Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of normolipemic and hyperlipemic humans

Teizo Sata,* Richard J. Havel, and Albert L. Jones

Cardiovascular Research Institute and Departments of Medicine and Anatomy, University of California School of Medicine, and the Cell Biology Section, Veterans Administration Hospital, San Francisco, California 94122

Abstract As judged from measurements of the diameters of particles fixed with osmium tetroxide and shadowed with platinum, gel chromatography on 2% agarose has been shown to be an effective quantitative method for separating triglyceride-rich lipoproteins according to particle size. Particles in the size range of chylomicrons, uncontaminated by lipoproteins smaller than about 700 Å or by other serum proteins, emerged in the void volume of the column, and very low density lipoproteins with diameters between 400 and 700 Å were separated into fractions with average standard deviation of 71 Å from the mean. Systematic comparison of the relationship between diameter and chemical composition of fractions obtained from subjects with various hyperlipoproteinemic disorders demonstrated a precise correlation consistent with a spherical model for these lipoproteins in which phospholipids, free cholesterol, and protein occupy a surface monolayer with an invariant thickness of 21.5 Å surrounding a liquid core of triglycerides and cholesteryl esters. The chemical composition of very low density lipoproteins of given particle size in most recognized types of hyperlipemia was similar to that of normolipemic subjects, but particles in the size range of chylomicrons sometimes had higher contents of cholesteryl esters and free cholesterol. Results obtained in subjects with dysbetalipoproteinemia were consistent with the presence of three populations of particles. Two of these, with mean diameters of about 850 and 350 Å, had unusually high cholesteryl ester content and reduced triglyceride content and may represent "remnants" of the metabolism of structurally normal chylomicrons and very low density lipoproteins, respectively. The third, a heterogeneous group with intermediate range of particle size and pre-β mobility, may represent a population of very low density lipoproteins with relatively normal composition.

Supplementary key words very low density lipoproteins · chylomicrons · electron microscopy · dysbetalipoproteinemia · lipoprotein structure

The triglyceride-rich lipoproteins constitute two heterogeneous groups of apparently spherical particles with diameters between 300 and 5000 Å that transport large amounts of fat in the blood. They include chylomicrons secreted from the small intestinal mucosa which enter the blood from the thoracic duct during active fat absorption, and VLDL which enter the blood continuously from the liver and to a limited extent from the small intestinal mucosa via the thoracic duct. One or both of these two classes of lipoproteins accumulate in the blood in a variety of disease states (1). Subfractionation of chylomicrons and VLDL has generally been achieved by repeated ultracentrifugations at progressively increasing centrifugal force (2) or by ultracentrifugation through a density gradient (3–5). The first of these methods requires repeated washings to provide fractions of constant chemical composition and is accompanied by substantial loss of material. The second is technically demanding, particularly as applied to VLDL, and provides only limited amounts of material.

Gel filtration on columns of agarose appears to offer a simple means for obtaining large quantities of VLDL subfractions with virtually no losses (6). The degree of

Abbreviations: VLDL, very low density lipoproteins.

* Dr. Sata was a Research Fellow of the Bay Area Heart Association; his present address is Research Institute of Angiocardiology and Clinics for Cardiovascular Disease, Kyushi University School of Medicine, Fukuoka, Japan.

Downloaded from www.jlr.org by guest, on June 19, 2012

heterogeneity of the fractions obtained with this method has not been determined. To provide more definitive data about the usefulness of this method, we have used electron microscopy to evaluate the heterogeneity of fractions obtained from mixed triglyceride-rich lipoproteins of healthy subjects and individuals with various forms of hyperlipoproteinemia. Comparison of chemical composition of the fractions with their particle size has demonstrated a regular relationship for all subjects which is consistent with the "pseudomicellar" model in which phospholipids, free cholesterol, and protein form a monolayer at the surface of an oil droplet.

METHODS

Ultracentrifugation and gel chromatography

Blood samples were obtained in the postabsorptive state from five healthy, normolipidemic men and from eight subjects with various types of primary hyperlipoproteinemia (one with exogenous, two with endogenous and two with mixed hyperlipemia, and three with dysbetalipoproteinemia). Fat content of the diet was not regulated except as noted. Samples of freshly obtained serum were ultracentrifuged at density 1.006 in the 40.3 rotor of a Beckman preparative ultracentrifuge for $\sim 1.3 \times 10^8$ *g*-min (maximum value) at 12°C to separate VLDL and chylomicrons (7). The suspended triglyceride-rich lipoproteins were applied to a column of 2% agarose gel as described previously (6). Samples were applied in a volume of 15 ml or less to columns with total bed volume of 450 ml (90 × 2.5 cm) and eluted with 0.2 M NaCl in water containing 0.001 M EDTA (disodium salt) at pH 7.0; 15–16 fractions of 15 ml each were collected.

Chemical analysis

Portions of the eluates were extracted in chloroform–methanol 2:1 (v/v). Total and free cholesterol were precipitated as the digitonide according to the method of Sperry and Webb (8) and were determined with the ferric chloride reagent of Zak et al. (9). Cholesteryl esters were calculated as: (total − free cholesterol) × 1.67. Lipid phosphorus was determined after acid digestion (10), and phospholipid was estimated as lipid phosphorus × 25. Glyceride glycerol was determined by the method of Carlson (11), and triglyceride was estimated assuming a molecular weight of 860. Protein content of eluates was measured directly by a modification of the method of Lowry et al. (12). A 500-μl sample was transferred to a test tube, and 1 ml of double-strength alkaline copper reagent (12) was added and mixed well. After 10 min, 0.2 ml of Folin's phenol reagent was added and the solution was well mixed immediately. After an additional 30 min, 4 ml of diethyl ether was added and mixed with a tube buzzer. The test tube was centrifuged for 5 min at 2000 rpm to obtain a clear reaction mixture. This procedure was repeated once, or with particularly lipid-rich fractions, twice. Protein content was estimated using crystalline bovine serum albumin as standard. This procedure gave results closely similar to but more reproducible than those obtained when protein was precipitated with ethanol–acetone 1:1 (v/v) and dissolved in 2.5 M aqueous sodium hydroxide. In these studies, recovery of triglyceride between elution volumes of 100 and 350 ml was 88.3 ± 4.8% (mean ± SD), and of protein, 84.5 ± 11.3%. A small second peak of protein associated with virtually no lipid emerged later. Its elution volume (about 425 ml) coincided with that of albumin.

Electron microscopy

Samples of the eluate stored overnight were fixed with a solution of 1% osmium tetroxide and 0.05 M phosphate buffer at pH 7.35. A drop of this solution was placed on a Formvar-covered, carbon-coated, 300-mesh copper grid, allowed to stand for 3 min, and then processed according to Jones and Price (13). In the present study, however, the shadow angle was approximately 15°. The shadowed lipoproteins were examined in a Philips EM 300 electron microscope and photographed at an initial magnification of 9700. This magnification was checked each day with a calibration grid. All samples obtained from a given patient were processed together. Earlier studies demonstrated no difference in the size and shape of lipoprotein particles fixed immediately or stored overnight.

In order to determine accurately the thickness of the evaporated metal on the surface of the particles, polystyrene latex spheres, 0.109 μm diameter (SD 0.0027 μm) (Dow Chemical, Midland, Mich.), were used as a standard. Photographic negatives of the lipoprotein and unshadowed and shadowed polystyrene latex particles were magnified 50 times with an optical microcomparator equipped with a vernier scale for measurement of their diameters. For each sample, approximately 90–450 particles contained in one to four negatives were measured. After subtracting the thickness of the platinum coating on the latex particles, the mean size of the lipoproteins and their size distribution were determined.

