Studies of the Composition and Structure of Serum Lipoproteins: Isolation, Purification, and Characterization of Very Low Density Lipoproteins of Human Serum*

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ABSTRACT: A method has been developed for the fractionation of very low density (VLD) human serum lipoproteins (S_f >20) by angle-head preparative ultracentrifugation. A buffer solution of density 1.006 g/ml is layered over serum and both centrifugal force and time ("g min") are increased in each successive run. A procedure was standardized for the separation of VLD-lipoproteins into five subfractions. Each subfraction, freed of contaminating serum proteins, has been characterized by determination of flotation coefficient, anhydrous density, concentration dependence of S_f (k values), electrophoretic mobility on agar gel, by calculation of average diameter and molecular weight, and by determination of lipid and protein composition. The triglyceride content increases and protein content diminishes with increasing size and rate of flotation of the lipoprotein fractions. The physicochemical characterization of isolated subfractions provided the information necessary for correlating flotation rate and centrifugal force, thus supplying technical data for the isolation of a VLD-lipoprotein subfraction of any desired flotation coefficient.

Relatively little is known about the composition and structure of very low density (VLD)[1] lipoproteins, in comparison with the higher density α- and β-lipoproteins. Although the lipid moieties have been well characterized qualitatively, there is a paucity of information concerning the nature of the corresponding protein moieties. Amino acid analyses have indicated as many as four distinct proteins (Rodbell, 1958), while immunological studies suggest only one or two (Marsh and Whereat, 1959).

Very low density serum lipoproteins can be isolated by precipitation with natural or synthetic polymers. Heparin, in the presence of calcium or magnesium ions precipitates VLD-lipoproteins and β-lipoproteins (Burstein and Samaille 1955, 1958), whereas 5% polyvinylpyrrolidone (PVP) flocculates only VLD-lipoproteins (Burstein and Prawerman, 1958; Gordis, 1962). Separation of VLD-lipoproteins by various electrophoretic techniques is difficult because of the large amount of lipid present.

Major lipoprotein classes are readily isolated by differential preparative ultracentrifugation. By appropriate adjustment of solvent density, subfractionation of higher density lipoprotein classes is readily accomplished (Havel *et al.*, 1955). However, subfractionation of VLD-lipoproteins, which have hydrated densities less than that of water, is difficult with presently employed salt solutions. The application of various centrifugal forces and the adjustment of serum density by means of a NaCl solution of density 1.006 g/ml (Scanu and Page, 1959; Bragdon *et al.*, 1956), a NaCl density gradient (Lindgren *et al.*, 1962), or the use of unaltered serum (Jobst and Schettler, 1956; Lindgren *et al.*, 1959) resulted in the isolation of various fractions which have received little further study with respect to their chemical and physical properties. In only a few studies have attempts been made to characterize these fractions (Lindgren *et al.*, 1959, 1962) or to purify them further (Scanu and Page, 1959). The particles comprising these fractions are inhomogeneous in respect to their physical properties and protein moieties.

The importance of VLD-lipoproteins in the genesis of atherosclerosis and the role of the protein moieties in maintaining the structural stability of very low density, lipid-rich lipoproteins have prompted efforts to isolate and characterize this heterogeneous group of very low density complexes. This report, representing the initial phase of a broad study of the function and composition of VLD-lipoproteins, sets forth a method for their separation into five fractions, or into fractions of any desired flotation rate, by a differential preparative ultracentrifugal technique, and describes the

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[1] Abbreviations used: S_f, flotation rate, expressed as Svedbergs (10^{-13} cm sec/dyne per g) of flotation in NaCl medium density 1.063 g/ml at 26°; $S_f°$, flotation rate at infinite dilution; VLD-lipoproteins, very low density lipoproteins, lipoproteins of density <1.006 g/ml and S_f >20; α-lipoproteins, high density lipoproteins, lipoproteins of density 1.063–1.21 g/ml; β-lipoproteins, low density lipoproteins, lipoproteins of density 1.006–1.063 g/ml and S_f 0–20; PVP, polyvinylpyrrolidone.

