudosclerosis has been reported.[89]

In the hepatic forms of Wilson's disease, neurological signs are minimal.[90] This condition has been observed mainly in children. Jaundice, hepatosplenomegaly with liver cirrhosis, Kayser–Fleischer rings, and elevated liver copper levels are diagnostic of the disease.

A variation of Wilson's disease has been documented repeatedly, where the patient's first indication of the disease was some psychiatric manifestations.[91] Some of these patients have had a long history of psychiatric treatment before the diagnosis of Wilson's disease was made.

During the course of Wilson's disease, acute hemolytic episodes may occur. These are purported to be due to a sudden release of stored copper from the tissues and result from the toxic effects of copper.[81,92]

Ceruloplasmin levels are very low in all forms of Wilson's disease.[93] This distinguishes the condition from primary biliary cirrhosis (see Table 8.7).

TABLE 8.7

Comparison of Copper and Ceruloplasmin Concentration Found in Various Types of Liver Disease Compared to Wilson's Disease[a]

Subject	Serum cerulo-plasmin[b] (mg/liter)	Total serum copper (mg/liter)	Serum non-cerulo-plasmic copper (μg/liter)	Liver[c] copper (μg/g dry wt.)	Urinary copper (μg/liter)
Normal adult	170–360 (265)	0.80–1.18 (0.90)	84–117 (101)	15–57 (36)	< 70
Primary biliary cirrhosis[d]	380–860 (540)	1.14–2.58 (1.62)	125–271 (170)	90–940 (411)	25–250 (81)
Acute hepatitis	220–680 (390)	0.86–2.04 (1.17)	104–190 (147)	8–68 (24)	12–80 (48)
Chronic active hepatitis	200–400 (280)	0.89–1.20 (0.84)	90–126 (88)	15–80 (40)	7–46 (30)
Alcoholic cirrhosis[e]	300–420 (360)	0.90–1.26 (1.08)	95–133 (113)	17–72 (67)	0–238 (67)
Bile duct obstruction	380–680 (560)	1.14–2.04 (1.68)	97–225 (177)	18–106 (50)	40–140 (90)
Wilson's disease	0–150 (40)	0.30–0.80 (0.50)	184–274 (229)	250–1800 (700)	100–1500 (400)

[a] The values are a composite from several reports. Values in parentheses are mean values.

[b] By radial immunodiffusion.

[c] The moisture content of liver is about 80%. Divide by five to get the copper content per wet weight. Fetal liver contains about eight times the copper concentration than adult liver.[41]

[d] *Primary biliary cirrhosis* is a rare form of cirrhosis of the liver occurring without obstruction of the major bile ducts, affecting chiefly middle-aged women. The disease is characterized by pruritis, jaundice, hypercholesterolemia with xanthomas and malabsorption. It occurs often after treatment with certain drugs such as chlorpromazine and arsenicals. It is distinct from *secondary biliary cirrhosis* which results from chronic obstruction of the bile duct caused by congenital atresia or stricture.

[e] *Cirrhosis of the liver* refers to liver disease with loss of lobular structure with fibrosis, parenchymal cell destruction and regeneration to form nodules.

Normally, about 95% of the copper in the serum is complexed with ceruloplasmin. The rest is bound to albumin. In Wilson's disease, serum copper concentration is actually decreased but the *percentage of copper bound to albumin may reach levels as high as 30% of the total.*[94,95] This copper is bound to albumin. This is typical of Wilson's disease. For example, in 19 normal adults, the serum contained 100 ± 16 μg of

nonceruloplasmic copper per liter. When divided as to sex, females were lower (92 μg/liter) and males higher (107 μg/liter). In patients with Wilson's disease, 229 ± 45 μg/liter were found, in spite of the fact that ceruloplasmin levels were lower in these cases.[94] As a result, if the nonceruloplasmic copper level of the patient with Wilson's disease, is divided by his serum ceruloplasm level, the result will be about four times greater than that observed in the normal, or patient with some other liver disease (see Table 8.7).

A characteristic symptom of Wilson's disease is impaired tubular reabsorption in the kidney. Thus serum urate, phosphate, and calcium levels are low with increased amounts of these substances being found in the urine.[96] For the same reason, aminoaciduria and glycosuria are noted.[97]

The Nature of Wilson's Disease

The basic problem in Wilson's disease is the relentless accumulation of copper in the liver and other tissues in the body until severe symptoms of copper intoxication occur, and the patient dies. *Underlying this condition is the inability of the patient to synthesize ceruloplasmin at an adequate rate.*[98]

When copper is absorbed, it is first bound to albumin (Figure 8.7). It is then carried to the liver and released for ceruloplasmin synthesis. Studies with ^{64}Cu (half-life 12.88 hr) and ^{67}Cu (half-life 61.8 hr) have clearly demonstrated that the copper continues to circulate adsorbed to albumin in the patient with Wilson's disease long after it would have disappeared in the normal. As a result the nonceruloplasmic Cu fraction in the serum is elevated (see Table 8.7), and urinary copper is increased since, distinct from ceruloplasmin, albumin will readily exchange its copper.

In the patient with Wilson's disease the paucity of ceruloplasmin available interferes with the transport of the copper to the bile for excretion. Coupled to this is the decreased biliary excretion of copper probably because of some biliary defect. The net result is a continuous accumulation of copper, since the diet contains excess copper. It has also been proposed that the gene for the synthesis of ceruloplasmin is defective and a condition resembling thalassemia interferes with the rate of ceruloplasmin synthesis. Alternatively, the ceruloplasmin synthesized is aberrant and is synthesized more slowly or carries copper less

effectively. This would explain the various genetic types of Wilson's disease.[99,100]

In primary biliary cirrhosis, the rate of ceruloplasmin synthesis is not decreased. As a result, the percentage of circulating copper bound to ceruloplasmin is normal ($\sim 5\%$) and serum ceruloplasmin levels are elevated. For this reason, urinary copper is not as markedly elevated as in Wilson's disease.

8.7.6 *Management of the Patient with Aberrant Copper Metabolism*

1. *Copper Overload.* In the management of the patient with copper intoxication, it is of prime importance to ascertain the cause for the accumulation of the excess copper. It may originate as a result of an occupational hazard or from exposure to copper contaminated foods or foods stored in copper vessels. This needs to be distinguished from the copper accumulated from the acquired diseases such as in liver disease, or the genetic disorders such as the various forms of Wilson's disease.

Copper overload responds to treatment with chelate compounds which complex the copper and are excreted, mainly in the urine.[101]

Although BAL [1,2-dithioglycerol, $CH_2(SH)CH(SH)CH_2OH$] and other chelating agents, such as triethyltetramine have served, penicillamine is the substance of choice for this purpose.[102] In adults, approximately 1 g of penicillamine is administered orally per day. Along with this, the patient is put on a low copper diet. Some use cation exchange resins orally to decrease the copper absorbed, especially in acute conditions.[103] With this treatment it is advantageous to supply pyridoxine since penicillamine acts as a pyridoxine antimetabolite, especially since pyridoxine is required for the activity of certain copper-containing oxidases.

In the case of Wilson's disease, penicillamine treatment is remarkably effective. The Kayser–Fleischer rings disappear, and behavior approaches that of the normal. *At least six cases have been reported in the literature where young girls, placed on a penicillamine regimen, have matured, and given birth to normal children.*[104]

To test the efficacy of the treatment, it is advisable to collect the urine and assay for copper. Huge quantities of copper will be found in the urine which helps to confirm the diagnosis of excess copper deposits being present.

2. *Copper Deficiency.* With the widespread use of intravenous hyperalimentation, there has been an increase of the incidence of anemias resulting from copper deficiency.[56,105] For this reason, I.V. fluids, when administered over extended periods of time, should contain, for the adult, from 150 to 300 μmol Cu/day, usually as copper sulfate.[106]

When copper deficiency has been shown to be due to malnutrition, including the hypocupremia seen in certain milk fed children, oral administration of copper salts such as the acetate or sulfate are effective. Where the copper deficiency is due to a malabsorption syndrome, the copper may be administered parenterally along with iron, B_{12}, and other hematinics, administered to correct an anemia.[57,107] Where a copper deficiency exists, the recommended initial adult dose is 630 μmol Cu/day.[56,58] Ascorbic acid should also be administered since it is required for copper metabolism at several levels. It is also advisable to administer pyridoxine since some of the copper oxidases require pyridoxine as a coenzyme.

In following these patients with copper deficiency, serum ceruloplasmin, as well as serum copper levels need to be measured. If the copper is absorbed and utilized, this should be reflected in an increase of ceruloplasmin levels to the normal range.

In Menkes' kinky hair syndrome, copper administration parenterally will raise the serum and erythrocyte copper levels but will not relieve the symptoms.[68] This is also true in animals such as sheep and mice where a similar genetic disease occurs.[108] Treatment of this congenital copper deficiency in mice, with copper salts parenterally, will correct a mottled appearance of the coat, raise noradrenaline production to the normal range and cytochrome-c oxidase level but not quite to the normal level. However, copper levels will remain low in liver and brain. The copper administered will accumulate in the kidney, with most of the hepatic and neurological symptoms remaining.

3. *Simulated Copper Deficiency* (*Lathyrism*). Certain compounds occurring in natural products react to bind copper tightly when ingested, simulating a copper deficiency, in the presence of what seems to be adequate amounts of copper in the tissues and diet.[122] One such widely studied compound is β-aminopropionitrile ($H_2NCH_2CH_2CN$). Others are aminoacetonitrile, bis-β-iminodipropionitrile, and β-cyano-L-alanine.[123,124] These compounds occur in the seeds of certain members of the pea family (e.g., Lathyrus Sativus, blue vetch) in high

concentration. On ingestion of these seeds, the patient develops a spastic paraplegia, hyperesthesia and paresthesia.[128–130] The amine oxidases, such as lysine oxidase, lose their activity, and the elastin of, e.g., the trachea and blood vessels, begin to disintegrate due to lack of cross-linking. This condition is referred to as *lathyrism*[109] or *odoratism*, if acquired from ordinary peas.[123,125,127] This has been known for centuries. Hippocrates refers to it as follows, "At Ainos, all men and woman who ate peas continuously became paralyzed in the legs, and remained in that condition."[122] In times of famine in France, Algeria, Italy, India, Spain, and Greece, epidemics of lathyrism have been reported due to a diet consisting mainly of peas.[126] It is a serious hazard in raising fowl such as turkeys.[110] In experimental animals, lathyrism has been induced to relieve stricture of the esophagus from any of several causes.[111]

Removal of the offending food will eventually relieve the symptoms of lathyrism. During the recovery period, ascorbate, pyridoxine, and copper administration help to relieve the symptoms.

8.8 *CERULOPLASMIN AND COPPER ASSAY*

Major stimulus was given to ceruloplasmin assay when it was noted in the 1950s that ceruloplasmin levels were high in certain mental disorders. The technique used was to incubate serum with *p*-phenylenediamine, measuring the brown color which developed.[112] This assay became routine in the clinical laboratory and was applied extensively. It was soon found that ceruloplasmin levels were elevated in pregnancy to higher levels than that noted in schizophrenics. This was followed with the observation that the ceruloplasmin level was elevated in certain types of neoplastic diseases,[113] particularly Hodgkin's disease,[114] and certain types of multiple myeloma.[115] Ceruloplasmin levels are elevated in almost every disease, other than Wilson's disease, malnutrition, nephrosis, or malabsorption (see Table 8.8).

Since ceruloplasmin contains almost all of the copper in the serum there is direct correlation between serum copper and ceruloplasmin levels. A typical example is psoriasis where the copper and ceruloplasmin levels both rise as the condition worsens. On remission, they fall together, depending upon the severity of the condition.[116]

TABLE 8.8

Conditions Where Serum Ceruloplasmin and Copper Levels have been Reported as being Significantly Elevated or Depressed

Level	Condition
Lowered	Malnutrition, malabsorption, nephrosis, Wilson's disease
Elevated	*Neoplastic diseases* such as various types of tumors, Hodgkins disease, leukemia, and multiple myeloma. *Autoimmune diseases* such as rheumatoid arthritis. *Copper intoxication, pregnancy*, and with oral contraceptives, *liver diseases. Infections. Psychiatric diseases* such as schizophrenia and delerium tremens. *Myocardial infarct* with tissue necrosis, infections. *Trauma* such as postoperative; *ataxia telangiectasia; prolonged physical exercise*

Assay for ceruloplasmin by its oxidase properties also yields high results in ascorbate deficiency. Thus this type of assay has been abandoned. Instead, immunochemical techniques are now widely employed and kits are available for its assay from numerous commercial companies.[48]

Other techniques which have been used are the saturation of serum with Cu^{+}, permitting it to oxidize and separating the ceruloplasmin. The blue color is read at 610 nm, the Cu^{2+} is reduced, and the bleached solution used as a blank.[117]

The procedures for serum copper assay use a complexing agent which produces a color such as cuprizone, phenanthroline derivatives, diethyldithiocarbamate, and numerous others.[118–120] The method of choice is by atomic absorption.[121] Of major importance is the fractionation into ceruloplasmin bound and albumin bound copper, before assay.[94,95] If an X-ray spectrometer is available, the ceruloplasmin copper can be readily separated from other copper electrophoretically on cellulose acetate, and both read *in situ* nondestructively.[74]

8.9 *RECAPITULATION*

Ceruloplasmin (Cp) is the major copper transport protein in the serum, carrying all but 5–10% of the serum copper. The remainder is bound to albumin.

Ceruloplasmin has a molecular weight of about 134,000 and moves at the head of the α_2 globulins on electrophoresis. One molecule of ceruloplasmin carries six copper atoms, tightly bound, and a variable amount of a seventh copper atom which can be readily removed by chelating resins. The copper in ceruloplasmin is in the bivalent state. Two of the copper atoms are held in a coordinate type of linkage which is responsible for the blue color of ceruloplasmin. The other four are tightly chelated.

Ingested inorganic copper binds to albumin. The copper is then carried to the liver where apoceruloplasmin is synthesized. The copper is reduced to the monovalent state, and it is this form which adsorbs to apoceruloplasmin. The Cu^+ is then oxidized to Cu^{2+} and the ceruloplasmin is released to the plasma.

On electrophoresis, ceruloplasmin shows two variants in almost all individuals. More than 90% is the CpB form. A more rapidly moving fraction, occurring in small amounts, is the CpA form. Other variants have been identified, mainly in Blacks. In general, Caucasians show from 96–99.4% of CpB, the rest being CpA. Blacks often contain other variants showing greater and lesser mobility than CpB.

Ceruloplasmin is an oxidase. It can oxidize polyphenols and aromatic polyamines to quinones, generating H_2O_2 in the process. It also serves to bring copper to the tissue oxidases, such as cytochrome-c oxidase, tyrosinase, and lysyl oxidase. It also supplies copper to the cytocupreins in the various tissues which are superoxide dismutases. Copper from ceruloplasmin also serves to activate dopamine-β-hydroxylase.

Because of its role in supplying copper to the oxidases, ceruloplasmin deficiency results in symptoms of oxidase deficiency. These include a decrease in brain catecholamine levels, depolymerization of elastin and collagen, loss of color and kinking of the hair, and various neurological manifestations.

A major function of ceruloplasmin is to oxidize iron to the ferric state for transport by transferrin. It is therefore involved in hemopoiesis and in the formation of the oxidases of the cells, especially the cytochrome oxidases. Copper deficiency therefore results in an anemia.

A congenital copper deficiency called Menkes' kinky hair syndrome, which occurs in animals and man, shows the symptoms of copper deficiency. However, oral or parenteral copper does not relieve all of the symptoms of this disease.

Copper intoxication, where copper pipes or utensils are used to carry water or prepare foods is not uncommon. Taken in gram quantities copper can be lethal. The symptoms of severe copper poisoning are extensive hemolysis, hepatic necrosis, nephropathy, coma, and if not treated, the patient dies. In the milder forms, the condition is associated with jaundice and abnormal liver function tests, such as elevated alkaline phosphatase, and transaminase. The serum copper and ceruloplasmin levels are markedly elevated, as well as the copper in the liver, as measured after a biopsy.

Excess copper is excreted mainly by way of the bile duct bound to bilirubin and other bile pigments, and eventually reaches the stool. Since the copper intake is much greater than the daily need, this system needs to be effective or copper will accumulate in the tissues. Copper is stored in the liver bound to a low molecular weight protein, copper thionein. As excess copper accumulates, it deposits in all the tissues in the body. In chronic copper overload, copper deposits in the cornea forming a brown ring around the pupil. These are called Kayser–Fleischer rings and are indicative of chronic copper overload.

A congenital condition occurs, in which the excretion of copper is not completely effective, and as a result copper accumulates. This is called *Wilson's disease.* In one form of the disease the lentiform nuclei in the basal ganglia of the brain degenerate and neurological symptoms dominate the clinical picture. In another form, neurological symptoms are mimimal and the condition is that of a severe liver cirrhosis. In Wilson's disease, serum ceruloplasmin levels are low. For this reason it is proposed that in this condition copper is not being transported to the tissues for its various functions, as a result the copper accumulates.

Laboratory findings in Wilson's disease are low serum copper and ceruloplasmin levels, a very high liver tissue copper level, and an increased percentage of albumin-bound serum copper. Kayser–Fleischer rings are seen in these patients.

Although liver copper deposits are increased in liver disease because of impaired copper excretion, serum ceruloplasmin and copper levels are elevated. This distinguishes liver disease from Wilson's syndrome. The percentage of albumin-bound copper remains low in liver disease.

When serum ceruloplasmin levels are determined by immunochemical techniques, they correlate with serum copper levels. Low ceruloplasmin levels are found in malnutrition, malabsorption, nephro-

sis, and Wilson's disease. In most other diseases serum ceruloplasmin levels are elevated.

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9

Haptoglobins: Hemoglobin Binding

9.1 *SURVEY*

Certain proteins appear in the plasma in increased amounts during inflammatory reactions. These are often referred to as the *acute phase reactants.*[1] See Section 5.7. Generally, these proteins are *glycoproteins* synthesized in the parenchymal cells of the liver which contain substantial amounts of carbohydrate attached to the protein molecule. They are precipitable by adding about 150 times the volume of 95% ethanol to serum.[2] The C-reactive protein of serum is a typical example (see Section 5.7.1).

A major component of the *acute phase proteins* are the haptoglobins (Hp). The haptoglobins are characterized by their ability to bind the globin part of the hemoglobin molecule in a 1:1 ratio and thus bind hemoglobin in this ratio. They are located by adding a hemoglobin solution to serum and then performing acrylamide or starch gel electrophoresis. The heme visualizes the area. The hemoglobin–haptoglobin complex exhibits peroxidase activity. It was this property of the haptoglobins which led to their discovery.[3] See Figure 9.1.

The peroxidase activity of the haptoglobin–hemoglobin complex (Hp–Hb), stems from the hemoglobin portions of the complex and not from the haptoglobin moiety. Oxyhemoglobin, carbon monoxide–hemoglobin (carboxyhemoglobin), methemoglobin, and cyanmethemoglobin are all bound tightly to haptoglobin. Haptoglobin has only a low affinity for deoxyhemoglobin.[4] Hemoglobin attached to haptoglobin does not exhibit the Bohr effect. This effect is a result of positional changes of the hemoglobin chains with respect to each other (see

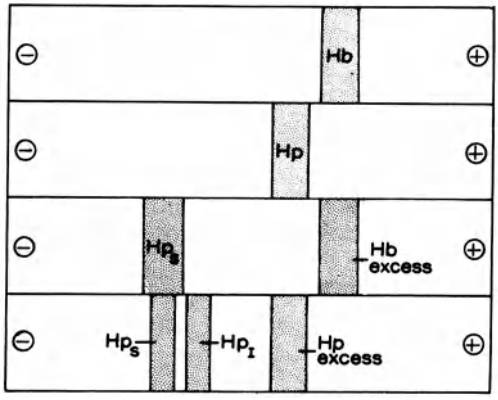


FIGURE 9.1 Comparative electrophoretic mobility in gel of saturated haptoglobin (Hp_s) with hemoglobin (Hb) excess, and the intermediate Hp–Hb complexes (Hp_I) with haptoglobin in excess.

Volume 2, p. 398, and Volume 1, pp. 42–49). This is impossible, due to the nature of the haptoglobin–hemoglobin complex (see Table 9.1).

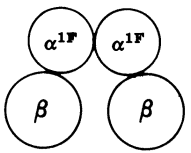
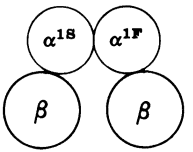
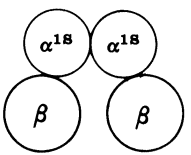
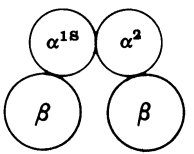
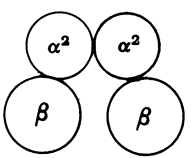
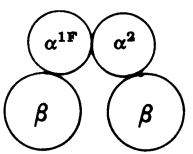
9.2 *HAPTOGLOBIN COMPOSITION*

Haptoglobin resembles the immunoglobulins, in that it is composed of two identical light (α) and two identical heavy (β) chains.[5] There the resemblence ends since there is very little amino acid sequence homology between the haptoglobin and immunoglobulin chains. Two distinct chromosomal loci, *Hp*α and *Hp*β, control the structure of these chains.[6] Genetic variation at the *Hp*β locus is extremely rare. However, there are three common alleles at the *Hp*α locus, namely, *Hp*α^{1F}, *Hp*α^{1S}, and *Hp*α^{2}. Thus, when the chains are separated from the haptoglobin molecule, from a homozygous individual, two Hpβ chains and two of any combination of two of the Hpα^{1F}, Hpα^{1S}, or Hpα^{2} chains are obtained. This is illustrated in Table 9.1. The designation F and S refers to chains which move faster and slower in the electrophoretic field, respectively.[7] The convention is to use lowercase letters to designate the chains.

In order to separate the various chains from the haptoglobin molecule, the protein is incubated with 8 M urea at pH 3.7, in the presence of a disulfide (—S—S—) reducing agent such as mercaptoethanol (HS—CH_2—CH_2—OH).[8]

TABLE 9.1

The Six Common Genotypes of Haptoglobin

Designation	Chain composition	Designation	Chain composition
Homozygote, Hpα^{1F} Hpα^{1F} (Hp-1)		Heterozygote, Hpα^{1S} Hpα^{1F} (Hp-1)	
Homozygote, Hpα^{1S} Hpα^{1S} (Hp-1)		Heterozygote, Hpα^{1S} Hpα^{2} (Hp2-1)	
Homozygote, Hpα^{2} Hpα^{2} (Hp-2)		Heterozygote Hpα^{1F} Hpα^{2} (Hp2-1)	

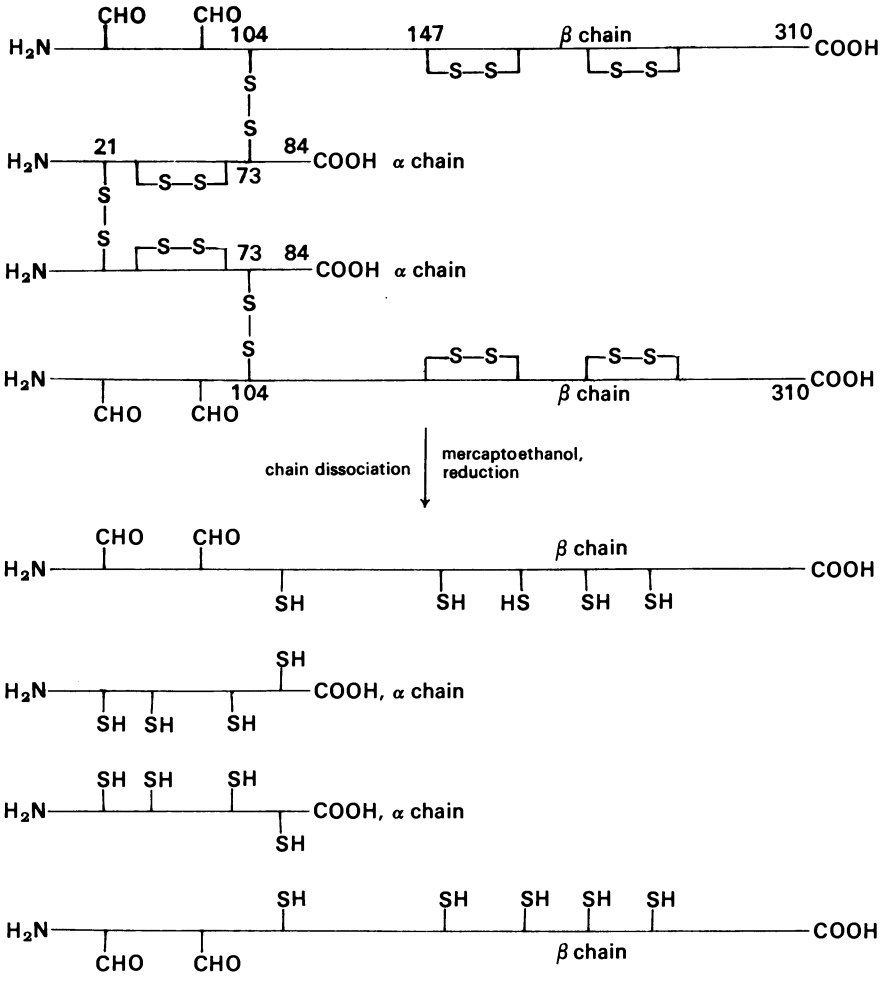


FIGURE 9.2 Relationship between haptoglobin and its separated reduced chains.

Figure 9.2 is a schematic representation of the process leading to the separation of the chains. It can be seen that the haptoglobin molecule is a dimer of two $\alpha\beta$ monomers, resembling hemoglobin. As indicated by the requirement for acid pH and 8 M urea, disulfide bonding is not the only force holding the chains together. Hydrogen bonding and secondary valences play a significant part.[8] This is discussed in connection with the quaternary structure of hemoglobin in Volume 2, p. 396 ff., this series.

9.3 *HAPTOGLOBIN ELECTROPHORESIS*

On electrophoresis, the haptoglobins move to the α_2 globulin region (see Figures 3.5 and 3.7) and are readily located by the peroxidase activity of their complex with hemoglobin.[9,10] On starch gel electrophoresis, three major phenotypes are noted in humans. The haptoglobin with the greatest mobility is referred to as Hp 1-1. A second type with lesser mobility, Hp 2-2 is also noted, and an intermediate type is designated Hp 2-1. The electrophoretic pattern of Hp 2-2 is quite complex, as indicated in Figures 9.3 and 9.4.

Hp 1-1 has an electrophoretic mobility of 4.5 Tiselius units at pH 8.6 and an isoelectric point at pH 4.25. It is soluble in 0.6 mol/liter perchloric acid but precipitates with phosphotungstic acid. This classifies it as a *mucoprotein.*[2,11] Hp 1-1 actually is any one of three proteins whose compositions are $2\beta \cdot 2\alpha^{1F}$, $2\beta \cdot 2\alpha^{1S}$, or $2\beta \cdot \alpha^{1S} \cdot \alpha^{1F}$ (see Table 9.1). Which of these proteins is present cannot be determined on the native protein at alkaline pH by starch or acrylamide gel electrophoresis.[11] For this purpose it is necessary to separate the chains, and identify them either by acrylamide or starch gel electrophoresis or by column chromatography.[12,13] In view of the above, Hp 1-1 acts as a homogeneous protein. It migrates as a single band on electrophoresis whether or not it is complexed with hemoglobin in excess and sediments as a single symmetrical peak in the analytical centrifuge. This can be seen in Figure 9.5.

Hp 1-1 has a molecular weight of about 110,000. When bound to hemoglobin, the molecular weight is 175,000 since binding to hemoglobin is in the ratio of 1:1 for the two molecules.[14]

As can be seen from Figure 9.4, the electrophoretic pattern of serum from a homozygote for slower-moving Hp 2-2 comprises a

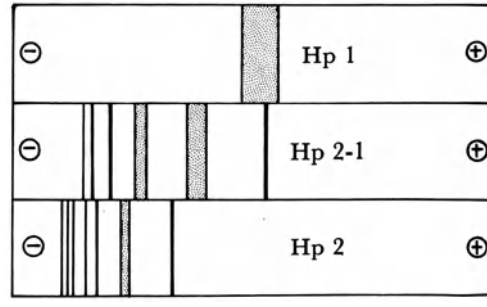


FIGURE 9.3 Electrophoretic pattern of the three haptoglobin phenotypes in gel at alkaline pH 8.6.

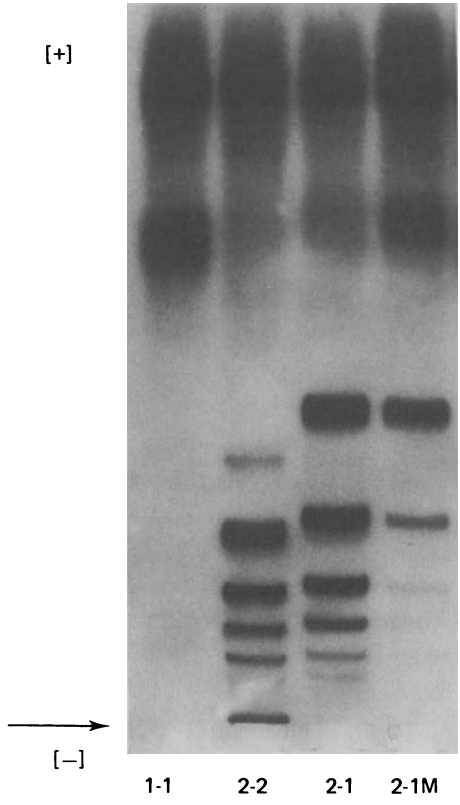


FIGURE 9.4 Gel electrophoretic patterns of the haptoglobin types.

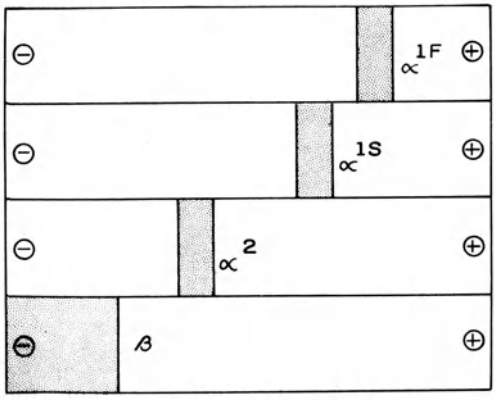


FIGURE 9.5 Relative mobility of the various haptoglobin chains in starch gel.

series of bands. These are polymers of the haptoglobin monomer, $\beta\alpha^2$. Six distinct polymers have been isolated and shown to be members of the series $(\alpha^2\beta)_3$, $(\alpha^2\beta)_4$, $(\alpha^2\beta)_5$, $(\alpha^2\beta)_6$, $(\alpha^2\beta)_7$, and $(\alpha^2\beta)_8$.[15] The $\alpha^2\beta$ monomers are tied together through disulfide bonds to form the polymers. These can be disrupted with mercaptoethanol. An α^2 chain has a molecular weight of about 17,300 and a β chain of about 40,000 for a total of 57,300 for the monomer.[15a]

Hp 1-1 is a dimer of $\beta\alpha^1$, and therefore can be written $(\beta\alpha^1)_2$. It should, therefore, have a molecular weight of about 114,600. For Hp 2-2, the trimer, $(\beta\alpha^2)_3$, would be 171,900 and the octamer 458,400. Since starch and acrylamide gel separate proteins not only by charge but also by molecular weight, it would be expected that the higher polymers would move more slowly. This explains the pattern obtained for Hp 2-2 in Figure 9.3.

Haptoglobin 1-1 is then $(\alpha^1\beta)_2$ as discussed above, and haptoglobin 2-2 is $(\alpha^2\beta)_n$, where n is a series, represented by $3 + m$, where m is a positive whole number including zero. The heterozygote Hp 2-1 is similar to the polymer model for Hp 2-2. It contains polymers made up of the $\alpha^1\beta$ and $\alpha^2\beta$ subunits. All haptoglobins can be represented by $(\alpha^1\beta)_p \cdot (\alpha^2\beta)_q$, where p and q can be any whole number including zero, and may or may not be different. Thus, when $q = 0$ and $p = 2$, we have the Hp 1-1 type of Figure 9.4.

The α^2 chain has cysteine residues which readily react with cysteine residues from other chains to form a disulfide link, resulting in polymer formation.[16,17] For this reason, molecular weights cited for Hp 2-2 of about 400,000 and for Hp 2-1 of 220,000 are average molecular weights of a group of polymers, and indicate an average polymer of $(\alpha\beta)_7$ for the Hp 2-2 and $(\alpha\beta)_4$ for Hp 2-1 (15). Among white Americans, 38% show the Hp 2-2 phenotype and 47% are Hp 2-1. The remainder (15%) are Hp 1-1 (Table 9.2).

9.4 *GENE FREQUENCY OF THE HAPTOGLOBINS*

In calculating the *gene frequency* for any characteristic in accordance with statistical practice, the gene frequency for the total population is 1.0. If there are only two alleles, such as Hp^1 and Hp^2, then the sum of the two gene frequencies, according to probability laws, must equal 1.0. A homozygote (Hp 1-1) carries two Hp^1 genes. A heterozygote,

TABLE 9.2

Distribution (%) of the Common Haptoglobin Types 1 and 2 Among Various Ethnic Groups, Showing the Gene Frequencies[14,44]

Ethnic group	Number tested	Hp 1-1	Hp 2-1	Hp 2-2	Hp 0	2-1 (mod)	Hp^1 frequency
Caucasians							
England	397	14.2	53.2	31.3	1.3		0.41
France	1750	15.5	46.9	37.3	0.4		0.38
Russia	205	13.7	46.8	38.1	1.4		0.37
Poland	6017	14.3	48.1	37.4	0.2		0.38
Scandinavia	2282	15.1	46.5	38.0	0.4		0.38
Spain	2763	16.3	48.3	35.4	—		0.40
Germany and Austria	1095	17.3	49.5	33.2	0		0.42
Italy	4310	14.0	45.1	40.9	—		0.37
Greece	649	9.4	42.1	46.5	2.0		0.31
United States	409	13.2	50.3	36.4	0		0.38
Israel							
Ashkenasi	499	8.6	40.5	50.3	0.6		0.29
Sefardic	345	5.8	41.1	52.2	0.9		0.26
Kurdish	113	10.6	50.5	37.2	1.7		0.36
India							
Punjabis	161	5.6	30.4	63.4	0.6		0.21
Bengalis	176	4.5	29.5	64.2	1.6		0.20
Irulas	74	0	13.5	79.7	6.8		0.07
Tamils	133	3.0	10.5	83.5	3.0		0.15
Iran	97	12.4	46.4	41.2	—		0.35
Australia	323	14.6	46.1	39.0	0.3		0.38

Mongols							
Chinese							
Taiwan	172	9.3	37.7	52.9	0		0.28
Hong Kong	122	13.9	48.4	37.7	0		0.39
Malay	167	10.8	34.1	53.9	1.2		0.28
Japanese	349	6.0	34.9	57.6	1.4		0.24
Eskimo (Alaska)	638	9.5	42.3	47.7	0.5		0.31
American Indians							
Apaches	98	34.7	48.0	17.3	—		0.59
Nez Perce	180	22.2	48.3	29.4	0		0.48
Navajo	169	23.1	47.3	29.6	0		0.47
Athabascan	284	18.0	31.3	47.9	2.8		0.42
Mayas	661	40.1	45.8	13.5	0.6		0.63
Mexican	386	22.0	48.4	24.9	4.7		0.49
Peru	250	35.5	49.7	14.8	0		0.60
Chile	229	59.4	34.1	6.5	—		0.76
Jivaro	222	41.4	45.0	12.6	0.9		0.65
Mixed groups							
Filipino	293	14.0	48.1	37.2	0.7		0.38
Thais	1095	5.3	37.4	55.5	1.8		0.24
Malays	236	5.1	36.0	58.1	0.8		0.23
New Guinea	1807	47.1	39.4	10.3	2.5		0.67
Solomon Islands	183	29.0	35.5	8.7	26.8		0.64
Easter Islands	36	72.2	27.8	0	—		0.86
Hawaiian natives	38	50.7	34.7	14.6	—		0.68
United States Blacks	573	27.5	36.2	22.3	4.2	9.8	0.51

continued overleaf

TABLE 9.2 (*continued*)

Ethnic group	Number tested	Hp 1-1	Hp 2-1	Hp 2-2	Hp 0	2-1 (mod)	Hp^1 frequency
Africa							
Pygmies	121	6.6	40.5	20.6	31.4	0.9	0.70
Nigerians	230	28.0	33.3	4.4	31.0	3.3	0.60
Uganda	115	28.5	38.7	8.5	24.2	6.1	0.63
Liberia	53	37.7	20.8	13.2	18.9	9.4	0.65
Central African Republics	353	24.1	22.7	16.7	33.1	3.4	0.56
Hottentot	59	30.5	42.4	27.1	0	0	0.51
Zulu	113	31.9	39.8	25.7	2.6	2.6	0.53

Hp 2-1, carries one Hp^1 gene. Thus, a weight of two must be given for Hp 1-1 as compared to Hp 2-1, in calculating gene frequencies.

The sum of twice the percentage of the homozygotes, plus the percentage of the heterozygotes is a measure of the frequency of occurrence of that gene. Since Hp 2-1 is being counted twice, once for each gene, the sum of the frequencies then comes to 200. To calculate gene frequencies, we then use the following formula, where $\overline{1\text{-}1}$, $\overline{2\text{-}1}$, and $\overline{2\text{-}2}$, represent the percentage of each genotype in the population. The symbol for gene frequency is always written in italics. Thus Hp^1 and Hp^2 represent the two gene frequencies under discussion.

$$\text{Hp 1, gene frequency } (Hp^1) = \frac{2(\overline{1-1}) + \overline{2-1}}{200}$$

$$\text{Hp 2, gene frequency } (Hp^2) = \frac{2(\overline{2-2}) + \overline{2-1}}{200}$$

Let us now compare the gene frequencies for Hp 1 and Hp 2, in Caucasians and Blacks in the United States. From Table 9.2, for Caucasians, we substitute in the formula for Hp^1, 2(13.2) + 50.3, and obtain 76.7. Dividing by 200, the gene frequency for Hp^1 calculates to 0.38. For Hp^2 it is then 0.62. For U.S. Blacks, from the table, Hp^1 calculates to 0.51 and Hp^2 is then 0.49.

The standard deviation is the same for both frequencies, since once one is determined, the other is also fixed.

Note from Table 9.2 that generally, the gene frequency in Mongols and Caucasians is higher for Hp^2 than for Hp^1. In Negroes, the reverse is the case. The occurrence of a high incidence of Hp^1 in central and South America and in the Polynesian islands is explained by the migration of African Negroes to these areas in ancient times. This concept is supported by archaeological evidence, and the physical characteristics of the natives.

