Studies of the Composition and Structure of Serum Lipoproteins. Isolation and Characterization of Very High Density Lipoproteins of Human Serum*

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ABSTRACT: High density lipoproteins of human serum, *d* 1.210–1.250 g/ml ($VDHL_1$), have been isolated from a parent fraction (*d* >1.210 g/ml) by preparative ultracentrifugation at 165,000*g* for 44 hr, utilizing cesium chloride, *d* 1.250 g/ml. The isolation of $VHDL_1$ represents the first demonstration of a very high density lipid–protein complex of reproducible chemical composition and immunochemical homogeneity. It was characterized by the determination of sedimentation and diffusion coefficients, hydrated density, molecular weight, peptide pattern, and lipid and protein analyses. The $VHDL_1$ and the *d* >1.250 g/ml (bottom) fraction containing $VHDL_2$ and serum proteins differed considerably in respect to the nature and composition of lipid moieties. The $VHDL_1$ contained 15–25% of the total phospholipid of the parent (*d* >1.210 g/ml) fraction (but only 1% of the protein). The $VHDL_1$ lipid was mainly phospholipid (77%). The principal phospholipid was lecithin with a small amount of lysolecithin. The fatty acid content of $VHDL_1$ was negligible. The cholesterol:phospholipid ratio was 0.12. The lipid in the *d* >1.250 g/ml fraction (presumably present as $VHDL_2$) was also principally phospholipid (54%). However, the chief phospholipid was lysolecithin with a smaller amount of lecithin. Unesterified fatty acids constituted a large portion (39%) of the *d* 1.250 g/ml lipid. The exchange reactions, peptide >patterns, and immunochemical studies showed that $VHDL_1$ contained the same protein moiety as high density (α) lipoproteins, namely apolipoprotein A. The protein components of the *d* >1.250 g/ml fraction ($VHDL_2$) consisted of traces of apolipoprotein A, albumin, and other globular proteins. It is suggested that very high density lipoproteins consist of lipid–protein complexes separable into two major groups characterized by the presence of apolipoprotein A and albumin as lipid-containing proteins. The first group of these complexes (of which $VHDL_1$ are representative) is probably a continuation of, or the upper hydrated-density portion of, a polydisperse system of HDL. The second group ($VHDL_2$) has albumin–fatty acid and albumin–lysolecithin complexes.

When human serum is subjected to ultracentrifugation in a solvent of density 1.21 g/ml at 105,000*g* for 17 hr or more, most of the lipoproteins undergo flotation. However, approximately 40–50% of total serum unesterified fatty acids bound to albumin (Lindgren *et al.*, 1955; Fredrickson and Gordon, 1958), 8–15% of the total serum phospholipid, and small amounts of triglyceride and cholesterol undergo sedimentation (Hillyard *et al.*, 1955; Havel *et al.*, 1955; Furman *et al.*, 1956; Bradgon *et al.*, 1956). Phillips (1959) reported that the principal phosphatide in the *d* > 1.210 g/ml fraction is lysolecithin which accounts for about one-half of total phospholipid in this fraction and about one-half of the total lysolecithin of serum. These very high density lipoproteins (VHDL)[1] are soluble in water and ethanol–acetone, precipitable with zinc hydroxide, and nondialyzable against 0.15 M sodium chloride (Havel *et al.*, 1955). They migrate with the α_1-globulin and albumin fractions in starch block electrophoresis (Kunkel and Trautman, 1956). Recently it has been shown by immunological methods (Levy and Fredrickson, 1965; Scanu and Granda, 1966) that the *d* > 1.210 g/ml fraction of serum contains a protein identical with the protein moiety of high density lipoproteins (HDL). In a study on trans-

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† Work done during tenure of a Cardiovascular Research Traineeship, supported by U. S. Public Health Service Grant HTS-5403. Present address: University of Michigan, Department of Medicine, Ann Arbor, Mich.

[1] Abbreviations used: LDL, low density lipoproteins; β-lipoproteins, lipoproteins of *d* 1.006–1.050 g/ml (1.050 g/ml rather than the conventional 1.063 g/ml was selected as the upper density limit for the β-lipoproteins to minimize contamination with α-lipoproteins); HDL, high density lipoproteins, α-lipoproteins, lipoproteins of *d* 1.095–1.210 g/ml (1.095 g/ml was selected to minimize contamination with β-lipoproteins); $HDL_{1.185}$, a subfraction of high density lipoprotein of *d* 1.185–1.210 g/ml; VHDL, very high density lipoproteins, liporproteins of *d* >1.210 g/ml; $VHDL_1$, very high density lipoproteins of *d* 1.210–1.250 g/ml; $VHDL_2$, very high density lipoproteins of *d* >1.250 g/ml; ApoA, apolipoprotein A, the protein moiety of HDL; ApoB, apolipoprotein B, the protein moiety of LDL.

