Effects of Ultracentrifugation on the Human Serum High-Density ($1.063 < \rho < 1.21$ g/ml) Lipoprotein*

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ABSTRACT: Recentrifugation in an angle rotor of ultracentrifugal isolates of human serum high-density lipoproteins HDL_2 ($1.063 < \rho < 1.120$) and HDL_3 ($1.120 < \rho < 1.21$) led to a redistribution of lipoprotein species throughout the preparative tube. The top milliliter contained about 70% of the starting material and was identical with it by all parameters studied (electrophoresis, ultracentrifugation, immunodiffusion, and chemical composition). The bottom milliliter contained only about 3% of the original lipoprotein protein, was poor in lipid, and had immunochemical characteristics of HDL protein; it was referred to as very high-density lipoprotein (VHDL). Upon delipidation with ethanol–diethyl ether, the protein of the top milliliter of either HDL_2 or HDL_3 and the corresponding VHDL forms had a tendency to polymerize readily and could be reduced into subunits of an average 2.15×10^4 molecular weight by either acetylation or addition of 0.05 M sodium dodecyl sulfate. VHDL forms were also detected immunochemically in density >1.21 serum residues and fresh serum. In the latter their production was increased by freezing and thawing, and addition of urea or sodium dodecyl sulfate. From the molecular weight of HDL_2 ($3.6 \times 10^5 \pm 2.1 \times 10^4$) and HDL_3 ($1.7 \times 10^5 \pm 1.2 \times 10^4$), their protein mass, and weight of the peptide subunits, it was estimated that HDL_2 has an average of seven peptide subunits as compared with HDL_3 which has an average of five. Based on this estimate and on the difference in lipid content of these subclasses it was postulated that HDL_3 derives from HDL_2 by removal of lipid and concomitant release of two protein subunits. The latter, in turn, contribute to the formation of VHDL. Since VHDL could reacquire lipid, it would appear that HDL can undergo a delipidation–relipidation cycle according to the scheme: $HDL_2 \rightarrow HDL_3 \rightarrow VHDL \rightarrow HDL_2$. Whether the cycle represents an ultracentrifugal artifact or may be operative *in vivo* was not established.

By the conventional technique of preparative ultracentrifugation (Lindgren and Nichols, 1960), the density of human serum is increased in stepwise fashion up to 1.21 g/ml to permit sequential flotation of the low- and high-density families of lipoproteins. It was recognized early (Hillyard *et al.*, 1955; Havel *et al.*, 1955) that not all plasma lipids are recovered in these lipoproteins and that approximately 10% of whole serum phospholipids and a small percentage of triglycerides are present in the density >1.21 g/ml residues. Whether these lipids are part of a single or an heterogeneous heavy lipoprotein species has not been established. Presence of a protein with immunochemical characteristics of HDL protein has been reported (Levy and Fredrickson, 1965; Scanu and Granda, 1964). Whether it can alone account for the lipids present in the density >1.21 residues remains to be established.

Scanu and Hughes (1960) reported that ultracentrifugation in angle rotors of human serum high-density lipoprotein promotes sedimentation of a small percentage of the lipoprotein protein. A similar observation has been reported recently by Furman *et al.* (1964) and Levy and Fredrickson (1965).

Because of its direct bearing on the problem of HDL structure, the present study was undertaken to elucidate the mechanism of formation and nature of this ultracentrifugally produced heavy density lipoprotein. Attempts were also made to determine whether, as suggested by Levy and Fredrickson (1965), an ultracentrifugal artifact may be responsible for the presence of heavy lipoproteins in the density >1.21 serum residues. A preliminary report of these studies has appeared (Scanu and Granda, 1964).

Materials and Methods

Source of Serum. Blood (500 ml) was obtained from healthy fasting (18 hr) white male donors, 20–30 years of age, group A, Rh positive. After clotting, the serum was separated by centrifugation in a Sorvall RC-2 Superspeed refrigerated centrifuge SS-34 rotor at 12,100*g* for 30 min. One milliliter of 1% EDTA (disodium ethylenediaminetetracetate) solution neutralized to pH 7 with 0.1 N NaOH was added to each 100 ml of serum. Separation of lipoproteins was initiated on the same day of blood collection.

* From the Department of Medicine, The University of Chicago, Chicago, Illinois. *Received May 21, 1965; revised October 26, 1965.* Supported by Grants HE 08727 from the U. S. Public Health Service, G-64-42 from Life Insurance Medical Research Fund, and 56-N64-6 from the Illinois and Chicago Heart Association. Presented in part at the 148th National Meeting of the American Chemical Society, Chicago, Ill., Aug 30–Sept 4, 1964.

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TABLE 1: Scheme of Separation of Serum Lipoproteins by Ultracentrifugal Flotation.[a]

Lipoprotein Class (Abbreviation)	Density Range (g/ml)	Rotor Model	Vol/ Tube (ml)	Gravitational Force (g)	Density of Medium (g/ml)	Salt	Time (hr)	Temp (°C)	Fraction Collected (1 ml)
Chylomicrons	1.006	30.2	9.3	8,832	1.006	—	1/6	16	Top
Very Low Density (VLDL) and Low Density (LDL)	1.006–1.063	30.2	9.3	79,488	Chylomicron infranates *d* 1.063	NaCl	20	16	Top
High Density (HDL_2)	1.063–1.120	40.3	6.4	114,480	VLDL–LDL infranates *d* 1.120	NaCl–NaBr	24	16	Top
Sub-classes (HDL_3)	1.120–1.21	40.3	6.4	114,480	HDL_2 infranates *d* 1.21	NaCl–NaBr	24	16	Top
Residue (BF)	1.21	40.3	6.4	114,480	As above	—	24	16	Bottom

[a] Density increments refer to infranates following removal of the lighter class of lipoprotein by the aid of a tube slicer.