9.5 *THE HAPTOGLOBIN CHAINS*

Haptoglobins represent only 2% of the serum proteins.[18,19] For this reason, other body fluids which are richer in haptoglobins have been used for its purification. Ascitic fluid and urine of patients with

lipoid nephrosis have been used as haptoglobin sources.[20] In animals, an acute phase reaction may be induced in the animal by injection of turpentine, before samples of the serum are taken for the preparation of haptoglobin.[21] Hp 1-1 has been crystallized, by precipitating first with rivanol, fractionating with ammonium sulfate, followed by preparative zone electrophoresis and gel filtration.[22]

The purified haptoglobin is subjected to reductive cleavage in acid–urea starch gels as mentioned above. This separates the α and β chains. For subsequent aminoacid analysis and sequencing, the chains may also be fractionated by the various techniques of column chromatography. Figure 9.5 shows the relative mobility of the various chains on starch or acrylamide gel.[23]

9.5.1 *Haptoglobin α Chains*

The Hpα^{1F} and Hpα^{1S} chains have a molecular weight of 9100. There are 84 amino acids which differ only at one point of substitution. In Hpα^{1S}, glutamic acid has replaced lysine at position 54 of the Hpα^{1F} chain. The sequence of these chains are shown in Figure 9.6.[16,17]

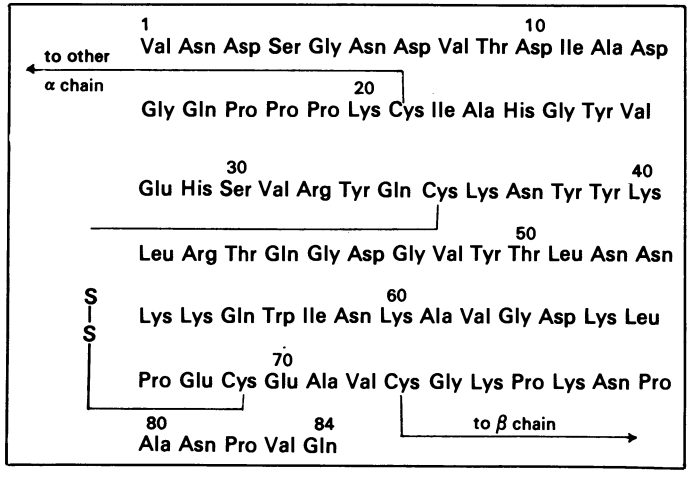


FIGURE 9.6 The 84 amino acid sequence of the Hpα^{1F} chain. The hpα^{1S} chain substitutes glutamic acid for lysine at position 54.

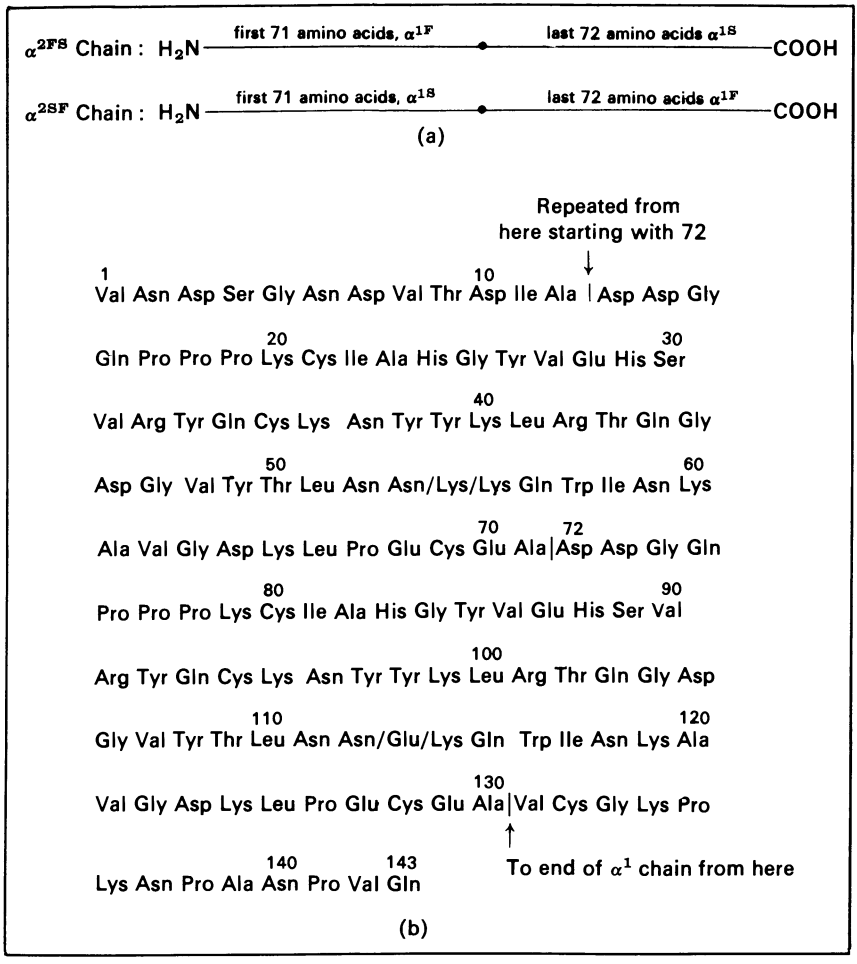


FIGURE 9.7 The composition of the α^2 chains. (a) Schematic representation. (b) amino acid sequence of the α^{2FS} chain. Compare the sequence of the first 71 amino acids with that of Figure 9.6.

The haptoglobin α^2 chain is almost twice as long as the Hpα^{1F} or Hpα^{1S} chains. Its molecular weight is 16,000. It consists of 143 amino acids. If it were twice as long as the Hpα^1 chains, then it would have a molecular weight of 18,200 and consist of 168 amino acids.

Sequence analysis of the α^2 chain presented the surprising fact that it was composed of the first 71 amino acids of the Hpα^{1F} or Hpα^{1S} chain, followed by the *last* 72 amino acids of the other Hpα^1 chain

(Hpα^{1S} or Hpα^{1F}, respectively.)[16] Thus, there were two α^2 chains, as indicated in Figure 9.7. These have a molecular weight of about 16,000 and are composed of 143 amino acids.

In order to explain the origin of the α^2 chain it has been proposed that there is duplication of genes for the production of the α chains of the haptoglobins.[16,24] The two *Hp*α^1 genes are located on different chromosomes. The difference between *Hp*α^{1F} and *Hp*α^{1S} is a point mutation resulting in a single substitution at position 54, which occurred in relatively recent times, speaking archeologically, since the apes show no such mutation.[25] A similar situation exists for the hemoglobins (see Volume 2, pp. 398–404).

In the case of Hpα^2, it is postulated that crossing over took place. This crossing over was unique in that it was unequal and during the process some of the codons were lost, and fusion then took place of the two genes, resulting in the synthesis of an elongated protein. This is

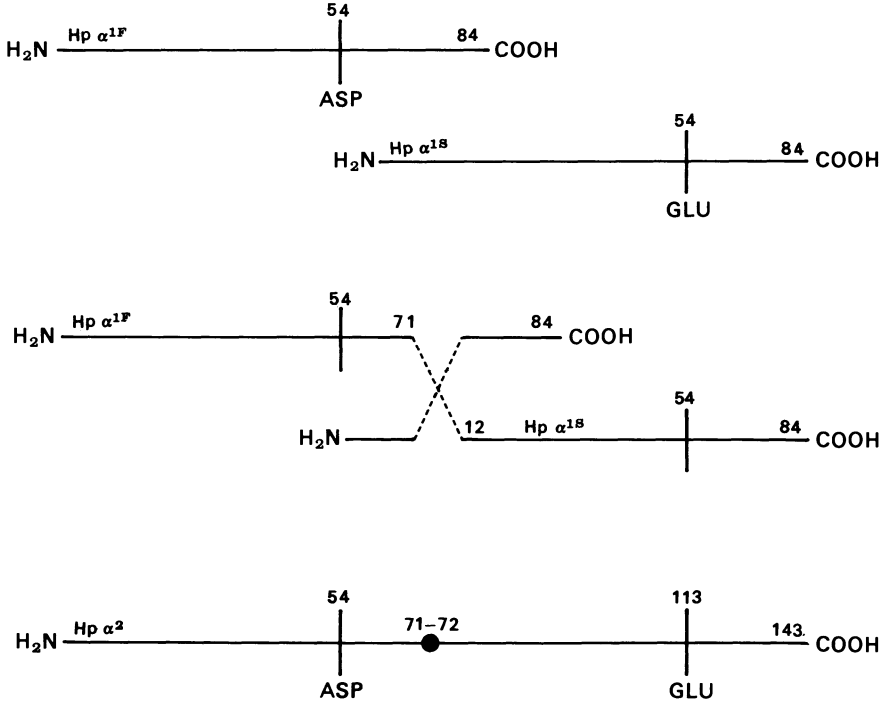


FIGURE 9.8 Representation of the unequal crossing over of the chromosomes carrying the genes for synthesis of Hpα^{1F} and Hpα^{1S} resulting in the formation of Hpα^2.

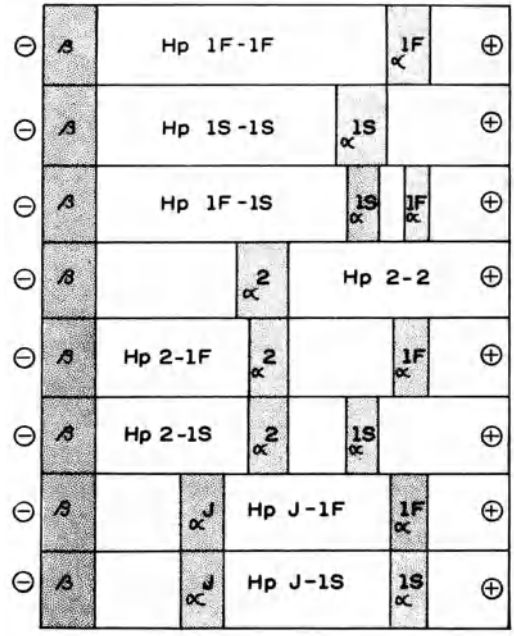


FIGURE 9.9 The three common phenotypes of haptoglobin after electrophoresis in gel in the presence of 8 mol/liter urea and mercaptoethanol at acid pH so as to release the α chains. The β chains remain at the origin (see Figure 9.5).

illustrated in Figure 9.8. A similar process is probably responsible for the *fusion hemoglobins*, such as hemoglobin Lepore (see Volume 2, p. 428).

On the basis of the discussion above, it is apparent that the haptoglobin types 1-1, 2-1, and 2-2 can be further classified on the basis of the type of α chains being carried. This is of significance, in finding the haptoglobin genotypes. This has been used in forensic medicine for determining parenthood. For this reason it is important to distinguish between Hp 1-1 which contains only α^{1S} or α^{1F} or is a hybrid of these two chains. For this purpose, the haptoglobins are first classified by their starch gel electrophoretic pattern at alkaline pH. The chains comprising the haptoglobin are then determined by electrophoresis at acid pH in the presence of urea and mercaptoethanol. Patterns for the α chains of the common phenotypes are shown in Figure 9.9. These are compared to the α chain's mobility of the *Johnson type* (*Hp J-1*) reported by many experimenters.[26] The Johnson α chain (α^J) is the result of unequal crossing over of genes producing a *partially triplicated* α^1 *chain.* This explains its slower mobility.

9.5.2 *Haptoglobin β Chains*

In all three types, Hp 1-1, 2-1, and 2-2, the β chain is identical, by electrophoresis, in acid–urea gels, immunochemically, and apparently in amino acid composition.[27] All of the carbohydrate attached to haptoglobin is attached to the β chain.[28]

The β chain contains 310 amino acids and has a molecular weight of about 45,000. The amino acid sequence indicates that there is about

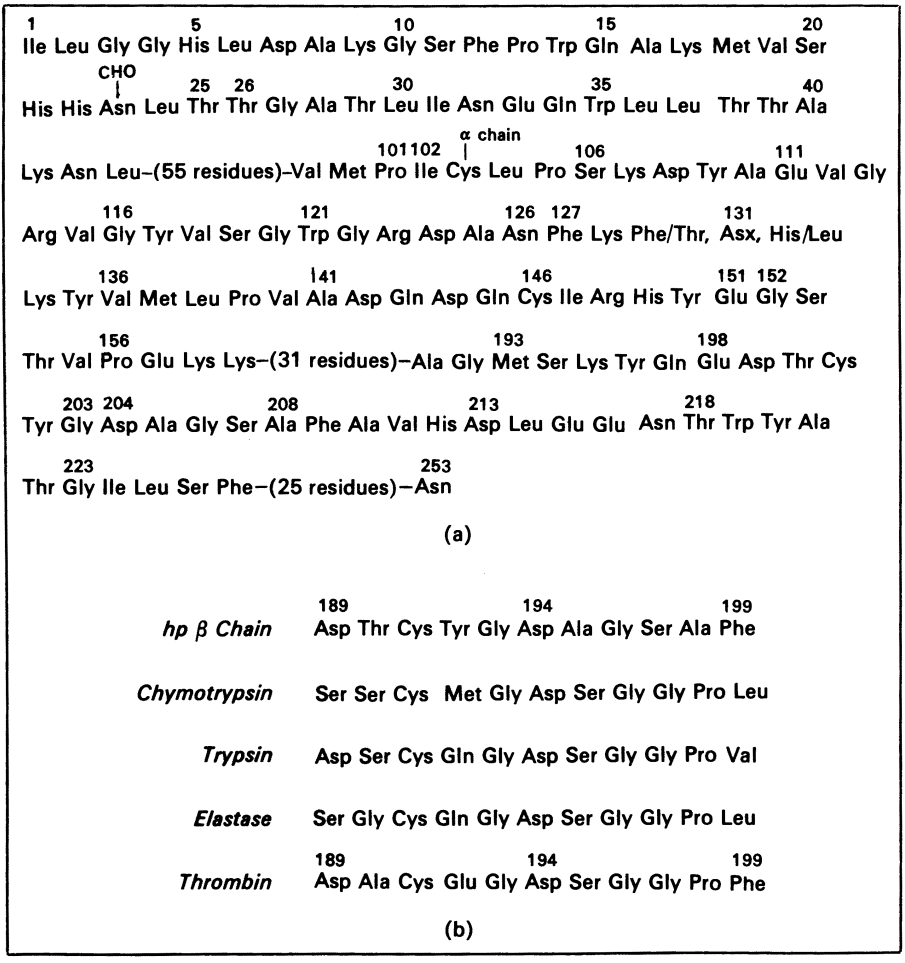


FIGURE 9.10 (a) Abbreviated sequence of the β chain of haptoglobin. (b) Sequence in the serine-195 region of Hpβ indicating homology with the serine proteases.

30–40% homology with the serine proteases, trypsin, thrombin, plasmin, chymotrypsin A and B, and elastase.[29] Haptoglobin, however, *per se*, does not have any protease activity. There is very little homology with the immunoglobulins.[30] Carbohydrate is attached to the chain at at least three positions, all being linked to asparagine residues (Figure 9.10). Carbohydrate is also attached to other sites such as asparagine at position 84.

Linkage between the β and α chains is at position 104 on the β chain. Linkage between the two α chains is at position 21. The β chain has two additional disulfide linkages, not utilized in binding the chains with each other (see Figure 9.2). The amino acid sequence of the β chain has been almost completely unraveled at this writing.[31] An abbreviated form of this is shown in Figure 9.10 so as to indicate a typical portion of the molecule with homology for the serine proteases.

9.6 *HAPTOGLOBIN CARBOHYDRATE*

Carbohydrate comprises about 20% of the weight of the haptoglobin.[19,31] It is attached only to the β chain.[32] Its presence increases the solubility of the protein and it is for this reason that the haptoglobins remain in solution when proteins are precipitated by 0.6 mol/liter perchloric acid. Galactose (5.3%) and mannose (2.5%) comprise the hexoses in the carbohydrate fraction. There is also 5.3% acetyl glucosamine and 5.3% sialic acid. Small amounts of fucose (0.2%) have been reported in this fraction.[28–33]

Neuraminidase removes about 70% of the sialic acid from haptoglobin. In the case of the haptoglobin–hemoglobin complex, this does not affect the peroxidase activity. This activity comes solely from the hemoglobin portion of the complex.

The carbohydrate seems to be linked to the haptoglobin molecule via an amide of aspartic acids (Figure 9.10). On hydrolysis of the β chain to smaller fragments, it appears that the carbohydrate is attached as oligosaccharides, at three or four sites on each β chain. Each oligosaccharide contains 5 to 6 moles of hexose (galactose and mannose), 3 to 4 moles of *N*-acetylglucosamine and 0 to 3 moles of sialic acid.[31]

9.7 *HEMOGLOBIN BINDING TO HAPTOGLOBIN*

Once formed, the haptoglobin–hemoglobin (Hp–Hb) complex is irreversible, *in vivo*. In this regard, haptoglobin is a "suicidal protein" rather than a transport protein.[4] The hemoglobin is bound tightly and is taken up by the parenchymal and RE cells by pinocytosis. The haptoglobin and hemoglobin are digested and the iron is recovered.

Free hemoglobin will tend to dissociate to form two $\alpha\beta$ dimers and filter through the glomeruli of the kidneys, resulting in the clogging of the tubules and kidney failure. Binding to haptoglobin produces a huge molecule, which cannot pass the basement membrane, and which also holds the heme more tightly, preventing its dissociation from hemoglobin.[34–36] The net result is the protection of kidney function, while recovering the iron, when hemoglobin is released into the blood stream, due to some hemolytic process.

The amount of haptoglobin normally present in the serum ranges widely, from 300 to 1900 mg/liter. The various types of haptoglobin bind about the same amount of hemoglobin (1 mg haptoglobin binding 0.77 mg hemoglobin.[37–39] The total amount of plasma (about 5% of the body weight) for a 70-kg man is approximately 3.5 liters. Assuming 1 g haptoglobin per liter, there would be a total of 3.5 g haptoglobin. This would bind 2.7 g hemoglobin at any one time. This calculates to about half the daily turnover of hemoglobin. Thus, normally, there is enough haptoglobin to bind any released hemoglobin. Since the affinity of haptoglobin for hemoglobin is great, there is normally no measurable free hemoglobin in the serum.

The Nature of the Hemoglobin–Haptoglobin Complex

Haptoglobin combines with globin and not with the heme part of the hemoglobin molecule. Haptoglobin will adsorb the globin of hemoglobin whether it carries the heme or not.[36] When heme is present, binding of heme to the globin is enhanced when hemoglobin is bound to haptoglobin.[4,34,35] In order for this binding to take place, it is necessary first to split the hemoglobin tetramer at the linkages which hold the $\alpha\beta$ complexes (dimer) of hemoglobin together (see Figure 9.5, Volume 2, p. 396). Thus the reaction for hemoglobin may be represented as $(\alpha\beta)_2 \leftrightarrow 2(\alpha\beta)$. The haptoglobin now binds the two hemoglobin $\alpha\beta$

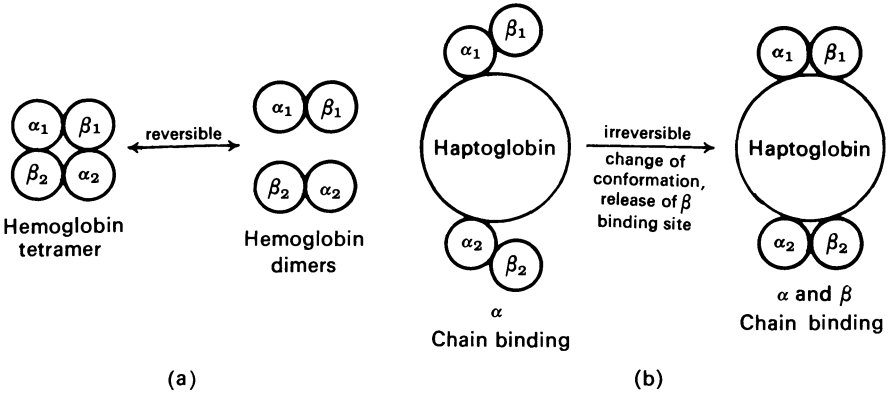


FIGURE 9.11 Steps in the binding of hemoglobin to haptoglobin. (a) Dissociation of hemoglobin to two $\alpha\beta$ dimers. (b) Binding first of α chain of the Hb dimer followed by binding of the β chain.

dimers at two different sites. The binding is first to the α chain of hemoglobin.[4,40] When partially saturated, the haptoglobin will then bind the β chain. Separated α chains, but not β chains will bind to haptoglobin. Only when partially saturated with α chains will haptoglobin begin to bind the β chain.

Hemoglobins which cannot form an $\alpha\beta$ dimer will not bind. For example, hemoglobin Barts (β_4) does not contain an α chain and does not bind to haptoglobin.[41] For similar reasons, myoglobin will not bind to haptoglobin. Binding to haptoglobin involves the tyrosine residues in haptoglobin.[42]

The steps, in the binding of hemoglobin to haptoglobin, are illustrated in Figure 9.11.

On electrophoresis the Hp–Hb complex moves somewhat slower than either haptoglobin or hemoglobin in gels, since the complex has the higher molecular weight.[43] Partially saturated haptoglobin moves at an intermediate rate. This is shown in Figure 9.12.

9.8 *HAPTOGLOBIN VARIANTS*

The mutations which have resulted in the variant haptoglobins, except for a few rare exceptions, are mutations in the α chain.[14]

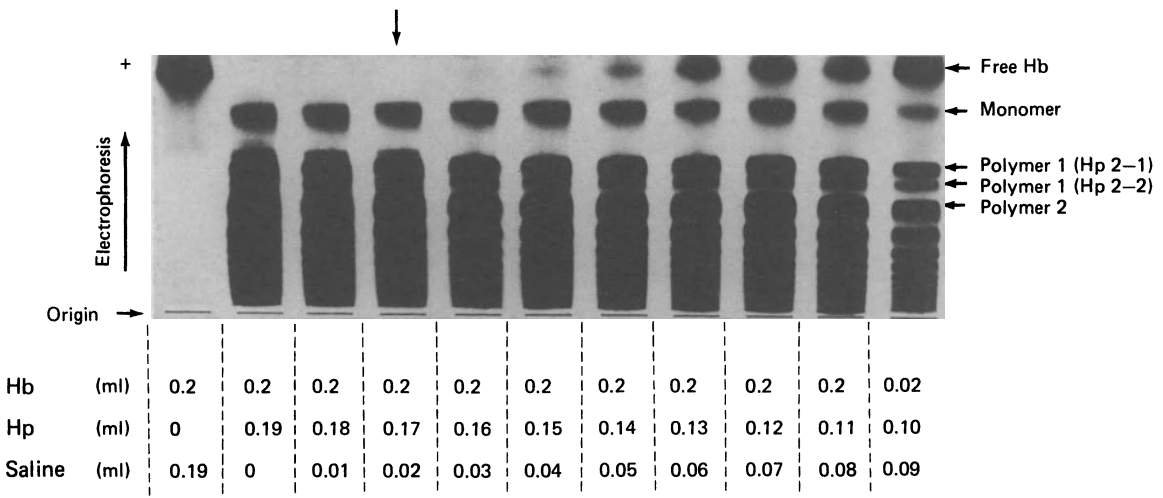


FIGURE 9.12 Estimation of haptoglobin binding capacity with acrylamide gel. Hemoglobin concentration is kept constant. Haptoglobin concentration is varied. When no excess Hb is apparent (0.17 ml Hp, arrow), then the binding capacity of the haptoglobin is determined. Note the presence of Hp-1 monomer and polymers. (Courtesy of Dr. Satoshi Funakoshi, Green Cross Co., Osaka 534, Japan.)

The types of mutation are analogous to those which resulted in the hemoglobin variants and may be listed as follows:

1. *A point mutation resulting in the exchange of one amino acid for another.* This is the case with the α^{1F} and α^{1S} chains. Examples in the hemoglobin series would be the S, C, and D hemoglobins.
2. *A chromosomal unequal crossing over and fusion resulting in the α^2 chain.* This is analogous to the Lepore and Antilepore hemoglobins.
3. *Change in control, leading to a change in the rate of synthesis of the α or β chains of haptoglobin.* This is analogous to the thalassemias.

About 25 abnormal haptoglobins of genetic origin have been reported around the world.[44] Most often, an unusual haptoglobin is encountered in a heterozygote. If the heterozygote is Hp 2-J, for example, then impressed upon the complex pattern for Hp 2, resulting from polymer formation, is that of the abnormal haptoglobin which may also form polymers. Add to this the fact that the electrophoretic pattern changes when the haptoglobin is complexed with hemoglobin, and the problem becomes even more complex. In addition, some haptoglobins like Hp 2-1D are not distinguishable when complexed with hemoglobin.[45] This is explained on the basis that hemoglobin is binding at the site of the mutation, resulting in a normal electrophoretic mobility. Others like Hp 1-P, Hp 2-P, and Hp 1-L show different electrophoretic mobility only when complexed with hemoglobin.[46–48] Hp 1-H is a variant observed both when complexed and not complexed with hemoglobin.

The *Johnson haptoglobin*, reported as the heterozygote with the α^1 or α^2 chain, such as Hp 2-J and Hp 1-J, has been referred to.[18,26] The α^J chain has molecular weight of 24,000 as compared to 8000 for the α^1 chain and 17,400 for the β^2 chain.[18]

A relatively common variant among Blacks is the *Hp 2-1M haptoglobin*, where M signifies the word *modified*. This variant shows the α^1 and first β^2 migrating bands but lacks the slower bands, showing an absence of the higher polymers (see Figure 9.3).[49,50]

Hp 2-1 (Marburg) is a rare β chain variant, demonstrated by immunochemical techniques, showing a β chain which will not cross react with normal β chain antibodies.[51] Similarly, *Hp 2-1 Bellevue* has also been demonstrated as a β chain variant, distinct immunochemically.[52] *Haptoglobin Porto Alegre* (Brazil) cross reacts immunochemically with normal β chain antibody but *is unable to bind hemoglobin.*

TABLE 9.3

Characteristics of Some of the Rarer Haptoglobin Variants

Phenotype found in gel at pH 8.6	Gene symbol	Comment and references
Hp-B	Hp^{B}	Contains an α^{B} chain with a mobility slower than α^{S} but faster than α^{2}. Hp 1-B gives three bands on gel, two with rates similar to Hp 1 and a third somewhat retarded.[101]
Hp 2-1 Bellevue	Hp^{Bell}	β Chain variant, discovered in American Negro, α chains are normal, β chain has higher mobility than the normal.[52]
Hp 2-1 Carlberg	Hp^{1Ca}	First rare haptoglobin observed. Discovered in Denmark, named after family. Result of decreased synthesis of Hp α^{1} chain.[99]
Hp 2-1D	Hp^{1D}	Observed in a British family. Not detectable when complexed with hemoglobin, Hp 1 band moves faster in gel than normal Hp 1. This difference disappears if complexed with Hb. Contains an α polypeptide α^{D} which moves faster than α^{1} in gel.[45]
Hp 1-H Hp 2-H	Hp^{H}	Observed in a British family. When complexed with hemoglobin, Hp 1-H resembles that of Hp 2-1, with extra band, just behind Hp 1. Slow moving bands of Hp 2-1 are missing, Hp 2-H resembles Hp 2-2, with an extra band midway between the first and second bands of a normal Hp 2-1 pattern.[48]
Hp 2-1 Johnson	Hp^{J}	Second haptoglobin discovered. Found in American Negroes. Observed also in Australia, Holland, Scandinavian countries, Hawaiian Chinese, and Kurdish Jews. The gene results from an unequal crossing over between chromatids of a homozygous 2-2, yielding a partially triplicated cistron, resulting

TABLE 9.3 (*Continued*)

Phenotype found in gel at pH 8.6	Gene symbol	Comment and references
		in an elongated α chain, about 3 times the length of α^1. Structure can be, FSS, SFF, etc., resulting in eight possible variants.[7]
Hp 2-L	Hp^{L}	Observed on gel only when complexed with hemoglobin. Noted in West Indies in individuals with Creole and Chinese ancestors. Pattern similar to Hp 2-2 with slightly increased mobility, when free of Hb. Mobility of α^2 bands, same as normal α^2.[100]
Hp 1-Marberg Hp 2-Marberg	Hp^{Mb}	Discovered in a German family. No difference in α^1 or α^2 chains. Difference noted in β chain. First β chain variant discovered.[51]
Hp 1-P Hp 2-P	Hp^{P}	Observed on gel only when hemoglobin is added. Mobility of α chain of Hp 1-P is the same as α^{1S} when free of hemoglobin. Difference apparently in Hb binding site. Observed in English family.[48]
Hp 2-1 (mod)	Hp^{mod}	Decreased synthesis of α^2 chain so higher polymers cannot be seen on gel. Occurs in 10% of American Negroes, α^1, β, and α^2 chains of normal mobility.[95,102] See Figure 9.4.
Hp 0	Hp^{0}	Almost complete suppression of synthesis of α^1, α^2, and β chains. Occurs in about 4% of adult American Negroes. No clinical symptoms associated with the deficiency.[49,102,103]
Hp 2-1 (Trans)	Hp^{2Tr}	Found in orientals. Pattern similar to Hp 2-1 in gels but with a change in concentration of bands. Bands 3 and 4 darker than in Hp 2-1 (mod). A decrease in rate of synthesis of α^2 chains.[54]

continued overleaf

TABLE 9.3 (*Continued*)

Phenotype found in gel at pH 8.6	Gene symbol	Comment and references
Hp 2-1 (Haw)	Hp^{2Haw}	Found in samples from Hawaii. Resembles Hp 2-1 (mod) but somewhat more of the higher polymers are formed. A defect in rate of synthesis of α^2 chain.[54]
Hp AB	Hp^{Ab}	Decreased synthesis of α^2 chain. Found in serum of Boston woman. Similar to Hp 2-1 (mod) but additional minor components moving ahead of the major bands. Seems to be a combination of a decrease in the rate of synthesis of the α^2 chain and an alteration in Hb binding.[54]

Whereas the normal β chain has a molecular weight of about 40,000, the Porto Alegre β chain has a molecular weight of only 30,000. The terminal residue is tyrosine for this variant, whereas the normal terminal amino acid is isoleucine. Thus it seems there is deletion resulting in a shorter chain.

Table 9.3 lists some of the characteristics of selected rare haptoglobins.

9.9 *QUANTITATIVE HAPTOGLOBIN VARIANTS*

Several haptoglobin variant genes have been noted, where the defect is in the rate of synthesis of the α^1 or α^2 chains. A common such variant, mentioned earlier, is Hp 2-1 (mod) where the slower moving polymeric bands are not observed. This results from a decreased rate of synthesis of the Hp α^2 chain. The higher polymers then occur in such low concentration that they are not observed. Hp 2-1 (mod) is noted in 10% of Blacks both from Africa and the United States.[49,50,53] The Hp 2-1 Carlberg variant results from diminished rate of synthesis of the α^1 chain.[54] Two other variants, which result from a decreased synthesis of the α^2 chain are Hp 2-1 (Trans) and Hp 2-1 (Haw). Hp 2-1 (Trans)

gives a pattern transitional between Hp 2-1 and Hp 2-1 mod and hence its designation. Hp 2-1 (Haw) gives patterns intermediate to that of Hp 2-1 and Hp 1-1.[54]

9.10 *ANHAPTOGLOBINEMIA*

Some individuals go through life without any haptoglobin in their blood serum. This is designated Hp 0.[54] About 32% of Nigerians and 4% of American Negroes have no haptoglobin. About 12% of Negro children have no haptoglobin, but some is detectable as they mature.[14,44,54] Generally, individuals with low haptoglobin levels of genetic origin are homozygous with two $Hp\alpha^2$ genes of the mod type. Since these individuals often do not show any disease symptoms, it has to be assumed that haptoglobin is not essential for survival.

Anhaptoglobinemia occurs in 80–90% of infants up to 3 months of age.[55] This results from the consumption of haptoglobin by the destruction and disappearance of fetal hemoglobin in the first few months of life. It also results from immaturity of the reticuloendothelial system. It can be detected in infants serum, however, in small amounts, immunochemically.[56]

After almost any hemolytic episode the haptoglobin in the serum disappears.[57,58] This is observed after heart lung surgery and as a chronic condition in individuals with artificial heart valves, even though the valves are functioning normally.[59] This results from continuous rupture of erythrocytes by these valves in their normal function.

9.11 *SERUM HAPTOGLOBIN CONCENTRATION*

In measuring haptoglobin levels, some use chemical techniques to measure the actual haptoglobin level to be expressed as mg/liter.[60] More commonly, the haptoglobin binding capacity (HpBC) is measured.[61] In this procedure, an excess of hemoglobin is added to serum. On electrophoresis the free hemoglobin moves away from the Hp–Hb complex and the latter may be measured colorimetrically. Using the formula, serum Hp concentration = HbBC × 1.3, the haptoglobin level may also be estimated (Figure 9.12).

The haptoglobin HbBC in men has been determined to be 1130 mg/liter ± 300.[36] On this basis, the average male would have in his serum 1469 mg Hp/liter; for women this value would be 1222 mg Hp/liter. Direct measurement shows a mean value for haptoglobin of 1280 ± 250 mg/liter for a pooled sample of men and women.[36]

The haptoglobin concentration in serum also varies with the phenotype. Values of 1360 ± 370 mg/liter and 1080 ± 370 mg/liter for Hp 1-1 and Hp 2-1, respectively, have been reported. For Hp 2-2, affinity for hemoglobin is substantially lower, and 820 ± 340 mg hemoglobin per liter are bound.[60,61]

Haptoglobin diffuses into the interstitial fluid, including ascitic, synovial, and blister fluid.[62–65] For Hp 1-1, the amount in the extracellular spaces is about the same as for plasma. For Hp 2-2, with its higher molecular weight, only a small percentage diffuses into the extravascular spaces.

Haptoglobin is synthesized in the parenchymal cells of the liver and has a half-life in serum of 5 days.[58,66,67] *Hp–Hb complex administered to humans intravenously leaves the blood stream with a half-life of 9 min.*[58,68] *On the other hand, the half disappearance time for hemoglobin per se is 5 days.* The Hp–Hb complex is taken up not only by the Kupffer cells[69] but also the parenchymal cells of the liver,[70,71] which probably accounts for its rapid disappearance from the serum. Some haptoglobin is also metabolized in the spleen and bone marrow.

When hemoglobin, in amounts sufficient to complex all of the haptoglobin, was administered intravenously to volunteers, the serum haptoglobin level fell to zero in 6–12 hr. It did not return to normal for about 5–7 days.

Haptoglobin synthesis is accelerated in the presence of stress and, as has been pointed out, is the major component of the acute phase proteins. Various mechanisms have been proposed for this phenomenon. One factor is the fact that the prostaglandins, whose production increases in stress, stimulate haptoglobin synthesis in the liver.[71,72] Insulin and cortisone will also stimulate haptoglobin synthesis in the liver.[73]

9.12 *HAPTOGLOBIN FUNCTION*

In acute and chronic infection, the serum haptoglobin along with the ceruloplasmin levels and other acute phase proteins rise

TABLE 9.4

Changes in Serum Haptoglobin Levels in Disease

Diseases in which serum haptoglobin levels are often elevated[a]	Diseases in which serum haptoglobin levels are normal[b] or low
Acute and chronic infections	Hemolytic disease (low)
Rheumatoid arthritis	Megaloblastic anemia (low)
Lupus erythematosis	Acute hepatitis (normal)
Acute rheumatic fever	Thalassemias (low)
Carcinoma	Trauma with hemolysis (low)
Aplastic anemia (normal to very high)	Nonspherocytic hemolytic anemias (low)
Biliary obstruction	Sickle cell anemia (low)
Chronic hepatitis	After transfusion (low)
Drug hepatitis	After cardiac surgery (low)
Acute and chronic nephritis	After renal dialysis (low)
Chronic pyelonephritis	
Peptic ulcer (mildly elevated)	
Ulcerative colitis	
Myocardial infarction	
Diabetes mellitus	

[a] Values above 2200 mg/liter are considered elevated.

[b] The normal value in human serum is 930 $\pm$ 1400 mg/liter (2σ).

sharply.[74] Serum haptoglobin levels also rise with certain malignancies.[75] In renal disease such as the nephrotic syndrome, urine levels of haptoglobin as high as 9 g/liter have been noted.[64] This has served as a means of obtaining large quantities of haptoglobins. The conditions associated with markedly elevated serum haptoglobin levels may be classified as diseases associated with inflammation. In these conditions there is often increased erythrocyte destruction and hemoglobin turnover. *This suggests that a major function of haptoglobin is to keep the plasma clear of hemoglobin so as not to interfere with the immunochemical processes which need to be activated in defense against disease.* Table 9.4 indicates changes in haptoglobin levels to be expected in disease.

When the haptoglobin system is saturated, hemoglobin, released into the plasma, circulates as such. Hemoglobin then dissociates into two $\alpha\beta$ dimers, each of molecular weight 32,000, which readily pass the basement membrane entering the glomerulus. Most of the hemoglobin is reabsorbed in the proximal tubule. The rate of tubular reabsorption in adults is about 1.43 $\pm$ 0.96 mg/min.[76] If more than 1.4 mg is

presented per minute, hemoglobin will appear in the urine. This occurs when serum hemoglobin levels exceed the 200–600 mg/liter concentration.[77,78] With inflammation, this may occur readily. For this reason, *another major haptoglobin function is to keep the kidneys clear of hemoglobin so that they can function more efficiently in clearing the body of toxic substances generated in the acute infectious process.*

A third function for haptoglobin is the conservation of iron. By preventing the loss of hemoglobin into the urine, it returns the iron to the liver where it can be reclaimed (see Figure 10.2).

Haptoglobin has other characteristics which suggest that it has other protective functions in inflammation. For example, it is a powerful noncompetitive inhibitor of some serine proteases especially cathepsin B, which is released with tissue injury.[79] Haptoglobin can protect the erythrocytes against agglutination by influenza virus.[80]

With these vital functions of haptoglobin, conditions of apparently normal metabolism in Blacks with genetic anhaptoglobinemia needs to be explained. In these individuals, reliance has to be on tubular reabsorption for serum hemoglobin iron conservation.

9.13 *MEDICAL APPLICATIONS*

Haptoglobin typing has been used for blood identification for forensic purposes. Early studies on the genetics of haptoglobin types established the fact that the haptoglobin types and subtypes were independent of the various blood groupings such as A, B, and O. This provided additional evidence for blood identification in cases of suspected crime or for purposes of establishing parenthood.[81,82] The ease with which the chains may be separated in acrylamide gel, places genotyping of haptoglobin within the capability of many laboratories.[83] Since we have a combination of α^F, α^S, and α^2 chains, six genotypes exist, and these have been shown in Table 9.1.

Serum haptoglobin levels have been used for diagnostic purposes. Elevated levels due to increased synthesis are noted in the inflammatory diseases, neoplasia, and severe stress.[44,74,75,84] In these conditions, elevation of levels to ten times the normal are not uncommon. On the other hand, haptoglobin concentration is decreased with severe hepatocellular damage. Since it is synthesized in the liver, it has been proposed as a

liver function test.[14] In hemolytic diseases, such as the thalassemias and sickle cell anemia, serum haptoglobin concentration has been proposed as a measure of the state of the disease. Thus if levels dropped to zero, the patient was going into a crisis. This has been shown to be of limited value, for even with a mild hemolytic episode, haptoglobin levels will drop to zero and not return to normal for about one week. Thus serum haptoglobin levels are chronically low in patients with the hemolytic anemias even when the condition is relatively mild.