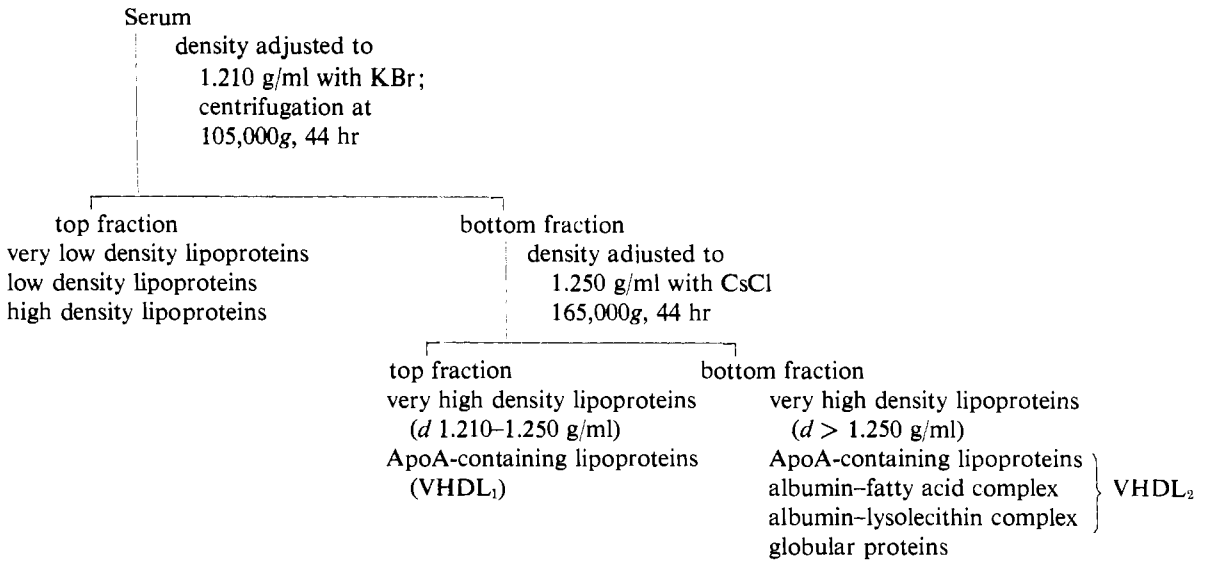

FIGURE 1: Procedure for the isolation of two fractions ($VHDL_1$ and $VHDL_2$) of very high density human serum lipoproteins ($d > 1.21$ g/ml).

port of serum lysolecithin, Switzer and Eder (1965) concluded that as much as 96% of the total serum lysolecithin is bound to albumin in the $d > 1.21$ fraction and suggested that the negligible amount of phospholipid, other than lysolecithin, is carried by a ultracentrifugally altered HDL. However, since VHDL have not been isolated as a single entity, the controversy regarding their origin, chemical composition, and the nature of their protein moiety or moieties has not been resolved.

The purpose of this study was to isolate and identify the protein and lipid moieties of VHDL in the serum of human subjects, to determine the chemical composition of VHDL, and to establish the relationship of VHDL to HDL (d 1.095–1.210 g/ml) by a comparison of these factors. This report describes the first isolation and physical-chemical characterization of an albumin-free VHDL fraction of d 1.210–1.250 g/ml ($VHDL_1$) and establishes its protein moiety as apolipoprotein A (ApoA, the protein moiety of HDL). These studies suggest that at least a part of VHDL represents the continuation of the polydisperse system of HDL and that the protein moieties of HDL and $VHDL_1$ are identical.

Experimental Section

Blood samples were obtained from healthy young men and women who had fasted overnight. The blood was allowed to clot and the serum was recovered by low-speed centrifugation at room temperature. Disodium EDTA was added to the serum to a final concentration of 0.01 M and individual or pooled samples were subjected to ultracentrifugation within 2–3 hr after venapuncture.

Isolation of High Density Lipoproteins. HDL was isolated from serum by preparative ultracentrifugation at 4°, utilizing the Spinco Model L ultracentrifuge and the no. 40 rotor. To avoid the contamination of HDL with low density lipoproteins (LDL) which were repeatedly demonstrated immunochemically and by the N-terminal amino acid analysis in the subfraction d 1.063–1.095 g/ml, serum density was adjusted to 1.095 g/ml by the addition of KBr and the LDL was separated by flotation at 105,000g for 22 hr. The density of the remaining bottom portion was then increased to 1.210 g/ml by the addition of KBr and centrifuged at 105,000g for 44 hr. The top layer was removed by a tube-slicing technique, resuspended in the NaCl–KBr solution (1.210 g/ml) and recentrifuged under identical conditions. This procedure was repeated two more times yielding HDL (d 1.095–1.210 g/ml) free of apolipoprotein B (ApoB)-containing LDL, albumin, and other globular proteins, as demonstrated by immunochemical analyses and starch gel electrophoresis. The preparation was then dialyzed against 0.15 M NaCl and 0.01 M disodium EDTA solution for 48 hr at 4°.

Isolation of $HDL_{1.185}$. By raising successively the solvent density of each remaining bottom fraction, five subfractions (d 1.095–1.110, d 1.110–1.130, d 1.130–1.150, d 1.150–1.185, and d 1.185–1.210 g/ml) of purified HDL were separated by flotation at 105,000g for 22 hr. The top layers obtained at each density were removed by a tube-slicing technique and purified by repeated ultracentrifugations in the KBr solution of appropriate density. After two recentrifugations at solvent density 1.210 g/ml the subfraction $HDL_{1.185}$ (d 1.185–1.210 g/ml) was dialyzed against 0.15 M NaCl and 0.01 M disodium EDTA solution for 48 hr at 4°

and characterized by lipid and protein analyses and by determination of hydrated density and molecular weight.

Isolation of Very High Density Lipoproteins. The isolation of VHDL is shown in Figure 1. Serum density was adjusted to 1.210 g/ml by the addition of KBr and VLDL and HDL were separated in the no. 40 rotor of the Spinco Model L ultracentrifuge by flotation at 105,000*g* for 44 hr. The tubes were cut through the clear zone approximately 1.5–2.0 cm below the solution surface with the Spinco tube slicer. Bottom fractions were combined and the density was raised to 1.250 g/ml by the addition of CsCl. The mixture was then centrifuged in the titanium rotor, Ti-50, of the Spinco Model L2-50 preparative ultracentrifuge at 165,000*g* for 44 hr. The top fractions accumulating in the upper centimeter of each tube were removed by the tube-slicing technique and resuspended in the *d* 1.250 g/ml CsCl solution. The combined bottom fractions containing *very high density lipoproteins of d > 1.250 g/ml* ($VHDL_2$) were concentrated to approximately one-half of their original volume by dialysis against a 40% dextran solution and stored at 4°. The top fractions were recentrifuged at 165,000*g* for 44 hr and the thin layers of floating lipoproteins were recovered by the slicing technique yielded albumin-free *very high density lipoproteins of d 1.210–1.250 g/ml* ($VHDL_1$).

Delipidization Procedures. Purified HDL was delipidized by extraction with ethanol–diethyl ether (3:1, v/v) followed by diethyl ether at −20°, by a modification (Sanbar, 1963) of a method of Scanu *et al.* (1958). Removal of lipids resulted in the isolation of the lipid-free protein moiety ApoA. Lipids present in $VHDL_1$ and $VHDL_2$ were extracted with chloroform–methanol (2:1, v/v) and the lipid extracts were washed with 0.58% NaCl solution (Folch *et al.*, 1957), using an amount equal to 20% of their volume.