Electrophoresis and gas–liquid chromatography

Each sample examined by electron microscopy was also subjected to electrophoresis in agarose gel by the method of Noble (14). Triglycerides were separated from cholesteryl esters and phospholipids on columns of silicic acid (15), and fatty acid compositions of their

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 19, 2012

methyl esters were determined by gas–liquid chromatography (16).

RESULTS

Measurement of particle diameter

Uptake of osmium by triglyceride-rich lipoproteins appeared to be dependent upon the degree of saturation of the constituent fatty acids (Fig. 1). Lipoprotein particles obtained from a subject with mixed hyperlipemia appeared darker and were better defined when he had been on a diet containing 65% of calories from corn oil for 3 days than those obtained when he was on his usual diet. Triglyceride fatty acid composition of d < 1.006 lipoproteins in this subject when he was consuming a diet rich in corn oil was 16:0, 13.1%; 18:1, 28.3%; and 18:2, 54.5%. When on his usual diet, corresponding values of: 16:0, 24.9%; 18:1, 42.2%; and 18:2, 20.1% were similar to those of the other subjects whose intake of fat was not regulated. Fatty acid composition of triglycerides in particles of various sizes was determined in five normolipemic and four hyperlipemic subjects. Composition was similar in subfractions of VLDL from all subjects, but content of polyunsaturated fatty acids was appreciably lower in chylomicron-sized particles from three subjects.

The margin of shadowed particles was visualized more easily than that of unshadowed ones (Fig. 1). The thickness of the shadowed material varied from 43 to 64 Å from batch to batch but was similar among samples shadowed in the same batch. With latex beads, the boundary between the particle proper and the platinum coating could easily be defined, but this was often difficult in the case of lipoproteins. Since the overall diameter of shadowed particles was determined more easily and the thickness of the metallic layer was almost identical for particles of different sizes, the diameter of chylomicrons and VLDL was estimated by subtracting the thickness of the platinum deposited on polystyrene latex beads which were included with each group of particles.

Fig. 2 shows electron micrographs of fractions obtained from a subject with dysbetalipoproteinemia (type III) and demonstrates that particle size decreased progressively with increasing elution volume. Fig. 3 shows histograms of the distribution of particle diameter in six fractions obtained from this subject and from a subject with mixed hyperlipemia (type V). The largest particles (fraction A), which were eluted at the void volume of the column, are a heterogeneous group exceeding 700 Å diameter. This heterogeneity was substantially less in two subjects with dysbetalipoproteinemia (SD 100 and 146 Å) than in five other subjects (SD 197–418 Å). Particle diameter was more narrowly distributed in the subsequent fractions (SD 71 ± 21 Å, n = 31) and fell rapidly in fractions B–D. Subsequently, particle size changed relatively little, especially in subjects with dysbetalipoproteinemia. These results indicate that the column fractionates particles most effectively in the range of 400–700 Å diameter. The results shown in these figures are typical for those obtained in all subjects. The relation between elution volume and particle size differed from column to column (see Fig. 3) and even changed slightly when the same column was used repeatedly, so elution volume was not a reliable determinant of particle size.

Relationship between particle diameter and chemical composition

The measurements presented in Fig. 4 are typical of those obtained in normal subjects. Results in two subjects with endogenous hyperlipemia (type IV), one with exogenous hyperlipemia (type 1), and two with mixed hyperlipemia (type V) were similar except that the peak of the VLDL distribution was usually shifted to the left. To examine systematically the composition of particles with the same mean diameter (see below), chemical compositions of fractions whose summed content of protein, free cholesterol, and phospholipids was closely similar were compared. Table 1 shows that the composition of particles of given size was generally similar in normal subjects and in subjects with these types of hyperlipemia. Triglyceride content fell progressively with decreasing particle diameter while that of phospholipids and protein increased. The pattern for cholesteryl esters and free cholesterol varied. When their contents were low in large particles, they tended to rise as size decreased; otherwise, they tended to remain constant. The latter pattern was most common in hyperlipemic subjects. Except for chylomicron-sized particles in some hyperlipemic subjects, the free cholesterol–phospholipid ratio was generally less than 0.5 (molar ratio slightly below unity) (Table 1).

The pattern in three subjects with dysbetalipoproteinemia was strikingly different (Table 1 and Fig. 5). Percentage content of triglycerides remained constant at about 50 until diameter had fallen to 400–500 Å, and then it fell. Content of cholesteryl esters was about 30% in particles that were excluded from the column, fell temporarily to about 25% with decreasing diameter, and then tended to rise back to near 30%. Percentage content of free cholesterol changed little with diameter while that of phospholipids and protein increased. The free cholesterol–phospholipid ratio exceeded unity in the largest particles and was close to unity in all others.

Fig. 6 shows agarose gel electrophoretograms of serum and its ultracentrifugal fractions obtained from one of