Low serum haptoglobin values are especially difficult to evaluate in Blacks because of the genetic characteristics common to these individuals. These result in anhaptoglobinemia or chronically low serum haptoglobin levels because of the presence of the low rate of haptoglobin synthesis gene.

When patients have received massive transfusions or have been subjected to artificial heart–lung circulation or treatment with an artificial kidney, the haptoglobin level drops to zero because of the extensive hemolysis experienced. Some of these patients develop oliguria with substantial amounts of hemoglobin appearing in the urine which may eventually result in anuria. In some of these patients, administration of haptoglobin has served to clear the circulation of free hemoglobin and return normal kidney function.[59]

In one case, as an example, haptoglobin, prepared on a large scale from Cohn fractions IV and IV-1 was administered to a 53-year-old male who had a pelvic fracture, urethral rupture, and was in hemorrhagic shock. Before treatment, the daily output of urine was less than 100 ml with intensive hemoglobinuria which persisted for 12 days after orthopedic treatment. When 200 ml of a solution of haptoglobin (Hp to bind 2 g Hb/100 ml) was administered to the patient, the urine became yellow within 1 hr and the volume of urine in the next 12-hr period was over 500 ml. The patient recovered.[59] Similar observations were made on five of nine critically ill patients in this study.

These authors[59] have devised a system where blood containing excessive amounts of serum hemoglobin is circulated over haptoglobin immobilized on a gel, to remove free hemoglobin, returning the cleared blood to the patient. If this technique is found to be practicable it could have wide application in the treatment of the numerous cases of trauma, resulting in extensive hemolysis and kidney malfunction, which are presented to the trauma centers throughout the world, daily.

9.14 *HAPTOGLOBIN ASSAY*

The most commonly used method for haptoglobin assay uses hemoglobinometry, after the serum has been saturated with hemoglobin and the excess hemoglobin removed.[61,85] The hemoglobin can be removed by electrophoresis on starch or acrylamide gel, cellulose acetate or agarose,[85–88] or by gel filtration.[89,90] A more sensitive procedure is to add a substrate so as to measure the enhanced peroxidase activity of added hemoglobin. In this case separation of the hemoglobin may be avoided. For screening, this procedure may be automated[91] or carried out kinetically.[92–94]

In measuring the peroxidase activity of the Hp–Hb complex, the action of a peroxide on a suitable substrate is measured. The substrates used have been iodide ion, guaiacol, dianisidine, leucomalachite green, and others. The peroxides used are most commonly hydrogen peroxide or ethyl hydroperoxide. In these cases the reactions may be formulated as follows:

$$\underset{\text{Ethyl hydroperoxide}}{C_2H_5OOH} + 2I^- \xrightarrow[\text{complex}]{\text{Hp–Hb}} \underset{\text{Ethanol}}{C_2H_5OH} + I_2 + H_2O$$

$$H_2O_2 + o\text{-dianisidine} \xrightarrow[\text{complex}]{\text{Hp–Hb}} \text{colored quinone} + H_2O$$

When using [^{59}Fe]-hemoglobin, the separated haptoglobin–hemoglobin complex may be detected by its radioactivity.

Immunochemical methods, such as radial immunodiffusion, may be used for haptoglobin assay. By use of a polyvalent antibody for Hp 1-1 and Hp 2-2, the total haptoglobin serum level may be readily assayed. If individual types are to be assayed then a monovalent serum is utilized.[96] The immunochemical system may also be automated by measuring turbidity formed by the antigen–antibody complex.[97]

Rare variants of haptoglobin are identified by gel electrophoresis both at alkaline and acid pH in the presence of 8 M urea and mercaptoethanol.[97] Sodium dodecyl sulfate electrophoresis in acrylamide gel is used to note changes in molecular weight. Changes in hemoglobin binding affinity and enhanced peroxidase activity also serve to distinguish the variants. Changes in precipitation by rivanol also distinguish

some variants.[44] When a variant seems to be present, its immunochemical properties will usually specifically distinguish it from other variants. In the latter case, there are exceptions which have been presented above.

Haptoglobin protects hemoglobin from acid denaturation. This property has been used to assay for serum haptoglobin.[98] It may serve also to distinguish between haptoglobin variants.

9.15 *RECAPITULATION*

Haptoglobin (Hp) is a major component of the *acute phase reactants* which appear in the serum in increased amounts as a result of inflammatory reactions. It is a glycoprotein which is composed of four chains. Two are light chains labeled α and two are heavier labeled β.

Hp is characterized by its strong affinity for hemoglobin (Hb). It forms a complex with hemoglobin by adsorption of the α chains of the globin portion of the molecule. In the process, hemoglobin, which is tetramer, dissociates to two $\alpha\beta$ dimers. Haptoglobin adsorbs the α portion of the hemoglobin dimers first and then the β portion, forming a complex which is irreversible *in vivo*. This complex is then metabolized, mainly in the parenchymal and RE cells of the liver. Some is also metabolized in the spleen and bone marrow. In this way the iron is recovered at the expense of the destruction of haptoglobin.

There are three common phenotypes of the haptoglobins, labeled Hp 1-1, 2-1, and 2-2. They are the result of the fact that, commonly, there are two types of α chains, labeled α^1 and α^2. The β chains are identical in the three phenotypes. Thus Hp 1-1 may be written $2(\alpha^1\beta)$, Hp 2-2 is $2(\alpha^2\beta)$, and Hp 2-1 is the hybrid which may be written $(\alpha^2\beta)(\alpha^1\beta)$.

The α^1 chains can be broken down further into two common subtypes labeled α^{1S} and α^{1F}. The α^{1F} chain is so labeled because it has the faster mobility in the electrophoretic field. These chains differ by only one amino acid substitution in the chain. The α^2 chain is a result of an unequal crossing over of the $Hp\alpha^{1F}$ and $Hp\alpha^{1S}$ genes and a fusion of a major portion of the α^{1F} chain to a major portion of the α^{1S} chain. This results in a polypeptide (α^2) with a molecular weight that is almost double that of the α^1 chain. This explains the lower electrophoretic

mobility, in gels, of the α^2 chain and thus of Hp 2-2. The α^2 containing haptoglobins tend to polymerize, resulting in a complex electrophoretic pattern of the polymers.

White Europeans have a preponderance of α^2 chain in their haptoglobin. On the other hand, Blacks of Nigeria and the United States have a preponderance of α^1 chains in their serum haptoglobin. Mongols, such as the Chinese and Japanese, have an even higher percentage of α^2 chain than white Europeans.

Rare variants of the haptoglobins have been reported, mainly with mutations occurring in the α chain. A few with β chain differences have also been recorded.

A substantial percentage of the black population have no measurable haptoglobin (anhaptoglobinemia). Thus 12% of black children and 4% of adults show this genetic anomaly. This condition is referred to as Hp 0 and is most likely the result of the presence of a gene suppressing haptoglobin synthesis. This would account for the various gradations of this condition, from no Hp to moderately reduced amounts in different individuals. This would correspond to the thalassemia genes for hemoglobin.

Anhaptoglobinemia is also observed in a variety of pathological conditions. After a hemolytic episode, the haptoglobin often completely disappears from the plasma. In this case the haptoglobin is completely removed from the plasma by the excess hemoglobin. In newborns, haptoglobin does not appear in significant amounts in the plasma until 3 months of age. After surgery or trauma resulting in substantial hemolysis, plasma haptoglobin levels are reduced to the point where they are not detectable.

Increased serum haptoglobin levels are noted in inflammatory diseases, such as infection, the rheumatic diseases, the nephrotic syndrome and with malignancies. In these conditions, haptoglobin serves to clear the plasma of any hemoglobin released during the disease process. This is advantageous in preventing interference with the immunochemical reactions taking place in serum, such as antigen antibody reaction and complement fixation. Haptoglobin protects erythrocytes against agglutination by certain viruses. In defense against disease, it keeps the tubules of the kidney free of hemoglobin, so as not to interfere with excretion of toxic substances. Haptoglobin also inhibits certain proteases released during the inflammation reaction.

Reduced haptoglobin levels, in the absence of a genetic defect, suggest a hemolytic episode. For example, if the urine contains a heme-colored pigment and the serum haptoglobin level is low, it suggests hemoglobinuria. On the other hand, if the serum haptoglobin level is normal, it suggests myoglobinuria or excretion of a nonheme pigment.

Serum haptoglobin typing is useful for forensic purposes. Where a crime is suspected or in the blood typing for the determination of parenthood, determination of the nature of the haptoglobin chains adds an additional dimension to the process of blood identification.

The administration of purified haptoglobin to patients with hemorrhagic trauma has been successfully applied to clearing the blood rapidly of serum hemoglobin and improving kidney function. Where there is anuria in these patients it has been suggested that circulation of the patient's blood over haptoglobin, immobilized on an inert support, could serve to remove serum hemoglobin.

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10

Hemopexin: Iron Recycling

10.1 *HEMOPEXIN (β HAPTOGLOBIN)*

There is a significant amount of methemoglobin circulating in human plasma at all times. In this compound, the iron in the iron–porphyrin complex is in the trivalent state and does not participate in oxygen exchange. The prosthetic group is called ferriprotoporphyrin IX. The free base is called *hematin*, and the chloride salt *hemin* (see Volume 2, p. 469). Some use the expressions ferriheme for ferriprotoporphyrin IX and ferrihemoglobin to signify methemoglobin.

The affinity of ferriprotoporphyrin for the globin portion of the hemoglobin molecule is less than that for heme. As a result, the ferriprotoporphyrin has a tendency to dissociate from the molecule. Since it is rather insoluble, it would precipitate, except for the fact that it binds with albumin and with a glycoprotein called *hemopexin.* This name is derived from the Greek words *hemo*, meaning blood, and *pexis*, meaning fixation. Since it appears in the β globulin region on electrophoresis, and appears reddish, it was considered at first thought to be related to the haptoglobins and called *β haptoglobin.*[1–4]

Hemopexin is distinct from the haptoglobins, not only in the fact that it is located in the β globulin region on electrophoresis, but also in the fact that it does not bind the globin part of the hemoglobin molecule. It binds heme, ferriprotoporphyrin, protoporphyrin, deuteroporphyrin, mesoporphyrin, and numerous other porphyrins, which circulate in the blood, and which are devoid of iron.[1,2] It is located by saturating the plasma with heme and performing electrophoresis on

agarose, cellulose acetate, or acrylamide gel.[1–4] It moves at the head of the β region in the electrophoretic field (see Figure 3.5).

10.2 *HEMOPEXIN COMPOSITION*

Hemopexin, distinct from haptoglobin, is composed of a single polypeptide chain with a molecular weight of about 57,000.[5] Hemopexin contains no free sulfhydryl groups.[6] All the half cysteines form intramolecular disulfide bridges, and apparently take no part in porphyrin binding. They serve to maintain the conformation of the folded chain. The hexopexin molecule contains a very compact hydrophobic core.[7]

The amino acid sequence of hemopexin has not been completely worked out (as of 1980), but a substantial portion of the molecule from the amino nitrogen end has been elucidated.[6] There are 16 histidine and 15 tryptophane residues in the molecule. Amino acid analysis indicates the presence of about 552 amino acid residues, including 62 amide residues. Aspartic acid (45 residues), glycine (45 residues), glutamic acid (48 residues), and leucine (42 residues) are the most abundant of the common 18 amino acids present.[2,7]

About 22% of the hemopexin molecule is made up of two identical oligosaccharides, each with a molecular weight of about 5000 daltons. The linkages are *N*-glycosidic to aspartate as in haptoglobin. The hexoses comprise 9%, acetylhexosamines 7.4%, sialic acid 5.8%, and fucose 0.4% of the molecule. There are eight sialic acid residues on the carbohydrate chains.[8]

The extinction coefficient ($E^{1\%}_{1\,cm}$) of the apoprotein at 280 nm is 19.7. When bound to heme, the extinction coefficient at 180 nm is 21.8 and for the Soret band at 414 nm is 19.2[5,8,9] The partial specific volume (inverse of the specific gravity) for hemopexin is 0.702 ml/g.

10.3 *HEME BINDING TO HEMOPEXIN*[10–12]

Ferriheme and the various porphyrins bind with hemopexin in a 1:1 ratio. The dissociation constant (K_d) for the ferriheme–hemopexin complex is 10^{-13} M, indicating a strong affinity between these two

molecules, the free energy of the reaction being about 15 kcal/mol. For heme (Fe^{2+}), the porphyrins, protoporphyrin IX, deuteroporphyrin, and mesoporphyrin the dissociation constant is about 10^{-6} to 10^{-8},[10–14] indicating a much lower binding affinity for these substances. Bilirubin binds only very weakly to hemopexin.[15]

The Mossbauer and electron spin resonance spectra show that the iron in heme–hemopexin and ferriheme–hemopexin is of low spin, indicating the stability of the molecule.[13] This is in contrast to the less stable ferriheme–albumin complex (methemalbumin) whose iron is of high spin.[13,16]

An important observation is that in the binding of ferriheme to hemopexin a significant conformational change takes place. This is important in connection with the fact that the parenchymal cells of the liver, which metabolize the hemopexin–hemoglobin complex, must be able to distinguish it from the native hemopexin molecule.[17–19]

There is strong evidence that tryptophan residues of hemopexin are involved in the binding of heme to hemopexin.[19] When heme is bound to hemopexin, the fluorescence of the tryptophan residues are quenched, and their absorption spectrum is perturbed.[20] There is evidence that histidine residues are also involved in the binding of ferriheme.[21] This may account for the marked increase in the free energy of binding, when Fe^{3+} is incorporated into the porphyrin molecule.[22]

Hemopexin also binds the corrin ring (see Volume 2, p. 511) of vitamin B_{12} and the dye Rose Bengal.[23,24]

In view of the wide range of porphyrins which are bound, it is apparent that the binding site is sterically nonrestrictive. These facts are illustrated in Figure 10.1. In the figure, the hemopexin molecule is shown, exposing a single binding site. The tryptophan molecules then bind to the porphyrin part of the methemoglobin molecule, and the histidine residues bind to the Fe^{3+}. This forms a tightly bound complex. In the absence of histidine binding to Fe^{3+}, as with the porphyrins, the linkage is weaker. *This binding then changes the conformation of the molecules of hemopexin.* Figure 10.1 illustrates the requirement that there be a high affinity, specific binding site, and two coordinate colinkages with the Fe moiety of the ferriheme.[13] In support of the latter statement, carboxymethylation of the histidine residues of hemopexin prevents hemopexin coordination with the Fe of heme.[13,21]

The heme–hemopexin complex is removed from the circulation

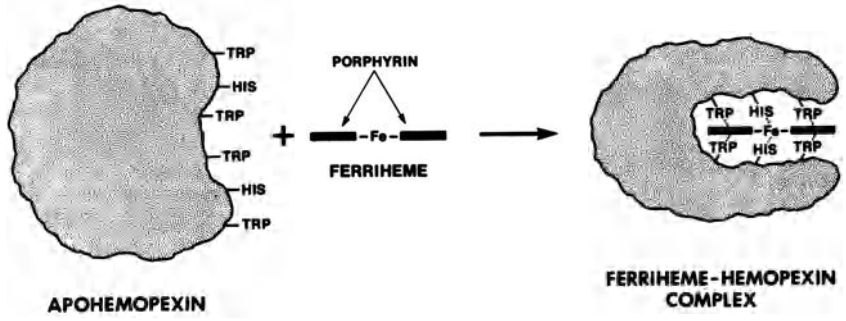

FIGURE 10.1 Schematic representation of the interaction of ferriheme with hemopexin, showing the change in conformation of the hemopexin molecule on binding. Trp and His represent tryptophane and histidine residues, respectively.

by the parenchymal cells of the liver.[25,26] Recognition of the complex results probably from the change of conformation of the hemopexin molecule. There is a quantitative difference between the rate of [^{125}I]-hemopexin and [^{3}H]-heme uptake from the heme–hemopexin complex by the parenchymal cells. After 1 hr, 60% of labeled heme is taken up but only 10% of labeled hemopexin, the remainder continuing to circulate.[26] This can be only partly explained by the exchange of the added complex with endogenous hemopexin. It suggests a possibility that unlike haptoglobin, hemopexin may be reutilized and is not metabolized by the hepatocytes. If this is so, then it would be a true transport protein like transferrin or ceruloplasmin.

Human serum hemopexin has a half-life of about 7.1 days.[26–28] When heme is injected intravenously, the hemopexin half-life drops to 0.8 days in healthy subjects. In patients with hemolytic problems, the hemopexin turnover rate is increased. For example, with sickle cell disease, the half-life of hemopexin is about 4 days. With erythropoietic portoporphyria, the half-life is about 6 days, and with porphyria cutanea tarda, about 4 days.

10.4 *HEMOPEXIN FUNCTION*

An obvious function of hemopexin is to remove circulating heme, ferriheme, and porphyrins. In this way it maintains the blood clear of substances which would interfere with the normal function of the plasma, such as

immunochemical defense against disease. The iron, in the circulating heme, is salvaged for reutilization. In this function, it complements the functions of transferrin and the haptoglobins.

Heme, released into the plasma, is taken up rapidly by albumin and hemopexin. Because of the high concentration of albumin, (40,000 mg/liter, as compared to about 750 mg/liter for hemopexin) most of the heme reacts with the albumin. This is then transferred to hemopexin. For example, in studies with [^{59}Fe]-heme incubated with albumin and then injected intravenously, 50% of the [^{59}Fe]-heme was transferred to hemopexin within 10 min. After 4 hr, only about 8% of the heme was still attached to the albumin.[29]

The heme–hemopexin complex, in distinction to haptoglobin, has no enzymic activity. It is not a peroxidase.[1–40,30] However, it has an important function in relationship to P-450 and B_5 metabolism.

The prosthetic group of the B cytochromes is heme, as found in hemoglobin (see Volume 2, pp. 484–495). P-450 is a B type cytochrome which occurs not only in the mitochondria but also in the microsomes. Cytochrome B_5 occurs in the microsomes. Both are prosthetic groups for the hydroxylation of numerous substances, such as steroids, barbiturates, hydrocarbons, and others. *Both occur in the erythrocyte.* When cytochrome P-450 is denatured, it goes to cytochrome P-420 (the numbers refer to a maximum wavelength absorbance of the CO–cytochrome complex). If apohemopexin is present, the heme of P-420 is transferred rapidly to hemopexin. Thus hemopexin has a role in the degradation of P-450, transferring the heme to the hepatocytes, where it is oxidized to biliverdin by heme oxygenase.[31]

There is another relationship between P-450 and hemopexin. As indicated in the pages cited in Volume 2 of this series, administration of certain substrates induces increased synthesis of P-450. Thus, for preparative purposes, the animal under study is given barbiturate, or a carcinogenic hydrocarbon, in order to induce an increase in P-450 levels. *When this is done, serum hemopexin levels are increased significantly.*[32] This is further evidence that hemopexin is involved, not only in heme transport, but also porphyrin transport to sites of heme synthesis.[33]

In the hemolytic diseases, serum hemopexin levels drop, since the hemopexin is being utilized to transport released ferriheme and heme to the liver.[34–36] Serum hemopexin levels are lowered markedly also in the porphyrias, for a similar reason. In these cases, the hemopexin is involved in the transport of the porphyrins to the liver for

catabolism. In porphyria cutanea tarda, serum hemopexin is almost undetectable.[37] Low levels are seen in erythropoietic protoporphyria, acute intermittent porphyria and other porphyrias. In patients with cutanea tarda, in one study, the amount of hemopexin in the blood was related inversely to the coproporphyrin level in the urine.[37] *Thus, hemopexin also functions in porphyrin transport.*

In its transport of heme and the porphyrins and exchange in the liver cells, hemopexin should probably be compared to transferrin and ceruloplasmin, rather than the haptoglobins, since hemopexin is probably reutilized after delivering the heme or porphyrin for catabolism.[38]

In the synthesis of δ-aminolevulinic acid (ALA), the action of ALA-synthetase, from glycine and succinate, is inhibited by the heme formed. This serves to regulate the rate of ALA synthesis (see Volume 2, p. 322). Apohemopexin inhibits the action of heme on the ALA synthetase system.[39] *Thus hemopexin is also involved, indirectly, in regulating the rate of heme synthesis.*

10.5 *SERUM HEMOPEXIN*

The hemopexin concentration in the healthy adult ranges from 500 to 1000 mg/liter.[3,4,40] Males and females have about the same concentration of hemopexin in their serum. Children older than 6 months also fall into the adult normal range.

Hemopexin concentration in the serum of the fetus, at 10 to 26 weeks of gestation, ranges from 1.0 to 7.2% of the mean adult level. This level rises so that in the neonate, serum hemopexin levels are about 30% of that found in the adult.[42] Hemopexin levels then continue to rise, until at 6 months of age adult hemopexin levels are reached. Pregnant mothers have high hemopexin levels but umbilical cord blood contains only about 320 ± 3 mg hemopexin per liter.[43]

In older individuals, especially after age 60, hemopexin levels tend to fall to somewhat lower values.

Hemopexin levels are low in the compartmentalized fluids, such as amniotic,[44] synovial, and cerebrospinal fluids.[45,77] However, in multiple sclerosis[46] the hemopexin concentration in cerebrospinal fluid rises markedly. It has been suggested that hemopexin be determined in this condition, as an aid in diagnosis.

Hemopexin is synthesized in the parenchymal cells of the liver.[47] Other organs do not produce this protein, and only liver cells are capable of hemopexin synthesis. In liver cells the rate of albumin synthesis compared to that of hemopexin is 10:1. Since the concentration of albumin in the serum is about 40,000 mg/liter and that of hemopexin is about 750 mg/liter, the ratio in the serum is about 53:1. This has suggested to some investigators that there exists a hemopexin pool which is involved in porphyrin and cytochrome metabolism and only part of the hemopexin formed is employed in clearing the serum of heme.

Hemopexin, because of its high polysaccharide content, will not precipitate with low concentrations of perchloric acid.[1] In serum, hemopexin is stable at 6°C for about two weeks. If removed from serum, it tends to polymerize lowering its heme binding capacity and releasing heme.[48] Apohemopexin polymerizes even more readily. On electrophoresis, polymer formation will occur, and heme will be released, if the temperature reaches 60°C.

The serum hemopexin concentration is under genetic control. When serum hemopexin levels were studied in identical twins (monozygotic), the values found differed from each other only within the reproducibility of the analytical system. This was not true for nonidentical twins (dizygotic). The variance of the values found for the latter were about that found in the normal population.[50]

10.6 *HEMOPEXIN IN IRON REUTILIZATION*

In Section 7.2.3, dealing with transferrin, the mechanism for the reutilization of iron in the human was outlined. With the explanation of the function of haptoglobin, ceruloplasmin, and hemopexin in this regard, an overall picture of iron disposal can now be presented.

Senescent deformed cells and cell fragments (*schistocytes*) are taken up by the cells of the reticuloendothelial system (RE cells, Kupffer or sinusoidal cells in the liver), and the hemoglobin is oxidized, so as to reclaim the iron. This is transported to various organs by transferrin.

Those cells that are lysed intravascularly will release hemoglobin which will give rise to oxyhemoglobin. The oxyhemoglobin will gradually be converted to methemoglobin. The oxyhemoglobin has

two possible pathways of disposal. One is to break up to two $\alpha\beta$ dimers, and pass the kidney basement membrane. Some will be reabsorbed, and some will pass out into the urine as recombined hemoglobin. A substantial portion of the hemoglobin, $\alpha\beta$ complex will be adsorbed to haptoglobin irreversibly. Some methemoglobin will also bind to haptoglobin. The Hb–Hp complex will reach the hepatocytes to be catabolized, so as to release the iron.

A substantial part of the methemoglobin generated will lose its ferriheme which will be picked up by albumin, and be transferred to hemopexin, if adequate amounts of hemopexin are present. The ferriheme–hemopexin complex will move to the hepatocytes where the ferriheme will be oxidized to biliverdin, releasing the iron for reuse. The globin portion of the methemoglobin molecule will also bind to haptoglobin and move to the hepatocytes for catabolism.

The above mechanism is outlined in Figure 10.2.

10.7 *SERUM HEMOPEXIN IN DISEASE*

Although only a mild degree of hemolysis almost completely depletes the blood stream of haptoglobin, this is not true for hemopexin. Only when plasma heme levels are elevated, does the hemopexin concentration fall significantly.[51]

With severe hemolysis, due to malfunction of grafted cardiac valves, severe iron deficiency and kidney malfunction may result. Various tests have been proposed for setting the guidelines as to when surgical intervention is necessary. These include, measuring the plasma hemoglobin concentration, counting the number of deformed or fragmented erythrocytes, measuring serum lactate dehydrogenase, urinary hemosiderin excretion, or following the half-life of isotope-labeled erythrocytes. The serum hemopexin concentration may also serve this purpose. This level correlates inversely with the plasma heme level, schistocyte count, or hemosiderin excretion.[7]

In the amniotic fluid of a normal pregnancy, the hemopexin level rises rapidly with advancing gestation relative to albumin. In the presence of significant hemolysis, the hemopexin:albumin ratio falls. If an increase in the bilirubin level is noted in the amniotic fluid (see Volume 2, p. 361) associated with a rapidly dropping hemopexin–

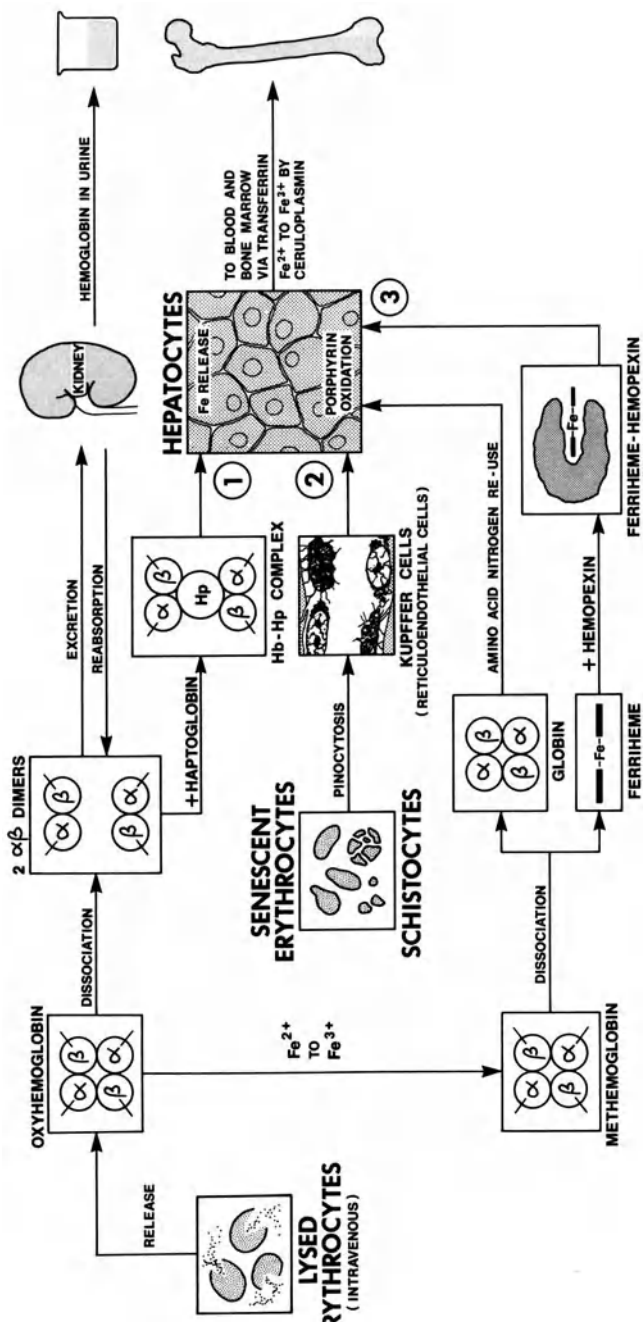

FIGURE 10.2 The role of haptoglobin, hemopexin, transferrin, and ceruloplasmin in iron conservation. 1, 2, and 3 represent the source for iron reclamation by the hepatocyte.

albumin ratio, an additional dimension is available for noting significant hemolysis in the fetus in erythroblastosis fetalis.

Hemopexin is not an acute phase reactant as is haptoglobin, in spite of its high carbohydrate content. Although C-reactive protein, and haptoglobin levels rise in severe infections and after trauma, serum hemopexin concentration does not change significantly.[52,53] This indicates a rigid control of the rate of synthesis and catabolism of hemopexin.

Low hemopexin levels are noted in the hemolytic anemias, including the thalassemias, sickle cell disease, and other genetic diseases resulting in the formation of abnormal hemoglobins, associated with severe hemolysis.[35,53] In hemorrhagic pancreatitis and in dengue with hemorrhagic fever, serum hemopexin levels are markedly reduced. Hemopexin levels are also low in paroxysmal nocturnal hemoglobinuria and pernicious anemia.[51,54,55]

When there is extensive liver damage, hemopexin levels are low because the polypeptide is synthesized in the liver. Thus, in severe alcoholic cirrhosis and with certain tumors invading the liver, serum hemopexin levels are lowered.[54] This is also true where there is malnutrition and decreased protein synthesis with low serum albumin levels as in kwashiakor.[36]

In kidney disease, with albumin excretion in the urine, hemopexin is also lost in the urine since its molecular weight is less but on the order of that of albumin. This results in low serum hemopexin levels.

Low serum hemopexin levels are also noted in the porphyrias. This is observed with cutanea tarda,[37] acute intermittent porphyria, erythropoietic protoporphyria, and with coproporphyria.[57]

Elevated hemopexin levels are found in diabetes mellitus,[58] in the muscular dystrophies,[59] and in various types of cancer.[60,61] Very high serum hemopexin levels are encountered in patients with rapidly proliferating melanomas.[60]

High hemopexin serum levels are also found in patients with Duchenne disease.[59,62] Duchenne's disease is a form of hypertrophic muscular dystrophy. A careful study of Duchenne carriers, which included definite, probable, and possible carriers, showed a mean serum hemopexin level of 663 mg/liter in 26 healthy controls and 964 mg/liter in the Duchenne carriers. These differences were statistically significant. There is no rise of hemopexin levels in other neuromuscular diseases.[62] A suggested reason for this elevation is the observation that there is alteration of the erythrocyte membrane in Duchenne patients

and carriers.[63,64] Release of small amounts of heme could act as an inducing agent to stimulate hemopexin synthesis.

The explanation for the increased serum hemopexin concentration in the conditions cited above probably stems from the fact that in some manner they induce increased hemopexin synthesis. It has been pointed out, that certain compounds other than heme[49,65] are inducers of hemopexin synthesis. In this group are the carcinogens 3,4-benzpyrene and 3-methylcholanthrene,[66] the porphyrinogens, heme, allylisopropyl acetamide, barbiturates, steroids, and others.[66,67] Lead salts are also inducers of hemopexin synthesis, since lead intoxication results in elevated serum and erythrocyte porphyrin levels.[68]

In contrast to the above compounds, diphenylhydantoin (dilantin), used in the treatment of epileptics, results in decreased serum hemopexin concentration.[52] Ethanol in small amounts induces hemopexin synthesis. With chronic use and severe alcoholic cirrhosis, hemopexin levels are lowered. Steroids tend to stimulate porphyrin synthesis. In this regard they simulate the effect of barbiturates (see Volume 2, p. 328). Steroids also cause an increase in serum hemopexin concentration.

TABLE 10.1

Some Conditions Which Result in Elevated or Depressed Serum Hemopexin Levels

Elevated serum hemopexin concentration	Depressed serum hemopexin concentration
Diabetes mellitus	Thalassemias
With neoplasms such as melanomas and other types of cancer	Sickle cell disease
Duchenne disease	Paroxysmal hemoglobinuria
Lead poisoning	Pernicious anemia
On treatment with certain drugs such as barbiturates	Severe hepatic cirrhosis
With steroid therapy	Iron deficiency anemia
With industrial poisons such as tars and other carcinogens	Erythroblastosis fetalis
	Hemorrhagic pancreatitis
	Dengue hemorrhagic fever
	Alcoholic cirrhosis of the liver
	Malnutrition
	Kidney disease
	Cutanea tarda
	Acute intermittent porphyria, proto- and coproporphyria
	Dilantin therapy

A summary of the diseases associated with increased and decreased serum hemopexin concentration is given in Table 10.1.

10.8 *HEMOPEXIN PREPARATION*

Methods of hemopexin preparation usually involve precipitation with rivanol so as to remove albumin, followed by precipitation with ammonium sulfate, ethanol, or a combination of these two substances. Fractionation is then done on DEAE-cellulose or Sephadex columns.[2,22,41,69–71]

Affinity chromatography has been shown to be a rapid method for preparing pure samples of hemopexin.[72] In this procedure, hematin (hemin) is immobilized on polyacrylamide gel (Bio-Gel, p-200). The hemopexin is selectively adsorbed to the column and the other proteins are eluted. The hemopexin is eluted from the column with 0.35 M glycine–HCl buffer, pH 2.4. It is interesting to note that if albumin remains in the solution to be eluted, it will also adsorb to some extent to the hematin. The hemopexin comes off the column complexed with some hematin since the buffer strips some of it off the column.[72]

10.9 *HEMOPEXIN ASSAY*

A practical method for hemopexin assay is by radial immunodiffusion. Kits for this purpose can be purchased from any of several commercial companies.[73] Two-dimensional immunoelectrophoresis (rocket electrophoresis) kits are also available commercially (Dako-immunoglobulins Ltd., Copenhagen).[74,75] Conventional immunoelectrophoresis is also practicable.[2]

A test used for determining methemoglobin in blood is also a measure of the blood hemopexin level. This is referred to as the *Schumm test*. In this procedure, ether is added to plasma, followed by a few drops of conc. ammonium sulfide. A hemochromogen forms with a sharp band at 558 nm, probably due to sulfmethemoglobin formation.[4,76]

10.10 *RECAPITULATION*

Ferriheme, derived from methemoglobin in the blood, is transported to the liver parenchymal cells, for iron recovery, by hemopexin.

Hemopexin is a glycoprotein containing about 2% carbohydrate, including sialic acid, hexose, hexosamine, and small amounts of fucose. The molecular weight of hemopexin is about 57,000 and it is made up of a single chain.

Hemopexin binds porphyrins, and the porphyrin part of the hematin or heme molecule at tyrosine residues. It also binds the iron in the heme or ferriheme (hematin or hemin) with histidine residues, producing a stable linkage.

Methemoglobin released into the blood stream dissociates, so as to release a globin and ferriheme. The globin is metabolized by the parenchymal cells of the liver so as to recover the amino acid nitrogen. The ferriheme is first adsorbed, weakly, to albumin which then transfers the ferriheme to the hemopexin. The hemopexin–ferriheme complex then goes to the parenchymal cells of the liver where it is oxidized to form biliverdin and Fe^{3+}. The iron is reduced and picked up by transferrin. The Fe^{2+} is oxidized to Fe^{3+} by ceruloplasmin and the transferrin–Fe^{3+} complex moves to the blood stream from where it reaches the bone marrow for erythrocyte production.

There is some evidence to indicate that hemopexin, like transferrin and ceruloplasmin is reutilized and not destroyed after hematin release. The hemopexin–ferriheme complex has no peroxidase or other enzymic activity.

Serum hemopexin concentration ranges from 500 to 1000 mg/liter in adults. Newborns have lower serum levels which reach adult values at 6 months postpartum. Very low levels occur in the compartmentalized fluids such as the cerebrospinal, synovial, and amniotic fluid.

Serum hemopexin levels are reduced with the hemolytic diseases such as the thalassemias and sickle cell disease due to the utilization of the hemopexin in heme and ferriheme transport. In the porphyrias, hemopexin serum levels are lowered since porphyrins are also transported to the liver by hemopexin. In severe liver disease or malnutrition, hemopexin synthesis is lowered and low levels will be found in serum. Dilantin therapy results in reduced serum hemopexin levels.

Increased synthesis of hemopexin, resulting in *high serum levels*, can be stimulated by a variety of inducers such as heme, carcinogens, barbiturates, griseofulvin, steroids, and a number of others which also induce P-450 production. It is postulated that the stimulation results from the need for hemopexin to transport heme to and from the site of P-450 production. This suggests a role for hemopexin in porphyrin synthesis.

Elevated serum hemopexin levels are noted in diabetes mellitus, with certain rapidly proliferating tumors such as melanomas and in the muscular dystrophies. Very high levels are found with Duchenne's disease, and it is claimed that Duchenne carriers can be detected by their high serum hemopexin levels.

Hemopexin production is under genetic control since serum hemopexin levels of identical twins are closer than would be expected from the normal distribution. At present, no hemopexin variants have been reported, nor has any individual been found without any hemopexin, even with a disease process.

Hemopexin is not an acute-phase reactant. Hemopexin serum levels remain unchanged during infection or inflammation.

Hemopexin, along with haptoglobin, transferrin, and ceruloplasmin comprise a system for conservation of iron and copper, and transport of the necessary materials for the synthesis of hemoglobin, myoglobin, and the cytochromes.

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11

Prealbumin I: Iodothyronine-Binding Proteins

11.1 *PREALBUMIN*

Prealbumin is predominantly a complex of *thyroxine-binding prealbumin* and *retinol-binding protein.* Thyroxine-binding prealbumin is well characterized and will be discussed first. For convenience, the nature of thyroxine binding globulin will also be discussed since the function of these two proteins is interrelated.

11.2 *THYROXINE-BINDING PROTEINS*

Other than albumin, thyroxine is transported by two proteins in serum. One comprises the major portion of the prealbumin fraction and is called *thyroxine-binding prealbumin* (*TBPA*). A second, which travels in the α_1 region on electrophoresis, is thyroxine-binding globulin (TBG).

Although the existence of a fraction which has a higher mobility than albumin was well known as early as 1942 and was demonstrated with the Tiselius apparatus, it was not isolated until 1962 by Schultz.[1,2] It was soon recognized that TBPA was bound to a second protein, retinol-binding protein (RBP), from which it could be separated.

11.3 *THYROXINE-BINDING PREALBUMIN (TBPA)*

TBPA contains no carbohydrate and is made up of four chains of equal composition, each comprising 127 amino acids. The amino acid sequence of the monomer has been determined.[3,4] From the amino acid composition, the molecular weight of the monomer is calculated to be 13,745. The tetramer has a molecular weight of four times this value or 54,980. This agrees with data obtained by sedimentation equilibrium and gel electrophoresis.[5] See Figure 11.1.

