Structural Studies of Rat Plasma Lipoproteins*

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ABSTRACT: The very low density (VLDL) and high density (HDL) lipoproteins from rat plasma were partially and totally delipidated by a *n*-heptane and ethanol–acetone extraction. The partially delipidated lipoproteins contained only phospholipid and protein. HDL, originally sedimenting as a homogeneous boundary with $s_{20,w}$ of 5.8 S and mol wt 246,000, dissociated after partial extraction into two products with sedimentation coefficients of 5.4 and 1.9 S. These products were separated by density gradient centrifugation and only the faster contained bound phospholipids. After ethanol–acetone extraction the lipid-free protein moiety of HDL was quantitatively recovered in a soluble form that sedimented as a single boundary with $s_{20,w}$ of 2.1 S and had mol wt 27,000. No changes in immunochemical properties of HDL were observed after partial or total lipid removal, although modifications were detected in optical rotatory dispersion parameters and in the response of the absorption spectra to pH changes. The properties of the major component isolated by partial or total lipid extraction from VLDL (immunoelectrophoretic behavior, sedimentation pattern, and antigenic determinants) were very similar to those observed for the phospholipid–protein residue and the lipid-free protein residue obtained from HDL. It appears that rat HDL and probably VLDL are formed by association of the small 1.9S protein with different proportions of phospholipids and neutral lipids, and that an appreciable part of the peptide chain is covered by lipids. An apparent relationship between immunochemical determinants in HDL and rat plasma albumin was found, but differences in amino acid composition and molecular size ruled out identity of these proteins.

The voluminous literature dealing with the structure of human plasma lipoproteins has been recently reviewed by Scanu (1965a) and Fredrickson *et al.* (1967). Knowledge of lipoproteins from other species is fragmentary, except for the detailed information concerning the metabolism of plasma lipoproteins using the rat as experimental subject (Marsh and Whereat, 1959; Radding and Steinberg, 1960; Haft *et al.*, 1962; Roheim *et al.*, 1965; Bungenberg and Marsh, 1966). Little is known about the structure of rat plasma lipoproteins beyond their lipid composition (Ugazio and Lombardi, 1965) and partial amino acid analysis (Trams *et al.*, 1966). In this paper are reported studies on some of the physical and chemical properties of intact rat plasma lipoproteins and of the proteins obtained by partial and total removal of the lipid.

Experimental Procedure

Animals. Adult Sprague–Dawley male and female rats (300–400 g) were used. A regular diet was fed of Purina Laboratory Chow, and water *ad libitum*. Blood was collected *between 8 and 10 AM* by cannulation of the abdominal aorta under ether anesthesia. Clotting was prevented with 2 mg of Na_2EDTA/ml of blood. Cells were separated by low-speed centrifugation at 4°, and the clear plasma was collected.

Isolation of Lipoproteins. VLDL,[1] LDL, and HDL were isolated from five pools of 100 ml of rat plasma using a modification of the procedure of Havel *et al.* (1955). Plasma was centrifuged for 24 hr at 105,000*g* at 4° using a Spinco rotor no. 40. The fraction with density less than 1.006 g/ml was collected in the top 1 ml of the tubes, combined, and recentrifuged twice for 24 hr at 105,000*g* at 4°. The fraction in the upper 1 ml of the tubes after the second recentrifugation constituted the purified VLDL. The remaining plasma was centrifuged at 105,000*g* for 24 hr at 4° and the upper 2 ml in each tube was discarded. The infranatant was adjusted to density 1.063 g/ml and centrifuged 24 hr at 105,000*g* at 4°. The upper 2 ml in each tube was collected and recentrifuged twice for 24 hr at 105,000*g*. The top 1 ml in each tube after the last centrifugation contained purified LDL. The infranatant of the plasma solutions with density 1.063 g/ml was centrifuged for 24 hr at 105,000*g* at 4° and the top 2 ml in each tube was discarded. The density of the infranatant solution was raised to 1.210 g/ml and centrifuged for 24 hr at 105,000*g* at 4°. The lipoproteins which collected in the top 1 ml were pooled and recentrifuged twice for 24 hr at 105,000*g*. The top 2 ml in each

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[1] Abbreviations used: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; S_f (1.210), flotation coefficient in a medium with background density of 1.210 g/ml.

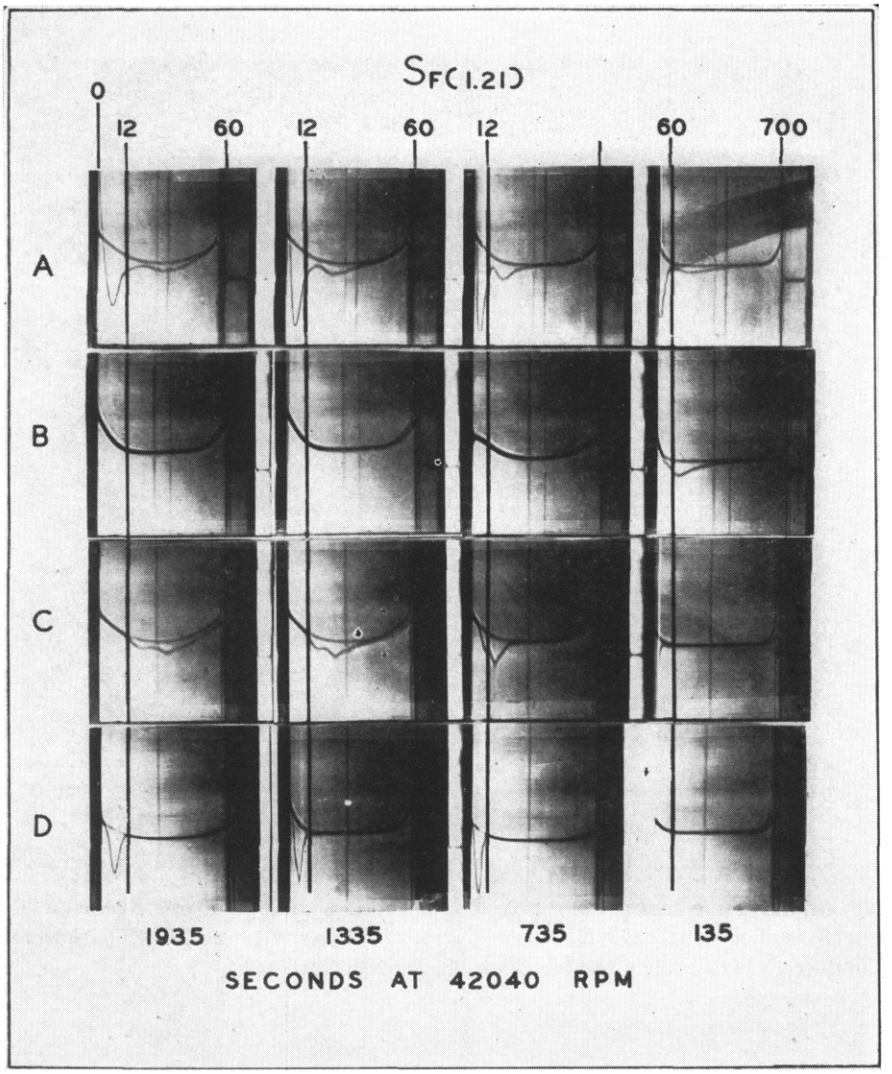

FIGURE 1: Flotation profiles of (A) total rat plasma lipoproteins, (B) purified VLDL, (C) purified LDL, and (D) purified HDL. Time reported as seconds of centrifugation after reaching 42,040 rpm, plus the equivalent up to speed time (135 sec).

tube after the last recentrifugation contained purified HDL.

The plasma proteins with density greater than 1.21 g/ml were used as source of rat plasma albumin. This was prepared according to the method of Schwert (1957).

Analytical Ultracentrifugation. Flotation analysis was carried out in the total lipoprotein fraction with density less than 1.210 g/ml isolated from individual animals (Del Gatto *et al.*, 1959). Analyses of purified lipoprotein fractions were performed after adjusting the density of their solutions to 1.210 g/ml by addition of appropriate volumes of aqueous NaBr, density 1.416 g/ml.