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 19, 2012

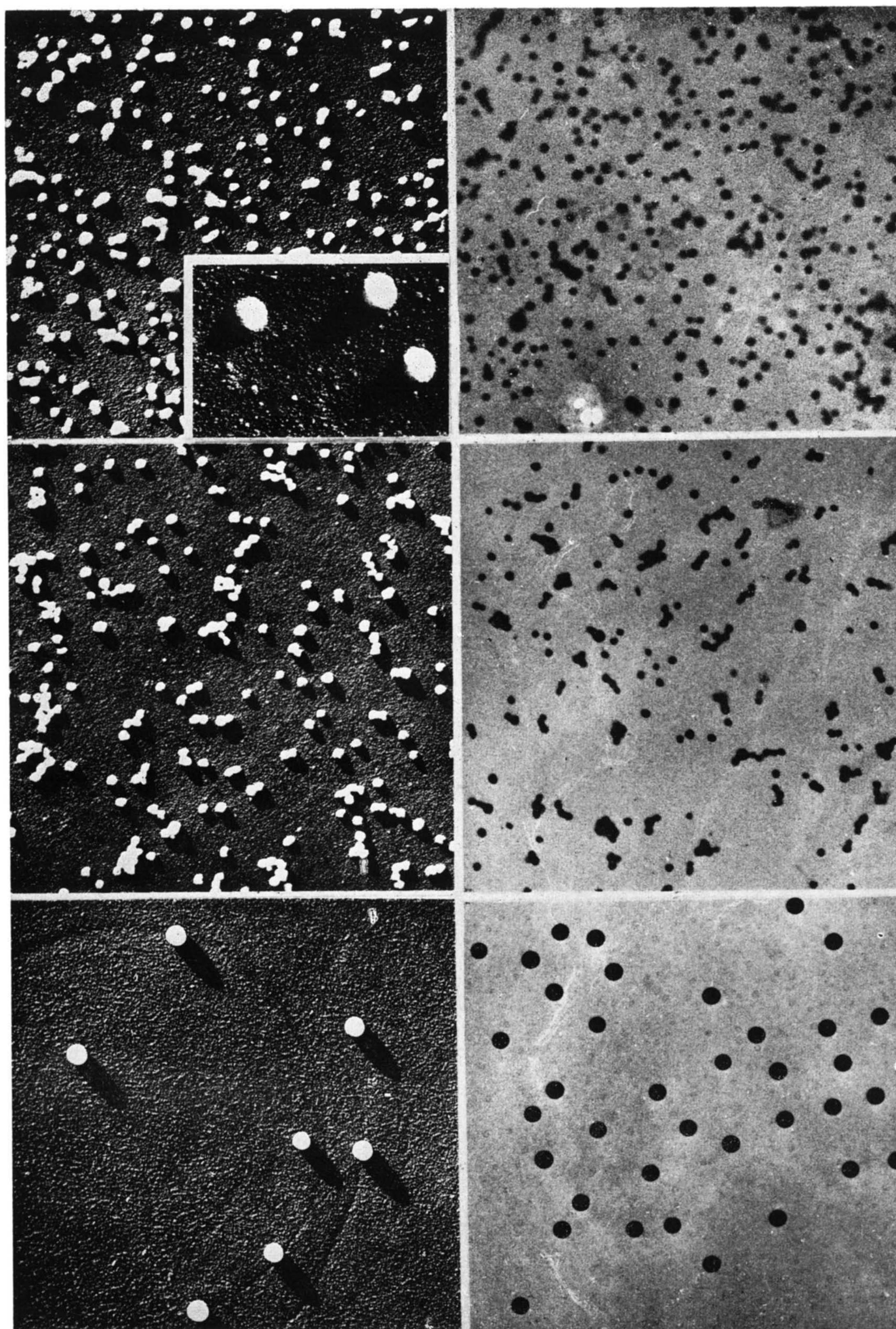

FIG. 1. Shadowed (*left*) and unshadowed (*right*) electron micrographs of latex beads (*bottom*), subfractions of VLDL from a subject with mixed hyperlipemia when he had been on a diet rich in polyunsaturated fatty acids (*middle*), and the same subject on an ordinary diet (*top*). Note the increased density of osmium-fixed particles rich in polyunsaturated fatty acids. × 29,100. Inset (× 48,500) shows the variation in shadow length of particles of similar size (the shorter the shadow length the greater the flattening).

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

JOURNAL OF LIPID RESEARCH

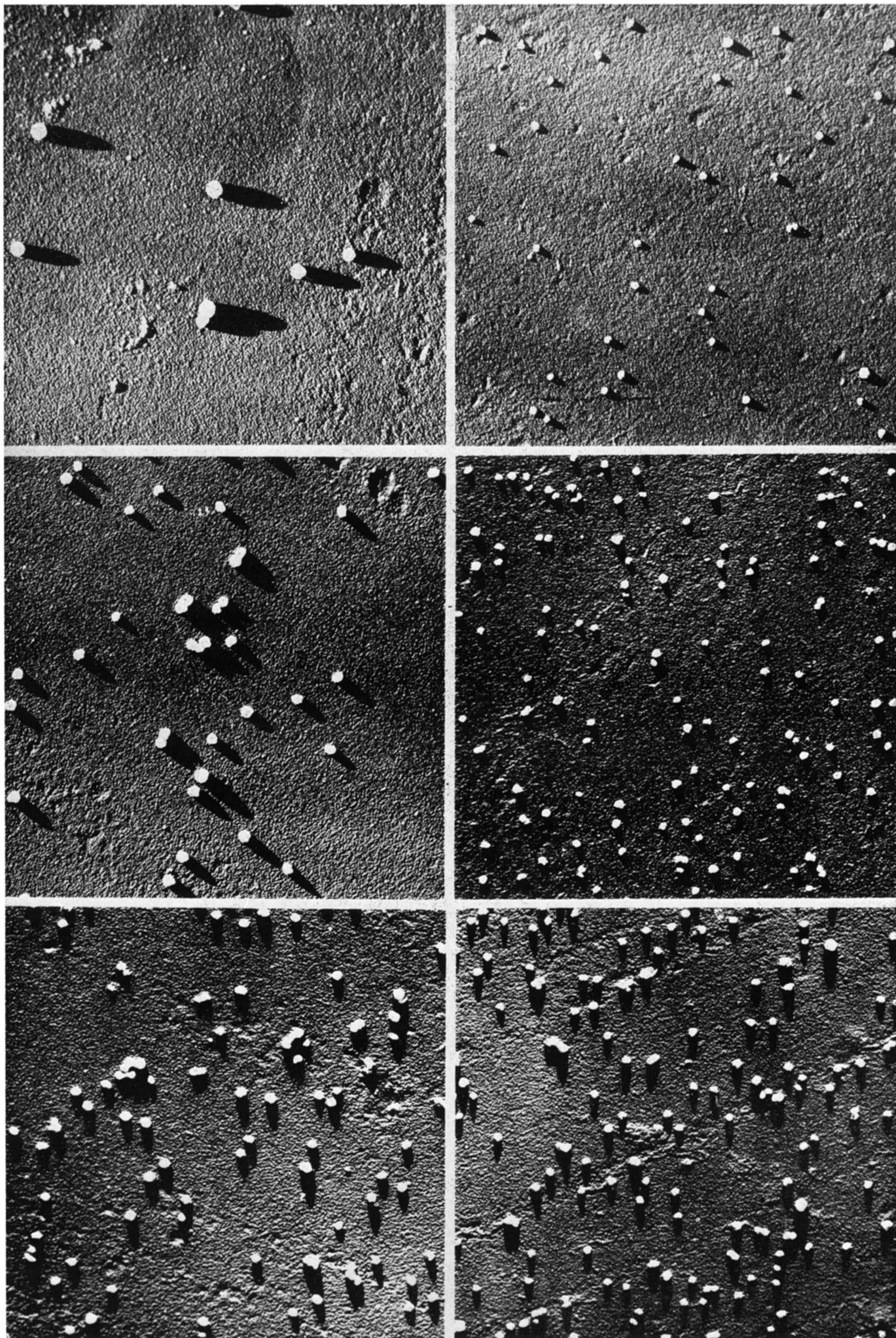

FIG. 2. Electron micrographs of subfractions obtained by gel chromatography of triglyceride-rich lipoproteins from a subject with dysbetalipoproteinemia. Elution volumes for these fractions increase from top down in left panels and then from bottom up in right panels. The distribution of diameters for each fraction is shown in left panel of Fig. 3.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB
JOURNAL OF LIPID RESEARCH

