[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. XXV. Composition and Properties of Human Serum β-Lipoprotein[1,2,3]

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The lipids of blood and other tissues have long been recognized to be present in combinations with other substances rather than in a free state.[4,5] Numerous investigators, most notably Hardy,[6] Haslam,[5] Chick,[7] Theorell[8] and Sorensen,[9] studied the distribution of lipids in the course of the precipitation of serum protein fractions. Hardy, Haslam and Chick emphasized the presence of lipids in the serum protein fraction that is insoluble in the absence of salts, the euglobulin fraction.

The first preparation isolated from plasma or serum that contained reproducible proportions of lipids and protein, and that could therefore properly be called a lipoprotein, was obtained from horse serum in 1928 by Macheboeuf.[10] The purified material contained 18% cholesterol and 23% phospholipid, yet dissolved freely in water to give clear solutions.

In the system of plasma fractionation developed by Cohn, *et al.*,[11] the lipids of human plasma are concentrated almost exclusively in two protein fractions, Fraction IV-1 and Fraction III-0, from which it has been possible to purify two distinct lipoproteins, α-lipoprotein[12] and β-lipoprotein. These lipoproteins migrate in electrophoresis with the α_1-globulins and β_1-globulins, respectively. Their isolation explains the earlier finding by Blix, Tiselius and Svensson[13] that the lipids were present in highest concentration in the electrophoretically separated α-globulin and β-globulin fractions of human serum. The present report describes the composition of the β-lipoprotein and extends the previous description of its physical properties.[14]

Methods

Determination of dry weight was used as the primary standard measure of concentration of β-lipoprotein. Preparations of β-lipoprotein were dialyzed for at least four days against several daily changes of 0.15 *M* sodium chloride. In addition to the samples taken for the various analytical procedures described below, duplicate aliquots were withdrawn into weighing bottles and dried *in vacuo* first from the frozen state and then to constant weight over phosphorus pentachloride in the Abderhalden apparatus maintained at 76–77° with boiling carbon tetrachloride.[15] The dry weight of the sample was corrected by subtraction of the dry weight of an equal volume of dialysate.

Nitrogen was determined by micro-Kjeldahl analysis.[16]

The amino acid content of the purified β-lipoprotein was calculated from the nitrogen values on the assumption that only monoaminomonophospholipids were present, and hence that Lipid N = Lipid P × 14/31. The classical factor of 6.25 was then applied to the value for Total N − Lipid N to give the peptide content. The essential correctness of the foregoing method of calculating the amount of peptide was shown by direct measurement with the biuret reaction. A sample of dried β-lipoprotein (Preparation No. 7) was extracted with hot 1:1 absolute methanol–chloroform mixture in an apparatus patterned after that of Thannhauser and Setz.[17] Extraction for sixteen hours was followed by two hours of drying *in vacuo*. The biuret determination was carried out on the residue by a modification of the procedure of Mehl.[18] Crystalline human serum albumin[19] was used as protein standard. The value of 23.7% peptide in β-lipoprotein obtained in this way was in satisfactory agreement with 24.8% as determined from the nitrogen analysis and the factor 6.25 (Table II).

Lipid phosphorus was determined in an aliquot of an alcohol–ether extract prepared according to Bloor.[20] The procedure of Gortner[21] for ashing and color development

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(3) Presented before the Division of Biological Chemistry at the 116th meeting of the American Chemical Society, Atlantic City, September 18–23, 1949. A preliminary report of this investigation was presented at the 109th Meeting of the American Chemical Society, Atlantic City, N. J., April 8–12, 1946. Much of this work was taken from a thesis submitted by F. R. N. Gurd to the Graduate School of Arts and Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Harvard University, 1949.

(4) J. Nerking, *Arch. ges. Physiol.*, **85**, 330 (1901).

(5) H. C. Haslam, *Biochem. J.*, **7**, 492 (1913).

(6) W. B. Hardy, *J. Physiol.*, **33**, 251 (1905).

(7) H. Chick, *Biochem. J.*, **8**, 404 (1914).

(8) H. Theorell, *Biochem. Z.*, **175**, 297 (1926).

(9) S. P. L. Sørensen, *Compt. rend. Trav. Lab. Carlsberg*, **18**, No. 5 (1930).

(10) M. A. Macheboeuf, *Bull. chim. Biol.*, **11**, 268 (1929).

(11) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(12) E. J. Cohn, M. H. Blanchard and L. E. Strong, unpublished results.

(13) G. Blix, A. Tiselius and H. Svensson, *J. Biol. Chem.*, **137**, 485 (1941).

(14) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 156 (1947).

(15) S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison and M. Hasson, THIS JOURNAL, **69**, 1747 (1947).

(16) F. Pregl, "Quantitative Organic Microanalysis," The Blakiston Co., Philadelphia, Pa., 1930.

(17) S. J. Thannhauser and P. Setz, *J. Biol. Chem.*, **116**, 533 (1936).

