Forms of human serum high density lipoprotein protein

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ABSTRACT Delipidation by ethanol–diethyl ether at −10°C of human serum high-density lipoprotein (HDL, d 1.063–1.21) or of its subclasses HDL_2 (d 1.063–1.120) and HDL_3 (d 1.120–1.21), yielded proteins—αP, αP_2, and αP_3—containing 3% phospholipid (largely lecithin) and 3.3% carbohydrate (glucosamine : L-fucose : D-galactose, D-mannose : sialic acid, 1.00 : 41 : 0.56 : 0.31).

Solubility data and analytical ultracentrifugal analyses indicated that, upon lipid removal, HDL protein aggregates readily; the aggregation is dependent upon pH and ionic strength of the solvent medium. Subunits of 21,000 mol wt were obtained by acetylation or addition of sodium dodecyl sulfate (SDS).

HDL and αP elicited in the rabbit a similar immunological response. By agar gel immunoelectrophoresis both anti-HDL and anti-αP sera detected a major and two minor antigenic determinants in HDL, HDL_2, αP, αP_2, and αP_3. HDL_2, antigenically homogeneous, gave an immunoelectrophoretic pattern of HDL_3 upon mixing with αP. αP, αP_2, and αP_3 exhibited a single antigenic determinant after treatment with SDS (0.5 M) or upon acetylation.

Native or delipidated forms of HDL, HDL_2, and HDL_3 were separated by vertical starch gel electrophoresis into several components, which showed identical reactions against anti-HDL or anti-αP sera.

The data suggest that (*a*) the proteins of HDL, HDL_2, and HDL_3 are made of subunits, probably identical, of an average molecular weight of 21,000; (*b*) the difference in antigenic behavior between HDL_2 and HDL_3 is due to the presence in the latter of a lipid-poor protein; (*c*) antigenic polymorphism of αP is probably related to the presence in solution of monomeric and polymeric forms having different reactivity against anti-HDL and anti-αP sera.

KEY WORDS serum · high density lipoprotein · delipidation · carbohydrate content · solubility · amino acid analysis · ultracentrifugal properties · immunoelectrophoresis · starch gel immunoelectrophoresis · protein subunits

A TECHNIQUE FOR SEPARATING a water-soluble, essentially lipid-free protein from the high density lipoprotein class (HDL) of human serum has been previously reported (1). This protein (αP) was considered homogeneous by electrophoretic, ultracentrifugal, and immunochemical criteria (2, 3); it exhibited marked avidity for lipids (4, 5). Recently, Shore and Shore (6), using a different delipidation technique, recovered from HDL a protein whose monomeric unit had a molecular weight of 36,500 ± 1000, about half the size reported by Scanu, Lewis, and Bumpus (1) for the whole αP (75,000). The possibility of a monomer–dimer relationship between the two products was suggested (6). Polymerization of αP has been also shown in a preliminary study by Sanbar and Alaupovic (7).

Subunit formation and polymerization phenomena have now been described for many globular proteins (8) and search into their mechanism of occurrence has led to improved understanding of protein structure. In the light of these recent developments, further studies on HDL protein were undertaken in an attempt to define in detail the dependence of its chemical, physical, and immunological properties on the nature of the solvent medium. A preliminary account of the immunological findings has been given elsewhere (9).

MATERIAL AND METHODS

Source of Serum

Blood obtained from healthy fasting (18 hr) white male

Abbreviations: HDL, high density lipoproteins of d 1.063–1.21; HDL_2, subclass of HDL of d 1.063–1.120; HDL_3, subclass of HDL of d 1.120–1.121; LDL, low density lipoproteins; αP, αP_2, and αP_3, partially delipidated products of HDL, HDL_2, and HDL_3; SDS, sodium dodecylsulfate.

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donors, 20–30 years of age, group A, Rh-positive, was collected in 50-ml lusteroid tubes and allowed to clot at 26°C for approximately 1 hr. Serum was separated by centrifugation in a Servall RC-2 Superspeed refrigerated centrifuge (SS-34 rotor) at 12,000 × *g* for 30 min. One milliliter of 1% EDTA (disodium salt) solution neutralized to pH 7 with 0.1 N NaOH was added to each 100 ml of serum. Separation of lipoproteins was begun on the day of blood collection.

Preparation of Lipoproteins

After the removal of chylomicrons (9800 × *g*, 10 min, 16°C), low density lipoproteins (LDL) were separated by ultracentrifuging the serum, brought to d 1.063 g/ml with solid NaCl (densities were checked by pycnometry at 26°C) in a Spinco Model L ultracentrifuge, with the use of a 30.2 rotor at 79,488 × *g* for 24 hr at 16°C. The tubes were sectioned in the middle clear zone and the bottom fractions pooled. From them, either whole high density lipoprotein of d 1.063–1.21 (HDL) or its subfractions of d 1.063–1.120 (HDL_2) and d 1.120–1.21 (HDL_3) were obtained by ultracentrifugation (40.3 rotor, 114,480 × *g*, 16°C) after suitable adjustment of densities by addition of solid NaBr. To remove serum protein contaminants—predominantly albumin and α_1-globulin—HDL, HDL_2, and HDL_3 were diluted 1:3 with NaCl–NaBr solution of d 1.21 and recentrifuged twice for 24 hr in a 40.3 rotor at 114,480 × *g* at 16°C. A second washing was found necessary to remove the trace contamination by albumin indicated by immunoelectrophoretic studies. Preparations of LDL were recentrifuged twice at d 1.063 for 24 hr. The pure fractions were combined and dialyzed at 4°C against several changes of either 0.15 M NaCl containing 0.05% EDTA adjusted to pH 7.0 with 0.1 N NaOH or with buffer solutions, as reported in the Results section. Specimens were stored at 4°C. If storage was to be for longer than 24 hr, HDL or LDL was passed through sterile Millipore filters contained in Swinny hypodermic adapters (Millipore Co., Bedford, Mass.).

Delipidation of HDL, HDL_2, and HDL_3

Details of the procedure, a modification of that described by Scanu et al. (1), are shown in Fig. 1. The solvents were cooled at 10°C before use. The extraction was conducted in a Multi-Purpose Rotator, Model 150 V (Scientific Industries, Inc., Springfield, Mass.) kept in a freezer at −10°C.

Presence of a protein component in the organic solvent phase was detected by the casual observation that ethanol–diethyl ether extracts and diethyl ether washes, clear when kept separate, became turbid upon mixing (see Fig. 1). After 24 hr standing at 4°C a fine, white precipitate could be separated from the clear supernatant fraction by centrifugation. The precipitate was dried under N_2 and stored at 4°C until use. More studies on this phenomenon are reported in the Results section.