Preparation of ApoA-^{131}I and HDL-^{131}I. The ApoA and HDL were labeled with iodine-^{131}I according to the procedure of Jones and Gardner (1962). The radioactive proteins were passed through an ion-exchange column, Ioresin (Abbott Laboratories, Chicago, Ill.), to remove excess iodine. Labeled ApoA and HDL showed the same migration rates during starch gel electrophoresis as their unlabeled counterparts and the radioactivity was associated with only the ApoA and HDL bands. When the protein moiety was precipitated with 50% trichloroacetic acid, more than 95% of the total radioactivity was recovered in the precipitate. The radioactivity of ApoA-^{131}I and HDL-^{131}I was measured in a well-type, sodium iodide crystal scintillation counter (Picker) with an efficiency of 52% and a background of about 140 cpm.

Electrophoresis. Horizontal starch gel electrophoresis was performed according to Smithies (1955) utilizing a discontinuous buffer system, pH 8.2 (Poulik, 1957). Gels were sliced longitudinally into two layers for separate staining of protein and lipid with Amido Black 10B (K & K Laboratories, Inc., Plainview, N. Y.) and Oil Red O (Allied Chemical & Dye Corp., N. Y.).

Immunochemical Methods. The immunochemical properties of lipoproteins were studied by double diffusion (Ouchterlony, 1953) in agar gels employing barbital buffer, pH 8.6, ionic strength 0.05. The microslides were covered with 1% Special Agar Noble (Difco Laboratories, Detroit, Mich.) in barbital buffer, pH 8.6, and the wells were stamped out with a commercial device (Gel Punch Set, LKB Instruments, Inc., Washington, D. C.). Precipitin lines of undiluted antigens and antibodies appeared usually after a development time of 12–16 hr at room temperature. The plates, allowed to develop for additional 24 hr, were washed several times with 0.15 M NaCl and distilled water, and dried at room temperature. They were stained for protein and lipid with Amido Black 10B and Oil Red O, respectively. Horse antihuman serum and rabbit antihuman serum HDL, LDL, albumin, 7S γ-globulin, α_1-acid glycoprotein, and fibrinogen (Behringwerke AG, Marburg an der Lahn, Germany) were used. The antibodies to HDL present in the rabbit serum gave two precipitin lines (Levy and Fredrickson, 1965) with human serum HDL (*d* 1.110–1.210 g/ml); antibodies to LDL gave a single precipitin line with human serum LDL (*d* 1.006–1.050 g/ml); antibodies to HDL and LDL showed no reaction with human serum albumin. Rabbit serum containing antibodies to human serum albumin, α_1-acid glycoprotein, 7S γ-globulin, and fibrinogen gave no reaction with HDL or LDL. The characterization of antibodies was performed by double diffusion in agar gels.

Ultracentrifugal Analyses. Refractometric ultracentrifugal analysis of $VHDL_1$ was carried out in a Spinco Model E ultracentrifuge equipped with a phase-plate schlieren diaphragm and an automatic temperature control unit. Plate measurements were made with a Nikon microcomparator (Nikon Co., Japan). The sedimentation coefficient was determined by centrifugation of a 0.15 M NaCl solution of $VHDL_1$ (5 mg/ml) in a single-sector cell at 56,100 rpm at 25°. The observed sedimentation coefficient was corrected to a value in water at 20° by the usual method (Schachman, 1957).

Hydrated density was determined by measuring the sedimentation coefficients at three different NaCl solution densities (1.006, 1.042, and 1.069 g/ml) and extrapolating (ηS as a function of ρ) to the solution density of zero sedimentation. The values for the densities and relative viscosities of solutions were taken from the International Critical Tables. The value for the apparent partial specific volume, $\bar{V}$, was obtained from the relation $\bar{V}$ = 1/hydrated density.

The apparent diffusion coefficient of $VHDL_1$ was determined in 0.15 M NaCl solution in a synthetic boundary cell, at rotor speed of 12,590 rpm, by applying the height–area method. The diffusion coefficient, corrected to zero time, was converted to standard conditions, $D_{20,w}$, in the usual manner (Schachman, 1957). The average molecular weight of $VHDL_1$ was calculated by utilizing the Svedberg equation ($M = RTs/D(1 - \bar{V}\rho)$) or by the Archibald's method of approach to sedimentation equilibrium as described by Schachman (1957).

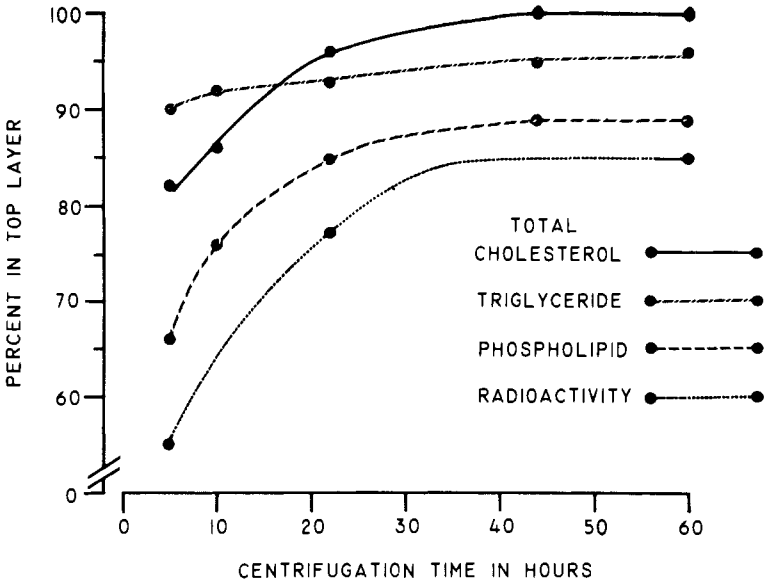

FIGURE 2: The per cent recovery of HDL lipid components and radioactivity as a function of the time of centrifugation of serum previously incubated with ApoA-^{131}I or HDL-^{131}I.