Preparation of Lipoproteins. Lipoproteins were separated by flotation in a Spinco Model L preparative ultracentrifuge as summarized in Table 1. In each step, the lipoprotein fraction contained in the top milliliter was separated by aspiration, the tube was cut in the middle clear zone by a Spinco tube slicer, and the infranate was adjusted to a higher density by either solid NaCl or NaCl–NaBr. HDL_2 and HDL_3 were freed of serum protein contaminants (Scanu *et al.*, 1958b) by refloating them in a salt medium (NaCl–NaBr) of density 1.21 g/ml in a 40.3 rotor, 114,848*g*, 16°, for 48 hr (step I–II, Figure 1). To further investigate the effect of ultracentrifugation of these HDL isolates, the top milliliter fractions of step II (see Figure 1) were pooled, diluted 1:3 with a NaCl–NaBr solution of density 1.21, and respun for an additional 48 hr (step II–III, Figure 1).

The top and bottom milliliters were collected for analysis and will be referred to as HDL_2 (top) and HDL_3 (top) and HDL_2 (bot) and HDL_3 (bot). The bottom milliliter fractions (BF) obtained after separation of all lipoprotein floating at density 1.21 (step I, Figure 1) were pooled and then spun again for 48 hr (step $I–I_A$, Figure 1). Before analysis, all of the ultracentrifugal isolates were dialyzed for 48 hr at 4° against several changes of 0.15 M NaCl, pH 7, containing 0.05% EDTA adjusting to pH 7.0 with 0.1 N NaOH. In certain instances (see Results) dialysis was conducted against Tris buffer, pH 8.6, ionic strength 0.1.

Delipidation Techniques. Extraction of lipids from HDL, products thereof, or bottom fractions by a 3:2 mixture of ethanol–diethyl ether at −10° was conducted by a slight modification of the method previously described (Scanu *et al.*, 1958a). Protein recoveries were from 80 to 90%.

Analytical Ultracentrifugation. Sedimentation and flotation analyses were conducted in a Model E Spinco analytical ultracentrifuge using an An-D rotor and a 12-mm single-sector cell with aluminum centerpiece. Runs were made at 52,640 rpm at 20° with schlieren patterns photographed at 16-min intervals with a bar angle of 65°. Flotation (DeLalla and Gofman, 1954) and sedimentation constants (Schachman, 1957) were calculated from schlieren patterns, magnified by a Nikon Shadowgraph Model 6 C optical comparator (Nikon Co., Japan). Each sample was run at three different dilutions to permit extrapolation to zero

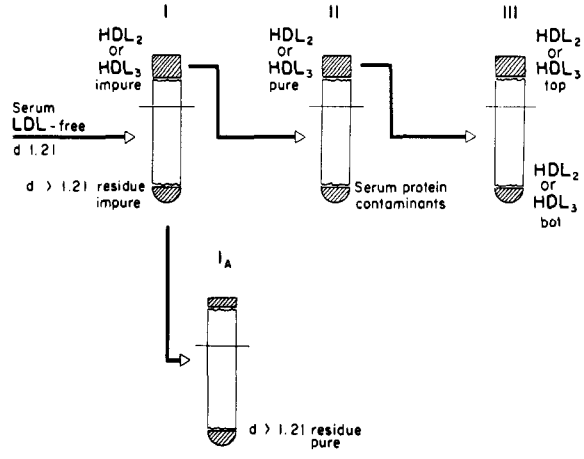

FIGURE 1: Steps for ultracentrifugal fractionation of HDL_3 and HDL_2. I–II and II–III: top milliliters were separated and diluted 1:3 with NaCl–NaBr solution of density 1.21 and respun for 48 hr in a Model L ultracentrifuge, 40.3 rotor, 114,480 *g*, 16°. $I–I_A$: bottom milliliters were pooled and respun undiluted under the same conditions as in step I–II. In each step the tubes were sliced in the clear zone.

protein concentration. Appropriate corrections were made for viscosity and density of the solvent medium and for the apparent partial specific volume of the solute. The partial specific volume, 0.723, of the delipidated high-density lipoprotein (HDL)[1] was calculated from its amino acid content (Schachman, 1957). The values for HDL_2 and HDL_3 at infinite dilution, 0.914 and 0.870, were calculated from density data obtained with a 5-ml pycnometer. Total lipoprotein concentration in each dilution was estimated from the chemical analysis of the protein, cholesterol, phospholipid, and triglyceride constituents. The values are slightly higher than those reported by Hazelwood (1958), 0.905 and 0.867 for HDL_2 and HDL_3, respectively.

The Archibald (1947) method of approach to sedimentation equilibrium as outlined by Schachman (1957) was used for molecular weight determinations.

Vertical starch gel electrophoresis was conducted according to Smithies (1959) using a discontinuous system of Tris–boric acid buffers. Amido Schwartz and oil red O were used for protein and lipid stain (Scanu and Hughes, 1960).

Immunization Program and Immunochemical Techniques. Antisera against whole serum HDL, delipidated HDL, HDL (bot), and LDL before and after delipidation (Granda and Scanu, 1965) all suspended in complete Freund's adjuvant (Difco Lab., Detroit, Mich.) were prepared in rabbits as described before (Scanu, 1965a). Rabbit anti human serum albumin was a commercial product (Hyland Laboratories, Los Angeles, Calif.). Quantitative immunoprecipitin reactions were studied according to Kabat and Meyer (1961). Immunoprecipitin reactions in prepurified agar gel (Bacto Agar, Difco Lab.) or agarose (Fisher Co., Chicago, Ill.) were conducted according to the double-diffusion method of Ouchterlony (1949) or the immunoelectrophoretic technique of Scheidegger (1955). Details were reported elsewhere (Scanu, 1965a).

