Observations on the Protein Components of Human Plasma High- and Low-Density Lipoproteins*

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ABSTRACT: Human plasma high-density (α-) lipoprotein and low-density (β-) lipoprotein were prepared by ultracentrifugation and the purity of the preparations was demonstrated by electrophoresis on cellulose acetate membranes and by immunodiffusion. Both lipoproteins were delipidated by lyophilization followed by extraction of lipid with ethanol–acetone (1:1). Over 99% of cholesterol and phospholipids was removed. The delipidated protein moiety of α-lipoprotein moved as a single band on cellulose acetate electrophoresis, and precipitated as a single line on immunodiffusion against rabbit antiserum to human α-lipoprotein, but was resolved into three fractions by gel filtration on Sephadex G-200. The distribution coefficients on G-200 were 0.12 for fraction 1, 0.49 for fraction 2, and 0.64 for fraction 3. Quantitative amino acid analyses showed major differences between the three fractions in the content of arginine, half-cystine, histidine, isoleucine, leucine, lysine, phenylalanine, and serine; this observation establishes that more than one protein species is present in α-lipoprotein. The three fractions were further analyzed by electrophoresis, immunodiffusion, fingerprinting of the tryptic digest, paper chromatography, and rechromatography on G-200 columns in the absence and presence of sodium dodecyl sulfate. The results are compatible with the following interpretation. α-Lipoprotein contains two subunits, A and B, of mol wt 20,000–25,000; fraction 1 is an aggregate containing predominantly subunit A; fraction 3 is the monomeric form of subunit B; and fraction 2 is an aggregate containing both subunits. Treatment of α-lipoprotein with trypsin did not alter the lipoprotein's electrophoretic or immunologic properties. Delipidation of trypsin-treated α-lipoprotein and examination of the protein moiety showed that all three fractions of α-lipoprotein were still present, less than 10% of the lipoprotein's protein content had been cleaved to low molecular weight (<2000) peptide fragments, and reactivity with rabbit anti-α-lipoprotein was retained. In contrast, treatment with trypsin *after* delipidation rapidly cleaved all protein components into peptide fragments <2000 in molecular weight and abolished the antigenic property. Delipidation of β-lipoprotein by lyophilization followed by ethanol–acetone extraction yielded a preparation which was totally insoluble in aqueous buffers. Treatment of β-lipoprotein with trypsin before delipidation released less than 10% of the protein moiety in the form of low molecular weight (<2000) fragments; 90% of the protein content of β-lipoprotein was recovered in the form of a water-soluble material which moved as a single band on electrophoresis, and which, in the presence of sodium dodecyl sulfate, emerged from G-200 columns with a distribution coefficient corresponding to mol wt 15,000–20,000.

The protein components of human high-density (*d* 1.063–1.21), or α-, and low-density (*d* 1.1019–1.063), or β-, lipoproteins are now under investigation in several laboratories. Scanu and coworkers have delipidated aqueous solutions of α-LP[1] (Scanu, 1966; Scanu and Hughes, 1962; Scanu *et al.*, 1958) and β-LP (Scanu and Hughes, 1960) by extraction with ethanol–diethyl ether at −10°. The protein moiety (α-P) of α-LP thus obtained contained 3% phospholipid and was soluble in aqueous buffers below pH 4 or above pH 6; β-P was totally insoluble in aqueous systems. The α-P of Scanu *et al.* showed three bands in starch gel electrophoresis and three precipitation arcs in immunoelectrophoresis, but sedimented in 0.05 M sodium dodecyl sulfate solution as a single component with $s_{20,w}$ = 2.0 S; they concluded that α-P was an aggregate of a subunit with mol wt 23,710. Shore and Shore (1962, 1967) delipidated α-LP with ethanol–diethyl ether at 2°, with removal of over 99% of the lipid. The resulting α-P in aqueous solution contained major and minor components with $s_{20,w}$ values of 2.3–2.6 and 4.2–4.6 S, respectively, as well as more highly aggregated material. In the presence of sodium dodecyl sulfate, however, this preparation of α-P dissociated into subunits with mol wt 30,000–31,000 as determined by sedimentation equilibrium; homogeneity of the subunits with respect to size suggested the presence of only one subunit species. Shore and Shore (1967) also separated α-LP

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[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: α-LP, α- or high-density lipoprotein; β-LP, β- or low-density lipoprotein; α-P, protein moiety of α-LP; β-P, protein moiety of β-LP.

into two fractions with *d* 1.081–1.123 and 1.123–1.195; in these experiments, they observed differences in the amino acid composition of the protein moieties of these two high-density lipoprotein fractions, suggesting the presence of more than one protein species. Similarly, Alaupovic *et al.* (1967) noted differences in the tryptic fingerprint and in the polyacrylamide gel electrophoretic pattern of the protein moieties of subfractions of α-LP prepared by sequential preparative ultracentrifugation, and suggested that nonidentical protein subunits might be present in α-LP. Sodhi and Gould (1967) prepared α-P according to Scanu's delipidation method. Their preparation migrated as a single band on filter paper electrophoresis but sedimented as two components in the ultracentrifuge; they suggested that the heavier component might be an aggregate of the lighter one. Rodbell and Fredrickson (1959) delipidated aqueous solutions of α-LP and β-LP by adding 50 volumes of ethanol–acetone (1:1). The resulting α-P was free of phospholipid, soluble in 6 M urea solution, and migrated as a single band in filter paper electrophoresis. The β-P prepared by Rodbell and Fredrickson again was totally insoluble in aqueous solvents. Similarly insoluble in aqueous systems was the β-P preparation obtained by Margolis and Langdon (1966a) through delipidation of β-LP solutions with methylal–methanol.

Thus two current problems in the field are the possibility that α-P may be made up of more than one type of protein and the difficulty of obtaining β-P in soluble form. The following information, relevant to these problems, forms the basis of this report: (a) a method for delipidating α-LP and β-LP by freeze drying the lipoproteins, then extracting the lipids with ethanol–acetone; (b) the solubility properties, amino acid composition, and lipid content of α-P and β-P thus obtained; (c) characterization of α-P by electrophoresis on cellulose acetate, immunodiffusion, paper chromatography, gel filtration, and fingerprinting the tryptic digest; (d) demonstration of the presence of two different proteins in α-P by examination of subfractions of this material obtained through gel filtration; (e) comparison of the action of proteolytic enzymes on α-P and β-P before and after removal of the lipoprotein's lipid components, leading to demonstration that the action of trypsin or chymotrypsin on β-LP releases 90% of the protein moiety as a H_2O-soluble, electrophoretically homogeneous material with a distribution coefficient on gel filtration corresponding to a molecular weight of approximately 20,000.

Material and Methods

Preparation of α-LP and β-LP. During the course of this investigation, six healthy male laboratory workers (age 21–40) each donated, after an 18-hr fast, 500 ml of blood into heparin-containing plastic bags; 250 ml of each individual's plasma was fractionated by the method of Havel *et al.* (1955) with minor modifications. After the addition of 1 mg of disodium ethylenediaminetetraacetate/ml of plasma, the specific gravity was adjusted to 1.019 by the addition of solid KBr. The plasma was subjected to ultracentrifugation in a Spinco Model L ultracentrifuge, using either a rotor no. 40 at 40,000 rpm for 20 hr or a rotor no. 30 at 30,000 rpm for 24 hr. The tubes were cut high, the top layer was discarded, the infranatant was adjusted to a specific gravity of 1.063 and centrifuged as described above. The tubes were then cut in the center to separate the low-density lipoprotein fraction (top) from the bottom fraction containing the high-density lipoprotein fraction together with albumin and other plasma proteins. The β-lipoprotein fraction was washed twice by layering one volume of ice-cold β fraction below two volumes of a KBr solution of specific gravity 1.063, filling the tubes carefully to the top with this KBr solution, and centrifuging at 40,000 rpm for 20 hr.

The α-LP plus albumin fraction was adjusted to specific gravity 1.21 with solid KBr and centrifuged in a rotor no. 40 at 40,000 rpm for 60 hr or in a rotor no. 30 at 30,000 rpm for 72 hr. The tubes were cut in the center and the top fraction (α-LP) was washed twice by layering one volume of ice-cold α fraction below two volumes of KBr solution of specific gravity 1.21 and centrifuging for 40 or 48 hr, respectively, depending upon the rotor used.

The purity of each preparation of α-LP and of β-LP was verified by electrophoresis on membranes of cellulose acetate and of Cellogel at pH 8.6 and 3.9 and by immunodiffusion in agar against rabbit and horse antisera to human α-LP, to human β-LP, to human serum, and to various purified human plasma proteins.

Electrophoresis of α-LP, β-LP, α-P, subfractions of α-P, and fragments of β-P was carried out at pH 8.6 (sodium barbital buffer, ionic strength 0.1, containing 0 or 2 moles of urea per l.) or at pH 3.9 (in a buffer containing per l. 2 moles of urea, 3 ml of pyridine, and 30 ml of acetic acid) in a Buchler electrophoresis cell on 2.5 or 5 × 17 cm membranes of cellulose acetate (Millipore) or of Cellogel (Colab). A potential gradient of 200 V for 1.5 hr was employed. The strips were fixed in sulfosalicylic acid solution (20% in H_2O) for 2 min and then stained in Coomassie blue solution (0.25% in H_2O) for 5 min.