(18) J. W. Mehl, *ibid.*, **157**, 173 (1945).

(19) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(20) W. R. Bloor, "Biochemistry of the Fatty Acids," Reinhold Publishing Corp., New York, N. Y., 1943.

(21) W. A. Gortner, *J. Biol. Chem.*, **159**, 97 (1945).

was usually followed, although in some cases a method employing perchloric acid[22] was substituted. Total phospholipid was obtained by multiplying the lipid phosphorus value by the factor 25.0.

Free and total cholesterol was usually determined by the method of Schoenheimer and Sperry[23] employed without change, except for the following modification of the color development. The cholesterol digitonide was dissolved first in 2 ml. of glacial acetic acid to which was then added 5 ml. of a mixture of 20 parts by volume of acetic anhydride to 1 part of concentrated sulfuric acid. Color development was carried out for thirty minutes in the dark at 25° and the measurement made in a Coleman Universal Spectrophotometer set at 635 mμ. In some of the early determinations the digitonide was not prepared: the procedure of Bloor, Pelkan and Allen[24] was followed, and a Klett photoelectric colorimeter was employed. On the assumption that the esterified cholesterol was present in the form of cholesterol oleate, the value for esterified cholesterol was multiplied by the factor 1.73 to obtain the amount of cholesterol esters ("total esters") present.

Solubility determinations were made by mixing a dialyzed solution of β-lipoprotein with the required buffer solutions to achieve the desired final conditions of *p*H, ionic strength and of concentration of lipoprotein, ethanol and glycine. The buffers employed were of known *p*H and ionic strength.[25] The *p*H was measured in the glass electrode at 25° by diluting 1 ml. of unknown solution with 4 ml. of 0.02 *M* sodium chloride. After equilibration for twenty hours at 0°, the precipitate was separated by centrifugation at 0°. The concentration of β-lipoprotein remaining in solution was determined either by analysis for total cholesterol or by measurement in the Beckman quartz spectrophotometer of the optical density at 275 mμ with simultaneous measurements on the stock solution of β-lipoprotein standardized by dry weight determination. The two methods for determining concentration generally agreed within 2%.

Preparation of β-Lipoprotein.—The purified β-lipoprotein was prepared from lipoprotein-rich fractions obtained from normal human plasma by the use of ethanol, low temperature, low ionic strength and accurate control of *p*H and protein concentration.[11,26] The method involved the separation of the low density β-lipoprotein from contaminating proteins in the preparative head of the ultracentrifuge, using a medium of sufficiently high density to cause the β-lipoprotein to rise slowly toward the surface.

The starting material for the preparation of β-lipoprotein was either Fraction III-0 from method 6[11] and 9,[26] or a corresponding fraction obtained in the newer fractionation method 10.[27] No differences were ever observed in the properties of the β-lipoprotein prepared from these crude fractions. To each kilogram of lipoprotein-containing paste, preserved at −5° until used, was added 1 kg. of partially frozen distilled water, and the suspension was stirred for one hour. The suspension was then poured slowly with stirring into 20 kg. of distilled water containing a small amount of ice, and after stirring for another hour was allowed to stand overnight. The suspension was now centrifuged at a rate of 10 l. per hour through a small Sharples continuous supercentrifuge refrigerated to 0°. The supernatant solution, which contained small quantities of pseudoglobulins, was not further studied. When Fraction III-0 was used as starting material, about 80% of the total solids were precipitated in this greenish-yellow lipoprotein-rich euglobulin paste.

The euglobulin paste was then dissolved in either 1 *M* sodium chloride, or in 0.15 *M* sodium chloride and 1.14 *M* glycine, the temperature being maintained at 0°. This was done by stirring 135 g. of the euglobulin paste either with 112 ml. of 4 *M* sodium chloride and 200 ml. of distilled water, or with 17 ml. of 4 *M* sodium chloride, 38 g. of solid glycine and 300 ml. of distilled water. In most cases this solution was dialyzed against the corresponding salt, or glycine–salt solution, to remove the remaining traces of ethanol and assure the desired density. The density of the glycine–sodium chloride solution was approximately equal to that of the molar sodium chloride. The glycine containing medium was introduced to avoid any possible denaturing effect of the strong salt solution.

Before the dialyzed solution was transferred to the preparative ultracentrifuge, it was first treated in either of two ways to remove the greater part of some aggregated material present. The first method was to centrifuge the solution at about 25,000 g,[28] after which the clear portion underneath a yellowish scum was drawn off with the aid of a large hypodermic syringe. A somewhat less satisfactory alternative method was to filter under pressure successively through asbestos pads D-0, D-6 and D-10. The concentration of the solutions so obtained was about 8 g./100 ml. These procedures were also carried out at 0°.