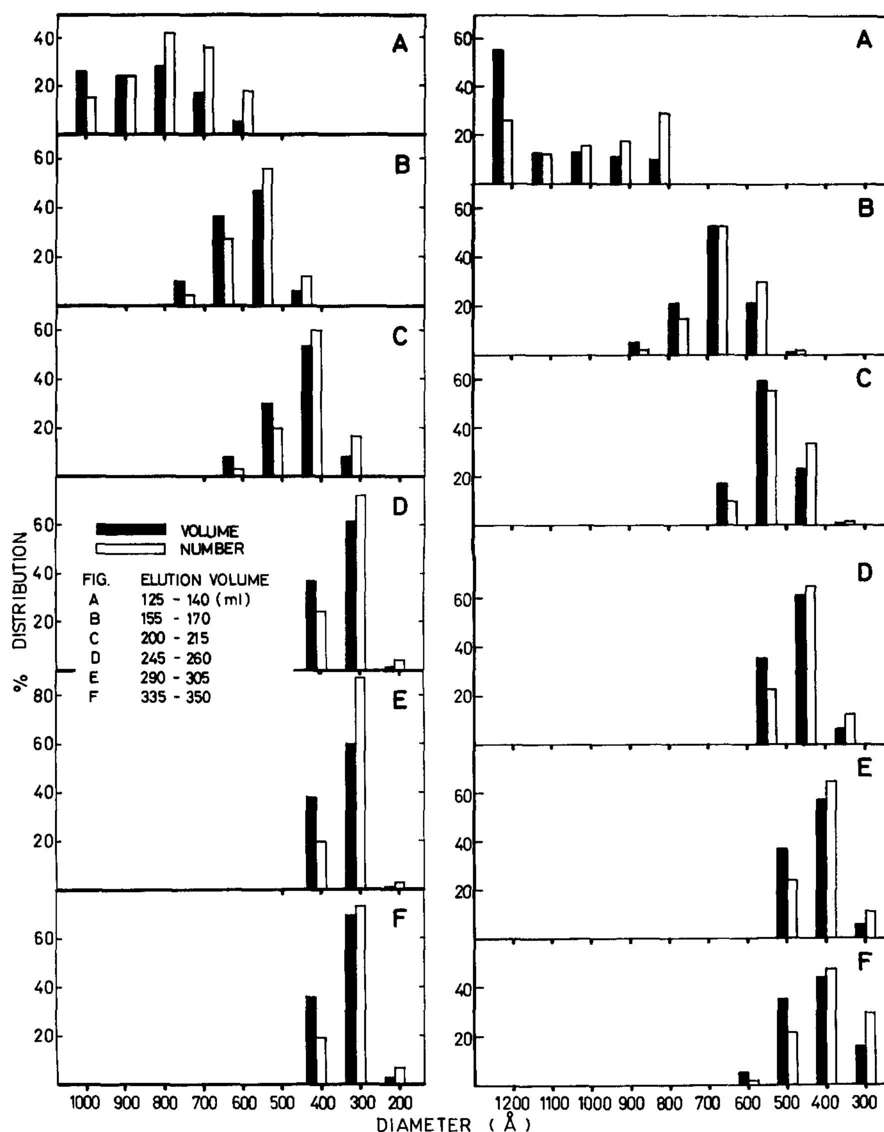

Fig. 3. Percentage distribution of particles of a given diameter by number and by volume (for spheres) from a subject with dysbetalipoproteinemia (*left*) and a subject with mixed hyperlipemia (*right*). Individual bars refer to particles with diameters ranging between 50 Å above and below the value indicated on the abscissa (for fraction A, the first bar refers to all particles with diameters equal to or exceeding the indicated value). Different columns were used for the two samples of triglyceride-rich lipoproteins. The skewed distribution apparent in some of these graphs may be attributable in part to contamination with material from earlier fractions that had been trapped in the syphon of the fraction-collecting device.

the subjects with dysbetalipoproteinemia when serum lipid concentrations were high and when they had fallen close to normal after the subject had a weight loss of 9 kg. The mobility of VLDL on the first occasion was typical for this disorder but on the second it was mainly pre-β, although the composition of fractions obtained by gel chromatography, shown in Fig. 5, was indistinguishable from that of the other two subjects with this disorder. On both occasions, the electrophoretic pattern suggested heterogeneity of the triglyceride-rich lipoproteins. This heterogeneity was more clearly seen in fractions obtained by gel chromatography. Fig. 7 shows the electrophoretic mobility of fractions obtained by gel chromatography of triglyceride-rich lipoproteins from one of the other subjects with this disorder. Mobility increased progressively in the early fractions with a predominant β component in fractions 3–5, and two distinct bands were observed beginning with fraction 6. Thereafter, the relative amount of the pre-β component appeared to decrease and that of the β component to increase. Only the β component could be detected in fractions 13–16. Electrophoretic patterns of fractions from subjects with other forms of hyperlipemia showed only a single component with increasing mobility up to

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB
JOURNAL OF LIPID RESEARCH

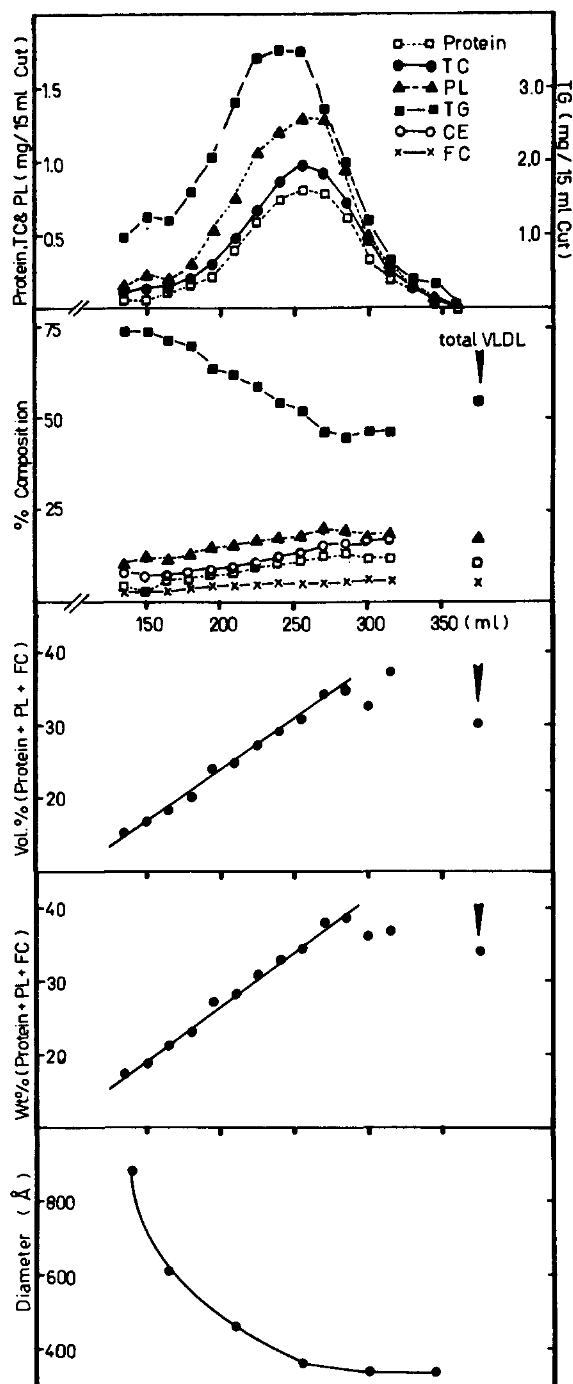

Fig. 4. Gel chromatographic elution pattern of triglyceride-rich lipoproteins from a normolipidemic subject. Individual points represent values obtained in a single 15-ml fraction. Elution volumes are shown at bottom of second panel. Points at right of middle three panels represent values for unfractionated triglyceride-rich lipoproteins (the value for protein in unfractionated material includes proteins which are eluted after about 400 ml). Diameters shown in bottom panel are numerical averages. TC, total cholesterol; PL, phospholipids; TG, triglycerides; CE, cholesteryl esters; FC, free cholesterol.

the pre-β region in fractions 3 and 4 and no change thereafter.

