Particle size and protein content of six fractions of the $S_f > 20$ plasma lipoproteins isolated by density gradient centrifugation

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ABSTRACT A procedure is described for the separation of plasma $S_f > 400$ and S_f 20–400 lipoproteins each into three fractions. Serum samples are overlayered with a sodium chloride density gradient in a preparative ultracentrifuge tube and thin layers are removed at the top of the tube after successive centrifugations at different speeds in a swinging bucket rotor.

The procedure was evaluated by electron microscopy of the $S_f > 400$ lipoprotein fractions and schlieren analysis of the S_f 20–400 lipoprotein fractions. Protein content of each fraction was measured by elemental N, C, H, and lipid-P analysis. Protein coverage was calculated for all fractions on the assumption that there is a surface layer 20 A thick.

For the entire $S_f > 400$ lipoprotein spectrum and for a part of the S_f 20–400 lipoprotein distribution the proportion of surface covered by protein was constant (approximately 20% coverage). Therefore, for these portions of the lipoprotein spectrum, the increase in surface:volume ratio as particle size decreases is approximately compensated for by an increase in the weight percentage of protein.

SUPPLEMENTARY KEY WORDS chylomicrons · very low density lipoproteins · electron microscopy · analytical centrifugation · protein coverage

PLASMA (or serum) lipoproteins of density <1.006 g/ml, the $S_f > 20$ lipoproteins, are generally divided into (*a*) S_f 20–400, the very low density (VLD) lipoproteins, and (*b*) $S_f > 400$ lipoproteins. This division is in part operational, since S_f 400 marks approximately the upper limit for effective quantification of lipoproteins by schlieren analysis. It is also in part physiological, since S_f 20–400 lipoproteins are generally found in low concentration in the serum of normal humans at all times, whereas $S_f > 400$ lipoproteins can be detected in the serum of normal persons only during a restricted interval after the consumption of fat. $S_f > 400$ lipoproteins are therefore frequently called chylomicrons [the term coined by Gage in 1920 (1) to describe particles that appear in plasma after a fatty meal and are readily discernible with the dark-field microscope]. These large lipoproteins are however, perhaps better referred to as the chylomicron-containing class, since in a number of hyperlipoproteinemic states endogenous particles are apparently included in this group.

The $S_f > 400$ lipoproteins are spherical particles consisting of about 85% triglyceride and small amounts of cholesterol, phospholipid, and protein (2). Accurate and reproducible measurement of the protein content of this class of lipoproteins has been hampered by the difficulty of isolating them free from contaminating protein or higher density lipoproteins by preparative ultracentrifugation. Convective disturbances that arise during centrifugation in isodense salt solutions in angle-head rotors preclude the isolation of these lipoproteins as uncontaminated fractions after a single centrifugation. Laborious washing procedures, usually resorted to for the isolation of these lipoproteins, may remove natural surface components (including protein), alter surface properties, and induce clumping of particles. However, this class of lipoproteins, precisely defined in terms of included S_f range, can, after a single centrifugation in a new procedure (3), be isolated free from significant contamination

Abbreviations: VLD, very low density; EDTA, disodium ethylenediamine tetraacetate.

with serum protein or lipoproteins of lower flotation rates. This procedure uses a swinging bucket rotor and a density gradient to eliminate convective disturbances; a computer program calculates S_f recoveries. The values for the weight percentage of protein in $S_f > 400$ lipoproteins isolated by this technique from the plasma of hyperlipemic humans (3) and from the plasma and thoracic duct lymph of normal rats after fat loading (4) were found to be on the average 2.3, 2.2, and 0.67 respectively. All of the protein of chylomicrons appears to be associated with a membrane-like surface coat, of which the principal constituent is phospholipid (5).

The S_f 20–400 plasma lipoproteins are also spherical (6), lipid-rich particles consisting of approximately 55% triglyceride, 15% cholesterol, 20% phospholipid, and 10% protein (2). Recent electron microscopic studies of lipoproteins in the S_f 20–400 size range synthesized during liver perfusion also suggest the existence of an outer membrane-like layer (7). Thus the S_f 20–400 lipoproteins, or at least a portion of them, appear to be structurally similar to the $S_f > 400$ lipoproteins.

If the assumption is made that the protein of all $S_f > 20$ plasma lipoproteins is confined to a surface layer of constant thickness and if the protein were to constitute a fixed proportion of such a surface covering, the increase in surface to volume ratio with decreasing particle size should be just compensated for by an increase in weight percentage of protein. The calculated percentage of the surface covered with protein would be the same for particles of all sizes. Gradual changes or discontinuities in the relationship between particle size and protein coverage calculated for these conditions could be accounted for by one of the following: (*a*) changes in the proportion of protein in a membrane-like coat of constant thickness; (*b*) changes in surface coat thickness and, perhaps, protein conformation; or (*c*) changes in protein–lipid orientation. Any discontinuity might suggest one of the latter two possibilities.