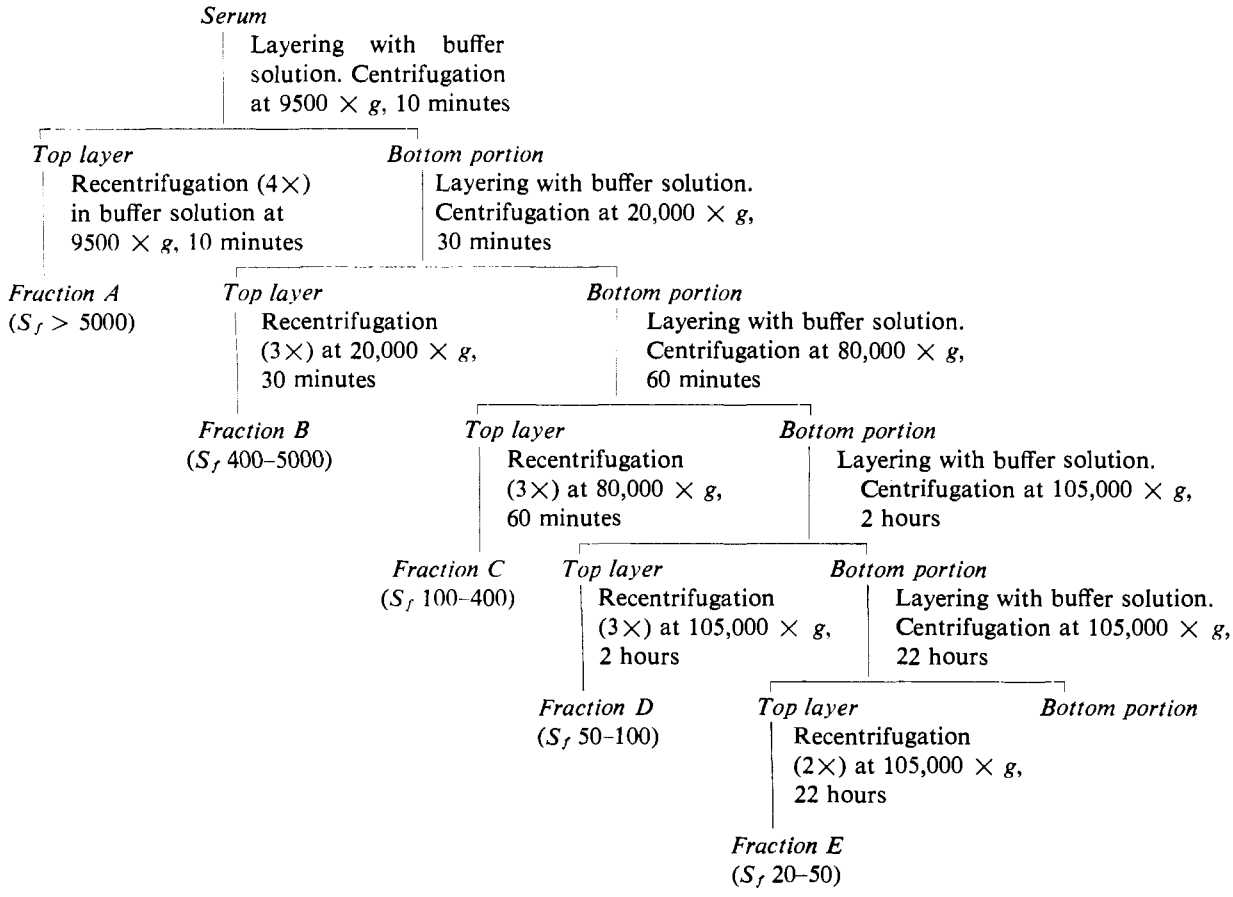

FIGURE 1: Procedure for the isolation and purification of five fractions of VLD human serum lipoproteins ($d \leq$ 1.0055 g/ml).

physical properties and chemical composition of each fraction.

Experimental

Blood samples were obtained from a healthy subject after a high-fat meal and from patients with hyperlipidemia after an overnight fast. The blood was allowed to clot, and serum was recovered by low-speed centrifugation.

Ultracentrifugal Fractionation. Five fractions of VLD-lipoproteins were isolated utilizing successively increased speeds and times of ultracentrifugation, as shown in Figure 1. Centrifugations were carried out in the No. 40 rotor of the Spinco Model L ultracentrifuge at approximately 4°. The buffer solution (1.42 g anhydrous disodium phosphate, 7.27 g sodium chloride, and 0.1 g disodium EDTA in 1-liter solution) was adjusted to *p*H 7.0 with 1 N HCl and had a density of 1.0055 g/ml at 25°. Specific gravity was determined with a hydrometer (Fisher Scientific Co.) calibrated by the U.S. National Bureau of Standards. Each fraction accumulating at the top of the plastic centrifuge tube as an opaque, creamy layer was removed by a tube-slicing technique, aspirated in a syringe, and resuspended by repeated extrusion through a 20-gauge needle into 4 volumes of buffer solution for further purification.

Five ml of fresh serum were layered under 5 ml of the buffer solution in a plastic tube and centrifuged for 10 minutes at 12,000 rpm (9500 × *g*). The top layer was removed, resuspended in the buffer solution, and recentrifuged under identical conditions. This washing procedure was repeated four times, yielding purified *fraction A* ($S_f > 5000$). Five ml of the bottom portion remaining after the separation of fraction A was layered under an equal volume of buffer solution and centrifuged for 30 minutes at 17,500 rpm (20,000 × *g*). The top layer was removed and recentrifuged three times, yielding *fraction B* (S_f *400–5000*). The bottom portion of fraction B was layered under an equal volume of buffer solution and centrifuged for 60 minutes at 35,000 rpm (80,000 × *g*). The top layer contained *fraction C* (S_f *100–400*) which was obtained in purified form after three successive washings by resuspension and recentrifugation. *Fraction D* (S_f *50–100*) was obtained after layering the bottom portion of fraction C under an equal volume of buffer solution and centrifuging for 2 hours at 40,000 rpm (105,000 × *g*). The washing procedure was repeated three times. Finally, the remaining bottom portion of fraction D was layered under the buffer solution and,

after centrifuging for 22 hours at 40,000 rpm (105,000 × *g*), a faintly yellow-colored top layer was recovered. Two successive washing cycles yielded the purified *fraction E* (S_f *20–50*). *Parent fraction* ($S_f > 20$) containing all VLD-lipoproteins was obtained by layering 5 ml of fresh serum under 5 ml of the buffer solution and centrifuging for 22 hours at 40,000 rpm (105,000 × *g*). The top layer was recentrifuged twice under identical conditions. All fractions were concentrated to a small volume by repeated centrifugation.

Electrophoresis. Cellulose acetate electrophoresis of purified VLD-lipoprotein fractions was performed according to the technique suggested by Kohn (1958) employing barbital buffer, *p*H 8.6, ionic strength 0.075. Nigrosin, 0.001% in 2% acetic acid, was used to stain protein. Agar-gel electrophoresis was performed according to Williams and Grabar (1955) utilizing a modified discontinuous buffer system (Laurell *et al.*, 1956). Six separate rectangular basins were perforated and used as filling reservoirs. Amido Schwartz 10B (Bayer) and Oil Red O (Spinco) were employed to stain protein and lipid, respectively.

Ultracentrifugal Analyses. These studies were carried out at 25–26° and at various rotor speeds in a Spinco Model E ultracentrifuge equipped with a phase-plate schlieren diaphragm. Single-sector cells were used. Plate measurements were made with a Nikon microcomparator having a sensitivity of 0.001 mm. The flotation coefficient, S_f, was determined as outlined by Schachman (1957). The flotation coefficient at infinite dilution, $S_f°$, was calculated from values obtained at three different protein concentrations.

