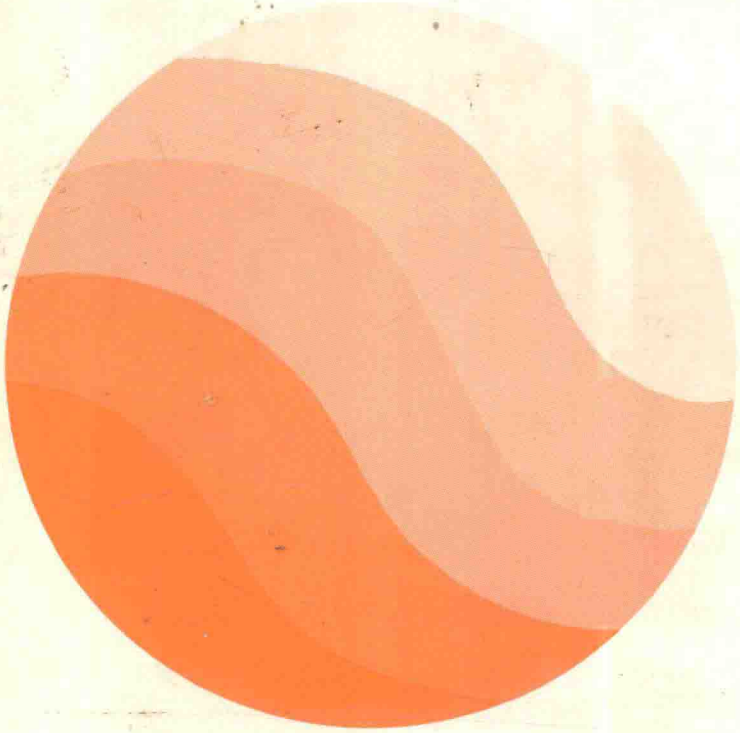
STRUCTURE AND FUNCTION OF PLASMA PROTEINS

Volume 1



Edited by A.C. Allison

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Preface

Plasma proteins are of interest from many points of view. Biochemists have separated and purified numerous plasma proteins and studied their physical properties, aminoacid composition and sequence, the carbohydrate components of some, and binding of metals, hormones and other materials. Much work has also been carried out on the synthesis, rates of turnover and degradation of plasma proteins.

Many plasma proteins show inherited variations, some of which (e.g. those of heptoglobins and transferrins) are common in various human populations while others (e.g. absence of lipoproteins or immunoglobins) are rare but important because of their association with clinical syndromes. Since blood is the most accessible bodily constituent, geneticists have made good use of serum protein differences as genetic markers in family and population studies.

Physiologists have long been interested in plasma proteins in relation to colloid osmotic pressure, transport of lipids, iron, hormones and other materials, the activities of renal glomeruli and tubules, the function of the liver, and many other bodily activities. Plasma proteins are also widely studied in relation to malnutrition and undernutrition, particularly that associated with defective intake of protein.

One of the routine activities of clinical chemistry laboratories is the analysis of plasma proteins. Although used primarily in relation to the diagnosis and assessment of prognosis of liver and kidney diseases and lymphoreticular malignancies, plasma proteins can provide information of value in many disorders. Detailed studies of particular groups of plasma proteins, such as the blood clotting factors, immunoglobulins and complement components, are of basic importance to haematologists and immunologists.

Developmental biologists are interested in the ontogeny of the plasma proteins and the reappearance in malignant disease of proteins which are normally confined to the foetus. Regulation of the synthesis of plasma proteins, for example acute-phase reactants, is a problem of general interest as well as importance in limiting the progress of inflammation. Several protease inhibitors circulate in the plasma and their role *in vivo* is still imperfectly understood.

Plasma proteins have been used, for the most part empirically, in media for culturing cells and tissues, and it is becoming clear that their interactions with various cell types are complex. Many stimulatory and inhibitory effects of particular plasma protein components on cell motility, division, enzyme synthesis and other aspects of cell function

have been reported. Indeed, study of the interactions of various plasma proteins with different cell types are now a major branch of cell biology, as well as having an important bearing on the pathogenesis of such common diseases as atherosclerosis and cancer. Specific interactions of plasma proteins with cells can be illustrated by the attachment of immunoglobulins and complement components to leucocytes and the elimination by the liver of plasma proteins with certain sugar groups exposed by removal of terminal sialic acid groups.