Analytical Ultracentrifugation

Sedimentation or flotation analyses were conducted in a Model E Spinco analytical ultracentrifuge using an An-D rotor and a 12 mm single sector cell. Runs were made at 52,640 rpm at 20°C with schlieren patterns photographed at 16-min intervals with a bar angle of

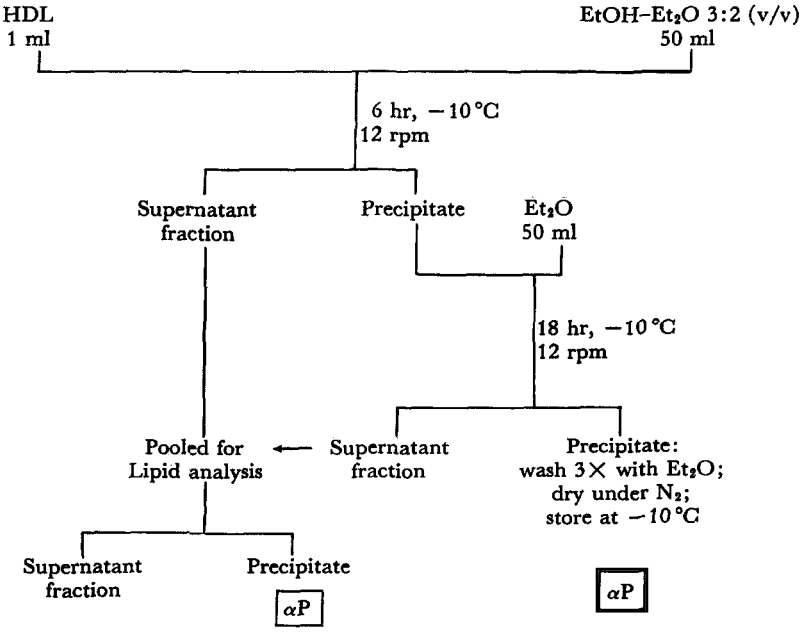

FIG. 1. Scheme of delipidation of HDL.

65°. Flotation (10) and sedimentation constants (11) were calculated from schlieren patterns, magnified by a Nikon Shadowgraph Model 6C optical comparator (Nikon Co., Japan). Appropriate corrections were made for solvent viscosity, density, and protein concentrations.

The Archibald method (12) of approach to sedimentation equilibrium as outlined by Schachman (11) was used for molecular weight determinations. Runs were conducted in 12 mm synthetic boundary cells, 12,480 rpm, 20°C, bar angle 65° for periods usually no longer than 2 hr. The partial specific volume of αP, 0.723, was calculated from its amino acid content (13). Concentrations at the meniscus were used for computation. Average molecular weights were calculated according to Yphantis (14) by extrapolating to zero time a plot of molecular weights versus the square root of time of centrifugation.

Starch Gel Electrophoresis

The vertical technique of Smithies (15) with a commercial apparatus (Buchler Instruments Inc., Fort Lee, N. J.) was used. Tris–boric acid buffer of pH 8.6 (0.9 M Tris, 0.5 M boric acid stock solution, diluted 1:20 prior to use) was the buffer for the starch gel block and Tris–boric acid pH 8.6 (0.54 M Tris, 0.3 M H_3BO_3, final dilution 1:4) was the buffer on the electrode vessels [Tris = tris (hydroxymethyl) amino methane]. Electrophoresis was conducted with a potential gradient of 7.3 v/cm, and a constant current of 14 ma for 18–20 hr at 4°C. The block was sliced with a commercial gel slicer (Buchler Instruments Inc., Fort Lee, N. J.). Staining procedures for protein (Amido Schwartz) and lipids (Oil Red O, both dyes from Allied Chemical Co., New York) have been previously described (2) with the exception that the excess of dye in the protein-stained patterns was removed by a commercial electrical destainer (Otto Hiller, Madison, Wis.).

Immunization Program and Immunochemical Techniques

Male albino rabbits, 2–3 kg in weight, were allowed to acclimatize in the laboratory kennels before use. They were injected intramuscularly at weekly intervals alternately in the right and left legs, with 6–8 mg of antigen suspended in 0.2 ml of Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.). Four injections were given. One week after the last injection the animals were bled by heart puncture under light ether anesthesia. The antigens were sterilized by passage through Millipore filters in Swinny hypodermic adapters. Sixty animals were injected: 24 with HDL, 12 with αP, 12 with LDL, and 12 with whole serum.

To determine the amount of antibody in each antisera, the quantitative immunoprecipitin technique of Kabat and Meyer (16) was used, in which the antiserum is challenged with various dilutions of its homologous antigen. For protein determination, the precipitate was dissolved in carbonate buffer, pH 10. A maximum peak of precipitation was noted at equivalence, with minima in the regions of antibody or antigen excess. Antibody titers were determined by the dilution method (16) using as an endpoint the highest dilution of serum at which a precipitin reaction was noted after 48 hr at 4°C. In each series, the first tube contained 1 ml of undiluted antiserum and 4 mg of antigen. Only antisera with titers above 1:100,000 or having an antibody protein content of 3–4 mg/ml were used for study. They were encountered in 4–5 of each 12 immunized animals. As estimated from the quantitative precipitin curve and the dilution technique, the potency of anti-HDL, anti-αP, and anti-whole serum, when challenged against constant amounts of either HDL or αP, was in the following order: anti-HDL > anti-αP > anti-whole serum.

Immunoprecipitin reactions according to Ouchterlony (17) and agar gel immunoelectrophoresis (18) were conducted on 3 × 7 cm microslides covered by a 1 mm layer of 1.5% prepurified agar gel (Bacto-Agar, Difco Laboratories) in Veronal buffer pH 8.6, ionic strength 0.04. Wells of constant diameter and distance were made by special gel cutters (Gel punch set 6807 A, Die 6866 A, LKB-Products AB, Stockholm, Sweden). Agar electrophoresis was conducted in an LKB Immunophor apparatus using Veronal buffer, pH 8.6, ionic strength 0.04, with a potential gradient of 6 v/cm and a constant current of 40 ma. The time of a run in an air conditioned room (25°C) was 1.75–2 hr, depending upon the specimen analyzed.

Antigens, with the exception of whole serum which was kept undiluted, were studied in solutions of an average concentration of 4 mg/ml of protein in either 0.15 M NaCl or Tris buffer pH 8.6, ionic strength 0.1. Antisera were studied undiluted. A 12–14 hr period was allowed for diffusion of antigens and antibodies in the agar microplates at 4°C in humid chambers. This time was chosen after it was established that no further resolution or additional arcs of precipitation were obtained by prolonging the time of diffusion.

Initially, the arcs of precipitation on wet plates were photographed according to Reed (19). Photography of stained patterns was later found to be more satisfactory and was used almost exclusively. For this technique, the unreacted antigen and antibodies were first removed by several washings with 0.15 M NaCl and a final one with distilled water, the plates were dried at ambient temperature and then stained for protein with Amido Schwartz (20) or for lipids with Oil Red O (saturated solution in 60% ethanol; staining time 12 hr at 37°C; rinsing solution, methanol–acetic acid–water 50:5:50). An MP-3 Polaroid Camera (Cambridge, Mass.) was used for photographic recording. Identical staining pro-

cedures were used for the detection of electrophoretic bands.

Starch gel immunoelectrophoresis was conducted according to the method of Poulik (21) as modified by Korngold (22). Time of diffusion of the protein from starch into agar gel was about 3 days. The arcs of precipitation were stained as for agar immunoelectrophoresis.

