Particle-size distribution of very low density plasma lipoproteins during fat absorption in man

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ABSTRACT   The size distributions of electrophoretically isolated subfractions of the very low density human plasma lipoproteins have been determined using electron microscopy. The primary and secondary particles observed in plasma of normal subjects after fat ingestion appear to have similar size distributions. Particles produced by corn oil feeding can be fixed by the osmium tetroxide reaction while those produced by butter fat feeding could not be fixed or made visible by this technique. Good agreement between particle size as measured by electron microscopy and particle size as predicted by ultracentrifugal analysis was obtained.

KEY WORDS   absorption · fat · primary · secondary · particles · size · plasma · very low density lipoproteins · chylomicrons · electron microscopy · corn oil · butter fat · osmium fixation · ultracentrifugation · man

THE VERY LOW DENSITY plasma lipoproteins (S_f 20–10^5) are important in the transport of lipids, especially newly absorbed fat, in man. They range in size from approximately 250 to 10,000 A and include the large "fat particles" (1, 2) (approximately 750 A and above; $S_f > 400$), that produce turbid or lactescent aqueous solutions. The electron microscope is particularly suitable for study of lipoproteins in this size range (3–7).

Lipoproteins can be fractionated according to differences in properties other than particle size, e.g., zone electrophoresis on starch block (8, 9). Very low density lipoproteins migrate with both α_2- and β-globulins. The α_2-lipoproteins (8) are present normally in small quantities in postabsorptive plasma and are considered to have a flotation rate in the S_f 20–400 range (approximately 250–750 A). Ultracentrifugal subfractionation of this group of lipoproteins has been described (2). Their plasma concentration and estimated size may be greatly increased in some patients with hyperlipemia maintained on fat-free diets (10). After fat feeding, plasma may contain large "primary particles" which appear to be identical with particles in lymph (chylomicrons), and also migrate in the α_2-globulin region. They can be readily isolated from the smaller S_f 20–400 lipoproteins by brief ultracentrifugation in saline (d < 1.006; 10^6 g-min). In addition, variable quantities of "secondary particles" also may appear in the circulation after fat meals. Presumably, they are derived from primary particles (11), but, in contrast, migrate in the β-globulin region (9). Subsequent ultracentrifugal separation from β-lipoproteins (S_f 0–20) presents little difficulty. On paper electrophoresis, both primary and secondary particles adhere to the origin and do not migrate (9, 13). Fractionation of particles also has been achieved by differential flocculation in polyvinyl-pyrrolidone solutions (12).

Preliminary estimates (9) of the physical properties for both types of particle appearing in plasma during fat absorption have suggested that they are in the size range of 700–2000 A. In the present study, electron microscopy, coupled with density gradient centrifugation and analytical ultracentrifugation, has been used to obtain a more definitive analysis of both particle-size and flotation-rate distribution of these two electrophoretically distinct kinds of large, very low density lipoprotein.

METHODS

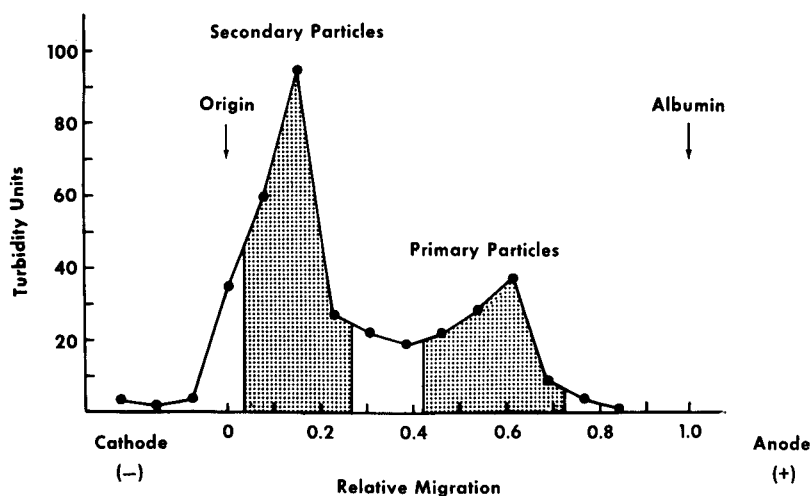Postprandial human plasma was obtained from seven normal male subjects 3–4 hr after the feeding of 250