Since the opalescence of fraction A prevented visualization of a schlieren pattern, the minimum flotation coefficient was estimated from movement of the edge of the opalescence as flotation occurred. The five fractions were analyzed at the following densities and speeds: fraction A at 1.0055 g/ml, 12,000 rpm; fraction B at 1.063 g/ml, 16,500 rpm; fractions C and D at 1.063 g/ml, 35,500 rpm; and fraction E at 1.0055 g/ml and 1.045 g/ml, 52,640 rpm. Corrected values for the flotation coefficients of fractions A and E were obtained from the equation by Svedberg and Pedersen (1940). In these calculations the values of 1.075 and 1.014 were used for the partial specific volumes of fractions A and E, respectively. They were calculated utilizing the anhydrous density of 0.930 g/ml for fraction A and the experimentally determined hydrated density of 0.986 g/ml for fraction E. The values for the densities and relative viscosities of solutions were taken from the International Critical Tables.

Concentration Dependence of S_f (*Determination of k Values*). The *k* values were calculated from the equation:

$$S_f° = \frac{S_f}{1 - kc}$$

where S_f and $S_f°$ are flotation coefficients, *c* is the lipoprotein concentration in g/ml, and *k* is the factor for concentration dependence in ml/g (Shore, 1957). The *k* values were determined for fractions B, C, and D in the lipoprotein concentration range of 0.003–0.030 g/ml.

Calculation of Density. The anhydrous densities of the five VLD-lipoprotein fractions were calculated from the average chemical composition of each fraction and the following approximate densities for protein and lipids as suggested by Oncley (1958, 1963): protein, 1.33 g/ml; triglyceride, 0.915 g/ml; free cholesterol, 1.06 g/ml; cholesterol ester, 0.99 g/ml; and phospholipid, 0.97 g/ml. The hydrated density of fraction E was estimated also from the flotation rates measured at several solvent densities and extrapolated to the solvent density corresponding to a zero flotation rate.

Estimation of Diameter. The diameters of lipoproteins of several fractions were estimated, assuming spherical particles, according to the equation (Oncley and Gurd, 1953):

$$S_f° = \frac{1.063 - \rho_o}{188} \cdot d^2$$

where ρ_o is the solution density in g/ml, and *d* is the lipoprotein diameter in A. A variation of ±0.01 g/ml in the density value would result in an error in the calculated diameter of ±10%.

Estimation of Molecular Weight. Approximate average molecular weights were calculated according to the equation (Oncley, 1963):

$$M = 0.317 \cdot \rho \cdot d^3$$

where ρ is the lipoprotein density in g/ml, and *d* is the lipoprotein diameter in A.

Lipid Analyses. The lipoprotein fractions were extracted with chloroform-methanol (2:1, v/v), the lipid extracts were washed with 20% of their volumes of 0.58% NaCl solution (Folch *et al.*, 1957), and the two phases were allowed to separate overnight. The chloroform phase was dried with anhydrous Na_2SO_4, filtered, and evaporated to dryness *in vacuo* under nitrogen. The residue was redissolved in diethyl ether and aliquots were taken for lipid analyses. Total lipid content was determined gravimetrically in aliquots dried to constant weight over P_2O_5 in a vacuum desiccator. Aliquots contained approximately 5 mg of total lipids and the accuracy of weighing was ±0.1 mg. Cholesterol was determined according to the method of Sperry and Webb (1950) and lipid phosphorus by the wet digestion procedure of Youngburg and Youngburg (1930) with phosphorus measured by the method of Fiske and Subbarow (1925). The factor 25 was used to convert lipid phosphorus to phospholipids. Values for cholesterol esters were calculated as cholesteryl oleate (mw 651). The values for triglycerides were obtained by subtracting the sum of cholesterol esters, cholesterol, and phospholipids from total lipids.

Protein Analysis. Protein was determined by a slight modification of the Lowry method (Lowry *et al.*, 1951). The turbidity caused by high lipid concentrations was cleared by extracting the solution with diethyl

TABLE I: Per Cent Composition of Fraction A after Repeated Washing and Recentrifugation.

	Total Cholesterol (%)	Phospho-lipid (%)	Tri-glyceride[a] (%)	Protein (%)	Per Cent Loss of Total Lipid per Recen-trifugation[b]	Protein Impurities[c]
Initial preparation	11.7	4.9	81.1	2.3		Serum proteins, VLD-lipo-proteins
First washing	12.4	4.3	82.2	1.1	39.7	Albumin
Second washing	10.3	4.0	84.9	0.8	18.6	None
Third washing	10.0	4.2	85.0	0.8	16.1	None
Fourth washing	10.1	4.6	84.5	0.8	17.5	None
Fifth washing	9.3	4.2	85.7	0.8	11.7	None
Sixth washing	9.7	4.0	85.5	0.8	22.7	None

[a] Determined according to Van Handel and Zilversmit (1957). [b] Per cent loss of lipid from top layer of preceding run. [c] Presence of impurities detected by cellulose acetate electrophoresis.

ether at room temperature after color development was complete. The accuracy of this modification of Lowry's method was verified by analysis of duplicate samples by the biuret method (Gornall *et al.*, 1949). The protein values were standardized by determining the nitrogen content of purified samples of human albumin (micro-Kjeldahl method) and multiplying by 6.25.

Results

Purification Procedure. Rigorous application of this isolation technique provided a highly reproducible fractionation of human serum VLD-lipoproteins (S_f >20). Cellulose acetate electrophoresis of ultracentrifugally separated lipoprotein fractions demonstrated that, prior to washing, the preparations were contaminated with lipoproteins of lower flotation rates or with globular proteins. Only by repeated washing and recentrifugation were preparations of constant composition and electrophoretic homogeneity obtained. Fraction A was submitted to six consecutive recentrifugations under the same conditions employed in its initial isolation and the chemical composition and electrophoretic pattern were determined after each washing (Table I). Two washings were necessary for complete removal of lipoproteins of adjacent VLD-fractions and of other serum proteins, including albumin. The constancy of the lipid and protein composition of fraction A after the second and subsequent washings (Table I) indicated a chemically homogeneous preparation. Similar treatment of other fractions also resulted in preparations of constant composition devoid of serum proteins. Three consecutive washings of each of the five VLD-lipoprotein fractions resulted in a total loss of 45–50% of the original lipid content.

Physicochemical Properties. Physicochemical characterization of the five subfractions was performed only on samples the chemical composition of which was found to be constant after three washings.