Proteolysis of Proteins and Two-Dimensional Separation of Peptides. The protein moieties obtained by total delipidization of HDL and $VHDL_1$ were dissolved in distilled water (5 mg/ml) and kept at 75–80° for 30 min. The solution was cooled to room temperature and digestion with pepsin (twice crystallized, Worthington Biochemical Corp., Freehold, N. J.) carried out for 24 hr at 28° employing an enzyme: substrate ratio of 1:40 in a solution adjusted to pH 2.0 by 0.1 N HCl. The pepsin digests were dried *in vacuo* over P_2O_5 and NaOH and the residual peptides were dissolved in distilled water. They were then subjected to two-dimensional paper chromatography and electrophoresis according to the techniques of Katz *et al.* (1959).

Lipid and Protein Analyses. Esterified and unesterified cholesterol was determined according to the method of Sperry and Webb (1950), lipid phosphorus by the method of Fiske and Subbarow (1925), the triglycerides by the procedure described by Van Handel and Zilversmit (1957), and the unesterified fatty acids by the method of Dole (1952). Quantitative analysis of individual phospholipids was performed by two-dimensional thin layer chromatography by a modification (Gustafson *et al.*, 1966) of the method described by Wagner *et al.* (1961). Phosphorus was determined by the micromethod of Gerlach and Deuticke (1963). The recovery of phosphorus was 90–95%. Protein was determined by the method of Lowry *et al.* (1951).

Results

The Effect of Centrifugation on Flotation Characteristics and Lipid Composition of HDL and VHDL. The data in Figure 2 indicate that the amounts of cholesterol, triglyceride, and phospholipid present in the top fraction (the uppermost centimeter of the contents of the ultracentrifuge tube) increased with duration of centrifugation until a plateau was reached after 44 hr of centrifugation at 105,000*g*. Since prolongation of centrifugation had no further effect on the distribution of lipids between top and bottom fractions, lipoproteins floating at d 1.21 g/ml after 44 hr were considered as HDL and those sedimenting at this density as VHDL. The latter contained 11% of the total serum phospholipid, 5% of the total serum triglyceride, and 2–3% of the cholesterol. A comparison of the lipid composition (Table I) of HDL with that of $d > 1.21$ fraction shows a greater content of unesterified fatty acids and a smaller content of unesterified and esterified cholesterol in the VHDL. The phospholipid content is slightly less, although it remains the major lipid component of VHDL. The difference between the HDL and $d > 1.21$ fractions with respect to the content of cholesterol and phospholipid is clearly demonstrable in the cholesterol:phospholipid (C:P) ratios for these fractions. Although the C:P ratio diminishes with increasing hydrated density and approaches zero in VHDL (Table I), triglyceride, on the other hand, shows no particular correlation with either cholesterol or phospholipid. Moreover, there was little difference between the HDL and VHDL in respect to triglyceride content. The presence of the albumin–fatty acid complex in the $d > 1.21$ fraction (Fredrickson and Gordon, 1958) accounts for the high content of unesterified fatty acids.

The phospholipid composition of the *d* 1.185–1.210 g/ml subfraction of HDL and of VHDL is shown in Table II. Lysolecithin accounted for more than 20% of the lipid phosphorus in VHDL and for only a relatively negligible amount in $HDL_{1.185}$. Since the relative amounts of phosphatidylethanolamine, phos-

TABLE I: Per Cent Lipid Composition of Human Serum High Density and Very High Density Lipoproteins.

Lipoprotein Fraction Density Range (g/ml)	Cholesterol Ester (%)	Unesterified Cholesterol (%)	Phospholipid (%)	Triglyceride (%)	Unesterified Fatty Acids (%)	Cholesterol: Phospholipid	Triglyceride: Phospholipid
HDL,[a] 1.095–1.210	24.1 (15.4–28.0)	8.1 (7.3–10.5)	51.1 (46.0–58.0)	11.9 (11.2–14.4)	4.8 (2.2–5.6)	0.62	0.23
VHDL,[a] $d > 1.210$	4.9 (4.1–5.2)	1.6 (1.3–1.8)	45.5 (35.5–50.3)	13.2 (11.5–20.5)	34.6 (26.5–42.5)	0.14	0.28

[a] HDL fraction represents the mean value of eight separate samples and VHDL the mean value of four separate samples obtained from fasting male and female subjects. The range of values is given in parentheses.

TABLE II: Per Cent Phospholipid Composition of Human Serum Very High Density Lipoproteins.[a]

Liproprotein Fraction Density Range (g/ml)	Lysolecithin (%)	Sphingomyelin (%)	Lecithin (%)	Phosphatidyl-inositol (%)	Phosphatidyl-ethanolamine (%)
$HDL_{1.185}$, 1.185–1.210	1.9 (1.0–2.7)	10.6 (9.8–11.4)	81.4 (78.2–85.9)	5.0 (4.8–5.2)	1.9 (1.1–2.3)
VHDL, $d > 1.210$	21.8 (17.0–25.6)	12.3 (9.1–15.4)	59.5 (57.9–62.4)	4.6 (2.0–8.5)	1.7 (1.0–2.0)
$VHDL_1$, 1.210–1.250	3.5 (3.0–4.8)	11.8 (9.2–13.8)	71.6 (69.9–73.3)	10.2 (7.5–12.8)	2.9 (2.0–3.7)
$VHDL_2$, >1.250	64.1 (57.0–70.8)	7.7 (4.8–10.3)	28.1 (24.3–32.6)	—	—

[a] Expressed as per cent of sum of individually determined phosphorus values. Figures for each fraction represent mean values of three separate samples.

phatidylinositol, and sphingomyelin in $HDL_{1.185}$ and VHDL were similar, the content of lecithin and lysolecithin concentrations in these two closely related lipoproteins varied inversely. The lysolecithin content of several VHDL preparations usually ranged between 17 and 22% although occasional values (not included in Table II) as high as 55% were noted, in agreement with results reported previously by Phillips (1959).