FIG. 1. Starch block electrophoresis. Case 2. Electrophoretic separation of fat particles from plasma obtained 4 hr after corn oil feeding (2 g/kg). Electrophoretic conditions: barbital buffer, pH 8.6, 0.1 μ; 5 v/cm for 26 hr at 25°. Migration rate of turbid peak relative to that of albumin peak: primary particles, 0.62; secondary particles, 0.16. Stippled areas indicate fractions pooled for subsequent analysis.

ml of corn oil blended with skim milk. In addition, seven subjects were given butter fat in place of corn oil; two of these subjects also had received corn oil in another experiment. Samples (8–17 ml) of fresh, unrefrigerated plasma were fractionated by starch block electrophoresis under the conditions previously described (9). The turbidity peaks of primary and secondary particles, as determined by nephelometry (Coleman Model 14) of eluted starch block segments, migrated (relative to albumin, 1.00) within the range previously reported (mean ± SD for 23 observations = 0.60 ± 0.10 and 0.23 ± 0.08 respectively) (9).

Large starch segments containing primary particle and secondary particle fractions (Fig. 1) were eluted with 0.9% saline by aspiration through a coarse sintered glass filter or by vortex mixing, centrifugation, and decantation. Eluates were concentrated and washed twice by ultracentrifugal flotation through 0.9% saline for approximately 3 × 10⁶ g-min (35,000 rpm; 20°) in a Spinco SW 39 rotor. In most experiments, to reduce convection, the concentrated fractions were mixed with sucrose solutions to a final sucrose concentration of 5% prior to ultracentrifugation through saline. The fractions were stored at 4–10° until analysis.

Flotation rates of these primary and secondary particle fractions were determined by analytical ultracentrifugation and particle-size distribution by electron microscopy. Total lipid content of each fraction was determined from a gravimetric assay of an extract patterned after the method of Sperry and Brand (14).

One primary and three secondary particle fractions were studied in the analytical ultracentrifuge to deter-

mine, if possible, the lower limit of the S_f rate distribution. For this purpose, 87.1 mg of solid NaCl was added to 1 ml of each fraction to bring the solvent density at 26° to 1.0613 g/ml (1.700 molal NaCl). Although a diffuse turbidity boundary rapidly migrated through each fraction during acceleration of the ultracentrifuge, no resolvable concentration of lipoproteins less than S_f 400 was observed. Unfortunately, the turbidity of particles of 750 A and larger (corresponding to S_f values of 400 and greater) effectively interferes with resolution in the schlieren optical system. Nevertheless, these ultracentrifugal findings definitely indicate that the S_f rates of the primary and secondary particle fractions studied are essentially > 400.

In order to evaluate further the particle-size distribution as well as lipoprotein stability to preparative ultracentrifugation, a secondary-particle fraction was subfractionated in a density gradient (15). A secondary-particle fraction (case 3) in 1.70 molal NaCl (0.5 ml) was introduced at the bottom of a previously formed nonlinear NaCl gradient by using a spinal needle and syringe. The conditions of low-speed centrifugation (3800 g at the midpoint of the tube for 23 min at 20°) would allow recovery of lipoproteins S_f > 20,200 in the top milliliter of the preparative tube. In all, 17 fractions (1 × 1 ml and 16 × 0.5 ml fractions) were taken by serial aspiration from the 0.5 × 3.5 inch preparative tube. Precision refractometry of a control NaCl gradient after ultracentrifugation was used to evaluate the density[1] and viscosity in each region of the preparative

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[1] Unless otherwise indicated, all densities are given at 20°

tube. Initially, the top of the gradient tube had a density of 1.0065 g/ml and the bottom 1.0650 g/ml.

Particle-size distributions for each fraction were determined using a model HU-11 Hitachi electron microscope. The fixation process for electron microscopy utilized 1% OsO_4 in a buffer at pH 7.4 after the particle fractions had come to room temperature. After exposure for 30 min, the fixed lipoproteins were placed on the electron microscope grid for viewing. Measurements of particle diameter were made from prints w'th a total magnification of about 15,000 ×. Data obtained in this way were analyzed in order to yield particle-size distribution, mass distribution, and sedimentation-rate

distribution as well as average values of these parameters.