The electropherograms of fractions A–E are shown in Figure 2. Fractions A (S_f >5000) and B (S_f 400–5000) remained at the origin, whereas fractions D (S_f 50–100) and E (S_f 20–50) migrated to a pre-β-lipoprotein position. Fraction C (S_f 100–400) showed an incompletely resolved pattern of migration between these two positions. Smith (1957) observed similar migration rates for VLD-lipoprotein fractions on paper electrophoresis and established that the VLD-lipoproteins of S_f 18–130 migrated to the pre-β-lipoprotein position and the VLD-lipoproteins of S_f >400 remained at the origin. The low or absent electrophoretic mobility of macromolecular species rich in lipid is probably due to their adsorption to the medium rather than to paucity of protein moieties. For example, adsorption was not observed in a starch medium (Kunkel and Trautman, 1956) in which all VLD-lipoproteins (S_f >12), regardless of size, migrated to the α_2 position. Recently, further separation of the α_2-lipoproteins into three fractions was achieved by starch-block electrophoresis (Bierman *et al.*, 1962; Bierman, 1963).

The values for the flotation coefficients at infinite dilution ($S_f°$) for all five subfractions are given in Table II. The ultracentrifugal schlieren patterns of fractions B, C, D, and E showed a single flotation peak (Figure 3). In contrast to the symmetrical peaks of fractions D and E, the broad diffuse boundaries of fractions B and C indicated most probably the presence of a variety of chemically similar lipoproteins of differing sizes and degrees of aggregation. As mentioned previously, the opalescence of fraction A caused by the large size of the lipoproteins (S_f >5000) prevented the visualization of a schlieren pattern. This was also observed by Lindgren *et al.* (1962) with lipoproteins of S_f >800. Lactescense or opalescence caused by reflection and scattering of

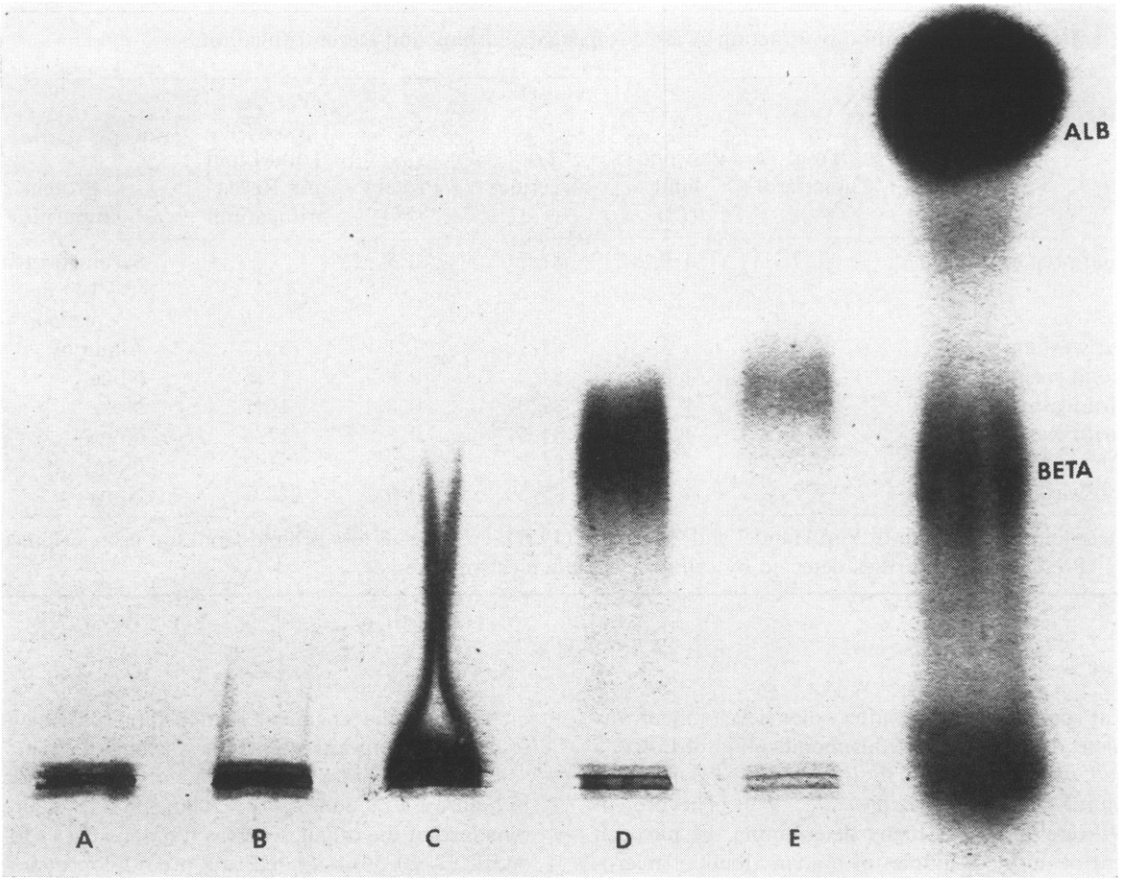

FIGURE 2: Agar-gel electropherogram of fractions A, B, C, D, E (lipid stain), and normal serum (protein stain).

TABLE II: Physical Characteristics of Five VLD-Lipoprotein Fractions.

Lipoprotein Fraction			Calculated Anhydrous Density (g/ml)			Lipoprotein Concentration Range[a] (g/ml)	Calculated Diameter (A)	Calculated Molecular Weight M × 10^6
	Isolated at "g min"	S_f Range		$S_f°$	k (ml/g)			
Fraction A	0.1 × 10^6	>5000	0.928	>4750		0.005–0.030	>2572	>5005
Fraction B	0.6 × 10^6	400–5000	0.936	573.5	2.3	0.003–0.010	922	233
Fraction C	4.8 × 10^6	100–400	0.957	111.6	7.6	0.003–0.025	445	27
Fraction D	12.6 × 10^6	50–100	0.977	61.4	7.3	0.003–0.010	366	15
Fraction E	139 × 10^6	20–50	0.989	29.7		0.003–0.010	275	6

[a] Concentration range used for measurements of $S_f°$ and k.

light from the surface of particles was also noticed in fractions B, C, and D. Flotation of lipoprotein particles larger than 350 A (S_f >50) by a centrifugal force of 12.6 × 10^6 "g min" resulted in complete clearing of hyperlipemic, lactescent sera. Since the particle size of opalescent colloids varies between 500 and 5000 A (Jirgensons, 1958) and since opalescence is observed in fraction C (445 A), lipoproteins of the minimum particle size necessary for opalescence probably are present in the S_f 100–400 fractions.