Solutions clarified in either of the above ways were then transferred to tubes in the preparative head of the ultracentrifuge[29] and centrifuged for six hours at 45,000 r.p.m. (*ca.* 120,000 g.). The rotor was precooled to 0° and kept as near this temperature as possible except for the duration of the centrifugation when the temperature rose to perhaps 15°. After centrifugation the following fractions were withdrawn: (1) a yellow layer on the surface was removed with a small spatula and usually discarded; (2) the clear yellow solution below the solid material on the surface was next drawn off, followed by (3) a greenish-yellow intermediate layer which was usually not further treated; (4) the green solution at the bottom of the tube was decanted off as a separate fraction, leaving (5) a greenish precipitate which could be largely redissolved in 0.15 *M* sodium chloride.

Representative yields of total non-dialyzable solids (as estimated from refractive index measurements) and of total cholesterol in these fractions are given in Table I. The surface material was found to be composed of β-lipoprotein, probably at least partially denatured. The quantity separated increased with the age of the lipoprotein preparation. The proportion of lipid to protein in this material was usually somewhat higher than in β-lipoprotein, indicating that some free lipid may be present. The material could not easily be dissolved in water, and in contrast to the native β-lipoprotein, much of the cholesterol, phospholipids and carotenoids were readily extracted by stirring in ether in the absence of ethanol.

Spectrophotometric analysis was in accord with the conclusion from the chemical analyses that the middle solution (3) was a mixture of the lipid-rich (2) and almost lipid-free (4) fractions.

The properties of the lipid-free proteins separated in the two lower fractions (4) and (5) are under study. These include the two lipid-free β_1-globulins described by Oncley, Scatchard and Brown,[14] and in addition certain β_2-globulins. Small quantities of a blue protein probably identical

(22) W. M. Sperry, personal communication.

(23) R. Schoenheimer and W. M. Sperry, *J. Biol. Chem.*, **106**, 745 (1934).

(24) W. R. Bloor, K. F. Pelkan and D. M. Allen, *ibid.*, **52**, 191 (1922). We are indebted to P. M. Gross, Jr., for some of these preliminary determinations.

(25) A. A. Green, THIS JOURNAL, **55**, 2331 (1933).

(26) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., *ibid.*, **71**, 541 (1949).

(27) E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma, *ibid.*, **72**, 465 (1950).

(28) A multispeed attachment, running at 18,000 r. p. m., was used. International Equipment Co., Boston, Mass.

(29) A. V. Masket, *Rev. Sci. Instruments*, **12**, 277 (1941). We are indebted to C. G. Gordon and Mrs. B. A. Koechlin for performing the ultracentrifugal separations.

to the caeruloplasmin of Holmberg and Laurell[30] have been recognized, as well as a pale yellow component shown to be a heme protein by the fact that the typical hemin absorption spectrum was obtained in the acid-acetone extract.

TABLE I

DISTRIBUTION OF SOLIDS AND CHOLESTEROL AMONG FRACTIONS SEPARATED IN THE ULTRACENTRIFUGE

	Fraction	% total non-dialyzable solids[a]	% total cholesterol	Lipids extracted by ether alone
(1)	Surface layer	19	(37)	+
(2)	Top solution (β-lipoprotein)	30	50	−
(3)	Middle solution	14	10	−
(4)	Bottom solution	27	2	−
(5)	Bottom paste	10	1	−

[a] Estimated from refractive index measurements.

The preparations of β-lipoprotein considered in this communication were isolated in the upper solution (2). Since the material was found to be denatured by freezing, it was necessary to store it in solution at a temperature just above freezing. Glycine was first removed by dialysis at 0° for four days against several daily changes of 0.15 *M* sodium chloride. Unless the material was to be used immediately, it was then sterilized by filtration through asbestos sterilizing pads and dispensed aseptically into several small bottles which were stored at 0°.

All preparations of β-lipoprotein were alike, and all were homogeneous in the ultracentrifuge when tested at various concentrations and in media of various densities. A considerable degree of boundary spreading was observed in the media of density very near that of β-lipoprotein, an observation that we have taken to indicate some small differences in the composition of the liquid moiety in different β-lipoprotein molecules. The unlikelihood of a contaminant being present to the extent of much more than 2% (the limit of interpretation of the ultracentrifuge diagrams) was further increased by the consistency of the analytical data, presented below, for the various preparations of β-lipoprotein.

Attempts to prepare β-lipoprotein by chemical means have not been successful. The ultracentrifugal method of isolation was undertaken only after numerous failures had made it clear that a knowledge of the properties of the various components of the crude material would be a necessary prerequisite to successful chemical separations.

Composition of β-Lipoprotein.—Detailed information about the composition of β-lipoprotein is of interest for two reasons. First, for an improved knowledge of fat metabolism it is important to know the nature and quantity of the lipids carried in this form of plasma. Second, knowledge of the nature and number of the functional groups in the molecule of β-lipoprotein is required for an understanding of its physical chemical behavior, of which the most important aspect will probably prove to be the equilibria into which its lipids enter with the surroundings. In order to make the information about the composition of β-lipoprotein as useful for these purposes as possible, it will be presented not only in terms of per cent. by weight of the various components (Table II), but also as the number of moles of each component per mole of β-lipoprotein (Table III).