Particle-diameter distributions were converted to mass and flotation-rate distributions by means of a computer. The experimental data, consisting of measurements of each individual particle diameter, were punched on Standard IBM cards and analyzed by a program for the IBM 7094 computer. The computer procedure was approximately as follows: (a) reading the data for a single electron microscope plate; (b) performing a desired calculation on each datum, e.g., conversion of measured diameter to actual diameter in angstroms, calculation of molecular weight and flotation rate; (c)
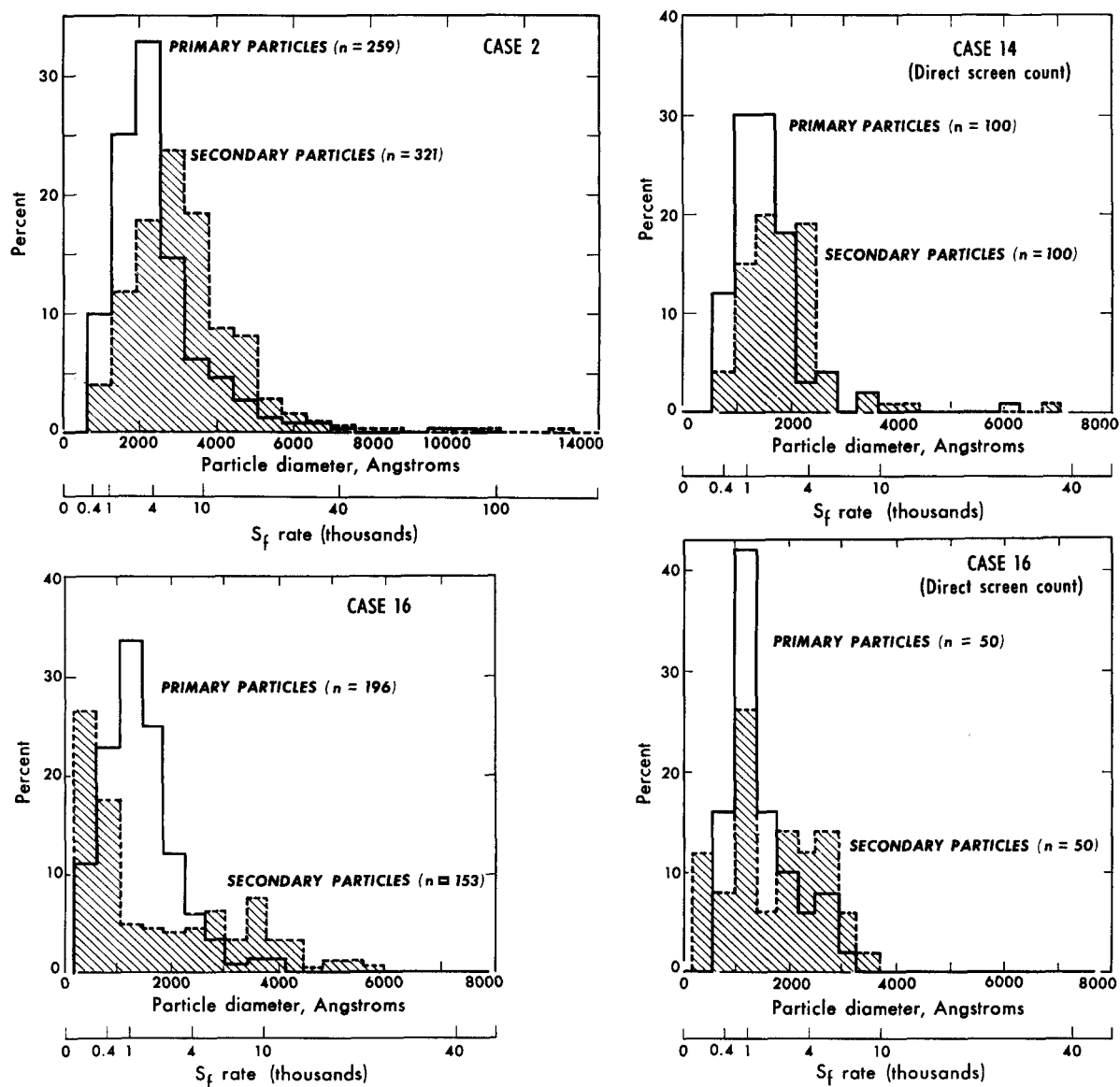