The k values and corresponding concentration ranges of fractions B, C, and D are also presented in Table II. Shore (1957) has determined k values of 5 ml/g and 10 ml/g for the lipoproteins of S_f 20–60 and S_f 6–8, respectively.

Since the calculation of densities (Table II) based on chemical composition did not include a correction factor for the hydration of lipoproteins, the calculated values for anhydrous densities are somewhat higher than the experimentally determined hydrated densities, particu-

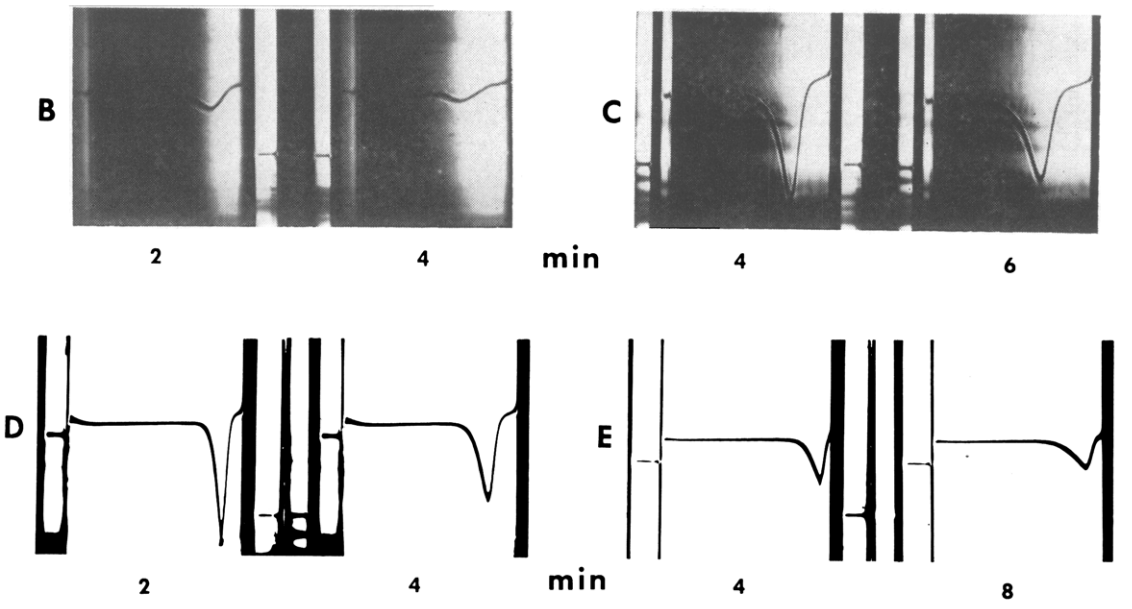

FIGURE 3: Ultracentrifugal schlieren patterns of fractions B, C, D, and E. Ultracentrifugal conditions: fraction B in NaCl, 1.063 g/ml, 16,500 rpm; fractions C and D in NaCl, 1.063 g/ml, 35,500 rpm; fraction E in NaCl, 1.0055 g/ml, 52,640 rpm. Time in minutes after rotor attained desired speed.

larly for lipoproteins with higher flotation rates. However, the hydrated density established for fraction E (S_f 20–50) agrees very well with that observed by Lindgren *et al.* (1962). The reported values for anhydrous densities of lipoprotein fractions of S_f 30 and S_f 100 (Oncley, 1963) and of lymph particles of approximate size 0.1 μ and S_f >400 (Pinter and Zilversmit, 1962) are in good agreement with the values obtained in the present study (Figure 4).

The calculated average diameters and molecular weights of fractions A, C, and E approximate very closely values suggested by Oncley (1963) for fractions of similar flotation coefficients. Similarly, the calculated value of 910 A for the lipoproteins in fraction B (S_f 573.5) are in good agreement with the value of 800 A obtained by electron microscope measurement of S_f >400 lipoproteins (Lindgren and Nichols, 1960).

The Correlation of Centrifugation and Flotation Rates. Determination of the flotation rates of the purified VLD-lipoprotein fractions provided the experimental data required for constructing a curve relating flotation rate to centrifugal force (Figure 5). The amount of centrifugation, given by the product of relative centrifugal force and time, is expressed in units of "g min," as suggested by Dole and Hamlin (1962). These authors, using a similar nomogram to solve the equation:

$$\frac{X}{d^2} = (3.27 \times 10^{-10}) \left(\frac{\Delta\rho}{\eta} \times gt \right)$$

were able to predict particle size (d) from the distance of flotation (X) under known centrifugal conditions [$(\Delta\rho/\eta) \times gt$]. Using S_f values and corresponding "g min" values from their nomogram (for X = 10 mm) a straight

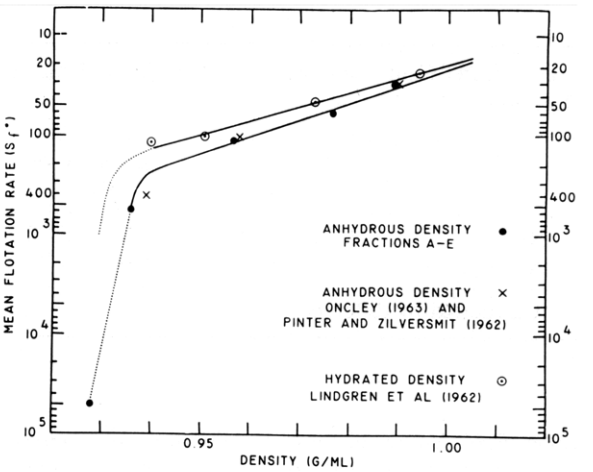

FIGURE 4: Mean flotation rate (S_f°) as a function of density of VLD-lipoproteins (S_f >20).