The results of the analyses of four different preparations of β-lipoprotein are given in Table II. The constituents which were measured directly, comprising free and total cholesterol, lipid phosphorus and total nitrogen, are marked with an asterisk. From these values it was possible to arrive at a nearly complete description of the composition of β-lipoprotein in terms of the main categories of its constituents—amino acid and fatty acid analyses, etc., have not yet been performed.

TABLE II

COMPOSITION OF β-LIPOPROTEIN

Grams per 100 g. of β-lipoprotein

Constituent	Preparation number 2	3	6	7	Average
Cholesterol					
Total*	31.2	30.4	32.9	29.0	30.9
Esterified	23.1	22.4	23.5	21.2	22.5
Free*	*8.12*	*7.95*	*9.41*	*7.75*	*8.31*
Total esters	*40.0*	*38.8*	*40.7*	*36.7*	*39.1*
Phospholipid					
Lipid phosphorus*	0.95	1.21	1.32	1.21	1.17
Phospholipid	*23.7*	*30.2*	*33.0*	*30.2*	*29.3*
Polypeptide					
Total nitrogen*	4.22	3.96	4.12	4.51	4.20
Lipid nitrogen	0.42	0.55	0.60	0.55	0.53
Peptide nitrogen	3.80	3.41	3.52	3.96	3.67
Polypeptide	*23.7*	*21.3*	*22.0*	*24.8*	*23.0*
Totals	95.5	98.3	105.1	99.5	99.6

* These values were obtained by direct measurement. Only the italic values are included in the summations.

Studying an impure preparation containing β-lipoprotein, Mehl[31] pointed out in 1944 that the color was primarily due to carotenoids. A fairly fresh preparation of the highly purified β-lipoprotein used in the present study gave the absorption spectrum shown in Fig. 1. The absorption spectrum of pure β-carotene published by Devine, Hunter and Williams[32] has been included for comparison. The similarity of the two spectra may be taken as evidence for the presence of carotenoids, probably chiefly β-carotene, in β-lipoprotein. It is difficult to assess the significance of the precise locations of the maxima, since they are known to be quite strongly affected

(30) C. G. Holmberg and C. B. Laurell, Abstract No. 291/8, 1st International Congress of Biochemistry, August 19–25, 1949; *Acta Chem. Scand.*, **1**, 944 (1947); *Nature*, **161**, 236 (1948).

(31) J. W. Mehl, personal communication.

(32) J. Devine, R. F. Hunter and N. E. Williams, *Biochem. J.*, **39**, 5 (1945).

TABLE III

ESTIMATED MOLAR CONTENT OF VARIOUS COMPONENTS AND GROUPS PER MOLE OF β-LIPOPROTEIN

Component or group	Moles/mole β-lipoprotein
Cholesterol	
Total	1040
Free alcohol	280
Esters	760
Phospholipids	500
Carotenoids	0.5
Estriol[a]	0.02
Fatty acid residues	
Cholesterol	760
Phospholipid	1000
Total	1760
Amino acid residues[b]	2500
Cationic groups	
Basic amino acid residues	400
Phospholipid[c]	500
Total	900
Anionic groups	
Acidic amino acid residues	500
Phospholipid	500
Total	1000

[a] Calculated from the data of Roberts and Szego.[33] [b] Assuming molecular weight of the protein moiety of 300,000 (23% of 1,300,000) and average residue weight of 120. [c] This value is minimal, since account has not been taken of diaminophospholipids. A single analysis by the procedure of Hack[34] indicated that less than 10% of the phospholipid was sphingomyelin.

by the solvent.[32] From absorption measurements on an alcohol–ether extract of the β-lipoprotein taken up in cyclohexane, it was calculated that this preparation of β-lipoprotein contained about 0.02% carotenoids as β-carotene. However, as shown in Table III, this low concentration of carotenoid cannot enter stoichiometrically into the composition of β-lipoprotein. The problem of the distribution of estriol, another trace component, appears to be even more exaggerated, since only one-fiftieth of the minimum stoichiometric amount was found to be present.[33] These facts do suggest that the lipid moieties of the lipoprotein molecules are not of constant composition.

The computations summarized in Table II account satisfactorily for the composition of β-lipoprotein without the necessity of assuming the presence of significant quantities of triglycerides, cerebrosides or unesterified fatty acids.

Physical and Chemical Characteristics

Solubility.—The solubility characteristics of β-lipoproteins are those of a typical euglobulin, highly insoluble in water in the absence of salts,

(33) S. Roberts and C. M. Szego, *Endocrinology*, **39**, 183 (1946). Serious losses of estrogenic activity may have been incurred in this assay, enhancing the uncertainty in the nature and amount of the estrogens involved [N. T. Werthessen, C. F. Baker, and B. Borci, *Science*, **107**, 64 (1948); R. D. H. Heard, personal communication].