For *amino acid analysis*, delipidated specimens of HDL were hydrolyzed in 6 N HCl according to Moore and Stein (1963). In the initial phase of the experimentation, specimens were hydrolyzed for 18, 24, 48, and 72 hr at 110° to estimate amino acid losses due to hydrolysis by extrapolation to zero time. They were of the order reported by Moore and Stein (1963), 5% for threonine and tyrosine and about 10% for serine. Separation of the amino acids was conducted by ion-exchange chromatography using a single column packed with Dowex 50-X8 (Chromobeads, type A, Technicon, Chauncey, N. Y.) in a Technicon automatic amino acid analyzer, according to Piez and Morris (1960). Cysteine and cystine were determined as cysteic acid after performic acid oxidation according to Moore (1963). Tryptophan, destroyed by acid hydrolysis, was determined spectrophotometrically according to Goodwin and Morton (1946). The method of Laki *et al.* (1954) modified by Hirs *et al.* (1954) was used for determination of amide ammonia.

The technique of Sanger (1945, 1949) as described by Fraenkel-Conrat *et al.* (1955) was used for the determination of the N-terminal residues. Standard DNP-amino acids were obtained commercially (Nutritional Biochemical Corp., Cleveland, Ohio). The COOH-terminal residues of delipidated HDL products were determined according to Niu and Fraenkel-Conrat (1955).

Lipid Analysis. Lipoproteins were extracted with chloroform–methanol, 2:1 v/v, and the extracts were chromatographed in 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 (Abell *et al.*, 1952), free cholesterol (Sperry and Webb, 1950), and triglycerides (Van Handel and Zilversmit, 1957). Phospholipids were eluted with absolute methanol. The factor 25 was used to convert lipid P (Fiske and Subbarow, 1925) into phospholipids. Separation of phospholipid fractions was obtained by ascending thin layer chromatography in silica gel H (Brinkman Instrument Co., Westbury, N. Y.) using chloroform–methanol–water, 8:35:5 as the developing solvent. Fractions were detected by the molybdenum blue spray of Dittmer and Lester (1964). Purified phospholipid standards were obtained commercially (Applied Science Lab., State College, Pa.). For quantitative analysis, the spots were detected by iodine vapors (Mangold, 1961), and the gel powder was scraped off the glass and aspirated into special glass funnels (Kopp Laboratory Supplies, New York, N. Y.) from which the phospholipids were eluted with absolute methanol.

Carbohydrate Analysis. In acid hydrolysate of the delipidated HDL protein the following determinations were performed: neutral sugars (Roe, 1955), L-fucose (Dische and Shettles, 1948), D-galactose and D-mannose (Stahl and Kaltenbach, 1961), hexosamine (Boas, 1953), and sialic acid (Svennerholm, 1957). Details are reported elsewhere (Scanu, 1966).

Iodination Procedure, Radioautographic and Counting Technique. Labeling of the protein moiety of HDL with ^{125}I was conducted according to a modification of the method of McFarlane (1958). A solution of iodine monochloride was prepared according to Vogel (1961). The stock solution contained 0.42 mg of iodine/ml and was 0.01 N with respect to HCl and 1 M with respect to NaCl. It was diluted before use in glycine buffer, pH 9.0, to contain 1 atom of iodine/mole of protein. A molecular weight of 75,000 (Scanu *et al.*, 1958a) was assumed for the HDL protein. Carrier-free $Na^{125}I$, reagent grade, was obtained on the day of iodination from Volk Chemical Co. (Skokie, Ill.). In a typical experiment, the diluted ICl solution in a volume usually not exceeding 0.2 ml was rapidly mixed with 0.1 ml of ^{125}I (specific activity of 1 mcurie/ml) and to this mixture 0.1 μmole of protein in 1 ml of glycine buffer, pH 9.0, was added. All operations were conducted at 4°. Free iodine was removed through an anion-exchange resin (Ioresin, Abbott Labs., Chicago,

[1] Abbreviations used: HDL, high-density lipoprotein; VHDL, very high-density lipoprotein; SDS, sodium dodecyl sulfate.

TABLE II: Per Cent Distribution of Protein, Cholesterol, Phospholipid, and Triglycerides of HDL_2 and HDL_3 and of Top and Bottom Milliliters after Recentrifugation for 48 Hours.

Lipoprotein Class	Distribution, mg/100 ml of Serum Av of 6 Detrmn ± SE[a]			
	Total Protein	Phospholipids Whole	Total Cholesterol	Triglycerides
HDL_2	38.0 ± 1.6 (40.6)	35.0 ± 1.5 (36.2)	17.0 ± 1.0 (18.7)	3.8 ± 0.8 (4.5)
HDL_3	150.0 ± 3.4 (56.0)	70.0 ± 2.6 (26.2)	33.3 ± 1.9 (12.9)	14.2 ± 1.0 (5.1)
		Top		
HDL_2	20.0 ± 1.4 (42.6)	16.6 ± 1.0 (35.3)	8.80 ± 0.8 (18.7)	1.6 ± 0.3 (3.4)
HDL_3	107.6 ± 4.1 (49.6)	66.0 ± 2.8 (30.5)	37.10 ± 1.8 (17.1)	6.2 ± 0.9 (2.8)
		Bottom		
HDL_2	0.9 ± 0.2 (96.6)	0.04 ± 0.01 (3.4)		
HDL_3	3.7 ± 0.6 (96.8)	0.13 ± 0.07 (3.2)		

[a] Values in parentheses indicate percentages.

Ill.) column, 0.5 cm in diameter and 2 cm high, previously washed with distilled water and equilibrated with Tris buffer, pH 8.6, 0.1 ionic strength, also used as eluent. Monitoring of the effluent allowed collection of the radioiodinated protein in a 1–2-ml volume. Specific activity values were of the order of 10 μcurie/μmole of protein. The iodinated material exhibited the same electrophoretic and immunochemical properties as the unlabeled one.

The radioiodinated protein, usually 0.1-ml aliquots, was dissolved in 15 ml of Bray's solution (1960) and counted in polyethylene vials in a Packard liquid scintillation spectrometer, Model 2000.

For radioautographs, Kodak X-ray films without screen were used. Time of contact between these films and the gels containing the radioactive protein varied between 3 and 5 days. The films were developed and fixed using commercial solutions (Kodak Co., Rochester, N. Y.).

Total protein determinations were made according to the method of Lowry *et al.* (1951). In acid hydrolysates of the HDL protein, microdetermination of Kjeldahl nitrogen was performed according to Lang (1958).