As a result of all these studies an enormous body of knowledge about plasma proteins has accumulated. Only a small part of this knowledge has penetrated to textbooks of biochemistry, physiology and medicine. Much of the information is either in large, expensive and inaccessible handbooks or in volumes on specialized subjects. The need was recognized for a convenient handbook on plasma proteins in which the main observations would be clearly set out with enough detail for most purposes, together with references for interested readers to obtain access to more detailed studies on any topic.

For the convenience of authors and readers the subject has been divided into two volumes, each with ten chapters. Each chapter will provide some guide to techniques appropriate for the isolation or characterization of the proteins, and one chapter in the second volume is devoted to methods. This handbook is intended for senior and postgraduate students in biochemistry, medicine and other subjects as well as hospital biochemists, physicians and research workers. I have confidence that the knowledge and skill of the authors in presenting so complex a subject in so lucid a fashion will appeal to a wide range of readers.

A. C. Allison
March 1974

Contents for Volume 1

Chapter 1

Plasma Lipoproteins

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Lipoproteins are macromolecules in which the water insoluble lipids are transported in plasma. Four main lipoprotein classes have been defined in human plasma. Each of these contains esterified and non-esterified cholesterol, phospholipid and triglyceride, though in differing and characteristic proportions. Non-esterified fatty acid (NEFA) is transported as a complex with albumin; the albumin-fatty acid complex is not, however, conventionally regarded as one of the lipoproteins. The terminology used for the classification of the lipoprotein classes depends upon the methods used for their separation; a description of the methodology is thus a prerequisite to any discussion of terminology or physiology.

1.1. METHODOLOGY

1.1.1. SEPARATION OF LIPOPROTEINS

The main methods used for the separation of lipoproteins depend upon either the electrical charge (electrophoresis), the density (ultracentrifugation), the immunochemical characteristics of the protein moiety, the selective precipitation of individual lipoproteins by chemical reagents, or the size of the particle.

(a) *Electrophoresis*

A variety of techniques have been described; paper, cellulose acetate and agarose are the most commonly used support media, with subsequent staining of the lipid moiety of the lipoprotein with Oil Red O or Sudan Black.

Electrophoretic separation on paper in barbitone buffer at pH 8.6[1,2] permits identification of four lipoprotein bands (Fig. 1.1). The fraction which remains at the origin consists of chylomicrons, betalipoprotein has the mobility of beta-globulin, alphalipoprotein has the mobility of alpha$_1$-globulin and pre-betalipoprotein moves just ahead of betalipoprotein, from which it is not completely separated. The

simultaneous running of a duplicate strip which can be stained for protein may aid identification of the lipoprotein bands.[1] The addition of 1% albumin to the buffer is claimed to improve resolution of the beta and pre-beta fractions.[2]

Cellulose acetate[3] and agarose[4] both have the advantage over paper in that smaller samples of serum can be loaded, the time taken for separation reduced from 16 hours (overnight) to a few hours, and that improved separation is obtained between beta and pre-betalipoproteins.

Attempts have been made to make electrophoretic techniques quantitative by measurement of the uptake of the fat stain by dye elution[1] or by scanning[5] but because of the inherent problems arising from differential dye uptake by the various lipids making up the lipoproteins, electrophoresis should probably be used to give qualitative information only.

(b) *Ultracentrifugation*

The various classes of lipoproteins differ in the proportion of lipid compared with protein, and the greater the proportion of lipid, the lower the density of the lipoprotein complex. The differing densities allow separation of lipoproteins by the application of accelerated gravitational force, and in the ultracentrifuge forces of the order of 100 000 × gravity (g) can be obtained. Under such conditions lipoproteins will either float or sediment according to the differences between the density of the lipoprotein and that of the fluid environment.