The Exchange Reaction between ^{131}I-Labeled HDL and VHDL. Previously reported *in vitro* (Scanu and Hughes, 1960) and *in vivo* (Furman *et al.*, 1964) studies involving incubation of ApoA-^{131}I with serum indicated similar patterns of ultracentrifugal distribution of radioactivity for HDL and VHDL. In both experiments, more than 30% of the radioactivity remained in the VHDL fraction. To gain further insight into the nature of the protein moiety of VHDL a series of exchange reactions was performed. Samples of 200 μg of HDL-^{131}I or ApoA-^{131}I (sp act. 0.2 μc/mg) were incubated with 11.0 ml of serum for 30 min at 20°. After adjusting the density to 1.21 g/ml by the addition of KBr, the incubation mixtures were centrifuged at 105,000*g*. The bottom fractions (VHDL) contained 23–37% of total radioactivity after centrifugation for 22 hr. However, after centrifugation of the same incubation mixtures for 44 hr the VHDL fraction contained only 10–15% of the total radioactivity. The distribution of HDL-^{131}I radioactivity between the top and bottom fractions paralleled that of the phospholipid content (Figure 2).

A 1.210 g/ml bottom fraction, containing VHDL and various proteins, was dialyzed against 0.15 M NaCl. The dialyzed mixture was labeled with ^{131}I in the usual manner and incubated with nonradioactive HDL for 30 min at 20°. The incubation mixture was centrifuged for 22 hr at d 1.21 g/ml to separate floating lipoproteins from other labeled proteins. The top fraction was resuspended in NaCl–KBr solution (1.21 g/ml) and twice recentrifuged under identical conditions to obtain purified HDL. Following the last centrifugation 71% of the remaining radioactivity was in the top fraction (HDL) and 29% in the bottom fraction. This distribution pattern is very similar to that obtained by the exchange reaction between HDL-^{131}I or ApoA-

TABLE III: Per Cent Composition of Human Serum Very High Density Lipoproteins.

Liproprotein Fraction Density Range (g/ml)	Cholesterol Ester (%)	Unesterified Cholesterol (%)	Phospholipid (%)	Triglyceride (%)	Unesterified Fatty Acids (%)	Protein (%)
$HDL_{1.185}$,[a] 1.185–1.210	10.4 (8.5–13.2)	2.2 (1.8–2.5)	23.4 (18.5–26.8)	4.2 (3.2–5.8)	1.3 (1.2–1.5)	58.5 (55.2–61.8)
$VHDL_1$,[a] 1.210–1.250	3.2 (3.0–3.4)	0.3 (0.1–0.4)	28.0 (26.0–30.0)	4.6 (3.5–7.5)	0.6 (0.5–0.7)	62.4 (61.8–62.9)]
$VHDL_2$, >1.250	0.05 (0.04–0.06)	0.02 (0.02)	0.83 (0.80–0.85)	0.05 (0.04–0.05)	0.60 (0.55–0.65)	98.45 (97.0–98.8)
$HDL_{1.185}$[b]	25.1	5.3	56.2	10.1	3.1	—
$VHDL_1$,[b] 1.210–1.250	8.5	0.9	76.7	12.4	1.5	—
$VHDL_2$,[b] >1.250	3.2	1.3	53.5	3.2	38.7	—

[a] Expressed as sum of individually determined mean lipid and protein values of three separate samples. The range of values is given in parentheses. [b] Expressed as per cent of the sum of the individually determined mean lipid values only.

^{131}I and serum after only 22 hr of centrifugation. Scanu and Hughes (1960) also found 70% of radioactivity in the top fraction after centrifugation of an incubation mixture of ApoA-^{131}I and serum, *d* 1.21 g/ml, at 79.420*g* for 18 hr. The top fraction was subjected to starch gel electrophoresis and the radioactivity was detected in the band characteristic of HDL. The radioactivity of VHDL-^{131}I, obtained following incubation of either ApoA-^{131}I with whole serum or fraction $d > 1.21$-^{131}I with nonradioactive HDL, was detected in the immediately postalbumin region. The exchange reactions, in concert with the electrophoretic differentiation between HDL and VHDL, suggest strongly that VHDL contain apolipoprotein A as the protein moiety.

Isolation and Chemical Characterization of $VHDL_1$ and $VHDL_2$. The possibility that interactions between unlabeled HDL and either ^{131}I-labeled VHDL or albumin resulted in the formation of a complex rather than an exchange of identical protein moieties necessitated the isolation of a VHDL fraction of characteristic chemical composition and containing a distinct and specific protein. As shown in Figure 1 the serum density was adjusted to 1.210 g/ml and the centrifugation was carried out for 44 hr to ensure a complete removal of floating very low, low, and high density lipoproteins. Since prolongation of centrifugation time had no further effect on the distribution of lipids (Figure 2) the density of the bottom fraction was raised to 1.250 g/ml by the addition of cesium chloride and the mixture was centrifuged at 165,000*g* for 44 hr. This procedure resulted in the separation of very high density lipoproteins into a top fraction, *$VHDL_1$* (*d* 1.21–1.25 g/ml), and a bottom fraction containing *$VHDL_2$* ($d > 1.25$ g/ml). The thin layer chromatographic analysis of top and bottom fractions revealed the presence of unesterified and esterified cholesterol, triglyceride, phospholipid, and unesterified fatty acids.

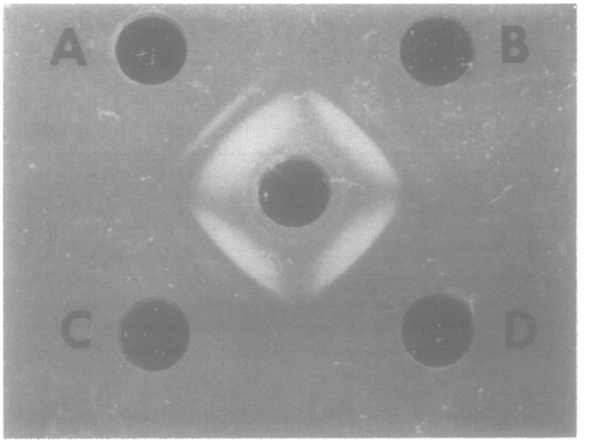

FIGURE 3: Immunodiffusion patterns of HDL and VHDL with anti-HDL. (A) HDL (*d* 1.185–1.210 g/ml); (B) $VHDL_2$ ($d > 1.25$ g/ml); (C) $VHDL_1$ (*d* 1.21–1.25 g/ml); (D) HDL (*d* 1.063–1.120 g/ml). Rabbit serum containing antibodies to human HDL is in the central well. Antibody:antigen protein ratio, 10:1.