(34) M. H. Hack, *J. Biol. Chem.*, **169**, 137 (1947).

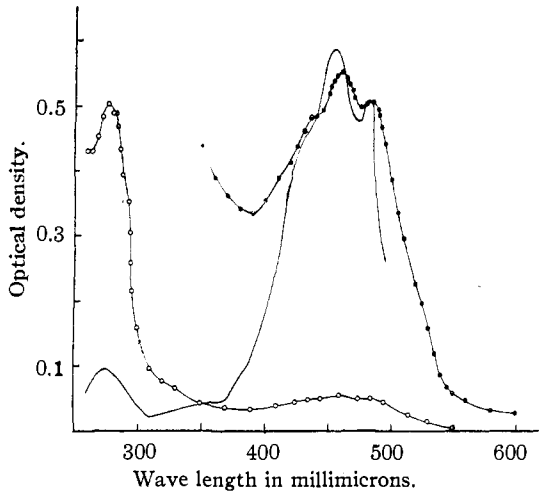

Fig. 1.—Absorption spectra of β_1-lipoprotein and β-carotene: O, β_1-lipoprotein, 0.1% solution; ◒, β_1-lipoprotein, 1% solution; —, β-carotene, 2.4×10^{-4} % in cyclohexane.

and with a minimum solubility near *p*H 5.4. Solubilities were dependent upon the amount of saturating body, and the measurements reported were made with about 10 mg. of lipoprotein per ml. The solubility of β-lipoprotein as a function of *p*H for three different ionic strengths is represented in Fig. 2. The solubility at a given *p*H

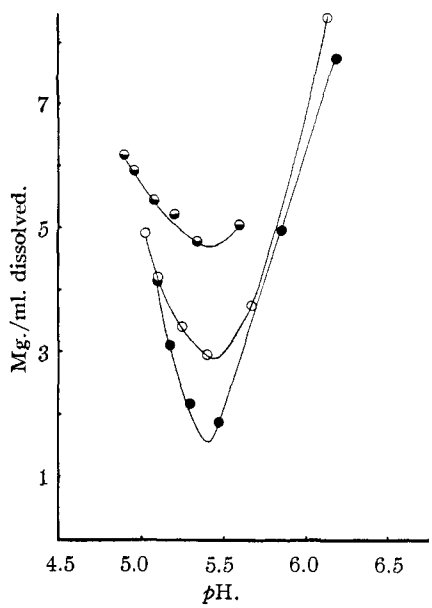

Fig. 2.—Solubility of β_1-lipoprotein in acetate buffers, temperature 0°: ◒, Γ/2 = 0.02; O, Γ/2 = 0.01; ●, Γ/2 = 0.005.

increases with increasing ionic strength (Fig. 3, Curve I). Similarly characteristic of a protein are the effects of ethanol in reducing the solubility (Fig. 3, Curve II), and of glycine in increasing the solubility (Fig. 3, Curve III), at constant *p*H and ionic strength. Such effects

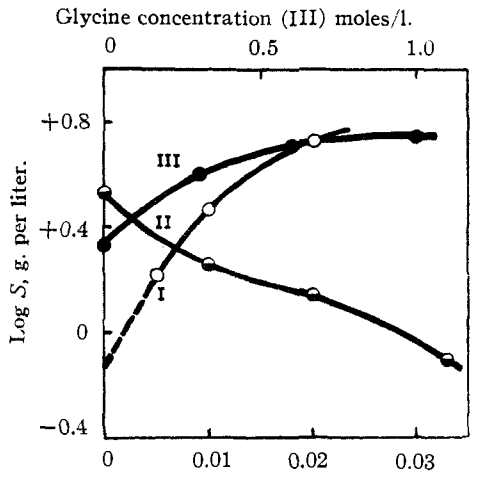

Fig. 3.—Variation of solubility of β-lipoprotein with ionic strength and with concentrations of ethanol and of glycine, temperature 0°: curve I, ○, varying ionic strength at *p*H 5.4; curve II, ◒, varying ethanol concentration at *p*H 5.3, ionic strength 0.01; curve III, ●, varying glycine concentration at *p*H 6.0, ionic strength 0.005.