Immunodiffusion was done at 25° in agar of the following composition: Difco special noble agar, 2%; glycine, 7.5%; sodium chloride, 1%; and sodium azide, 0.1% (pH 7.0–7.2) (Ouchterlony, 1949). Antisera against human serum, human α-LP, human β-LP, and various human plasma proteins were obtained from Behringwerke and from Hyland;[2] lot numbers are listed in Table I. Sample concentration was 1–2 mg of delipidated protein/ml or 3–15 mg of lipoprotein/ml. Immunoplates were photographed at 24 or 48 hr.

Chemical methods used in the characterization of

[2] The specificity of the various commercial antisera employed was checked by testing each in double diffusion against human serum, human albumin, human 7S γ-globulin, and human α- and β-lipoproteins; judging by the formation of only a single line of precipitation with serum, and by failure to react with the purified plasma proteins except when this protein was the antigen used to develop the antiserum, the commercial antisera appeared to possess the appropriate specificity.

TABLE I: Summary of Immunodiffusion Results.[a]

Animal	Source (lot no.)	Antigen	Reactivity							
			α-LP	α-P	Fraction 1 of α-P	Fraction 2 of α-P	Fraction 3 of α-P	α-LP[b]	β-LP	β-LP[b]
Rabbit	Behringwerke (927/C67)	Human plasma α-LP	+	+	0	0	+	+	0	0
Rabbit	Behringwerke (1228B/G67)	Human plasma α-LP	+	+	0	0	+	+	0	0
Rabbit	Behringwerke (9713/C67)	Human plasma α-LP	+	+	0	0	+	+	0	0
Rabbit	Behringwerke (927R/C67)	Human plasma β-LP	0	0	0	0	0	0	+	0
Horse	Hyland (8431C001A1)	Human plasma β-LP	0	0	0	0	0	0	+	0
Horse	Hyland(8040D001A1)	Human serum	+	+					+	0
Rabbit	Hyland (S121D1)	Human serum	+	+					+	0
Rabbit	Hyland (8107D001AL)	Human serum albumin	0						0	
Rabbit	Hyland (8117D004A1)	Human α_2-haptoglobin	0						0	
Horse	Hyland (8402D002B1)	Human immunoglobulins	0						0	
Rabbit	Hyland (8101D001A1)	Human γ-globulin	0						0	
Rabbit	Hyland (8108D007A1)	Human fibrinogen	0						0	
Rabbit	Hyland (8115E001A1)	Human transferrin	0						0	

[a] + indicates visible precipitin line; 0 indicates no visible reaction. Where no symbol is given, reactivity was not tested. [b] Protein moiety, trypsin treated.

α-P and β-P were as follows. For determination of amino acid composition, 3–4 mg of protein was dried at 25° *in vacuo* over P_2O_5, weighed, and hydrolyzed in 6 N HCl under N_2 at 110° for 24 hr; the hydrolysate was dried *in vacuo* over KOH and analyzed in the Model 120C Beckman–Spinco quantitative analyzer. Half-cystine and methionine were determined as cysteic acid and methionine sulfone after oxidation with performic acid (Moore, 1963). No correction for destruction or incomplete liberation of amino acids during acid hydrolysis was made and tryptophan was not determined. Phospholipid and total cholesterol content of both ethanol–acetone (1:1) and chloroform–methanol (2:1) extracts of α-P and β-P were determined according to Fiske and Subbarow (1925) and Abell *et al.* (1952), respectively. For measurement of solubility of α-P, β-P, and derivatives of β-P in various solvents, an excess of the protein preparation was shaken in the solvent for 6 hr and after centrifugation the clear supernatant was analyzed for protein content with the method of Lowry *et al.* (1951); a known quantity of α-P or of the protein moiety of trypsin-treated β-LP (both of which protein preparations are soluble in aqueous buffers) served as the standard.

Paper chromatography of α-P and β-P was done on Whatman No. 4 paper with the descending technique in various solvent systems to be described. The solvent front was allowed to flow up to the origin before 0.1–0.5 mg of the protein, dissolved in 30 μl of the chromatographic solvent, was applied to the origin. The chromatogram was stained overnight with 0.002% nigrosin in 2% acetic acid.

Gel filtration of α-P and of proteolytic digests of α-P and β-P was done at 25° on a 1 × 100 or 2 × 200 cm column of Sephadex G-75 or G-200 (Pharmacia). The gels were prepared according to Andrews (1964), the eluting solvent was generally 1.0 N acetic acid (which was also used to dissolve 2–10 mg of the protein or peptide preparation, in a volume of 0.5–3 ml), and the effluent was collected in 3–10-ml fractions. Aliquots 0.1–1.0 ml from each effluent fraction were dried *in vacuo* at 80°, hydrolyzed for 4 hr at 90° in 2.5 N NaOH, and analyzed with the colorimetric ninhydrin reagent (Hirs *et al.*, 1956). Fractions constituting a peak of ninhydrin-reactive material were then pooled and lyophilized. The relationships between molecular weight, distribution coefficient, K_d, and elution volume on the G-75 and G-200 Sephadex columns were determined, as described by Andrews (1964), with the following purified substances from Mann Research Laboratories: leucine, urea, tetraglycine, glucagon, oxidized B chain of insulin, insulin, cytochrome C, lysozyme, ribonuclease, chymotrypsinogen, soy bean trypsin inhibitor, ovalbumin, bovine plasma albumin, bovine plasma γ-globulin, and Dextran blue (Pharmacia). In certain experiments 10 mg of the lipoprotein–protein sample was dissolved in 3 ml of 0.05 M Na_2HPO_4 (pH 9.0) containing 0.05 mmole of sodium dodecyl sulfate/ml and the solution was placed on a 2 × 200 cm column of G-200 equilibrated with 0.05 M Na_2HPO_4; the column was eluted with 0.05 M Na_2HPO_4 and 1 ml of each successive 10-ml fraction of effluent