Boundary sedimentation experiments were effected at 59,780 or 56,100 rpm in a Spinco Model E ultracentrifuge. Rotor temperature was maintained at 25 ± 0.2°. Samples to be analyzed were dissolved in 0.17 M NaCl (pH 7.6) and density 1.006 g/ml. Sedimentation coefficients were calculated for these conditions. Corrections were applied to obtain $s_{20,w}$ values when the partial specific volumes were known. Molecular weights were measured by approach to sedimentation equilibrium, according to procedures described by Schachman (1957). The apparent partial specific volume of HDL was evaluated by pycnometry and for the HDL protein residue from the amino acid composition (McMeekin and Marshal, 1952). Solutions used for molecular weight determinations contained 5 mg/ml of protein dissolved in 0.17 M NaCl adjusted to pH 7.6 or 8.6.

Density gradient centrifugations were performed using the procedure suggested by Charlwood (1963). Sucrose (20–10%, w/v) was used in the preformed density gradient experiments. Approximately 5 mg of lipoprotein dissolved in 0.17 M NaCl (*d* 1.006) was

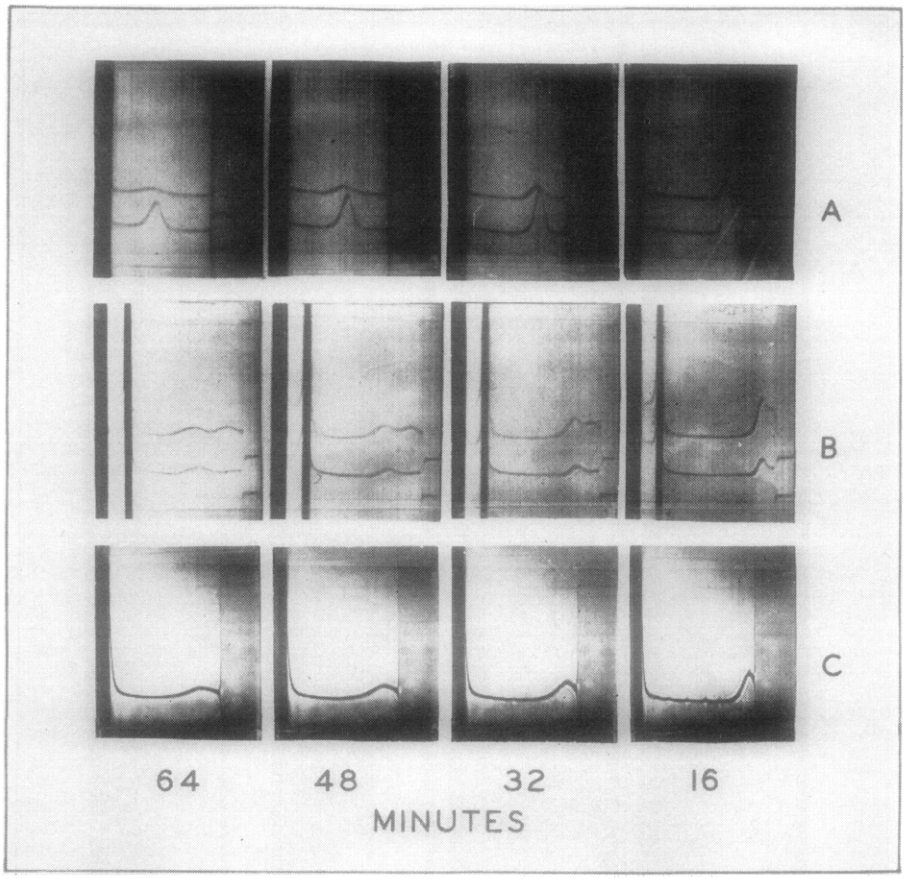

FIGURE 2: Boundary sedimentation analyses of rat plasma lipoproteins and lipid-extracted residues (A) HDL, 3 and 6 mg/ml, 56,100 rpm; (B) heptane-extracted HDL (upper tracing) and heptane-extracted VLDL (lower tracing), 5 mg/ml, 56,100 rpm; (C) lipid-free VLDL protein residue, 5 mg/ml, 59,780 rpm.

layered on these sucrose gradients. The samples were centrifuged for 16 hr at 105,000*g* at 4° in the Spinco no. 40 angle rotor; 0.5-ml fractions were collected from the bottom of the tubes and aliquots were used for analysis of protein and lipid phosphorus. Field-formed density gradient runs were carried out after 0.5 ml of 0.17 M NaCl containing HDL or rat plasma albumin was placed in separate tubes on top of 12 ml of NaBr solution of density 1.150 g/ml. The samples were centrifuged for 24 hr at 105,000*g* at 4° in a no. 40 rotor. At the end of the run 0.5-ml fractions were collected from the bottom of each tube and aliquots were used for analysis of protein and lipid phosphorus. Fractions from a tube containing only the salt solutions were used for establishing the gradient at the end of the run.

Optical Rotatory Dispersion. Experiments were performed in a Cary Model 60 spectropolarimeter at a temperature of 25 ± 2°. Protein concentrations of 1 and 0.2 mg/ml were used, respectively, for the runs from 600 to 280 mμ and from 280 to 225 mμ. The same cell, optical path of 1 cm, was used in all experiments. HDL and its lipid-free residue were dissolved in phosphate buffer (pH 7.6) adjusted to ionic strength 0.10 with NaCl.

Difference Spectra. Absorption and difference spectra were recorded with a Cary Model 14 M spectrophotometer at 25 ± 2°. Measurements were made in matched tandem double cells with optical path length of 1 cm, designed to subtract the solvent contribution to the difference spectra directly (Herskowitz and Laskowski, 1962). The proteins were dissolved (200 μg of protein/ml) in NaCl solutions, and adjusted to pH 7.0 and 12.0 by addition of NaOH, at a final ionic strength 0.20. Solutions at pH 7.0 were placed in the reference cell and those at pH 12.0 in the sample cells.

Immunochemical Procedures. Antisera against rat plasma were obtained by injecting four New Zealand rabbits with 0.5 ml of rat plasma intramuscularly every 5 days for 45 days. The rabbits were bled from the ear artery and the collected sera were stored at 2° after addition of merthiolate (1:10,000). Immunoelectrophoresis was effected as described by Scheidegger (1955) on microscope slides with 2% w/v Noble (Difco) agar and 1% w/v agarose gels (Bausch and Lomb) in barbital–barbiturate buffer (pH 8.6), ionic strength

0.05. The electrophoretic chamber was filled with the same buffer. Generally 2 ma/slide was applied during 2 hr. Undiluted antiserum (10–15 μl) was usually placed in the troughs in the gels and diffusion was allowed to proceed for at least 12 hr at room temperature in a humid chamber. After washing with 0.17 M NaCl the gels were dried and stained with Amido Black in acetate buffer (pH 4.5) for 2 min. Excess dye was removed by washing in 2% acetic acid. Some slides were prestained in a saturated solution of Oil red "O" in 60% ethanol for 2 hr at 37°. Pictures of the stained slides were taken with a Polaroid camera using transmitted light. Specific absorption of anti-rat plasma sera was effected by the procedure of Burtin (1964), using purified preparations of HDL and rat plasma albumin that showed no contaminants when analyzed by immunoelectrophoretic, ultracentrifugal techniques, gel electrophoresis, and gel filtration.

Gel Electrophoresis. The supporting gel was prepared from a mixture of 0.4% w/v agarose (Bausch and Lomb) and 0.1% w/v hydrolyzed potato starch (Connaught) melted in boric acid–NaOH buffer (pH 8.9), ionic strength 0.036. The melted gel (4 ml) was poured on microscope slides and allowed to solidify at room temperature in a humid chamber. Slots for the samples were cut in the gel with strips of filter paper. The buffer in the electrophoretic chamber was sodium barbital–barbituric acid (pH 8.6), ionic strength 0.050. There was an application of 4 ma/slide during 3 hr for these runs. After electrophoresis the slides were fixed for 1 hr in 5% acetic acid in 60% methanol, covered with wet filter paper, and dried under a stream of hot air. Staining of the dried gels was effected as described for immunoelectrophoresis.