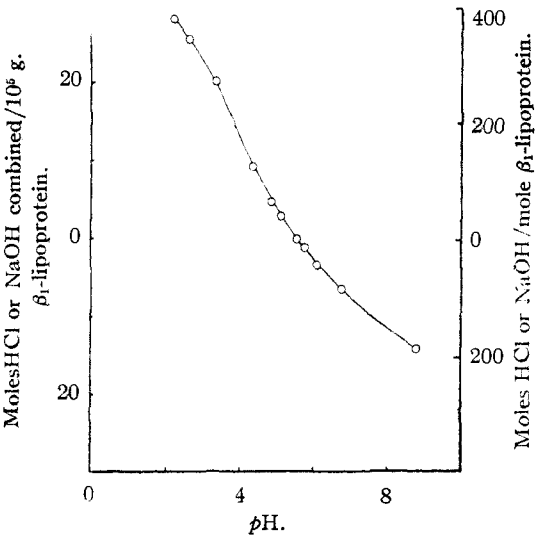

Fig. 4.—Titration curve of β_1-lipoprotein.

of ethanol and of glycine were observed over a wide range of values for *p*H and ionic strength. By contrast, insolubility in water or solutions of neutral salts or dipolar ions, and solubility in organic solvents are characteristic properties of lipids.[20] The magnitudes of the variations of solubility of β-lipoprotein with *p*H, ionic strength, and ethanol and glycine concentration are thus sufficiently characteristic of a typical protein to rule out any considerable contribution of the lipids to the over-all solubility behavior. This is the more striking in view of the fact that the lipids form more than three-fourths of the anhydrous weight of the lipoprotein (Table II).

Acid- and Base-Binding.—A partial titration curve of β-lipoprotein is presented in Fig. 4. The usual procedures employed in this Laboratory were followed,[35] except that the *p*H measurements were made with the glass electrode. The binding of acid and base is expressed both as moles per 10^5 g. β-lipoprotein and as moles per mole β-lipoprotein, assuming a molecular weight of 1,300,000.[14] Over the range covered, *p*H 2.2–8.8, the curve is typical of a protein titration curve.

The determination of the acid-binding capacity of the polypeptide moiety of the β-lipoprotein is complicated by the dissociation of the secondary phosphate groups in the phospholipids, with estimated *pK* near 1.[36,37] These groups may be expected to overlap in their dissociation with the carboxyl groups of the aspartic and glutamic acid residues in the peptide moiety, thereby concealing the point at which all the amino acid residues have been titrated. Accordingly, the apparent acid-binding capacity obtained in the conventional way[38] about *p*H 2 will be only a rough measure of the total cationic groups of the polypeptide, and will certainly be very much less than the total cationic groups of the lipoprotein. The estimate in Table III of 400 basic amino acid residues per mole of β-lipoprotein is based on the assumption that a slight undertitration of the amino acid residues was compensated by the inclusion of a part of the phosphate groups. A somewhat less reliable estimate of free carboxyl groups was made by measuring the amount of base binding from *p*H 2 to about 6, yielding approximately 500 groups per mole. The purpose of these estimates is to show the equal or greater numerical importance of the charged groups borne by the lipids compared with those of the polypeptide. Over the entire *p*H range of stability of the β-lipoprotein (*p*H 5.0–7.5) the phospholipid charges remain fixed in number and presumably also in location on the molecule.

Extraction of Lipids.—Only traces of lipids are extracted when serum or serum protein fractions are shaken with ethyl ether.[4,5] A number of studies on the use of more drastic tech-

TABLE IV

EXTRACTION OF LIPIDS FROM β-LIPOPROTEIN

	% separated in phase					
	McFarlane procedure				Hardy-Gardiner procedure	
Constituent	Extract	Emulsion	Residue Aqueous	Total	Extract	Residue
Polypeptide	0	44	43	87	0	78
Cholesterol	76	2	1	3	3	0
Phospholipid	(48)	34	18	52	51	1
Estriol[a]	0	..	..	100	..	..

[a] Analysis by Roberts and Szego.[38]

(35) A. A. Green, THIS JOURNAL, **60**, 1108 (1938).
(36) T. H. Jukes, *J. Biol. Chem.*, **107**, 783 (1934).
(37) H. Fischgold and E. Chain, *Biochem. J.*, **28**, 2044 (1934).
(38) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, *cf.* p. 446.

niques for the extraction of the lipids from solutions of serum β-lipoprotein have been made. Many of these studies were made with preparations containing 30 or 40% of impurity (consisting of lipid-free β- and α-globulins). The results of a typical experiment are presented in Table IV.

An extraction procedure modified from that of McFarlane[39] was first followed, using ten volumes of ethyl ether as the extraction solvent. This was cooled to a temperature of about −50°, and then the solution of lipoprotein at just above its freezing point was added dropwise with gentle stirring. Thereafter the mixture was warmed to 0° over a period of several hours, during which gentle rotation of the container was maintained. This extraction was always accompanied by the formation of a considerable emulsion phase which was not easily broken to form aqueous and ether phases. The lipid extracted by ether in this procedure was found to consist of approximately 28% phospholipid, and to represent about three-quarters of the lipid present in the original lipoprotein.

The aqueous and emulsion phases from the above extraction were then treated by a modification of the procedure of Hardy and Gardiner[40] with ten volumes of solvent consisting of three parts by volume ethanol and one part ethyl ether, using the same temperatures and stirring arrangements as in the previous step. The alcohol–ether extract was found to contain approximately 90% phospholipid, and, when the residue was treated with hot alcohol–ether, less than 1% of the original lipid was found to have remained unextracted by the second step.