Characterization of Antisera

The reactivity of each antiserum against various antigens was studied by immunodiffusion techniques in agar gel (Ouchterlony and immunoelectrophoresis). The sera were allowed to react against various dilutions of the antigen. A summary of the results is presented in Table 1. It can be noted that all anti-HDL or anti-LDL sera had similar behavior and reacted only with their homologous antigen. The reactions between anti-HDL and anti-αP were reported elsewhere(9). The patterns showed two arcs of precipitation, giving a reaction of partial identity, which were interpreted to represent a lipid-rich and a lipid-poor form of HDL.

Amino Acid Analysis

Conditions of hydrolysis were essentially those described by Moore and Stein (23). In the initial phase of the experimentation specimens were hydrolyzed for 18, 24, 48, and 72 hr to estimate by extrapolation to zero time amino acid losses due to hydrolysis. These were of the order reported by Moore and Stein (23), namely 5% for threonine and tyrosine and about 10% for serine.

The amino acids were separated by ion-exchange chromatography on a single column packed with Dowex 50-X8 (Chromo-beads type A, Technicon, Chauncey, N. Y.) in a Technicon automatic amino acid analyzer, according to Piez and Morris (24). Cysteine and cystine were determined as cysteic acid after performic acid oxidation according to Moore (25). In the computation a 5% correction factor was applied (26). Tryptophan, destroyed by acid hydrolysis, was determined spectrophotometrically according to Goodwin and Morton (27). Amide ammonia was determined by the method of Laki et al. (28) modified by Hirs, Stein, and Moore (29).

Lipid Analysis

Lipoproteins were extracted with chloroform–methanol 2:1 (v:v) and the extracts chromatographed on silicic acid (Unisil 100–200 mesh, Clarkson Chemical Co., Williamsport, Pa.) columns (1 × 14 cm). The chloroform eluates were used for the determination of total cholesterol (30), free cholesterol (31), and triglycerides (32). Phospholipids were eluted with absolute methanol. The factor 25 was used to convert lipid P (33) to phospholipid. The various phospholipid fractions were separated by thin-layer chromatography. A 0.5 mm layer of Silica Gel H (Brinkman Instruments Co., Westbury, N.Y.) was spread on 200 × 200 mm glass plates by means of a Desaga applicator (Brinkman Co.). Before use, the plates were activated at 110°C for 1 hr. Ascending chromatograms were developed with chloroform–methanol–water 80:35:5 and the plates were dried at room temperature. For quantitative analysis, the spots were detected by iodine vapor (34) and the gel powder scraped off the glass was aspirated into a special sintered glass funnel (Kopp Laboratory Supplies, New York, N. Y.). Phospholipids were eluted with absolute methanol and transferred to a volumetric flask for analysis.

TABLE 1 CHARACTERIZATION OF ANTISERA

Animal	Antigen	Reactivity				
		LDL	HDL	Albumin	Other Proteins*	Whole Serum
Rabbit No.						
R 2, 3, 7, 9	HDL (d 1.063–1.21)	−	+	−	−	+
R 41, 44, 47, 50, 52	"	−	+	−	−	+
R 15, 17, 19, 22, 23	αP (delipidated HDL)	−	+	−	−	+
R 26, 27, 30, 31, 34	LDL (d < 1.063)	+	−	−	−	+
R_{Hy}†	Human serum albumin	−	−	+	+	+
R_{Sy}‡	Human serum orosomucoid	−	−	−	+	+
Goat‡						
G^{I}_{Sy}	Human serum 7S and β-macroglobulin	−	−	−	+	+
G^{II}_{Sy}	Human serum albumin–glycoprotein	−	−	+	+	+
G^{III}_{Sy}	Human serum transferrin	−	−	−	−	+
G^{IV}_{Sy}	Human serum ceruloplasmin	−	−	−	+	+
Horse						
H§	Whole human serum	+	+	+	+	+

* Fractions V and VI (Calbiochem., Los Angeles, Calif.).
† Hyland Laboratories, Los Angeles, Calif.
‡ Antisera obtained from Sylvana Co., through Fisher Chemical Co., Chicago, Ill.
§ Lot No. 306, Pasteur Institute, Paris.

Phospholipid fractions were detected by the molybdenum blue spray of Dittmer and Lester (35). Identification of the various phospholipid fractions was based on the R_f values of purified standards (Applied Science Laboratories, State College, Pa.).

Carbohydrate Analysis

For analysis of neutral sugars, αP was hydrolyzed with 1 N HCl for periods of 3–6 hr at 100°C and the sugar released was monitored by the method of Park and Johnson (36). Optimum time of hydrolysis was 4 hr. For quantitative analysis, the 4-hr hydrolysates were brought to pH 6 with sodium carbonate and centrifuged, and the supernatant fraction was passed through a Dowex X_2 (200–400 mesh) column prepared according to Whitehouse and Zilliken (37). In the water eluates neutral sugars were determined by the anthrone reaction according to Roe (38), with a D-galactose–D-mannose mixture (Nutritional Biochemicals Corporation, Cleveland, Ohio) as a standard. Methyl pentose was determined by the method of Dische and Shettles (39) with fucose (Nutritional Biochemicals Corporation) as a standard. Separation of the reducing sugars was accomplished by ascending thin-layer chromatography on Kieselguhr G (E. Merck, Darmstadt, Germany) according to Stahl and Kaltenbach (40) with ethyl acetate–isopropanol–water 65:23.4:11.6 as the developing solvent. The sugars were detected by a 9:0.5:0.5 mixture of 95% ethanol–H_2SO_4–anisaldehyde (Eastman Organic Chemicals, Rochester, N. Y.). Hexosamine was determined in αP hydrolysates (2 N HCl, 6 hr, 80°C) by the method of Boas (41). Amino sugars were identified by chromatography on Dowex 50-X8 columns in a Technicon automatic amino acid analyzer, with glucosamine, galactosamine, and mannosamine (Nutritional Biochemical Corporation) as standards. Losses due to hydrolysis were estimated from the analyses of 2, 4, 6, and 8-hr hydrolysates.

Sialic acid was determined by the resorcinol method of Svennerholm (42) in αP hydrolysates (2 N HCl, 6 hr, 100°C) with *N*-acetylneuraminic acid (CalBioChem. Corp., Los Angeles, Calif.) as a standard.

Total protein determinations were made according to the Lowry method (43). In the acid hydrolysate of αP, microdetermination of Kjeldahl nitrogen was performed according to Lang (44).

Chemicals were reagent grade. Salts used in the ultracentrifugal procedures (NaCl and NaBr) were kept in a desiccator at room temperature. All organic solvents were freshly distilled. Urea and sodium dodecyl sulfate (SDS) were recrystallized from ethanol before use.