Gel Filtration. Molecular sieving chromatography was performed in a Sephadex G-200 column (2 × 50 cm) previously equilibrated with phosphate buffer (pH 7.6), ionic strength 0.1. Flow rates were adjusted to 2–3 ml/hr. Protein was determined (Lowry *et al.*, 1951) on aliquots from the 5-ml fractions. Lipid phosphorus analyses were carried out on extracts (Folch *et al.*, 1957) of aliquots from fractions containing protein.

Removal of Lipids from Lipoprotein Fractions. The *n*-heptane-extraction method of Gustafson (1965) was used to obtain the residues of VLDL and HDL free of neutral lipids. Thin layer chromatography was utilized to monitor the extent of lipid removal. The insoluble potato starch used for these extractions was a purified commercial preparation which was washed of soluble contaminants by repeated cycles of suspension in 1% NaCl, water, and centrifugation discarding the supernatants. These cycles were repeated until no positive anthrone or iodine tests were obtained with the supernatants. After evaporation of the heptane, the protein–phospholipid residue was separated from the insoluble starch by extraction with 0.17 M NaCl adjusted to pH 8.6 and centrifugation. The procedure was repeated until no further protein could be detected in the supernatants. The combined extracts were centrifuged once more (6000*g*, for 30 min) to eliminate any insoluble particles. Iodine tests conducted in samples of the last supernatant failed to detect any starch. Total lipid extraction of HDL and VLDL was obtained by forcing 1 ml of a solution of heptane-extracted lipoprotein (2 mg/ml) into 50 ml of ethanol–acetone (1:1, v/v). The flocculated protein was collected after 2 hr at 25° by low-speed centrifugation, washed twice with ethanol–acetone mixture, and twice with acetone. The acetone was evaporated under a stream of nitrogen. The dried protein dissolved readily in 0.17 M NaCl adjusted to pH 8.6 and 7.6.

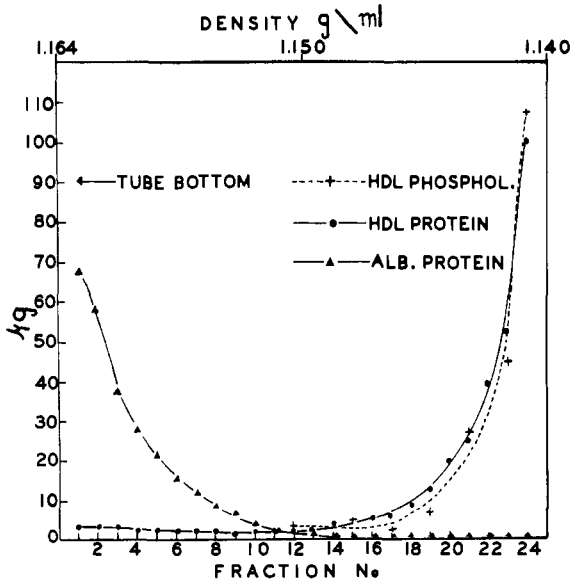

FIGURE 3: Distribution of HDL and rat plasma albumin (separate tubes) at the end of a field-formed density gradient centrifugation. NaBr, initial density of 1.150 g/ml.

Peptide Mapping. Freeze-dried protein (2 mg) was dissolved in 0.50 ml of formic acid, 0.05 ml of 30% hydrogen peroxide was added, and the oxidation was allowed to proceed for 30 min at 25°. After addition of 5 ml of distilled water the oxidized protein was freeze dried. The procedure of Katz *et al.* (1959) was used for the enzymatic digestion and isolation of the peptides. Peptides were separated by thin layer chromatography and electrophoresis on silica gel plates (Ristchard, 1964). In order to obtain reproducible patterns, the two plates to be compared were placed in the electrophoretic chamber facing each other. Whatman 3MM paper, used to establish electrical contact with the buffer, served as spacer. With this arrangement the silica gel surfaces formed a narrow humid chamber and no cooling was necessary. The two patterns obtained in this way with the same protein were therefore mirror images.

Analytical Methods. All solvents and chemicals used were reagent grade. Lipid analyses were conducted on extracts obtained following the procedure of Folch *et al.* (1957). Phosphorus was measured by the method

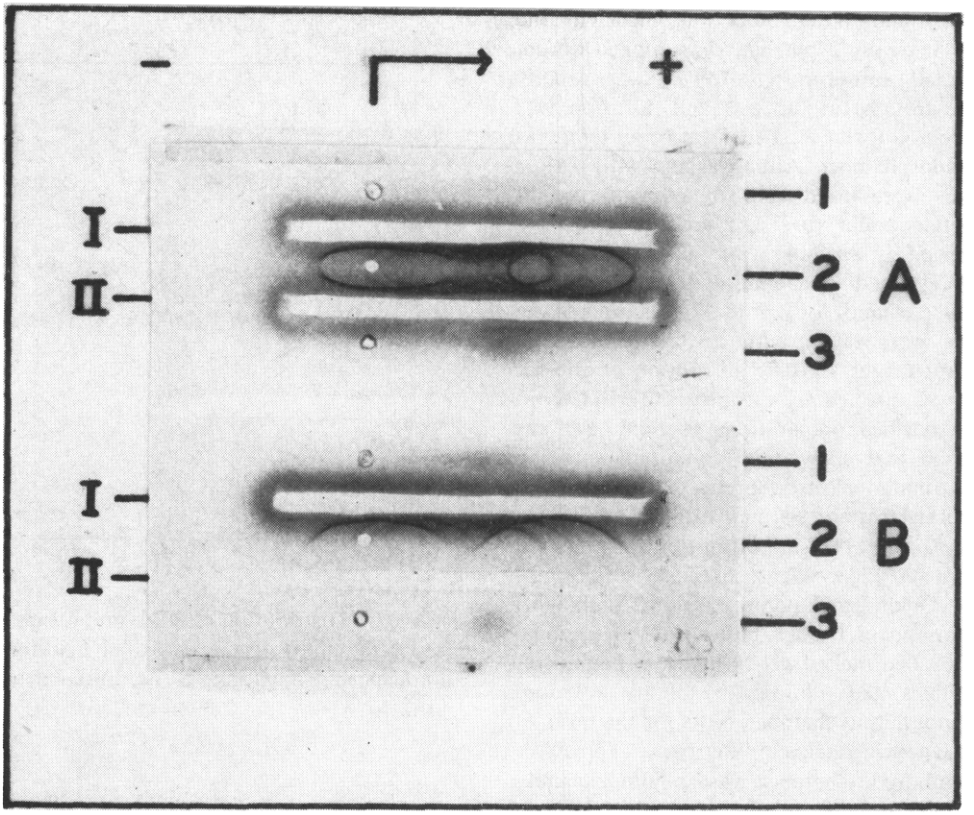

FIGURE 4: Agarose immunoelectrophoresis of rat plasma VLDL and LDL fractions. Slide A: antigen 1, VLDL; antigen 2, rat plasma; antigen 3, LDL; antisera I and II, anti-rat plasma. Slide B: antigen 1, VLDL; antigen 2, rat plasma; antigen 3, LDL; antiserum I, anti-rat plasma; antiserum II, none.

of Beveridge and Johnson (1949) after perchloric acid digestion. Protein was measured by the procedure of Lowry *et al.* (1951), using rat plasma albumin as standard. Glycerides were estimated according to Van Handel *et al.* (1957), and cholesterol according to Abell *et al.* (1952). Amino acid analysis was performed on a Phoenix VG-6000 analyzer. Weighed samples of the dried proteins were hydrolyzed according to Moore and Stein (1963). Corrections for decomposition were applied to the values of threonine, serine, and tyrosine. Tryptophan was measured spectrophotometrically (Goodwin and Morton, 1946).