FIG. 2. Particle-size distributions of primary and secondary particles electrophoretically isolated from plasma after corn oil feeding, as determined by electron microscopy. "Direct screen counts" were done in the electron microscope utilizing a scale in the binocular eyepiece to measure the image formed on the fluorescent screen. All other counts were obtained by measuring particle diameter from photographs.
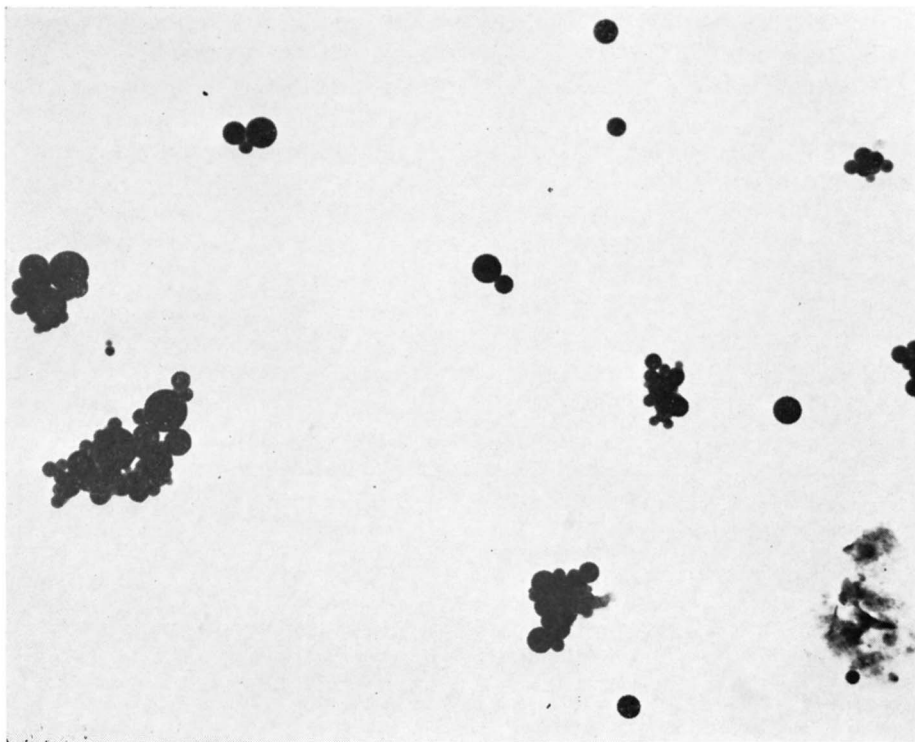
FIG. 3. Typical electron micrograph of primary-particle fraction (case 2). ×11,400. A similar preparation of the secondary-particle fraction from the same case appeared identical.
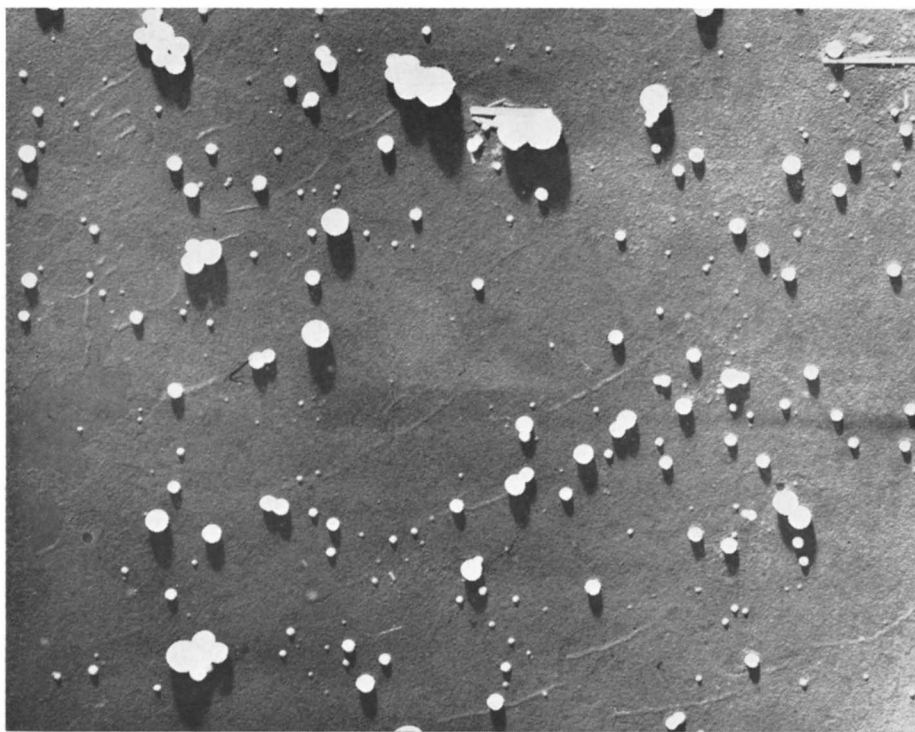