TABLE 2 CHEMICAL COMPOSITION OF HDL AND αP

	HDL	αP
	mg	*mg*
Protein	*100.00*	*100.00*
Lipid		
Free cholesterol	6.00	
Cholesterol esters	28.00	
Phosphatidyl choline	30.10	0.60
Phosphatidyl ethanolamine + Phosphatidyl serine	2.51	
Sphingomyelin	5.40	0.31
Lysolecithin	4.80	
Glycerides	16.00	
Carbohydrate		
Glucosamine		1.51
Methylpentose (as L-fucose)		0.60
D-Galactose + D-Mannose		0.85
Sialic acid		0.48

RESULTS

Delipidation Technique

Protein recoveries were 95–98%. Losses could be partially accounted for by the presence in the lipid extracts of a protein (see below) which precipitated when ethanol–ether and ether phases were pooled (Fig. 1). The precipitate occurred when HDL, prior to delipidation, was dialyzed against NaCl, 0.15 M, pH 7 (or buffers of the same hydrogen ion concentration) but not when the HDl was dialyzed against an alkaline buffer (i.e., Tris buffer, pH 8.6, ionic strength 0.1). In the latter case, a precipitate could be produced by adding NaCl 0.15 M, pH 7 (or buffer solutions of the same pH), in the ratio of 1 ml/50 ml of lipid extract. The precipitate was completely soluble in buffers of pH 7.5–11.0, gave a maximum UV absorbance at 280 mμ, was not dialyzable, and had electrophoretic and immunochemical properties of αP. Lipid analysis, conducted after passage of the protein through a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (1 × 25 cm) to eliminate the possible presence of unbound lipids, revealed the presence of phospholipids, 1–2 mg/100 mg of protein. It was estimated that during each extraction procedure about 0.5% of the HDL protein was recovered in the organic solvent phase.

Chemical Analysis of HDL and αP (Table 2)

Delipidation removed essentially all cholesterol and glycerides and left about 3 mg of phospholipid per 100 mg of protein. Thin-layer chromatography identified phosphatidyl choline as the major phospholipid component. Attempts were made to prepare αP completely free from lipids by increasing the time of delipidation up to 48 hr, changing the ratio of the solvent mixture, or raising the temperature to 4°C. The phospholipid content of αP remained within the range reported in Table 2. Also shown in Table 2 are the results of the carbo-

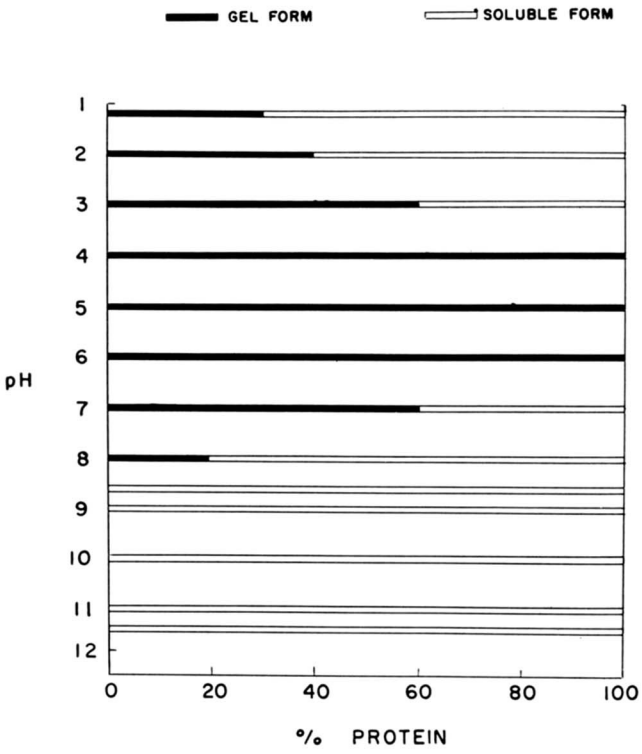

FIG. 2. Dependence of solubility of αP on pH. The buffers employed in these studies were pH 1.2 (KCl–HCl); 2.0 and 3.0 (glycine–HCl); 4.0, 5.0, and 5.8 (acetic acid–acetate); 7.0, 7.5, 8.0, and 8.6 (Tris); 9.8 and 11.4 (carbonate–bicarbonate). All buffers had an ionic strength of 0.1. Experiments were conducted at 25°C. The amount of αP in each tube was that obtained from the delipidation of HDL containing 10 mg of protein. Determination of the protein content in the soluble and gel portion (upon solubilization with 6 M urea) of αP was by the Lowry method (43).

hydrate analysis. Glucosamine was the major component. Quantitative, individual determination of D-galactose and D-mannose was not attempted. An equal distribution of these two neutral sugars could be estimated by the intensity of the spots on the thin-layer chromatogram.

The data on the amino acid analysis were in close agreement with those by Shore and Shore (6) and Levy and Fredrickson (45). The values for tryptophan and amide N, not reported previously, were 23.4 ± 1.7 and 105.2 ± 4.4 per 100,000 g protein.

Solubility of αP

Solubility tests were conducted at room temperature (25°C) using αP aliquots of about 10 mg, as estimated from the HDL protein content before delipidation. One milliliter of buffer was added to the dry protein and the mixture gently shaken by hand for a few seconds approximately every 10 min. Maximum solubility, whether total or partial, was obtained within 50–60 min. Similar results were obtained with larger volumes (2 ml or more) of buffer. No changes in solubility were noted when αP solutions at 25°C were stored at 4°C.

The solubility of αP was pH-dependent (Fig. 2). The protein gelled completely between pH 4 and 6, whereas it was totally soluble at pH above 8.6. At pH 1.2, 2, 3, 7, and 8 only partial solubility was observed. The solubility of HDL was not affected by pH changes nor was its behavior in the analytical ultracentrifuge (Fig. 3). The soluble portion of αP gave a single peak at pH 7 with an $S^{\circ}_{20,w}$ of 4.2. Between pH 1 and 3 and between 7.5 and 10.4 a light and a heavy component were noted, the latter having a peak area about one-third of the former. The heavy component showed a steep decrease in S values as the pH of the medium was either increased or decreased (Fig. 4). At pH 11.4 (carbonate buffer) a peak that was apparently symmetrical during the first 72 min was resolved into two components ($S_{20,w}$ 2.0 and 2.6) after 2 hr of ultracentrifugation. At this time, a third component was occasionally noted.

Increase in ionic strength (0.05 to 0.2, in stages) of a Tris buffer at pH 8.6 led to a decrease in the percentage of the heavy component. Addition of urea (4–6 M) or

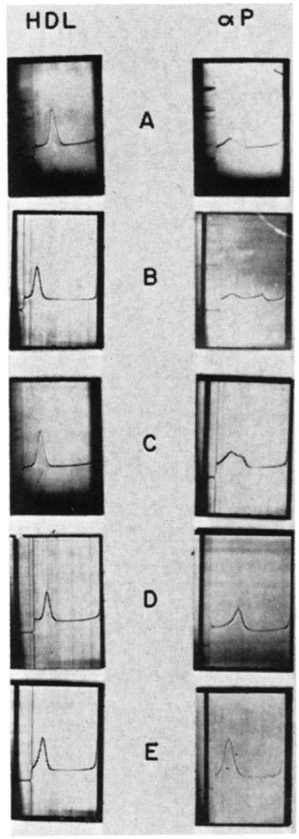

FIG. 3. Behavior in the analytical ultracentrifuge of HDL and αP in various solvent media of ionic strength 0.1. *A*, NaCl, 0.15 M, pH 7.0; *B*, Tris, 0.1 M, pH 8.6; *C*, Na carbonate, 0.1 M, pH 10.6; *D*, Tris, 0.1 M, pH 8.6 and urea 6 M; *E*, Tris, 0.1 M, pH 8.6 and SDS, 0.05 M. The protein was extensively dialyzed against each buffer before analysis. Speed 52,640 rpm, 20°C, bar angle 65°. Schlieren patterns were taken 64 min after rotor reached full speed. Protein content: 4–5 mg/ml.