Results

Properties of Purified Rat Plasma Lipoproteins. ULTRACENTRIFUGAL ANALYSES. Flotation profiles of the lipoprotein spectrum with density below 1.210 g/ml are shown in Figure 1A. Also in Figure 1 are presented the flotation patterns of purified rat plasma lipoprotein fractions. The VLDL fraction (Figure 1B) appeared as a continuum with S_f (1.21) values ranging from 60 to 700 and 220 at the mid-area ordinate. The LDL fraction was found to be least abundant in rat and showed a complex flotation pattern (Figure 1C). The S_f (1.21) value for the mid-area term was 23. Rat HDL (Figure 1D) floated as a single peak with S_f (1.21) in the range 0–12 and a mid-area ordinate of 4.0. Centrifugation of purified HDL up to 64 min did not result in distortion of the peak. Boundary sedimentation analysis of HDL (Figure 2A) supports the homogeneity of this lipoprotein fraction. No time dependence of its apparent diffusion coefficient was observed (Schachman, 1957). The partial specific volume of HDL was 0.907 and the molecular weight obtained by approach to equilibrium sedimentation was found to be 246,000 with a standard deviation of 15,000. The S value was 5.6 and the $s_{20,w}$ = 5.8 S. In Table I is presented the percentage composition of the purified lipoproteins.

Analyses of fractions obtained from field-formed density gradient experiments (Figure 3) indicated that HDL preparations did not contain proteins or lipoproteins with densities above 1.210, and that the rat plasma albumin prepared according to the procedure of Schwert (1957) was free of measurable lipoprotein contaminants. This albumin preparation gave a homogeneous peak with $s_{20,w}$ of 4.50 S.

IMMUNOELECTROPHORETIC ANALYSES. Rat plasma VLDL and LDL did not move freely in agar gels and gave streaky patterns moving toward the anode. In agarose gels these fractions still showed streaks but immunoelectrophoretic mobilities were higher (Figure 4). VLDL did not show a defined precipitin line, but

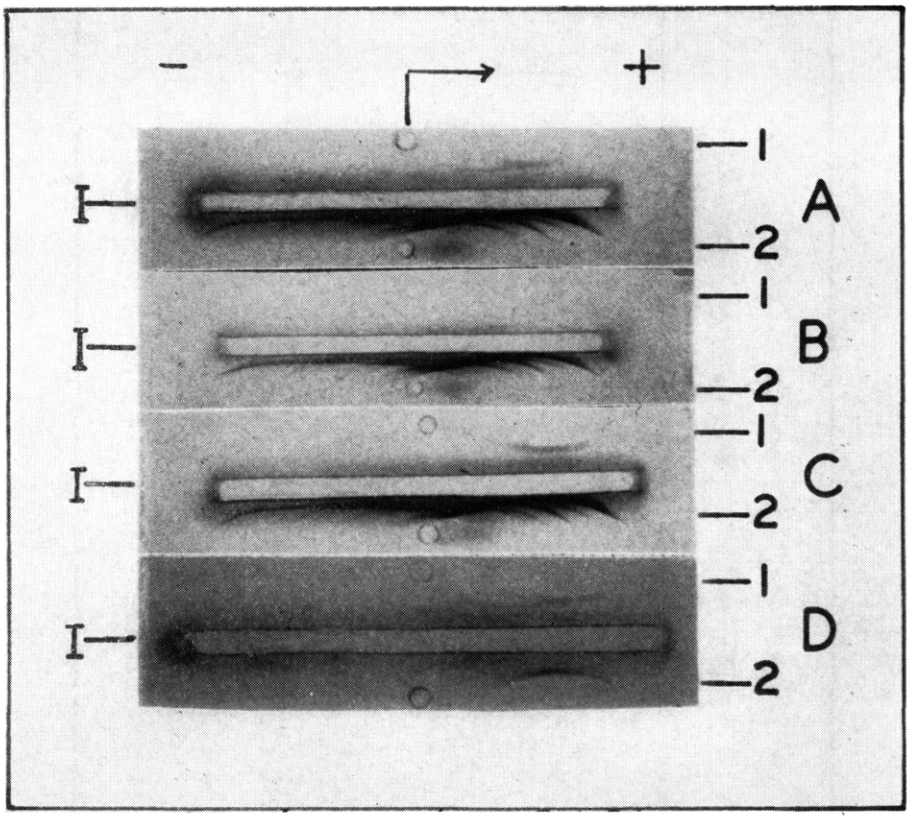

FIGURE 5: Agar immunolectrophoresis of rat plasma lipoproteins and heptane-extracted residues. Slide A: antigen 1, heptane-extracted VLDL; antigen 2, rat plasma. Slide B: antigen 1, heptane-extracted LDL; antigen 2, rat plasma. Slide C: antigen 1, HDL; antigen 2, rat plasma. Slide D: antigen 1, heptane-extracted VLDL; antigen 2, heptane-extracted HDL. Antiserum I in all slides, anti-rat plasma.

TABLE I: Composition of Rat Plasma Lipoproteins and Lipid-Extracted Residues.[a]

Fraction	Protein (%)	Phospholipids (%)	Cholesterol (%)	Glycerides (%)
VLDL	12.3	20.2	8.7	58.7
LDL	22.0	21.8	47.7	7.8
HDL	41.7	39.6	18.7	*b*
VLDL protein–phospholipid residue	50.0	50.0	*b*	*b*
VLDL protein residue	>99.0	*c*	*b*	*b*
HDL protein–phospholipid residue	55.0	45.0	*b*	*b*
HDL protein residue	>99.0	*c*	*b*	*b*

[a] Average of duplicate determinations on fractions obtained from the pooled plasma of 30 animals. [b] Below the limit of detection of the method used, approximately 5%. [c] Below the limit of detection of the method used, approximately 1%.

rather a diffuse zone from the β to α-1 region (slides A1 and B1). LDL appeared as a defined arc in the α-2 region (slide A3). Purified HDL gave a sharp arc with mobility in the α-1 region in agar gels (Figure 5, slides C1 and D2). In agarose, HDL moved into the albumin region, and showed a single arc (Figure 6, slide A1). Purified rat plasma albumin gave only one precipitin line when tested against antiserum to rat plasma (Figure 6, slide C1).

GEL ELECTROPHORESIS AND GEL FILTRATION. In agarose–starch gel, HDL moved faster than albumin (Figure 7, slide A). Purified HDL moved as a single

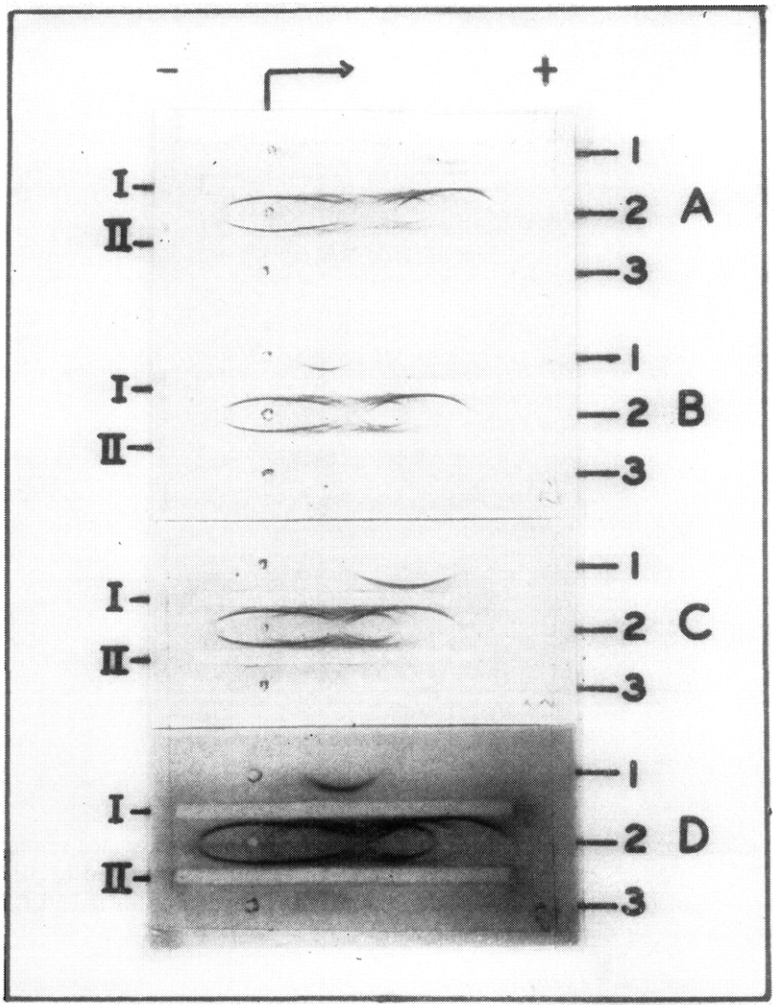

FIGURE 6: Agarose immunoelectrophoresis. Slide A: antigen 1, HDL; antigen 2, rat plasma; antigen 3, HDL; antiserum I, anti-rat plasma; antiserum II, HDL-absorbed anti-rat plasma. Slide B: antigen 1, HDL–protein residue; antigen 2, rat plasma; antigen 3, HDL–protein residue; antiserum I, anti-rat plasma; antiserum II, HDL-absorbed anti-rat plasma. Slide C: antigen 1, rat plasma albumin; antigen 2, rat plasma; antigen 3, rat plasma albumin; antiserum I, anti-rat plasma; antiserum II, HDL absorbed anti-rat plasma. Slide D: antigen 1, VLDL–protein residue; antigen 2, rat plasma; antigen 3, VLDL–protein residue; antiserum I, anti-rat plasma; antiserum II, HDL-absorbed anti-rat plasma.