Relationship between content of polar material and particle diameter

Figs. 4 and 5 also show the relationship between content of the more polar constituents of the lipoproteins and particle diameter. As particle diameter fell in a roughly exponential manner with increasing elution volume, percentage content of the sum of phospholipids, free cholesterol, and protein increased linearly from about 15 to 35%. The pattern was similar whether percentage content was expressed as weight or volume %.[1] This relationship was examined systematically in 34 fractions from seven subjects. A close relationship existed between diameter and content of these three constituents or the content of the two most polar of the three, phospholipids and protein, in five of these subjects who had trigylceride-rich lipoproteins of ordinary composition (Figs. 8 and 9). The values for the two subjects with dysbetalipoproteinemia were indistinguishable from the others when content of all three polar constituents was related to diameter (Fig. 9). However, when free cholesterol was eliminated (Fig. 8), most of the values for these subjects deviated to one side. Under the assumption that all of these constituents occupy the surface of the lipoprotein particles and that the particles are perfect spheres, the thickness of the shell containing them that would evenly cover the lipoproteins was calculated for each fraction in which composition and diameter were known from the formula: $t = \frac{d\,(1 - (1 - F)^{1/3})}{2}$, where t is the thickness of the shell of a sphere of diameter d when F is the fractional volume of the sphere occupied by shell.[2] The results are shown in Fig. 10. The calculated thickness of the shell varied over a narrow range with a mean value of 21.5 Å and a standard deviation of 1.66 Å. The line shown in Fig. 9 for a shell of this constant thickness appears to provide an excellent fit for particles over the entire range of diameters between 250 and 1000 Å.

[1] Partial specific volumes were taken to be: triglycerides, 1.093; cholesteryl ester, 1.044; free cholesterol, 0.968; phospholipids, 0.970; and protein, 0.705. Values for cholesteryl oleate, free cholesterol, and egg lecithin dispersed in water were kindly provided by Dr. Donald Small; those for triglycerides and protein were taken from Oncley (17).

[2] $F = \frac{\frac{4}{3}\pi\left(\frac{d}{2}\right)^3 - \frac{4}{3}\pi\left(\frac{d-2t}{2}\right)^3}{\frac{4}{3}\pi\left(\frac{d}{2}\right)^3}$. This calculation holds strictly only for individual particles (18), and it cannot be applied to a population of particles as heterogeneous in size as unfractionated triglyceride-rich lipoproteins.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 1. Chemical composition of subfractions of triglyceride-rich lipoproteins[a]

Fraction	Normolipemic Subjects (n = 5)						Hyperlipemic Subjects (n = 5)[b]						Dysbetalipoproteinemic Subjects (n = 3)					
	TG	CE	Prot	FC	PL	FC/PL	TG	CE	Prot	FC	PL	FC/PL	TG	CE	Prot	FC	PL	FC/PL
			%						%						%			
I	76.0[c]	8.6	4.1	3.2	8.3	0.39	74.4	10.8	3.0	4.0	7.8	0.55	48.2[d]	35.0	2.7	5.9	8.4	0.70
	2.0	1.9	0.7	0.6	1.0	0.06	3.2	3.4	1.1	1.1	1.4	0.23						
II	71.8[e]	7.1	6.1	3.3	11.7	0.28	69.3[c]	10.7	4.7	4.3	11.4	0.39	46.4	33.4	3.2	6.9	10.2	0.67
	2.3	0.9	0.6	0.6	0.8	0.05	4.0	4.0	0.7	1.3	1.2	0.14	2.0	2.1	0.8	0.8	0.4	0.07
III	67.3	7.6	7.6	4.5	12.9	0.35	62.8	12.0	6.9	5.1	13.6	0.36	45.7	29.0	6.1	6.9	12.3	0.57
	1.1	0.7	0.8	1.1	1.4	0.12	3.0	2.4	0.8	1.1	1.1	0.04	2.3	2.8	0.4	1.2	0.8	0.14
IV	60.2	9.4	9.5	5.1	15.9	0.32	57.7	12.5	9.2	5.3	15.8	0.33	45.5	25.1	7.2	7.1	15.6	0.46
	1.6	1.2	0.8	0.7	0.8	0.06	3.8	2.9	0.6	0.8	0.5	0.06	1.5	1.1	0.5	0.6	0.5	0.03
V	52.3	13.0	11.5	5.7	17.4	0.33	51.8[c]	13.1	12.0	5.1	18.0	0.29	34.9	29.3	10.0	7.8	18.1	0.43
	2.6	2.7	0.7	0.7	0.7	0.05	3.9	3.9	1.0	0.9	0.2	0.05	2.8	2.4	0.5	0.4	0.7	0.01
VI	45.2[c]	15.7	14.5	6.1	18.7	0.33							29.3	31.0	11.6	8.6	19.5	0.44
	2.6	2.4	1.0	1.2	1.2	0.08							4.1	4.2	0.5	0.5	0.7	0.02

TG, triglycerides; CE, cholesteryl esters; Prot, protein; FC, free cholesterol; PL, phospholipids.

[a] Samples were selected which most closely gave percentage content of protein + free cholesterol + phospholipids of 15, 20, 25, 30, 35, and 40% for successive fractions. Values are means (above) ± SD (below).

[b] Includes one subject with exogenous hyperlipemia and two each with endogenous and mixed hyperlipemia.

[c] n = 4.

[d] n = 2.

[e] n = 3.

DISCUSSION

The direct measurements of particle diameters that were made in this study extend previous observations (6) on the fractionation of triglyceride-rich lipoproteins by columns of 2% agarose gel. This method is shown to provide effective fractionation over most of the usual size range of VLDL, and particles in the size range of chylomicrons are conveniently separated as a group emerging in the void volume of the column. Another highly useful feature of the method is that it yields lipoproteins essentially free of material of smaller particle size. For example, chylomicrons separated from whole lymph or plasma have essentially the same content of protein as those obtained after preliminary ultracentrifugal separation of triglyceride-rich lipoproteins.[3] Thus, there is no need to subject fractions to washing procedures that might alter their compositions (2, 18). Substantial quantities of material can be fractionated so that additional studies of the properties of the lipoproteins and their constituents are facilitated.

The direct comparison of particle size and chemical composition of subfractions of VLDL provides strong support for the concept that these particles, like chylomicrons (19), consist of an oil droplet of triglycerides and cholesteryl esters surrounded by a monomolecular film of constant thickness composed of phospholipids and free cholesterol together with protein. Up to equimolar quantities of cholesterol can be incorporated into lamellar forms of lecithin (20), so that the quantity of cholesterol found in subfractions of VLDL could easily exist in such a surface film, as pointed out earlier by Gustafson, Alaupovic, and Furman (2). Additional evidence for the surface location of at least the bulk of the free cholesterol is provided by the improved fit of the surface volume–diameter relationship for particles from subjects with primary dysbetalipoproteinemia with those from subjects with triglyceride-rich lipoproteins of normal composition when free cholesterol is assumed to be included in the surface film. According to Small (21), very little cholesterol can be incorporated into mixtures of triolein and cholesteryl oleate at 38°C. Zilversmit (19) concluded from studies of the membranous material produced when chylomicrons from lymph of dogs, rats, and humans were subjected to repeated freezing and thawing that about two thirds of the free cholesterol was present, together with phospholipids and protein, in a surface monolayer. He also found some of the triglycerides but none of the cholesteryl esters in this material. Gustafson et al. (2) concluded from studies with subfractions of VLDL separated by repeated angle head ultracentrifugations that inclusion of free cholesterol in a surface film is required to provide complete coverage of spherical particles. They assumed that the film was in a maximally condensed state and that it had a thickness of 14 Å. The values they obtained for composition of subfractions were admittedly subject to error, since content of both free cholesterol and cholesteryl esters in all subfractions was less than that of the original unfractionated lipoproteins. Their values for phospholipid content of subfractions are also lower than ours, and they did not exceed that of the unfractionated material. Their estimates of molecular diameter were calculated from S_f rates. For these reasons, detailed comparison of their results with ours is not

[3] Alexander, C., and R. J. Havel. Unpublished observations.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