line was obtained. Relatively good agreement was found between calculated and observed values in the range of S_f 100–4000. To test the applicability of the curve, two additional lipoprotein subfractions, B-2 and D-2, were isolated and their mean flotation coefficients were determined experimentally. Fraction B-2 (approximate range S_f 150–400), isolated from the bottom of fraction B at 35,000 rpm (80,000 × g) for 30 minutes (2.4 × 10^6 "g min"), had a measured mean S_f value of 194; and fraction D-2, obtained from the bottom portion of fraction D after centrifugation at 40,000 rpm (105,000 × g) for 5 hours (35.5 × 10^6 "g min"), had a measured mean S_f

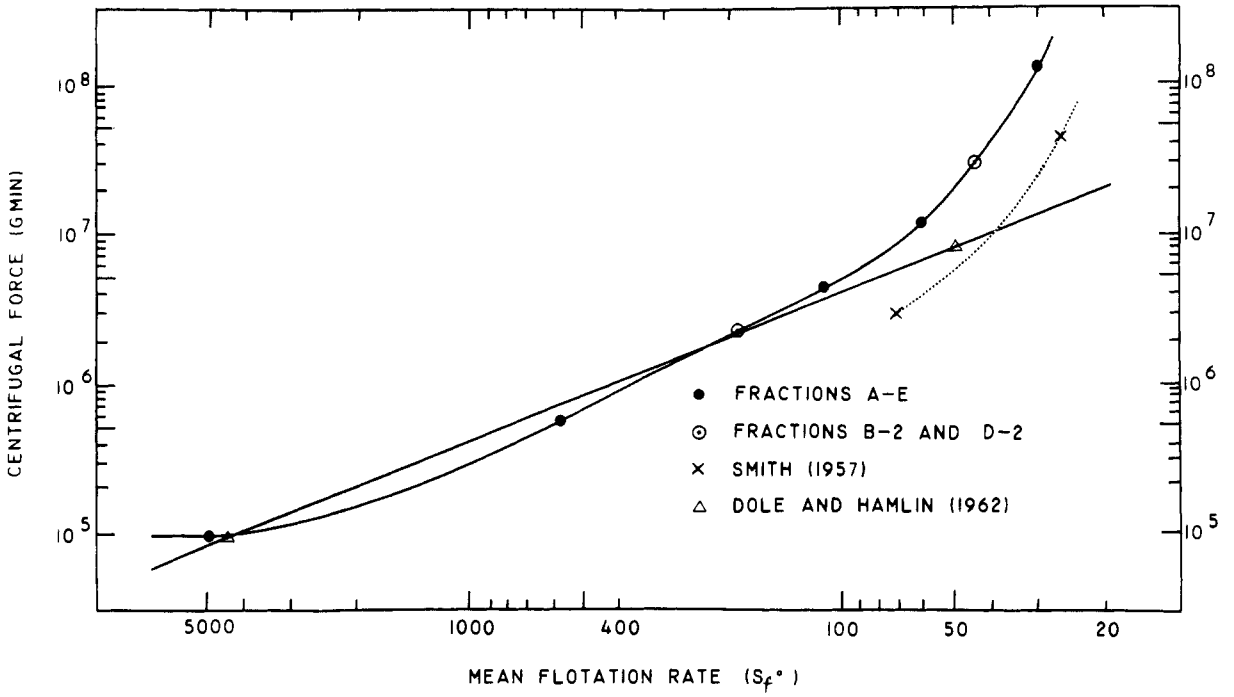

FIGURE 5: Centrifugal force ("g min") as a function of mean flotation rate (S_f°) of VLD-lipoproteins (S_f>20°).

TABLE III: Per Cent Composition of Fraction A Isolated from Serum Obtained from the Same Donor on 13 Different Occasions.

	Free Cholesterol	Cholesterol Ester	Phospholipid	Triglyceride[a]	Protein
Range	1.51 - 3.42	1.92 - 6.23	2.62 - 6.06	85.30 - 92.12	0.84 - 1.73
Average	2.11 ± 0.14[b]	3.90 ± 0.33	4.28 ± 0.28	88.65 ± 0.59	1.06 ± 0.07

[a] Calculated by difference. [b] ±SE.

value of 44. Values of measured flotation rates agreed well with those predicted from the curve. A curve representing experimentally determined mean flotation rate values ($S_{f,\rho 1.007}$) of two fractions (Smith, 1957) is also shown in Figure 5. As expected, a lower "g min" value was necessary to obtain a fraction of the same S_f value at the higher density. The parallelism of the two experimentally obtained curves is noteworthy.

Chemical Characterization. The reproducibility of the isolation procedure, as exemplified by the per cent lipid and protein composition of one lipoprotein fraction, is demonstrated in Table III. A total of thirteen serum samples was obtained on different occasions from the same donor. Fraction A, isolated from each serum sample and analyzed for protein and lipid content after four washings, showed a remarkable consistency of composition.

The average protein and lipid composition of the five VLD-lipoprotein fractions and of their parent fraction (S_f >20) is presented in Table IV. As was expected, the triglyceride content increased and the protein and phospholipid content decreased with increasing flotation rate. Free cholesterol and cholesterol esters also tended to decrease with flotation rate. The content of cholesterol esters varied more than any other component within an individual fraction. Each fraction can be characterized by a phospholipid/protein ratio, the values for which increase with flotation rate. The relatively high values for free and ester cholesterol in the parent fraction are owing most probably to the fact that the washing procedure results in a relatively greater loss of sterols when applied to fractions than when applied to the parent fraction.

Discussion

The results of this study indicate that by layering a buffer solution of density 1.006 g/ml over serum and increasing both centrifugal force and time ("g min") in each successive run, one may obtain by angle-head pre-

parative ultracentrifugation any theoretical number of subfractions of VLD-lipoproteins (S_f >20). The application of these techniques resulted in a standardized procedure for the separation of VLD-lipoproteins into five subfractions of characteristic chemical composition and physical properties. This procedure thus simplifies and extends a similar method of fractionation of VLD-lipoproteins by Lindgren *et al.* (1962) in which a NaCl density gradient and a specially designed swinging-bucket rotor had been employed for separation of three subfractions. (These three subfractions were characterized only by lipid analyses and determination of hydrated density.) Although the layering of a buffer solution of known density over serum is of primary importance for the achievement of separation, based on flotation rate and the elimination of undesirable contamination by serum components, subsequent purification is necessary to obtain fractions of constant composition. This is especially important in the determination of the protein content of subfractions which always contain traces of other globular proteins if not subjected to repeated washings.