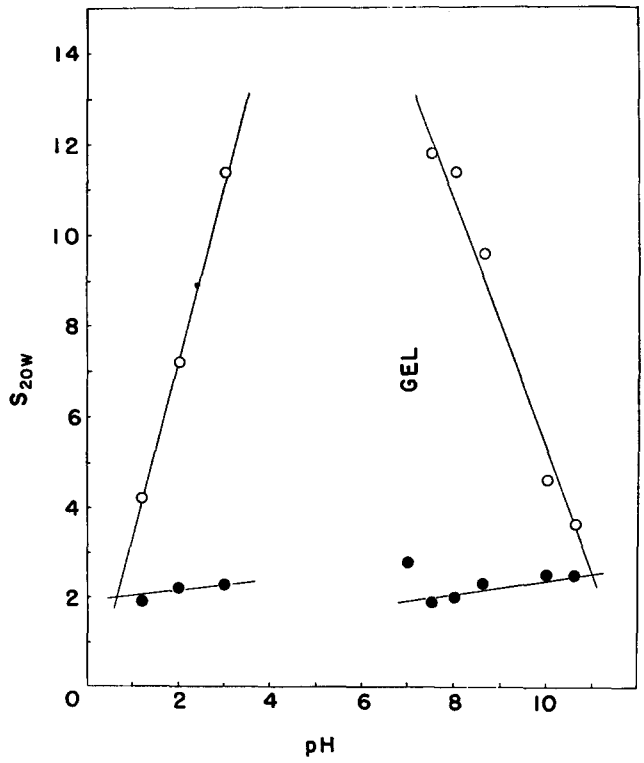

FIG. 4. Variation of S values of the slow and the fast components of αP as a function of pH changes of solvent medium. Protein concentration: 4–5 mg/ml. ●, heavy component; ○, light component.

SDS (0.02–0.05 M) to buffers of pH 1.2–8.0 led to prompt solubilization of the gelled portion of αP. In turn, formation of the gel was prevented by urea or SDS dissolved in the buffer prior to addition of the dry αP. In the presence of either of the two hydrogen bond-breaking agents (regardless of the pH of the buffer) αP gave a single peak (Fig. 3) in the analytical ultracentrifuge with $S°_{20,w}$ values of 2.0 and 3.4 for SDS and urea respectively.

The values of molecular weight of αP, obtained by the approach to sedimentation equilibrium method, are reported in Table 3. In the SDS experiments, calculations were made with the assumption that all of the detergent was bound to the protein (46). No corrections were made for the acetylated (49) product. Calculation of the minimum molecular weight of αP from its amino acid and carbohydrate composition gave a figure of 23,710.

Electrophoretic and Immunological Studies

By *agar electrophoresis* αP showed a single band, close to the origin, which stained only for protein (Fig. 5). HDL exhibited a major, fast band detected by protein and lipid stains and a minor one (3–5% of the major band by densitometric estimation) close to the origin, detected clearly by protein stain and poorly by lipid stain.

TABLE 3 MOLECULAR WEIGHTS OF αP BY THE SEDIMENTATION EQUILIBRIUM METHOD

	Solvent	$S°_{20,w}$	Molecular Weight
αP_2	NaCl, 0.15 M, pH 7	3.80	41,500 ± 2000*
αP_2–SDS†	Tris‡	1.96	22,300 ± 1500
αP_2-acetyl§	Tris‡	2.10	22,800 ± 2200
αP_3	NaCl, 0.15 M, pH 7	3.90	42,600 ± 2500
αP_3–SDS†	Tris‡	2.00	23,000 ± 2400
αP_3-acetyl§	Tris‡	2.10	22,000 ± 2000

* Average of 4 determinations ± SEM.

† αP was dissolved in Tris buffer pH 8.6, ionic strength 0.1, made 0.05 M with respect to sodium dodecyl sulfate. The solution was dialyzed against several changes of Tris buffer to remove unbound SDS. SDS content of αP (100 mg/g protein) was determined according to the colorimetric method of Karush and Sonenberg (47) and by sulfate analysis after combustion of αP-SDS complex (48). αP_2 and αP_3 are the delipidated products of HDL_2 and HDL_3.

‡ Tris buffer, pH 8.6, ionic strength 0.1.

§ Acetylation was carried out according to Fraenkel-Conrat (49).

To determine the chemical composition of each of the electrophoretic components, sections of agar from several electrophoretic runs, containing either the fast or the slow band (stained patterns were used as markers) were separately pooled. Gel blanks served as a control. In acid-digests of the gels, proteins were determined according to Lang (44). Cholesterol (20), phospholipids (33), and triglycerides (32) were determined in 2:1 chloroform–methanol extracts of agar gel homogenates. In agreement with the densitometric data, the protein content of the minor band was about 3% of the major one. The percentage protein:phospholipid:cholesterol:glyceride distribution was as follows: major band, 55:23:17:5; minor band, 85:15:0:0.

By agar immunoelectrophoresis, the reaction between anti-HDL or anti-αP sera and HDL resulted in one heavy and one or two light arcs of precipitation, which stained for protein and had a reaction of partial identity. Dilution of the antigens led to disappearance of these minor components. The lipid stain detected clearly only the major arc; the minor ones were seen faintly. One major and two minor arcs of precipitation were also seen with αP. These arcs remained near the origin and were stained only for protein. The double diffusion studies by the Ouchterlony technique (bottom of Fig. 5) supported the immunoelectrophoretic findings. Anti-HDL or anti-αP sera absorbed with either αP or HDL failed to react with HDL or αP respectively.