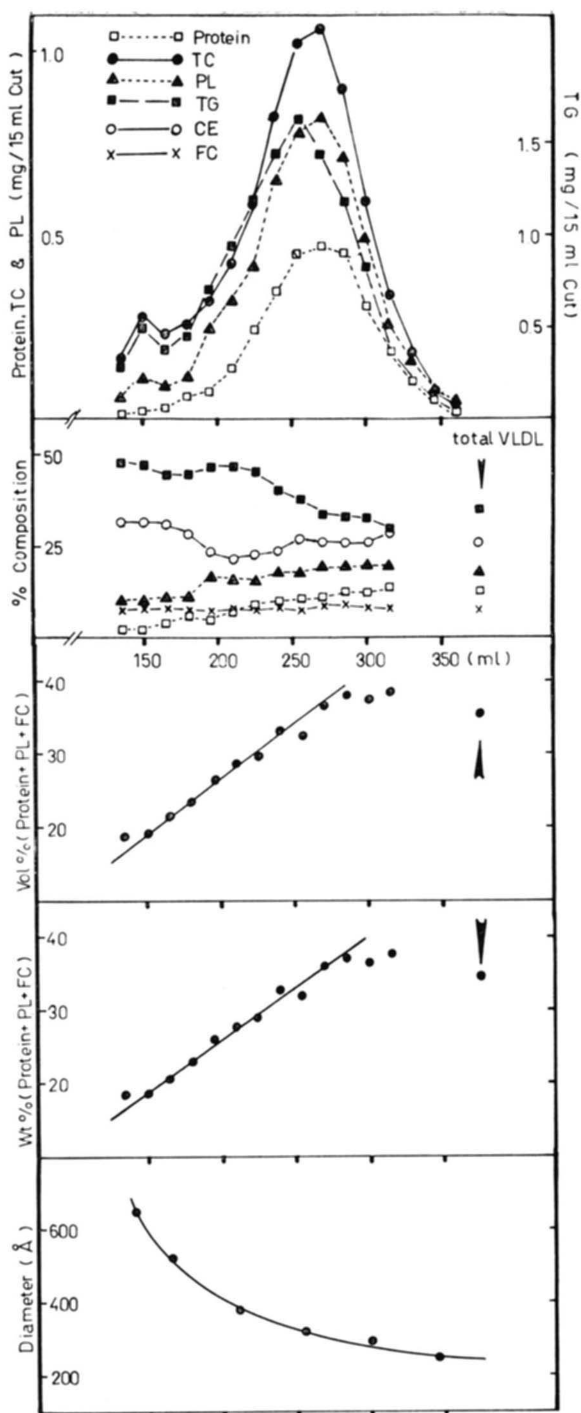

FIG. 5. Gel chromatographic elution pattern of triglyceride-rich lipoproteins from a subject with dysbetalipoproteinemia. Abbreviations as in Fig. 4.

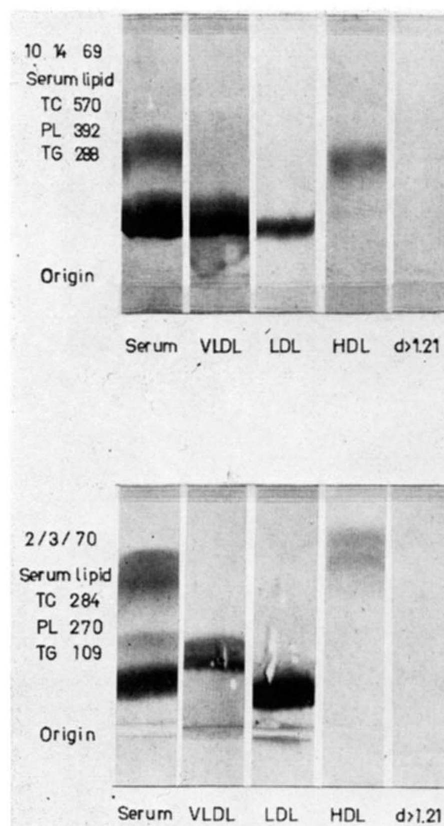

FIG. 6. Agarose gel electrophoretograms from a subject with dysbetalipoproteinemia before (*above*) and after (*below*) weight loss of 9 kg. Serum lipid values are in mg/dl. Note that two bands are present in VLDL on both occasions although the mobility of the slower band is clearly in the β region only in the first pattern. The gel chromatographic pattern shown in Fig. 5 represents the sample taken after weight loss.

warranted, although they agree well in a qualitative sense.

Our estimate of about 21.5 Å for the thickness of the surface film is about half of the value derived from X-ray diffraction patterns for the thickness of bilayers of lecithin (22) or lecithin and cholesterol (23) in water. Adsorption of protein to such lamellae may increase the thickness somewhat (24), but the structural relationship between protein and other surface constituents is uncertain (25). The present results are consistent with the concept that protein forms an intimate part of the film itself. The value of 21.5 Å may overestimate the actual thickness of the surface material since the actual diameters of the lipoproteins may be somewhat smaller than those measured in the electron micrographs. In the samples obtained from the subject on a polyunsaturated fat-rich diet, particles with a given percentage of surface material were about 11% (range 6–16%) smaller than those obtained when he was on his usual diet. At least two explanations can be offered for this apparent discrepancy. First, the particles containing larger amounts of unsaturated fatty acids may have been less subject to flattening because they had taken up more osmium (Fig. 1). Second, the molecular volume of the lipids on the surface monolayer may have been larger because of greater content of unsaturated acids at the β position of phospholipids (this would result in reduced

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB
JOURNAL OF LIPID RESEARCH

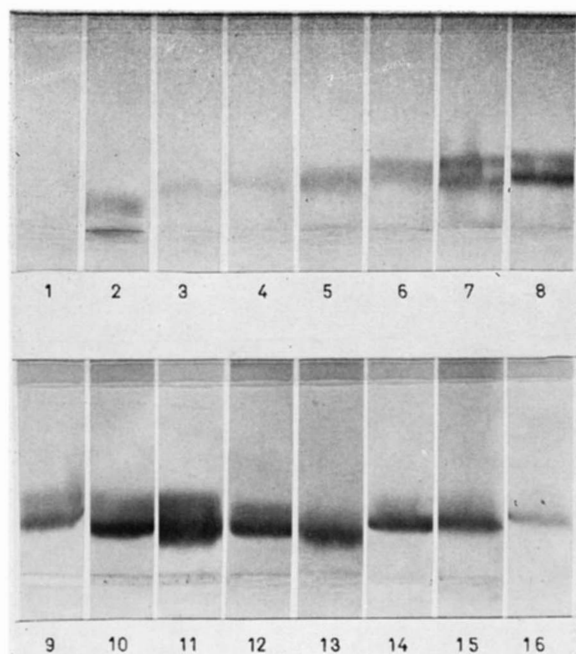

FIG. 7. Agarose gel electrophoretograms of triglyceride-rich lipoproteins contained in each of 16 fractions obtained from a subject with dysbetalipoproteinemia. Note that fraction 2 has a distinct component at the origin and that the migrating band increases in mobility up to fraction 6, at which two migrating bands are first seen. The faster of these bands gradually decreases in intensity and is no longer evident after fraction 12.