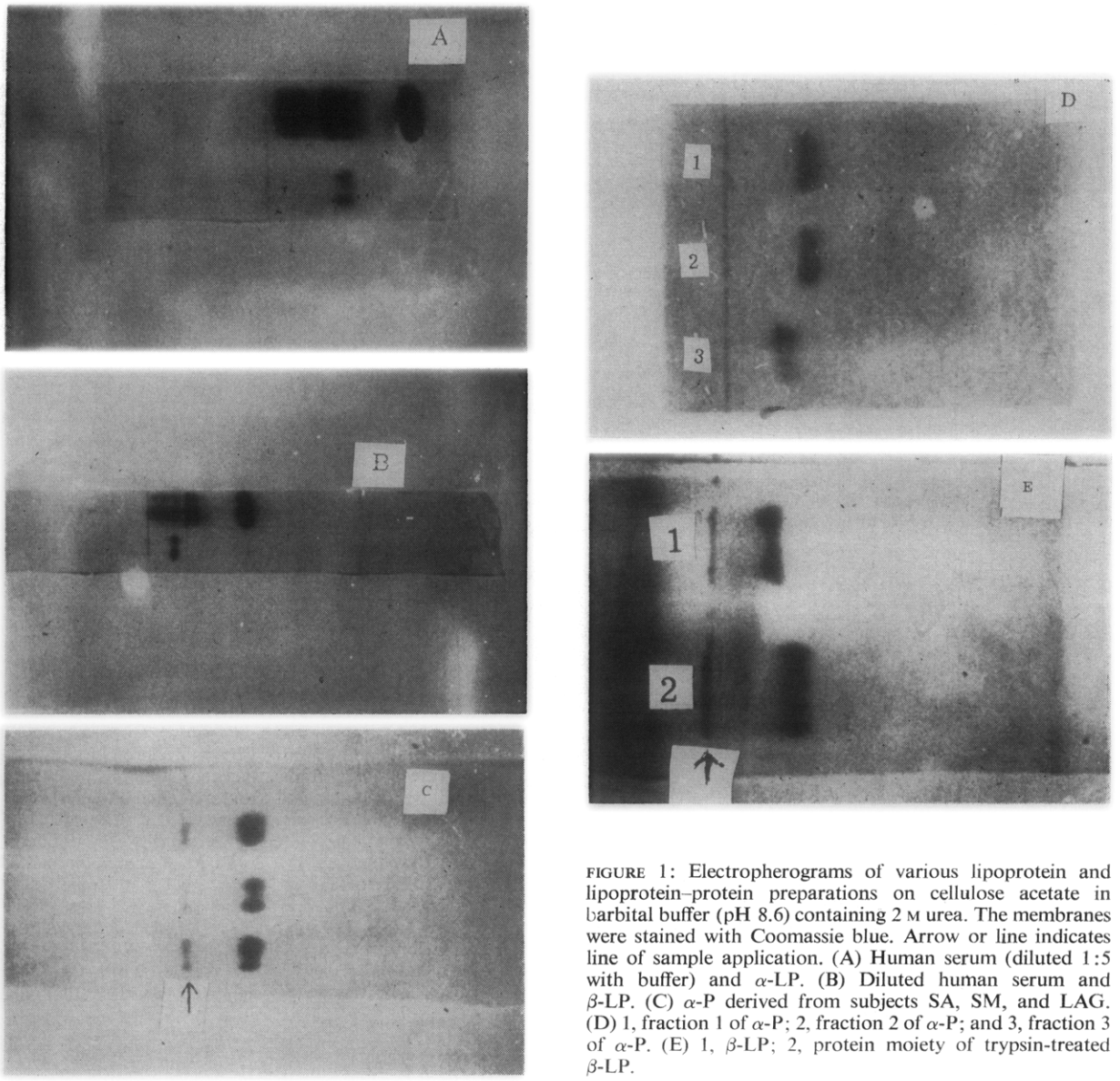


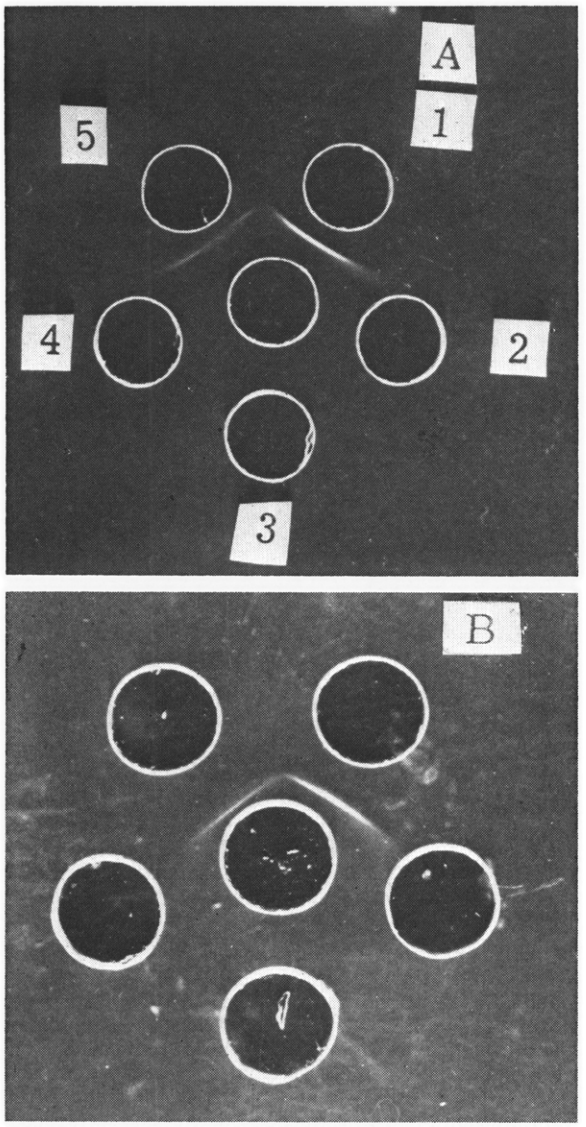
FIGURE 1: Electropherograms of various lipoprotein and lipoprotein–protein preparations on cellulose acetate in barbital buffer (pH 8.6) containing 2 M urea. The membranes were stained with Coomassie blue. Arrow or line indicates line of sample application. (A) Human serum (diluted 1:5 with buffer) and α-LP. (B) Diluted human serum and β-LP. (C) α-P derived from subjects SA, SM, and LAG. (D) 1, fraction 1 of α-P; 2, fraction 2 of α-P; and 3, fraction 3 of α-P. (E) 1, β-LP; 2, protein moiety of trypsin-treated β-LP.

was analyzed for protein content by the Folin–Lowry method.

Proteolytic digestion of α-LP, of β-LP, of α-P, and of various fractions of α-P and β-P was done by incubating the lipoprotein or protein with two-times-crystallized trypsin (10,000 units/mg) or three-times-crystallized α-chymotrypsin (9000 units/mg) (Mann) in 0.1 M ammonium acetate solution at pH 9.0 for 6 hr at 25° with a protein to peptidase ratio of 100:1, after which the incubation mixture was lyophilized.[3]

Fingerprinting of tryptic digests of α-P and of its subfractions was done on 25 × 59 cm sheets of MN chromatography paper (Brinkmann Instruments). The tryptic digest of 3 mg of the protein preparation in 30 μl of buffer was applied in the center of the right-hand margin of the paper; 10 μg of arginine, applied in the center of the left-hand margin, served as a marker for calculation of the electrophoretic mobility of the peptides. The first dimension of the fingerprinting procedure constituted high-voltage electrophoresis for 2 hr at 3000 V, in the Brinkmann Pherograph II apparatus, with pyridine–acetate buffer (pH 5.3) (pyridine–acetic acid–H_2O, 40:20:4000, v/v). After electrophoresis, the paper was allowed to dry, then rolled into a cylinder, and with the original right-hand margin now dependent was placed in a cylinder containing 150 ml of 1-butanol–acetic acid–H_2O (12:3:5); the solvent was allowed to rise 30 cm (16–20 hr), just short of the position of arginine on the original left-hand margin (now uppermost) of the paper. After drying, the paper was sprayed with 0.2% ninhydrin in acetone.

[3] Preliminary experiments showed these to be the minimal duration and enzyme concentration which led to cleavage of all susceptible bonds in α-P. Higher concentration of peptidase or more prolonged digestion did not detectably alter the tryptic or chymotryptic fingerprint; with lower enzyme concentration or shorter duration, α-P was not totally converted into peptides <2000 in molecular weight, as judged by gel filtration of the digest on Sephadex G-75.



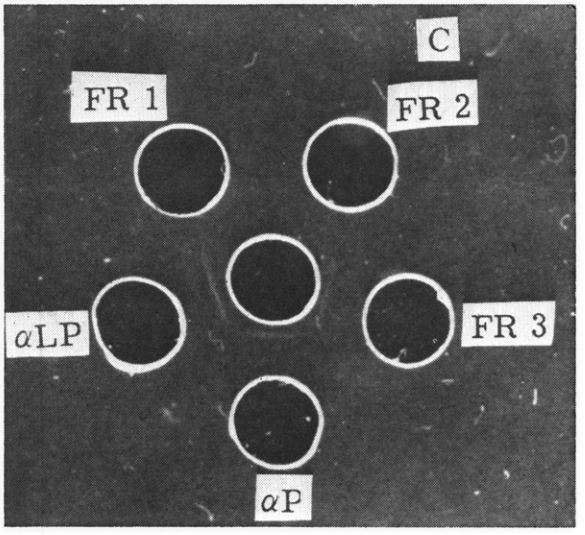


FIGURE 2: Immunodiffusion of various lipoprotein and lipoprotein–protein preparations. In each case, the peripheral upper right well is designated 1 and the remaining peripheral wells in clockwise arrangement designated 2, 3, 4, and 5 (as illustrated in panel A). (A) Center well: anti-β-LP. Peripheral wells: 1, β-LP of subject SM; 2 and 3, α-LP of subjects DR and SM; 4, α-P of subject SM; 5, β-LP of subject SA. (B) Center well: anti-α-LP. Peripheral wells: 1, α-LP of subject SM; 2, β-LP of subject SM; 3, β-LP of subject SA; 4, β-LP of subject LG; 5, α-P of subject SM. (C) Center well: anti-α-LP. Peripheral wells: well 1, fraction 2 of α-P; well 2, fraction 3 of α-P; well 3, α-P; well 4, α-LP; well 5, fraction 1 of α-P.

Results

Purity of α-LP and β-LP Preparations. Electrophoresis at pH 8.6 in the absence or presence of 2 M urea and at pH 3.9 in 2 M urea showed only one component in each preparation of α-LP and β-LP (Figure 1A,B). With the lots of antiserum employed, the following immunodiffusion results were obtained (Figure 2 and Table I). α-LP formed a single precipitin arc in immunodiffusion against antihuman serum and against antihuman α-LP and did not react with antihuman β-LP; β-LP formed a single line of precipitation with antihuman serum and with antihuman β-LP and did not react with anti-α-LP. Neither α-LP nor β-LP reacted detectably with antisera against human haptoglobin, γ-globulin, fibrinogen, transferrin, or albumin.

Delipidation of α-LP and β-LP. CHEMICAL COMPOSITION AND SOLUBILITY PROPERTIES OF α-P AND β-P. All preparations of α-LP and β-LP, after documentation of purity by electrophoresis and immunodiffusion, were dialyzed for 24 hr against a total of 18 l. of H_2O. In the initial experiments, the delipidation technique of Rodbell and Fredrickson (1959) (addition of 50 volumes of ethanol–acetone to the aqueous solution or suspension of lipoprotein) was used. We then found that the following modification yielded apparently identical[4] results but was more convenient for large-scale preparations. The aqueous solution or suspension of dialyzed α-LP or β-LP was divided into 5–15-ml aliquots in tared 25-ml conical test tubes and lyophilized. The residual powder was then extracted overnight at room temperature with 25 ml of ethanol–acetone (1:1, v/v); the residue was washed three times with 25 ml of the same solvent, suspended in 3 ml of H_2O, and lyophilized to yield lipid-free α-P and β-P as fluffy white powders. Yields from six normal plasmas were 1.8–2.5 mg of α-P and 0.4–0.7 mg of β-P per ml of plasma.