band even after prolonged runs. Since a mixture of 96% HDL and 4% albumin could be resolved (slide B), the purified HDL was therefore uncontaminated above this level. Rat plasma albumin did not show other bands (slides A2 and D2). VLDL and LDL moved as diffuse bands in these agarose–starch gels. The elution pattern of HDL from a Sephadex G-200 column (Figure 8) shows a large symmetrical peak containing 90% of the lipoprotein followed by a smaller peak that also contained phospholipids. This second peak was found to be larger in aged HDL preparations. The sample shown had been stored at 2° for 1 week. This type of column proved to be very efficient for the resolution of HDL–albumin mixtures. It was found that 0.16 mg of albumin could be recovered completely as an independent peak (Figure 8, arrow) when a mixture of 0.16 mg of albumin and 8.20 mg of HDL was placed on the column. The methods used to monitor the effluent could readily detect 0.070 mg of albumin (OD at 230 mμ and protein determination); however, when 16.4 mg of HDL was placed on the column no albumin was detected (Figure 8) indicating that the level of contamination with albumin was less than 0.4%.

Properties of Lipid-Extracted Lipoproteins. ULTRACENTRIFUGAL ANALYSIS AND COMPOSITION. After extraction of HDL and VLDL with *n*-heptane, soluble phospholipid–protein residues were obtained. From HDL and VLDL the recoveries of protein soluble in 0.17 M NaCl (pH 7.6 or 8.6) were over 90% whereas from LDL the recovery was only 40–60%. Boundary sedimentation analyses of these phospholipid–protein

residues are presented in Figure 2B. Two components with S values of 1.9 and 5.4 were obtained from HDL (upper trace). Sucrose gradient centrifugation showed that only the faster component of *n*-heptane-extracted HDL contained phospholipids (Figure 9). The composition of the residues (Table I) shows that 40% of the phospholipids is removed by the *n*-heptane extraction of VLDL and practically none by extraction of HDL. Essentially lipid-free protein residues were obtained from HDL and VLDL phospholipid–protein complexes after ethanol–acetone extraction. These proteins were soluble in 0.17 M NaCl (pH 7.6)–phosphate buffer (pH 7.6), ionic strength 0.01 or 0.10, but they precipitated upon dialysis against water when the pH fell below 5.5. The precipitate could be redissolved by raising the pH above 7. Recoveries of lipid-free protein from HDL and VLDL were consistently better than 90%. No soluble lipid-free protein could be obtained from LDL. Boundary sedimentation analysis of the VLDL–protein residue showed a single component with sedimentation coefficient of 1.9 S (Figure 2C). The patterns obtained from HDL and HDL–protein residue prepared from the same batch of rat plasma are compared in Figure 10. The sedimentation coefficient of HDL–protein was 1.9 S and its $s_{20,w}$ value was 2.1 S. The calculated partial specific volume was 0.724 and the molecular weight was found to be 26,800 with a standard deviation of 2600.

IMMUNOCHEMICAL STUDIES. Agar immunoelectrophoresis of phospholipid–protein residues from VLDL, LDL, and HDL is shown in Figure 5. The VLDL phospholipid–protein residue gave a major arc in the α-1 region and two faint arcs in the α-2 and β regions, respectively (slides A1 and D1). LDL–phospholipid–protein residue did not give precipitin lines when tested against anti-rat plasma antiserum (slide B1), this probably caused by denaturation as indicated by the low solubility of the residue. HDL and VLDL protein residues gave streaking patterns in agar gels; however, arcs with mobilities in the β region were obtained in agarose (Figure 6, slides B1 and D1).

In Figure 6 are also presented the results obtained in immunoelectrophoretic experiments using anti-rat plasma antisera absorbed with HDL or plasma albumin. With the HDL-absorbed antisera the arcs from HDL, HDL protein residue, and VLDL protein residue were no longer visible, also the plasma albumin arc disappeared (slides A–D). When rat plasma albumin was used for the absorption of the antiserum the arcs given by plasma albumin, HDL, and the protein residue from HDL and VLDL disappeared (Figure 11), although eight to ten times more HDL than albumin was required to remove the antibody directed against the apparent common determinant.

GEL ELECTROPHORESIS. Agarose–starch gel electrophoresis also showed the difference between the mobilities of HDL and its protein residue. A comparison of these products with rat plasma and rat plasma albumin is shown in Figure 7. Although the absolute mobilities are not the same from slide to slide (different runs), the relative values can be compared. Purified HDL moved as a prealbumin (slides A1, B1, and C1) and HDL–protein moved as a β-globulin (slides C2, D1, and E1).

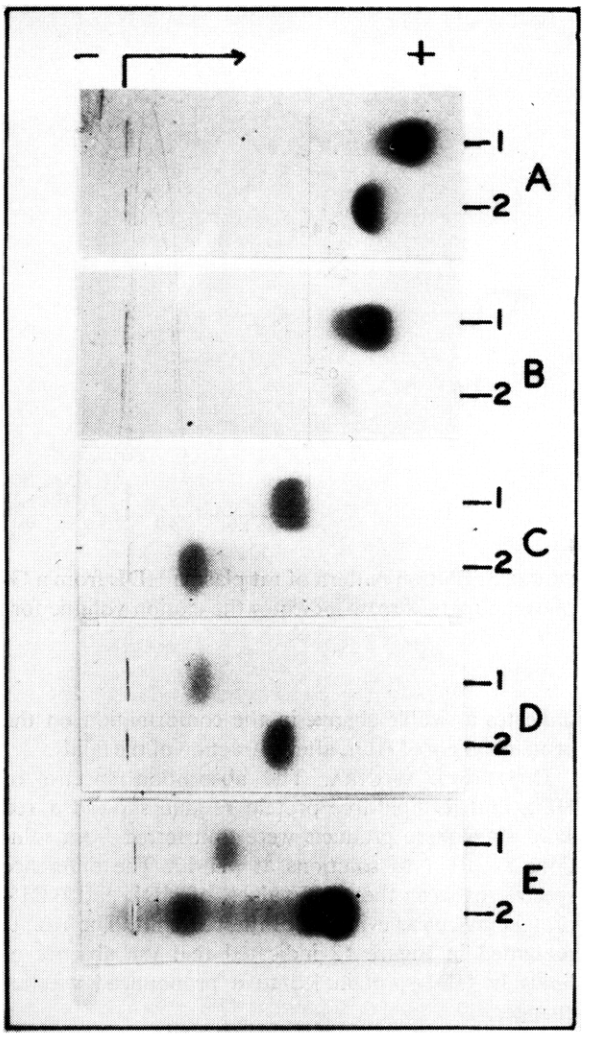

FIGURE 7: Agarose–starch electrophoresis. Slide A: sample 1, HDL; sample 2, rat plasma albumin. Slide B: sample 1, HDL (21 μg) plus albumin (1.5 μg); sample 2, plasma albumin (1.5 μg). Slide C: sample 1, HDL; sample 2, HDL–protein residue. Slide D: sample 1, HDL–protein residue; sample 2, rat plasma albumin. Slide E: sample 1, HDL–protein residue; sample 2, rat plasma.