The $VHDL_1$ contained *15–25%* and the $VHDL_2$ *75–85%* of the total phospholipid of the parent $d > 1.21$ g/ml fraction. The $VHDL_1$ produced no precipitin line with antibodies to human LDL, albumin, α_1-acid glycoprotein, 7S γ-globulin, and fibrinogen but produced two precipitin lines with the antibodies to human HDL (Figure 3C) and human whole serum. On the other hand, the $VHDL_2$ reacted with the antibodies to human serum albumin, α_1-acid glycoprotein, 7S γ-globulin, and whole serum, and developed also two precipitin lines with antihuman HDL (Figure 3B). Both fractions showed identity reactions with HDL subfractions of 1.185–1.210 and 1.063–1.120 g/ml

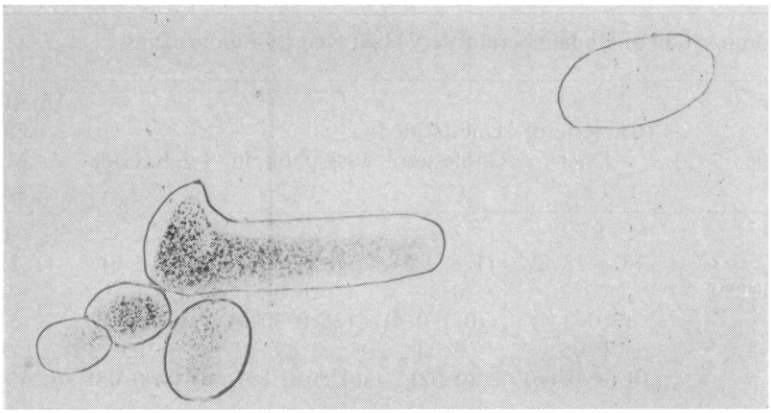

FIGURE 4: Two-dimensional thin layer chromatographic pattern of phospholipids extracted from $VHDL_1$. Spots from the lower left corner to the upper right corner are: lysolecithin, sphingomyelin, lecithin, phosphatidylinositol (spot below lecithin), and phosphatidylethanolamine. Developing solvents: in the first dimension (from left to right), chloroform–methanol–water (65:25:4, v/v); in the second dimension, chloroform–methanol–acetic acid–water (65:25:8:4, v/v).

TABLE IV: Lipid and Protein Content of Human Serum Very High Density Lipoproteins.

Lipoprotein Fraction Density Range	Cholesterol Ester[a]	Unesterified Cholesterol[a]	Phospholipid[a]	Triglyceride[a]	Unesterified Fatty Acids[a]	Protein[a]
$VHDL_1$, 1.210–1.250	0.69	0.06	6.24	0.99	0.13	13.0
$VHDL_2$, >1.250	1.50	0.60	24.96	1.50	18.0	2950
Total	2.19	0.66	31.20	2.49	18.13	2960

[a] Given in mg/100 ml of serum.

densities (Figure 3A, D, respectively). The average protein and lipid composition of VHDL fractions is presented in Table III. In comparison with $HDL_{1.185}$, $VHDL_1$ contained less cholesterol and slightly more triglyceride, phospholipid, and protein. The unesterified fatty acid content was negligible. The $VHDL_2$ contained less cholesterol, triglyceride, and phospholipid and more unesterified fatty acid than $VHDL_1$. The much greater content of fatty acids is due to the presence of well-documented (Fredrickson and Gordon, 1958) albumin–fatty acid complex in the >1.21 fraction. The high content of protein reflects the presence of nonlipid carrying globular proteins. Quantitative chromatographic analysis of individual phospholipids (Table II) revealed a remarkable difference between $VHDL_1$ and $VHDL_2$. The expected high lysolecithin (64.1%) and lower lecithin (28.1%) contents were noted only in $VHDL_2$. The phospholipid composition of $VHDL_1$ (Figure 4), with a characteristically high percentage of lecithin (71.6%) and a negligible amount of lysolecithin (3.5%), differed very little from that of an ultracentrifugally preceding HDL subfraction (Table II).

The content of lipid and protein of $VHDL_1$ and $VHDL_2$ is shown in Table IV. The $VHDL_1$ contained approximately 20% of the total phospholipid of the parent $d > 1.21$ fraction or 2–3% of the total serum phospholipid. Corresponding values of $VHDL_2$ were 80 and 9%, respectively. Since the average protein value for high density lipoproteins ($HDL_2 + HDL_3$) is 250–300 mg/100 ml of serum (Lindgren and Nichols, 1960) the $VHDL_1$ accounts for 6–8% of the total apolipoprotein A containing lipoproteins.

The results of physical–chemical characterization of the $HDL_{1.185}$ and $VHDL_1$ are summarized in Table V. A slightly higher protein content of $VHDL_1$ in comparison with that of $HDL_{1.185}$ was clearly reflected in its physical properties. The $VHDL_1$ fraction showed a single, symmetrical sedimenting boundary (Figure 5), $s_{20,w}$ of 4.5 S, hydrated density 1.155 g/ml. By substituting a value for $s_{20,w}$ of 4.5 S, a value for $D_{20,w}$ of 5.4×10^{-7} cm²/sec, and a value of 0.866 g/ml for the partial specific volume on the Svedberg's equation, an apparent weight-average molecular weight of 148,000 was obtained which is in fair agreement with the value of 153,800 obtained by sedimentation equilibrium experiments.

Peptide Pattern of Delipidized HDL and $VHDL_1$.

TABLE V: Physical–Chemical Characterization of Human Serum High Density and Very High Density Lipoproteins.

Liproprotein Fraction Density Range (g/ml)	Hydrated Density (g/ml)	Partial Sp Vol. (ml/g)	$S_{20,w}$ (S)	$D_{20,w} \times 10^{-7}$ (cm²/sec)	Mol Wt (from S and D)	Mol Wt (sedimentation equil)
$HDL_{1.185}$, 1.185–1.210	1.146	0.873	4.6	5.2	166,000	175,000
$VHDL_1$, 1.210–1.250	1.155	0.866	4.5	5.4	148,000	153,800

To show chemically the identity of the protein moieties, HDL and $VHDL_1$ fractions were subjected to complete delipidization. The peptide patterns of pepsin digests are shown in Figure 6. Both hydrolysates contained 63 spots arranged in almost identical patterns. Since the chromatographic and electrophoretic runs could not be performed simultaneously the relative positions of some peptides differed slightly. Because of very low concentrations the presence of peptides circled in dotted lines could not be ascertained unequivocally.