Ethanol–acetone (1:1) and chloroform–methanol (2:1) extracts of α-P and β-P did not contain a detect-

[4] As indicated by solubility properties, electrophoretic behavior, and amino acid composition of the delipidated protein preparations.

able quantity of cholesterol or phospholipid (less than 0.01 mg of either lipid/mg of protein).[5] The quantitative amino acid compositions of α-P and β-P are given in Table II. These data are in general agreement with those of Scanu and Granda (1966) and Margolis and Langdon (1966a). The solubility properties are shown in Table III. All preparations of α-P were soluble in aqueous buffers of pH 2–9 to a maximal concentration of 0.8–3.9 mg/ml, while β-P was insoluble at any pH tested, even in the presence of 6 M urea. α-P was also soluble in mixtures of primary or secondary butyl alcohol, acetic, or formic acid, and H_2O;[6] β-P was insoluble in these mixtures.

Studies on α-P. A. ELECTROPHORESIS AND IMMUNODIFFUSION. On cellulose acetate strips, in the presence or absence of 2 M urea, α-P moved as a single band[7] with the same mobility as α-LP. No difference in electrophoretic mobility of α-P from different subjects was detectable (Figure 1C). α-P gave a single line o precipitation with rabbit antiserum to human α-LP and did not react with rabbit or horse antihuman β-LP (Figure 2A–C).

B. TRYPTIC FINGERPRINT. On a 1 × 100 cm column of Sephadex G-75, α-P emerged at a volume of 28–52 ml (K_d = 0.0–0.4) (Figure 3A). After incubation with trypsin, α-P was totally converted into a mixture of peptides eluting at 70–90 ml (K_d = 0.66–0.92) (Figure 3B); calibration of the column with standard substances (Figure 4A) showed that this K_d range corresponds to a molecular weight of <2000. Twenty-four spots were visualized with ninhydrin[8] in the tryptic digest of α-P by the fingerprinting technique; at pH 5.3, nine had a positive charge (spots 1–9), seven showed little migration (spots 10–16), and eight were negatively charged (spots 17–24) (Figure 5A).

C. PAPER CHROMATOGRAPHY. In view of the solubility of α-P in butanol-containing mixtures, paper chromatography in a variety of solvent systems was attempted. Several solvents employed by Light and Simpson (1956) for the paper chromatography of insulin proved to be useful. The results are summarized in Table IV. α-P remained at the origin in 1-butanol-containing systems (Figure 6A, sample 4); it moved as a single component close to the solvent front in *sec*-butyl alcohol–acetic acid–H_2O (4:1:5) (Figure 6C, sample 4), in *sec*-butyl alcohol–formic acid–H_2O (4:1:5), and in concentrated aqueous solutions of phenol (80 g/100 ml of solution). In *sec*-butyl alcohol–acetic acid–H_2O (4:0.2:5), however, a partial separation of at least two components was suggested: a broad zone with R_F 0.1–0.4 and a compact band at R_F 0.6 (Figure 6B, sample 4).

D. GEL FILTRATION OF α-P. Since paper chromatography in *sec*-butyl alcohol–acetic acid–H_2O (4:0.2:5) suggested the presence of more than one protein in the α-P preparation, attempts were now made by gel filtration to separate the suspected different subunits. A variety of gel columns were tested (G-75, G-100, G-150, and G-200) and a 2 × 200 cm column of the latter was found most effective. Calibration of this column with polypeptides of known molecular weight is shown in Figure 4B. When 10 mg of α-P was dissolved in 1.0 N acetic acid[9] and introduced on a 2 × 200 cm G-200 column, it emerged as three peaks (labeled fractions 1–3 in Figure 7A) with K_d values 0.12, 0.49, and 0.64, respectively. These K_d values correspond to molecular weights of approximately 150,000, 70,000, and 25,000 (see Figure 4B). (It must be emphasized at this point that estimations of molecular weight by gel filtration on calibrated columns are only approximate, since the K_d values can be influenced by shape of the protein molecule and its possible affinity for the gel.) Over 90%

[5] Further evidence for the removal of virtually all lipid by the delipidation technique employed was obtained as follows. α-P or β-P (2 mg) was placed in 2 ml of 14% BF_3 in methanol under nitrogen. The sealed tubes were placed in a boiling-H_2O bath for 1 hr, then cooled to 0°. H_2O (2 ml) and hexane (2 ml) were added; after mixing and centrifuging, the hexane layer was removed. The aqueous layer was extracted again three times. The pooled hexane extracts were evaporated to dryness under nitrogen, redissolved in 10 μl of hexane, and a 1-μl aliquot was analyzed by gas–liquid partition chromatography for content of fatty acid methyl esters (Dr. A. Rosenberg, Department of Biochemistry, Columbia University, kindly performed these analyses). The results showed only 0.04 mg of fatty acid/100 mg of α-P, and only 0.02 mg of fatty acid/100 mg of β-P.

[6] The possibility was considered that the apparent solubility of α-P in mixtures of primary or secondary butyl alcohols, acetic acid, and H_2O could result from hydrolysis of covalent bonds catalyzed by these solvent systems. Therefore α-P was dissolved in *sec*-butyl alcohol–acetic acid–H_2O (4:1:5), dialyzed overnight against 1 N acetic acid, and recovered as the white powder by lyophilization. When this preparation was examined by electrophoresis, immunodiffusion, paper chromatography, and gel filtration on G-200, it was indistinguishable from the original α-P. Thus solubility of α-P in mixtures of butanol, acetic acid, and H_2O did not appear to involve cleavage of covalent bonds.

[7] These experiments were done with cellulose acetate membranes from Millipore. Satisfactory electrophoretic patterns of α-P were not obtained with membranes from other manufacturers.

[8] When these ninhydrin-stained fingerprints were counterstained with Ehrlich's reagent, followed by Pauly's or Sakaguchi's reagent, to visualize tryptophan-, tyrosine-, histidine-, and arginine-containing peptides, respectively, it was apparent that at least 8 of these 24 ninhydrin-positive spots were actually composites of 2 or more peptides, since in these instances only one section of the ninhydrin-positive spot reacted with the new stain. Thus the total number of peptides in the tryptic digest of α-P was uncertain but was greater than 32.

[9] Freshly made solutions of α-P were employed. Recent experiments have shown that when solutions of α-P in 1.0 N acetic acid are allowed to stand at 5° for 5 days before gel filtration, the resulting elution profile reveals extensive dissociation of fractions 1 and 2 to material with K_d values of 0.49–0.64 corresponding to mol wt 70,000–25,000. No material with molecular weight lower than that of fraction 3 was observed. When solutions of α-P after standing at 25° in 1 N acetic acid for 5 days, were lyophilized, freshly dissolved in 1 N acetic acid, and analyzed by gel filtration on G 200, the usual pattern of fractions 1–3 in proportions of about 20, 60, and 20% was observed. Again no ninhydrin-reactive material with molecular weight lower than fraction 3 (K_d = 0.64) was present. These observations indicate that the change in elution pattern of α-P after exposure to 1 N acetic acid for 5 days was due to disaggregation rather than to hydrolysis of covalent bonds. Since the peptide subunits of α-P are known to possess a molecular weight in the range 23,000–31,000 (Scanu, 1966; Shore and Shore, 1967), hydrolytic cleavage of peptide bonds would be expected to release fragments with molecular weights below this value.