Chemicals were reagent grade. All organic solvents were freshly distilled. Urea and sodium dodecyl sulfate (SDS) were recrystallized from ethanol before use.

Results

Comparative Effects of Ultracentrifugation on HDL_2 and HDL_3. When purified specimens of HDL_2 and HDL_3 (Figure 1, II) were respun for 48 hr, at 114,480*g* (step II–III, Figure 1), 74% of HDL_2 protein and 72% of HDL_3 protein was recovered in the top milliliter

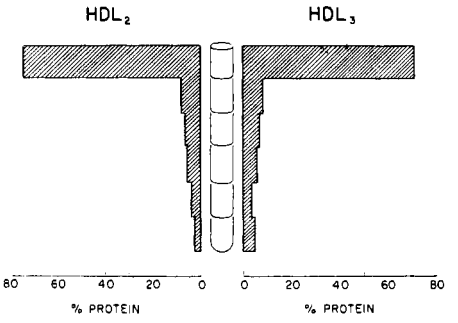

FIGURE 2: Protein distribution of ultracentrifugal isolates of HDL_2 and HDL_3 after 48-hr recentrifugation in a Spinco Model L ultracentrifuge, 40.3 rotor, 114,480 *g*, 16°. Protein content was estimated by absorbancy readings at 280 mμ and by the Lowry (1951) method.

(Figure 2). The remainder was distributed between the bottom milliliter and the intermediate layers. Similar results were obtained using HDL_2 or HDL_3, labeled in the protein moiety with ^{125}I. The specific radioactivity (2000 dpm/μg of protein) was the same in all layers. In all of these experiments 1–2% of the original lipoprotein protein remained unaccounted for and was related to losses in the walls of the preparation tube.

For analysis, top and bottom milliliters derived from 50 ml of serum were pooled. Because of the low protein content, the infranates were concentrated against Carbowax (polyethylene glycols, Union Carbide Co., New York, N. Y.) and then dialyzed against 0.15 M NaCl, pH 7, containing 0.05% EDTA. No protein losses were noted in this step.

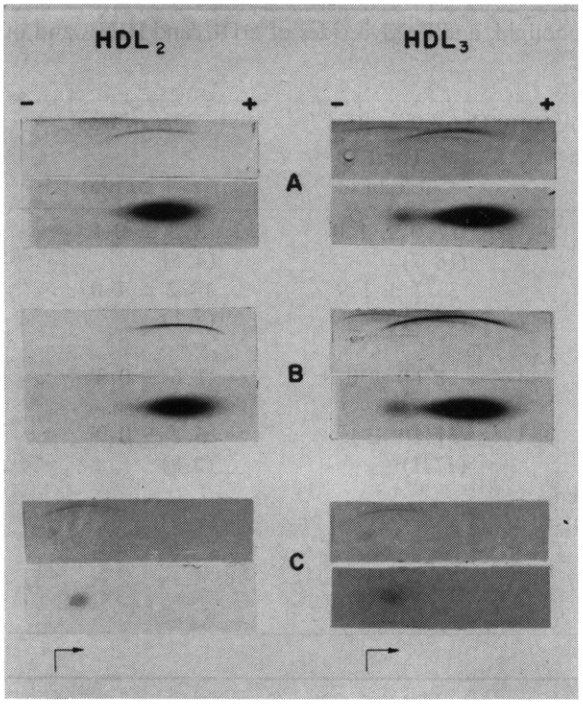

FIGURE 3: Agar electrophoresis and immunodiffusion patterns of HDL_2 and HDL_3, whole, top, and bottom, against anti-HDL sera. Agar electrophoresis: Veronal buffer pH 8.6, 6 v/cm, 40 ma, 20°. Antibody: antigen: protein ratio 10:1, time of diffusion 16 hr, 4°. Staining: amido Schwartz.

TABLE III: Flotation and Sedimentation Constants of HDL_2 and HDL_3, Whole, Top, and Bottom.

Lipoprotein Species	HDL_2		HDL_3	
	$S_{f(1.21)}$	$S^0_{20,w}$	$S_{f(1.21)}$	$S^0_{20,w}$
Whole	6.0	4.79	3.78	5.00
Top	5.90	4.74	3.82	4.98
Bottom	—	4.30	—	4.20

The chemical analysis of purified HDL_2 and HDL_3 (Table II, whole), in accord with previous reports (Lindgren and Nichols, 1960), showed a higher protein percentage in HDL_3 than in HDL_2. After ultracentrifugation, top milliliters of HDL_2 and HDL_3 had a percentage distribution of protein and lipid similar to that of whole HDL_2 or HDL_3. The bottom milliliters contained approximately 3% phospholipids, predominantly as lecithin. In the intermediate fractions the percentage protein–lipid distribution was similar to that observed in the top milliliter.

As shown in Table III, no significant differences in flotation and sedimentation constants were noted between whole HDL_2 and HDL_3 and their corresponding top fractions. Molecular weights were also in the same range (Table IV). The bottom fractions failed to float at density 1.21 and sedimented as a single symmetrical peak with $S^0_{20,w}$ of about 4.

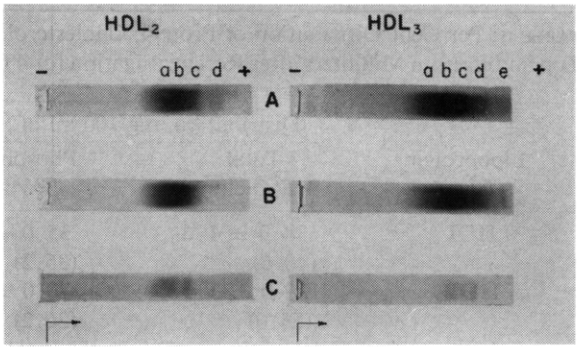

FIGURE 4: Starch gel electrophoretic patterns of HDL_2 and HDL_3, whole, top, and bottom. A, whole; B, top; C, bottom. Conditions of electrophoresis: discontinuous Tris–boric acid buffer, pH 8.6, 7.3 v/cm, 14 ma, 20 hr, 4°. Staining: amido Schwartz.