There are two types of ultracentrifuge: the analytical and the preparative. The analytical ultracentrifuge is used for the determination of the rate of flotation of lipoproteins at a fixed density of 1.063 g/ml.[6] The flotation of lipoprotein fractions through the medium is recorded photographically in the form of Schlieren patterns, and the rate of flotation is measured in Svedberg units (10^{-13} cm/sec/dyne/g); a subscript to denote flotation is added to give S_f units. The lipoproteins of lowest density, that is those carrying the most lipid relative to protein, have the highest flotation rates. The analytical ultracentrifuge is also used for determining total lipoprotein concentrations by reference to the area under the curve in the Schlieren pattern.[7] The instrument does not, however, give information about lipoprotein composition.

The preparative ultracentrifuge is used to prepare fractions which contain lipoproteins of a particular density; these fractions are obtained in sufficient quantity for detailed analyses of composition to be made. The main advantage of this method is that it allows definition of the composition of plasma lipoproteins in addition to giving information about concentrations. Lipoproteins are inherently somewhat unstable and, therefore, the use of lengthy procedures to achieve complete fractionation is accompanied by the risk of physico-chemical changes. Such changes may be potentiated by exposure to dilution with the various salt solutions which are used to achieve the required density.

However by careful selection of the conditions of time and temperature for ultra-centrifugation, and in some instances by the addition of certain chemical reagents, it is possible to minimise the risks of degradation. Various schemes of preparative ultracentrifugation have been described;[8] sequential spins at progressively increasing density, with collection of the required lipoprotein fraction at the meniscus after each spin, may require several days of ultracentrifugation for complete fractionation. The use of density gradient techniques[9] reduces the time required for ultracentrifugation. Although the preparative ultracentrifuge is the only method available for complete analysis of plasma lipoproteins, its use is limited by its cost together with the size of blood sample required (about 10 ml), the sophisticated technical support needed, and the fact that the method is not amenable to automation.

(c) *Immunochemical Methods*

Human serum lipoproteins behave as protein antigens and can be used for the production of antibodies in various animal species. When polyvalent antihuman antiserum is prepared, specific antibodies are produced which react with betalipoprotein and with alphalipoprotein. The use of a purified lipoprotein is necessary for the production of a single specific aniserum.

Several techniques have been described for the immunochemical estimation of betalipoprotein, but because the specific antiserum also reacts with pre-betalipoprotein the latter fraction must be removed by prior ultracentrifugation, or misleading results will be obtained, especially with abnormal plasma. A satisfactory immunoassay for betalipoprotein, based on radial immunodiffusion in agarose of serum from which pre-betalipoprotein was removed by ultracentrifugation, has been described.[10]

Immunochemical methods are also useful for the characterisation of different lipoprotein species. In combination with electrophoresis both qualitative[11] and quantitative[12] information can be obtained.

(d) *Chemical Precipitation Methods*

Lipoproteins react with certain polyfunctional anions to form insoluble complexes. Heparin in the presence of manganese ions,[13] or dextran sulphate in the presence of magnesium ions,[14] or sodium phosphotungstate in the presence of magnesium ions[15] have all been described for the selective precipitation of beta together with pre-betalipoproteins. The amount of precipitate formed has been used for the estimation of the total beta and pre-betalipoprotein concentration by a turbidimetric method;[14] the main problems of this type of method are the failure to differentiate between beta and pre-betalipoproteins, together with the difficulties of standardisation. Alphalipoproteins are not precipitated under the conditions described and can be estimated directly

in the supernatant; this forms the basis of the method described by Fredrickson *et al.*[13]

(e) *Gel Filtration*

It is possible to make use of materials which behave as molecular sieves (for example various cross-linked dextrans) to resolve mixtures of lipoproteins, as well as the other plasma proteins, according to their molecular size. Columns of Sephadex G200 have been used to produce fractions containing betalipoproteins and alphalipoproteins.[16]

(f) *Nephelometry*

Some lipoproteins are sufficiently large to scatter light and nephelometry has been used to measure the concentration of these particles which include pre-betalipoproteins and chylomicrons. Stone and Thorp[17] have described a method in which ultrafiltration through cellulose ester membranes is used to separate chylomicrons from pre-betalipoprotein prior to their estimation by nephelometry.