The protein residue obtained from pure β-lipoprotein after the two extractions in the cold was largely soluble in distilled water, and the soluble protein appeared in the ultracentrifuge not to be highly aggregated. The increased solubility in the absence of salts after removal of the lipids from the purified β-lipoprotein explains the findings of many investigators that the removal of lipids from impure preparations abolished euglobulin characteristics.[7,40,41,42]

Discussion

Concentration in Plasma.—The complexity of the present procedure for the separation of β-lipoprotein makes very difficult a direct estimation of its concentration in plasma by isolation. From Table I it may be seen that about half of the cholesterol in the crude β-lipoprotein fractions was lost during the ultracentrifugal purification. Part of this loss was due to denaturation and aggregation undoubtedly promoted by prolonged previous storage, since in fresh plasma very little material may be recognized with the properties of the insoluble part of the layer separated at the surface of the centrifuge tubes. A large part of the loss was due to the intrinsic fault of such a preparative method, in that the native β-lipoprotein originally in the upper third of the centrifuge tube was packed into the surface layer where separation from the denatured material was difficult.

An indirect estimate of the concentration of β-lipoprotein in normal fasting human plasma may be made from the proportion of the total plasma cholesterol recovered in the crude β-lipoprotein fraction separated quantitatively from α-lipoprotein in a one-stage procedure.[37] This estimate agrees closely with the finding by the earlier procedure[11] that about 75% of the total cholesterol[43] is separated in the β-lipoprotein fraction. Taking an average value of 2.0 g. of total cholesterol per 100 g. of total plasma protein and the observed content of 31% total cholesterol in β-lipoprotein (Table II), the concentration of β-lipoprotein in normal pooled plasma may be estimated at 5% of the plasma protein.[44] There are several places in the procedure we have outlined, where fairly large losses of β-lipoprotein occur, and we have actually isolated only about one-third of the figure calculated above. We have seen no evidence that the material lost in this process has properties different from the β-lipoprotein we actually obtain, however. The repeated failure to detect other lipoproteins in the β-lipoprotein fraction supports the assumption that all the cholesterol in this fraction is present in the form of β-lipoprotein. The presence of any chylomicrons in the crude β-lipoprotein preparations[45] does not affect the argument, because cholesterol is not a quantitatively important constituent of these triglyceride-rich droplets.[46]

Striking confirmation of these estimates of the concentration of β-lipoprotein in human plasma has recently been furnished by the direct ultracentrifugal measurements of Gofman, Lindgren and Elliott.[47] Using the same principle as we have employed in the preparative procedure, namely, that of causing the β-lipoprotein to rise in a medium of sufficiently high density, these workers obtained the concentration of β-lipoprotein directly from the quantity of the rising component. In a series of 10 normal plasmas the concentration of β-lipoprotein varied from 2.0 to 4.5 g. per liter, corresponding to 2.5–6.0% of the total protein. This work has the added importance of giving conclusive support to the explanation of Johnston and Ogston[48] for the anomalously high concentrations of "X-protein" found by Pedersen.[49] Studies in this Laboratory[50] on various preparations containing β-lipoprotein and on mixtures of β-lipoprotein with human serum albumin have likewise shown the "X-protein" phenomenon to be due to a boundary anomaly of the type described by Johnston and Ogston.[48]

(39) A. S. McFarlane, *Nature*, **149**, 439 (1942).

(40) W. B. Hardy and S. Gardiner, *J. Physiol.*, **40**, lxviii (1910).

(41) T. H. Jukes and H. D. Kay, *J. Exp. Med.*, **56**, 469 (1932).

(42) J. Polonovski, M. Faure and M. A. Macheboeuf, *Ann. Inst. Pasteur*, **72**, 67 (1946).

(43) Calculated from Tables VII, VIII and IX of ref. 11.

(44) E. J. Cohn, *Experientia*, **3**, 125 (1947).

(45) Any chylomicrons in the crude fractions are removed along with the aggregated material as a surface film during the ultracentrifugal purifications, leaving the purified β-lipoprotein solution very clear.

(46) A. C. Frazer, *Trans. Faraday Soc.*, in press.

(47) J. W. Gofman, F. T. Lindgren and H. Elliott, *J. Biol. Chem.*, **179**, 973 (1949).

(48) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

(49) K. O. Pedersen, "Ultracentrifugal Studies on Serum and Serum Fractions," Almqvist and Wiksells, Upsala, 1945.

(50) J. L. Oncley and F. R. N. Gurd, unpublished results.