Since the five subfractions obtained in this study were isolated in amounts sufficient for subsequent purification and extensive characterization, it was of considerable interest to compare their properties with those of corresponding VLD-lipoprotein fractions compiled from the literature (Table V). It must be kept in mind that these latter fractions were isolated by methods varying from precipitation to ultracentrifugation, with or without subsequent washing procedures, and that in most cases a complete physicochemical characterization was not performed. Although for these reasons an objective comparison of data is not possible, the similarities in the chemical composition of several fractions isolated under different conditions are remarkable. Thus, the three VLD-lipoprotein fractions (samples 4, 5 and 7, Table V) isolated by fractional precipitation with PVP are similar to fractions B, C, and D in respect to protein and phospholipid composition. The lipoprotein fraction S_f 20–50 (sample 9, Table V), obtained by dextran-sulfate precipitation and subsequent ultracentrifugation, is similar to that of fraction E in respect to per cent composition. There is also good agreement between the composition of the parent fraction (S_f >20) and that of comparable fractions obtained by Hayashi *et al.* (1959) and by Korn (1959) (samples 10 and 11, Table V).

The first four fractions in Table V, isolated under conditions similar to those for fraction A, have been called "chylomicrons," "lipomicrons," "primary particles," (Bierman *et al.*, 1962), or "alimentary particles" (Dole and Hamlin, 1962). Because of the controversy concerning its chemical composition and, more specifically, the question of whether a protein moiety is an integral component of the particle, special attention was given to the isolation and purification of this fraction. Repeated recentrifugation of fraction A (Table I) indicated that several consecutive washing procedures were required to remove serum proteins and lipoproteins of lower flotation rates. Cornwell *et al.* (1961) concluded from electron microscopic studies that chylomicrons isolated at 9300 × *g* for 30 minutes were contaminated by serum proteins. Scanu and Page (1959) showed by immunoelectrophoresis that several protein impurities were removed after one recentrifugation. The relatively high protein content of some chylomicron fractions in Table V could be accounted for primarily by the presence of serum proteins such as albumin. However, the constant protein content of fraction A, persisting through six consecutive recentrifugations, supports the concept that a specific protein is an essential component of the particle and contributes structural stability. Considering the small but characteristic content of a specific protein found in particles ranging in diameter from 900 to 5000 A (S_f >400), as well as their structural similarities to

TABLE IV: Per Cent Composition of VLD-Lipoprotein Fractions.

Lipoprotein Fraction		No. of Samples	Cholesterol Free (%)	Cholesterol Ester (%)	Phospholipid (%)	Triglyceride[a] (%)	Protein (%)	Phospholipid/Protein Ratio (mg/mg)
Fraction A	S_f >5000	18	2.0	4.0	4.2	88.7	1.1	4.1
Fraction B	S_f 400–5000	5	2.9	7.3	6.6	81.4	1.8	3.8
Fraction C	S_f 100–400	7	3.9	10.9	11.6	68.3	5.3	2.4
Fraction D	S_f 50–100	4	4.2	13.3	13.9	59.3	9.3	1.5
Fraction E	S_f 20–50	4	4.4	13.5	13.1	56.8	12.2	1.1
Parent fraction	S_f >20	7	5.4	14.4	13.0	60.6	6.6	2.0

[a] Calculated by difference.

TABLE V: Average Per Cent Composition of VLD-Lipoprotein Fractions Isolated by Various Methods.

Sample or Fraction	Approx S_f Range	Isolated at "g min" ($\times 10^6$)	Recentrifugations	Total Cholesterol (%)	Phospholipid (%)	Triglyceride (%)	Protein (%)	Phospholipid/Protein Ratio	Reference
				Chylomicrons					
1	>5000	0.1[a]	0	9.2	7.1	81.3	2.5	2.8	Bragdon *et al.* (1956)
2	>5000	0.1[a]	5	4.3	9.0	84.8	2.0	4.5	Scanu and Page (1959)
3	>800	0.1[b]	0	23	7	70			Lindgren *et al.* (1962)
4		[c]		5.4	8.1		1.7	4.8	Burstein and Prawerman (1959)
A	>5000	0.1[a]	4	6.0	4.2	88.7	1.1	4.1	
B	400–5000	0.6[a]	3	10.2	6.6	81.4	1.8	3.8	
				VLD-Lipoproteins, S_f 100–400					
5		[c]		8.7	10.3		4.5	2.3	Burstein and Prawerman (1959)
6	25–600	9.6[b]	0	18.8	14.3	66.8			Lindgren *et al.* (1962)
C	100–400	4.8[a]	3	14.8	11.6	68.3	5.3	2.4	
				VLD-Lipoproteins, S_f 50–100					
7		[c]		11.8	14.7		6.7	2.2	Burstein and Prawerman (1959)
D	50–100	12.6[a]	3	17.5	13.9	59.3	9.3	1.5	
				VLD-Lipoproteins, S_f 20–50					
8	20–150	50[b]	0	30.5	18.0	51.5			Lindgren *et al.* (1962)
9	20–50	[d]	1	16.0	19.0	55.0	10.0	1.9	Oncley *et al.* (1957)
E	20–50	139[a]	2	17.9	13.1	56.8	12.2	1.1	
				VLD-Lipoproteins, S_f >20					
10	20–400	150[a]	0	17.4	21.1	53.1	8.4	2.5	Hayashi *et al.* (1959)
11	>20	144[a]	0	23	17	54	7	2.4	Korn (1959)
Parent fraction	>20	139[a]	2	22.0	14.3	56.2	7.5	2.0	

[a] Preparative centrifugation in angular rotor. [b] Density-gradient centrifugation in swinging-bucket rotor. [c] Precipitation with PVP and subsequent centrifugation. [d] Precipitation with dextran-sulfate and subsequent ultracentrifugation (114×10^6 "g min").

lipoprotein complexes of lesser flotation rates (S_f 20–400), it seems appropriate to classify chylomicrons as VLD-lipoproteins. In a theoretical treatment of the protein density on various lipoprotein surfaces, Vandenheuvel (1962) came to a similar conclusion. The isolation and characterization of the protein moiety of chylomicron (S_f >400) particles will be reported in future communications.