The three-dimensional structure of TBPA has been studied by the X-ray crystallographic method.[6]

The binding of thyroxine to TBPA is not very tight, the association constant being $1.3 \times 10^8\ M^{-1}$ for thyroxine (T_4). There is only one binding site for T_4 and one for triiodothyronine (T_3). *Complexing with the retinol-binding protein has no effect on thyroxine binding.*[7,8]

TBPA is synthesized in the liver. In the fetus, synthesis by the liver and yolk sac begins as early as 4 weeks after conception and soon reaches a level of 10 mg/100 ml. At delivery, in cord blood it is 13 mg/100 ml and in the mother's serum 23 mg/100 ml.[9]

On electrophoresis, in acrylamide gel, no polymorphism of TBPA has been observed in humans. However, in Rhesus monkeys three

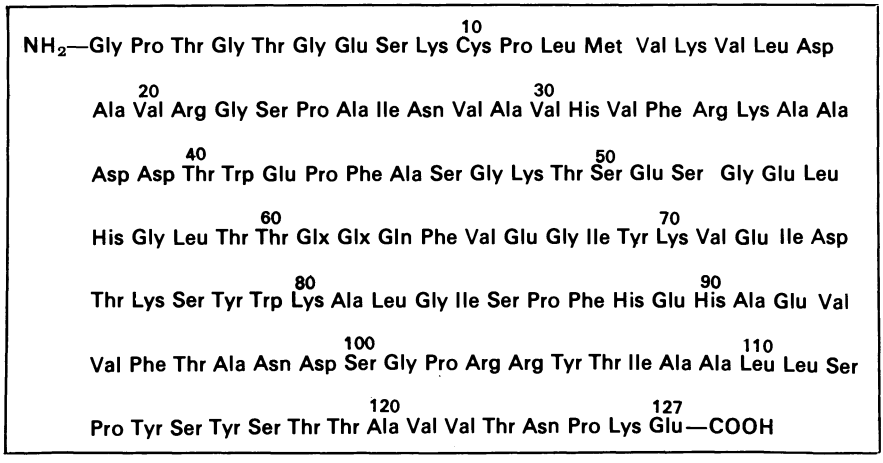


FIGURE 11.1 Sequence of subunit of thyroxine-binding prealbumin (TBPA). The molecule is composed of four of these subunits with an overall molecular weight of 54,980.

polymorphic forms have been distinguished. Serum from most monkeys exhibit a single band, similar to that observed in humans. As a result of a point mutation in the structural gene controlling TBPA synthesis, a valine–isoleucine exchange takes place, giving rise to an abnormal chain. Since TBPA is a tetramer, five possibilities exist. The tetramer may be made up of all normal chains (A_4), all abnormal chains (B_4) or intermediate conglomerates such as A_3B_1, A_2B_2, and A_1B_3. This results in five bands on the electropherogram similar to that observed with the isoenzymes of lactate dehydrogenase. This was the evidence which lead to the discovery that TBPA was a tetramer. The genetic variants of TBPA all react normally with the retinol binding proteins.

11.4 *THYROXINE-BINDING GLOBULIN (TBG)*

Thyroxine-binding globulin (TBG) is a plasma α_1 globulin. Distinct from TBPA, which contains no carbohydrate, TBG is a glycoprotein containing 6.7% of neutral hexoses, 2.4% sialic acid, 3.3% glucosamine, and 0.7% galactosamine.

The binding of thyroxine to thyroxine-binding globulin (TBG) is 10 times that of TBPA. The binding constant (measure of avidity) for TBG is 10^9 M^{-1} as compared to 10^8 M^{-1} for TBPA. Although TBG is present in small amounts (10–20 mg/liter) as compared to 100–400 mg/liter for TBPA, both proteins are of importance. TBPA carries 25% of the circulating T_3 and T_4 and TBG carries 75% of these substances. TBG moves with the α_1 globulins in the electrophoretic field at pH 8.6. It has been highly purified.[10,11]

In contrast to TBPA, thyroxine-binding globulin variants have been observed in man. These abnormalities seem to be linked to the X chromosome.[12,13] Female offspring of affected males all carry the trait. Male offspring of female heterozygotes are abnormal in half the cases, showing the trait in full expression, and do not transmit it to male offspring.

Three types of male are seen. Those with *no* TBG, those with *decreased* TBG levels and those with *increased* TBG levels. In the latter cases the TBG levels are at least three to four times the normal.[13] In one family, elevated TBG levels was found associated with goitre.[14]

Variation of TBG levels may not be associated with any effect

on thyroid hormone action. Individuals with TBG levels ranging from zero to four times the normal have shown no physiological effects.[15,16]

It must be kept in mind that albumin, TBPA, and even the lipoproteins bind thyroxine to varying extent, and can substitute for TBG.[17]

In addition, the active forms of thyroxine (T_4) and triiodothyronine (T_3) circulate free in the plasma. These comprise a very small portion of the total and it should also be noted that TBG from patients with genetic alteration has been shown to react normally to antibodies to TBG. Its electrophoretic mobility, heat stability, and thyroxine affinity is normal. Thus it is postulated that the defect in these patients is a defect in the structural gene which results in increased, decreased, or no synthesis, the product formed being normal in structure.[18]

11.5 *PLASMA THYROXINE-BINDING PROTEINS AND THYROID FUNCTION*

Historically, attempts to evaluate thyroid function depended on measuring the basal metabolic rate (BMR) and the serum levels of various lipids, thus total lipid cholesterol and carotene are elevated in hypothyroidism and depressed in hyperthyroidism. *The reasons for this is that thyroxin stimulates the oxidative cycle. Slow down of this system of metabolism results in accumulation of acetate.* This then condenses with itself to form acetoacetate (2 molecules) or mevalonate (3 molecules). The mevalonate gives rise to isoprene derivatives which form squalene and eventually cholesterol. This is illustrated in Figure 11.2.

Certain enzymes associated with normal liver function are also affected by the state of the thyroid. These include creatine phospho-

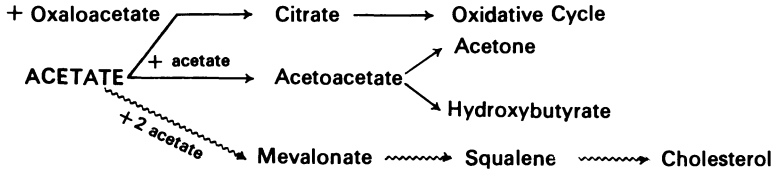


FIGURE 11.2 Pathway to cholesterol from acetate. The oxidative cycle is slowed down in hypothyroidism.

kinase, glutamic oxaloacetate, transaminase, and hydroxybutyrate dehydrogenase. However, the chemical tests are not specific and are not relied upon for diagnostic purposes except as screening or confirmatory tests. The BMR and Achilles tendon relaxation time (accelerated in thyrotoxicoses and delayed in myxedema), used for evaluation of the thyroid, are also altered in many nonthyroidal disease states. To confirm the diagnosis, biopsy had to be resorted to.

11.6 *ASSAY FOR THYROID FUNCTION*

The introduction of radioisotope techniques, particularly first the use of ^{131}I and then ^{125}I, ^{123}I, and ^{99}Tc, permitted a more scientific approach to the problem of chemical evaluation of thyroid function. Measurement of radioiodine and ^{99}Tc uptake by the thyroid and scintiscanning permitted the mapping of the thyroid with the location of malfunctional areas. This technique continues in use today.

Development of radioimmunoassay procedures permitted the testing for the presence of abnormal substances in the blood such as antibodies against thyroid antigens and the presence of the *long-acting thyroid-stimulating hormone* (*LATS*). These are of value in establishing the cause of thyroid dysfunction.

LATS is a 7S γ-globulin produced by lymphocytes within the thyroid, and is actually an antibody with hormonal action. Its action resembles that of TSH on the thyroid, except that its effects are prolonged. Ninety percent of hyperthyroid cases of the diffuse Graves' type and 60% of the uni- or multiadenomatous type show LATS activity in the plasma.

The ability to measure the thyroid-stimulating hormone of the pituitary (TSH) by radioimmunotechniques permits the measurement of the integrity of the pituitary–thyroid feedback system. This is done by measuring the ability of T_3 to suppress TSH release and thus thyroid function (T_3 suppression test). In addition, TSH may be administered and the release of thyroxine measured, to monitor the responsiveness of the thyroid gland to TSH. TRH (thyrotropin-releasing hormone from the hypothalamus) may be administered and TSH release measured as an index of the responsiveness of the pituitary to TRH. These tests permit the identification of the nature of the

problem in thyroid disease which may be secondary to pituitary or hypothalamic malfunction.[19] However, there was a need for a direct measurement of thyroxine released from the thyroid.

The concentration of thyroxine in plasma was first estimated from the amount of protein-bound iodine (PBI) present. The protein was precipitated, removing the inorganic iodine and other contaminants. After digestion, the amount of iodine present was estimated by its catalytic effect on the reduction of ceric ion by arsenite.[20] The system was automated and widely employed. The normal range is 4–8 μg PBI/100 ml.[21]

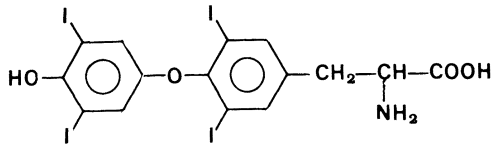
The iodothyronines (thyroxine and triiodothyronine) are extractable into butanol. This phenomenon gave rise to the test for *butanol extractable iodine* (*BEI*). The method of assay was as for PBI after butanol extraction. It was argued that by this procedure there was less interference from inorganic iodine and iodinated proteins. Normal values, by this method, are 3.5–6.5 mg BEI/100 ml.[22]

The laborious procedures used for PBI and BEI and ease of contamination with iodine resulted in the gradual reduction of the use of these procedures for thyroid evaluation. Need was felt for a simple method for the measurement of thyroxine in plasma directly. The introduction of radioligand procedures, for assay of trace substances in the plasma, was ideal for this purpose. Thyroxine-binding protein was first utilized as the binding protein for this purpose.

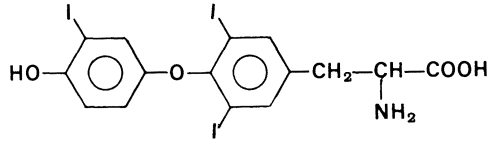
The competitive binding of T_4 and T_3 to the thyroxine-binding protein measures the total thyroid hormone concentration with a high degree of specificity. This is generally referred to as the *competitive protein binding test* or CPB. The formulas for T_3 and T_4 are shown in Figure 11.3.

In a typical procedure, serum is extracted with two volumes of ethanol. A portion of the extract is evaporated to dryness and dissolved in diluted human serum containing TBG and $^{125}IT_4$. After equilibrium has been established, the unbound T_4 is removed by adsorption to an anion exchange resin, often in the form of a sponge, and the supernatant is counted. The lower the percentage of radioactive T_4 which remains in solution (adsorbed to TBG), the more nonradioactive T_4 was present in the patient's serum to displace it. The normal range by this procedure is 4.2–9.4 μg T_4/100 ml.[23]

This procedure was gradually replaced by radioimmunoassay procedures using antisera specific for T_3 or T_4. Recently, with the



Thyroxine (3,5,3′,5′-Tetraiodothyronine T_4)
[*O*-(4-Hydroxy-3,5-diiodophenyl)-
3,5-diiodo-L-tyrosine]



3,5,3′-triiodo-thyronine (Liothyronine, T_3)
[*O*-(4-Hydroxy-3-iodophenyl)-
3,5-diiodo-L-tyrosine]

FIGURE 11.3 Structures of T_4 and T_3.

isolation and availability of purified TBG, there is renewed interest in total thyroxine procedures with the use of TBG.

In the procedure, using antibodies to T_3 and T_4, serum is first treated so that T_4 and T_3 are liberated from TBG and other proteins normally present in serum. This is done by removing, or inactivating the proteins, or by adding a binding competitor, such as barbital, diphenylhydantoin, or 8-anilino-1-naphthalene sulfonic acid. ^{125}I–T_3 or ^{125}I–T_4 and the antibody to T_3 or T_4 on a solid support, such as a resin or the wall of a plastic tube, are added. Endogenous hormone competes with the added radioactive hormone for binding sites. After equilibrium is attained, the solution is removed from the solid support and the solid support is counted. The higher the radioactivity on the solid support the lesser the amount of T_3 or T_4 present in the serum.[24,25] Alternatively, a solution of the antibody to T_3 or T_4 is added, and after equilibrium is established the antibody–T_3 or –T_4 complex is precipitated by a second antibody to the Fc portion of the antibody molecule. This is referred to as the *double antibody technique.*

Although T_3 and T_4 assays are quantitative measures of total serum thyroxine, it is clearly recognized that there are many *euthyroid subjects who give abnormal results. Conversely, there are many abnormal patients who*

yield normal results with these tests.[25,26] The reason for this is that T_3 and T_4 are bound, for the most part, to TBG, TBPA, and albumin. Less than 0.04% of T_4 circulates freely and this is the metabolically significant fraction. This is more difficult to quantitate and dialysis or chromatography needs to be employed to separate the free from the bound hormones. Recently introduced methodology for direct free T_4 assay has presented some difficulties.[26a,b] These procedures have been improved.

The extent to which TBG and other proteins are unsaturated is often measured by the *resin T_3 uptake test* (RT_3U). In this test, ^{125}I-T_3 is added to the serum and after equilibrium is established, the unbound ^{125}I-T_3 is removed with an anion exchange resin, or an antibody to T_3 on a solid base (affinity chromatography). The T_3 uptake is usually expressed as the percent of labeled T_3 which is not bound to the patient's TBG but is adsorbed to the resin and counted. The *free thyroxine index* is defined as the total T_4 multiplied by the ratio of the patient's *resin T_3 uptake* (RT_3U), divided by the control RT_3U.[26] Conventionally the procedures measure total T_4 and RT_3U.

$$\text{free thyroxine index (FTI)} = \text{total } T_4(TT_4) \times \frac{\text{patient's } RT_3U}{\text{control } RT_3U}$$

It can be shown, by mathematical derivation, that the FTI is a measure of the circulating free thyroxine. It is thus more closely correlated with thyroid function than any of the individual tests. The normal range of the FTI is 3.5 to 9.5. This parameter correlates very closely with free thyroxine levels determined by dialysis. The percent free T_4 as determined by dialysis is 0.03–0.045 of the total T_4, and for free T_3 is 0.22–0.46% of the total T_3. The normal range for TT_3 is about 90–160 ng/100 ml and for TT_4 is 4.2–9.4 μg/100 ml.[27]

Another test which purports to estimate free thyroxine concentration is the *effective thyroxine ratio* (ETR) test. After the T_4 is extracted from the patient's serum, a small amount (5 μl) of the patient's serum is added to the T_4 extract. Radioactive thyroxine plus TBG, as binder, is added and the mixture is incubated. The radioactive thyroxin adsorbed by a resin strip is directly proportional to patient's T_4 and inversely proportional to the patient's unoccupied thyrobinding protein sites, and thus proportional to free T_4. This kit can be purchased from Mallinckrodt Nuclear Chemical Co., St. Louis, Missouri.[27a]

Table 11.1 outlines the tests used to measure various facets of thyroid function. The concentration of the circulating hormones is measured

TABLE 11.1

Commonly Used Thyroid Function Tests

Thyroid gland uptake:	Radioiodine, technetium
Circulating thyroid hormones:	1. Total hormones: PBI, BEI, T_4, T_3
	2. Free hormones: T_3, T_4; T_4 and T_3 indexes; T_3, T_4 in urine; ETR
	3. Thyroid-stimulating hormone (TSH), LATS
Functions of thyroid hormone:	BMR, cholesterol, Achilles' reflex time, creatine kinase, serum tyrosine, red cell sodium, glucose tolerance

by the chemical tests, mainly by radioimmunoassay. The scanning techniques use radioiodine or technetium. For overall function, mainly on the oxidative cycle, the BMR test is still employed. Abnormal serum cholesterol levels and glucose tolerance curves often lead to the diagnosis of malfunction of the thyroid. Measurement of the thyroid-stimulating hormone helps in defining the nature of the thyroid defect.

11.7 *ABNORMAL SERUM TBG LEVELS*

Genetic abnormality in the composition of TBG may result in either increased or decreased TBG activity. In either case, inheritance is linked to the X chromosome. Since males possess only one X chromosome, their condition must be severe if they show the abnormality at all (hemizygous). Homozygous females are rare. More commonly they are heterozygous. Since one chromosome is normal, the condition is much milder than when it occurs in the male.[16,28]

Loss of TBG may be the result of disease. Thus in nephrosis, TBG is lost in the urine. *In liver disease TBG synthesis is diminished*, resulting in a lowered level in plasma.[33] *In thyrotoxicosis*, there is an increase in the rate of TBG destruction.

Increased plasma TBG levels may result from a congenital condition or may be acquired. *In hypothyroidism there is a decreased rate of synthesis of TBG.*[29] In certain diseases, such as acute hepatitis, myeloma and the collagen diseases, where increased metabolic activity is taking place in the liver, TBG synthesis is increased.[27]

Pregnancy results in increased TBG synthesis and the resin T_3 uptake test (a measure of free thyroxine) decreases. *If a decrease does not take*

TABLE 11.2

Relationship Between Changes in Plasma Thyroxine-Binding Globulin Concentration and TT_4, RT_3U, and TBPA

TBG level	TBG	TBPA	TT_4	RT_3U
Cause for decrease				
Inherited TBG deficiency (X-linked)				
Males (hemizygous)	0–trace	Normal	Very low	Very high
Females (heterozygous)	Low	Normal	Low	High
TBG loss (e.g., nephrosis)	Low	Low	Low	High
Increased destruction TBG (e.g., thyrotoxicosis)	Low	Low	Very high	Very high
Decreased synthesis TBG (e.g., liver cirrhosis)	Low	Low	Normal to low	High
Increased androgens from tumor or administered	Low	Increased	Normal to low	Normal to low
Increased adrenal steroids from tumor or administered	Low	Elevated	Low normal	Normal to high
Prolonged illness and inanition (nonspecific)	Low	Very low	Normal to low	High
Cause for increase				
Inherited TBG excess (X-linked)				
Males (hemizygous)	Very high	Normal	Very high	Very low
Female (heterozygous)	High	Normal	High	Low
Hypothyroidism (decreased TBG destruction)	High normal	High normal	Very low	Very low
Increased synthesis (e.g., viral hepatitis, myeloma, collagen, diseases)	High	Normal	Normal to high	Normal to low
Pregnancy (increased synthesis)	High	Low	High	Low
Oral contraceptives (estrogens)	High	Low	High	Low
Choriocarcinoma	High	Low	High	Low
Porphyria (acute)	High	Low normal	High	Low

place by the 10th week the fetus is in jeopardy.[31] The increased metabolism of pregnancy requires an increase in TBG, to handle the increased T_4 and T_3 production by the thyroid.

The dosing with contraceptive agents such as estrogenic steroids results in increased TBG production and T_4 levels. The resin T_3

uptake test is decreased. Similar phenomena are seen with choriocarcinoma and porphyria.

Hormone substitution therapy such as T_4 (synthroid), will raise TT_4 levels and lower resin T_3 uptake. With adequate dosage, patients on synthroid will correct T_4 and T_3 levels by peripheral conversion of T_4 to T_3. Administration of T_3 (cytomel) will suppress production of T_4 and TT_4 levels become very low.[31] However, it has a short biological half-life.

Table 11.2 summarizes the effect of abnormal concentrations of TBG on the TT_4 and resin T_3 uptake tests. TBPA levels are also indicated in the various conditions.

Certain drugs will bind to TBG binding sites, in effect, producing a TBG deficiency. This results in decrease in total thyroxin level and an increase in the resin uptake of T_3. Free T_3 levels are unaffected and symptoms, if any, are mild. Typical drugs with this effect are diphenylhydantoin (dilantin) and related compounds, salicylates, phenylbutazone (Butazolidin), clofibrate, the phenothiazines, and halofenate (MK-185). TBG and TBPA levels are normal when measured by radioimmunoassay in the presence of these drugs.

Assay for TBG in plasma is now readily performed by immunoassay procedures.[30–32]

11.8 *RECAPITULATION*

The proteins which transport thyroxine (T_4) and triiodothyronine (T_3) in plasma are *thyroxine-binding prealbumin* (*TBPA*), *thyroxine-binding globulin* (*TBG*), and albumin. Other proteins such as the lipoproteins transport small amounts of the hormones. Most of the transport is done by TBG ($\approx 75\%$) and TBPA ($\approx 25\%$).

TBPA is a tetrapeptide made up of four equal chains. The sequence of these chains has been elucidated. Although genetic variants of TBPA have been observed in monkeys, no variant has been reported in humans at this writing. TBPA binds the low molecular weight *retinol binding protein* (RBP) and prevents its loss in the urine. Most of the prealbumin fraction in serum is composed of the TBPA–RBP complex. TBPA contains no carbohydrate.

TBG is a glycoprotein which is found in the α_1 fraction on electrophoresis. Inherited defects in TBG synthesis have been reported. These

may result in decreased or elevated TBG levels in the plasma. The defect is X-linked and males are hemizygous. For this reason the condition is more severe in males.

Congenital TBG deficiency, associated with very low T_4 levels and hypothyroidism, have been reported. Conversely, congenital TBG increased synthesis and plasma levels have been observed associated with very high T_4 levels and hyperthyroidism. However, most of the cases reported of congenital aberration in the TBG plasma level are euthyroid. The reason for this is that the amount of free, circulating hormone is of the order of less than 0.04% of the total T_4 and 0.4% of the total T_3. It is these fractions which are biologically significant.

In TBG deficiency, TBPA and albumin can supply the necessary binding sites for transport.

TBG deficiency may be acquired. In Graves' disease (thyrotoxicosis) increased destruction of TBG is noted and plasma TBG levels are low. Since TBG is synthesized in the liver, severe liver disease with cirrhosis is usually associated with decreased TBG synthesis. This also results from malnutrition.

During pregnancy, the TBG level rises, along with increase in T_4 levels, required for the increased metabolism taking place. Some have used this observation as an indication of the well-being of the fetus. Elevated TBG levels are also noted in choriocarcinoma and with porphyria.

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12

Prealbumin II: Vitamin A(Retinol)-Binding Protein

12.1 *RETINOL-BINDING PROTEIN*

Vitamin A circulates in the blood in the form of the alcohol called *retinol*. Retinol is found bound to prealbumin on electrophoresis.[1] It was soon shown that the retinol-binding protein (RBP) of prealbumin which held the retinol was tightly bound to thyroxine-binding prealbumin (TBPA) in a 1:1 molar ratio. This complex of RBP and TBPA is the retinol transport system.[2–4] Figure 12.1 is a schematic representation of this complex.

The retinol-binding protein can be split from the TBPA and has a molecular weight of 21,000 and a sedimentation coefficient of 2.3S. It is a single polypeptide chain of 181 residues.[5] Some RBP circulates in the free form and is readily excreted in the urine. In proteinuria the retinol-binding protein appears in the urine in increased amounts.

The amino acid sequence of most of the retinol-binding protein has been determined and it seems to be unrelated to thyroxine binding prealbumin. Its carboxyl terminal sequence is Lys-Arg. Some of the retinol-binding protein in the urine has lost the terminal arginine. In this case it cannot bind to TBPA.[6,7]

A major advantage of the RBP being bound to TBPA is that in this form it cannot be readily excreted in the urine, since the molecular weight of the complex is 85,000. The half-life of RBP bound to TBPA in the human is 12 hr. When not bound, it is only 3.5 hr.[8]

The RBP–TBPA complex is the only carrier of retinol in the

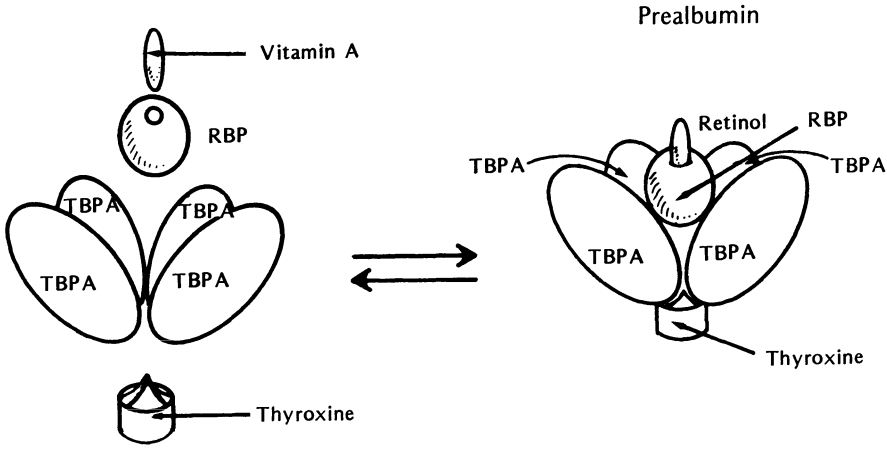

FIGURE 12.1 Schematic representation of the complex formed between thyroxine-binding prealbumin (TBPA), thyroxine, retinol-binding protein (RBP), and retinol (vitamin A).

blood. Normally, it is saturated to the extent of 75%. In proteinuria, sufficient RBP may be lost, to pose a serious threat to the transport of retinol to the tissues.

12.2 *RETINOL-BINDING PROTEIN AND VITAMIN A TRANSPORT*

RBP and TBPA are apparently synthesized separately by the liver and combine in the plasma. Vitamin A is obtained in the diet either as such or as β-carotene. In the intestine, esters of fatty acids are formed, especially with palmitic and oleic acids. These retinyl esters are absorbed via the lymphatic pathway in association with the chylomicrons[9] (see Volume 1, p. 202).

The vitamin A esters are then extracted from the lymph by the liver and stored. When mobilized, the esters are hydrolyzed to the free vitamin A alcohol (retinol) and the retinol combines with RBP, also synthesized by the liver, and secreted into the circulation. From there it goes to the various tissues where the vitamin A is consumed and the RBP is returned to the circulation. Any surplus free RBP may then be cleared by the kidney.

Normally, the filtered RBP is reabsorbed or catabolized in the renal tubules. In kidney tubular disease, the RBP cannot be metabolized and therefore increased amounts appear in the urine. The process described is summarized in Figure 12.2.

The various tissues where vitamin A is to be utilized, such as the retina, contain proteins with specific binding sites for the retinol binding protein and vitamin A.[10,12]

12.3 *RETINOL-BINDING PROTEIN IN DISEASE*

The normal level of retinol-binding protein (RBP) is 40–50 μg/ml, as compared to 200–300 μg/ml of TBPA. Generally, the concentration of RBP in serum correlates with the level of TBPA and vitamin A.[13] Since TBP is synthesized in the liver, it is not unexpected that *the level of RBP is low in liver disease.*[14] Since determination of RBP by radioimmunoassay is practicable, some have suggested its assay as a means of assessing the course of acute hepatitis.

Serum RBP concentration is low in hyperthyroidism but normal in hypoparathyroidism.

In *chronic renal disease*, the levels of RBP and Vitamin A are markedly elevated, although the TBPA level remains normal. This is explained in Figure 12.1. Since free circulating RBP is cleared by the kidney, it is felt also that in kidney disease, resulting in decreased filtration rate, the free RBP continues to circulate, which partially accounts for its higher concentration.

When oral vitamin A is given to patients with *cystic fibrosis of the pancreas*, vitamin A is not retained, and plasma levels are low.[15] Plasma RBP levels are also low. The exact relationship between the symptoms of cystic fibrosis and this phenomenon need to be clarified.

In malnourished children, such as with kwashiorkor, all plasma protein levels are low, including RBP and TBPA, as well as vitamin A levels. This condition is corrected with normal nutrition[16] even if supplemental vitamin A is not given. The rise in RBP levels is associated with a rise in vitamin A levels. Thus even if adequate stores of vitamin A are present in the liver they cannot be effectively utilized unless transported by the plasma proteins.

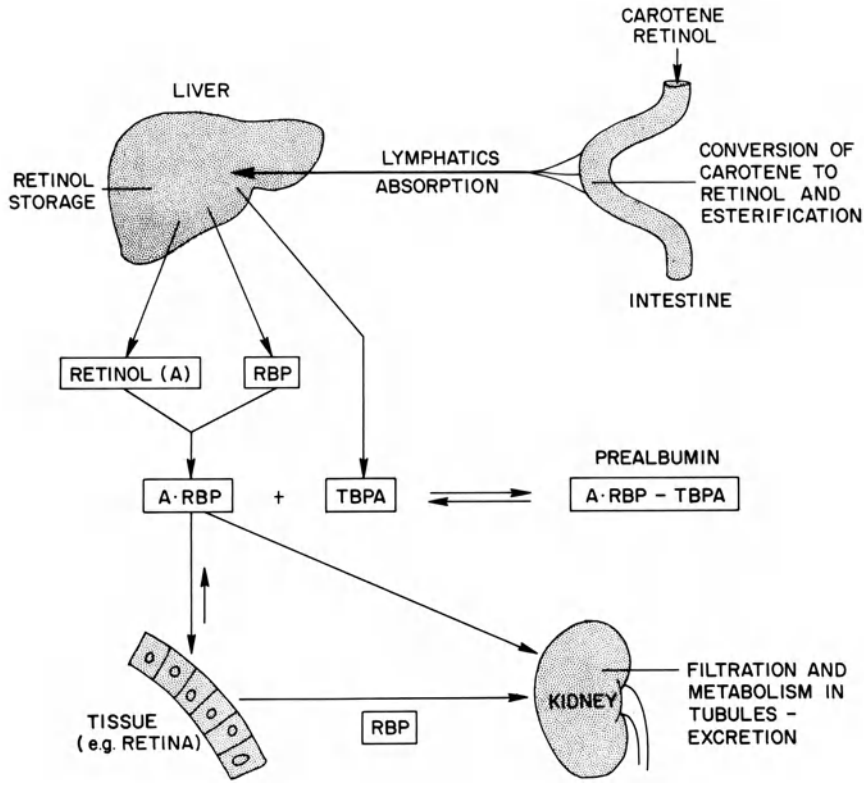

FIGURE 12.2 Absorption of vitamin A and transport by retinol-binding protein (RBP) and thyroxine-binding prealbumin (TBPA) complex. RBP is synthesized in the liver. Retinol is stored in the liver mainly as the ester with long-chain fatty acids (oleic and palmitic).

In certain ophthalmic disorders such as *retinitis pigmentosa* it has been postulated that a defect in the delivery of retinol to the retina may be involved.[17] Serum RBP levels are normal in this condition.[18]

In hypervitaminosis A, retinol, unbound to RBP, will accumulate in the lipid milieu of the various membranes in the body, resulting in their destruction. Thus retinol in excessive amounts is *membranolytic*.[19,20] In embryonic skeletal tissue, grown in organ culture, retinol bound to serum proteins other than RBP caused the degradation of the extracellular matrix of chick limb-bone rudiments. This apparently was the result of the rupture of lysosomal membranes by the retinol,

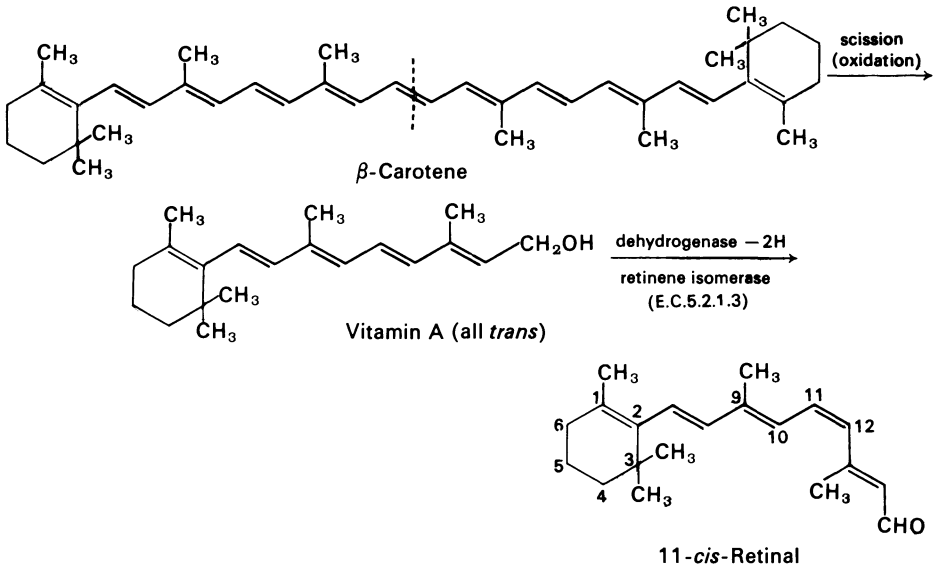

FIGURE 12.3 Relationship between carotene, retinol, and the active form of vitamin A, retinal. The point of scission to produce two vitamin A molecules from one carotene is indicated by a dotted line.

releasing the lysosomal hydrolases.[21] This could be prevented by adding RBP to the medium. Thus RBP serves to deliver retinol to specific binding sites where Vitamin A is to be utilized while protecting biological membranes against its effect.

The symptoms of hypervitaminosis A follow from the discussion above. Dry rough skin, cracked lips, and alopecia are noted early. Most of the observations have been noted in children receiving more than 100,000 USP units of vitamin A for several months. In these cases hepatomegaly and splenomegaly may occur. Cortical hyperostosis and arthralgia are commonly observed in these children.

The diagnosis is usually made with the aid of a history of high vitamin intake and the laboratory observation that serum vitamin A levels exceed 100 μg/100 ml. One microgram of retinol is equivalent to 3.57 U.S.P. units. Levels as high as 20,000 μg/100 ml have been reported. Normal serum vitamin A levels are 15–60 μg/100 ml.[22,23]

Carotene, ingested in excessive amounts, does not seem to cause hypervitaminosis A. However, serum levels in substantial excess of 400 μg carotene/100 ml impart a deep yellow color to the plasma (*carotenemia*).

This may result in *carotenosis*, in which condition the skin (not the sclera, however) assumes a deep yellow color, especially on the palms and soles of the feet. Normal carotene levels in the plasma, in the adult, range from 40 to 400 μg/100 ml depending upon the diet. In infants on a milk regimen, the carotene level rarely exceeds 80 μg/100 ml.

Figure 12.3 illustrates the structures of β carotene and its conversion to vitamin A and finally retinal (vitamin A aldehyde).

Vitamin A deficiency results from any of three major causes, a dietary deficiency, malabsorption, or a defect in the carotene conversion mechanism. Malabsorption occurs in the celiac syndrome, sprue cystic fibrosis, ulcerative colitis, cirrhosis of the liver, bile duct obstruction and related conditions.

Retinol (vitamin A) deficiency causes impairment of dark adaptation, xerosis of the conjuctiva and cornea, xerophthalmia, keratomalacia, and kerotinization of the epithelia of the lungs, intestines, and urinary tubules. Malformation of the endothelium which occurs results in greater susceptibility to infection. These symptoms result from the several functions of retinol in the body, especially its involvement in glycoprotein synthesis. See Section 5.4.1.

12.4 *RETINOL AND VISION*

Retinol is necessary for the conversion of photons to electrical signals at the retina which signal the central nervous system to translate the flow of electrons to form a visual image. In the dark, the retina is purple. The purple material, which comprises a retinal protein complex, is bleached when exposed to light. Several theories have been proposed for this conversion (see Figure 12.4). Vitamin A deficiency therefore results in "night blindness" as a first symptom.[24] Retinol, by changing its absorption spectrum when illuminated, is responsible for color vision.[24a]

Maintenance of normal differentiated epithelia and mucus secretions requires retinol. Retinol deficiency therefore results in dryness of the cornea and conjunctiva (*xerophthalmia*).[25] Vitamin A helps to resist the induction of squamous cell metaplasia in experimental cancer of the lung.[26] In pregnancy, the implantation of the fertilized ovum requires retinol. *Retinol deficiency therefore results in sterility* or, where a fetus develops, early abortion.[27,28]

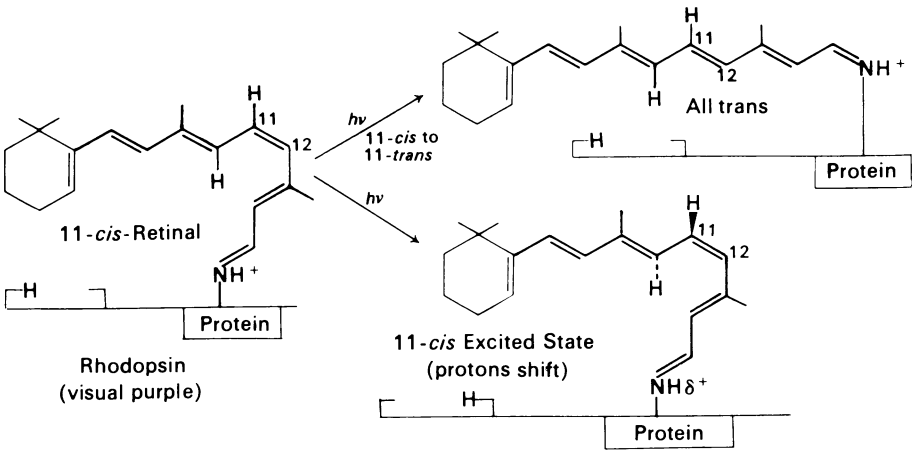

FIGURE 12.4 Two theories proposed for the action of a photon in bleaching rhodopsin, (visual purple) at the retina, resulting in signals to the central nervous system. One assumes conversion of the 11-*cis*-retina, to all-*trans*-retinal. The all-*trans*-retinal is then released from the protein. The second proposes that the configuration remains the same, but that a change in charge distribution occurs, with a shift in the protons of the protein, producing an excited state, thus stimulating the nerve ending. The retinal can then return to the ground state. Formation of *trans*-retinal is considered a thermal reaction, unrelated to the seeing mechanism [*Chem. Eng. News* **57**:25–26 (1979)].

12.5 *RECAPITULATION*

Retinol (vitamin A) is carried in the plasma by the retinol-binding protein (RBP). This complex is bound to thyroxine-binding prealbumin (TBPA) to form the prealbumin of the serum. RBP has a relatively low molecular weight and free of TBPA it can be cleared by the kidney. It serves to carry vitamin A to the various tissues.

In the intestinal tract carotene is converted to retinol. As the retinol is absorbed by the lacteals it is esterified and stored in the liver. As it is needed, the ester is hydrolyzed, and the released retinol binds with RBP synthesized in the liver. This complex then combines with TBPA and is transported to the various tissues. In the tissues, specific binding sites at the cellular level bind the RBP–retinol complex and release the RBP, retaining the retinol.

In the retina the retinol is oxidized to the vitamin A aldehyde,

retinal, which combines with a protein to form rhodopsin (visual purple). The bleaching of the rhodopsin by light stimulates the nerve endings, sending a message to the central nervous system which translates these signals to a visual image. Retinol is also involved in color vision.

RBP is synthesized in the liver. Serum levels are low in liver disease, malnutrition, cystic fibrosis, celiac disease, hyperparathyroidism, and related conditions. In these conditions, even though adequate stores of vitamin A are present in the liver, serum retinol levels are low and the patient shows symptoms of hypovitaminosis A.

In renal disease, RBP serum levels are elevated and this is associated with higher levels of circulating retinol.