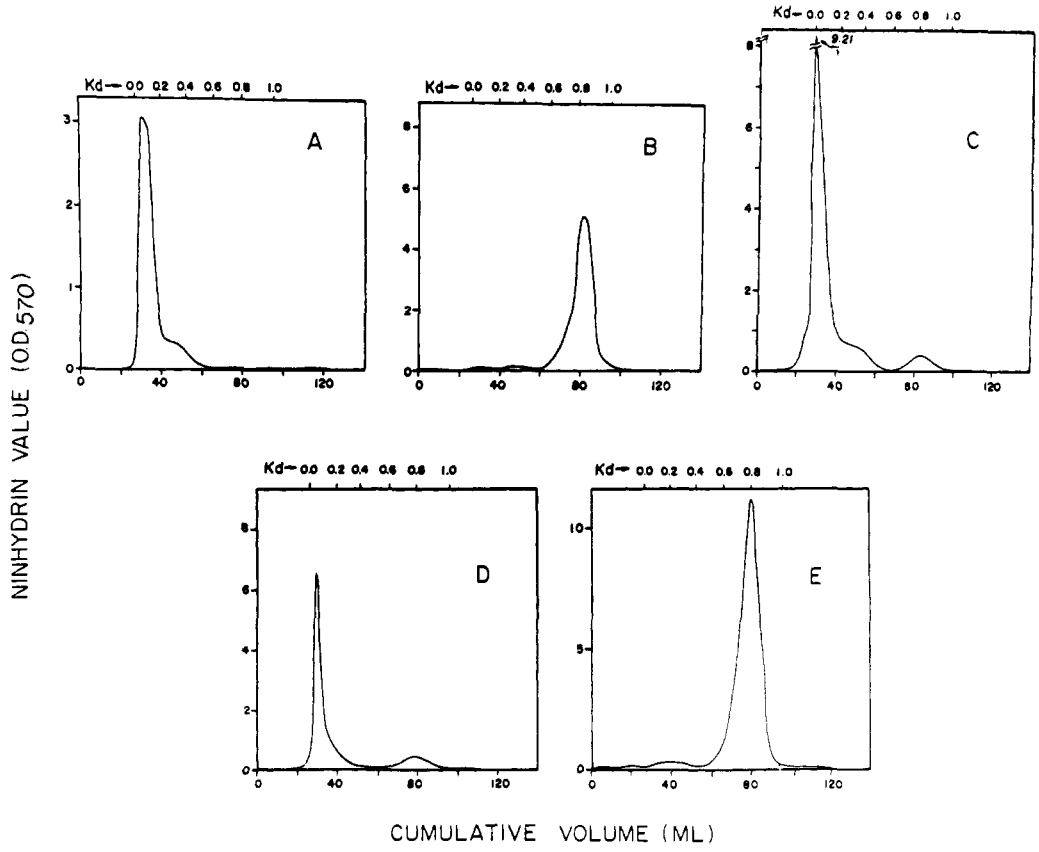


FIGURE 3: Gel filtration of various lipoprotein-protein preparations on 1 × 100 cm columns of G-75. Abscissa shows cumulative volume of elution. Ordinate represents optical density (570 mμ) of colorimetric ninhydrin reaction mixture per milliliter of hydrolyzed effluent. K_d values (in intervals of 0.2) at various cumulative volumes of effluent are also shown (upper horizontal axis). (A) 2 mg of α-P. (B) 5 mg of tryptic digest of α-P. (C) 7 mg of protein moiety of trypsin-treated α-LP. (D) 7 mg of protein moiety of trypsin-treated β-LP. (E) 5 mg of tryptic digest of protein moiety of trypsin-treated β-LP.

TABLE II: Amino Acid Composition of α-P, Fractions of α-P, and β-P.

	Moles of Amino Acid/100,000 g of Protein (av of 4 detn[a] ± SE)				
Amino Acid	α-P	Fraction 1 of α-P	Fraction 2 of α-P	Fraction 3 of α-P	β-P
Aspartic acid	59.6 ± 2.7	64.5 ± 2.3	60.0 ± 2.9	53.5 ± 1.2	88.7 ± 2.9
Threonine	33.2 ± 1.4	30.7 ± 0.9	32.9 ± 2.1	40.2 ± 1.7	47.0 ± 1.5
Serine[b]	45.8 ± 0.9	40.0 ± 1.2	49.5 ± 1.8	70.9 ± 2.0	66.5 ± 2.6
Glutamic acid	131.7 ± 1.6	126.5 ± 1.9	133.4 ± 2.3	140.0 ± 2.4	102.7 ± 2.7
Proline	40.1 ± 2.1	38.8 ± 1.9	42.7 ± 2.5	40.5 ± 2.7	33.4 ± 1.3
Glycine	35.0 ± 1.7	37.3 ± 2.0	35.3 ± 1.6	39.4 ± 1.7	36.9 ± 2.1
Alanine	58.3 ± 1.1	56.6 ± 0.9	59.4 ± 1.9	55.8 ± 1.5	50.5 ± 2.5
Valine	42.5 ± 2.2	39.1 ± 1.9	41.0 ± 1.9	50.6 ± 2.3	41.9 ± 1.9
Half-cystine[b]	5.8 ± 0.5	5.0 ± 0.6	7.3 ± 0.8	13.8 ± 0.7	5.0 ± 0.4
Methionine	6.5 ± 0.3	6.8 ± 0.9	6.9 ± 0.5	8.8 ± 0.7	12.8 ± 0.7
Isoleucine[b]	5.1 ± 0.5	4.2 ± 0.9	7.9 ± 0.8	14.2 ± 0.4	42.0 ± 1.8
Leucine[b]	108.7 ± 1.9	111.9 ± 2.5	104.1 ± 3.1	80.3 ± 2.6	94.8 ± 3.5
Tyrosine	25.0 ± 0.8	22.5 ± 1.2	26.3 ± 1.2	30.4 ± 1.0	22.6 ± 1.0
Phenylalanine[b]	26.6 ± 1.5	17.1 ± 1.7	23.8 ± 2.4	37.7 ± 0.9	37.7 ± 1.0
Lysine[b]	64.5 ± 1.1	55.6 ± 0.9	69.7 ± 2.1	75.8 ± 1.7	53.8 ± 1.9
Histidine[b]	15.9 ± 0.6	17.7 ± 0.9	12.5 ± 1.4	3.2 ± 0.2	20.0 ± 0.9
Arginine[b]	37.3 ± 1.3	44.6 ± 1.5	38.2 ± 1.4	14.6 ± 0.4	26.5 ± 0.7

[a] α-P, its three fractions, and β-P were prepared from four different subjects. [b] Difference between fraction 1 and fraction 3 significant at $p < 0.001$.

TABLE III: Solubility (Micrograms per Milliliter) of α-P, β-P, and the Protein Moiety of Trypsin-Treated β-LP in Various Solvents.

Solvent	α-P	β-P	β-LP[b]
1 N acetic acid (pH 2.4)	3900	0	820
0.1 M sodium phosphate (pH 4.0)[a]	848	0	512
0.1 M sodium phosphate (pH 7.0)[a]	1280	0	480
0.1 M sodium phosphate (pH 9.0)[a]	1860	0	814
0.1 M sodium phosphate (pH 7.0)[a]–7 M urea	Not tested	0	1485
0.1 M sodium phosphate (pH 9.0)[a]–7 M urea	Not tested	0	1730
sec-Butyl alcohol–acetic acid–H_2O (4:1:5)	1800	0	580
sec-Butyl alcohol–formic acid–H_2O (4:1:5)	1650	0	820

[a] Molarity based on sodium ion concentration. [b] Protein moiety, trypsin treated.

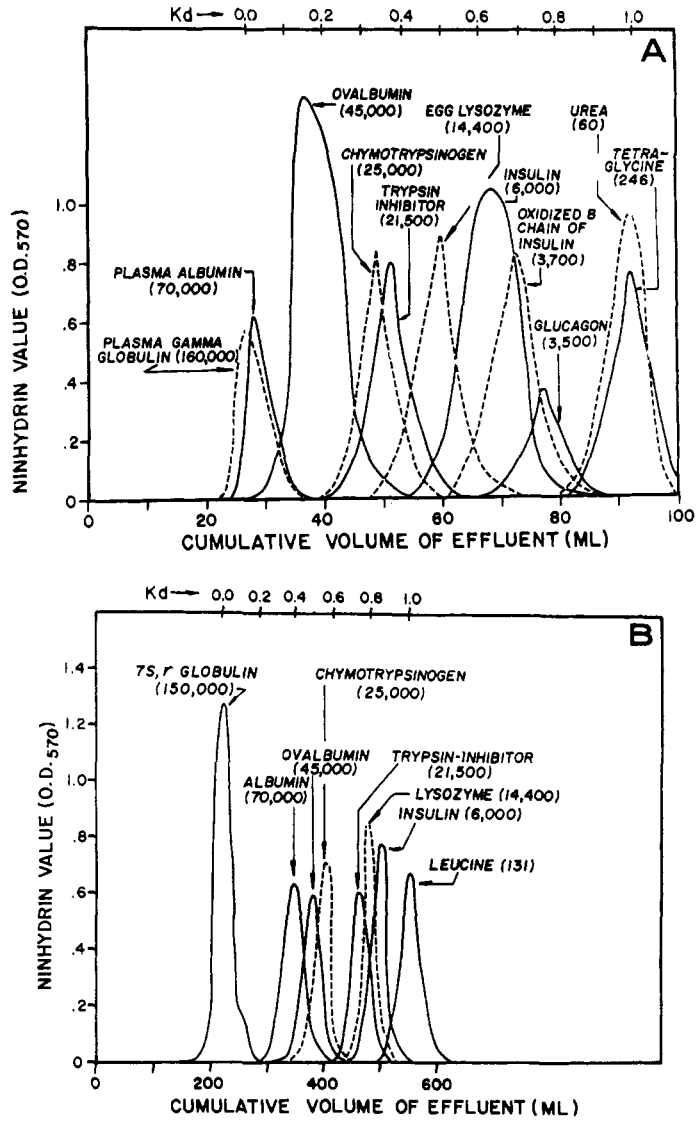


FIGURE 4: Calibration of a 1 × 100 cm column of G-75 (panel A) and a 2 × 200 cm column of G-200 (panel B) with pure polypeptides of known molecular weight. The columns were equilibrated with 1.0 N acetic acid. In series A, 1 mg of each polypeptide was dissolved in 1 ml of 1.0 N acetic acid and introduced on the column; ordinate represents ninhydrin value per milliliter of hydrolyzed effluent. In series B, 5 mg of each polypeptide was dissolved in 3 ml of 1.0 N acetic acid and placed on the column. Ordinate and abscissa as in Figure 3.