1.1.2. ANALYSIS OF THE LIPID MOIETY OF LIPOPROTEINS

The chemical nature of the main plasma lipids is indicated in Fig. 1.2. Analytical methods for estimating plasma lipids were developed before techniques became available for the separation and estimation of lipoproteins. Estimation of the concentration of individual lipids in plasma gives only limited information regarding the concentration of individual lipoproteins, but when taken in conjunction with qualitative information on the lipoproteins separated by electrophoresis it is possible to make a meaningful interpretation of the lipoprotein status. Analysis of the lipids in lipoprotein fractions isolated by preparative ultracentrifugation is necessary for the determination of the composition of the fractions; also the lipid content is used as a measure of the lipoprotein concentration for each density class. The methods used for the estimation of lipids in whole plasma and in isolated lipoprotein fractions are essentially the same.

(a) *Lipid Extraction*

It is doubtful whether methods of chemical analysis are sufficiently specific to allow omission of the preparation of a lipid extract. The treatment of plasma with either ethanol/diethyl ether, chloroform/methanol, or isopropanol, results in denaturation of the lipoproteins and quantitative release of the bound lipids into solution. Aliquots of this solution are used for the analysis of individual lipids.

(b) *Cholesterol*

Satisfactory colorimetric analyses based on the reaction with ferric chloride/sulphuric acid and on acetic anhydride/sulphuric acid are available for total (esterified plus unesterified) cholesterol. Versions

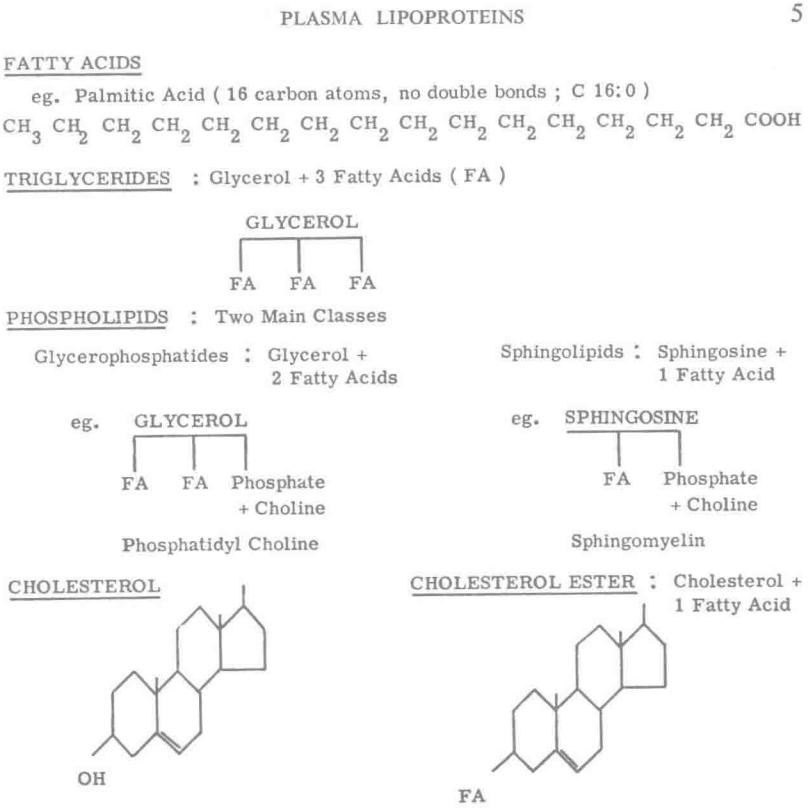


FIG. 1.2. Chemical nature of main plasma lipids. (Reproduced from Archives of Disease in Childhood, 1968, **43**, 393 by permission of the editors.)

of these methods suitable for the autoanalyser are available, and precision has been improved by the use of automated methods. Estimation of the relative amounts of esterified and unesterified cholesterol can be made using digitonin precipitation of unesterified cholesterol, or perhaps most conveniently by the use of thin-layer or column chromatography to separate the two fractions prior to their colorimetric determination.

(c) *Triglyceride*

The analysis of triglyceride has in the past presented difficulties because of the lack of a direct colorimetric reaction. The development of alternative techniques such as gas-liquid chromatography, fluorimetry, infra-red absorptiometry and enzymology now enables triglyceride to be reliably measured. An automated version using fluorimetry is available.