By agar electrophoresis whole HDL_2 and HDL_3 had in common a major fast band which stained for protein and lipid (Figure 3). In addition, HDL_3 exhibited a lipid-fast protein-stainable band, which remained close to the origin. Immunoelectrophoretic analysis using anti-HDL sera put into evidence a major and a minor arc of precipitation in HDL_3. Only the major arc was seen in HDL_2. HDL_2 (top) and HDL_3 (top) had agar electrophoretic and immunodiffusion patterns qualitatively similar to those of whole HDL_2 or HDL_3. The amount of the slow component in HDL_3 (top) was decreased with respect to whole HDL_3 (Figure 3). HDL_2 (bot) and HDL_3 (bot) exhibited a similar immunoelectrophoretic pattern characterized by a single arc of precipitation, close to the origin and stainable only for protein (Figure 3). In experiments where HDL_2 or HDL_3 had been labeled with ^{125}I, radioautography of the immunoelectrophoretograms of top and bottom milliliters gave patterns similar to those detected by protein stain.

By starch gel electrophoresis whole HDL_2 and HDL_3 (Figure 4A) had four bands in common (a, b, c, d). HDL_3 had an additional fast component, e, which, compared with the other bands, was only faintly stained for lipid. After 48 hr of ultracentrifugation, this e component was less prominent. The bottom fractions of HDL_2 or HDL_3 had similar patterns characterized by two major bands stained only for protein.

Comparative Analysis of Top and Bottom Milliliters of HDL_2 and HDL_3 after Delipidation with Ethanol–Diethyl Ether. Because of the marked difference in lipid between top and bottom milliliters of HDL_2 and HDL_3 these fractions, prior to analysis, were delipidated by an ethanol–diethyl ether mixture at −10° (see Methods). All delipidated fractions exhibited identical solubility properties characterized by: (a) formation of a gel in the region of the isoelectric point (pH 4.6), (b) partial

TABLE IV: Molecular Weights of HDL_2 and HDL_3, Whole, Top, and Bottom.

Liproprotein Species	HDL_2	HDL_3
Whole	$3.6 \times 10^5 \pm 2.1 \times 10^4$	$1.7 \times 10^5 \pm 1.2 \times 10^4$
Top	$3.6 \times 10^5 \pm 2.3 \times 10^4$	$1.7 \times 10^5 \pm 1.1 \times 10^4$
Bottom[a]	$3.8 \times 10^4 \pm 1.2 \times 10^3$	$3.8 \times 10^4 \pm 1.6 \times 10^3$

[a] The partial specific volume of 0.723 corrected for the presence of 3% phospholipid was 0.733.

TABLE V: Amino Acid Composition of the Protein of Top and Bottom Fractions of HDL_2 and HDL_3.

	Moles of Amino Acids/100,000 g of Protein Av of 4 Determinations ± SE			
	HDL_2		HDL_3	
Amino Acid	Top	Bottom	Top	Bottom
Aspartic acid	57.4 ± 1.2	60.2 ± 1.0	58.4 ± 1.2	61.0 ± 1.0
Threonine	36.5 ± 1.0	37.0 ± 0.8	37.2 ± 0.9	36.4 ± 1.0
Serine	48.6 ± 0.8	48.4 ± 0.8	48.6 ± 0.6	47.9 ± 0.6
Glutamic acid	134.7 ± 1.0	141.1 ± 1.6	140.0 ± 1.3	136.8 ± 1.3
Proline	36.6 ± 0.8	37.0 ± 0.6	36.8 ± 0.7	37.1 ± 0.6
Glycine	32.2 ± 1.3	33.0 ± 1.2	32.8 ± 1.2	33.4 ± 1.3
Alanine	55.1 ± 1.3	55.3 ± 1.4	56.4 ± 1.2	54.1 ± 1.4
Valine	46.1 ± 1.3	48.0 ± 1.2	46.4 ± 1.4	47.3 ± 1.3
Cystine	3.8 ± 0.2	3.6 ± 0.3	3.5 ± 0.3	3.5 ± 0.3
Methionine	7.5 ± 0.6	7.5 ± 0.5	7.0 ± 0.6	6.8 ± 0.4
Isoleucine	6.2 ± 0.8	6.3 ± 0.7	6.5 ± 0.7	6.2 ± 0.7
Leucine	105.2 ± 2.2	104.8 ± 2.5	103.6 ± 2.2	104.2 ± 2.0
Tyrosine	29.1 ± 1.6	30.6 ± 1.4	30.6 ± 1.5	30.2 ± 1.4
Alanine	28.2 ± 0.7	26.9 ± 0.6	27.2 ± 0.6	27.3 ± 0.6
Lysine	69.5 ± 2.4	70.1 ± 2.0	69.2 ± 2.2	70.3 ± 2.0
Histidine	12.5 ± 0.2	10.9 ± 0.3	11.4 ± 0.4	11.0 ± 0.3
Arginine	33.1 ± 0.8	34.1 ± 0.6	33.4 ± 0.7	33.0 ± 0.6
Tryptophan	23.4 ± 1.8	23.8 ± 1.6	22.9 ± 1.0	23.0 ± 1.6
Amide NH_3	105.2 ± 4.3	104.9 ± 4.1	102.6 ± 3.6	104.6 ± 4.0

solubility at pH <4 and complete solubility at pH >8.6, (c) solubilization of the insoluble residues by either 4–6 M urea or 0.1–0.5 M sodium dodecyl sulfate.

All delipidated fractions after addition of SDS (0.05 M) or acetylation (Fraenkel-Conrat, 1957) could be reduced to a single ultracentrifugal component with an $s^0_{20,w}$ value of 2.0 and a molecular weight of 21,500 ± 1500.