To study the relationship between lipoprotein size and protein coverage, one must isolate in as natural a state as possible discrete subfractions of the $S_f > 400$ and S_f 20–400 lipoprotein classes, precisely defined in terms of their included S_f range. In the present report we describe a density gradient procedure for the isolation of three fractions each of the $S_f > 400$ and S_f 20–400 lipoproteins. These procedures were evaluated by electron microscopy of the $S_f > 400$ lipoprotein fractions and by schlieren analysis of the S_f 20–400 lipoproteins. The percentage weight of protein of fractions isolated from human plasma was determined by elemental (N, C, H) analysis and measurement of phospholipid phosphorus and the coverage of the surface of representative particles in each fraction was calculated for an outer coat assumed to be 20 A thick.

METHODS

Fractionation of the $S_f > 400$ Lipoproteins

$S_f > 400$ fractions were isolated from the plasma of two patients, one a diabetic with normal blood lipid levels, the other a hyperlipemic (Type V) subject. Both patients consumed a mixture containing 100 g of safflower oil, 300 g of egg white, 20 g of sugar, and added flavoring before blood was drawn (8 hr after the meal in the case of the diabetic and 12 hr after in the case of the Type V patient).

The procedure used represents a modification (3) of an earlier (8) cumulative flotation procedure. In the first step of the procedure 3 ml of plasma, raised to a background density[1] of 1.065 g/ml with solid NaCl, was placed in a cellulose nitrate heavy-walled preparative ultracentrifuge tube ($^5/_8$″ O.D. × 4″) and specially overlayed (3) with a 14 ml density gradient of NaCl solutions. The solutions were added in volumes (from highest to lowest density) of 1 ml each for the first two and 3 ml each for the remaining four. These densities were 1.0464, 1.0336, 1.0271, 1.0197, 1.0117, and 1.0064 g/ml. The gradient is shown in Fig. 1.

Three slight modifications in the previously-described procedure (3) for the preparation of the density gradients (apart from scaling up the salt volumes) have been introduced in this step. They are as follows. (*a*) Solid epoxy hemispherical inserts which fit snugly into the bottom of the centrifuge tubes were placed into the tubes (over a few drops of a NaCl solution of density 1.065 g/ml) before the sample was added. This eliminated the curvature in the lower boundary of the sample. (*b*) A lucite sleeve (see Fig. 1) was inserted into the centrifuge tube before transfer of sample to avoid contact of the transfer pipette with the inside wall of the tube. (*c*) All of the salt solutions contained EDTA (3 mg/100 ml). We have found that the isolated fractions can be stored for longer periods of time without showing signs of deterioration, such as clumping of particles, when EDTA is present.

In the next step of the procedure 0.5 ml layers were removed from the tops of the tubes after three successive centrifugations in a swinging bucket rotor under conditions calculated to bring into the top 0.5 ml 100% of the $S_f > 3200$, $S_f > 1100$, and $S_f > 400$, respectively, after the first, second, and third centrifugations. Our principal reasons for fractionating in this manner, rather than by centrifuging once and removing a number of layers, are as follows. (*a*) Removal of thin layers at the top of the tubes after density gradient centrifugation avoids the necessity for either isolating the total $S_f > 400$ lipoprotein class free of contaminating protein prior to the fractionation or washing each fraction after the fractionation.

[1] All densities are given at 20°C.