The electrophoretic and immunodiffusion patterns (anti-HDL or anti-αP sera) did not change significantly when HDL or αP were, before analysis, dialyzed or dissolved in buffer solutions of different pH (1–4 and 6–11) and ionic strength (0.5–2) containing 4–6 M urea. Electrophoresis was conducted in Veronal buffer, pH 8.6. This may have favored aggregation of αP. Specimens

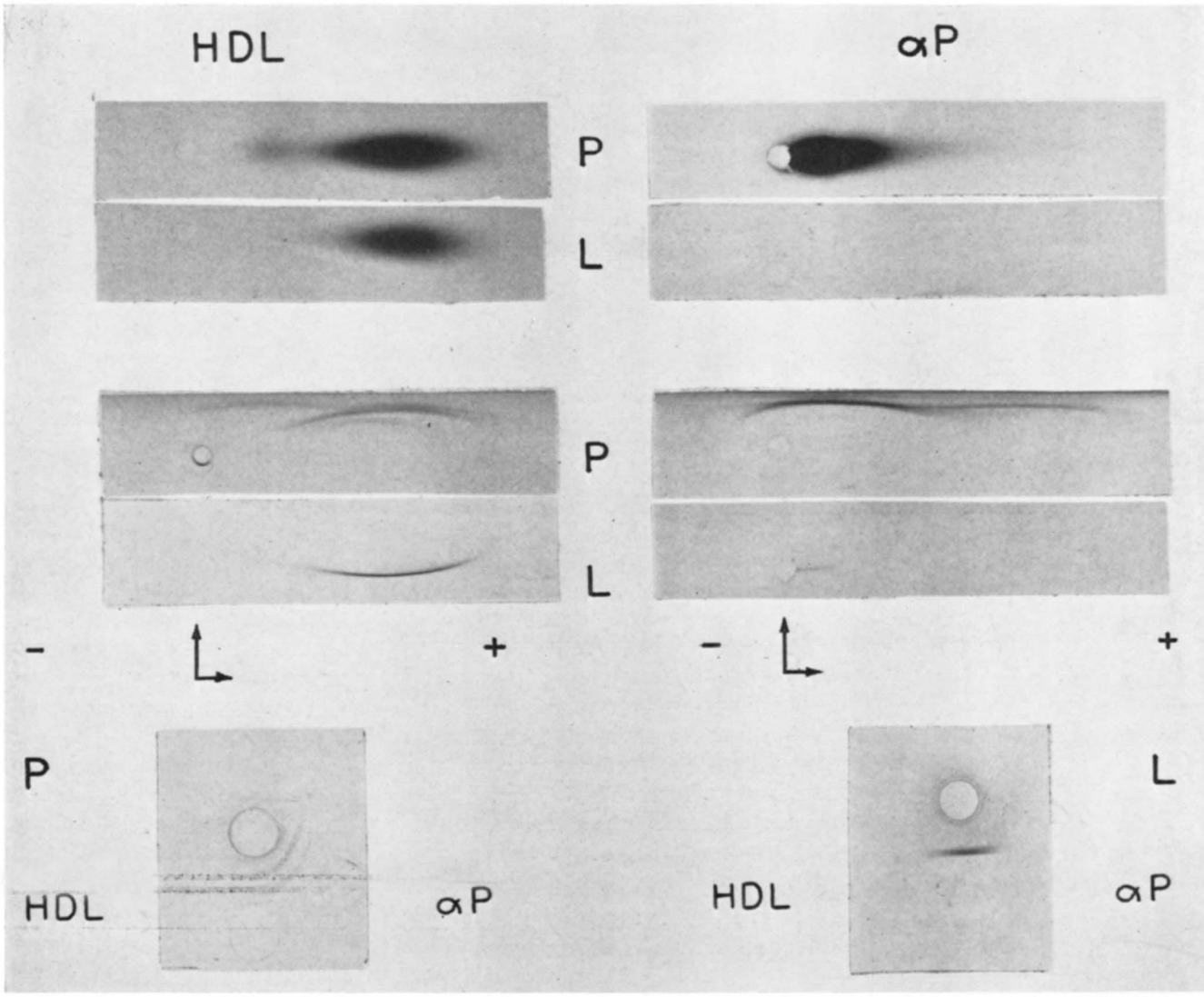

FIG. 5. Agar electrophoresis and immunodiffusion patterns of HDL and αP. Agar electrophoresis: Veronal buffer pH 8.6, 6 v/cm, 40 ma, 20°C. Antibody (anti-HDL, R 3, see Table 1): antigen protein ratio, 10:1. Time of diffusion 16 hr, 4°C. Staining: Amido Schwartz (*P*), Oil Red O (*L*).

dissolved in SDS solutions showed a marked increase of the rate of anodic migration. The immunological pattern varied according to the concentration in SDS of the buffer employed. In Tris pH 8.6–0.05 M SDS, two arcs of precipitation having partial identity were observed. A single arc was noted in Tris–0.5 M SDS. All acetylated specimens of αP were antigenically homogeneous.

The nature of the buffer against which either HDL or αP was dialyzed before analysis markedly influenced the results by *starch gel electrophoresis* (Fig. 6). The best resolution was obtained with either Tris or glycine buffer pH 8.6, ionic strength 0.1. With HDL, five bands, provisionally named *a*, *b*, *c*, *d*, and *e* were noted. All stained for protein and lipid. The bands of αP, lipid-fast were well defined. Two faint bands not detected in the native product were noted in the pre-albumin area.

By *starch gel immunoelectrophoresis* the reactions between HDL and anti-HDL, HDL and anti-αP, αP and anti-αP, and anti-HDL elicited a single, prolonged arc of precipitation (Fig. 7). The minor arcs, noted in agar gel, were not observed even with high concentrations of antigen. No reaction was obtained with either anti-HDL or anti-albumin sera. Using a stained pattern as a marker, sections of unstained gel containing the various fractions of either HDL or αP were cut and placed in an agar gel medium as shown schematically in Fig. 7. By the Ouchterlony technique, a reaction of identity was elicited using either anti-HDL or anti-αP area.

Studies on HDL_2 and HDL_3 and delipidated products αP_2 and αP_3. Comparative studies of these two HDL subclasses were conducted before and after delipidation with ethanol–diethyl ether. Before delipidation they differed in pro-

tein:lipid ratio, with an average percentage lipid content of 60 and 40 for HDL_2 and HDL_3, respectively. By agar electrophoresis and immunodiffusion techniques (Fig. 8) HDL_2 exhibited a single, lipid-rich component in contrast to HDL_3, where an additional lipid-poor fraction was noted. Mixture of HDL_2 and αP gave immunoelectrophoretic patterns of HDL_3. Anti-HDL or anti-αP_2 sera absorbed with either HDL_2 or αP_2 failed to react with either HDL_3 or αP_3. Similarly, following absorption with αP_3 or HDL_3, they showed no reaction with either HDL_2 or αP_2. On starch gel electrophoresis (Fig. 6) HDL_3 behaved similarly to HDL. HDL_2 migrated slightly less than HDL_3; bands *a*, *b*, and *c* were less well

lipid-free protein (αP) can be obtained from human serum high density lipoprotein by extraction with cold ethanol–ether. Recoveries (1, 6) and solubility properties (1, 50, 6) of the delipidated products varied in each study, probably because of differences in the extraction procedure employed. The technique used in the present investigation gave yields of αP significantly greater than those previously reported by Scanu et al. (1). Further, it provided evidence that a small portion of the protein is lost in the organic solvent phase, from which it may be precipitated by suitably changing the pH.