(d) *Phospholipids*

Lipid phosphorus can be determined after oxidation to inorganic phosphate by a colorimetric reaction using a "molydenum-blue" procedure, and the application of an average molecular weight factor allows calculation of phospholipid. The same method can be used for estimation of the different phospholipids after their separation by chromatography. The major plasma phospholipids are phosphatidyl choline, sphingomyelin, lysophosphatidyl choline, and phosphatidyl ethanolamine.

(e) *Fatty Acids*

Non-esterified fatty acids (present as an albumin complex) comprise only a small fraction of total plasma fatty acids and can be estimated by a number of procedures including titrimetric methods, colorimetry and gas-liquid chromatography. Nearly all the plasma fatty acids are present in esterified cholesterol, in the various glycerides of which triglyceride constitutes about 90–95% of the total, and in the phospholipids. The estimation of total esterified fatty acid concentration is of limited value. The introduction of gas-liquid chromatography, which allows separation and estimation of the individual fatty acids has enabled studies of fatty acid composition to be made. Because the lipid classes have different fatty acid compositions (Table 1.1) separation of the

TABLE 1.1
Composition of the plasma esterified fatty acids†

Major fatty acids (>5% total)			Triglyceride	Cholesterol Ester	*PC	S	PE
Trivial name	C Atoms	Double bonds	(Fatty acids, g/100g total fatty acids)				
palmitic	16	0	31	14	35	40	30
palmitoleic	16	1	5				
stearic	18	0	5		15	10	15
oleic	18	1	44	25	14		9
linoleic	18	2	10	45	21		9
arachidonic	20	4		6	9		20
behenic	22	0				16	
	22	6					13
lignoceric	24	0				9	
nervonic	24	1				15	

*PC = phosphatidyl choline
S = sphingomyelin
PE = phosphatidyl ethanolamine
† Mean values obtained in our laboratory from a group of healthy children and young adults.

individual lipids should be made before their analysis by gas-liquid chromatography. The method can be made quantitative by the addition of an unphysiological fatty acid as an internal standard.

Table 1.1 shows the percentage distribution of esterified fatty acids found in the various lipid classes in plasma of subjects eating a normal "Western-type" diet. Cholesterol ester is especially rich in linoleic acid, triglyceride contains mainly palmitic and oleic acids, and phosphatidyl choline contains mainly palmitic and linoleic acids. Sphingomyelin contains a large proportion of long-chain saturated acids, whereas phosphatidyl ethanolamine has the highest content of long chain polyunsaturated acids.

1.1.3. ANALYSIS OF THE PROTEIN MOIETY OF LIPOPROTEINS

The main difficulty in determining the protein concentration of lipoproteins lies in the preparation of the fraction free from the other plasma proteins. Prolonged ultracentrifugal procedures are required to prepare the lipoprotein fractions and their purity should be checked by immunochemical methods. Protein determination can then be made by the sensitive Lowry colorimetric method or the Kjeldahl method. Estimation by measurement of ultraviolet absorption at 280 mμ is only semi-quantitative, probably because of interference in the absorption spectrum by the lipid moiety. The amino acid composition of the protein can be determined by standard techniques of hydrolysis and chromatography, and endgroup analyses carried out by the appropriate techniques.