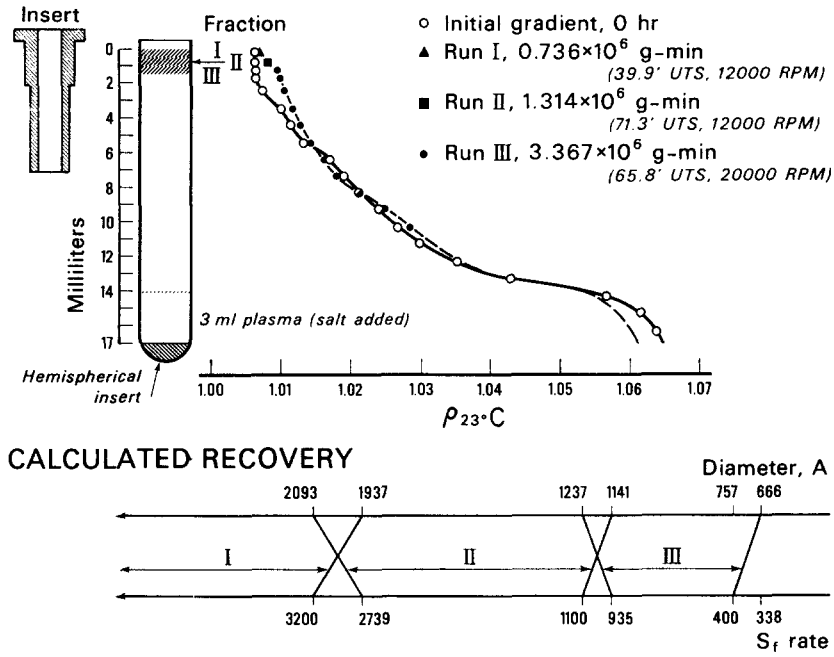

FIG. 1. Sodium chloride density gradient used for the isolation from plasma of three fractions of the $S_f > 400$ lipoproteins (17 ml gradient, SW 25.3 rotor, 23°C). The *g*-min values listed for each successive centrifugation are for ideal runs at the equivalent up-to-speed (*UTS*) time given. Diagramed below the gradient are the calculated ranges of S_f rates for particles recovered under these ideal conditions and the calculated particle diameters at each S_f boundary. For example, S_f 338 corresponds to a diameter of 666 A; fraction III has a calculated S_f range of 338–1100 for the outside limits (at which threshold or 0% recovery is calculated); for particles completely (100%) recovered, the calculated S_f range is 400–935.

(*b*) As a result of increasing the radial distance the lipoproteins in each fraction must travel by bringing them to the top and by concentrating each fraction into a thin 0.5 ml layer, the calculated difference in S_f rate between the 100 and 0% (threshold) recovery for a given fraction is reduced. This also means that the theoretical overlap among fractions is reduced. Moving the rotor to and from the centrifuge to collect fractions, of course, risks mechanically disturbing the gradient, but this risk is compensated for by the lesser contamination of the layer removed with its underlying layer when thin rather than broad layers are removed.

The centrifugations were carried out at room temperature (23°C) in the Spinco model L2-65 ultracentrifuge (modified to achieve temperature stability to ± 0.2°C during a run and rotor speed stability to ± 30 rpm) in the 25.3 rotor. The rotor speed settings for the first two centrifugations were 12,000 rpm and for the third, 20,000 rpm. At a mean radial distance of 11.43 cm, these centrifugations, including acceleration and deceleration, are equivalent to 0.736×10^6 *g*-min, 1.314×10^6 *g*-min, and 3.37×10^6 *g*-min, respectively, for the first, second, and third centrifugation. Lipoprotein recovery depends on the density and viscosity of regions in the gradient. Therefore, the initial rotor temperature (approximately equal to the gradient temperature) of the particular run determined the equivalent up-to-speed times (full-speed times plus 1/3 of the acceleration and deceleration times); they were generally in the order of 40, 71, and 66 min, respectively. After the third fraction had been collected, an additional 0.5 ml was removed for measurement of the background salt density and evaluation of possible plasma protein contamination. The precise 100% and 0% recovery of each of the three fractions for each run was calculated (3) from the actual centrifugal conditions, including the mean rpm, the initial and final rotor temperatures, and the up-to-speed, acceleration, and deceleration times.

Fractionation of the S_f 20–400 Lipoproteins

S_f 20–400 lipoproteins were obtained for fractionation from the plasma of two fasted hyperlipoproteinemic patients (Type IV). (If the fractionations had been continued with the samples used for the $S_f > 400$ fractionation, fresh aliquots of plasma would have been taken.) The $S_f > 400$ lipoproteins were first removed from the undiluted plasma sample. One can conveniently accomplish this, even when the sample is heavily laden with $S_f > 400$ lipoproteins, by centrifuging 3–6 ml in a swinging bucket rotor under conditions calculated to bring the $S_f > 400$ lipoproteins into the top 0.5 ml and collecting the subnatant fraction by puncturing the bottom of the tube. If a beam of light is directed at the tube in a darkened room, the light-scattering top layer can be readily

seen during the collection of the subnatant. (At 23°C, centrifugation of 6 and 4 ml of plasma for 4.54 × 10^6 *g*-min and 3.55 × 10^6 *g*-min, respectively, in a 25.3 Spinco rotor fitted with special 6-ml buckets, mean r = 10.44 cm, brings the $S_f > 400$ lipoproteins into the top 0.5 ml.)

2 ml of the subnatant plasma fractions, raised to a salt background density of 1.065 g/ml, was transferrred to a heavy-walled $^9/_{16}$" O.D. × $3^1/_2$" preparative ultracentrifuge tube and overlayered with the same six NaCl solutions used for the $S_f > 400$ fractionation, in the following volumes (from the highest to lowest density): 1 ml for the first two and 2 ml for the next four. The gradient is shown in Fig. 2. The same modifications listed for the $S_f > 400$ fractionation were used in this step of the procedure. 0.5 ml layers were then removed at the top of the tube after each of three successive centrifugations in a Spinco swinging bucket SW 41-Ti rotor under conditions calculated to bring up first $S_f > 100$, then $S_f > 60$, and finally $S_f > 20$ lipoproteins into the top 0.5 ml. All centrifugations were carried out at room temperature (23°C) at 35,000 rpm for approximately 2.40, 1.69, and 18.6 hr, respectively, for the first, second, and third centrifugations. At a mean distance of 10.76 cm, the equivalent *g*-min for these three centrifugations were 21.2 × 10^6 for the first, 15.0 × 10^6 for the second, and 164.7 × 10^6 for the third. An additional 0.5 ml was taken for measurement of the background salt density and possible protein contamination after collection of the third fraction.