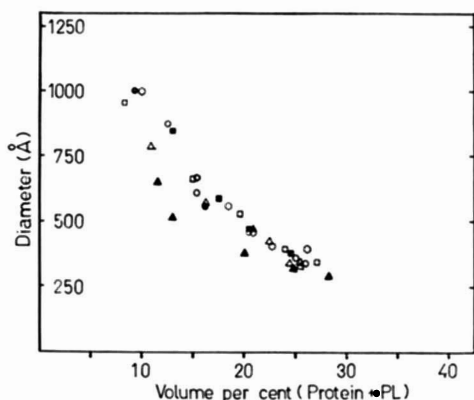

FIG. 8. Relationship between observed diameter (numerical average) and sum of content (volume %) of protein and phospholipids of 34 fractions of triglyceride-rich lipoproteins from seven individuals. Open and closed triangles represent subjects with dysbetalipoproteinemia.

particle size provided that the partial specific volumes of the core lipids were unchanged) (26). Comparison of the relationship between diameter of the particles and the length of their shadow suggested that some particles from subjects on ordinary diets were slightly flattened on the grid, thus increasing the apparent diameter. If the diameters of all particles were indeed about 11% less than that observed, the estimated thickness of the

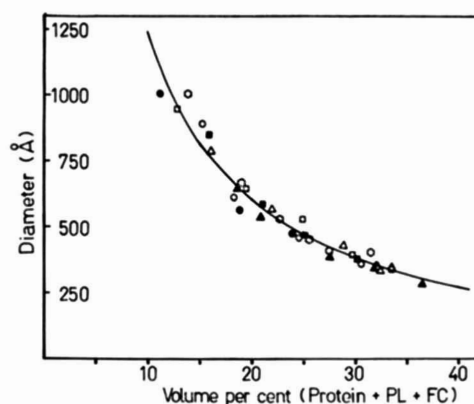

FIG. 9. Relationship between observed diameter and sum of content (volume %) of protein, phospholipids, and free cholesterol of 34 fractions from the seven subjects of Fig. 8. The curved line is that for a sphere having a surface shell with constant thickness of 21.5 Å.

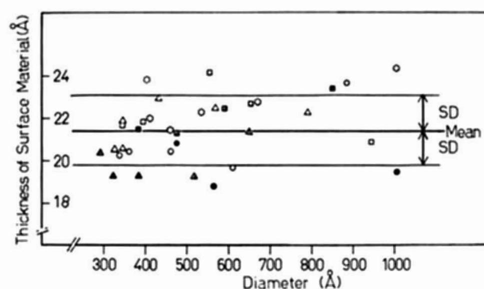

FIG. 10. Calculated thickness of the shell that would evenly cover spheres having the diameters and volume percentages of shell material indicated for each of the points of Fig. 9. No systematic difference in calculated thickness is apparent for particles of various diameters. The mean thickness ± SD is 21.45 ± 1.66 Å.

surface material would be about 19 Å. Our calculations are also subject to uncertainty related to the molecular volumes of the constituents of the surface material. We have taken them to be equal to the values observed in aqueous dispersions of model compounds. Mixed monolayers or lamellae of cholesterol and lecithin are known to be more condensed than those of lecithin alone (20, 26).

Application of gel chromatography to the study of triglyceride-rich lipoproteins in subjects with hyperlipemia provides information about their size distribution and about the composition of particles of defined size. The mean diameter of any subfraction can be calculated from summed fractional content of protein, free cholesterol, and phospholipids. Distribution of particle size is also provided by analytical ultracentrifugation, but this method gives no information about the content of chylomicrons or about chemical composition. A bimodal distribution indicating the presence of chylomicrons may be observed even in normolipemic individuals (Fig. 4), and it is the rule in subjects with exogenous

Downloaded from www.jlr.org by guest, on June 19, 2012

and mixed hyperlipemia and in subjects with dysbetalipoproteinemia. The peak of the VLDL distribution is often shifted to the left when VLDL of normal composition accumulate. Although there is evidence that the size of VLDL tends to increase in parallel with their rates of synthesis in the liver (27, 28), our observations indicate that particle size of VLDL in plasma may also be increased in states known to be accompanied by impaired removal of triglyceride-rich lipoproteins.

Our data confirm previous observations which have shown that triglyceride-rich lipoproteins in subjects with dysbetalipoproteinemia have increased content of cholesteryl esters and decreased content of triglycerides (29–31). In contrast, two of the surface constituents of the lipoprotein, phospholipids and protein, were present in relatively normal amounts for particles of a given size. Content of free cholesterol was consistently greater only in the largest particles, whose mean diameter was in the range of 660–850 Å. A lesser increase was observed in chylomicron-sized particles from most of the hyperlipemic subjects. Minari and Zilversmit (32) have reported that the free cholesterol content of chylomicrons obtained from thoracic duct lymph of dogs increased gradually when they were incubated with chylomicron-free serum or with high density lipoproteins. Thus, the increased content of free cholesterol could be a consequence of retention of these particles in the blood of subjects with these disorders. Porte, O'Hara, and Williams (33), who found increased content of total cholesterol in $S_f > 400$ lipoproteins from plasma of postabsorptive hyperlipemic subjects, came to a similar conclusion. In the subjects with dysbetalipoproteinemia, the molar ratio of free cholesterol to phospholipids in the large particles was consistently above unity. Since this exceeds the known capacity of lamellae of lecithin to accommodate free cholesterol (20), it is probable that some of the free cholesterol in these particles is in the central oil droplet.

Another feature of the lipid distribution of subjects with dysbetalipoproteinemia was the similar content of cholesteryl esters in the largest and smallest populations of triglyceride-rich lipoproteins. The large particles, which were excluded from the gel filtration column, were more homogeneous than those from other hyperlipemic subjects and contained few particles in the usual size range of chylomicrons (greater than 1000 Å diameter). In particles of intermediate size, the content of cholesteryl esters was consistently lower, so that their compositions more nearly resembled that of normal subjects and subjects with other types of hyperlipemia. In this intermediate population, two species of VLDL evidently were present since two bands with β and pre-β mobility were observed on electrophoresis in agarose gel (Fig. 6). The peak of the VLDL distribution in this disorder is close to 350 Å (Figs. 2 and 5). This compares with a value of about 400 Å in normolipemic subjects. There was a substantial population of these small particles in each of the subjects with dysbetalipoproteinemia, and they had an unusually narrow range of size (Figs. 2 and 3). Taken together with available information on the lipoprotein abnormality in dysbetalipoproteinemia (29–31), the present results are consistent with the presence of three populations of triglyceride-rich particles in this disorder. The two populations at the extremes of particle size are unusually rich in cholesteryl esters and may also differ in the composition of the protein at the surface when compared with ordinary lipoproteins of equivalent size. Respectively, the populations with mean diameters of 800 and 350 Å may constitute "remnants" of the metabolism of chylomicrons and VLDL from which the bulk of the triglycerides has been removed (34–37) and in which much or all of the "C" apoproteins have been transferred to HDL (38, 39). In part, their high content of cholesteryl esters may also reflect reciprocal transfers of the core constituents, cholesteryl esters and triglycerides, related to the action of lecithin:cholesterol acyltransferase on high density lipoproteins (40).[4] The third population may represent particles having a normal distribution of size (and chemical composition) that overlaps the abnormally accumulating large and small remnants.

We thank A. Frank and J. Quan for expert technical assistance. These studies were supported by U.S. Public Health Service grant HL-06285 and a grant from the Veterans Administration.

Manuscript received 24 April 1972; accepted 3 July 1972.