FIG. 4. Chromium shadowed preparation of secondary-particle fraction (case 2). ×11,400.

generating a histogram for each series of newly calculated values. The size of the histogram intervals was determined by an input card at the beginning of each use of the program. The program was written in Fortran and was easily modified to calculate any values for which histogram distributions were desired.

RESULTS

A comparison (Fig. 2) of the particle-size distribution of primary- and secondary-particle fractions obtained from the same plasma (case 2; corn oil) shows a range of particle diameters of 750–6000 A in each fraction. Two additional pairs of primary- and secondary-particle fractions obtained from other subjects after corn oil feeding were analyzed; the particle-size distribution for all these fractions is similar (Fig. 2). In each of the pairs, secondary particles appear to have a somewhat larger median particle diameter than primary particles. Typical electron micrographs used in the study of a primary-particle fraction (Fig. 3) and a secondary-particle fraction (Fig. 4) from the same subject (case 2) are shown.

In none of the cases of butter fat feeding could the fat particles isolated from plasma be made sufficiently visible for particle-size measurements in the electron
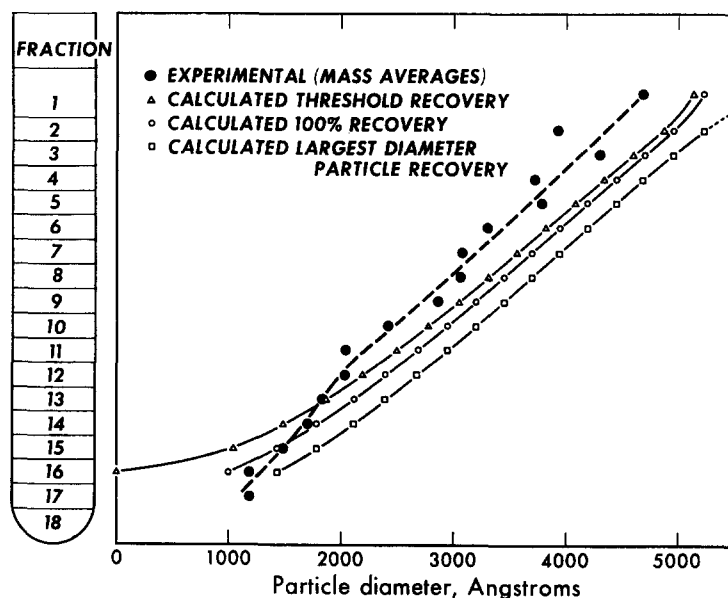

FIG. 5. Comparison of the recovery of particles experimentally obtained from electron micrographs with various recoveries calculated from ultracentrifuge theory for the density-gradient flotation experiment.
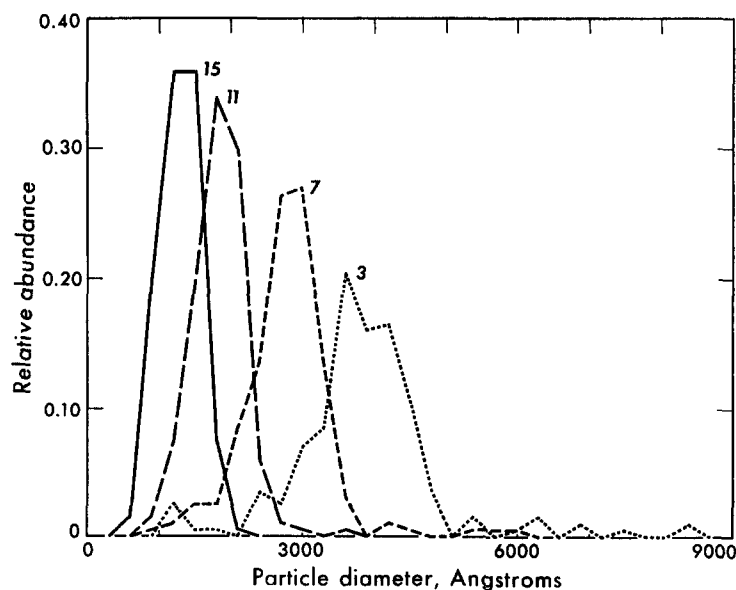