Evaluation of the $S_f > 400$ Fractionation by Electron Microscopy

Aliquots of each fraction were diluted 1:100 with glass-distilled water. Drops, containing approximately 0.005 ml, were placed on collodion-covered stainless steel grids and fixed with OsO_4 vapor in a humidity chamber. After being air dried they were shadowed with uranium in a vacuum evaporator. Electron micrographs were taken at known magnifications with an RCA-2D microscope. The diameters of the lipoprotein particles were measured on $8^1/_2$" × 11" prints (representing a total magnification of 24,280) of each photomicrograph, with the aid of a pair of dividers and a vernier caliper calibrated to 0.02 mm. A computer program converted these measurements into actual particle size in Angstroms, sorted all particles of a given fraction into an ascending sequence of diameters, and calculated the molecular weight of each particle, on the assumption of a uniform hydrated density of 0.93 g/ml. From these data, a mass vs. particle size histogram at 250 A intervals was constructed by a Cal-Comp plotter. In this histogram each fraction was normalized to its proportion of the total mass by virtue of the inclusion in the computer program of values for lipoprotein mass obtained by N, C, H analysis.

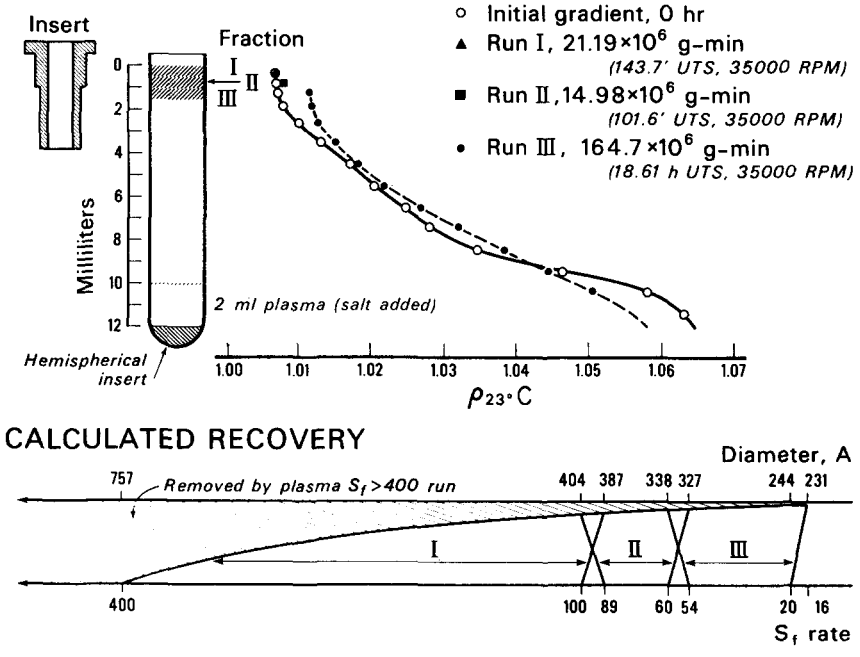

FIG. 2. Sodium chloride density gradient used for isolating three fractions of the S_f 20–400 plasma lipoproteins (12 ml gradient, SW 41 rotor, 23°C). The values of *g*-min are for ideal conditions. The S_f ranges calculated for such conditions and the particle diameters at the boundaries calculated from these conditions are diagramed below the gradient.

Schlieren Analysis of the S_f 20–400 Lipoprotein Fractions

Aliquots of the total VLDL sample and of each of its subfractions from which the $S_f > 400$ lipoproteins had been removed were raised to a density of 1.063 g/ml with solid NaCl before being subjected to analytical centrifugation. The procedures used for the analytical centrifugation and the computer analysis of the schlieren patterns have been described elsewhere (9).

Analytical Procedures

The total mass of each fraction was determined by elemental N, C, H analysis (3). Phospholipid content of the lipid extracts (10) of the $S_f > 400$ lipoproteins and of the solutions of the S_f 20–400 lipoproteins was determined by a phosphorus assay (11). The percentage by weight of protein in each fraction was calculated, as previously described (3), from the N/(N + C + H) ratios determined by the elemental analysis and lipid-P measurements. These calculations assumed the mass of the lipid and protein moities to be composed of 88.9% and 76.1% elemental N + C + H, respectively.

RESULTS AND DISCUSSION

The electron micrographs of the three $S_f > 400$ lipoprotein fractions of the plasma sample listed as No. 735 in Table 1 (below) show three distinct groups of particles (Fig. 3). Diagramed in the upper part of Fig. 4 are the expected S_f values for the recovery in that sample of 100% and 0% of the lipoproteins in each fraction and the particle diameters at the fraction boundaries calculated from these recoveries. For example, we calculated for fraction I that all (100% recovery) of the $S_f > 3176$ (diameter 2089 A) and none (0%) of the $S_f > 2719$ (diameter 1935 A) should have been recovered. The lower part of Fig. 4 shows the mass vs. particle size histogram derived from the electron microscopy measurements.