Since so little is known about the protein moieties of VLD-lipoproteins it was hoped that a preparative method of fractionation of this group of protein-lipid aggregates might lead to the separation of subfractions differing not only with respect to density, determined primarily by lipid components, but also with respect to characteristic proteins necessary for structural stability of lipoproteins. However, the gradual, rather than abrupt, changes in lipid and protein composition and physical properties, typified, for example, by the broad flotation boundaries of fractions A, B, and C, are probably caused by the presence of a mixture of heterogeneous but structurally similar particles in all fractions. It was then hypothesized that each subfraction might contain a mixture of heterogeneous lipoproteins, differing not only in size and density, but also with respect to the protein moiety. To test this hypothesis the VLD-lipoproteins were subjected to partial delipidization by heptane and the resulting mixture was separated by Pevikon-zone electrophoresis and preparative ultracentrifugation into three phospholipid-protein residues characterized by distinct protein moieties (Gustafson *et al.*, 1964).

The application of a controlled, standardized procedure in the fractionation of VLD-lipoproteins and the chemical and physicochemical characterization of purified subfractions provides valuable information on the distribution of particles differing in size, density, and composition. The usefulness of the procedure as a diagnostic tool and in establishing a lipoprotein classification

system of various hyperlipemic states will be published in a separate communication. Such procedures, however, are still inadequate for the isolation of a single homogeneous lipoprotein species and provide no further improvement in the classification of VLD-lipoproteins based on lipid composition.

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References

Bierman, E. L. (1963), *J. Clin. Invest. 42*, 917.
Bierman, E. L., Gordis, E., and Hamlin, J. T., III (1962), *J. Clin. Invest. 41*, 2254.
Bragdon, J. B., Havel, R. J., and Boyle, E. (1956), *J. Lab. Clin. Med. 48*, 36.
Burstein, M., and Prawerman, A. (1958), *Rev. Hematol. 13*, 329.
Burstein, M., and Prawerman, A. (1959), *Pathol. Biol. Semaine Hop. 7*, 1035.
Burstein, M., and Samaille, J. (1955), *Compt. Rend. 241*, 664.
Burstein, M., and Samaille, J. (1958), *Presse Med. 66*, 974.
Cornwell, D. G., Kruger, F. A., Hamwi, G. J., and Brown, J. B. (1961), *Am. J. Clin. Nutr. 9*, 41.
Dole, V. P., and Hamlin, J. T., III (1962), *Physiol. Rev. 42*, 674.
Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.
Gordis, E. (1962), *Proc. Soc. Exptl. Biol. Med. 110*, 657.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.
Gustafson, A., Alaupovic, P., and Furman, R. H. (1964), *Biochim. Biophys. Acta 84*, 767.
Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955), *J. Clin. Invest. 34*, 1345.
Hayashi, S., Lindgren, F., and Nichols, A. (1959), *J. Am. Chem. Soc. 81*, 3793.
Jirgensons, B. (1958), Organic Colloids, Amsterdam, Elsevier, p. 86.
Jobst, H., and Schettler, G. (1956), *Intern. Conf. Biochem. Probl. Lipids 3rd, Brussels, 1956*, 136.
Kohn, J. (1958), *Clin. Chim. Acta 3*, 450.
Korn, E. D. (1959), *Methods Biochem. Analy. 7*, 145.
Kunkel, H. G., and Trautman, R. (1956), *J. Clin. Invest. 35*, 641.
Laurell, C. B., Laurell, S., and Skoog, N. (1956), *Clin. Chem. 2*, 99.
Lindgren, F. T., and Nichols, A. V. (1960), *Plasma Proteins 2*, 1.
Lindgren, F. T., Nichols, A. V., Hayes, T. L., Freeman, N. K., and Gofman, J. W. (1959), *Ann. N.Y. Acad. Sci. 72*, 826.
Lindgren, F. T., Nichols, A. V., Upham, F. T., and Wills, R. D. (1962), *J. Phys. Chem. 66*, 2007.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marsh, J. B., and Whereat, A. F. (1959), *J. Biol. Chem. 234*, 3196.
Oncley, J. L. (1958), *Symp. Res. Advan. Appl. Med. Pract. 3*, 14 (Lipoproteins Methods Clin. Significance).
Oncley, J. L. (1963), *Brain Lipids Lipoproteins, Leucodystrophies, Proc. Neurochem. Symp., Rome, 1961*, 1.
Oncley, J. L., and Gurd, F. R. N. (1953), *in* Blood Cells and Plasma Proteins, Tullis, J. L., ed., New York, Academic, p. 337.
Oncley, J. L., Walton, K. W., and Cornwell, D. G. (1957), *J. Am. Chem. Soc. 79*, 4666.
Pinter, G. G., and Zilversmit, D. B. (1962), *Biochim. Biophys. Acta 59*, 116.
Rodbell, M. (1958), *Science 127*, 701.
Scanu, A., and Page, I. H. (1959), *J. Exptl. Med. 109*, 239.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Shore, B. S. (1957), *Arch. Biochem. Biophys. 71*, 1.
Smith, E. B. (1957), *Lancet 2*, 910.
Sperry, W. M., and Webb, M. (1950), *J. Biol. Chem. 187*, 97.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford.
Vandenheuvel, F. A. (1962), *Can. J. Biochem. Physiol. 40*, 1299.
Van Handel, E., and Zilversmit, D. B. (1957), *J. Lab. Clin. Med. 50*, 152.
Williams, C. A., Jr., and Grabar, P. (1955), *J. Immunol. 74*, 158.
Youngburg, G. E., and Youngburg, M. V. (1930), *J. Lab. Clin. Med. 16*, 158.