OPTICAL ROTATORY DISPERSION. The a_0 and b_0 values were calculated, for HDL and its lipid-free protein residue, from a plot of the Moffit and Yang (1956) equation as suggested by Urnes and Doty (1961). The following values were obtained: (a_0) HDL −150, HDL–protein −350; (b_0) HDL −250, HDL–protein −130. The mean residue rotation at 233 mμ, where a minimum in the dispersion curves of both preparations was found, was −6400 for HDL and −3950 for HDL–protein. This increase in b_0 and $(m')_{233}$

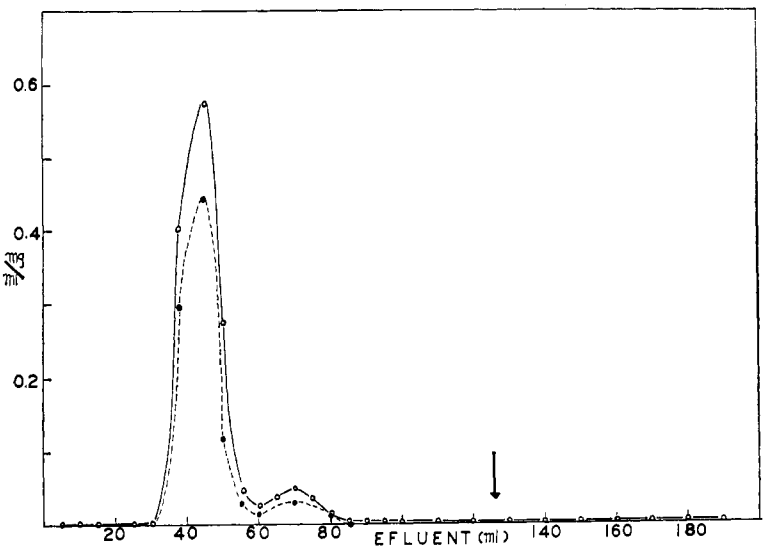

FIGURE 8: Elution pattern of rat plasma HDL from a G-200 Sephadex column (2 × 50 cm). (—O—) Protein; (- - ● - -) phospholipids. Arrow indicates the elution volume for rat plasma albumin.

indicates a sizable change in the conformation on the protein moiety of HDL after extraction of the lipids.

DIFFERENCE SPECTRA. The absorption spectra of HDL and its lipid-free protein residue showed a red shift when these products were transferred from solutions at pH 7 to solutions at pH 12. The difference spectra, between these pH values, of HDL and HDL-protein give clear evidence of the red shift. The results presented in Figure 12 indicated that the absence of lipids in HDL-protein led to a pronounced spectral change.

AMINO ACID ANALYSIS AND PEPTIDE MAPS. Table II presents the amino acid composition of rat HDL and rat plasma albumin. For comparison the data obtained by Scanu and Granda (1966) for human HDL-protein are included. As in its human counterpart, was found in HDL, a high content of aspartic acid, glutamic acid, and leucine, and very low content of half-cystine. Peptide maps of performic acid oxidized HDL-protein and rat albumin are shown in Figure 13. Although not identical the peptide patterns showed some resemblance. The number of peptides obtained from HDL-protein (22) was close to the theoretical number of 28/molecule calculated from the content of basic amino acids. From albumin only 25 out of the calculated 58 could be separated, in agreement with the known resistance of performic acid oxidized plasma albumin to total tryptic hydrolysis (Fried, 1963).

Discussion

The density ranges used for preparative fractionation of human plasma lipoproteins were selected according to discontinuities in the flotation profiles observed during analytical ultracentrifugation (de Lalla and Gofman, 1954; Havel *et al.*, 1955). These schemes of fractionation have been widely used for the preparation of lipoproteins from other mammals despite the fact that Lewis *et al.* (1952) showed that lipoproteins from different species present dissimilar flotation patterns. I have applied the method of Havel *et al.* (1955) with minor modifications to prepare rat

TABLE II: Amino Acid Composition of Rat Plasma HDL and Albumin.

Amino Acid	Protein (moles of amino acid/100,000 g)		
	Rat Albumin	Rat HDL	Human HDL[a]
Aspartic	72.9	85.4	60.2
Threonine	52.6	43.2	37.0
Serine	33.1	36.3	48.4
Glutamic	117.5	132.6	141.1
Proline	39.3	22.7	37.0
Glycine	25.6	39.2	33.0
Alanine	87.3	52.5	55.3
Valine	47.7	41.2	48.0
Cystine (half)	50.4	2.4	3.6
Methionine	8.3	20.5	7.5
Isoleucine	18.6	11.3	6.3
Leucine	77.5	80.0	104.8
Tyrosine	33.3	18.1	30.6
Phenylalanine	36.7	23.2	26.9
Lysine	49.2	57.9	70.1
Histidine	20.6	13.9	10.9
Arginine	39.5	49.4	34.1
Tryptophan		14.2	23.8

[a] Scanu and Granda (1966).

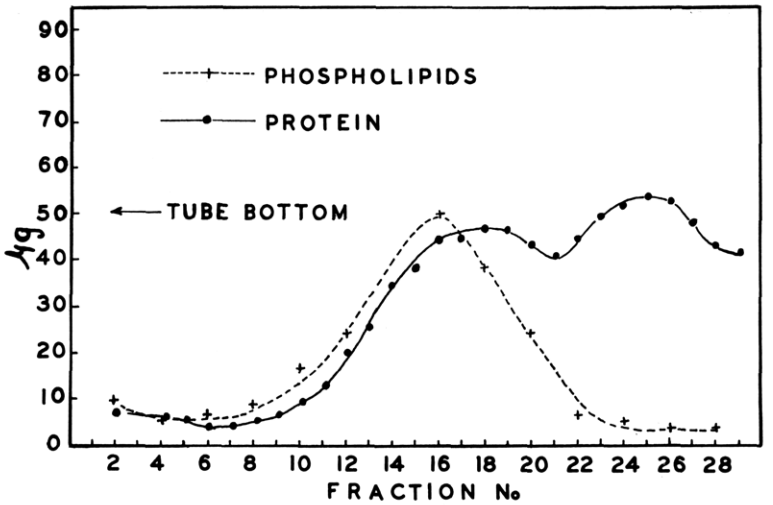

FIGURE 9: Distribution of heptane-extracted rat HDL after a preformed density gradient centrifugation. Sucrose 20–10% (w/v). Localization of phospholipid on the protein residues obtained.