Comparison of the predicted mean diameters at the fraction boundaries with the histogram reveals the following. The predicted mean particle diameters at the boundaries between fraction I and II and between II and III were 2012 A and 1175 A, respectively. According to the histogram, 97.7% of the mass of fraction I was recovered as particles with diameters larger than 2012 A, and 84.4% of the mass of fraction II was recovered as particles smaller than that dimension; 97.3% of the mass of fraction II was recovered as particles larger than 1175 A in diameter. The one major discrepancy between the predicted and measured particle size distribution occurred with fraction III. In this case, although essentially 100% of the mass was recovered as particles with measured diameters larger than predicted for the lower

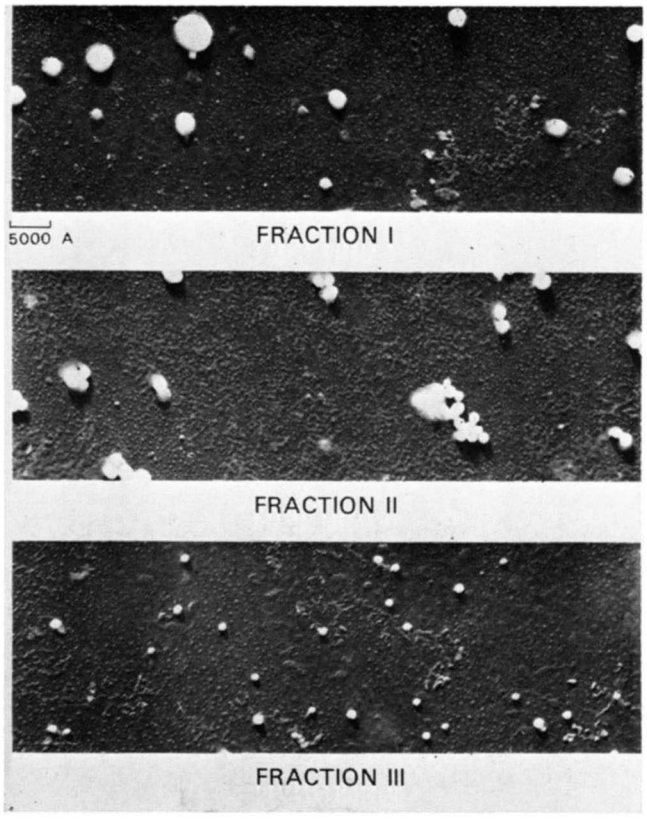

FIG. 3. Electron micrographs of the three $S_f > 400$ lipoprotein fractions isolated from plasma of sample designated No. 735 in Table 1.

boundary of that fraction, approximately 45% of the mass included particles with diameters larger than 1175 A, the particle diameter predicted for the boundary between fractions II and III. There appears to be no obvious explanation for the discrepancy. We considered the possibility that some of the large particles in fraction III were actually two or more coalesced smaller particles, but careful examination of the electron micrograph prints could not establish whether this was the case. In any event, the observed particle size distribution appears to be reproducible. An almost identical histogram was derived from measurements made on fractions isolated from the plasma sample designated No. 726 in Table 1.

The predicted S_f recoveries for sample No. 795 and the schlieren patterns of that sample for the S_f 20–400 lipoproteins and the three isolated fractions of this class, traced from computer-derived printouts from a cathode ray tube (12), are shown in Fig. 5. As can be seen, the corrected schlieren patterns of the three individual fractions agree well with expected recoveries for different S_f rates. Further it is apparent that the sum of the three fractions yields a lipoprotein distribution that is very similar in profile to the total S_f 16–403 lipoproteins.

The protein and phospholipid contents of all of the isolated fractions are recorded in Tables 1 and 2. The per-