Retinol functions in the maintenance of certain epithelial tissues and its deficiency results in xerophthalmia. It is also required for normal pregnancy and its deficiency results in sterility or early abortion of the fetus. It is involved in glycoprotein sythesis and thus the integrity of the membranes.

In hypervitaminosis A, retinol accumulates in the lipid membrane surrounding the lysosomes. This results in their disruption and release of hydrolytic enzymes. This causes disintegration of the cell membranes, cartilage, and the matrix of bone.

Intake of large amounts of carotene results in a carotenemia and may result in deposits of carotene in the skin. These do not cause any significant discomfort.

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13

Vitamin D Transport: Gc Globulins

13.1 *VITAMIN D AND HYDROXY-VITAMIN D*

After absorption, vitamin D is converted to 25-hydroxy-vitamin D in the liver (see Volume 1, pp. 149–154). From there it is transported in the plasma to the kidney and other organs for further metabolism (Figure 13.1).

Vitamin D and 25-hydroxy-vitamin D circulate in the plasma bound to a single protein.[1] The molecular weight of the protein is 52,000 and it travels postalbumin in the electrophoretic field in gels.[2] The sedimentation coefficient (s) determined in the ultracentrifuge is 3.49. Its isoelectric point is at about 4.8.[3] Serum concentration ranges from 400 to 450 μg/ml ($\approx 8\ \mu$mol/liter). The absorptivity, $E^{1\%}_{1\,cm}$, at 280 nm is 9.1.

Binding studies indicate that 1 mol of vitamin-D-binding protein (DBP) will bind 1 mol 25-hydroxy-vitamin D. Normally, even with good nutrition, only about 20% of the protein is saturated with 25-hydroxy-vitamin D.[4] 25-hydroxy-vitamin D concentration in the plasma is approximately 50 nmol/liter.[5] Vitamin D concentration in the plasma is approximately 160 nmol/liter.[6]

Amino acid analysis of DBP indicates that it is distinct from other proteins. Purified DBP cross reacts with the group-specific component (Gc) of plasma.[5,6] *Its amino acid composition is identical to that of Gc globulin and it is probably identical to that protein, to which, up until recently, no function was ascribed.*[4,12]

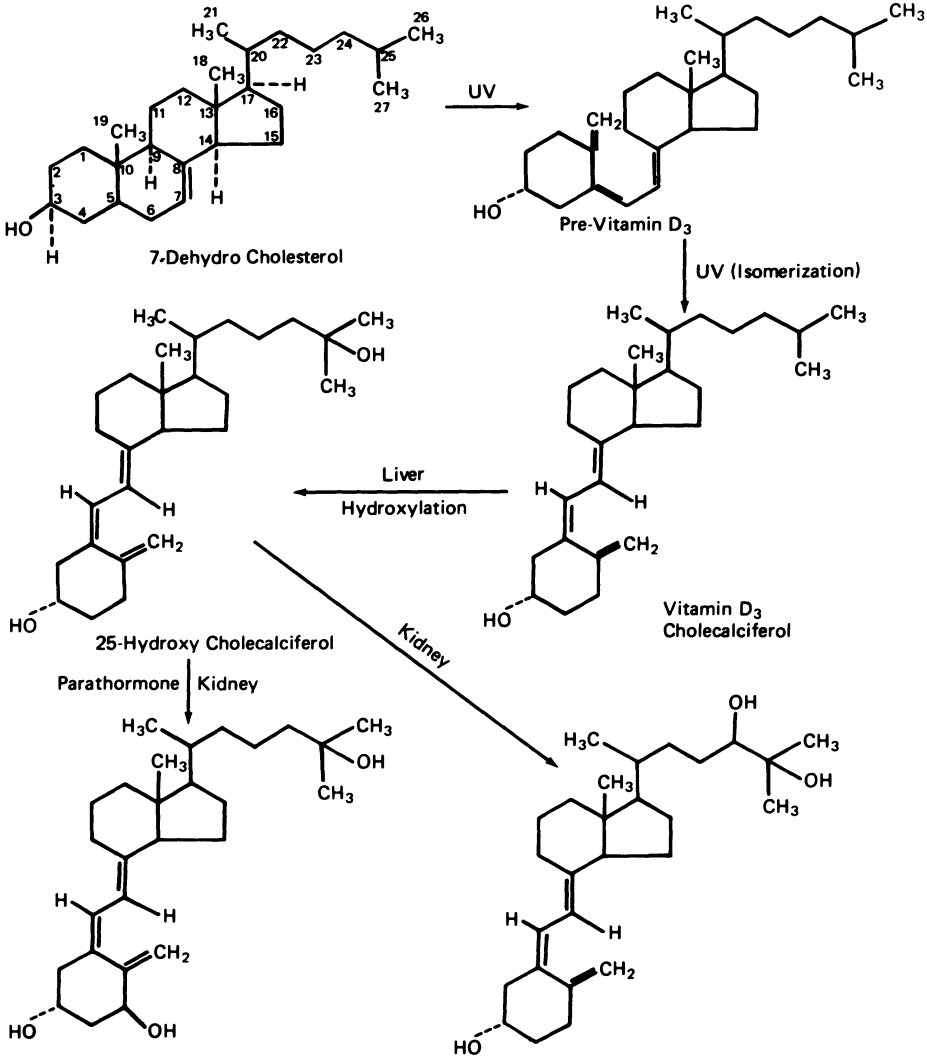

FIGURE 13.1 Conversion of 7-dehydrocholesterol to vitamin D and oxidation of vitamin D in the liver to form 25-hydroxy-vitamin D. Further oxidation takes place in the kidney under the influence of the parathyroid hormone to form 1,25-hydroxy-vitamin D which is the active form. DBP transports vitamin D to the liver and 25-hydroxy-vitamin D to the kidney (from Volume 1, p. 152).

13.2 *GROUP-SPECIFIC COMPONENT OF PLASMA, Gc GLOBULIN*

This glycoprotein was first studied by Hirschfeld.[7] The term Gc was given as signifying "group-specific component." This term was used because it was noted that there were three bands on electrophoresis, and the intensity of the various bands exhibited inheritable differences.

The Gc globulins contain approximately 4.2% carbohydrate, including 2% hexoses, 2% acetylhexosamine, and 0.2% fucose. Sialic acid is apparently absent.[8] The amino acid composition is apparently identical to that of the vitamin D-binding protein (DBP). It has the same molecular weight (52,000, approximately 434 amino acid residues), the same sedimentation constant (3.49S), and the same isoelectric point (4.8). Immunologically, it reacts with antibodies against DBP. A single precipitin line is obtained by any of various techniques for Gc globulin and DBP with antibodies prepared against either protein.[5]

The electrophoretic mobility of DBP and Gc globulins depends upon the electrophoretic conditions used. Thus DBP was first reported moving postalbumin and the Gc globulins with the α_2 proteins. However, when electrophoresis is carried out with both proteins under the same conditions, they have the identical mobility. Thus DBP also show α_2 mobility and the microheterogeneity of Gc globulin.[5,6]

The Gc globulin molecule has been reported to consist of three identical subunits two of which comprise the homozygotes Gc 1-1 and Gc 2-2. The third differs from these two forms.[9]

Gc globulin synthesis is detected in the fetus at approximately 4 to 10 weeks of gestation.[10] At 12 to 12 weeks it reaches about 7 mg/100 ml and 23 mg/100 at term. Maternal blood at term is about 46 mg/100 ml. Normally adult serum contains about 26 mg/100 ml of Gc globulin. The level in children's blood at 6 months of age is higher (32 mg/100 ml) than that found in adult blood.[11]

Until recently, no biological function was assigned to Gc globulin. In view of the above, it has major significance as the vitamin D and 25-OH-vitamin D transport system.

13.3 *Gc GLOBULIN VARIANTS*

Gc globulin has been studied extensively, especially for forensic purposes, by immunoelectrophoresis because of its polymorphism.

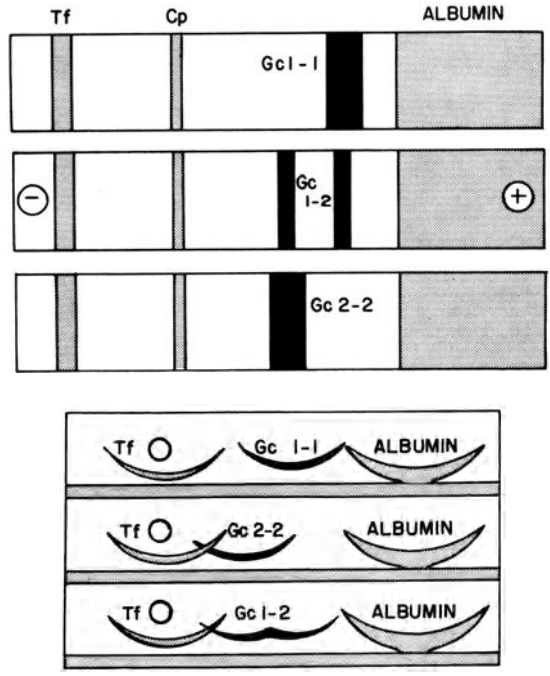

FIGURE 13.2 Schematic representation of Gc globulin polymorphism by electrophoresis on starch gel and by immunoelectrophoresis, showing location of the Gc 1-1, Gc 1-2, and Gc 2-2 bonds. Tf stands for transferrin.

Three common phenotypes are observed, Gc 1-1, Gc 2-1, and Gc 2-2. There is a faster component, designated Gc 1-1, which moves post-albumin. A second type, designated Gc 2-2, migrates in the α_2 region. A third phenotype, Gc 2-1, contains both forms in equal amounts (see Figure 13.2).

The polymorphism is apparently created by two autosomal codominant alleles, *Gc-1* and *Gc-2*. The *Gc-2* is rarer. Numerous population studies have been reported for almost all races in the world. Generally there is approximately 75% of Gc-1 and 25% Gc-2 among Europeans.[13] This ratio is approximately 90% Gc-1 and 10% Gc-2 among African Negroes.[14] Curiously, the Navajo American Indians have 98% Gc-1, whereas the Chippewas have only 69% Gc-1, the remainder being Gc-2 in both cases.[15] Gc globulin is present at birth and even cord blood shows the inherited phenotypes.[14,15]

Variants other than Gc-1 and Gc-2 have been described and have been variously labeled such as Gc-x, intermediate in mobility between Gc-1 and Gc-2, and Gc-y with an only slightly slower mobility than Gc-1.[14–16]

Gc-1 and Gc-2 have been further resolved and have been shown by acrylamide electrophoresis to be heterogeneous.[17] These probably represent, to some extent, variations in the carbohydrate portion of the molecule.

TABLE 13.1

Gene Frequency of the Gc Alleles Among the Major Ethnic Groups

Ethnic group	Number of cases	Gc^1	Gc^2
Caucasians			
Arabs	48	0.74	0.26
Czechs	254	0.72	0.28
French	221	0.73	0.27
Germans	4074	0.74	0.26
Jews (Ashkenazi, Poland)	99	0.66	0.34
Jews (Sephardic, N. Africa, Yemen, Persia)	48	0.77	0.23
Oraons (N.E. India)	125	0.74	0.26
Poles	1930	0.66	0.34
Punjabis	90	0.71	0.29
Scandinavians	2419	0.74	0.26
Mongols			
Chinese	210	0.77	0.23
Chippewas (Indians)	159	0.69	0.31
Eskimos	67	0.70	0.30
Koreans	122	0.70	0.30
Lapps	79	0.87	0.13
Navajos (Indians)	197	0.98	0.02
Negroes			
U.S.A.	361	0.91	0.09
African (Nigeria, Bondei, Congo, Baganda, Massai)	491	0.93	0.07
Mixed			
Aurukun (Australia)	74	0.74	0.26
Carolines (Afro-Mongol)	37	0.89	0.11
Irulas (Southernmost India)	61	0.90	0.10
Kurumbas (S. India)	52	0.64	0.36
New Guineans	45	0.72	0.28

The location of Gc-1 and Gc-2 is usually done by immunoelectrophoresis using sera with antibodies specific for these proteins. With the discovery that Gc globulin binds 25-OH-vitamin D, it is practicable to add [^{14}C]-labeled 25-OH-vitamin D to the serum to be tested and then perform acrylamide gel electrophoresis. The vitamin-D-binding proteins are then detected by scanning for radioactivity.[19] In this manner new variants are readily detected.

The polymorphism of the Gc globulins has served in forensic medicine for the exclusion of paternity and identification of blood stains and urines.[14,15] The two codominant gene frequencies (Gc^1 Gc^2) have been extensively studied throughout the world. In every case, Gc^1 is the more common variant. Table 13.1 lists the approximate distribution of the two alleles among various ethnic groups.[12–18]

Table 13.1 indicates that generally the Gc^2 allele is lowest among Negroes (10%) and highest among Poles, including Ashkenazi Jews (34%). In the Pacific Islands, the percentage of Gc^2 drops as the islands approach the African continent. Thus in the Carolines the percentages of Gc^1 and Gc^2 approach that of the Africans. This seems to be true also for the southern Indians (Irulas) who in the past have trafficked extensively with South Africa.

It will be noted that the percentage of the Gc^1 allele increases with the temperature of the climate. Since dehydrocholesterol is converted more readily to vitamin D under these conditions, it raises the question as to whether there is any advantage to the African having a higher percentage of the Gc^1 allele. The binding of both the 1-1 and 2-2 proteins for 25-OH-vitamin D seems to be about the same.

13.4 *VITAMIN-D-BINDING PROTEIN AND DISEASE*

The vitamin-D-binding proteins and 25-OH-vitamin D are synthesized in the liver. It is therefore to be expected that with chronic liver disease plasma DBP and 25-OH-vitamin D levels would be low. That this is the case can be seen from Table 13.2. With chronic renal disease and hypocalcemia however, plasma DBP levels were from normal to high. Hyperlipidemia tends to result in a slight elevation of DBP levels.

Serum DBP concentration does not seem to be correlated with

TABLE 13.2

Correlation between Serum DBP and 25-OH-Vitamin D Levels in Human Serum[a]

Subjects	Age range	DBP Concentration, mg/liter			25-OH-D Concentration, μg/liter		
		Number of cases	Mean	Range	Number of cases[b]	Mean	Range
Normal[c]	14–70	35	422	355–473	7	22.3	13.5–39.0
Hyperlipidemic[c]	24–72	66	451	354–551	6	17.6	5.1–31.4
Chronic liver disease[c]	40–74	5	202	148–262	5	2.6	0.6–5.4
Chronic renal disease[c]	19–71	9	418	286–524	4	23.4	15.7–31.4
Paget's disease[c]	44–71	6	432	410–466	6	20.9	16.8–25.2
Hypoparathyroid plus vitamin D[d]							
Hypercalcemic	F 40 M 43	2	422	329–515	2	286[d]	236–336
Normocalcemic	M 15	1	494	—	1	100[d]	—
Primary hyperparathyroidism	M 46	1	503	—	1	15.5	—

[a] See reference 5.

[b] Patients where 25-OH-D was measured.

[c] Males and females.

[d] 50,000–100,000 I.U. per day.

serum levels of 25-OH-D or of calcium. Major changes can occur in the concentration of these components without a corresponding change in DBP. This can be seen from Table 13.2, where large doses of vitamin D, resulting in a tenfold increase in 25-OH-D, did not change DBP levels significantly. In a single case of hyperparathyroidism the DBP level was slightly elevated.

At this writing there has not been sufficient time elapsed to completely explore the significance of DBP levels in various pertinent diseases such as rickets and related conditions. The effect of *variants* of DBP on 25-OH-vitamin D utilization and the health of the patient also requires exploration.

13.5 *RECAPITULATION*

Recent studies seem to indicate that the vitamin D transport protein (DBP) and Gc globulin are identical.

DBP binds both vitamin D and 25-hydroxy-vitamin D and is responsible for transporting vitamin D to the liver where it is hydroxylated. From there it is carried to the kidney where it becomes activated by a second hydroxylation to form 1,25-dihydroxy-vitamin D. Thus DBP should have a major role in calcium metabolism. This has not been demonstrated. It should be pointed out that albumin also transports vitamin D.

DBP is synthesized in the liver and low levels of DBP associated with low levels of vitamin D and 25-OH-vitamin D are found in chronic liver disease.

There are two types of Gc globulin commonly found in all ethnic groups. These give rise to three phenotypes designated 1-1, 2-2, and the heterozygote 1-2. This classification is based on the mobility of the Gc globulin in starch gel. The proportion of the two types varies substantially from ethnic group to ethnic group and from individual to individual. Blacks from the United States and Africa show a preponderance (ca. 90%) of the *Gc* 1 and 10% of the *Gc 2* allele. Caucasians show about 70% of the *Gc* 1 allele and 30% of the *Gc 2* allele. This has been used in forensic medicine to determine parentage.

The estimation of the percentage of each allele is usually done by immunoelectrophoresis on agarose. Other radioimmunotechniques are also practicable.

Variants have been reported which differ from Gc 1 and Gc 2 in their electrophoretic mobility, amino acid, and carbohydrate content. How these variants affect the transport of Vitamin D and its metabolites needs to be explored.

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14

Steroid Hormone-Binding Proteins

Steroid hormones bind to albumin but with low affinity, that is, with a relatively high dissociation constant (about 10^{-4}). However, because of albumin's high concentration in the serum, this binding is of substantial significance.[1] *The binding capacity of plasma albumin for the steroid hormones is far in excess of the physiological steroid hormone concentration.*

In addition to albumin, there are at least two proteins in the plasma which bind and transport steroids with some specificity. One binds cortisol, progesterone, and compounds closely related in structure. This protein is called *corticosteroid-binding globulin* or CBG.[2] A common name given to this protein is *transcortin.*

There is also a second protein in the plasma which binds and transports the androgen testosterone, the estrogen estratriol, and closely related compounds. This protein has been given several names, such as testosterone-binding globulin, estrogen-binding globulin, steroid binding β globulin, sex steroid-binding globulin, and 17-β-hydroxysteroid binding protein. The name used most commonly at present is, *testosterone-binding globulin*, abbreviated TeBG.[3]

These two steroid binding proteins will be considered here. The structures and nomenclature of the steroids are discussed in the Appendix.

14.1 *CORTICOSTEROID-BINDING GLOBULIN (CBG): TRANSCORTIN*

In studying the location of cortisol on the serum electrophoretic pattern, it was found to travel with albumin but also, in greater amounts,

with the α globulins.[1,2] Further studies indicated that this protein was mainly responsible for cortisol and progesterone transport. The name ascribed to this protein, *corticosteroid-binding globulin*, describes one of its properties. We will refer to this protein as *CBG* or *transcortin.*

Human CBG was first isolated in pure form from plasma by column chromatography on DEAE-cellulose followed by chromatography on an apatite column. The molecular weight, determined at that time, is approximately the same as that reported recently by several investigators.[2,4–6] From the sedimentation constant, $s_{20,w} = 3.66$, and other measurements, a molecular weight of about 49,500 has been calculated. The specific volume ($\bar{v}$) is about 0.701. The absorptivity of CBG, $E^{1\%}_{1\,cm}$, at 280 nm is about 7.07, and the dissociation constant for the cortisol–CBG complex is about 2.5×10^{-8}. This indicates only a moderate affinity.* For example, the hemopexin–hematin complex has a dissociation constant of about 10^{-13}. Thus hematin binds to hemopexin with 5000 times the avidity that cortisol binds to CBG.

The frictional ratio for transcortin is $f/f_0 = 1.42$. This suggests an elongated molecule. A frictional ratio of 1.0 represents a sphere.

CBG is a glycoprotein, and 27% of the molecule is made up of carbohydrate.[3–6] This is composed as follows: 17% galactose, 16% mannose, 2% glucose, 1% fucose, 19% acetylglycosamine, and 10% *N*-acetylneuraminic acid (NANA, sialic acid). It is most probable that the oligosaccharides are attached by *N*-glycosyl linkages.

CBG is composed of about 295 amino acids. Table 14.1 lists the distribution of these amino acids.

The *N*-terminal amino acid sequence has been determined as Met-Asp-Pro-Asn-Ala-Ala-Tyr-Val--. The C-terminal amino acid is serine.[4]

* The dissociation constant, *Kd*, is derived from the reaction

$$\text{cortisol–CBG} \rightleftharpoons \text{cortisol} + \text{BCG}$$

where

$$K_d = [\text{cortisol}][\text{BCG}]/[\text{cortisol–BCG}]$$

It is apparent that the smaller the number, the less the complex is dissociated. In order to get a positive number, which increases with binding, some report *binding* or *association constants* (K_a) which is the inverse of K_d and is reported in liter/mol instead of mol/liter. Thus $K_a = 1/K_d$.

TABLE 14.1

Amino Acid Composition of Human Transcortin (CBG)

Amino acid	Number of residues	Amino acid	Number of residues
Lys	15	Ala	17
His	9	½Cys	2
Arg	9	Val	23
Asp	33	Met	10
Thr	19	Ile	17
Ser	24	Leu	34
Glu	30	Tyr	9
Pro	9	Phe	18
Gly	16	Trp	3
		Total	295

14.2 *STEROID BINDING TO TRANSCORTIN*

Table 14.2 classifies some steroids and steroid derivatives as to their affinity for transcortin. It will be noted that only cortisol, progesterone, and closely related structures have a relatively high affinity for transcortin. Even simple oxidation of cortisol (dihydrocortisone) to cortisone decreases the affinity of the steroid for CBG significantly.[8,9]

Evidence has been adduced to suggest that a methionine residue of transcortin binds to the 11β-hydroxyl and 21-hydroxyl groups on cortisol, and that histidine binds to the keto group at position 20 (see Figure

TABLE 14.2

Relative Affinity of Various Steroids to Transcortin (CBG)

High	Moderate	Low
Cortisol (hydrocortisone)	Cortisone	Estrone
2-Methylcortisone	Deoxycorticosterone	11-dehydrocorticosterone
21-Deoxycortisol	11α-Hydroxycortisol	Δ5-Pregnenolone
11-Deoxycortisol	Pregnandiol	9α-Fluorocortisol
Progesterone	Testosterone	Pregnane-3α,17α,20α-triol
Prednisolone	Dehydroepiandrosterone	Tetrahydrodesoxycorti-
17α-Hydroxyprogesterone	Prednisone	costerone
	Dexamethasone	

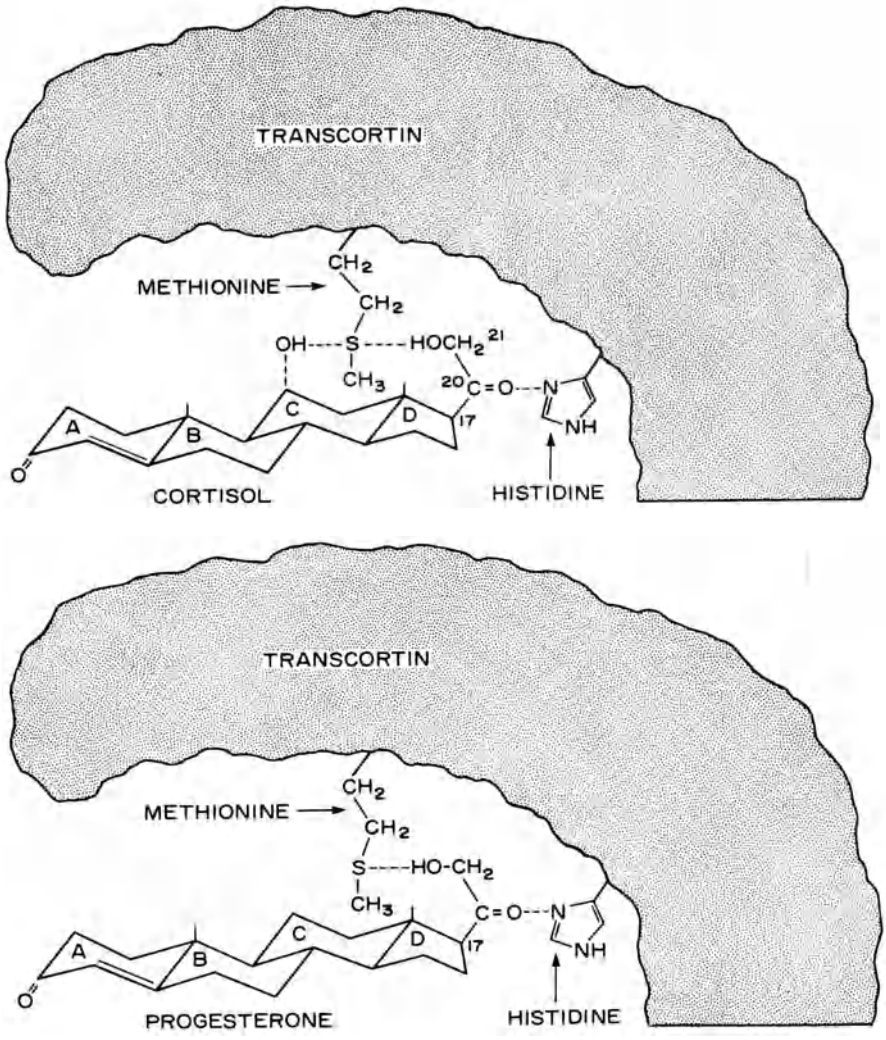

FIGURE 14.1 Schematic representation of binding of cortisol and progesterone to transcortin showing suggested involvement of methionine and histidine residues.

14.1). The steroid also seems to be held bound in the vicinity of the A and B rings.[9] Electron spin resonance studies suggest that the two-carbon chain attached at position 17 is oriented outward.[10] Since progesterone binds to transcortin but has no 11β-hydroxyl group binding at the A and B rings, the 21-hydroxyl and 20-keto groups probably

suffice to hold the steroid. Solution to this problem awaits elucidation of the amino acid sequence at the binding site.

In a recent report, the dissociation constants for cortisol and progesterone complexes with CBG were studied again. Values at 37°C, of 2.5×10^{-8} and 3.9×10^{-8} mol/liter were found for cortisol and progesterone, respectively.[21] In earlier studies, a value of 3.3×10^{-8} for cortisol was reported.[12] Testosterone and estradiol, with dissociation constants of 7×10^{-5} and 5×10^{-4}, are bound much more weakly.

From these studies it follows that cortisol is bound with 1.6 times the avidity to CBG as is progesterone. This difference may be attributed to the absence of the OH group at the 11 position for progesterone as indicated in Figure 14.1.

A technique used for measuring binding avidity comprises measuring the fluorescence of CBG. This fluorescence is due to tryptophan and tyrosine residues present in the protein. Certain steroids, such as 5β-pregnane-3,20-dione, bind and quench this fluorescence. If cortisol is present, it displaces the pregnanedione and restores the fluorescence. This suggests binding of cortisol to tyrosine and possibly tryptophane residues.[11]

CBG is partly helical in structure. The extent of this helicity decreases as the pH is lowered to pH 4–5. This is associated with a marked decrease in binding capacity. Below pH 4.0, the CBG molecule polymerizes.[13] Maximum binding occurs at about pH 8.

Apparently, the carbohydrate moiety is not involved in the binding of the steroids. Removal of the sialic acid has no effect on the binding effectiveness of CBG.[14]

14.3 *PLASMA TRANSCORTIN*

Most methods for measuring CBG concentration depend upon the saturation of CBG with cortisol and measuring the amount of bound cortisol. Results are expressed as μg CBG bound per 100 ml of serum or CBG binding capacity. Since the molecular weight of CBG is about 50,000 and that of cortisol is 362.5, and since there is only one binding site, the absolute concentration of CBG can be calculated.[15,16] Recently, however, the trend has been to determine transcortin directly by the use of a specific antibody and immunochemical techniques.[17]

The mean value found for CBG by radioimmunoassay in healthy adults in plasma is about 41.5 ± 5 mg/liter. The cortisol binding capacity ranges from 150–310 μg/liter, with a mean value of about 250 μg/liter. From this figure, multiplying by the ratio of the molecular weight of CBG divided by that for cortisone, a value of 50,000/362.5 × 0.250 = 34.5 mg CBG/liter is obtained. Thus, the binding capacity techniques and direct assay for CBG correlate with each other well enough so that the data can be utilized for clinical purposes.

14.4 *TRANSCORTIN PLASMA CONCENTRATION IN DISEASE*

Where plasma proteins are lost in the urine, as in the nephrotic syndrome, CBG is also lost in the urine, and plasma levels are reduced.[18] Plasma CBG levels are also low with malnutrition. In these cases, synthesis of CBG is decreased. In liver disease, CBG levels are lower.

A substantial percentage of the patients studied have low CBG levels, with no apparent direct cause. This has been noted in obese women, with overt or latent diabetes mellitus, and in some patients with hypertension. In one study of women with decreased plasma CBG levels of unknown etiology, 55% had some menstrual disturbance.[19] Low CBG levels have been also noted associated with gynecomastia, "pseudo-Frolich syndrome," and cryptorchidism.

Low CBG levels of genetic origin have been reported.[20,21] The mean concentration of CBG in one family was about 50% of normal. Plasma and urinary cortisol were in the normal range. No symptoms of hypo- or hyperfunction of the adrenals were noted in these cases.

Serum cortisol concentration varies widely during a 24-hr period. This is a result of episodic periods of secretion and resting during the day.[21] This is also reflected by changes in CBG synthesis rate at different times of the day. In a typical study, the serum CBG binding capacity was compared to the serum cortisol level for a 24 hr period.[22] The highest *serum cortisol level*, 142 μg/liter, was noted at 8 AM. This decreased gradually to a minimum of 38 μg/liter at midnight. From this minimum, cortisol levels rose gradually to 145 μg/liter by 8 AM of the next day. *CBG binding capacity for cortisol* was at a maximum of 214 μg/liter at 4 PM. This level dropped gradually to 105 by 4 AM, and rose

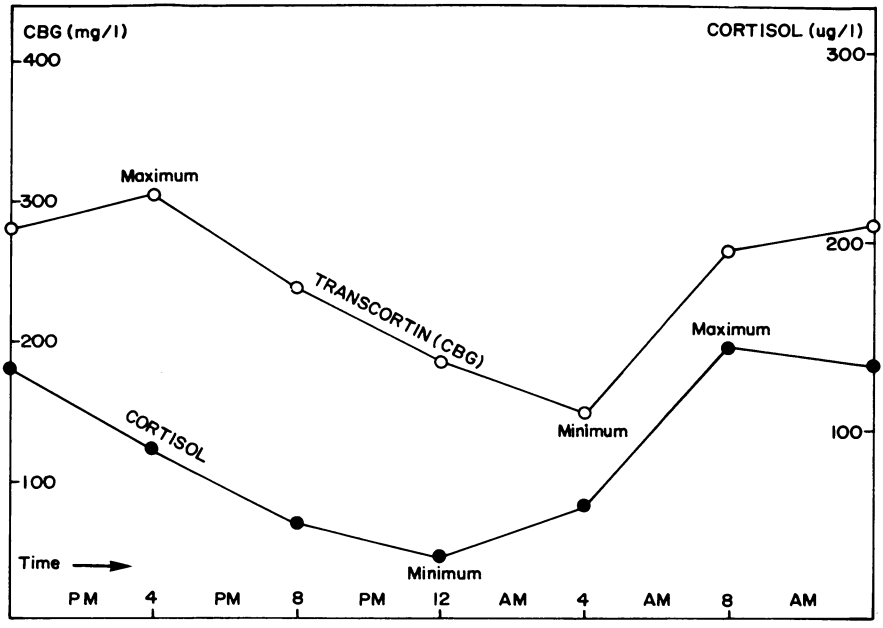

FIGURE 14.2 Diurnal variation of plasma transcortin and cortisol concentration.

gradually to the value of 214 g/liter by 4 PM. *Thus, there was correlation between the varying cortisol and CBG binding levels, but the CBG binding capacity lagged behind the serum cortisol levels* (see Figure 14.2).

No case of the complete absence of transcortin (atranscortinemia) has been reported. In one study, 10,000 individuals were screened but in no person was CBG completely absent.[23]

Oral or parenteral administration of estrogens results in an elevation of the plasma CBG level.[24,50,51] Pregnancy also results in a two- to threefold increase in plasma CBG levels. This results from increased synthesis of CBG, since its half-life remains the same.[25,26] In a typical study, cortisol levels rose from 170 μg/liter at the beginning of a pregnancy to a maximum of about 300 μg/liter at 6 months, and remained constant to term. CBG levels rose from 35 mg/liter to a maximum of 75 mg/liter at 6 months and remained constant until delivery.[27]

The use of contraceptive pills raises the plasma transcortin-binding capacity and the plasma cortisol concentration.[28–30]

In another study, the CBG, cortisol, and cortisone levels were

TABLE 14.3

Comparison of Serum CBG, Cortisol, and Cortisone Concentration in Maternal and Cord Blood with Normal (Vaginal) and Caesarean Delivery[a]

Serum sample source	Type of delivery	CBG (mg/liter)	Total cortisol (μg/liter)	Unbound cortisol (μg/liter)	Cortisone (μg/liter)
Nonpregnant women	—	29 ± 3	58 ± 6	2.9 ± 1.6	31 ± 6
Maternal	Normal	36 ± 3	296 ± 24	74 ± 17	44 ± 7
Maternal	Caesarean section	35 ± 2	232 ± 20	29 ± 7	25 ± 6
Cord blood	Normal	10.6 ± 0.5	82 ± 1	23 ± 6	99 ± 3
Cord blood	Caesarean section	12 ± 0.5	44 ± 12	3.3 ± 1	77 ± 7

[a] Adapted from Talbert *et al.*[27]

determined at the time of delivery, comparing maternal and cord blood obtained from mothers with normal (vaginal) and Caesarean delivery (Table 14.3). From the unbound cortisol levels it may be concluded that the stress on the mother and probably the fetus is less with Caesarean section. This has been found to be the case using other criteria such as serum and urine adrenaline levels.[27]

The tenfold increase of cortisol levels in pregnancy is not accompanied by any of the symptoms seen with cortisol overload such as Cushing's syndrome. This has been interpreted as indicating the significance of increased CBG levels in pregnancy. It has also been used to indicate that bound cortisol has no physiological activity.[28]

14.5 *TRANSCORTIN FUNCTION*

It has been well documented that steroid hormones, such as cortisol, estrogens, androgens, and progesterone, penetrate their target cells so as to form a complex with certain binding proteins within the cell.[31,32] It has also been shown that these steroids can readily penetrate the cell wall. On this basis, it is assumed that the CBG–cortisol and albumin–cortisol complexes serve to carry the steroid only to the

cell surface. Since these complexes are weakly bound, the cortisol dissociates and passes the cell wall and into the cell.

Some evidence that CBG can pass the cell wall before releasing the steroid has been presented by some. Using immunofluorescence antibody, a CBG-like protein was demonstrated to be present in the cytoplasm of human lymphocytes.[33] A protein, immunologically identical to CBG, has also been demonstrated to be present in the nuclei of liver cells.[34]

In the human uterus, a protein has also been demonstrated to be present, which had the binding characteristics of CBG and the same mobility on hydroxyapatite and Sephadex G-100 columns.[35] These authors postulate that CBG accumulates in the interstitial tissue of the myometrium, in support of its function, by regulating local concentrations of cortisol and progesterone. These authors did not present any evidence against the idea that some CBG may be intracellular.

A CBG-type protein is present in human milk. This seems to be identical to plasma CBG since it behaves like CBG on chromatography columns and by electrophoresis, in regard to its binding properties to cortisol and progesterone and immunochemically.[36] The CBG concentration in lymph is slightly lower than that in plasma.[34]

Regardless of whether CBG enters the cell to unload the cortisol or progesterone, or whether it unloads it extracellularly, transcortin function is significant in mediating cortisol and progesterone function.

The mechanism of steroid action on the cell is to stimulate protein synthesis.[38] Specific hormone receptors in the cell keep the free steroid concentration low so that diffusion of extracellular steroid is facilitated. On entry into the cell the steroid is bound to the specific hormone receptors. The steroid–receptor complex (S-R) is then transported into the nucleus of the cell. Here it is bound to the target cell genome. The target cell responds by an increase in a specific RNA synthesis. This then results in increased synthesis of a specific protein. This mechanism is outlined in Figure 14.3 and is based on studies showing that progesterone will stimulate the production of the protein avidin in the chicken.[37]

It is of interest to note that when estrogen is administered to an individual, *the plasma CBG concentration rises first and subsequently the cortisol level goes up.*[25] This supports the concept that estrogens stimulate protein synthesis, in this case CBG. As the increased supply of CBG binds the free cortisol in the plasma, reduced plasma cortisol levels

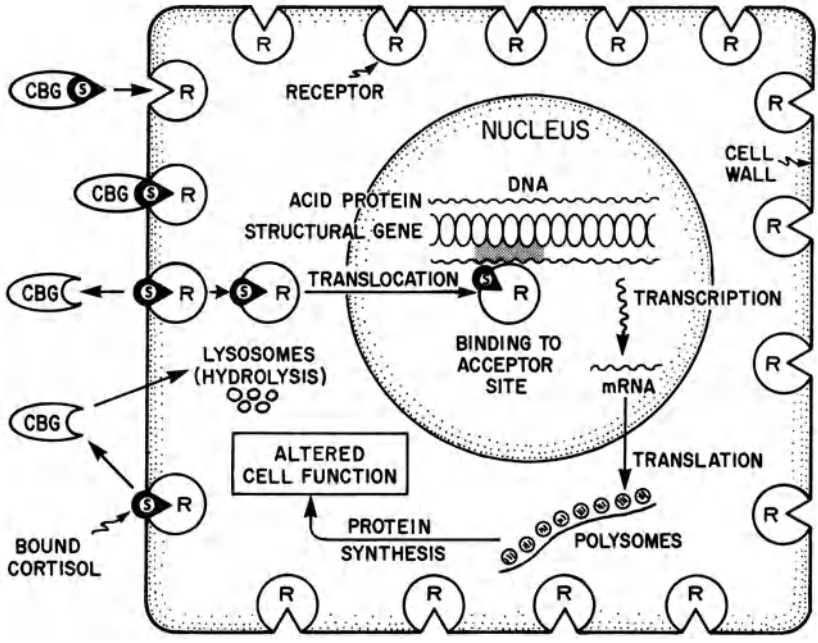

FIGURE 14.3 Schematic representation of the mechanism of cortisol action on the cell. The CBG–cortisol complex exchanges the cortisone with a protein receptor. The receptor–cortisol complex moves to the nucleus where DNA is activated to stimulate messenger RNA production which is translated by the polysomes in terms of protein synthesis. The CBG is hydrolyzed by the lysosomes of the cell.

stimulate the synthesis of more cortisol. Thus the total cortisol level increases markedly but the free cortisol is only slightly elevated.

Until relatively recently, the concept was held that protein hormones, like insulin, chorionic gonadotropin (HCG), and others bind to their receptors located at the surface of the cells. In some manner, this stimulated the cells to perform a particular function.