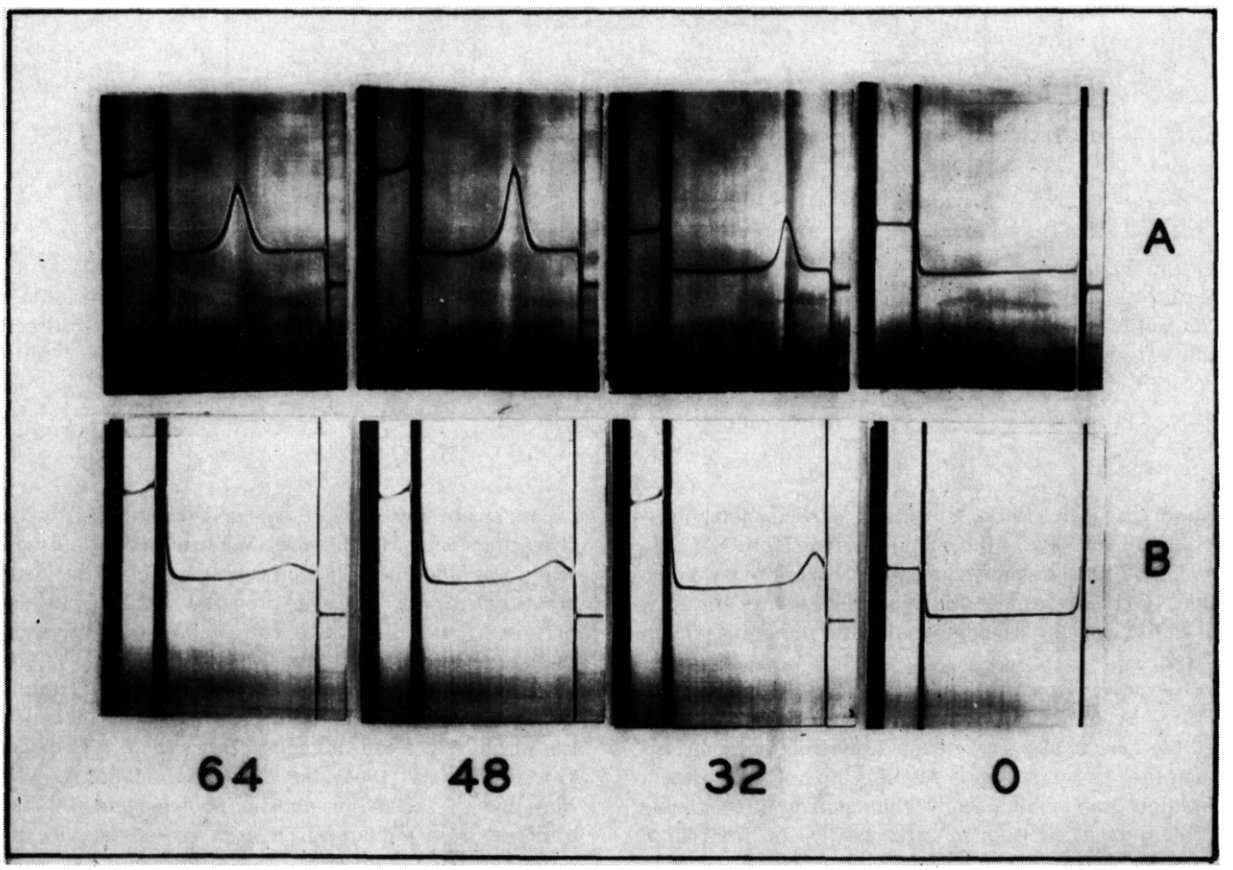

FIGURE 10: Boundary sedimentation analyses of rat plasma HDL (7 mg/ml) (A) and HDL–protein residue (5 mg/ml) (B), 59,780 rpm, solvent density 1.006 g/ml. Numbers at the bottom indicate time of centrifugation in minutes.

plasma lipoproteins. This procedure yielded fractions according to discontinuities observed in the flotation profiles of the lipoprotein spectra of rat plasma (Figure 1). These fractions appear to correspond closely to those detected in human plasma lipoproteins by the same methods (Del Gatto *et al.*, 1959; Small *et al.*, 1964). Both rat VLDL and LDL (Table I) were similar in composition to the corresponding fractions in human

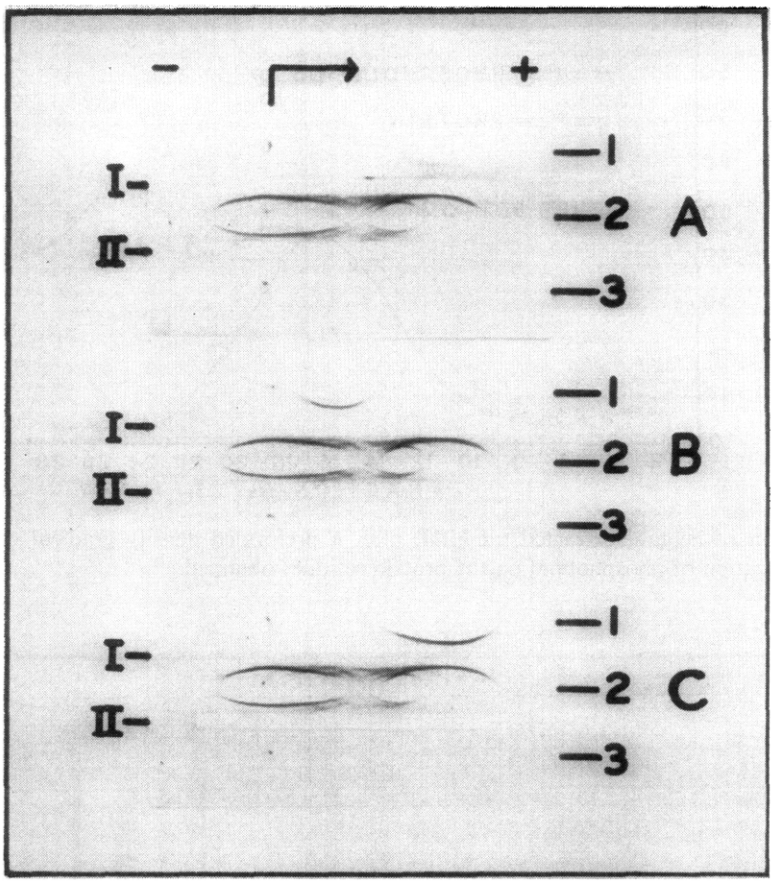

FIGURE 11: Agarose Immunolectrophoresis. Slide A: antigen 1. HDL; antigen 2, rat plasma; antigen 3, HDL; antiserum I anti-rat plasma; antiserum II, albumin-absorbed anti-rat plasma. Slide B: antigen I, HDL–protein residue; antigen 2, rat plasma; antigen 3, HDL–protein residue; antiserum I, anti-rat plasma; antiserum II, albumin-absorbed anti-rat plasma Slide C: antigen 1, rat plasma albumin; antigen 2, rat plasma; antigen 3, rat plasma albumin; antiserum I, anti-rat plasma; antiserum II, albumin-absorbed anti-rat plasma.

plasma (Oncley, 1963). Rat HDL showed more phospholipids and less protein than human HDL (Oncley, 1963). This is probably the cause of the lower average density (Figure 3) and the somewhat higher mean S_f (1.21) coefficient as compared with those of human HDL.

Recently, Gustafson *et al.* (1966), Scanu *et al.* (1958), Scanu and Granda (1966), and Shore and Shore (1964) have shown that detailed information, concerning the structure and relationship of lipoprotein fractions, can be obtained by characterization of soluble protein residues isolated after partial or total lipid removal. Using the method of Gustafson (1965) for extraction of neutral lipid followed by treatment with ethanol–acetone, I have obtained the protein of VLDL and HDL from rat plasma. The proteins isolated were soluble in neutral salt solutions; it was therefore possible to study some of their physical and chemical properties without the complication introduced by adding detergents or other solubilizing agents. The major protein components of VLDL and HDL were indistinguishable as judged by boundary sedimentation, gel electrophoresis, and immunochemical characteristics. Marsh and Whereat (1959) found that these lipoproteins (synthesized by rat liver slices) cross react with antisera prepared against purified plasma fractions, and Roheim *et al.* (1965) showed that a serum protein was incorporated into VLDL and HDL during perfusion of rat liver. Our results indicating the presence of a common apoprotein for rat VLDL and HDL are consistent with such findings. Gustafson *et al.* (1966) and Levy *et al.* (1966) showed that one of the major phospholipid–protein residues obtained from human VLDL was very similar to the residue isolated from HDL. In the rat, we have found that 90% or more of the VLDL protein residue is probably the same as that obtained from HDL.

Upon extraction of neutral lipids, HDL (5.6 S, mol wt 246,000) was found to dissociate into two components (Figure 2B). The faster component with sedimentation coefficient of 5.4 S contained all the phospholipid, whereas the slower, with sedimentation coefficient of 1.9 S, was found free of lipid. After total lipid extraction, the protein sedimented as a single