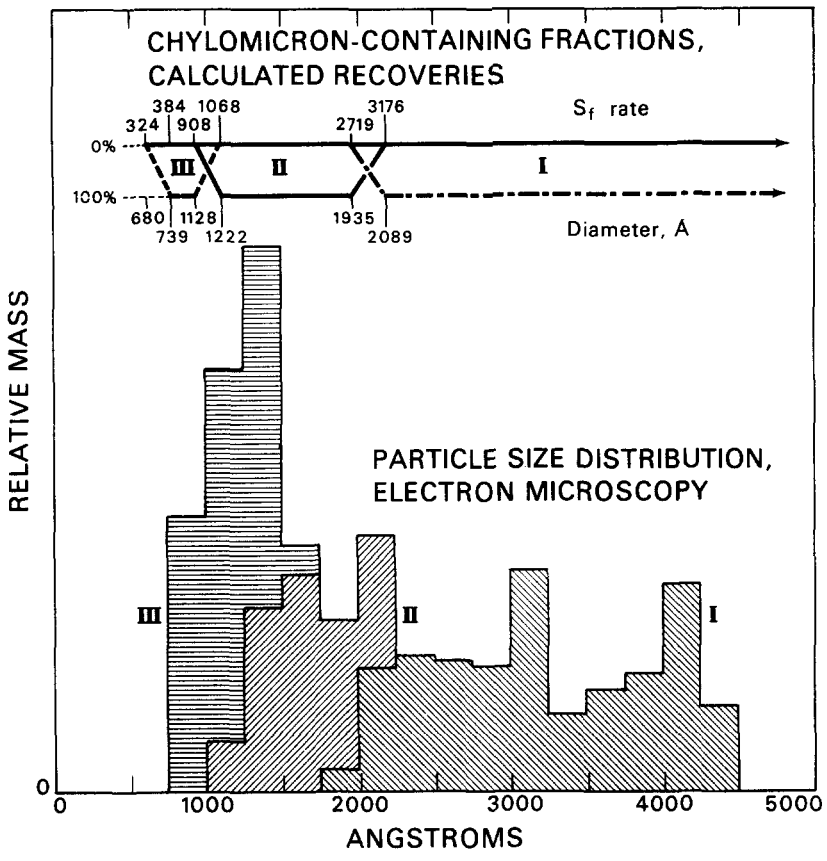

FIG. 4. *Upper part:* Diagrammatic representation of the predicted lipoproteins recovered in each of the three $S_f > 400$ fractions isolated from sample No. 735, and the calculated particle diameters at the boundaries. The S_f rates at each boundary were calculated from the recorded centrifugal conditions of this particular fractionation. For example, fraction III has a calculated S_f rate range of 324–1068 for the outside limits (at which threshold or 0% recovery is calculated); for lipoproteins completely recovered, the calculated S_f range is 384–908.

Lower part: Computer-constructed plot of lipoprotein mass vs. particle diameter derived from measurements for sample No. 735 of particle size on electron micrograph prints. Each fraction is normalized according to its total mass determined by elemental N + C + H analysis. Analyses of fractions I, II, and III were each made from five electron micrograph frames with measurement of 58, 67, and 138 particles, respectively.

centage compositions of both protein and phospholipid increased with decreasing particle size. However, the amount of protein per particle decreases greatly with decreasing size, in agreement with other data (16). Listed also in the tables are the values for the percentage of the surface covered with protein, calculated for representative particles in each fraction. We have taken as representative the particle with a surface:volume ratio equal to that of the whole fraction. After dividing the lipoprotein spectrum into narrow intervals (of diameter for the $S_f > 400$ and of S_f rate for the S_f 20–400 fractions), we calculated this as: $\Sigma n_i s_i / \Sigma n_i v_i$, where n_i = number of particles in the i-th interval; s_i = surface area of a single midinterval particle; and v_i = volume of the same particle. For the $S_f > 400$ fractions, the n_i are actual particle counts, while for the S_f 20–400 fractions $n_i = c_i/m_i$, where c_i is the concentration in the i-th interval of the schlieren analysis and m_i is the particle mass. Protein coverage was calculated as the ratio of the experimentally determined percentage weight of protein to the theoretical percentage weight of protein for spherical particles assumed to be completely covered with a protein layer 20 A thick. The density of the protein was assumed to be 1.33 g/ml. The density of whole particles was calculated from the experimentally determined percentage weight of protein and an assumed value of 0.95 g/ml for the density of the lipid moieties. The theoretical percentage weight of protein in particles assumed to be completely covered with protein was calculated from these densities and from the volumes of the outer layers and whole particles. Although the value of 20 A assumed for the thickness of the membrane-like coat is somewhat arbitrary, it is perhaps the best estimate that can be made at the present time. It is the value given in a preliminary report (13) for the thickness, estimated from electron micrographs, of the "membranes" of chylomicrons

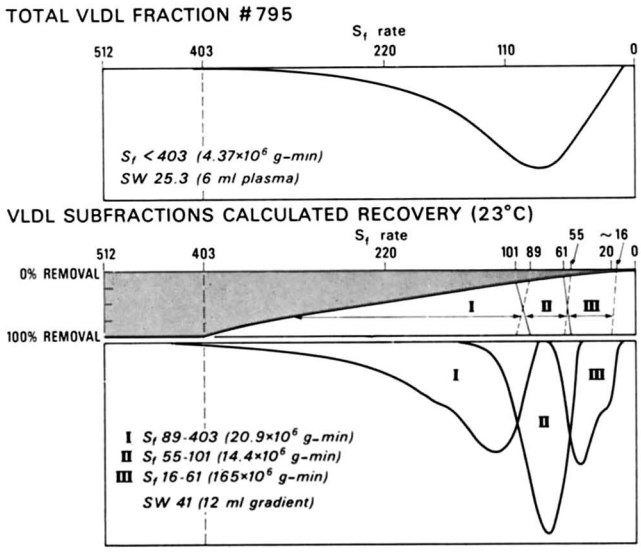

FIG. 5. Predicted S_f recoveries for sample No. 795 and the schlieren patterns of that sample obtained for the total very low density lipoproteins and the three isolated fractions. The upper pattern for the total VLDL was obtained on a sample from which all of the $S_f > 403$ lipoproteins were first removed (4.37×10^6 *g*-min) and the total VLDL then isolated in a single density gradient run (200.44×10^6 *g*-min). A continuously changing partial recovery across the entire S_f 20–400 spectra (resulting from $S_f >$ 403 removal) is indicated by the unshaded region.