Evidence has been developed to show clearly that after the receptor binds to insulin, for example, the receptor, which itself is a protein, enters the cell, reducing the number of binding sites on the surface.[39] It should be pointed out that receptors, on cells, are proteins which are, most often, readily removed and solubilized. Assay of various plasma components by the use of their specific receptors in place of antibodies is often employed in hormone assay by competitive binding.[40]

In the case of insulin, 40–50% of the insulin–receptor complex of liver cells enter the cell and move preferentially to the lysosomes, where the complex is degraded by the hydrolytic enzymes present. In the case of the CBG–cortisol complex, this would suggest that it exchanges its cortisol with a binding protein located on the cell wall or just under the cell wall within the cell. The receptor–cortisol complex now moves to the nucleus. The reduced number of receptor sites on or near the wall, decreases the probability of further binding, thus modulating the rate at which the cell is directed to synthesize a specific protein.

14.6 *TRANSCORTIN ASSAY*

Transcortin assay in serum was originally performed by adding [^{14}C]-cortisol, and measuring the amount of cortisol bound, as has been stated in Section 14.3.[15,16] This led to the assay of cortisol, and other steroids using transcortin as the receptor for radioimmunoassay.[40,41] For steroid assay, this was eventually replaced by specific antibodies which would distinguish between the different steroids.[42–47]

Antibodies were then made to transcortin (CBG), and kits are now available commercially for transcortin assay by radial-immunodiffusion or by competitive protein binding techniques.[17,48,49]

14.7 *RECAPITULATION: TRANSCORTIN (CBG)*

Steroid-binding proteins serve to carry various steroids synthesized in the adrenal, ovary, testes, and liver to the site of their utilization. Of great significance is albumin which binds these steroids weakly, but because of its huge concentration in plasma is significant in their transport. Cholesterol is transported by the lipoproteins and is discussed in Chapter 6.

There are two proteins in plasma which carry the corticoid and steroid hormones. These are *corticosteroid-binding globulin* (CBG), also called *transcortin*, and *testosterone-binding globulin* (*TeBG*). CBG binds cortisol and progesterone, whereas TeBG binds testosterone and estratriol.

Transcortin (CBG) travels with the α_1 fraction on electrophoresis. It has a molecular weight of about 49,500, and is a glycoprotein containing 27% of carbohydrate. The carbohydrate is not involved in binding, but helps in the solubilization of the CBG–cortisol complex.

Binding avidity of cortisol and progesterone for transcortin is substantially higher than that of albumin but not as great as that of other specific binding systems in the plasma, such as the hemopexin–hematin system. Thus the binding is classified as moderate. This permits the steroid to be released readily when brought to the cell where it is to act.

There are several theories as to the way the cortisol enters into the cell. One proposes that the steroid dissociates from the CBG and promptly moves into the cell, eventually reaching the nucleus where it acts on the DNA to stimulate protein synthesis. The CBG then enters the cell to be metabolized. In support of this idea, CBG has been found in liver cells and lymphocytes.

A more recent proposal is that the CBG–cortisol complex exchanges the cortisol at the cell surface with a protein receptor in the cell wall. The receptor–cortisol complex then moves into the cell. The cortisol moves to the nucleus, and the receptor is hydrolyzed by the lysosomes. This serves to remove receptors from the surface of the cell and decrease further stimulation while cortisol is acting on the DNA, so as to stimulate gluconeogenesis.

In healthy adults, the CBG concentration ranges from 30 to 50 mg/liter when measured by radioimmunoassay. Somewhat lower values are reported when measured by CBG–cortisol binding capacity. Since transcortin is synthesized in the liver, low values are observed in the various liver diseases. In the nephrotic syndrome, transcortin is lost in the urine and low values are observed. Low transcortin levels are often found in a variety of diseases such as malnutrition, diabetes mellitus, and cardiac disease. Some healthy individuals have low cortisol levels without apparent cause. In these cases, the low cortisol levels are occasionally observed in their kindred and this seems to be of genetic origin. No clinical symptoms are noted in these cases, attributable to the low CBG levels. However, no case of complete absence of CBG has been reported in any individual.

Estrogen administration, such as with contraceptives, results in elevated transcortin levels. The transcortin level rises and is followed later by a rise in cortisol levels. Plasma levels of transcortin and cortisol

vary diurnally with a 4–8 hr lag between the peak for CBG and that for cortisol.

In pregnancy, very high plasma transcortin levels are noted along with elevated cortisol levels. The free transcortin concentration is only moderately elevated. Thus, transcortin serves to buffer the high cortisol and progesterone concentrations during pregnancy.

Transcortin has been used as a binding protein to assay cortisol. Specific antibodies, for the different steroids, have replaced the less specific transcortin for this purpose. Transcortin is assayed by radial immunodiffusion or other immunochemical techniques, with materials available commercially.

14.8 *TESTOSTERONE-BINDING GLOBULIN (TeBG)*

During the late 1950s and early 1960s several reports noted that testosterone and the estrogens seemed to be bound to a protein which travelled with the β globulins on electrophoresis. This was distinct from albumin, which also binds testosterone, and from transcortin, which travels with the α_1 globulins. In 1965, this was identified as a protein distinct from other proteins.[52,53] Several reports appeared which confirmed this finding and showed clearly that the estrogen- and testosterone-binding proteins were a single entity.[54–57] While given several names, such as sex steroid globulin and 17β-hydroxysteroid-binding globulin, the original name of *testosterone-binding globulin* (*TeBG*) is now generally accepted.

14.9 *CHARACTERISTICS OF TeBG*

Testosterone binding globulin is present in serum in only low concentration. In young adult males it is present at only about 10 mg/liter, and in nonpregnant females, about twice that (20 mg/liter). Because of its low concentration it has been difficult to obtain adequate amounts for its characterization. In addition, it is relatively unstable and loses activity as it is purified. The protein is stabilized by calcium ion, dihydrotestosterone, *tris*-buffer, and glycerol, and in the presence of these substances a fairly pure protein has been isolated.[58]

TABLE 14.4

The Amino Acid and Carbohydrate Composition of TeBG

Amino acid	Number of residues	Amino acid	Number of residues
Lysine	43	Leucine	54
Histidine	14	Tyrosine	19
Arginine	19	Phenylalanine	23
Aspartate	67	Tryptophan	9
Threonine	26	Total	580
Serine	34		
Glutamate	56		
Proline	26	Carbohydrate	
Glycine	45		
Alanine	48	Fucose	2
Half-cystine	37	Mannose	25
Valine	36	Galactose	27
Methionine	9	*N*-Acetylglucosamine	45
Isoleucine	15	Sialic acid	35

The molecular weight of TeBG is about 94,000 as has been reported by several investigators.[58–61] It is thus about twice the weight of the transcortin molecule. It is a glycoprotein containing about 32% carbohydrate.

The carbohydrates present in the TeBG molecule are fucose, mannose, galactose, *N*-acetylglucosamine and sialic acid. There are also about 58 amino acid residues in the molecule. Table 14.4 lists the number of amino acid and carbohydrate residues in TeBG.

The sedimentation coefficient of TeBG is $s_{20,w} = 5.3$ and its partial specific volume, $\bar{v} = 0.665$ ml/g. The extinction coefficient at 280 nm, $E^{1\%}_{1cm} = 5.5$, and its isoelectric point, in the electrophoretic field, is at pH = 5.51. The frictional ratio for TeBG is $f/f_0 = 1.48$.

14.10 *STEROID BINDING TO TeBG*

There is only one binding site for the steroids on TeBG, and it is the same site for the estrogens as it is for testosterone. When both are present, they compete for the same site.[62,64]

A 17β-hydroxyl group is required for binding to TeBG. If the

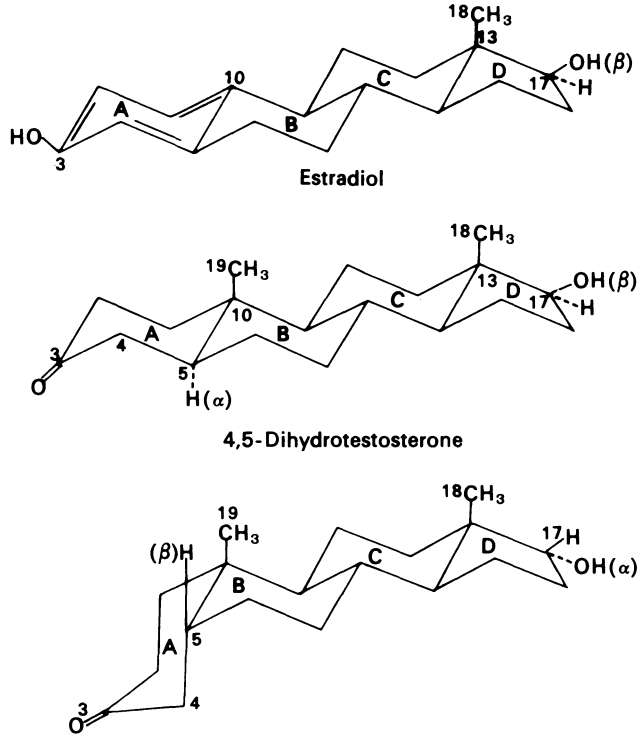

FIGURE 14.4 Comparisons of the configurations of estradiol and dihydrotestosterone (5α,17β hydroxy) and a testosterone isomer with low affinity for TeBG (5β,17α hydroxy).

hydroxyl group at position 17 is α, there is very little binding.[62] A 5α hydrogen in saturated ring A strengthens the binding, whereas with a 5β hydrogen configuration, binding is markedly decreased.[62–64] This is illustrated in Figure 14.4.

Although estradiol is bound tightly to TeBG, this is not so for the closely related estrone or estriol. (See Appendix for the comparative structures of the three estrogens.) Table 14.5 shows the effect of conformational changes of the steroid structures on the binding to TeBG. Oxidation of the OH group at the 17 position to the ketone, decreases binding. This occurs also with substitution in the 16 position.

The association constants (K_a) for the steroid hormones to albumin,

TABLE 14.5

Effect of Configuration Change of the Hydroxyl Groups at Positions 5 and 17 on the Binding to TeBG, Influence of the Oxidation of the 17-OH Group to a Ketone, and Substitution at the 16 Position in the Ring System

17β-OH		⟶	17α-OH	
Testosterone (β17-OH)	100[a]		*epi*-Testosterone (α17-OH)	4
17β-hydroxy-Δ⁴-pregnenedione,3,20	33		17α-Hydroxy-Δ⁴-pregnenedione,3,20	2.5
17β-OH		**oxidation ⟶**	**17-Keto**	
Testosterone	100		Δ⁴-Androstenedione	5
Estradiol	62		Estrone	4
5α-Androstane-3α-17-diol	160		Androsterone	3
5β-Androstane-3α-17β-diol	24		Etiocholanone	0
5α-OH		**⟶**	**5β-OH**	
5α-Androstane-3α,17β-diol	160		5β-Androstane-3α,17β-diol	24
5α-17β-OH-androstane-3-one	300		5β-17β-OH-androstane-3-one	10
Substitution in carbon at position 16				
Estradiol	62		Estriol	32
Δ⁵-Androstene-3-17β-diol	50		Δ⁵-Androstene-3β-17β-diol-16-one	1

[a] The numbers represent the relative amount bound, as compared to testosterone, which is listed as 100.

transcortin, and TeBG, are compared in Table 14.6. It will be noted that estradiol has 10,000 times the affinity for TeBG that it has for albumin, and testosterone has about 20,000 times the affinity for TeBG that it has for albumin.

TABLE 14.6

Approximate Association Constants, K_a, of the Steroid Hormones for Albumin, Transcortin, and TeBG

	Steroid			
Protein	Testosterone (liter/mol)	Estradiol (liter/mol)	Cortisol (liter/mol)	Progesterone (liter/mol)
Albumin	3.5×10^4	6×10^4	$\approx 10^{-4}$	$\approx 10^4$
Transcortin (CBG)	1.5×10^6	$\approx 10^6$	4×10^9	2.6×10^9
TeBG	8×10^8	6×10^8	$\approx 10^4$	$\approx 10^4$

14.11 *PLASMA TeBG*

In measuring plasma (or serum) TeBG concentration, the plasma is saturated with tritiated dihydrotestosterone or testosterone. The bound TeBG–steroid complex is separated and the amount is estimated with the scintillation counter. The results are then expressed in terms of the amount of androgen bound, or the TeBG binding capacity.[65,66]

In one study, the amount of testosterone bound to TeBG was found to be 4–14 μg/liter for healthy men, and for healthy women 6–55 μg/liter with a mean value of 15 μg/liter for women.[66] Other studies have reported values for dihydrotestosterone binding capacity, ranging from 9.2 to 13.3 μg/liter for men and 17.6 to 30.0 μg/liter for women.[65,70–73]

In preadolescent children, TeBG binding capacity values are the same for both sexes, and lower than that found after puberty, in *adult females.*[68,69,74,75] On the other hand, the plasma TeBG concentration *falls as the male passes through puberty.* This results from the fact that estrogens cause a marked increase in TeBG plasma levels, whereas androgens depress the plasma TeBG concentration. For example, in males beyond the age of 50, as the plasma testosterone levels fall, TeBG levels rise. Women with elevated plasma testosterone levels experience a depression of the plasma TeBG levels.[62,65,76]

In pregnancy, with a rise in plasma estrogen levels, TeBG levels increase almost eightfold. During the third trimester, mean values observed by several investigators ranged from 94 to 126 μg/liter, as compared to 15 μg/liter in nonpregnant women.[70,71,73]

14.11.1 *Depressed Plasma TeBG Levels*

Plasma TeBG levels vary inversely with plasma testosterone levels. Androgen therapy or administration of corticosteroids decreases the TeBG binding capacity.[70] In virilized females, TeBG binding capacity is also low.[55]

In hypothyroidism in women, low TeBG levels are noted. This is not the case in males.[65,77,78] Hypothyroid males show no significant decrease in serum TeBG concentration.

14.11.2 *Elevated TeBG Binding Capacity*

In male hypogonadism or senescence, androgen production falls and TeBG levels are elevated.[67] As mentioned above, pregnancy causes

TABLE 14.7

Conditions under which Plasma TeBG Levels Are Altered

Increased	Decreased
Pregnancy	Androgen administration
Estrogen therapy	Hirsute virilizing condition in females
Progesterone therapy	Hypothyroidism in women
Liver cirrhosis	Cortisone therapy
Thyrotoxicosis	High levels of endogenous corticoids or androgens as in adrenal tumors
Senescence	
Hypogonadism in males	

a rise in TeBG binding capacity of serum. Exogenous estrogen administration, such as contraceptive pills, causes a marked increase in plasma TeBG binding capacity.[78] It has also been mentioned that in senescence TeBG binding capacity is elevated.

In spontaneous thyrotoxicosis, a marked increase in plasma TeBG binding capacity occurs.[77–79] Thyroxine administration produces the same effect.[65]

Since TeBG is synthesized in the liver, it would be expected to be low in liver cirrhosis. The opposite is the case, and TeBG levels are high with cirrhosis of the liver.[55,65,71] It has been suggested that this indicates an increase in plasma estrogen concentration and decrease in androgen production. No evidence for this proposal has been adduced to date.

The mechanism whereby androgens lower and estrogens raise TeBG levels has been the subject of much discussion. Present thinking is that the effect is at the cellular level. It is proposed that estrogen binds to the genome so as to stimulate TeBG production, whereas androgens inhibit this effect by competitive inhibition. Definitive experiments at the cellular level have not been reported at this writing.

Table 14.7 lists the conditions under which TeBG binding capacity is increased or decreased.

14.12 *FURTHER STEROID HORMONE-BINDING GLOBULIN PROBLEMS*

From time to time different proteins have been proposed as being significant in steroid hormone transport. This is partly a result of the

fact that steroid hormones have reactive functional groups like double bonds, keto, aldehyde, and hydroxy groups. This results in some hydrogen bonding and other types of linkage to almost any protein, especially the glycoproteins.

There are three glycoproteins found in substantial quantities in serum from pregnant women which are present in only trace amounts in serum from nonpregnant women. These are called SP_1, SP_2, and SP_3. Of these, SP_2 has been identified as TeBG. The SP_3 *binds estriol more strongly than testosterone or estradiol.* Thus, there seems to be a specific estriol transport protein distinct from TeBG. This is important since estriol and not estradiol is the most active form of the estrogens.[80]

The question arises as to what is the major function of the pregnancy-associated steroid-binding globulins. Their low concentration raises a question as to their role as a transport protein. Several investigators have suggested that their real role is to protect against surges in *free* steroid hormone level. Thus, in pregnancy, the *free steroid hormone* levels are buffered, so as to prevent side effects from high concentration of these hormones which may terminate the pregnancy. Similarly, surges in free hormone levels during menstrual cycles are prevented from interrupting normal metabolism.

In line with this reasoning, several investigators have suggested that *free testosterone*, or *apparent free testosterone* concentration should be measured rather than total testosterone levels. They claim that there is a better correlation between free rather than total testosterone levels and the clinical condition, since it is unbound sex hormone which is physiologically active. For example, in hirsute women, the ratio of androgens to TeBG is claimed to be a better index of the clinical state of the patient than the concentration of androgen in plasma.[65,66,81,82]

14.13 *RECAPITULATION: TESTOSTERONE-BINDING GLOBULIN*

Testosterone-binding globulin (TeBG) is a protein with a molecular weight of about 94,000 which travels with the β globulins on electrophoresis. It has a moderately strong affinity for testosterone, progesterone and estradiol but not for estrone or estriol. Two other pregnancy-associated serum proteins, SP1 and SP3, bind estriol.

Plasma TeBG levels are about 15 mg/liter in adult males, and about 26 mg/liter in women. When estrogen plasma levels are increased, plasma TeBG is increased. Increase in plasma androgen levels causes a fall in plasma TeBG concentration. Thus in hypogonadism or senescence in males, with low androgen levels, TeBG plasma levels are elevated. In pregnancy or on estrogen therapy, such as with contraceptive pills, TeBG levels are also elevated.

In the presence of high concentrations of androgens, as in hirsute, virilizing condition in women, TeBG levels are low. Administration of cortisone or androgen also depresses plasma TeBG levels.

In hyperthyroid conditions such as in thyrotoxicosis, TeBG levels are elevated. With hypothyroidism, the plasma TeBG levels of women are lowered. This is not the case for males. TeBG plasma levels are also elevated in cirrhosis of the liver.

In preadolescent children plasma TeBG values are lower than those found in women. They increase after puberty. On the other hand, the TeBG levels of males decrease after puberty. This is the effect of the increase in plasma androgens.

It is proposed that TeBG serves not only to transport androgens and estradiol, but also to act as a buffer by complexing high concentrations of these substances when they occur because of physiological or abnormal changes in hormone secretion.

14.14 *SELECTED READING*

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15

Transcobalamins: Vitamin B_{12} Transport

In Volume 2 of this series, the transcobalamines were introduced, in connection with vitamin B_{12} function and transport (pp. 520–525). Here, a brief review will be given of this problem and of the developments which have taken place since this subject was discussed in Volume 2.

Vitamin B_{12} is the *extrinsic factor* which needs to be bound by an *intrinsic factor* in order to be absorbed in the ileum. *Congenital pernicious anemia* is the infantile form of a disease which results from a deficiency of the intrinsic factor. More commonly, the disease occurs in older people, especially after the age of 60 (see Volume 2, pp. 509, 510). The disease is characterized by megaloblastic erythroid precursors in the bone marrow, a macrocytic anemia, and neurological dysfunction.

The intrinsic factor is secreted by the parietal cells of the fundus of the stomach. Studies of intrinsic factor, revolve about its property of binding vitamin B_{12}. In 1966, it was reported that a B_{12}-binding protein was present in gastric juice which was devoid of any intrinsic factor activity. Since this polypeptide moved more rapidly than intrinsic factor on electrophoresis (with the α globulins), it was referred to as the *R-protein*, or more rapid protein.[1] Since it was apparent that the R-protein was involved in cobalamin (B_{12}) transport the R-protein was referred to as *transcobalamin.*

Affinity chromatography is used to isolate the R-protein and separate it from intrinsic factor. In this procedure, the propionamide of B_{12} is hydrolyzed to the free acid. This is then bound to Sepharose with

carbodiimide. By passing plasma, saliva, or buffered juice, as examples, through a column containing the Sepharose–B_{12} complex, the polypeptides which bind to B_{12} are adsorbed. Elution is usually with varying concentrations of guanidinium hydrochloride.[2]

In studying the elution patterns from a B_{12}–Sepharose column it was noted that there were at least three R-proteins in plasma, all different from intrinsic factor. They were then named transcobalamins I, II, and III, or TC-I, TC-II, and TC-III.

It was soon found that the granulocyte was the source of a transcobalamin which was then labeled TC-I. It has a molecular weight of about 65,000. A second, labeled TC-II, was present in plasma and had a molecular weight of about 38,000. This contained no carbohydrate.

A third transcobalamine, TC-III, was then found, which came off the Sepharose column with TC-I and had about the same molecular weight. Both TC-I and TC-III are glycoproteins but differ mainly in the composition of the attached oligosaccharide chains.[3,4] Blood TC-I and TC-III are found mainly in the granulocytes, whereas TC-II occurs in the highest concentration in the plasma.

Since TC-II has the major role in B_{12} transport, it is most often referred to as transcobalamin.[5] Transcobalamines I and III are referred to frequently as R-I and R-III. The R-proteins are also referred to as the *cobalophilins*, as distinct from transcobalamin (TC-II). In subsequent discussion, we will use only the designations transcobalamins, TC-I, TC-II, and TC-III.

15.1 *PLASMA TRANSCOBALAMINS*

Fractionation of human plasma on a Sephadex G-150 column, using gradient elution, results in two fractions which bind B_{12}. TC-II elutes with an apparent molecular weight of 38,000, whereas TC-I and TC-III come off with an apparent molecular weight of 150,000. The latter figure is artifactual, and characteristic of problems of fractionating glycoproteins on Sephadex.[3] The true molecular weights of TC-I and III are in the vicinity of 65,000. TC-I and TC-III are separated readily on DEAE-cellulose.[5]

With these techniques it has been found that a total of about 1500 ng/liter of vitamin B_{12} can be bound to the plasma transcobala-

mins. About 65% of this is normally due to TC-II. TC-III accounts for most of the remaining B_{12} binding capacity, less than 5% being accounted for by TC-I. Each of these proteins contains only a single binding site for B_{12}. From these data, the transcobalamin concentration in human plasma calculates to 15, 30, and 25 mg/liter for TC-I, TC-II, and TC-III, respectively. In contrast to these figures, TC-I accounts for most of the B_{12} binding in the granulocyte, only a small percentage being bound by TC-III. TC-II is present only in minute amounts in the granulocyte.

15.2 *PROPERTIES OF THE TRANSCOBALAMINS*

Plasma TC-I, TC-III, and the R-proteins of the granulocyte give single identical precipitin lines with immunoelectrophoresis or by immunodiffusion. These are distinct from those given by TC-II or intrinsic factor. TC-I contains 35% of carbohydrate, TC-II contains none, and TC-III contains about 35%. Intrinsic factor is also a glycoprotein and contains 15% of carbohydrate. Table 15.1 compares the carbohydrate composition of TC-I, TC-III, and the R-protein mixture obtained from the homogenized granulocyte.[5]

From Table 15.1 it can be seen that plasma TC-I and TC-III of the plasma resemble the R-protein of the granulocyte in carbohydrate composition. TC-III carbohydrate composition resembles the

TABLE 15.1

Carbohydrate Composition[a] of Plasma TC-I and TC-III Compared to That of the Granulocyte R-Protein

Carbohydrate	TC-I (mol/mol B_{12})	TC-III (mol/mol B_{12})	Granulocyte R-protein (mol/mol B_{12})
Sialic acids	18	11	11
Fucose	9	20	24
Galactose	41	51	46
Mannose	24	22	20
Galactosamine	2	2	2
Glucosamine	46	54	46
Total	140	160	149

[a] TC-II contains no carbohydrate.

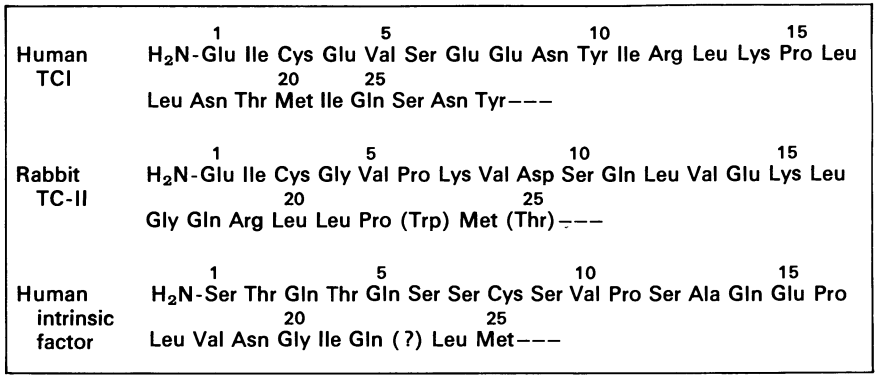

FIGURE 15.1 N-terminal amino acid sequence of human TC-I and rabbit TC-II compared with that for intrinsic factor.

R-protein more closely, especially in sialic acid content. This table supports the concept that the R-proteins of the plasma, TC-I and III, derive from the granulocyte.[5]

The amino acid composition of TC-I and III are similar. Substantial differences exist, however, between TC-I (III) and TC-II. Human intrinsic factor is distinct in its amino acid composition.[6,7,8] This can be seen from Figure 15.1 where the amino acid composition of the first 25 amino acids of human TC-I and intrinsic factor are compared to that of TC-II obtained from the rabbit.[6–10]

It was pointed out above that with affinity chromatography, TC-I and TC-III come off the Sepharose–B_{12} column together. On isoelectric focusing of this mixture three isoproteins are obtained at pH 3.2, 3.42, and 3.69. On ultracentrifugation TC-II sediments just after albumin, whereas TC-I and III come down just before IgG. Thus the sedimentation coefficient of TC-II is about 3.75, and TC-I and II are about 5.39. The molecular characteristics of the transcobalamins as given by one investigator, for porcine plasma transcobalamines, is given in Table 15.2.[11] The figures are given for the holotranscobalamins (transcobalamin-B_{12} complexes).

Variants of both transcobalamin I, II, and III occur in human plasma. For example, in Table 15.2 three bands are observed with TC-I(III) by isoelectric focusing. Some of this variation is due to differences in carbohydrate content. However, genetic variation in the polypeptide chain occurs also.[12]

TABLE 15.2

Molecular Characteristics of Serum Porcine Transcobalamins I and III (Cobalophilins), and Transcobalamin II (Transcobalamin), as the Vitamin B_{12} Complexes

Parameter	Cobalophilin–B_{12} complex [TC-I(III) B_{12}]	Transcobalamin–B_{12} Complex (TC-II B_{12})
Molecular weight (gel filtration)	135,000 ± 3000	38,000 ± 1000
Molecular weight (ultracentrifuge)	103,860	44,340
Sedimentation coefficient	5.39 ± 0.03	3.75 ± 0.04
Specific volume (ml/g)	0.710	0.747
Diffusion coefficient	4.34×10^{-7}	8.1×10^{-7}
Frictional ratio (f/f_0)	1.59	1.11
Isoelectric point (by isoelectric focusing)	3.23, 3.42, 3.69	3.47

15.3 *TRANSCOBALAMIN FUNCTION*

After being absorbed from the gut, vitamin B_{12} enters the blood stream attached to transcobalamin II. This transcobalamin II seems to be derived from the ileal enterocyte.[13] The B_{12}·TC-II complex has a half-life of only about 5 min, being rapidly removed by the RE cells of the liver and other tissues. The process has been followed in the granulocyte with [^{57}Co]-B_{12}. The B_{12} is transported to the granulocyte, and stored mainly as B_{12}·TC-I. This B_{12}·TC-I complex has a half-life of 9 to 10 days, and is not readily broken down as is the transcobalamin II complex. For these reasons, it has been proposed that TC-II is the major transcobalamin involved in B_{12} transport from the gut to the tissues.[14,15]

At any one time, about 20–30% of the B_{12} is carried as B_{12}·TC-II in the plasma.[15] Even after deprivation of B_{12}, TC-II will continue to transport B_{12}. This probably originates from the B_{12}·TC-I stores in the cells of the tissues, including the circulating granulocytes.[16] For these reasons, it is generally conceded that TC-II is the major transcobalamin involved in transport, and that TC-I and TC-III confine their activities to B_{12} storage.[17]

This problem is not as simple as would be suggested from the above. On analysis of the cobalamins attached to the transcobalamins, it has been determined that the B_{12} attached to TC-I is mainly methylcobalamin. On the other hand, TC-II carries 5′-deoxyadenosylcobalamin (coenzyme-B_{12}).[14] These cofactors have different functions. The methyl cobalamin is concerned with transfer of methyl groups in numerous reactions, such as to folic acid, methionine, and others (see Volume 2, pp. 510–515). The coenzyme-B_{12}, on the other hand, is involved in numerous mutase reactions. This suggests a different enzymatic function for TC-I and TC-II. This needs to be clarified.

Transcobalamin II, along with intrinsic factor are classified as *permeases*. That is they facilitate the penetration of B_{12} into the cells. For TC-II, the process has been studied with fibroblasts, HeLa cells, granulocytes, and lymphocytes.[18,19] The steps in the absorption of the B_{12}·TC-II complex comprise: (a) *binding of the complex to a specific cell-surface receptor protein* (this step has been shown to be Ca dependent); (b) *internalization by pinocytosis*; and (c) *degradation of the TC-II-protein receptor-B_{12} complex in the lysomes*, and release of B_{12} for cell utilization. This is similar to the process which has been described for the action of transcortin in bringing steroid hormones to their site of utilization. This may be illustrated as follows:

$$B_{12} + \text{intrinsic factor(IF)} \rightarrow B_{12}\cdot IF + \text{TC-II} \rightarrow B_{12}\cdot\text{TC-II} + IF$$

$$B_{12}\cdot\text{TC-II} + \text{binding protein (BP)} \rightarrow B_{12}\cdot\text{TC-II}\cdot BP \xrightarrow[\text{to lysosomes}]{\text{internalized}}$$

$$B_{12} + BP + \text{TC-II fragments}$$

The binding of the B_{12}·TC-II complex (holotranscobalamin II) and TC-II *per se* (apotranscobalamin II) are distinctly different, and apoTC-II does not compete with the internalization of holoTC-II.[19–20] The receptor for TC-II has been isolated from human placenta.[21] It has a molecular weight of about 50,000 and contains 33% carbohydrate. The TC-II receptor has an association constant of 56 nM^{-1} with holoTC-II. This is about twice that for B_{12}-free TC-II, apoTC-II, whose association constant is 28 nM^{-1}. There is only one binding site on the receptor protein and only one on TC-II. Thus they combine stoichiometrically.[21]

In summary, the functions of the transcobalamins may be listed as cell permeases for vitamin B_{12}, transport proteins for B_{12}, reserve storage

sites for excess B_{12} taken in the diet, and probably, involvement in the activation of B_{12} as a methylating and mutase cofactor.

15.4 *THE TRANSCOBALAMINS IN DISEASE*

Elevated plasma levels of TC-I and TC-III are observed in polycythemia vera, myelogenous leukemia, and related diseases.[22–25] It is proposed that most of this is due to TC-I and III released from the granulocytes. As a result of the proliferation of the cells of the bone marrow, TC-I and III are released into the plasma. Most direct studies of the release rate of TC-I and III from the granulocytes of these patients do not support the idea that increased cell permeability is at fault. Most likely, the increase follows from the increased concentration of cells in the plasma.

Unless precautions are taken, the concentration of TC-I and III will be higher in serum than in plasma. During the clotting sequence, some of the granulocytes, and erythrocytes, which also contain TC-I and III in substantial concentration, are disrupted, spilling their contents into the serum. In estimating transcobalamin concentration, it is therefore recommended that plasma be used and EDTA be used as the anticoagulant.[25]

TC-I concentration is also elevated with various types of neoplastic diseases such as carcinoma of the breast and with the hepatomas.[26,27] Normally, plasma TC-II binding capacity for cyanocobalamin (mol. wt. 1355) ranges from about 700 to 1200 ng/liter or somewhat less than 1 nmol/liter. For R-binder (TC-I + TC-III) in plasma, the level is given as 32–384 ng/liter by one investigator.[28] The mean values given by this author are 940 for TC-II and 213 for the R-binder. In the neoplastic diseases, especially in the myeloproliferative diseases such as in the leukemias, the total cyanocobalamin binding capacity can increase by as much as ten fold. This can be seen in Table 15.3.

With severe elevation of the R-binders in the plasma, symptoms of vitamin B_{12} deficiency may develop, in spite of high serum B_{12} level. The reason for this is that TC-I and TC-III bind B_{12} tightly and strip TC-II of its B_{12}. Polyneuropathy and megaloblastic anemia, characteristic of B_{12} deficiency, develop in some of these patients.[27]

In compensation for the severe elevation of plasma TC-I and III

TABLE 15.3

Unsaturated Vitamin B_{12} Binding Capacity[a] in the Normal Adult as Compared to Patients with Various Diseases Involving the Reticuloendothelial System[25]

Probands	Number in group	Total unsaturated vitamin B_{12} binding capacity	Vitamin B_{12} binding capacity		
			TC-I	TC-II	TC-III
Normal	15	811 ± 135	109 ± 12	696 ± 122	6 ± 13
Polycythemia vera	6	1022 ± 214	208 ± 80	742 ± 147	73 ± 79
Chronic myelogenous leukemia	5	2925 ± 736	1566 ± 859	1300 ± 168	59 ± 131
Acute leukemia	2	1477	324	1142	12
Pernicious anemia	6	1330 ± 233	324 ± 119	1066 ± 276	0
Leukocytosis	16	2182 ± 2084	781 ± 2132	1484 ± 514	34 ± 81
Eosinophilia	4	1233 ± 54	163 ± 105	1057 ± 140	12 ± 25
Cancer[b]	5	4804 ± 3891	3287 ± 4112	1521 ± 915	0

[a] ng cyanocobalamin per liter, bound; mean and SD values.
[b] Granulocytes > 8000/μliter.

levels, TC-II is also elevated. This is a constant finding in chronic lymphocytic leukemia, with lymphomas, and in carcinoma.[28] In the normal, TC-II plasma B_{12} binding is about 1000 ng/liter. In the neoplastic conditions, TC-II levels range from about 2000 in carcinoma to as high as 9500 with the lymphoproliferative diseases. This accounts for the fact that symptoms of B_{12} deficiency are not more common in the neoplastic diseases.

Transcobalamine synthesis has been demonstrated in rat liver.[29] In liver disease, such as in viral and infectious hepatitis and cirrhosis, plasma TC II concentration is more than double the normal level.[30,31] This has been presumed to result from increased synthesis during liver regeneration, and leakage from the liver into the blood stream. However, with infection and inflammation, such as in rheumatoid arthritis, and in the autoimmune diseases, lupus erythematosis, dermatomyositis, autoimmune hemolytic anemia, and in renal transplant patients,

transcobalamin II levels are also markedly elevated, especially during the active phases of the autoimmune diseases. In this respect, in spite of the fact that it contains no carbohydrate, TC-II behaves like an acute phase reactant. Elevation of TC-II plasma levels correlates better with the clinical course of the autoimmune diseases than complement (C3), antinuclear antibody titer or native DNA binding capacity.[32]

Transcobalamin Deficiency

In discussing the deficiency of the intrinsic factor, in Volume 2 of this series (p. 521), it was pointed out that pernicious anemia, a result of B_{12} deficiency, is seen in many older people, as an autoimmune disease, the patient generating antibodies against his own intrinsic factor. This has also been demonstrated for the transcobalamins.

When patients with pernicious anemia were treated with long-acting preparations of B_{12}, they developed antibodies against TC-II.[34] This has also been reported as having occurred spontaneously in several patients, resulting in abnormal B_{12} transport.[35]

Antigen–antibody complexes, with TC-I bound to IgG have been reported in four patients, all of whom had a history of chronic alcoholism. One of the patients showed the classic symptoms of pernicious anemia.[36]

Congenital atranscobalaminemia II is reported by several investigators.[37–39] The clinical course in these patients has differed substantially in that some have developed a typical pernicious anemia, whereas others were not affected significantly, as far as exhibiting a B_{12} deficiency was concerned. All had low plasma vitamin B_{12} levels.

A familial hyperstranscobalaminemia II has also been reported.[40] The probands did not seem to show any clinical symptoms which could be attributed to high TC-II plasma levels in the various members of this family.

15.5 *RECAPITULATION*

The transcobalamins are polypeptides which bind vitamin B_{12}. They are distinct from the intrinsic factor and cannot substitute for it.

At least three well-defined polypeptides are referred to as transcobalamins. These are labeled TC-I, TC-II, and TC-III. The TC-II is of lower molecular weight, and is the main transport protein for vitamin B_{12} in the plasma. TC-I and TC-III are present in substantial concentration in the cells of the reticuloendothelial system. Plasma TC-II seems to be derived from the granulocyte. TC-I and III bind B_{12} tightly and serve to store the B_{12} not required for immediate use. The B_{12} carried by TC-II is coenzyme B_{12}. B_{12} is stored with TC-I and III as methylcobalamin.

The vitamin B_{12} is absorbed in the ileum as a complex with intrinsic factor (IF), which is synthesized in the fundus of the stomach. From there it is transferred to transcobalamin II (TC-II) as it passes the wall of the ileum. The B_{12} then circulates as the $B_{12} \cdot$ TC-II complex in the plasma. At the cell wall, this complex binds with a binding glycoprotein (BP) to form a $B_{12} \cdot$ TC-II $\cdot$ BP complex. This complex enters the cell by pinocytosis and moves to the lysosomes. There it is acted upon by proteolytic enzymes so as to release the B_{12} for metabolism or for storage as a complex with TC-I and TC-III.

Transcobalamin plasma levels are decreased in B_{12} deficiency, the rate of synthesis of TC-II being somewhat related to the abundance of B_{12}. TC-II levels are increased on vitamin B_{12} therapy. TC-II levels are absent or very low in a condition resulting from a defect or complete absence of the codon for the synthesis of this protein. This condition, atranscobalaminemia II, results in a megaloblastic anemia, simulating vitamin B_{12} deficiency, in some of these patients.

Disruption or proliferation of cells of the reticuloendothelial system results in release of TC-I and TC-II into the plasma in increased amounts. This occurs in the myeloproliferative disorders, with hepatomas, cancer, and the autoimmune diseases. In these cases, a simulated B_{12} deficiency may occur because of the strong affinity of TC-I and TC-III for B_{12}. Thus TC-II is stripped of its B_{12} and less is available for transport to the tissues. In these cases, plasma TC-II levels will also be elevated significantly. This serves to compensate for the effect of increased TC-I and TC-III levels and serves to ameliorate the condition.

Plasma antibody formation to intrinsic factor is a major cause of pernicious anemia in older people. Plasma antibodies to the transcobalamins are also formed under certain conditions. For example, treatment with long-acting B_{12} preparations for extended periods results in the development of antibodies to TC-II, in some of these

patients. In chronic alcoholism, development of antigen–antibody complexes of the TC-I · IgG composition has also been reported.