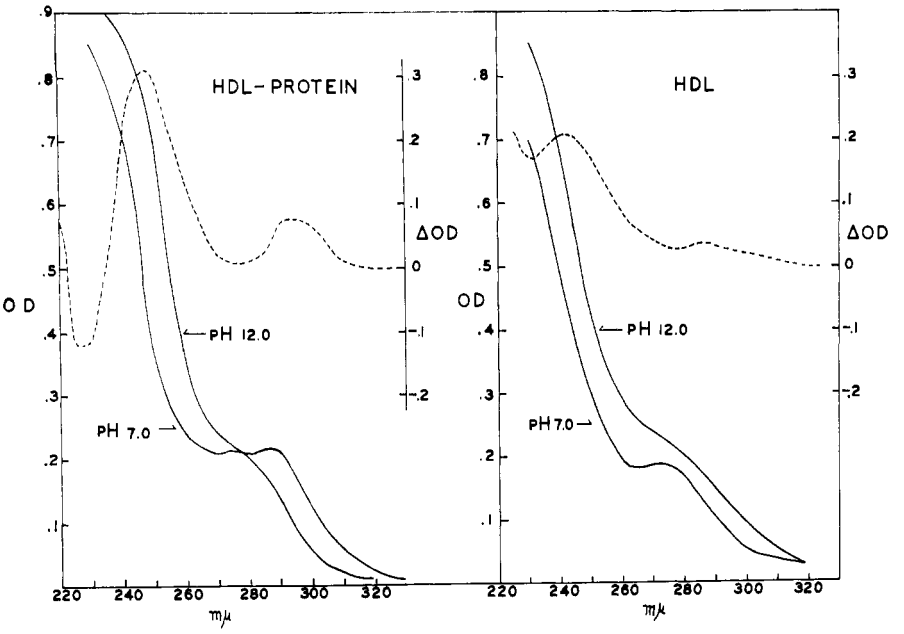

FIGURE 12: Absorption spectra of HDL–protein and HDL at pH 7 and 12 (solid lines) and difference spectrum of each preparation at these two pH values (dotted lines).

boundary with a sedimentation coefficient of 1.9 S; the same value of the lipid-free component of the neutral lipid-extracted fraction (Figure 10). The molecular weight of this subunit (26,800) suggests that rat HDL is made of four to six of these protein subunits plus an approximately equal weight of bound lipid. Scanu (1967) has described an analogous dissociation in human HDL, but his lipid-free protein showed two peaks during sedimentation with 2.4 and 12.3 S, respectively. Only after alkylation of the products was a homogeneous protein obtained. The molecular weight of HDL–protein from human sources has been reported as 75,000 (Scanu *et al.*, 1958) and 38,000 in a preparation solubilized with sodium dodecyl sulfate (Shore and Shore, 1964). Recently Scanu (1967) obtained 24,000 as the molecular weight of succinylated HDL–protein. This last value is very close to that found for rat HDL–protein.

The capacity for forming precipitin complexes with antisera was retained by rat VLDL and HDL after partial or total removal of the lipid (Figures 5, 6, and 11), thus indicating that most of their antigenic properties are carried in the protein moiety. The same phenomenon has been reported by Scanu and Granda (1966), Levy and Fredrickson (1965), and Gustafson *et al.* (1966) for human fractions. Upon total removal of the lipid the electrophoretic mobility in agarose gels of rat VLDL and HDL changed to the β region. This change was reported to occur in human HDL; Levy and Fredrickson (1965) found that upon aging, freezing, thawing, and lipid extraction, HDL was partially converted into a product with lower electrophoretic mobility. However, neither the degree of lipid removal nor the size of this slower moving product was reported.

The optical rotatory dispersion parameters evaluated for HDL and its lipid-free protein residue showed that removal of lipids produced a 40–50% increase in the b_0 and $(m')_{233}$ values. These changes indicated an appreciable alteration in conformation, probably caused by the exposure to the more polar aqueous environment of peptide regions normally shielded by lipid (Tanford *et al.*, 1960, 1962). The difference spectra studies seem to support this hypothesis (Figure 12). With lipid-free HDL at pH 12.0, a red shift of the absorption spectra of about 12 mμ was observed, leading to pronounced difference spectrum when compared with solutions at pH 7.0. On the other hand,

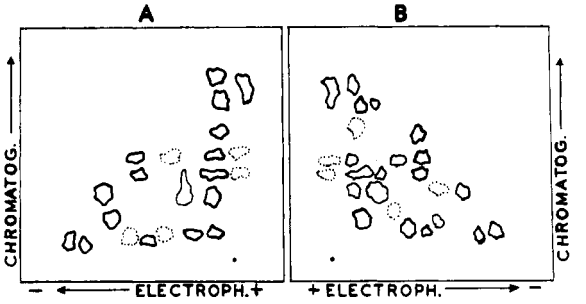

FIGURE 13: Peptide mapping of HDL–protein and rat plasma albumin (A, HDL–protein; B, plasma albumin) showing resemblance of the distribution patterns. Broken outlines indicate faint spots.

a smaller red shift was detected with intact HDL at pH 12.0, producing a less pronounced difference spectrum. Quantitative interpretation of these results is not yet possible (Wetlaufer, 1962), but it appears reasonable to conclude that peptide segments containing phenolic side chains became exposed to the aqueous environment after the extraction of lipids. Scanu (1965b) found an increase of only about 10% in the b_0 and $(m')_{233}$ values of human HDL after lipid extraction. This is in contrast with our findings, and could be due to real differences between the structural features of rat and human HDL-protein since Scanu (1967) has shown that only after alkylation is human HDL completely dissociated. However it is possible that differences in methods used for lipid extraction produced proteins with dissimilar conformations.

The experiments with absorbed antisera indicate that some common immunochemical determinants exist in both rat HDL and plasma albumin. It was found that methods, such as gel filtration, capable of detecting 0.4% of albumin in HDL preparations, and as field-formed density gradients and agarose–starch electrophoresis capable of detecting 2.5% of albumin in HDL preparations, failed to show any albumin in the HDL used for antigen absorption. It is therefore unlikely that the results observed were caused by contamination; since as indicated by the absorption experiments, quantitative precipitin curve, and relative intensity of immunoelectrophoretic arcs, a contamination of the order of 10% would be required to account for them. The possibility of a common amino acid sequence in human serum albumin and HDL was investigated by Avigan and Anfinsen (1959). These authors noted the similarities in N-terminal amino group, dye binding capacity, and solubility of these two proteins. Based on the results of unidimensional high-voltage electrophoresis of enzymatic hydrolysates of the proteins, they concluded that these similarities were not a reflection of common primary structure. Our preparation of rat plasma albumin appears to be free of all lipoprotein; however we have found that lipid-free HDL-protein has a strong tendency to associate with albumin, and when it constituted less than 5% of the mixture, it was no longer detectable. One may speculate that small amounts of HDL-protein exist in association with the albumin in plasma and that this type of tightly bound complex, with albumin as the minor component, may also exist in HDL fraction in a form not dissociable by the methods used. The occurrence of partially or totally lipid-free lipoprotein residues in association with other proteins (in rat plasma) had been suggested by Roheim *et al.* (1965), and by Levy and Fredrickson (1965) in human plasma. Differences in molecular weight, sedimentation coefficient, content of half-cystine, proline, glycine, methionine, and tyrosine eliminate the possibility of identity between HDL-protein and rat plasma albumin molecules. The results obtained from peptide mapping of the performic acid oxidized proteins are less conclusive. This parameter, however, cannot rule out the existence of some common peptide segments that could carry similar immunochemical determinants. A study of the composition and immunological properties of peptides obtained from these proteins after controlled partial hydrolysis, appears the only way to a definitive answer.

When compared with human HDL, rat HDL-protein exhibits dissimilarities in the content of methionine, isoleucine, tyrosine, and tryptophan. Despite the differences, it is evident that similarities exist between rat HDL and VLDL, on one hand, and the corresponding fractions isolated from human plasma on the other. This is probably a consequence of the use of a related lipoprotein-building block in both mammals, the HDL-protein. This protein can be isolated with relatively little effort and is a small molecule. As stated by Trams and Brown (1966), a study of its primary and secondary structure should prove rewarding for an understanding of the factors controlling lipid–protein associations, their metabolic function, and genetic control.

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Addendum

During the preparation of this manuscript, Windmueller and Levy (1967) clearly demonstrated differences in the metabolic behavior of rat LDL (lipoproteins) and HDL (lipoproteins). My results suggest that there is no structural relation between these lipoproteins. Windmueller and Levy found, in contrast to human lipoproteins, that the procedure of Havel *et al.* (1955) followed by recentrifugation did not entirely separate the rat LDL and HDL fractions. I have shown that rat HDL has a lower mean density and higher flotation coefficient than human HDL. This may explain difficulties observed in the separation of rat LDL from HDL. I have detected no cross-contamination between rat HDL and LDL when the centrifugations periods of the Havel *et al.* procedure are extended to 24 hr, and when each fraction is recentrifuged twice for 24 hr at 105,000*g*.

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