REFERENCES

1. Havel, R. J. 1969. Pathogenesis, differentiation and management of hypertriglyceridemia. *Advan. Intern. Med.* **15:** 117–154.
2. Gustafson, A., P. Alaupovic, and R. H. Furman. 1965. Studies of the composition and structure of serum lipoproteins: isolation, purification, and characterization of very low density lipoproteins of human serum. *Biochemistry.* **4:** 596–605.
3. Pinter, G. G., and D. B. Zilversmit. 1962. A gradient centrifugation method for the determination of particle size distribution of chylomicrons and fat droplets in artificial fat emulsions. *Biochim. Biophys. Acta.* **59:** 116–127.
4. Hatch, F. T., N. K. Freeman, L. C. Jensen, G. R. Stevens, and F. T. Lindgren. 1967. Ultracentrifugal isolation of serum chylomicron-containing fractions with quantitation by infrared spectrometry and NCH elemental analysis. *Lipids.* **2:** 183–191.
5. Lossow, W. J., F. T. Lindgren, J. C. Murchio, G. R. Stevens, and L. C. Jensen. 1969. Particle size and protein

[4] The larger content of cholesteryl esters in some particles of other hyperlipemic subjects (Table 1) may also reflect such transfers.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

content of six fractions of the $S_f > 20$ plasma lipoproteins isolated by density gradient centrifugation. *J. Lipid Res.* **10:** 68–76.
6. Sata, T., D. L. Estrich, P. D. S. Wood, and L. W. Kinsell. 1970. Evaluation of gel chromatography for plasma lipoprotein fractionation. *J. Lipid Res.* **11:** 331–340.
7. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
8. Sperry, W. M., and M. Webb. 1950. A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.* **187:** 97–106.
9. Zak, B., R. C. Dickenman, E. G. White, H. Burnett, and P. J. Cherney. 1954. Rapid estimation of free and total cholesterol. *Amer. J. Clin. Pathol.* **24:** 1307–1315.
10. Stewart, C. P., and E. B. Hendry. 1935. Phospholipins of blood. *Biochem. J.* **29:** 1683–1689.
11. Carlson, L. A. 1963. Determination of serum triglycerides. *J. Atheroscler. Res.* **3:** 334–336.
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
13. Jones, A. L., and J. M. Price. 1968. Some methods of electron microscopic visualization of lipoproteins in plasma and chyle. *J. Histochem. Cytochem.* **16:** 366–370.
14. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
15. Havel, R. J., J. M. Felts, and C. M. Van Duyne. 1962. Formation and fate of endogenous triglycerides in blood plasma of rabbits. *J. Lipid Res.* **3:** 297–308.
16. King, R. J., and J. A. Clements. 1970. Separation by gel chromatography of naturally occurring phosphatidylcholine mixtures according to number of ethylenic linkages. *J. Lipid Res.* **11:** 381–385.
17. Oncley, J. L. 1963. Lipid protein interactions. *In* Brain Lipids and Lipoproteins and the Leucodystrophies. J. Folch-Pi and H. Bauer, editors. Elsevier, Amsterdam. 1–17.
18. Dole, V. P., and J. T. Hamlin, III. 1962. Particulate fat in lymph and blood. *Physiol. Rev.* **42:** 674–701.
19. Zilversmit, D. B. 1965. The composition and structure of lymph chylomicrons in dog, rat, and man. *J. Clin. Invest.* **44:** 1610–1622.
20. Lecuyer, H., and D. G. Dervichian. 1969. Structure of aqueous mixtures of lecithin and cholesterol. *J. Mol. Biol.* **45:** 39–57.
21. Small, D. M. 1970. The physical state of lipids of biological importance: cholesteryl esters, cholesterol, triglyceride. *In* Surface Chemistry of Biological Systems. M. Blank, editor. Plenum Press, New York. 55–83.
22. Small, D. M. 1967. Phase equilibria and structure of dry and hydrated egg lecithin. *J. Lipid Res.* **8:** 551–557.
23. Levine, Y. K., and M. H. F. Wilkins. 1971. Structure of oriented lipid bilayers. *Nature New Biology.* **230:** 69–72.
24. Wilkins, M. H. F., A. E. Blaurock, and D. M. Engelman. 1971. Bilayer structure in membranes. *Nature New Biology.* **230:** 72–76.
25. Colacicco, G. 1970. Lipid monolayers: mechanisms of protein penetration with regard to membrane models. *Lipids.* **5:** 636–649.
26. Shah, D. O., and J. H. Schulman. 1967. Influence of calcium, cholesterol, and unsaturation on lecithin monolayers. *J. Lipid Res.* **8:** 215–226.
27. Schonfeld, G. 1970. Changes in the composition of very low density lipoprotein during carbohydrate induction in man. *J. Lab. Clin. Med.* **75:** 206–211.
28. Ruderman, N. B., A. L. Jones, R. M. Krauss, and E. Shafrir. 1971. A biochemical and morphologic study of very low density lipoproteins in carbohydrate-induced hypertriglyceridema. *J. Clin. Invest.* **50:** 1355–1368.
29. Hazzard, W. R., F. T. Lindgren, and E. L. Bierman. 1970. Very low density lipoprotein subfractions in a subject with broad-β disease (type III hyperlipoproteinemia) and a subject with endogenous lipemia (type IV). Chemical composition and electrophoretic mobility. *Biochim. Biophys. Acta.* **202:** 517–525.
30. Quarfordt, S., R. I. Levy, and D. S. Fredrickson. 1971. On the lipoprotein abnormality in type III hyperlipoproteinemia. *J. Clin. Invest.* **50:** 754–761.
31. Hazzard, W. R., D. Porte, Jr., and E. L. Bierman. 1970. Abnormal lipid composition of chylomicrons in broad-β disease (type III hyperlipoproteinemia). *J. Clin. Invest.* **49:** 1853–1858.
32. Minari, O., and D. B. Zilversmit. 1963. Behavior of dog lymph chylomicron lipid constituents during incubation with serum. *J. Lipid Res.* **4:** 424–436.
33. Porte, D., Jr., D. D. O'Hara, and R. H. Williams. 1966. Lipid composition of fat particles from normal man and patients with idiopathic hypertriglyceridemia. *J. Lipid Res.* **7:** 368–371.
34. Nestel, P. J., R. J. Havel, and A. Bezman. 1963. Metabolism of constituent lipids of dog chylomicrons. *J. Clin. Invest.* **42:** 1313–1321.
35. Redgrave, T. G. 1970. Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. *J. Clin. Invest.* **49:** 465–471.
36. Bergman, E. N., R. J. Havel, B. M. Wolfe, and T. Bøhmer. 1971. Quantitative studies of the metabolism of chylomicron triglycerides and cholesterol by liver and extrahepatic tissues of sheep and dogs. *J. Clin. Invest.* **50:** 1831–1839.
37. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1971. Abnormal metabolism of very low density lipoproteins (VLDL) in type III hyperlipoproteinemia (type III). *Circulation.* **44(Suppl. 2):** II-56 (Abstr.)
38. LaRosa, J. C., R. I. Levy, W. V. Brown, and D. S. Fredrickson. 1971. Changes in high-density lipoprotein protein composition after heparin-induced lipolysis. *Amer. J. Physiol.* **220:** 785–791.
39. Havel, R. J., M. L. Kashyap, and J. P. Kane. 1971. On the role of high density lipoproteins in the transport of exogenous fat. *Circulation.* **44(Suppl. 2):** II-9. (Abstr.)
40. Nichols, A. V., and L. Smith. 1965. Effect of very low-density lipoproteins on lipid transfer in incubated serum. *J. Lipid Res.* **6:** 206–210.

Downloaded from www.jlr.org by guest, on June 19, 2012