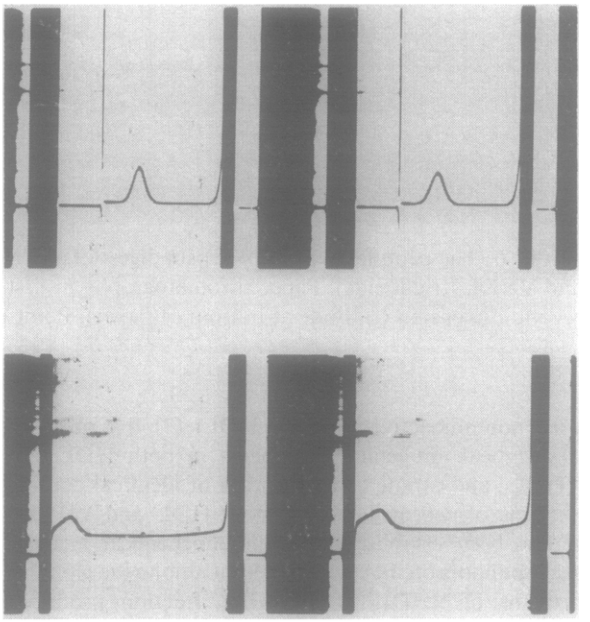

FIGURE 5: Ultracentrifugal schlieren patterns of $VHDL_1$. The upper frame: synthetic boundary cell. Photographs were taken at 34 and 50 min and a phase plate angle of 60° after reaching a rotor speed of 12,500 rpm. The lower frame: single-sector cell. Photographs were taken at 16 and 24 min after reaching a rotor speed of 56,100 rpm at an angle of 65° for the schlieren diaphragm. Both samples contained 0.45% $VHDL_1$ in 0.15 M NaCl, d 1.0055 g/ml.

Discussion

So far, the evidence for the existence of VHDL has been based on the lipid content of the serum fraction undergoing sedimentation during ultracentrifugation in a solvent density of 1.21 g/ml, and the demonstration of a lipid–protein entity having electrophoretic mobility in starch block and polyvinyl resin similar to that of HDL. The present quantitative and thin layer chromatographic analyses of lipids extracted from the $d > 1.21$ fraction confirmed the presence of 11% of the total serum phospholipid and 5% of the total serum triglyceride, and demonstrated that the 2–3% of the total serum cholesterol present occurred mainly as cholesterol ester. Furthermore, it was found that, in starch gel, the electrophoretic mobility of VHDL differed from that of HDL and was similar to that of lipid-free HDL. Thus, these experiments provide evidence for the occurrence in the $d > 1.21$ fraction of a lipid–protein complex differing in lipid composition and electrophoretic mobility from the HDL.

The difficulty in isolating very high density lipoproteins without contaminating proteins was overcome by applying a substantially greater centrifugal force and a longer period of centrifugation. Centrifugation of the $d > 1.21$ fraction for 44 hr in a solvent density of 1.25 g/ml at 165,000g resulted in the separation of VHDL into two distinct fractions differing considerably in lipid composition. The isolation of $VHDL_1$ represents the first successful demonstration of a very high density lipid–protein complex of a reproducible chemical composition and reacting only with the antibodies to human serum HDL. The very slightly different lipid and protein content and almost identical composition of individual phospholipids strongly suggested a close relationship between $VHDL_1$ and high density (1.185–1.210 g/ml) subfractions of HDL. The exchange reactions, electrophoretic mobilities, peptide patterns, and immunochemical studies of HDL and $VHDL_1$ are presented as proof for the presence of the same protein moiety in these two lipoproteins. The *in vitro* (Scanu and Hughes, 1960) and *in vivo* (Furman *et al.*, 1964) studies involving incubation of ApoA-^{131}I with serum have already shown similar patterns of distribution of radioactivity for HDL and VHDL. In both experiments, around 30% of the radioactivity remained in the VHDL. That the $VHDL_1$ fraction contains apolipoprotein A has been concluded on the basis: (1) the presence of radioactive VHDL in the starch gel electropherogram after incubation of ApoA-^{131}I with whole serum, (2) the presence of radioactive HDL in the starch gel electropherogram after incubation of the ^{131}I-labeled $d > 1.21$ fraction