isolated from human intestinal lymph, and is close to the value (18 A) we have calculated from the data of Zilversmit (5) for dog lymph chylomicrons. Since the purpose of our calculation is to examine the relationship between particle size and protein coverage for an outer coat of any given thickness, it is not essential for us to know with certainty the actual thickness of such a "membrane."

The calculated values for protein coverage for representative particles in the three fractions of the $S_f > 400$ lipoproteins were quite similar, ranging from 18 to 22%. This is consistent with the view that protein constitutes a fixed proportion of an outer membrane-like surface coat of all $S_f > 400$ lipoproteins. In the S_f 20–400 lipoprotein class, values calculated for protein coverage of the S_f 100–400 fractions of both samples and the S_f 60–100 fraction of one of the samples were in the same range as those calculated for the $S_f > 400$ fractions. Slightly higher values were calculated for the S_f 60–100 lipoprotein fraction of one of the samples and the S_f 20–60 lipoprotein fractions of both samples. The differences between the higher and lower values were technically significant. However, to establish a definite deviation in the protein–lipid relationship at the lower end of the VLD lipoprotein spectrum, more data from persons presenting a variety of lipoprotein distributions will be required.

In recent years, other investigators have fractionated chylomicron-containing and VLD lipoprotein classes of chyle and plasma and determined their chemical composition. Yokoyama and Zilversmit (14) used a single centrifugation with a sucrose density gradient in a swinging bucket rotor to separate chylomicrons, isolated from thoracic duct lymph of dogs, into three layered groups with estimated diameters of >2000 A, 1400–2000 A, and <1400 A. Although considerable variation in protein

TABLE 1 ELEMENTAL ANALYSIS, PROTEIN CONTENT, AND CALCULATED SURFACE PROTEIN COVERAGE OF THREE FRACTIONS OF THE CHYLOMICRON-CONTAINING CLASS OF PLASMA LIPOPROTEINS

Plasma Sample No.	Fraction	Calculated Recovered Range	N + C + H	$\frac{N}{N+C+H}$	Phospholipid*	Protein†	% of Surface Covered with Protein‡	Mass % of Total Lipoprotein in Fraction
			μg/0.1 ml			*weight %*		
735	I	S_f 2719–10^5	385	0.00267	2.8	1.24	20.9 (3051 A)	43.3
			395	0.00240		1.09 (1.17)		
	II	S_f 908–3176	206	0.00467	5.5	2.11	17.8 (1620 A)	23.5
			214	0.00365		1.50 (1.81)		
	III	S_f 324–1068	296	0.00710	9.4	3.09	19.8 (1127 A)	33.1
			295	0.00644		2.70 (2.90)		
726	I	S_f 2694–10^5	935	0.00218	3.0	0.94	18.9 (3146 A)	29.1
			947	0.00245		1.09 (1.02)		
	II	S_f 902–3146	1188	0.00381	2.1	1.99	20.2 (1709 A)	36.9
			1195	0.00376		1.96 (1.98)		
	III	S_f 324–1068	1108	0.00635	7.7	2.84	21.5 (1281 A)	34.0
			1091	0.00623		2.77 (2.81)		

* Mean of duplicate phosphorus analysis. Total phospholipid = 25 × P.

† The mean values are given in parentheses. The approximate standard error of measurement of 0.2% in weight % protein would introduce an uncertainty of approximately 2 and 0.5% in calculated protein coverage of the $S_f > 400$ and S_f 20–400 subfractions, respectively.

‡ Calculated for the representative particle (diameter in parentheses) with an assumed membrane-like surface layer 20 A thick (see text).