The protein from HDL_2 (top), HDL_3 (top), HDL_2 (bot), and HDL_3 (bot) had the same amino acid composition (Table V) and NH_2-terminal (aspartic acid) and COOH-terminal (threonine) residue. Their carbohydrate content was on the average of 3.3 mg/100 mg of HDL protein (by weight) with the following percentage distribution: glucosamine 45.0, methyl pentose 19.7, D-galactose–D-mannose 25.7, sialic acid 9.5.

After complete solubilization in Tris buffer, pH 8.6, ionic strength 0.1, the delipidated products showed by agar electrophoresis a single band, close to the origin, stainable only for protein. They gave also a similar immunoelectrophoretic pattern, characterized by a major and two minor arcs of precipitation giving a reaction of partial identity (Figure 5). By the double-diffusion technique of Ouchterlony, using anti-HDL sera, each of the fractions from either HDL_2 or HDL_3 gave an identical pattern with two distinct bands of precipitation.

To further ascertain the immunochemical relationship between top and bottom fractions, the latter were pooled, concentrated to a protein concentration of approximately 3 mg/ml, dialyzed against several changes of 0.15 M NaCl, pH 7, containing 1% EDTA, and used for antibody production in the rabbit (see Methods). When antisera so obtained were tested against HDL_2 and HDL_3 fractions, the immunoprecipitin reactions in agar gel were indistinguishable from those obtained with rabbit anti-HDL sera.

Effect of Repeated Ultracentrifugation on HDL_2 or

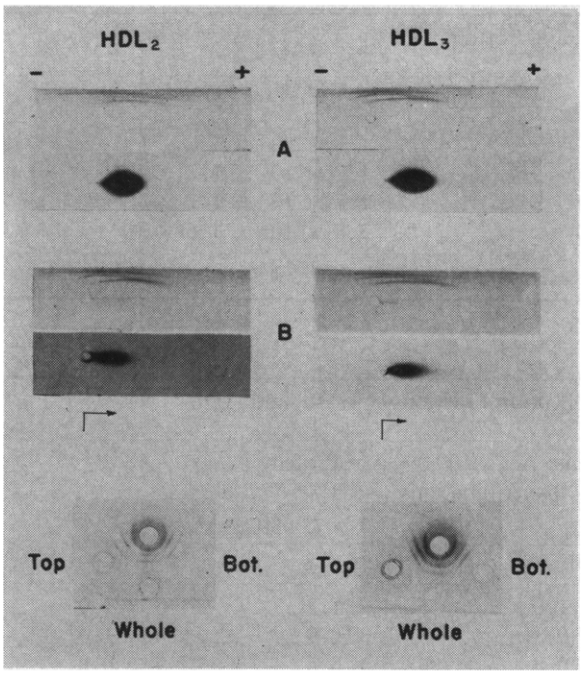

FIGURE 5: Agar electrophoresis and immunodiffusion patterns of HDL_2 and HDL_3, top and bottom, after delipidation with ethanol–diethyl ether. A, top; B, bottom. Conditions of electrophoresis: same as in Figure 3. Antiserum: anti-HDL. Two Ouchterlony patterns are shown.

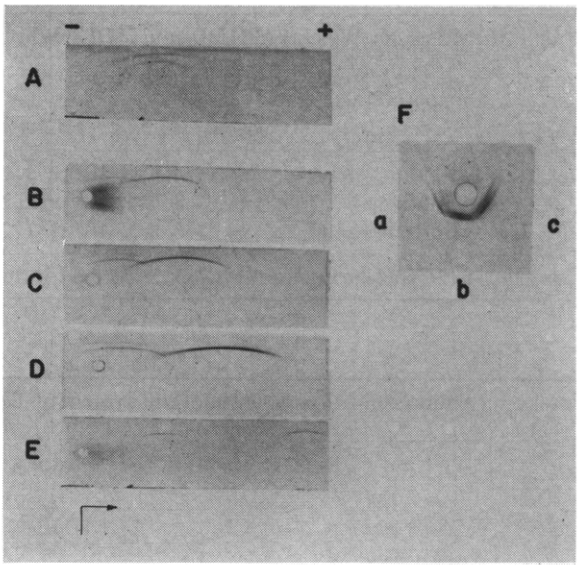

FIGURE 6: Immunodiffusion patterns of density >1.21 serum residues and whole serum against anti-HDL. A, density >1.21 residues; B, fresh, untreated whole serum; C, serum after freezing and thawing; D, serum in 6 M urea; E, serum in 0.05 M sodium dodecyl sulfate. Ouchterlony pattern at right. Conditions of electrophoresis: same as in Figure 3.

HDL_3. When top milliliters from either HDL_2 or HDL_3, obtained as outlined in previous sections, were pooled and then centrifuged again (114,480*g*, 48 hr, 16°), the distribution of HDL protein in the preparative tubes was similar to that shown in Figure 2. A close similarity between the proteins of top and bottom milliliters was again demonstrated. The same procedure was conducted for a total of five ultracentrifugal runs. In each instance, a small percentage of a lipid-poor HDL protein was demonstrated in the bottom milliliter.

Studies on the Density >1.21 Residues. Each milliliter of bottom residue (see Figure 1, step $I–I_A$) contained: 72 mg of protein, 0.30 mg of phospholipid, 0.12 mg of cholesterol, and 0.27 mg of triglycerides. Lysolecithin composed about 50% of the whole phospholipids. By immunoelectrophoresis, anti-HDL sera elicited one or two arcs of precipitation close to the origin (Figure 6A) and stainable only for protein. Anti low-density lipoprotein (LDL) sera elicited no immunoprecipitin reaction. A reaction between anti-HDL sera and density >1.21 residue was also obtained by the Ouchterlony technique (Figure 6E). The two lines of precipitation fused with the two antigenic components of delipidated HDL.