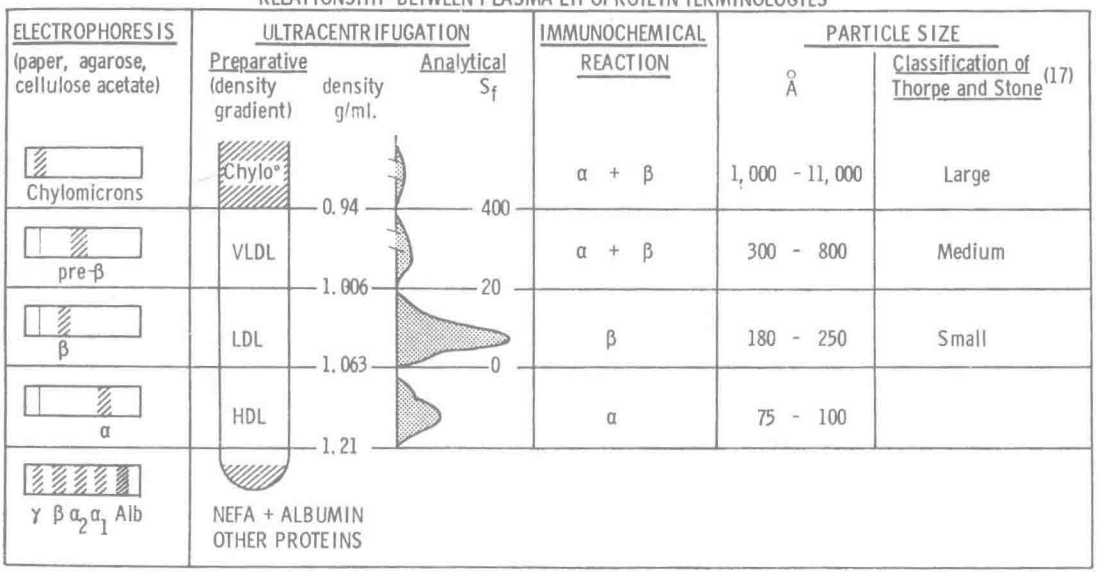
1.2. TERMINOLOGY, PHYSICAL CHARACTERISTICS AND CHEMICAL COMPOSITION

The nomenclature used to define the four main plasma lipoprotein classes is derived either from their electrophoretic mobility or their behaviour in the ultracentrifuge. The relationships between the various terminologies are shown in Fig. 1.3. Because of variations between the methods used for the analysis of lipoproteins it is important that results are accompanied by full details of the methodology, and caution should be exercised in comparing results obtained by different workers.

The chemical composition of plasma lipoproteins has been determined after isolation by preparative ultracentrifugation. The main characteristics of the four classes are summarised in Table 1.2. The values obtained by different workers are in broad agreement and the differences between various studies may arise from variations in the conditions used for isolation and analysis.

The identification of peptides with different carboxy-terminal amino acids has led to the use of nomenclature in which the apoprotein is

RELATIONSHIP BETWEEN PLASMA LIPOPROTEIN TERMINOLOGIES



pre-β = pre-betalipoprotein VLDL = very low density lipoprotein

β = betalipoprotein LDL = low density lipoprotein

α = alphalipoprotein HDL = high density lipoprotein

Chylomicrons are usually removed before ultracentrifugation.

FIG. 1.3. Comparison of lipoprotein terminologies.

designated according to the nature of its C-terminal group. Difficulties with this system, however, arise when different peptides have the same C-terminals.[17a] Furthermore, characterisation of many of the peptides is still incomplete, reinvestigation has already resulted in correction of previous observations,[17b,31] and other peptides may yet be discovered. We agree that the suggested nomenclature is provisional and temporary, and that a more acceptable and stable convention is required.[69]

TABLE 1.2
Composition of plasma lipoproteins (data of Oncley[24])

	Average percentage composition by weight				
Lipoprotein	Protein	Cholesterol	Cholesterol ester	Phospholipid	Triglyceride
Chylomicrons	2	2	5	7	84
Pre-betalipoprotein (VLDL)	8	7	13	19	51
Betalipoprotein (LDL)	21	8	37	22	11
Alphalipoprotein (HDL)	50	3	14	22	8

VLDL = very low density lipoprotein
LDL = low density lipoprotein
HDL = high density lipoprotein

1.2.1. CHYLOMICRONS

The term chylomicrons is used to refer to the large lipid particles in lymph or plasma which have an S_f greater than 400 and contain triglycerides primarily derived from dietary fat.[18] The density of chylomicron fractions as determined both by direct measurement and by calculation from the chemical composition is 0.94 g/ml. The particles are spherical and vary widely in diameter from approximately 1000–11 000 Å, although most of the chylomicrons during fat absorption are probably of the order of 1500–4000 Å.

Triglyceride is the predominant lipid in chylomicrons accounting for about 85% of the total lipid; its fatty acid composition shows a close similarity to that of the dietary fat.

Chylomicrons undergo certain chemical changes as they pass from their site of formation in the intestinal epithelial cell through the lymphatics to the blood stream. Interaction with plasma lipoproteins results in differences in composition between lymph chylomicrons and plasma chylomicrons. The triglyceride fatty acid composition of plasma chylomicrons does not parallel that of the dietary fat quite as closely as does that of lymph chylomicrons. This difference reflects the contribution of endogenous fatty acids to plasma chylomicrons.