TABLE 2 ELEMENTAL ANALYSIS, PROTEIN CONTENT, AND CALCULATED SURFACE PROTEIN COVERAGE OF THREE FRACTIONS OF THE VLD PLASMA LIPOPROTEINS

Plasma Sample No.	Fraction	Calculated Recovered Range	N + C + H	$\frac{N}{N + C + H}$	Phospholipid*	Protein†	%, of Surface Covered with Protein‡	Mass % of Total Lipoprotein in Fraction
			μg/0.1 ml			*weight %*		
795	I	S_f 89–403	1218	0.01411	16.7	6.32	20.4 (483 A)	46.8
			1225	0.01459		6.60		
			1200	0.01413		6.33		
			1208	0.01404		6.28 (6.38)		
	II	S_f 55–101	912	0.01985	18.7	9.42	23.6 (369 A)	35.1
			911	0.01985		9.42		
			905	0.01956		9.26		
			892	0.01988		9.44 (9.39)		
	III	S_f 16–60	468	0.02691	21.4	13.20	28.0 (295 A)	18.0
			462	0.02757		13.58		
			461	0.02689		13.19		
			454	0.02731		13.43 (13.35)		
	I–III	S_f 16–403	1413	0.01859	18.1	8.75		
			1377	0.01896		8.97		
			1416	0.01821		8.54		
			1413	0.01865		8.79 (8.76)		
781	I	S_f 90–385	270	0.01493	16.3	6.84	20.3 (435 A)	25.8
			269	0.01550		7.17 (7.01)		
	II	S_f 52–101	411	0.02346	19.9	11.37	29.1 (359 A)	37.6
			397	0.02467		12.07 (11.72)		
	III	S_f 16–58	393	0.02869	23.0	14.04	28.8 (283 A)	36.7
			390	0.02919		14.34 (14.19)		
	I–III	S_f 16–385	871	0.02258	19.9	10.87		
			868	0.02235		10.74 (10.81)		

* Mean of quadruplicate (No. 795) or duplicate (No. 781) phosphorus analysis. Total phospholipid = 25 × P.
† The mean values are given in parentheses.
‡ Calculated for the representative particle (diameter in parentheses) with an assumed outer membrane-like surface layer 20 A thick (see text).

content was observed for a given group, in general the percentage of protein was higher in the small than in the large chylomicrons. The smaller chylomicrons also contained higher percentages of phospholipid and cholesterol. From the analysis of isolated surface coats and cores of dog lymph chylomicrons Zilversmit has recently concluded that chylomicrons consist of a core of cholesteryl ester dissolved in triglyceride surrounded by a surface coat composed principally of phospholipid and protein; free cholesterol is partitioned between the core and the surface (15).

Gustafson, Alaupovic, and Furman (16) isolated five fractions of human serum $S_f > 20$ lipoproteins by anglehead preparative ultracentrifugation. Fractions were isolated at the top of the centrifuge tube after buffer had been layered over the serum sample or over the subnatant fractions remaining after successive centrifugations. The isolated fractions were washed as many as four times. Their approximate flotation rates were $S_f > 5000$, S_f 400–5000, S_f 100–400, S_f 50–100, and S_f 20–50. The percentage weight of protein increased and that of triglyceride decreased as the S_f rates and particle size decreased. The weight percentages of protein of these authors' three S_f 20–400 fractions, which were similar in flotation rates to the three S_f 20–400 fractions we isolated, were (in decreasing order of flotation rate) 5.3, 9.3, and 12.2. These values are similar to those recorded in Table 2 for our S_f 20–400 fractions. However, the phospholipid values for the three S_f 20–400 fractions isolated by Gustafson et al. were consistently lower than those recorded for the three S_f 20–400 fractions we isolated. Nevertheless, the fractions of the S_f 20–400 lipoproteins isolated by the two procedures appear to be similar in many respects.

The results of Yokoyama and Zilversmit (14), Gustafson et al. (16), and our present study therefore suggest that all the S_f 20–10^5 lipoproteins have roughly similar protein coverage, provided the protein configuration is the same. However, our data also indicate that within the S_f 20–400 fraction there might be an increase in protein coverage with decreasing S_f rate at the lower

end. This trend toward increased protein coverage would be consistent and comparable with protein composition (17) and physical data on the major component of the S_f 0–12 lipoproteins (18). Assuming the same protein configuration and spherical molecules, lipoproteins of the S_f 6–8 class, containing 20% protein, would have a protein coverage in the neighborhood of 30%.

As discussed previously (3), this method of lipoprotein isolation using an overlayering technique and density gradient minimizes contamination from both small and large molecules present in the initially layered plasma fraction. For meaningful quantitative and qualitative studies of lipoprotein protein, such absence of contamination is essential, especially for sensitive immunochemical studies. Another feature of this type of gradient preparation is its simplicity and reproducibility; it does not require special equipment for gradient formation. Although the method has been designed to obtain six specific subfractions within the total S_f 20–10^5 plasma lipoprotein spectrum, it can easily be modified to provide different S_f rate ranges of recovery and additional subfractions if desired. Further, although the application presented here involves fractionation of particles <1.006 g/ml by successive, cumulative centrifugation, other useful modifications are potentially possible. For example, by appropriate increase in the density of all the regions in the gradient by a fixed amount, lipoproteins of S_f 0–20, and perhaps even the high density lipoproteins, might be similarly fractionated. However, since their flotation rates would be much smaller, a swinging-bucket rotor with a higher performance and shorter radial path than the SW 41-Ti would be more appropriate.

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