of the material introduced into the column was recovered; in 12 such experiments, fraction 1 constituted 18–25% of α-P, fraction 2 constituted 40–60%, and the proportion of fraction 3 varied between 20 and 30%. When fractions 1 and 3 were rechromatographed on the same column, they emerged at the original volume of elution (Figure 7B,D); rechromatography of fraction 2, in contrast, gave a pattern similar to that of α-P in that 10–20% of the material was recovered in the positions of fraction 1 and of fraction 3, and 60–80% in the position of fraction 2 (Figure 7C). On cellulose acetate electrophoresis, fractions 1 and 2 moved as single bands with identical mobility; fraction 3 moved less rapidly (Figure 1D). On paper chromatography in the *sec*-butyl alcohol–acetic acid–H_2O (4:0.2:5) system, fraction 1 moved with an R_F of 0.1–0.4 (corresponding to the slower diffuse zone in α-P) and fraction 3 with an R_F of 0.6 (corresponding to the rapid compact zone in α-P); fraction 2 contained both slow and fast components and resembled α-P (Table IV and Figure 6B). The tryptic fingerprint of fraction 2 was generally similar to that of α-P, all of the acidic and basic spots in the latter's fingerprint being visualized with the occasional exception of spots 5, 18, and 20 (Figure 5C). In the fingerprint of fraction 1, five basic spots (3, 4, 7, 8, and 9) and three acidic spots (17, 22, and 24) were seen. All of these corresponded to spots which were present in the fingerprint of α-P but absent in that of fraction 3 (Figure 5B). The fingerprint of fraction 3 showed four basic spots (1, 2, 5, and 6) and four acidic spots (18–21), all of which were present in the tryptic digest of α-P but none of which were visualized in that of fraction 1 (Figure 5D). The neutral peptides in the various fingerprints were not resolved well enough for meaningful comparison of this region of the fingerprint of α-P and its subfractions. The comparative tryptic fingerprinting of fractions 1–3 was repeated five times during the course of this investigation with similar results.

The amino acid composition of fractions 1–3 are given in Table II. Fractions 1 and 3 differed from each other substantially in the content of arginine, half-cys-

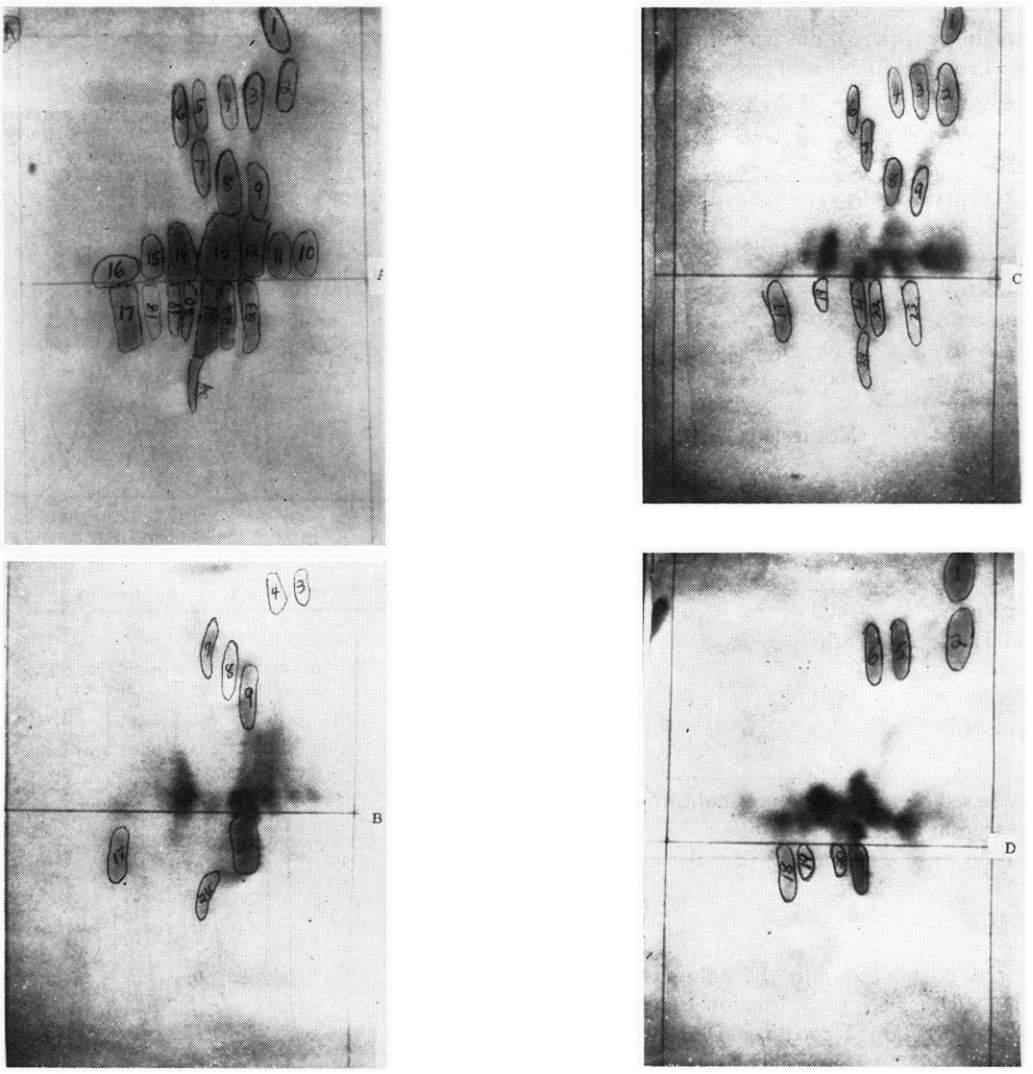


FIGURE 5: Fingerprints of tryptic digests of α-P (A) and of fractions 1 (B), 2 (C), and 3 (D) derived from α-P by gel filtration on Sephadex G-200. The sample was applied at the junction of the horizontal and right-hand vertical line. High-voltage electrophoresis was conducted in the vertical dimension (the cathode was above); for ascending paper chromatography, the left-hand margin of the paper was uppermost. The spot in the upper left corner of each sheet is arginine (labeled A) in panel A.

TABLE IV: Results of Paper Chromatography of α-P and of Fractions 1–3 Derived from α-P by Gel Filtration on Sephadex G-200.

Solvent System	R_F			
	α-P	Fraction 1	Fraction 2	Fraction 3
1-Butanol–acetic acid–H_2O (12:3:5)	0	0	0	0
sec-Butyl alcohol–1% acetic acid (1:1)[a]	0.1–0.6			
sec-Butyl alcohol–1% dichloroacetic acid (1:1)[a]	0.4–0.8			
sec-Butyl alcohol–acetic acid–H_2O (4:0.2:5)[a]	0.1–0.6	0.1–0.4	0.1–0.6	0.6
sec-Butyl alcohol–acetic acid–H_2O (4:1:5)	0.95	0.95	0.95	0.95
sec-Butyl alcohol–formic acid–H_2O (4:1:5)	0.95	0.95	0.95	0.95
80% phenol in H_2O[b]	0.95			
80% phenol in H_2O + 5 ml of concentrated NH_3 per l.	0.95			
80% phenol in H_2O–ethanol–H_2O (150:40:10)	0.95			

[a] Upper phase of two-phase system. [b] 80 g of phenol/100 ml of solution.

tine, histidine, isoleucine, leucine, lysine, phenylalanine, and serine. The compositions of fraction 2 and of unfractionated α-P more closely resembled that of fraction 1 than of fraction 3. Nevertheless, in the case of all eight amino acids mentioned, the abundance in fraction 2 and in α-P was intermediate between that in fraction 1 and in fraction 3.

Immunodiffusion of the three fractions of α-P *vs.* rabbit antihuman α-LP showed that only fraction 3 reacted with the antiserum (Figure 2C). This finding was confirmed with three different lots of rabbit antiserum to human α-LP (Table I) and with three different preparations of fractions 1–3 of α-P. None of the fractions reacted with anti-β-LP.

Since Scanu (1966) and Shore and Shore (1967) have shown that α-P is dissociated by sodium dodecyl sulfate into peptide units with molecular weight in the range 23,000–31,000, α-P and its subfractions 1–3 were each dissolved in 0.05 M Na_2HPO_4 containing 0.05 mmole of sodium dodecyl sulfate/ml (10 mg of protein in 3 ml of solvent) and analyzed by gel filtration on G-200 columns equilibrated with 0.05 M Na_2HPO_4; the columns were eluted with 0.05 M Na_2HPO_4 as well. All four preparations emerged as single, nearly symmetric peaks with K_d = 0.64–0.72 (Figure 7E). These distribution coefficients correspond to molecular weights of 20,000–25,000 (Figure 4B), provided the detergent does not alter the relationship between K_d and molecular weight. To investigate this point, six of the standardizing proteins (γ-globulin, ovalbumin, chymotrypsinogen, trypsin inhibitor, lysozyme, and insulin), whose K_d values on G-200 had already been determined in 1.0 N acetic acid (Figure 4B), were dissolved in 0.05 M Na_2HPO_4 containing 0.05 mmole of sodium dodecyl sulfate/ml (10 mg of protein in 3 ml of solvent) and subjected to gel filtration in columns of G-200 equilibrated and eluted with 0.05 M Na_2HPO_4. In no instance did the K_d values differ by more than 15% from those previously determined in 1.0 N acetic acid in the absence of sodium dodecyl sulfate.