Binding Capacity for Serum Lipids of the Protein Components of the HDL_3 (bot) and Density >1.21 Residues. These fractions, obtained by ultracentrifugation (see Methods), were labeled with ^{125}I and tested for their property to bind lipids according to the technique described previously (Scanu and Hughes, 1960). Briefly, mixtures of 100 μg of labeled protein and 1 ml of serum were incubated in an atmosphere of nitrogen with gentle agitation for 30 min at 37° and centrifuged in a Spinco Model L ultracentrifuge 40.3 rotor, 114, 480*g* at *d* 1.063 and 1.120 for 24 hr, 16°. Aliquots (1 ml) were then aspirated from top to bottom and analyzed for protein content and radioactivity. The labeled products, which alone sedimented at either *d* 1.063 or 1.120, floated at *d* 1.120 (not at *d* 1.063) as indicated by the recovery of about 75% of the total radioactivity in the top milliliter. The behavior of these top fractions by analytical ultracentrifugation, agar and starch gel electrophoresis, and immunodiffusion techniques in agar gel was that of HDL_2 (*d* 1.063–1.120). In control experiments, human serum albumin labeled with ^{125}I and mixed with whole serum did not interact with lipids, as suggested by the failure to float at either *d* 1.063 or 1.120.

The Reaction of Whole Serum with Anti-HDL Sera. Normal sera were studied under various experimental conditions: (1) immediately after separation from clotted blood by centrifugation in a Sorvall RC-2 Superspeed refrigerated centrifuge SS-34 rotor at 12,100*g* for 30 min, (2) after separation from retracted blood clots without centrifugation, (3) after a process of freezing (−10°) and thawing (20°), (4) after making the serum 6 M in respect to urea or 0.5 M in respect to sodium dodecyl sulfate. By immunoelectrophoresis, the reaction between fresh, untreated sera and anti-HDL resulted in the formation of two arcs of precipitation; the major one had migration characteristics of α_1-globulin, was

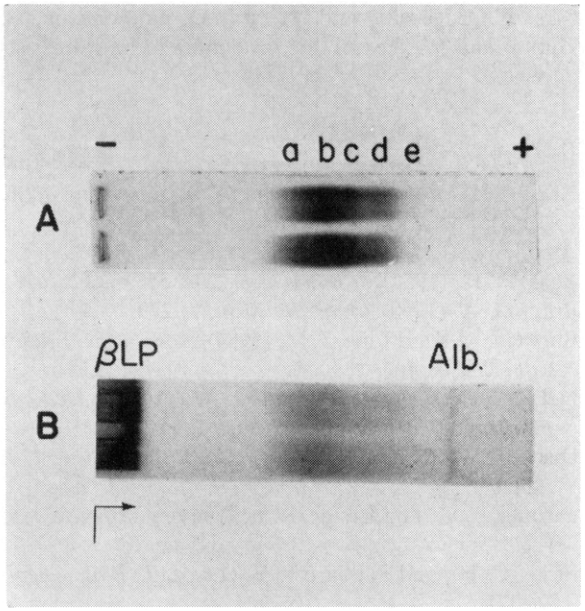

FIGURE 7: Starch gel electrophoretic patterns of whole serum and isolated HDL. A, HDL; B, whole serum. Specimens were dialyzed against either NaCl, 0.15 M NaCl, pH 7, or Tris buffer, pH 8.6, 0.1 M. Conditions of electrophoresis: same as in Figure 4. Staining: oil red O.

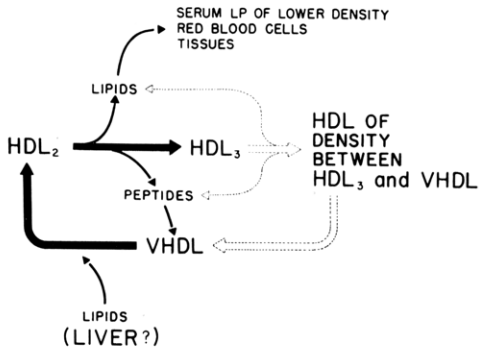

FIGURE 8: Hypothetical view of the "HDL cycle." It is postulated that during $HDL_2 \rightarrow HDL_3$ conversion, release of lipid-poor peptides contribute to the formation of a very high-density lipoprotein (VHDL). The lipid lost during the process is transferred to an "acceptor" which may be represented by serum lipoproteins of the very low- or low-density class, red cells or other blood elements, and/or body tissues. More VHDL may be formed during the conversion of HDL_3 to lipoproteins of higher densities. VHDL would become HDL_2 by uptake of lipids, a process which may occur in the liver.

stainable for protein and lipid, and most probably represented HDL; the second arc close to the origin could be detected only by the protein stain and, by analogy with the patterns reported in the previous sections, probably represented a delipidated product of HDL (Figure 6B). Repeated freezing and thawing of the serum or addition of urea led to a significant increase of the intensity of the second precipitin band (Figure 6C). Similar results were obtained with serum treated with urea (Figure 6D). In the presence of SDS, because of the increased surface charge secondary to the addition of the detergent, the two arcs of precipitation exhibited a high electrophoretic mobility. The fast band was far ahead of albumin (Figure 6E).

By the Ouchterlony technique, whole untreated serum, density >1.21 residues and HDL_3, tested against anti-HDL sera, had two antigenic components in common (Figure 6F).

To compare the electrophoretic properties of HDL in its native state and after ultracentrifugal isolation, whole serum and purified HDL both after dialysis against either 0.15 M NaCl, pH 7, or Tris buffer, pH 8.6, 0.1 M, were separated by starch gel electrophoresis and the patterns stained for lipids. The two preparations yielded similar patterns (Figure 7). It should be noted that the albumin band, identified by immunoprecipitation against rabbit anti human serum albumin, exhibited positive lipid staining.