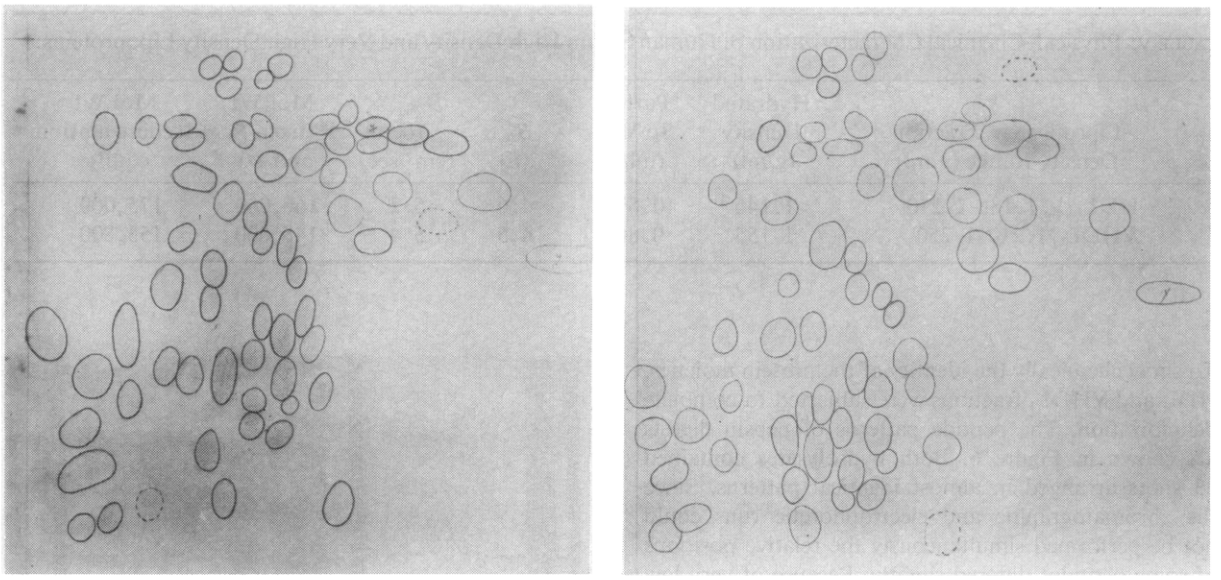

FIGURE 6: The peptide patterns of pepsin digest of apolipoprotein A obtained by total delipidization of HDL (left side) and $VHDL_1$ (right side). Paper chromatography in first dimension from left to right. High-voltage electrophoresis in second dimension (cathode at bottom of paper). Point of application in the left upper corner.

with nonradioactive, purified HDL, (3) the presence of identical antigenic components in both HDL and $VHDL_1$, and (4) the demonstration of identical peptide patterns of pepsin digests of both HDL and $VHDL_1$. It has been established by immunochemical analysis that apolipoprotein A is also present among the globular proteins of $VHDL_2$. The $VHDL_2$ fraction produced two characteristic precipitin lines with anti-HDL serum and showed an identity reaction with $VHDL_1$ and HDL.

Recently, several studies have revived interest and focused attention on the chemistry, formation, and possible metabolic role of VHDL. Several laboratories (Ayrault-Jarrier *et al.*, 1963; Burstein and Fine, 1964; Levy and Fredrickson, 1965; Scanu and Granda, 1966) presented evidence that ultracentrifugally isolated HDL exist in two forms differing in electrophoretic mobility in agar gel and cross-reacting only partially as antigens. Levy and Fredrickson (1965) considered the slower moving or "lipid-poor" form as a possible transformation artifact of the faster moving or "lipid-rich" form from which it could be obtained by prolonged ultracentrifugation, storage, or partial delipidization. They found in the HDL_2 (1.063–1.100 g/ml) only the "lipid-rich" form and the $d > 1.21$ fraction only the "lipid-poor" form. However, the results of our studies show that both antigenic forms are present in VHDL, and that the "lipid-rich" form of HDL_2 (1.063–1.100 g/ml) gives an identity reaction with one of the two antigenic forms of $VHDL_1$ and $VHDL_2$. A similar result was obtained by Scanu and Granda (1966) who reported immunochemical evidence for the presence of two common antigenic components obtained by reacting not only the HDL_3 (d 1.110–1.210 g/ml) but also whole untreated serum and $d >$ 1.21 fraction with the anti-HDL serum.

The actual *in vivo* occurrence of $VHDL_1$ and at least a portion of $VHDL_2$, is supported by: (1) the presence of two antigenic forms in both VHDL fractions, (2) the finding of both "lipid-rich" and "lipid-poor" forms in fresh untreated sera (Levy and Fredrickson, 1965; Scanu and Granda, 1966), (3) a close chemical resemblance of $VHDL_1$ to HDL (1.185–1.210 g/ml), (4) the recent report (Neff and Block, 1966) that HDL separated by Sephadex G-200 column chromatography contained two antigenic components, and (5) differences between the chemical composition of $VHDL_1$ and a VHDL fraction obtained by ultracentrifugation of HDL (Scanu and Granda, 1966). The latter VHDL preparation contained 96% protein and 4% phospholipid.

Switzer and Eder (1965) found, on the basis of electrophoresis, salt precipitation, and gel filtration experiments, that the major portion of plasma lysolecithin is bound to albumin. This finding is of particular interest since it may provide the explanation for the striking difference in the phospholipid composition of $VHDL_1$ and $VHDL_2$.

If the arbitrarily chosen solvent density of 1.210 g/ml is retained as the higher density boundary for the ultracentrifugal isolation of HDL, then the operational term *very high density lipoproteins* should be used as a designation for the existence *in vivo* of several lipid–protein complexes sedimenting under these experimental conditions. The very high density lipoproteins can be separated, at the present time, into two major groups characterized by the presence of apolipoprotein A and albumin as lipid-carrying proteins. The actual values for the protein mass calculated from the protein content and molecular weight (Tables III and IV) of

$HDL_{1.185}$ and $VHDL_1$ are very similar (102,000 and 96,000*g*, respectively). Since there is probably little or no difference in the structural make-up of these two protein moieties and since the slightly different physical–chemical properties of $VHDL_1$ are primarily a reflection of a lower lipid content, it is proposed that the first group of VHDL, *i.e.*, $VHDL_1$, represents a continuation of, or the upper hydrated-density portion of, a polydisperse system of HDL. They may play a role in the transport of cholesterol esters and triglyceride (Nichols *et al.*, 1964) or, alternatively, represent "lipid acceptor proteins" (Roheim *et al.*, 1965), the precursors of some lower density serum lipoproteins. This latter suggestion is supported strongly by the recent isolation of an ApoA-containing lipoprotein from very low density lipoproteins (Gustafson *et al.*, 1966).

The second group of VHDL, *i.e.*, $VHDL_2$, is represented primarily by albumin–fatty acid (Fredrickson and Gordon, 1958) and albumin–lysolecithin complexes (Switzer and Eder, 1965). Whereas the role of the former complexes in the transport of fatty acids is already accepted, the suggestion that the latter might be involved in the transport of lysolecithin into tissues to serve as a precursor of cellular lecithin remains to be established. The small amount of apolipoprotein A containing lipoprotein in $VHDL_2$ probably represents the remaining higher density portion of the polydisperse system of HDL characterized, in comparison with that of $VHDL_1$ by a further decrease in the cholesterol: phospholipid ratio.

Acknowledgments

The authors wish to express their gratitude to Drs. R. P. Howard and R. H. Bradford for valuable criticisms, and to Mrs. M. Farmer for assistance in the preparation and typing of the manuscript.

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