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Sec. IV

Appendix

Appendix

A.1 *STEROID NOMENCLATURE*

Studies of steroid structure were stimulated, originally, because of interest in the nature of cholesterol. In cholesterol there are four rings, three of these, labeled A, B, and C, comprise a phenanthrene type structure. The fourth (ring D) is a five-membered ring or cyclopentane structure. The ring skeleton structure of cholesterol, and all of the steroids, is called *sterane** or *gonane*. This is shown in Figure A.1.

In Figure A.1 it will be noted that single hydrogens are attached at carbons 5, 8, 9, 10, 13, and 14. These can be above the ring or below the ring, depending upon the relative configurations of rings A to B and C to D. There are two hydrogens at each of the other positions. Which is above or below the ring is of no consequence.

If a model is constructed, it will be seen that once the configuration of ring A relative to B is formed, the configuration of rings C and D fall in line. This is seen in Figure A.2.

Note that in the trans form, in Figure A.2, the hydrogen at position 5 is below the ring. This is referred to as an α linkage. In the *cis* form, the hydrogen at that position is necessarily above the ring, and this is referred to as a β linkage. Note how the position of the other hydrogens are then fixed. For example, in the *trans* form, the single hydrogens at positions 8, 9, 10, 13, and 14 are β, α, β, β, and α respectively.

How this applies to the structure of cholesterol and its isomers is shown in Figure A.3. *Note that a dotted line signifies a linkage below the ring,*

* Sterane (capitalized) is also the trade name of Chas. Pfizer and Co. for prednisolone (Δ^1-dehydrocortisone).

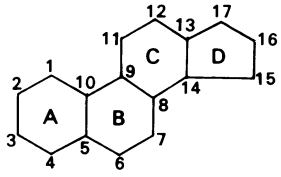


FIGURE A.1 Gonane (sterane).

and is designated α. A solid line signifies a linkage above the ring and is designated β. In Figure A.3, the structure of dihydrocholesterol is compared to that of dihydroallocholesterol. Dihydroallocholesterol (coprosterol) appears in the stool as the result of reduction and isomerization of cholesterol. *Allo* is a combining form meaning other than normal. It has no real structural significance.

Another type of isomerism is commonly encountered in steroid chemistry. *That is the two isomers formed by change of position of a particular hydroxyl group and hydrogen in the steroid molecule.* In the normal position in cholesterol, the —OH group is above (β), and the —H is below the ring or α (Figure A.3). If reversed, with the —OH in the α position, we have

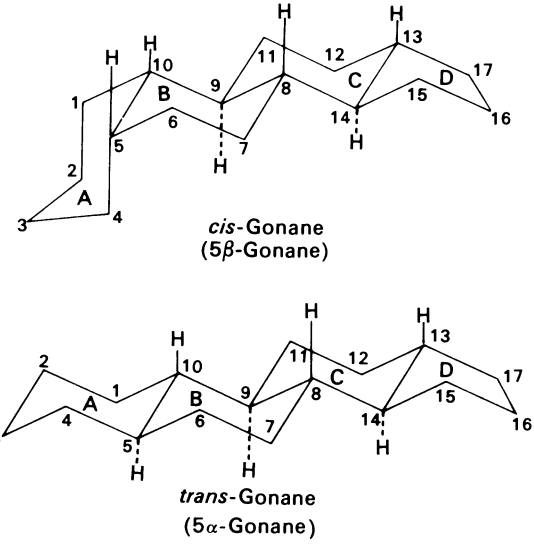
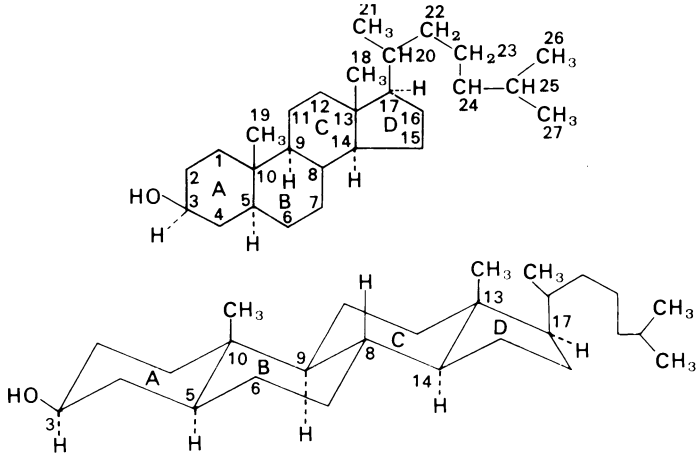


FIGURE A.2 Comparison of the *cis* and *trans* structures of gonane. In the *cis* form, the hydrogen at position 5 is below the ring and designated α. In the normal or *trans* form, this hydrogen is above the ring and labeled β.



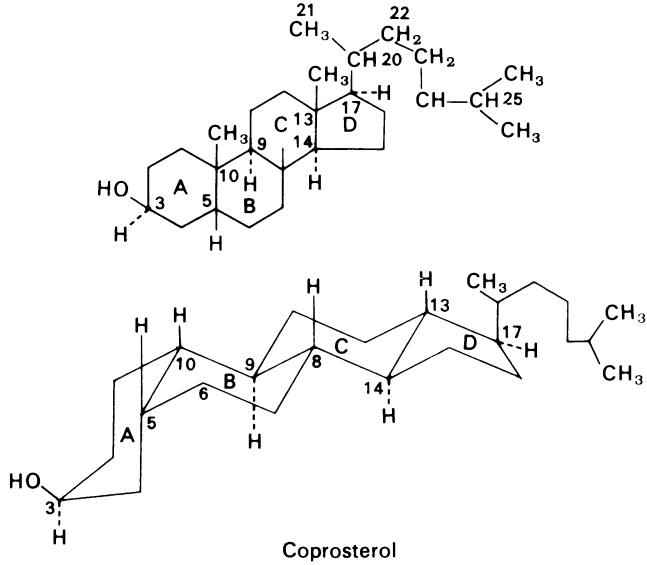


FIGURE A.3 Comparison of the structures of dihydrocholesterol and dihydroallocholesterol (coprosterol).

the *epi form*. Thus, we can have cholesterol and epicholesterol, allocholesterol and epiallocholesterol. This is indicated in Figure A.4. The hydroxyl group of *testosterone* in the 17 position is above the ring, and thus β. In epitestosterone, the 17 hydroxyl is below the ring or

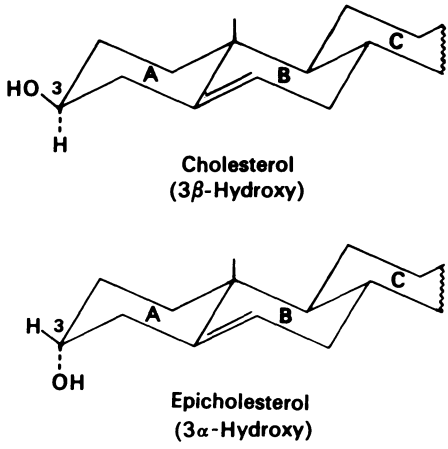


FIGURE A.4 Comparison of the normal and epi form of a steroid.

alpha. The term *epi* has no specific positional significance. It means the —OH configuration on a particular carbon other than that observed in the compound first identified, the ring structure remaining the same.

A *double bond* is indicated by the suffix *ene*. The location of the double bond is indicated by a delta (Δ) and a superscript, locating the double bond. Thus Δ^5-androst*ene*, would mean androst*ane* with a double bond at positions 5 to 6. Only the first carbon needs to be numbered, since the second is the following digit.

A.1.1 *Progesterone and the Corticosteroids*

In *progesterone* and the *corticosteroids* part of the side chain of cholesterol, from carbon 22 to 27, has been removed by oxidation. This

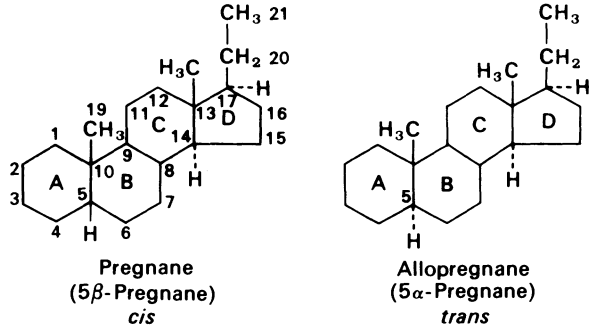


FIGURE A.5 The configurations of pregnane and allopregnane.

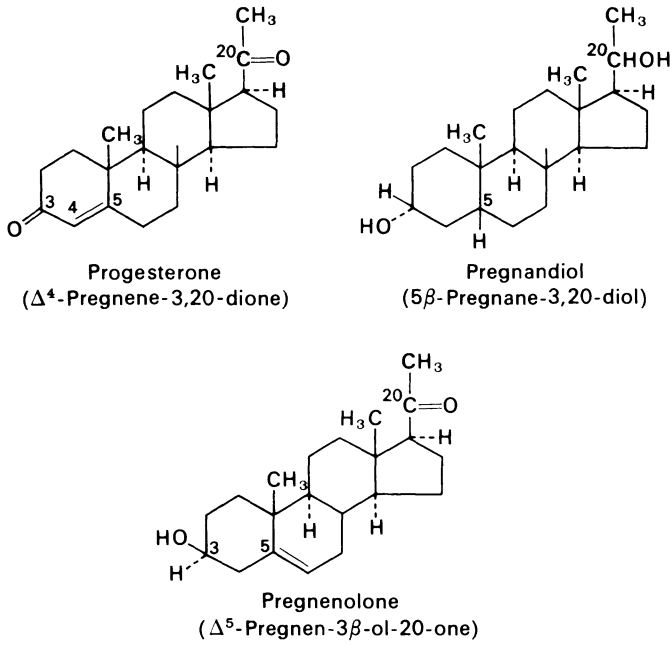


FIGURE A.6 Progesterone and structurally related compounds.

leaves a structure with a two-carbon side chain. This basic structure is called *pregnane*, and progesterone and the corticoids may be named as derivatives of pregnane. This is shown in Figure A.5. In the figure, it will be noted that when rings A and B have the *cis* configuration, the hydrogen at position 5 is β, this is *pregnane*. In the *trans* form the hydrogen at position 5 is α, and derivatives with this configuration are referred to as the *allopregnanes*. Alternatively, the designations, *5β- and 5α-pregnanes* are preferred by some. This nomenclature is applied to progesterone and related compounds in Figure A.6.

The *corticosteroids*, such as *cortisol*, are closely related to progesterone in structure but possess additional hydroxyl or keto groups. They are often referred to as *glucocorticoids* since the hydroxyl group at the 21 position resembles a ketose, $CH_2OH—(CO)—$, as observed in fructose. Thus, glucocorticoids reduce Fehling's solution. Some of the corticoids are shown in Figure A.7.

In *aldosterone*, the methyl group at position 18 has been oxidized to an aldehyde (see Figure A.8). This aldehyde may be hydrated and then

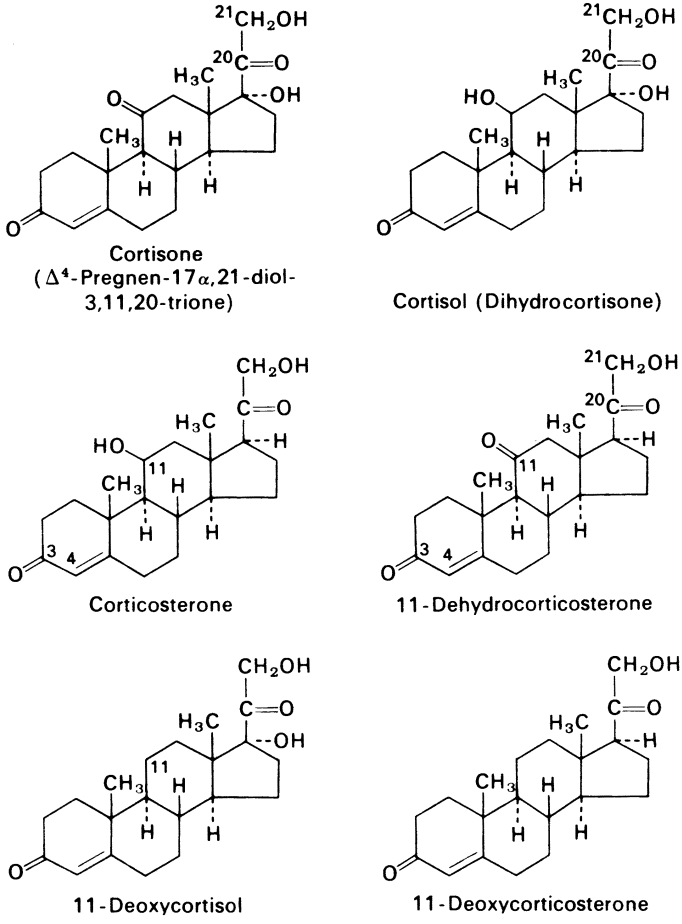
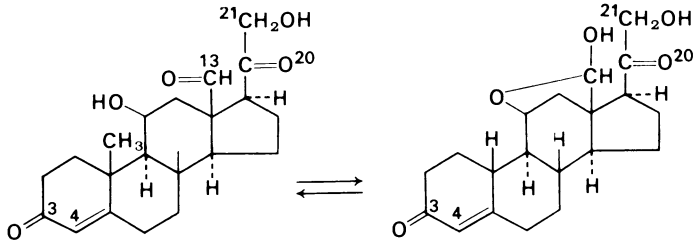


FIGURE A.7 Structures of some biologically active corticosteroids.

forms an anhydride with the hydroxyl group at the 11 position (a hemiacetal). Note that the hydroxyl group in the 11 position is above the ring or β. The hydroxyl group at position 21 can be either α or β since the $-CH_2OH$ group is free to rotate relative to the rest of the molecule.

In order to increase potency, and decrease side effects, a number of steroids have been synthesized with corticoid activity and are in common use. Some of these are referred to in the text, and their structures are therefore shown here (see Figure A.9).



Aldosterone
(Δ^4-Pregnene-11β,21-diol-3,20-dione-18-al)

FIGURE A.8 The alternative structures of aldosterone.

A.1.2 *The Steroid Sex Hormones*

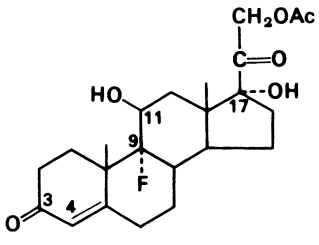
The female steroid sex hormones are referred to as the *estrogens* since they are important in maintaining the estrus cycle. These are distinguished from the other steroids in that ring A is aromatic in character. This results in the loss of the methyl groups at position 19. The most active estrogen is *estriol*, although *estradiol* and *estrone* also have estrogenic activity. Their structure is related to the hydrocarbon, 18-methylgonane which is given the name *estrane*. The nomenclature of the estrogens is based on this hydrocarbon. This is shown in Figure A.10.

The masculinizing steroid hormones are called the *androgens*. In these compounds, the structure retains the two methyl groups at positions 18 and 19. The basic hydrocarbon is 10,13-dimethylgonane or *androstane*. The structure of some of the androgens are shown in Figure A.11.

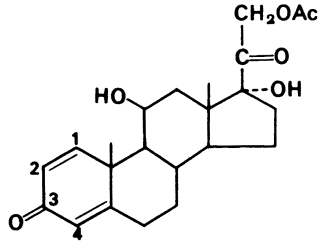
Similar to androstane in structure but with the rings A and B *trans* to each other, instead of *cis*, as in androstane, is *etiocholane*. This results in the hydrogen at the 5 position being above the ring system, or β. *Thus etiocholane is 5β-androstane*. Androsterone and epiandrosterone differ in the location of the —OH group at positions 3. Dehydroepiandrosterone also has a double bond at positions 5 to 6.

A.1.3 *The Bile Acids*

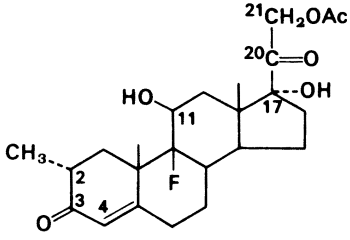
The bile acids are derivatives of cholesterol where the isopropyl group attached to C-24 has been removed by oxidation, leaving a five carbon side chain (Figure A.12).



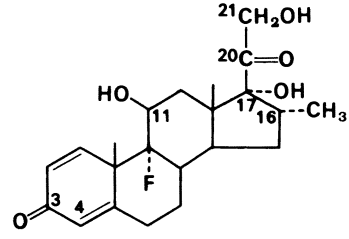
9α-Fluorocortisol Acetate



Prednisolone Acetate
(1,2-Dehydrocortisol Acetate)

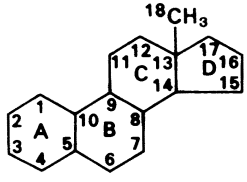


2α-Methyl-9β-
fluorocortisol Acetate

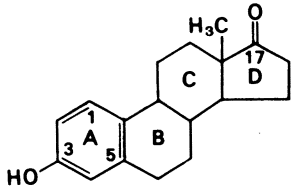


Dexamethazone
(16α-Methyl-1,2-dehydro-
9α-fluorocortisol)

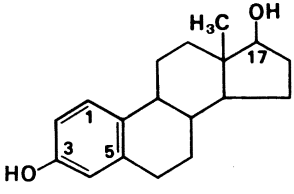
FIGURE A.9 The structure of several synthetic analogs of cortisol, in use for their corticoid activity.



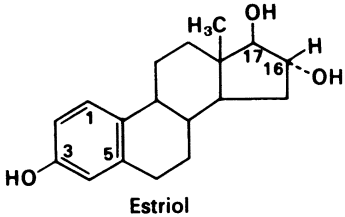
Estrane



Estrone
(1,2,5-Estratriene-3-ol-17-one)



Estradiol
(1,3,5-Estratriene-3,17β-diol)



Estriol
(1,3,5-Estratriene-3,16-α,17β-triol)

FIGURE A.10 The estrogens. Oxidation of ring A of estrane (the basic structure) forms a benzenoid ring which accounts for the phenolic properties and solubility in alkali of the estrogens. Estriol is the most active physiologically.

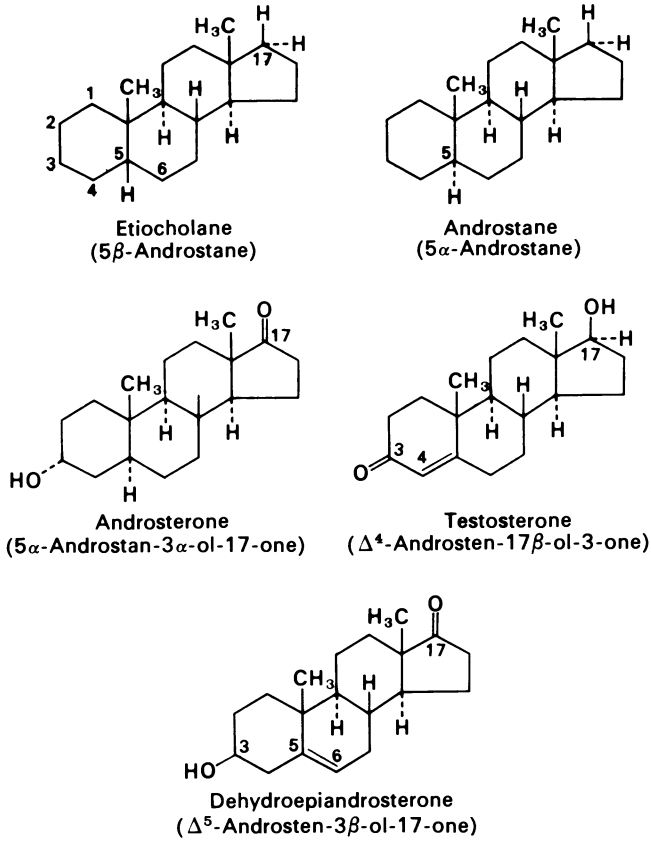
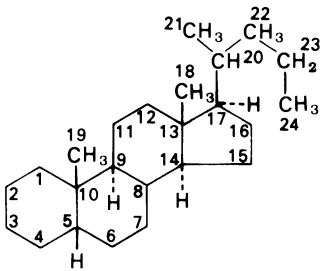


FIGURE A.11 The structural relationship between various androgens.

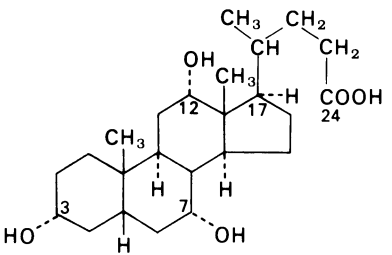
The skeletal hydrocarbon on which the bile acids are based is called *cholane.* If C-24 is oxidized to the acid we have *cholanic acid.* If the side chain is completely removed from cholane, we have *etiocholane* (see Figure A.11). The prefix *etio* means the cause or basis as in etiology of a disease. In this case it refers to the basic structure.

As can be seen from Figure A.12, the hydrogen at position 5 is above the plane of the ring or β. This means that rings A and B are *cis* to each other (see Figure A.2). This is the case for all the common bile acids.

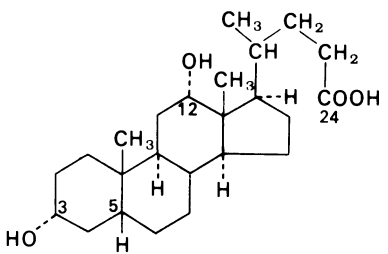
The most common bile acid is cholic acid, with three α-hydroxyl groups at positions 3, 7, and 12. In deoxycholic acid the hydroxyl groups at position 7 has been lost. Lithocholic, which is the least soluble, has



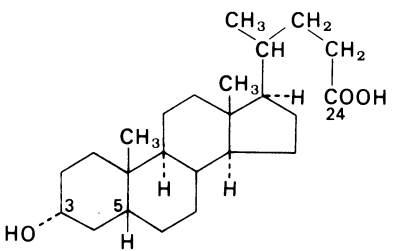
Cholane



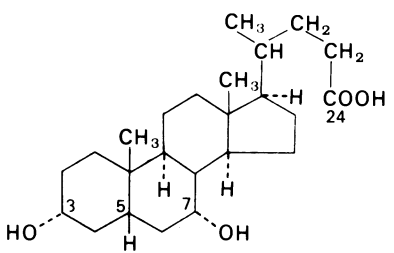
Cholic Acid
(3α,7α,12α-Trihydroxy-5β-cholanic Acid)



Deoxycholic Acid
(3α,12α-Dihydroxy-5β-cholanic Acid)



Lithocholic Acid
(3α-Hydroxy-5β-cholanic Acid)



Chenodeoxycholic Acid
(3α,7α-Dihydroxy-5β-cholanic Acid)

FIGURE A.12 Structure of the bile acids.

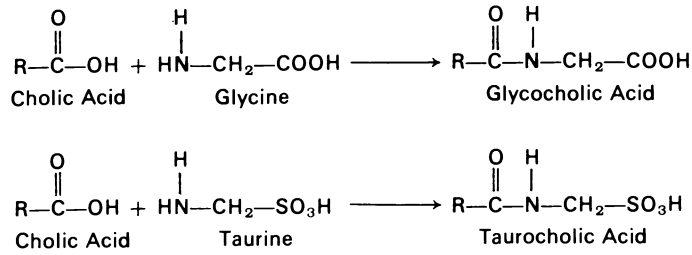


FIGURE A.13 Conjugation of cholic acid with glycine and taurine.

only one hydroxyl group, at position 3, and is the least soluble; thus its name, because of its tendency to form gall stones. Chenodesoxycholic [*chen* = goose] is cholic acid which does not have a hydroxyl group at position 12. It is found in the bile of poultry, such as the hen.

The bile acids occur conjugated with either glycine or taurine. Cholic acid conjugated with glycine is called *glycocholic acid*, and, when conjugated with taurine, *taurocholic acid*. The conjugation with glycine or taurine increases their solubility. This is shown in Figure A.13.

A.2 *MECHANISM OF CHOLESTEROL FORMATION*

Cholesterol is synthesized in the body from acetate originally generated mainly by degradation of lipids. Normally, acetate condenses with oxaloacetic to form citrate. The citrate is then metabolized to generate two molecules of H_2O and two of CO_2, both arising from the acetate. Oxaloacetate is regenerated to repeat the cycle.

$$\text{acetate} + \boxed{\text{oxaloacetate}} \xrightarrow[\text{(E.C. 4.1.3.7)}]{\text{citrate synthase}} \text{citrate}$$

$$\text{citrate} \xrightarrow{\text{Krebs cycle}} 2CO_2 + 2H_2O + \boxed{\text{oxaloacetate}}$$

Normally, some acetate does not enter the oxidative cycle but may take any one of several pathways. The acetate is in the form of acetyl-CoA when it reacts.

$$\text{acetyl-CoA} \xrightarrow{\text{fatty acid synthesis}} \text{stored fats}$$

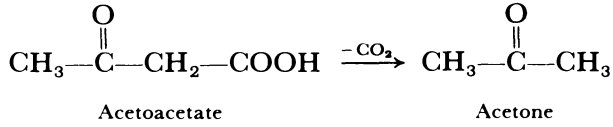
$$\text{acetyl-CoA} \xrightarrow{\text{oxidative cycle}} CO_2 + H_2O + \text{energy}$$

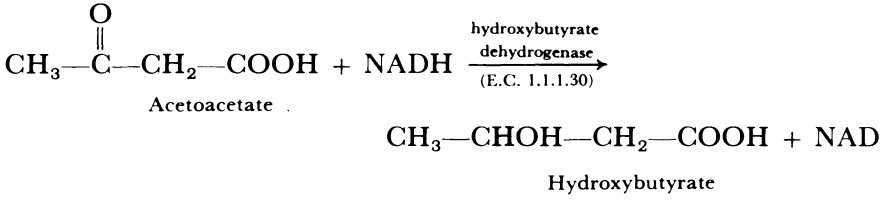
$$2 \text{ acetyl-CoA} \xrightarrow{\text{condensation}} \text{acetoacetate (ketone bodies)}$$

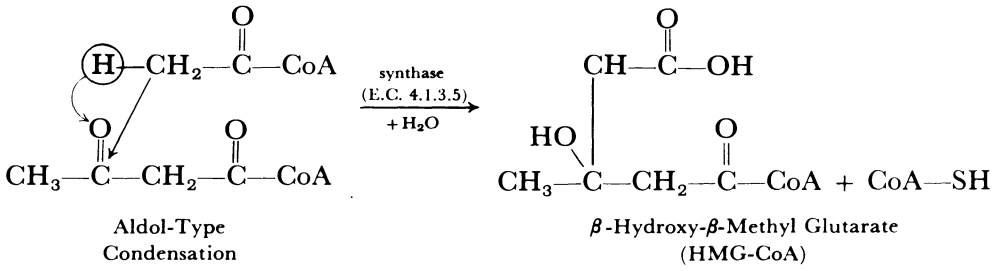
$$\text{acetyl-CoA} + \text{acetoacetate} \rightsquigarrow \text{mevalonate} \rightsquigarrow \text{cholesterol}$$

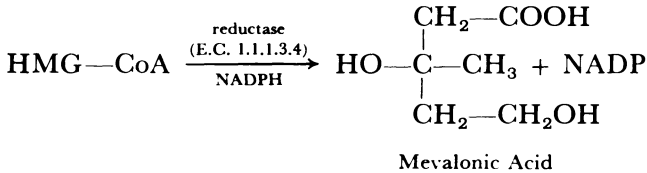
Acetoacetate may be reduced, to appear in the urine as hydroxybutyric acid, or it may be decarboxylated to form acetone. *Acetoacetate, acetone, and hydroxybutyrate are called the ketone bodies.*

Acetoacetate may be converted to cholesterol by a series of reactions, beginning with the condensation of a third acetate molecule with acetoacetate, followed by a reduction to form mevalonic acid. This takes place in the mitochondria.

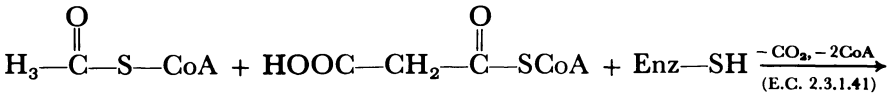




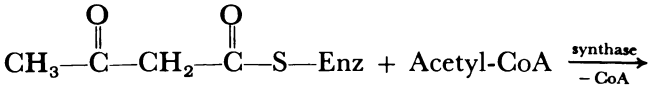


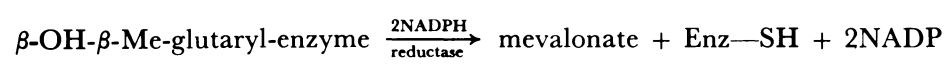


Mevalonic acid synthesis also occurs outside of the mitochondria. Acetyl-CoA condenses with malonyl-CoA, in the presence of a condensing enzyme containing an SH group, which binds the acetoacetate.



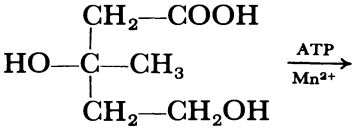
Acetate-CoA Malonate-CoA



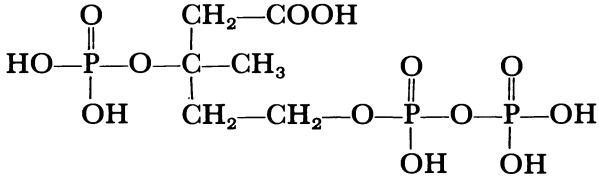


Reduction of β-hydroxy-β-methyl glutarate to mevalonate seems to be the rate-limiting step in the synthesis of cholesterol under hormonal control.

Mevalonic acid is then phosphorylated at the hydroxyl groups to form 3-phospho-5-pyrophosphomevalonic acid.

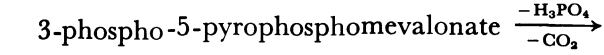


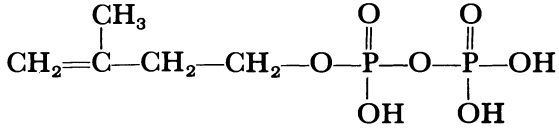
Mevalonic Acid



3-Phospho-5-pyrophospho Mevalonate

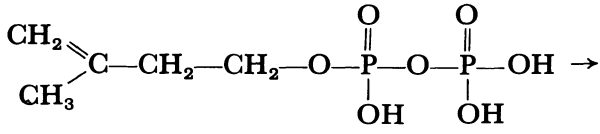
The phosphorylated mevalonic acid is then decarboxylated, the 3-phosphate group also being removed, to produce an isoprene derivative, isopentenyl pyrophosphate.



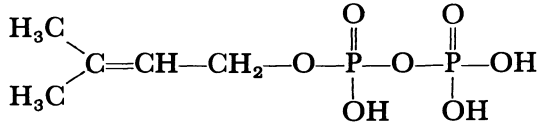


Isopentenyl Pyrophosphate

The double bond in isopentenyl pyrophosphate shifts to form dimethylallyl pyrophosphate.

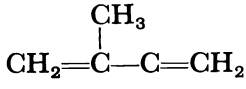


Isopentenyl Pyrophosphate

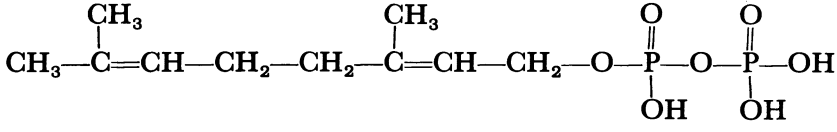


Dimethylallyl Pyrophosphate

Isoprene is a five-carbon compound originally isolated by destructive distillation of rubber. It has the formula:



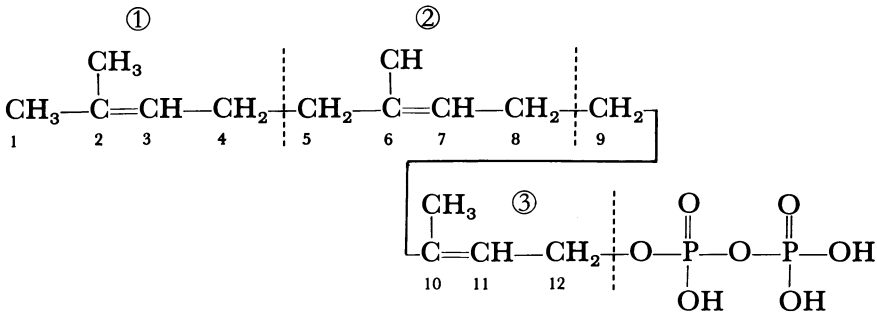
If two molecules of isoprene condense we have a *terpene.* Isoprene is then a *hemiterpene.* It can be seen that isopentenyl pyrophosphate is isoprene, to which a pyrophosphate radical has been added. If a molecule of isopentenyl pyrophosphate condenses with a molecule of dimethylallyl pyrophosphate, we would then have a *terpene.* In this case the terpene would be *geraniol* as the pyrophosphate.



Geranyl Pyrophosphate

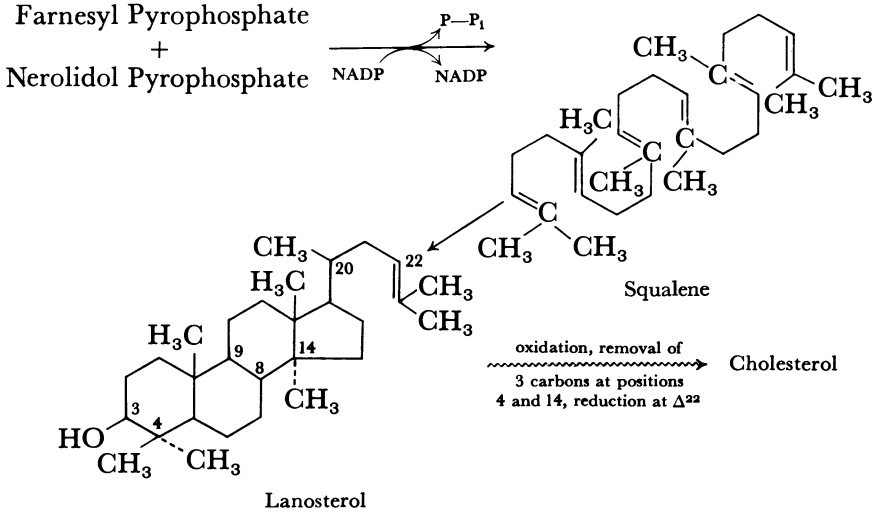
If another molecule of isopentenyl pyrophosphate is added, we obtain a *sesquiterpene* ($1\frac{1}{2}$ isoprene units) or in this case *farnesyl pyrophosphate.*

The three isoprene units are delineated above. A shift of a double bond at position 10–11 to 11–12, and the pyrophosphate to position 10, results in the formation of *nerolidol pyrophosphate.* Condensation of the



Farnesyl Pyrophosphate

farnesyl pyrophosphate and nerolidol pyrophosphate, followed by a reduction of a double bond, results in the formation of a triterpene (6 isoprene units) called *squalene.*



Geraniol, farnesene, and *squalene* all occur in both plants and animals. Squalene is present in substantial amounts in human liver. It is readily obtained in large amounts from shark's liver. Squalene is readily cyclized to form *lanosterol.* Lanosterol gets its name from its high concentration in wool grease. *Lanosterol* is readily converted to *cholesterol* by oxidation, resulting in the loss of three methyl groups. In each case the methyl group is converted by oxygen first to the alcohol, then the aldehyde and finally the acid before decarboxylation.

A.3 *NEUTRAL AND PHOSPHORYLATED GLYCERIDE STRUCTURES*

A.3.1 *Diglyceride Pathway for Glyceride Synthesis**

There are two major sources of the neutral and phosphoglycerides. They may be synthesized *de novo* or reassembled after hydrolysis of ingested lipids. In the former case, L-3-phosphoglycerate, from glucose metabolism (see Volume 2, p. 114) is acylated by the acyl-CoA formed from fatty acids which have been synthesized in the body or taken in the diet. The phosphatidic acid first formed, may then react with choline, serine, or inositol.

The phosphate of phosphatidic acid may be exchanged for another mole of fatty acid, resulting in the formation of neutral triglycerides. Alternatively, cytosine triphosphate reacts with phosphatidic acid to form *cytosine diphosphoglyceride* (*CDP*). Cytosine diphosphoglyceride can then react with serine to release cytosine monophosphate. The phosphatidyl serine can then be decarboxylated to form cephalin and methylated to form lecithin (see Figure 6.3). The sequence of reactions described is summarized in Figure A.14.

Cytosine diphosphoglyceride may also exchange the cytosine monophosphate group for inositol or glycerine to form phosphatidyl inositol or phosphatidyl glycerol.

$$\text{cytosine diphosphoglyceride} + \text{inositol} \rightarrow \text{phosphatidyl inositol} + \text{CMP}$$

$$\text{cytosine diphosphoglyceride} + \text{glycerol} \rightarrow \text{phosphatidyl glycerol} + \text{CMP}$$

Cytosine diphosphoethanolamine (CDP-ethanolamine) and CDP-choline are also synthesized in human tissue. These can react with a 1,2 diglyceride directly to form cephalin and lecithin.

$$\text{CDP-ethanolamine} + \text{1,2 diglyceride} \xrightarrow[\text{(E.C. 2.7.8.1)}]{\text{transferase}} \text{cephalin} + \text{CMP}$$

$$\text{CDP-choline} + \text{1,2 diglyceride} \xrightarrow[\text{(E.C. 2.7.8.2)}]{\text{transferase}} \text{lecithin} + \text{CMP}$$

The *plasmalogens* originate from the aldehydes formed by the reduction of the fatty acids.

* The structures of the glycerides are illustrated in the lipoprotein chapter, pp. 190 and 191.