Structure of β-Lipoprotein.—Certain inferences concerning the structure of β-lipoprotein may be drawn from the data on the composition and physical properties of the β-lipoprotein. Viscosity and ultracentrifugal data have shown the β-lipoprotein to be almost spherical and to have an anhydrous molecular weight of about 1,300,000.[14] Calculation of the hydration from the difference in density between the hydrated and anhydrous molecules yields the value 0.6 gram of water per gram of anhydrous β-lipoprotein.[14]

The solubility behavior of β-lipoprotein suggests rather strongly that the lipid, peptide and water are arranged in the spherical complex in such a way that the peptide moiety is for the most part exposed on the surface. It appears unlikely that the polypeptide could exert such a predominant influence in the interactions of the β-lipoprotein with the ions and uncharged molecules of the surrounding medium if it were deeply embedded in the lipids.

The assumption that the β-lipoprotein is composed of a sphere of lipids covered by an unbroken film of polypeptide, however, is not compatible with the relative proportions of lipid and peptide found to be present, as has been calculated by McFarlane.[51] Making a calculation[52] similar to that of McFarlane, one can estimate that not more than 42 to 57% of the exposed surface of the β-lipoprotein could consist of peptide material, depending on the location of the water of hydration in the interior or exterior of the spherical model assumed. It follows that the surface of the β-lipoprotein molecule must be made up of areas of lipids and peptide, possibly in a configuration reminiscent of the postulate of a mosaic structure in protoplasmic membranes.[54] If the lipid part of the surface consisted mainly of the hydrophilic phospholipids, these compounds would contribute at least as many charged groups as are present in the amino acid moiety (Table III). The presence of these positive and negative charges at the surface of the molecule would permit the protein-like solubility properties to predominate.

Water appears to be very important for the maintenance of the structure of β-lipoprotein. Drying from the frozen state, or indeed freezing alone, destroys the well-defined solubility properties. By contrast, these procedures do not affect such other serum proteins as albumin and γ-globulin, which are, indeed, best preserved in the dry state. Freezing in the presence of ether according to McFarlane[53] allowed the greater part of the lipids to be extracted with ether. Presumably an important part of the observed water of hydration[14] is involved in binding together the lipid and polypeptide.

This description of the composition and properties of the β-lipoprotein of human plasma is in keeping with the concept of its function as a carrier for certain lipids. By linkage with the polypeptide in the form of β-lipoprotein these lipids are rendered water-soluble and are freely transported in the blood stream without introducing any structure of radically different physical properties from those of the rest of the plasma proteins. The work of Frazer[55,43] as well as the phospholipid turnover studies of Zilversmit, Entenmann and Chaikoff[56] support the idea that the β-lipoprotein (and probably also the α-lipoprotein) is not a transport form for fat on its way to and from the fat depots, but rather is a specialized carrier molecule elaborated by the liver. The role of the β-lipoprotein as a carrier for vitamins and hormones is implied by the presence of carotenoids and estrogens in some of the molecules (Table III).

The ability to take up and to give off the material to be transported is an important property of a transport form, and provides a mechanism for the maintenance of definite plasma concentrations of various critical substances such as hormones. That some dissociation of lipids from purified β-lipoprotein takes place was concluded from the membrane leakage that occurred during the osmotic pressure measurements of Oncley, Scatchard and Brown.[14] Experiments in progress,[57] using isotopically labelled steroids, similarly indicate the ability of the β-lipoprotein to enter into equilibrium with compounds of high biological activity.

Summary

1. Approximately three-quarters of the plasma lipid is bound in the well-characterized β-lipoprotein, which represents 5% of the normal plasma protein.

2. A method for the purification of the serum β-lipoprotein of human blood is described.

3. The composition of β-lipoprotein is given in terms of polypeptide, phospholipid, esterified and unesterified cholesterol, and certain trace components.

4. Studies on the removal of the lipids by various means are described.

5. The solubility and amphoteric properties of the β-lipoprotein are typical of plasma proteins rather than of the component lipids, despite the fact that the lipid in the lipoprotein weighs more than three times as much as the amino acid moiety.

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(51) A. S. McFarlane, *Trans. Faraday Soc.*, in press.

(52) The value of 0.74 is taken as the specific volume of the protein moiety, and when combined with the composition data and the observed anhydrous partial specific volume of 0.95,[14] yields 1.01 for the partial specific volume of the lipids. The reasonableness of this result is shown by the value of 1.004 for the density of mixed plasma lipids obtained by Popjak and McCarthy,[53] and conversely such agreement helps establish the additivity of the volumes of lipids and protein in β-lipoprotein.

(53) G. Popjak and E. F. McCarthy, *Biochem. J.*, **40**, 789 (1946).

(54) A. Nathansohn, *Jahrb. wiss. Bot.*, **39**, 607 (1904).

(55) A. C. Frazer, *Physiol. Rev.*, **26**, 103 (1946).

(56) D. B. Zilversmit, C. Entenmann and I. A. Chaikoff, *J. Biol. Chem.*, **172**, 637 (1948).

(57) J. L. Oncley, A. K. Solomon and F. R. N. Gurd, unpublished results.