E. Proteolytic Cleavge of α-LP. As shown in Figure 3A,B, cleavage of α-P with trypsin or chymotrypsin rapidly went to completion with the disappearance of the high molecular weight components (K_d = 0.0–0.4 on G-75) and the recovery of 90–100% of α-P in the form of peptide fragments with mol wt <2000. Chymotrypsin produced a similar effect. Contrastingly, when α-LP was digested with trypsin or chymotrypsin, followed by delipidation, less than 10% of the protein moiety was recovered as peptide fragments and over 90% emerged with unchanged K_d = 0.0–0.4 on G-75 (Figure 3C). This high molecular weight fraction was indistinguishable from α-P on cellulose acetate electrophoresis or immunodiffusion against anti-α-LP. When examined on a 2 × 200 cm column of G-200, it was found to contain fractions 1–3 in the same general proportions as α-P. Trypsin-treated α-LP, examined before delipidation, was indistinguishable from untreated α-LP by electrophoresis and immunodiffusion against anti-α-LP.

Studies on β-LP and β-P. Delipidation of β-LP by the technique of lyophilization followed by ethanol–acetone extraction yielded a white powder which was totally insoluble at pH 3, 5, or 9 in aqueous buffers in the absence or presence of urea. This preparation was also insoluble in the *sec*-butyl alcohol containing systems in which α-P readily dissolved (Table III). Therefore β-P could not be studied by electrophoresis, immunodiffusion, or gel filtration.

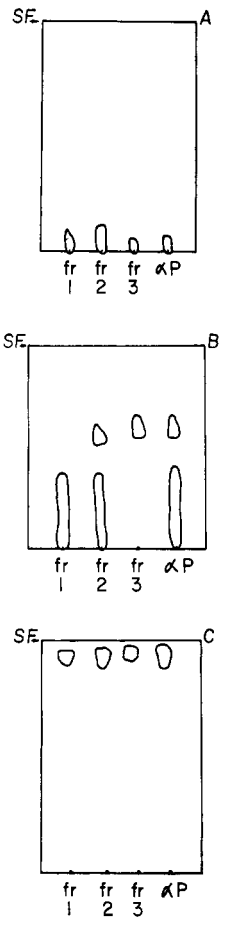


FIGURE 6. Tracings of paper chromatograms (descending technique) of α-P and of fractions 1–3 derived from α-P by gel filtration on Sephadex G-200. SF signifies solvent front. Solvent systems: (A) 1-butanol–acetic acid–H_2O (12/3/5) (one-phase system), (B) *sec*-butyl alcohol–acetic acid–H_2O (4/0.2/5) (upper phase), and (C) *sec*-butyl alcohol–acetic acid–H_2O (4/1/5) (one-phase system).

Incubation of β-LP with trypsin for 6 hr did not alter the electrophoretic mobility or immunologic reactivity of the lipoprotein (Table I). When the trypsin-treated β-LP was delipidated, the resulting white material was found to have considerable solubility in aqueous buffers; this was enhanced by the presence of 7 M urea (Table II). Gel filtration on G-75 (Figure 3D) showed that 5% of this preparation consisted of peptides with a K_d value corresponding to mol wt <2000 while 95% of the preparation was totally excluded from the gel (suggesting mol wt >70,000). Electrophoresis on cellulose acetate of the protein moiety of trypsin-treated β-LP revealed a single band with mobility 1.6 times greater than that of β-LP (Figure 1E); the mobility of this material prepared from the β-LP of four different subjects was identical. This high molecular weight protein fraction of trypsin-treated β-LP did not react in immunodiffusion with anti-β-LP, with anti-α-LP, or with antiserum to whole

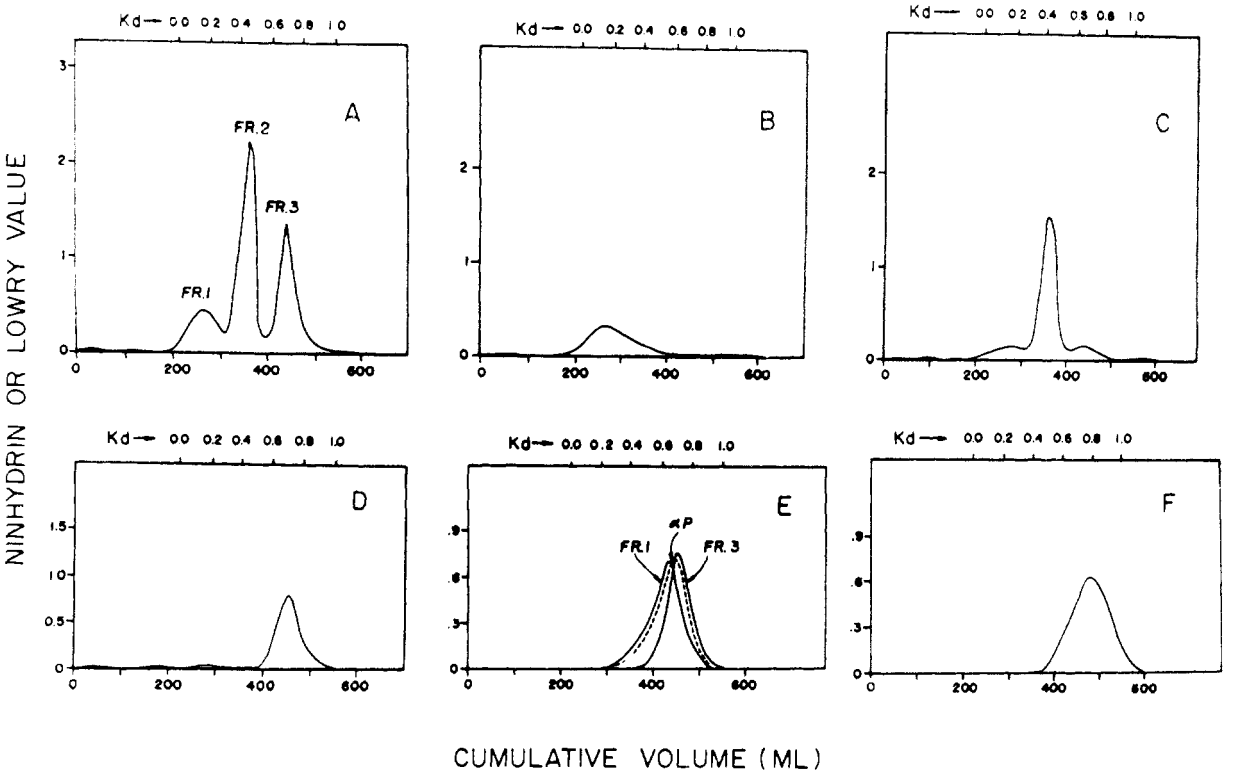


FIGURE 7: Gel filtration of various lipoprotein–protein preparations on 2 × 200 cm columns of Sephadex G-200. Columns A–D were equilibrated and eluted with 1.0 N acetic acid, and the samples were dissolved in this solvent. Columns shown in E and F were equilibrated and eluted with 0.05 M Na_2HPO_4 and the samples were dissolved in this solvent containing 0.05 mmole of sodium dodecyl sulfate/ml. Ordinate in A–D represents ninhydrin value (OD_{570}) per milliliter of hydrolyzed effluent. In E and F ordinate represents Folin–Lowry value (OD_{650}) per milliliter of unhydrolyzed effluent. (A) 10 mg of αP. (B) Rechromatography of 2 mg of fraction 1 from the experiment shown in panel A. (C) Rechromatography of 4 mg of fraction 2 from the experiment shown in panel A. (D) Rechromatography of 3 mg of fraction 3 derived from the experiment shown in panel A. (E) 6 mg of α-P, of fraction 1, or of fraction 3, run on separate columns but plotted on the same graph. (F) 5 mg of major protein component (Kd = 0 on G-75; see Figure 3D) of trypsin-treated β-LP, isolated by prior gel filtration in 1.0 N acetic acid of delipidated, trypsin-treated β-LP on G-75.

human serum (Table I). When this fraction was incubated with trypsin for 6 hr, it was totally cleaved to peptide fragments with K_d corresponding to mol wt <2000 (Figure 3E).

The protein moiety of chymotrypsin-treated β-LP was similar to that of the trypsin-treated lipoprotein in solubility properties, in elution pattern from the G-75 column, and in failure to react with anti-β-LP; on cellulose acetate electrophoresis, furthermore, the protein component of the chymotrypsin- and trypsin-digested β-LP showed an identical rate of migration.