Discussion

The present studies, in agreement with a previous report (Scanu and Hughes, 1960), have shown that recentrifugation in an angle rotor of ultracentrifugal isolates of serum HDL_2 or HDL_3 leads to the demonstration of a number of lipoprotein species which appear to assume an isopycnic distribution throughout the preparative tube. The lipoprotein species which was recovered in the top milliliter represented about 70% of the original material and was similar to it in all physical and chemical parameters employed (flotation and sedimentation constants, percentage of protein–lipid distribution). On the other hand, the lipoprotein recovered in the bottom milliliter, representing about 3% of the starting material, was markedly impoverished of its complement of lipid and had physical properties compatible with its lipid-poor state. The difference between the two lipoprotein species, in the top and bottom milliliters, appeared to reside in their lipid content, since their protein moiety was identical in terms of amino acid and carbohydrate composition and N-terminal, and COOH-terminal residues. Further, upon delipidation both exhibited identical patterns by agar gel electrophoresis and immunodiffusion techniques. The lipid-poor, heavy lipoprotein, recovered in the bottom milliliter, having immunochemical properties of HDL protein, will be referred to as very high-density lipoprotein (VHDL).

The demonstration of a VHDL in infranates of either HDL_2 or HDL_3 is of interest. Up to date, partially delipidated products of HDL had been obtained *in vitro* through the action of either organic solvents or surfactants (Scanu, 1965b). The fact that they have now been found through routine procedures of preparative ultracentrifugation suggests that they are probably components of native HDL, where they may exist in labile association with a lipid-rich moiety. Support for this hypothesis is the observation that dissociation between

a lipid-rich and a lipid-poor component in HDL can be achieved by agar electrophoresis, ether treatment (Hayashi *et al.*, 1959), freezing and thawing, or addition of urea (Levy and Fredrickson, 1965).

The present studies have shown that the protein moieties of HDL_2, HDL_3, and VHDL can be reduced, upon acetylation or treatment with SDS, to subunits of an average molecular weight of 21,500. Units about twice this size have been reported by Shore and Shore (1962) for human serum HDL_2 apoprotein. Differences in the amounts of SDS employed in the two studies may have accounted for the results. From the values of protein mass and molecular weight given in Tables IV and II, it can be estimated that HDL_2, HDL_3, and VHDL contain an average of 7, 5, and 2 subunits, respectively. This leads to the suggestion that in the process of $HDL_2 \rightarrow HDL_3$ conversion loss of lipid is associated with release of peptide chains which then contribute to the formation of the VHDL apoprotein. Whether this process is a simple ultracentrifugal artifact as suggested by Levy and Fredrickson (1965) or may also be operative *in vivo* cannot be defined by the present experiments. An answer to this question is likely to be obtained from comparative studies on serum HDL isolated by means other than ultracentrifugation, such as preparative column electrophoresis or chromatography, Cohn's alcohol fractionation, etc. Indirect evidence supporting the "*in vivo*" occurrence of the $HDL_2 \rightarrow HDL_3$ conversion derive from our own immunochemical data (Figure 6) and those of Burnstein and Fine (1964) showing that a lipid-poor form of HDL is present in fresh, uncentrifuged sera. A small amount of partially delipidated HDL, analogous to VHDL, has also been reported in fresh serum by Levy and Fredrickson (1965). Further, variations in the ratio of HDL_2–HDL_3 have been noted in various pathological states (Gofman *et al.*, 1954), and these may well reflect a derangement of the process of $HDL_2 \rightarrow HDL_3$ conversion. These data and the observation that VHDL may undergo relipidation suggest that HDL protein subunits lose and acquire lipid in cyclic fashion. A speculative view on the postulated "HDL cycle" is summarized in Figure 8.

The concept that the apoproteins of HDL_2 and HDL_3 are made of a number of similar, although not necessarily identical subunits, receives support by the polymorphism of these HDL subclasses by starch gel electrophoresis (Figure 4 and 7). An interpretation, which is suggested by the solubility studies of HDL protein and is compatible with reported chemical and physical data on HDL, is that the various electrophoretic bands represent a series of molecular aggregates of the same subunit. Such an interpretation agrees with recent findings (A. Scanu, unpublished observations) showing that the acetylated subunit of HDL, having a molecular weight of about 20,000, is immunologically homogeneous.

Acknowledgment

The authors are greatly indebted to Mrs. P. Brown, Mrs. C. Edelstein, and Messrs. D. Edmondson, S. Gupta, and W. Reader for invaluable technical assistance.

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The Paraffin Hydrocarbons of Wool Wax. Homologous Series of Methyl Alkanes*

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ABSTRACT: Several families of homologous paraffins with single methyl branching have been identified in wool wax. They were found to the extent of about 4.5% of the total saturated hydrocarbons and included compounds with 17–44 carbon atoms. For compounds of 30–44 carbon atoms with even numbers of carbon atoms, the methyl branch occurred mainly on carbon 13. Lesser amounts of the isomers branched at the 11 and 15 positions were found, with minor amounts of isomers branched at odd carbon atoms of higher number for the longer chain homologs. The general formula may be written: $C_nH_{2n+1}CH(CH_3)C_mH_{2m+1}$, where n is any even number from 10 to 20 and m is greater than n.

The homologs of 31–43 carbon atoms with odd numbers of carbon atoms consisted of mixtures of isomers branched on either even or odd carbon atoms, with the methyl branch occurring mainly on carbon 12. The general formula for these homologs may be written: $C_nH_{2n+1}CH(CH_3)C_mH_{2m+1}$ where n is any number from 11 to 17 and m is greater than n. The hydrocarbons with 17–29 carbon atoms were present in considerably smaller amounts and were more difficult to resolve from other classes in the gas–liquid partition chromatogram. These compounds appeared to be mixtures of isomers with single methyl branches at nearly every position but with the predominant isomers branched on carbon atoms 8–11.

The relative abundance of homologs of the series of normal, 2-methyl, and 3-methyl paraffin hydrocarbons for a sample of wool wax was reported previously (Mold *et al.*, 1964). In that report, the presence of homologous cyclic substituted paraffins and at least two other series of branched paraffins was noted. One of these groups of homologs (designated Series II in the earlier report) has now been separated and characterized by gas-liquid partition chromatography and mass spectrometry.

Results

The procedures used for the preliminary separation of the wool wax were the same as described in our earlier report (Mold *et al.*, 1964). Briefly, this involved chromatographic fractionation of the hexane extract on alumina to separate the saturated hydrocarbons from

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