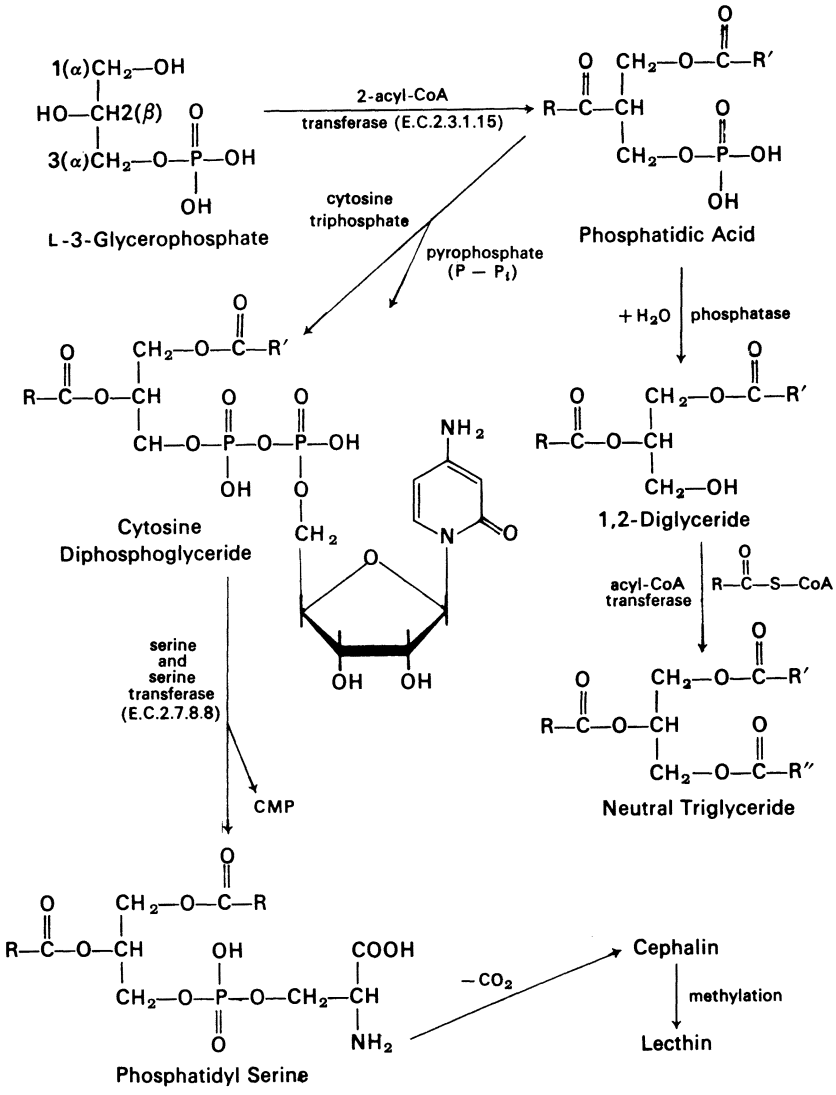


FIGURE A.14 Neutral triglyceride and phospholipid formation from glycerophosphate.

$$R{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}SCoA \xrightarrow{\text{reductase}} R{-}CH_2{-}CHO$$

Fatty Acid — Fatty Aldehyde

$$R{-}CH_2{-}CHO + \text{glycerophosphate} \longrightarrow$$

$$R{-}CH_2{-}\overset{\overset{OH}{|}}{CH}{-}O{-}CH_2{-}CHOH{-}CH_2{-}O{-}PO_3H_2 \xrightarrow{-H_2O}$$

$$R{-}CH{=}CH{-}O{-}CH_2{-}CHOH{-}CH_2{-}O{-}PO_2H_2 \xrightarrow{\text{choline}}$$

Vinyl Ether of Glycerol Phosphate

1-O-(1′ alk-1′ enyl)-glycero-3-phosphocholine + acyl-CoA

$$\xrightarrow[\text{(E.C. 2.3.1.25)}]{\text{Plasmalogen synthase}} \text{Plasmalogen}$$

The vinyl ether then react with acyl-CoA to produce the corresponding phosphatidic acid. This then reacts in a manner similar to that indicated in Figure A.14 to form the plasmalogens. Alternatively, the phosphocholine is formed first, and then the plasmalogen, by reaction with acyl-CoA as shown above.

A.3.2 *Monoglyceride Pathway for Glyceride Synthesis*

Fats taken into the diet can be reprocessed for storage or utilization without extensive intermediate metabolism. On hydrolysis of triglycerides in the intestines, specific intestinal lipases first hydrolyze the fatty acids in the end positions (1 and 3). This leaves a soluble, nonionic detergent-like structure with a hydrophilic (2 —OH groups of glycerol) and a hydrophobic (fatty acid chain) portion of the molecule. This compound is readily emulsified, to form micelles which pass into the lymph through the villi carrying the released fatty acids.

$$\text{triglyceride} \xrightarrow{\text{hydrolysis}} \text{2 fatty acids} + \text{2-acyl glycerol}$$

At the tissues, the fatty acids are converted to their acyl-CoA derivatives and react with the monoglyceride to form 1,2-diglyceride, and, finally, either the neutral triglycerides for fat deposition or phosphatidic acid for further processing.

$$\text{2-acyl glycerol} \xrightarrow{\text{acyl-CoA}} \text{1,2-diacyl glycerol}$$

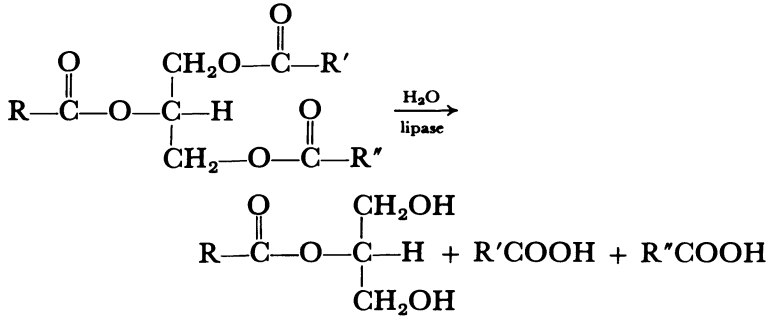
A.4 *NATURE OF THE LIPASES*

A lipase is distinguished from an esterase in that the normal substrate for the lipase is an insoluble fatty acid ester held in suspension in the form of a micelle. The esterase usually acts on water-soluble low-molecular-weight esters. However, there is considerable cross reaction between esterases and lipases. Lipases originate in all organs, and will now be discussed.

A.4.1 *Pancreatic Lipase*

Pancreatic lipases are synthesized in the acinar cells of the pancreas. They are a complex mixture of enzymes some of whose activity is stimulated by calcium ion and strongly inhibited by fluoride ion. Others are insensitive to these reagents and are activated by bile salts.

Pancreatic lipases have a broad pH optimum, ranging from 7.5 to 9.0. They are most active in emulsified substrates, the fat globules being suspended as micelles. The emulsifying agents in the human are the bile salts. The major substrates are the triglycerides derived from the diet. Hydrolysis takes place at the 1 and 3 positions, so that the soluble monoglyceride formed may be readily absorbed.



In addition to the lipases which attack the triglycerides, lipases exist in the pancreas which hydrolyze secondary alcohols such as cholesteryl esters.

Some pancreatic lipases will also attack synthetic substrates such as

phenyl or naphthyl laurate, linoleate, or oleate. Because of the ease of estimation of the phenol or naphthol generated, these substrates have been used in assaying serum lipase. *Serum lipase assayed with these substrates does not correlate with disease of the pancreas.* The lipase which finds its way into the plasma in pancreatitis is predominantly a triglyceridase. A triglyceride substrate such as olive oil is required for this purpose.

With chronic pancreatitis the serum lipase will remain elevated long after the amylase has returned to normal. Technical difficulties in lipase assay, however, have limited its uses, relative to amylase assay, as an indicator of pancreatitis.

A.4.2 *Lipoprotein Lipase*

The primary substrate of lipoprotein lipase is the triglycerides bound to the α and β lipoproteins. It also hydrolyzes the chylomicron triglycerides found in the plasma. Its pH optimum is at 8.5. Normally, albumin will inhibit lipase activity. However, it acts as an activator of lipoprotein lipase. This is attributed, probably erroneously, to its ability to bind free fatty acids and allow the reaction to go to completion. This idea does not explain why albumin inhibits the activity of other lipases. This enzyme or complex of enzymes, since it has not been clearly characterized, will hydrolyze both the α and β bonds of the triglycerides, albeit the α bonds are more rapidly hydrolyzed.

The lipoprotein lipase concentration of the plasma is normally very low. It seems to be bound to the capillary endothelium. On injection of heparin, it is released and serves to hydrolyze the fat in a lipemic serum. For this reason it has been called *clearing factor* by some, since heparin, *per se*, does not clear plasma.

Lipoprotein lipase is present in high concentration in adipose tissue and it has been suggested that its main function is to aid the uptake of triglycerides from the plasma to the tissues.

A.4.3 *Hormone-Sensitive Lipase (HSL)*

Hormone-sensitive lipase (HSL) is present in adipose tissue but is distinct from lipoprotein lipase. Epinephrine, norepinephrine, ACTH, thyroid stimulating hormone (TSH), and glucagon all serve to convert this enzyme to an "active form." Somatotrophin and cyclic AMP also act as activators of this enzyme. Only insulin serves to decrease the level of activity. An important role is assigned to this enzyme in mobilizing fat from the tissues and raising the plasma free fatty acid levels.

HSL catalyzes the hydrolysis of triglycerides to diglycerides. Adipose tissue also contains a monoglyceride lipase which hydrolyzes diglycerides very rapidly. This enzyme is not hormone dependent. It also acts on the β-monoglycerides but at a much slower rate. Apparently HSL starts the hydrolysis and once the diglyceride is formed this lipase, and possibly others, serve to complete the hydrolysis in the adipose tissue.

A.4.4 *Other Tissue Lipases*

The *liver* synthesizes several lipases, one of which is most effective in hydrolyzing triglycerides. This enzyme activity is stimulated by glucagon. These enzymes will also hydrolyze mono and diglycerides, phenyl and naphthyl esters of fatty acids. The latter substrates are very sensitive to fluoride and are readily inactivated by this substance. For this reason, liver lipase activity has been suggested as a possible test for fluoride in drinking water.

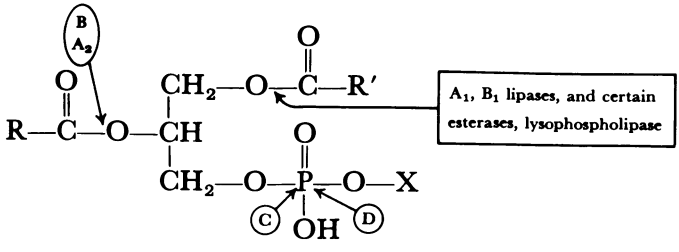
A lipase is also present in the *intestinal tissue* which hydrolyzes mono but not di- or triglycerides. This is of significance in fat absorption.

The *heart* also contains a hormone-sensitive lipase which is stimulated particularly by adrenaline. This is of significance in metabolism of cardiac tissue.

A.4.5 *Phospholipases*

The phospholipases all serve to hydrolyze the glycerophospholipids. There are at least six different phospholipases commonly found in living organisms. These include the phospholipases A_1, A_2, B, C, D, and lysophospholipase. In addition, lipases and certain esterases will also serve to hydrolyze acyl groups from the 1 position in the phospholipids.

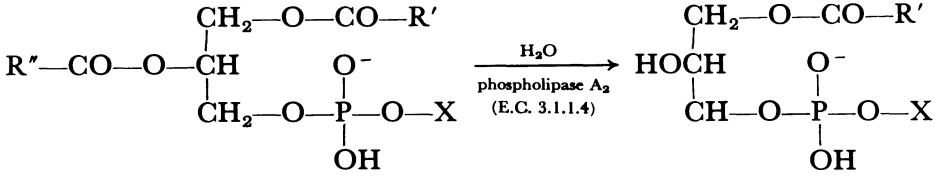
The position of attack of the various phospholipases and lipases is shown below (X is choline, ethanolamine, inositol, or serine):



A.4.5.1 *Phospholipase A_2*

This enzyme (phosphatide acyl hydrolase, E.C. 3.1.1.4) releases one mole of fatty acid from a diacyl phospholipid such as lecithin. It is present in all animals and plants but in especially high concentration in certain snake venoms. It releases the fatty acid only from the 2 position (β). It will also hydrolyze the fatty acid from the plasmalogens. The reason it hydrolyzes the fatty acid from the 2 position is because it seems to hydrolyze the acyl group adjacent to the phosphate ester linkage. This can be demonstrated with synthetic substrates. For example, if the 2 position is occupied by a phosphate linkage and the 1 position by an acyl group, the acyl group will be hydrolyzed off.

It will be noted that hydrolysis of the phosphatide by this enzyme results in a substance with a hydrophobic end (the fatty acid) and a hydrophilic end (the phospho ester). Thus, one has a detergent effective in hemolyzing erythrocytes. This adds to the toxicity of snake venom.



Lysolecithin

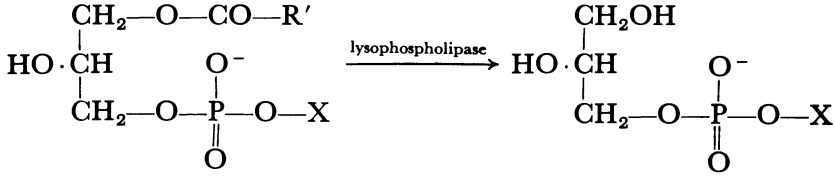
Phospholipase A_2 is also present in substantial quantitites in the pancreas and this accounts for the fact that small amounts of lysolecithin are found in human serum. This enzyme occurs in a form of low activity in the pig pancreas. On hydrolysis of small polypeptides from the molecule it is activated. This is similar to the activation of pepsinogen or trypsinogen. It serves to control the amount of active enzyme released since uncontrolled it could be dangerous, since lysolecithin is a detergent, and hemolyzes erythrocytes.

A.4.5.2 *Phospholipase A_1*

Phospholipase A_1 is present in the microsomal fraction of liver in all animals studied. It catalyzes the release of the fatty acid from the 1 position of the phosphatide. In a similar manner, lipase from the pancreas also releases the fatty acids from the 1 position. For this reason, phospholipase A_1 is often confused with pancreatic lipase.

A.4.5.3 *Lysophospholipase*

Lysophospholipase (E.C. 3.1.1.5) acts on the phosphatide molecule after the fatty acid in the 2 position has been hydrolyzed. Thus its natural substrate is *lysolecithin* or *lysocephalin.* In this way it serves to prevent accumulation of the lysolecithin.



A.4.5.4 *Phospholipase B*

Phospholipase B is claimed to hydrolyze the fatty acids at both the 1 and 2 positions of the phosphatide. Some believe that it is really an unresolved mixture of phospholipase A_2 and lysophospholipase.

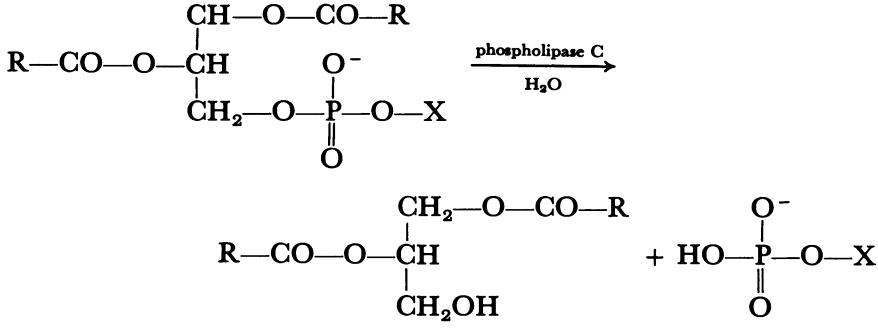
Phospholipase B from *Penicillium notatum* is reported to require activation by other lipids such as phosphatidyl inositol. Normally the micelles, which the phospholipases attack, are positively charged because of the choline or ethanolamine at the surface. These repel the enzyme. However, phosphatidyl inositol exhibits, at the surface of the micelle, only the negative charge of the phosphate linkage. For this reason the enzyme is able to approach the micelle and get at the fatty acid ester linkage so as to hydrolyze it. For similar reasons, low concentrations of detergents (such as the bile salts) change the charge at the surface of the micelle and activate the enzyme in a manner similar to that with phosphatidyl inositol.

A.4.5.5 *Phospholipase C*

This enzyme is quite distinct from the phospholipases discussed above in that it attacks a particular phosphate linkage. It hydrolyzes the phosphocholine at the ester linkage with glycerine. Thus it is called a phosphatidyl choline, choline hydrolase (E.C. 3.1.4.3). It is present in high concentrations in certain bacteria such as *Bacillus cereus* and *Clostridium perfringens.* Brain, kidney, liver, spleen, and other organs contain phospholipase C. The enzyme is not specific to

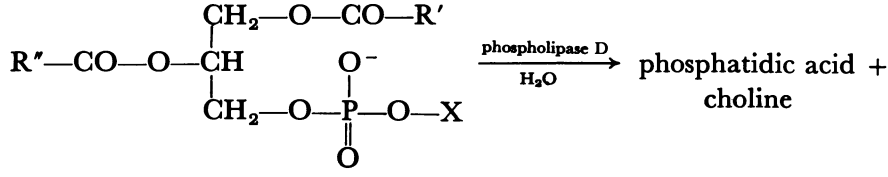
lecithin since phosphatidyl ethanolamine and serine are also hydrolyzed. In the following reaction, X may thus be choline, serine, or ethanolamine.

A different enzyme is required for the hydrolysis of phosphatidyl inositol, in a similar fashion. This is probably due to the difference in charge of the micelles formed as discussed above.



A.4.5.6 *Phospholipase D*

This enzyme which, at present, has been identified only in plants, will hydrolyze the phosphoglyceride at the phosphate ester linkage with choline. It is also called phosphatidyl choline, phosphatide hydrolase (E.C. 3.1.4.4). Its action is not specific for choline, and serine or ethanolamine will also be hydrolyzed from the phosphatide.



A.5 *PROTEIN PARAMETERS*

In discussing the various plasma proteins in the text, reference was made, from time to time, to the physical characteristics of proteins, such as molar absorptivity, sedimentation constant, diffusion constant, and electrophoretic mobility. A brief explanation will be presented here of the meaning of these terms.

A.5.1 *Absorptivity of the Plasma Proteins*

When monochromatic light passes through a solution, the fraction of light which emerges, % transmitted (%T/100), can be shown to be related to the light path (l), and the concentration of the solution (c) by the following relationship:

$$-\log \frac{\%T}{100} = (a)(l)(c) = (A) \text{ [absorbance (or absorbancy)]}$$

The constant (a) is called the absorptivity. When the concentration is molar (1 mol/liter) and the light path is 1 cm in length, then the absorbance (A) is equal to the absorptivity. This can be summarized as follows:

$$A = (a)(l)(c) = 2 - \log \%T \qquad \text{(A.1)}$$

In the older literature, the absorbance was called the optical density (OD) of the solution. The absorbance of a 1% solution was taken as standard, and for a light path of 1 cm, the term *extinction coefficient* was used. In this convention, still used by many protein chemists, the symbol, $E^{1\%}_{1\,\text{cm}}$ was used, to indicate these conditions, and the wavelength was stated. Others have continued to use the optical density designation. For example, gynecologists refer to the OD_{450} for the absorbance of amniotic fluid at 450 nm.

The International Union of Pure and Applied Chemistry (IUPAC) has recommended that where possible, the absorbance be given for a molar solution, recommending the term *molar absorptivity* with the symbol ϵ (epsilon). Where the molecular weight was uncertain, as for certain proteins, the absorptivity (a) could be used. The symbol a indicated a 1% solution and a 1-cm light path.

These conventions are not followed exclusively. In order to avoid stating the light path separately and to avoid the use of large numbers, as with molar absorptivity, *The Merck Index* uses its own convention. For example, the absorbance of heme is given for a millimolar solution and the wavelength is cited. Thus $E^{572}_{\text{mM}} = 5.5$ indicates the absorbance of a millimolar solution of heme at 572 nm. If a molar solution were used the value would be 5500.

The molecular weights of proteins are very high and many investigators use the absorbance of a 1% solution in reporting their

TABLE A.1

Absorptivity (Extinction Coefficient, $E^{1\%}_{1\,cm}$) for Some of the Plasma Proteins at 280 nm, Compared to their Tyrosine, Phenylalanine, and Tryptophan Content (mol/mol protein) in Order of Increasing Absorptivity

Protein	$E^{1\%}_{1\,cm,\,280\,nm}$	Tyrosine	Phenyl-alanine	Trypto-phan	Molecular weight
Albumin (human)	5.31	18	30	1	66,248
Albumin (bovine)	6.67	19	27	2	66,210
α_1-Acid glycoprotein	8.93	11	9	3	40,000
Transferrin	11.2	25	31	10	76,500
Haptoglobin 1-1 (Hb free)	12.0	34	16	14	103,772
IgM (hexamer)	13.3	258	269	152	950,000
IgA	13.4	43	40	28	160,000
IgG	13.8	62	47	33	160,000
Ceruloplasmin	14.9	74	55	20	134,000
Fibrinogen	15.5	104	95	55	340,000
Hemopexin	19.6	18	21	18	57,000

results. However, they seem to prefer the older designation of extinction coefficient, $E^{1\%}_{1\,cm}$, since it is easier to typewrite than the Greek epsilon.

Proteins show absorbance maxima at about 280 and 220 nm. The absorbance at 280 nm is due to the aromatic amino acids, tyrosine, phenylalanine, and tryptophan. The maximum at 220 nm derives from the peptide bond. If nucleic acids are present, a maximum absorbance peak will be at about 260 nm. Typical maxima for several proteins, as compared to their aromatic amino acid composition, are compared in Table A.1.

From Table A.1, it can be seen that the absorptivity of the proteins varies with their aromatic amino acid composition. Human albumin, with only one tryptophan per molecule, has a substantially lower absorbance than bovine albumin with two tryptophans per molecule. As the aromatic amino acid content increases, there is a corresponding rise in absorptivity at 280 nm, so that ceruloplasmin has about three times the absorptivity of albumin.

A most common procedure for following the proteins as they elute from a column is the measurement of their absorbance at 280 nm.

TABLE A.2

Partial Specific Volumes for Some of the Human Plasma Proteins in Order of Increasing Value

Protein	Specific volume, $\bar{v}$ (ml/g)
α_1-Acid glycoprotein	0.675
Apohemopexin	0.702
Fibrinogen	0.723
Apotransferrin	0.725
Apoceruloplasmin	0.730
Albumin	0.733
IgG	0.739
Apohaptoglobin 1-1	0.766

A.5.2 *Partial Specific Volume of Proteins*

For the purpose of relating the molecular weight of proteins to their sedimentation rate in a centrifugal field, and their rate of diffusion, the volume occupied by one gram of a protein, its *partial specific volume* ($\bar{v}$), is required. This is the reciprocal of the density of the protein. The standard temperature chosen by most of the contributors to this field is 20°C.

Since the density of proteins other than certain lipoproteins, in general, is greater than 1, the partial specific volume is usually less than 1 and of the order of 0.7 for most proteins.

The partial specific volume is an estimate of the space occupied by the protein in solution. This value can be obtained by a careful measurement of the density of the protein solution to four decimal places.[3] From this value, the density of the protein component of the solution can be calculated. Table A.2 lists the partial specific volume for some of the proteins in human plasma. The formula for calculating the partial specific volume ($\bar{v}$), from the density of the buffer (d_B), the density of the protein solution (d) and the concentration of the protein in g/ml (c), is given in Eq. (A.2).

$$\bar{v} = (1/d_B) - (1/c)\left(\frac{d - d_B}{d}\right) \tag{A.2}$$

A.5.3 *Viscosity and Frictional Resistance of Protein Molecules*

Viscosity, usually symbolized by the Greek eta (η), is the internal fluid friction of a liquid. When an insoluble particle moves through a medium such as water, a layer of water is irreversibly adsorbed to the particle. Thus, as the particle moves through water, it is the friction of the water layer on the particle with the surrounding water which resists the motion of the particle. Thus viscosity is a property of the solvent and not of the particle, and this is true regardless of whether the particle is a glass or steel ball, or a protein molecule.

The absolute unit of viscosity is the tangential force per unit area of two parallel planes, at a unit distance apart, when the space between them is filled with the fluid being studied, and one plane moves with unit velocity in its own plane relative to the other. If values are in the CGS system, the unit is the poise, in dynes.

When considering a *spherical* particle moving through a buffer solution, then the frictional force resisting this motion is the viscosity (η) multiplied by the effective surface ($6\pi r$). The symbol f_0 is used for the frictional force if the protein molecule is spherical and not hydrated. The formula for this frictional resistance is then given by $f_0 = 6\pi r\eta$.

If the particle is not spherical, or is hydrated, or both, the effective area being dragged through the solution will be different from that of a

TABLE A.3

Viscosity (η) for Water at Different Temperatures

Temperature (°C)	Viscosity (poise)[a]
5	0.015188
10	0.013077
15	0.011404
20	0.010050
20.20	0.010000
25	0.008937
30	0.008007
35	0.007225
40	0.006560

[a] The unit poise is the force, in dynes, required to maintain unit velocity difference between plates of unit area at unit distance apart.

sphere, and a correction factor, called the frictional ratio (f/f_0) needs to be applied. For a single protein molecule the frictional resistance then becomes, $f = 6\pi r\eta(f/f_0)$. For one mole, this number needs to be multiplied by Avogadro's number (N), to obtain the total frictional resistance. This results in Eq. (A.3).

$$\text{molar frictional resistance} = f = 6\pi r\eta N(f/f_0) \qquad \text{(A.3)}$$

This formula is used in calculating the molecular weight of proteins from data obtained by experiments in diffusion rate and rate of sedimentation in the ultracentrifuge. Values for the viscosity of water at different temperatures are given in Table A.3.

Many mucoproteins are highly hydrated. This results in a very high frictional ratio and marked increase in the frictional resistance. In contrast, tobacco mosaic virus also has a high frictional ratio, but this is due mainly to its elongated shape.

A.5.4 *Plasma Protein Diffusion Coefficient*

If a protein, dissolved in a dilute buffer, is permitted to communicate with the buffer solution without protein, in an apparatus resembling the Tiselius apparatus of Figure 3.1, then the protein will diffuse into the protein solution. If there is no agitation and constant temperature is maintained, a concentration gradient will be set up between the protein solution and the buffer. This can be recorded and quantitated by examining the solution with ultraviolet light (280 nm). The change in concentration at a distance (x) from the origin (dc/dx) can be obtained by observation and calculation. The relationship between the original protein concentration (Pr), the concentration gradient dc/dx, and the elapsed time (t), is given in Eq. (A.4). This is known as Fick's law of diffusion.[4]

$$-\frac{dPr}{dt} = D\frac{dc}{dx} \qquad \text{(A.4)}$$

This equation states simply that the rate at which protein will diffuse is directly proportional to the concentration gradient at the particular location being examined. The negative sign indicates that the protein concentration is decreasing. D is the proportionality factor and is called the *diffusion coefficient*. It is given in cm^2/sec.

The diffusion coefficient is constant for a particular substance, and is a function of the frictional resistance offered to the protein surface. As indicated above, this is a function of the frictional ratio of the particular protein molecule, which is a measure of the asymmetry of the protein molecule. The force with which the molecule pushes through the solvent is a function of its kinetic energy and this is related to its mass. This force is quantitatively the same as osmotic pressure, which will now be discussed.

A.5.5 *Osmotic Pressure and Molecular Weight of Proteins*

The pressure of 1 mole of an ideal gas like argon, occupying 22.4 liters, is 1 atmosphere, or equivalent to 760 mm Hg. Substances dissolved in water, may be considered as molecules in the gaseous state. If 1 mole of protein is dissolved in 22.4 liters of water, the pressure of the molecules of the protein on the wall of the container would be one atmosphere. If this solution is separated from water by a membrane pervious to water but not to the protein molecules, then an unbalanced situation would exist and 1 atmosphere of pressure would need to be applied to the protein solution to prevent the water from moving into the protein solution. This is called the *osmotic pressure* (Π).

From the gas laws, $PV = nRT$, where n represents the number of moles being considered, replacing the gas pressure (P) by the symbol for osmotic pressure, we have $\Pi V = nRt$, or $\Pi = (n/V)RT$. The number of moles divided by the volume can be replaced by the grams per liter (c), divided by the molecular weight. The equation then becomes $\Pi = (n/M)RT$. Rearranging the equation to solve for the molecular weight (M) yields Eq. (A.5), the van't Hoff equation:

$$M = (c/\Pi)RT \tag{A.5}$$

Equation (A.5) applies only where the proteins do not associate or dissociate or take up substantial quantities of water of hydration. By estimating the osmotic pressures of protein solutions at increasing dilution and extrapolating to zero concentration, valuable data have been obtained by the osmotic pressure method for estimating the molecular weights of protein.[6] For example, for Hb by amino acid analysis, a molecular weight of 66,248 is obtained. By osmotic pressure, values approximating 69,000 were reported early in this century. With modern

techniques, values within 5% of the true value have been obtained by osmotic pressure measurement.

A.5.6 *Molecular Weight of Proteins by Diffusion*

Energy is the product of an intensity by a capacity factor, such as force × distance, volts × no. of electrons or charge, or temperature × entropy, and, in the case of a gas it is pressure × volume, which in turn is equal to RT. The frictional resistance (f) multiplied by the diffusion coefficient (cm^2/sec) also has the dimensions of work or energy and can be equated to RT. For one mole we have

$$fD = 6\pi r\eta N(f/f_0)D = RT = \Pi M/c \tag{A.6}$$

Equation (A.6) links Eqs. (A.3)–(A.5).

Given the radius of a protein molecule, and the partial specific volume ($\bar{v}$), it can be shown that the radius (r) can be equated to a relationship including the molecular weight (M) and Avogadro's number. This is shown in Eq. (A.7):

$$\text{radius of protein molecule} = r = \left(\frac{3\bar{v}M}{4\pi N}\right)^{1/3} \tag{A.7}$$

Combining Eqs. (A.6) and (A.7), we can solve for the molecular weight (M) or the frictional ratio Eq. (A.8).

$$M = \frac{R^3T^3}{162\pi^2N^2\eta^3D^3\bar{v}}(f_0/f)^3 \tag{A.8}$$

Since the reaction is carried out in water at 20°C and Avogadro's number is known, the equation can be simplified to Eq. (A.9). From Table A.3, η, the viscosity of water is 0.010050 at 20°C.

$$M = \frac{2.42 \times 10^{-14}}{D^3\bar{v}(f/f_0)^3} \tag{A.9}$$

From Eq. (A.9) either the molecular weight or the frictional ratio can be determined, once the diffusion coefficient and partial specific volume have been determined. Frequently, the molecular weight is

estimated by some other method, and the frictional ratio is desired. In this case Eq. (A.9) is rearranged to yield Eq. (A.10):

$$f/f_0 = \frac{2.89 \times 10^{-5}}{D(M\bar{v})^{1/3}} \tag{A.10}$$

A.5.7 *Molecular Weight of Proteins by Sedimentation in the Ultracentrifuge*

The ultracentrifuge, for the determination of the molecular weights of proteins, has the capability of rotating at speeds that generate gravitational fields on the order of 250,000 $\times$ g. Under these conditions most of the plasma proteins will sediment. When the accelerating speed is such that the rate of sedimentation overcomes the frictional resistance and the tendency to diffuse, a constant rate of sedimentation will be reached. The rate of sedimentation (dr/dt), where r is the distance from the center of rotation, is proportional to the strength of the centrifugal field expressed by the product of the square of the velocity (in radians per second, ω^2), and the distance from the center of rotation (r). By using the proportionality factor (s), called the *sedimentation constant*, this can be summarized in the equation, $dr/dt = s\omega^2 r$. Rearranging this equation to $dr/r = s\omega^2 dt$ permits its integration from r_0, the initial protein front, to a point r to give $\ln(r/r_0) = s\omega^2 t$. Rearranging this equation, we get Eq. (A.11).

$$\ln r = \ln r_0 + s\omega^2 t \tag{A.11}$$

It is customary to report sedimentation constants corrected to 20°C in conformity with the convention for the diffusion coefficient and partial specific volume. The sedimentation constant unit is the reciprocal of the second, sec^{-1}. When this is done, most proteins of the plasma will have a sedimentation constant of the order of 10^{-13}. Thus for IgG, the sedimentation constant is about $7 \times 10^{-13}\ \text{sec}^{-1}$. The Svedberg unit dispenses with the 10^{-13} and would report a sedimentation constant of 7, in Svedberg units, for this protein. Table A.4 lists the sedimentation and diffusion constants at 20°C for some of the plasma proteins using this convention.

When determining the sedimentation constant, the movement of the protein front is followed with ultraviolet light synchronized with the

TABLE A.4

Diffusion and Sedimentation Coefficients for Some of the Plasma Proteins, in Increasing Order of Sedimentation Rate

Protein	$D_{20°,w}$ ($cm^2\ sec^{-1} \times 10^7$)	$s_{20°,w}$ ($sec^{-1} \times 10^{13}$)	Molecular weight
α_1-Acid glycoprotein	5.27	3.5	41,000
Haptoglobin 1-1	4.7	4.45	103,772
Albumin	6.1	4.6	66,248
Hemopexin	—	4.8	57,000
Transferrin	5.0	5.3	76,500
IgG	3.84	6.6	160,000
Ceruloplasmin	3.76	7.08	167,000
Fibrinogen	2.01	7.90	340,000

rotation of the rotor. Alternatively, schlieren patterns are followed as for electrophoresis with the Tiselius cell.

From the sedimentation and diffusion constants, it is possible to calculate the molecular weight of the protein.

The weight of the protein molecule in water, minus the weight of the water it displaces (buoyancy), is its true weight. For every gram of protein, the weight of an equal volume of water needs to be subtracted. This weight is numerically equal to the partial specific volume ($\bar{v}$) of the protein multiplied by the density of the buffer (d). For N molecules, one mole, the true weight is the molecular weight (M). Representing the effective weight of one mole by M_e, we obtain Eq. (A.12):

$$M_e = M(1 - \bar{v}d) \tag{A.12}$$

The sedimenting force (F) is the product of the mass and the acceleration. In the centrifuge, the acceleration is given by $r\omega^2$ where r is the radius and ω is the angular velocity in radians as before. Thus, $F = Mr\omega^2$. Combining this equation with Eq. (A.12), gives Eq. (A.13):

$$F = M(1 - \bar{v}d)r\omega^2 \tag{A.13}$$

In deriving Eq. (A.11) it was shown that $dr/dt = s\omega^2 r$. Substituting for $\omega^2 r$ in Eq. (A.13) gives Eq. (A.14):

$$F = \frac{M(1 - \bar{v}d)dr/dt}{s} \tag{A.14}$$

The Stokes relationship for the viscous drag exerted on spherical particles is given by the formula, frictional coefficient = $6\pi r\eta(dr/dt)$.

This states that the force resisting the fall of the particle is the frictional resistance, ($f = 6\pi r\eta$) multiplied by the rate of fall. From Eq. (A.6) we can substitute RT/D for f. When the viscous drag equals the sedimenting force, we get Eq. (A.15), after canceling dr/dt from both sides of the equation:

$$\frac{RT}{D} = \frac{M(1 - \bar{v}d)}{s} \tag{A.15}$$

Rearranging this equation to solve for the molecular weight, gives Eq. (A.16), the *Svedberg–Pederson equation*:

$$M = \frac{sRT}{D(1 - \bar{v}d)} \tag{A.16}$$

If slower centrifugal speeds are used, the protein will not sediment but reach a balance point where the sedimentation force is equal to the rate of diffusion. The equations for diffusion and sedimentation given above are combined, setting the diffusion force equal to the force of sedimentation. This results in Eq. (A.17), which is the *sedimentation equilibrium* equation:

$$M = \left[\frac{2RT}{(1 - \bar{v}D)}\right]\left[\frac{\ln C_2/C_1}{\omega^2(X_2^2 - X_1^2)}\right] \tag{A.17}$$

C_2 and C_1 are the protein concentrations at distances X_2 and X_1 from the center of rotation. The other symbols have the same designation as indicated previously.

This method is independent of the shape and degree of hydration of the molecules, but it is time-consuming. It takes 16–24 hr to establish equilibrium. There must be no denaturization of the protein during that time and the speed must be kept constant. Although still in use, most prefer other methods for estimating the molecular weights of proteins.

In deriving Eq. (A.16), we solved for molecular weight. Equation (A.8) relates the molecular weight to the frictional ratio (f/f_0). By mathematical manipulation, we can solve for f/f_0, combining Eqs. (A.8) and (A.16). This is given in Eq. (A.18):

$$f/f_0 = \frac{1}{6\pi\eta}\left[\frac{RT}{DN}\right]^{2/3}\left[\frac{4\pi(1 - \bar{v}d)}{3\bar{v}s}\right]^{1/3} \tag{A.18}$$

A.5.8 *Electrophoretic Mobility*

The rate at which a charge moves is a function of the voltage gradient of the field. In a solution, Ohm's law ($E = IR$), can be used to define the voltage gradient. In place of resistance, we use its inverse, the conductance. The specific conductance (K_s) is the conductivity of a 1 cm^3 of solution. Multiplying this by the cross-sectional area (A) will give the conductance of the particular cell being used. The voltage gradient is then represented by the equation, $E = I/AK_s$, where I is the current in amperes.

The mobility of a protein molecule in a buffer solution is affected by the viscosity of the solution, the temperature, the charge distribution on the surface of the molecule, its rate of diffusion, and other factors. To simplify the problem, with paper electrophoresis, for example, it is customary to give the voltage gradient in V/cm, and then give the distance traveled in a fixed time for a particular buffer at a defined pH value. If the ionic strength and pH of a particular buffer is used, then the rate of movement will be proportional to the voltage gradient. Tiselius used a pH of 8.6 in a barbiturate buffer, with ionic strength of 0.1. With these conditions, the mobility could be given as the movement (cm) of the protein, per unit time (sec), per volt. This is shown in Eq. (A.19):

$$\text{mobility} = m = \left[\frac{\Delta X}{\Delta t}\right]\left[\frac{AK_s}{I}\right] \tag{A.19}$$

Tiselius defined a unit as $cm^2\ V^{-1}\ cm^{-1}$, for his system, and this is used generally today in defining the mobility of proteins. The Tiselius apparatus, or its equivalent, is used in its determination (Figure 3.2). Results are of the order of 10^{-5}. For this reason, the Tiselius unit is usually multiplied by 10^5. Values for the mobility of some of the proteins are given in Table A.5. An important application of the Tiselius system is in estimating isoelectric points. The pH is varied and a plot of mobility with pH is made. Interpolation to zero movement yields the isoelectric point (pI).

When electrophoresis is carried out in gels, the mobility of the proteins varies with the surface charge distribution and their molecular weight. By adding dodecyl sulfate to the protein solution, the dodecyl group is held in the hydrophobic folds of the protein and the sulfate radical is exposed. Thus all proteins have the same charge distribution

TABLE A.5

Electrophoretic Mobility (m) in Tiselius Units, $cm^2/V\,sec$, and the Isoelectric Points (pI) of Some of the Plasma Proteins in Order of Decreasing Mobility

Protein	Mobility × 10^5	pI
Prealbumin, thyroxin binding	7.6	4.7
Albumin	5.9	4.7
α_1-Acid globulin	5.7	2.7
Ceruloplasmin	4.6	4.4
Haptoglobin 1-1	4.5	4.1
Hemopexin	3.1	—
Transferrin	3.1	5.5
Fibrinogen	2.1	5.5
IgM	2.1	—
IgG	1.1	5.8–7.3

on their surface. As a result they move in order of their molecular weights (Figure 3.12). By using known proteins, such as the cytochromes, or dyes, where the molecular weight is known, the distance traveled can be plotted against the logarithm of the molecular weight. This gives a straight line. The position taken by the unknown protein then gives the molecular weight. This is known as *SDS-electrophoresis* or *sieving*.

A related procedure is the use of the Sephadex column. The proteins leave the column in accordance with their molecular weight, with the heavier proteins coming off first. By the use of suitable standards the elution position taken by the unknown protein, as compared to the standards, yields its molecular weight.

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