In order to obtain a first approximation of the molecular weight of the major, electrophoretically homogeneous component which comprises >90% of the protein moiety of trypsin-treated β-LP, this material was isolated by gel filtration of delipidated, trypsin-treated β-LP on G-75 in 1.0 N acetic acid; the major peak with K_d = 0 was recovered by lyophilization. When this material was dissolved in 1.0 N acetic acid and subjected to gel filtration on Sephadex G-200 equilibrated with this solvent, it emerged as a single peak with K_d = 0, corresponding to mol wt >200,000. When, however, this preparation was dissolved in 0.05 M sodium dodecyl sulfate–0.05 M Na_2HPO_4 and subjected to gel filtration on G-200 equilibrated with 0.05 M Na_2HPO_4, it emerged as a single symmetric peak with K_d = 0.75 (Figure 7F), corresponding to mol wt 15,000–20,000 (see Figure 4B).

Discussion

The present results are in general agreement with those of previous workers on the amino acid composition of α-P and β-P (Scanu and Granda, 1966; Shore and Shore, 1967; Margolis and Langdon, 1966a), and on the markedly restricted cleavage of the protein components of the intact lipoproteins by proteolytic enzymes as compared with the extensive cleavage which occurs after the lipids are removed (Margolis and Langdon, 1966b; Bernfeld and Kelley, 1962; Banaszak and McDonald, 1962). β-P showed the same total insolubility in aqueous buffers as the preparations of other workers (Rodbell and Fredrickson, 1959; Scanu and Hughes, 1960; Banaszak and McDonald, 1962; Margolis and Langdon, 1966b). α-P differed from the preparation of Scanu (1966) in possessing greater solubility at pH 4 and lesser solubility at pH 9. These solubility differences may possibly be related to the fact that the present α-P preparations were totally delipidated, while that of Scanu contained 3% phospholipid.

The new information provided by the present experi-

ments is as follows: (1) a minor modification of the delipidation method of Rodbell and Frederickson (1959). The advantage of the present method of dehydrating the lipoprotein before extracting the lipids with ethanol–acetone lies in the complete removal of phospholipid and cholesterol, and in the suitability for handling large volumes (50–120 ml) of α-LP and β-LP solutions, such as result from fractionation of 250 ml of plasma samples with the repeated washing of the ultracentrifuge tube caps necessary for complete recovery of the floated material. (2) While this delipidation method leads to an insoluble β-P refractory to characterization by electrophoresis, immunodiffusion, or chromatography, the present experiments demonstrate that digestion of β-LP with trypsin or chymotrypsin (under the specified conditions of protein to peptidase ratio, pH, temperature, and duration of digestion) prior to the delipidation procedure leads to recovery of over 90% of the β-LP protein as an electrophoretically homogeneous component, with considerable solubility in aqueous buffers. This component appears to be a dissociable aggregate of a subunit, the distribution coefficient of which corresponds to mol wt 15,000–20,000. (This estimate depends upon the assumption that the relationship between K_d and molecular weight was not markedly influenced by the presence of the disaggregating agent sodium dodecyl sulfate; gel filtration results with several purified proteins of known molecular weight supported this assumption.)

The data do not tell how extensively the primary structure of β-P was altered during the exposure of β-LP to trypsin. Suggestive that only minor alteration was involved is the fact that in the analogous experiments with α-LP (where the protein moiety of untreated and trypsin-treated α-LP could be compared by electrophoresis, immunodiffusion, and gel filtration), the protein moiety appeared to undergo little change during the exposure of the lipoprotein to trypsin. The probability that this analogy is valid is strengthened by the fact that digestion of both α-LP and β-LP with trypsin converted a similar minor proportion (about 5%) of the mass of the lipoprotein–protein into low molecular weight peptides. Furthermore, the electrophoretic and immunologic properties of α-LP and β-LP were not altered by the exposure to trypsin under the conditions employed. On the other hand, Margolis and Langdon (1966b) found that the minor proportion of lipoprotein–protein released by trypsin from β-LP as peptide fragments differed substantially in amino acid composition from the total protein moiety, indicating that a specific region of the protein in β-LP is more accessible to peptidase action and is preferentially cleaved. Accordingly it seems likely that the electrophoretically homogeneous protein component obtained in soluble form from delipidated, trypsin-treated, or chymotrypsin-treated β-LP contains a major section of the unaltered primary structure of the β-P, but that specific regions of this structure have been removed. This preparation, which was reproducibly obtained with the same electrophoretic mobility from four normal persons, may provide a useful opportunity for partial characterization of β-P in various human subjects and for partial determination of the protein's amino acid sequence. (3) The solubility of α-P in *sec*-butyl alcohol containing solvents was described. An application of this finding in the present study was the paper chromatography of α-P and of its subfractions. Other possible applications are the purification and fractionation of α-P by partition chromatography and countercurrent distribution. (4) The presence of at least two different proteins in α-LP has been demonstrated. The line of reasoning leading to this conclusion can be summarized as follows. Scanu (1966) and Shore and Shore (1962, 1967) observed by analytical ultracentrifugation that α-P (prepared by ethanol–diethyl ether delipidation) at pH 2–4 contained a heavy and light component, but was dissociated by sodium dodecyl sulfate to a material with molecular weight in the range 23,000–31,000. In the present study, α-P totally delipidated with ethanol–acetone and freshly dissolved[9] in 1.0 N acetic acid appears to consist of a mixture of three types of material: a highly aggregated fraction 1; a moderately aggregated fraction 2; and fraction 3, probably monomeric since its K_d value corresponds to a molecular weight of about 25,000. As would be expected from Scanu's and the Shores' molecular weight measurements of α-P in the presence of sodium dodecyl sulfate, the present α-P preparation and its aggregated fractions 1 and 2 were found to be totally disaggregated by this detergent to material with a K_d value corresponding to mol wt 20,000–25,000. Quantitative amino acid analyses demonstrated that the protein components of the three fractions of α-P are not identical. If fractions 2 and 1 were successively larger aggregates of the monomeric protein (fraction 3), then the three fractions would have the same amino acid composition. Therefore it can be concluded that fractions 1 and 2 contain at least one additional protein of mol wt 20,000–25,000 either aggregated with that present in fraction 3, or aggregated with itself, or both. Evidence from the tryptic fingerprints and paper chromatography suggests that the additional subunit, deduced from the quantitative amino acid data, is the predominant component in fraction 1, while both types of subunit are represented in fraction 2. Thus in the fingerprints, 8 of the acidic or basic spots of α-P's digest were found in fraction 1 but not in fraction 3, while a different set of 8 acidic or basic spots showed the reverse relationship; nearly all of these 16 components were regularly observed in the fingerprint of fraction 2. On paper chromatography in *sec*-butyl alcohol–acetic acid–H_2O (4:0.2:5), fraction 1 exhibited only a slow component, fraction 3 only a fast component, while fraction 2 showed both. The quantitative amino acid data also support this interpretation, since the composition of fraction 2 is generally intermediate between that of fractions 1 and 3. The differing electrophoretic mobilities of fractions 1 and 3 and the fact that fraction 3 but not 1 reacted with the three lots of anti-α-LP serum employed are also consistent with the above hypothesis. Observation that fraction 2 possesses the same electrophoretic mobility as fraction 1 suggests that it contains a greater proportion of the subunit of fraction 1 (which will be called subunit A) than of the subunit found in fraction 3 (subunit B). The same conclu-

sion is suggested by the fact that the amino acid composition of fraction 2 more closely resembles that of fraction 1 than that of fraction 3 (Table II). The failure of fraction 2 to react with the available anti-α-LP sera may be due to this predominance of postulated subunit A; alternatively, the antigenic site(s) of subunit B may become shielded from the aqueous environment when it forms an aggregate, in the absence of lipid, with subunit A.

To summarize these conclusions: the quantitative amino acid data of the three fractions prove that more than one protein species is present in α-P. The simplest hypothesis consistent with the available evidence postulates two subunits of similar molecular weight differently distributed in fractions 1–3. Subunit A is unreactive to the anti-α-LP sera employed (with which B reacts); contains substantially more arginine, histidine, and leucine, and less half-cystine, isoleucine, lysine, phenylalanine, and serine, than does B; has a lower isoelectric point; moves more slowly on paper chromatography in *sec*-butyl alcohol–acetic acid–H_2O (4:0.2:5); and has a greater tendency to form aggregates in 1.0 N acetic acid. It must be emphasized, however, that the differing amino acid compositions of fractions 1–3, which establish the presence of more than one protein species in α-P, do not exclude the possibility that even more than two subunits of differing amino acid composition might be present.

The isolation by gel filtration of fractions with differing amino acid composition from α-P seems to depend upon the greater tendency of one protein species than of the other to form aggregates in 1.0 N acetic acid. The major fraction obtained (fraction 2), however, appears to represent a mixture of the two (or more) protein species present in α-P. It is evident that further progress on this subject will be facilitated by development of methods more efficient than gel filtration for isolating the different protein species of α-P, preferably in monomeric form.

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