The presence of a carbohydrate moiety in αP has been reported previously (1, 51, 52), but the moiety has been

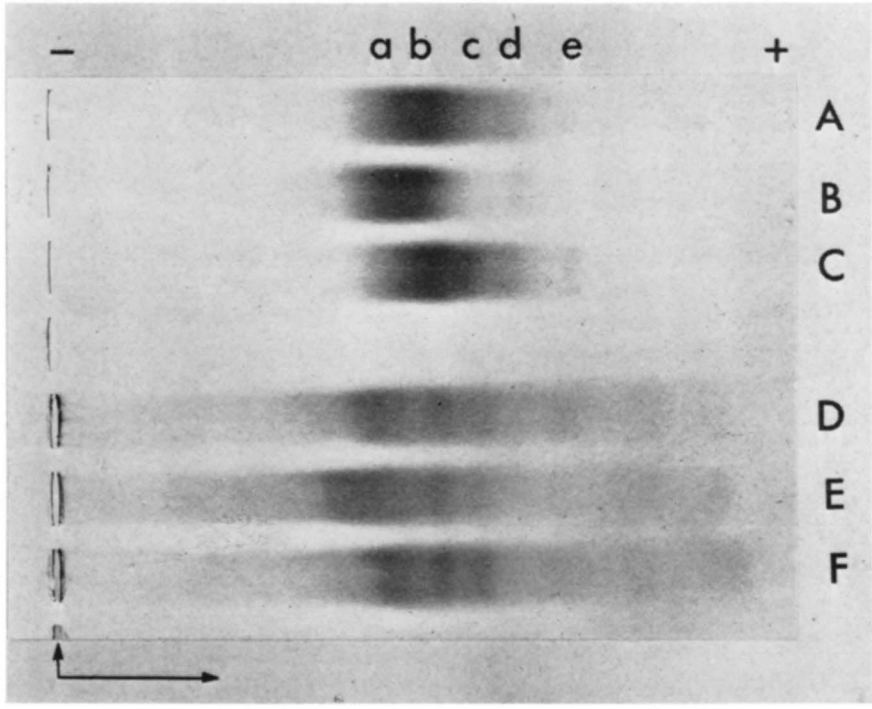

FIG. 6. Vertical starch electrophoresis of HDL and subclasses before and after delipidation. *A*, HDL; *B*, HDL_2; *C*, HDL_3; *D*, αP; *E*, αP_2; *F*, αP_3. Protein concentration: 4 mg/ml. Conditions of electrophoresis: discontinuous Tris–boric acid buffer pH 8.6, 7.3 v/cm, 14 ma, 20 hr, 4°C. Staining: Amido Schwartz.

defined and band *e* was absent. Upon extraction by ethanol–diethyl ether, the delipidated products, αP_2 and αP_3 were found to be identical with αP, in terms of solubility characteristics; lipid, carbohydrate, and amino acid composition; and immunoelectrophoretic behavior. By starch gel electrophoresis, αP_2 and αP_3 showed the same number of fractions as the parent αP. They were detected only by protein stain.

DISCUSSION

The studies of Scanu et al. (1), Avigan (50), and Shore and Shore (6) have shown that a water-soluble essentially

only partially characterized. The complete analysis furnished here indicates that its sugar composition is qualitatively similar to that reported for human α_1-glycoprotein (53). Since the presence of carbohydrate has also been noted in serum LDL (54), the term lipoglycoprotein (55) may be more appropriate for defining human serum lipoproteins.

Scanu and Hughes (3) have reported the absence of cystine from hydrolysates of human serum αP. With the oxidative procedure used in the present studies, cystine was found to represent about 0.5% of the total amino acid residues. This figure and the other data of the amino acid analysis are in agreement with the findings of

Shore and Shore (6) and Levy and Fredrickson (45), with the exception of tryptophan and amide N, for which data are not available for comparison.

The properties of αP were markedly influenced by the solvent medium. Solubility and ultracentrifugal data indicated that changes in pH or ionic strength of the medium, while ineffective on HDL, cause association–dissociation phenomena of αP. Polymerization was greatest at pH 4–6 at the isoelectric region of αP (51, 52) and was evidenced by the formation of a gel which was dissolved by either urea or SDS. At pH values higher than 7.0 or lower than 4.0 (Fig. 4) more discrete molecular aggregates were noted. These became progressively smaller as the pH shifted toward the alkaline or acid regions. The evidence that the protein of HDL_3 (d 1.125–1.20 g/ml) is made of three chains, each with an approximate molecular weight of 36,500 ± 1000, presumably having one mole of aspartic acid as *N*-terminal residue. In the studies reported here, a 2S subunit of an average molecular weight of 21,000 was obtained by acetylating αP or treating this protein with SDS. Whether this product represents the smallest subunit of αP or is a chemical artifact remains to be established.

HDL and HDL_3 could each be dissociated by agar electrophoresis into a lipid-rich and a lipid-poor component. This interesting finding, which is in agreement with that of Levy and Fredrickson (45), suggests that in ultracentrifugal preparations of HDL, lipid-poor lipoproteins

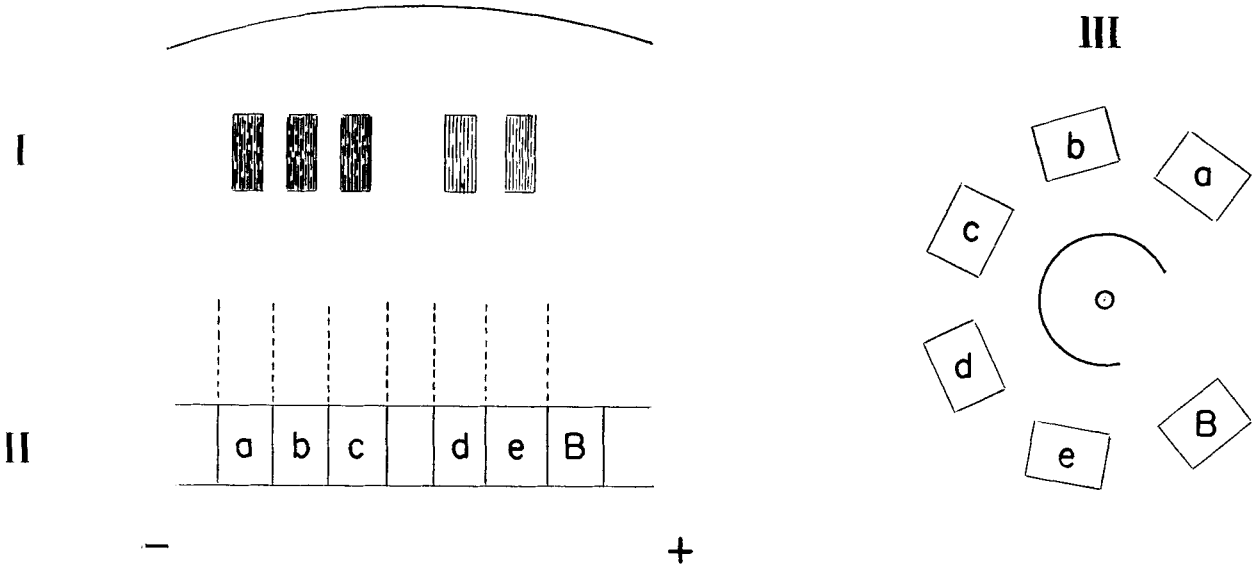

FIG. 7. Schematic representation of a starch gel immunoelectrophoretic analysis of HDL. I. Stained (Amido Schwartz) electrophoretogram and immunoprecipitin pattern (arc above), obtained after the unstained fractions of αP were allowed to diffuse in agar gel medium against anti-HDL serum. II. Segments containing bands *a*, *b*, *c*, *d*, and *e* were separated from a second section of the starch gel using pattern I as a marker. III. The segments were transferred to an agar gel medium and disposed around a central trough containing rabbit anti-HDL. Conditions of electrophoresis: same as in Fig. 6. Time of diffusion: 3 days, 4 °C. Staining of arcs of precipitation: Amido Schwartz.

observed dependence of the state of aggregation of αP on pH and ionic strength of the medium strongly suggests that the forces that cause αP to associate or dissociate are electrostatic. The tendency of HDL protein to aggregate when lipid is removed, earlier recognized by Shore and Shore (6) and Sanbar and Alaupovic (7), may account for the various molecular weights of αP reported (1, 6).

The average weight of the smallest subunit of αP has not been clearly established. In early studies Shore (56) reported that HDL_2 and HDL_3 contain *N*-aspartic acid, *C*-threonine peptide chains of identical amino acid composition and molecular weight—approximately 95,000. Recently, however, Shore and Shore (6) have presented may be held in labile association with lipid-rich species. In agreement with this concept are the data showing that release of lipid-poor protein from HDL is favored by ultracentrifugation (45, 57), freezing and thawing, and treatment with urea (45) or diethyl ether (58).

The presence of two forms of HDL with different lipid complements may account for the antigenic heterogeneity of HDL observed in the present studies and in those of Ayrault-Jarrier, Levy, and Polonovski (59), Burstein and Fine (60), and Levy and Fredrickson (45). The interpretation is supported by the observation that HDL_2, which exhibited a single antigenic component, acquired the immunoelectrophoretic heterogeneity of HDL_3 upon mixing with αP. That the difference in antigenic prop-

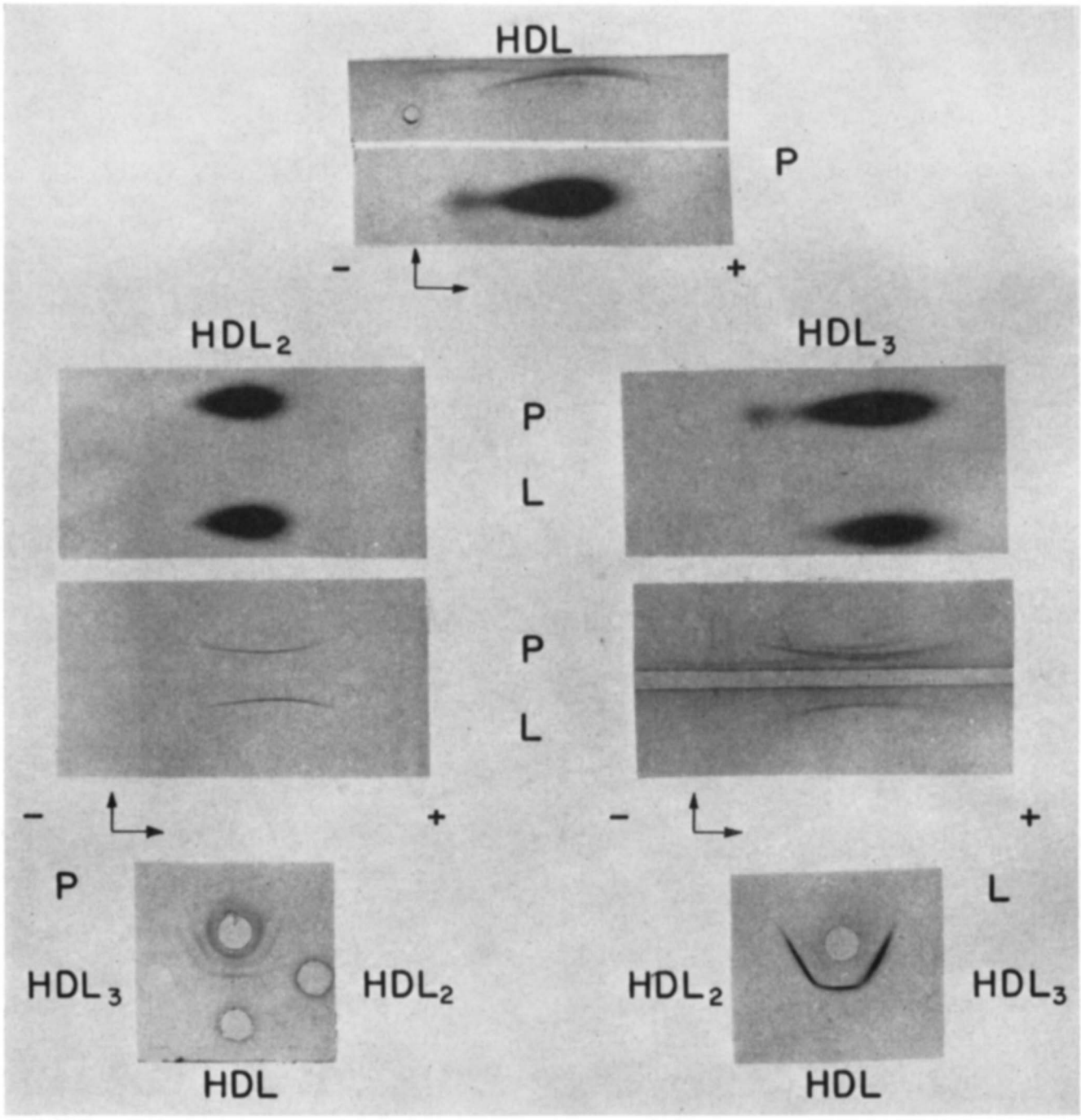

FIG. 8. Agar electrophoresis and immunodiffusion patterns of HDL, HDL_2, and HDL_3. Antiserum: rabbit anti-HDL. For experimental conditions see Fig. 5.

erties of these two forms may not be merely dependent on the amount of lipids they carry is suggested by the present observations and those of Ayrault-Jarrier et al. (59) showing that antigenic heterogeneity of HDL persists upon removal of lipid. Furthermore, HDL_2, which is antigenically homogeneous in its native state, became heterogeneous after delipidation. A possible interpretation for these findings is that the protein subunit of HDL, while undergoing polymerization in vitro as a consequence of removal of lipids, may assume antigenic properties different from its monomeric form. Thus, antigenic heterogeneity of HDL protein may be only apparent and dependent on the extent of its polymerization in solution. This hypothesis is supported by the observation (A. Scanu, unpublished data) that the 2S subunit obtained on a preparative scale from the protein of HDL, HDL_2,

and HDL_3 has a single antigenic determinant. Similarly, a single antigenic determinant was observed in αP treated with 0.5 M SDS or after acetylation. This was also true for the fractions of HDL separated by electrophoresis in starch gel. This medium acting as a molecular sieve may have permitted separation of αP into oligomeric units of similar antigenic reactivity. Studies to test this hypothesis are in progress.

The differences in electrophoretic and immunochemical behavior noted with HDL_2 and HDL_3 were not found for their delipidated products αP_2 and αP_3. These data support the previous conclusion (3) that these proteins have similar properties and carry different complements of lipid.

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