FIG. 6. Distribution of particle diameters for the 3rd, 7th, 11th, and 15th subfractions obtained by electron microscopy from the density-gradient flotation of a secondary-particle fraction (case 3; Table 1). For clarity of presentation, the histograms have been converted to a continuous particle-diameter distribution.

TABLE 1   Subfractionation of Secondary-Particle Sample. Physical Data Derived from Electron Microscopy

Fraction		No. of Particles Counted	Particle Diameter	Molecular Weight × 10⁻⁶	S_f
			A		
1	a*	52	$4,240 \pm 1,500$†	$30,500 \pm 25,700$†	$15,200 \pm 9,440$†
	b	80	$4,150 \pm 1,420$	$28,100 \pm 23,400$	$14,400 \pm 8,750$
2	a	58	$3,870 \pm\ \ \ 897$	$19,700 \pm 12,600$	$11,800 \pm 5,180$
	b	36	$3,510 \pm\ \ \ 959$	$15,400 \pm 10,100$	$9,850 \pm 4,740$
3	a	68	$4,010 \pm\ \ \ 984$	$22,400 \pm 19,600$	$12,700 \pm 6,650$
	b	52	$4,010 \pm 1,110$	$23,700 \pm 26,800$	$12,900 \pm 8,220$
4	a	30	$3,480 \pm\ \ \ 606$	$13,400 \pm\ \ 5,770$	$9,270 \pm 2,930$
	b	62	$3,550 \pm\ \ \ 966$	$16,500 \pm 19,900$	$10,100 \pm 6,560$
5	a	151	$3,440 \pm\ \ \ 864$	$14,300 \pm 13,500$	$9,350 \pm 5,040$
	b	85	$3,690 \pm\ \ \ 674$	$16,300 \pm\ \ 9,980$	$10,500 \pm 4,070$
6	a	94	$3,190 \pm\ \ \ 667$	$10,990 \pm\ \ 8,690$	$7,880 \pm 3,630$
	b	83	$3,060 \pm\ \ \ 824$	$10,100 \pm\ \ 6,440$	$7,440 \pm 3,530$
7	a	120	$2,930 \pm\ \ \ 698$	$8,790 \pm\ \ 8,950$	$6,710 \pm 3,760$
	b	54	$2,940 \pm\ \ \ 453$	$7,990 \pm\ \ 3,290$	$6,550 \pm 1,910$
8	a	67	$2,860 \pm\ \ \ 770$	$8,720 \pm 12,900$	$6,480 \pm 4,780$
	b	63	$3,840 \pm\ \ \ 577$	$7,700 \pm\ \ 8,060$	$6,200 \pm 3,270$
9	a	50	$2,720 \pm\ \ \ 747$	$7,690 \pm 12,700$	$5,890 \pm 4,710$
	b	46	$2,710 \pm\ \ \ 521$	$6,480 \pm\ \ 4,180$	$5,500 \pm 2,270$
10	a	78	$2,220 \pm\ \ \ 387$	$3,510 \pm\ \ 1,860$	$3,720 \pm 1,320$
	b	143	$2,430 \pm\ \ \ 482$	$4,820 \pm\ \ 5,930$	$4,510 \pm 2,480$
11	a	120	$1,950 \pm\ \ \ 376$	$2,420 \pm\ \ 1,350$	$2,880 \pm 1,100$
	b	139	$2,010 \pm\ \ \ 303$	$2,550 \pm\ \ 1,360$	$3,010 \pm\ \ \ 975$
12	a	90	$1,980 \pm\ \ \ 307$	$2,450 \pm\ \ 1,090$	$2,940 \pm\ \ \ 901$
	b	—	—	—	—
13	a	300	$1,700 \pm\ \ \ 324$	$1,630 \pm\ \ 1,450$	$2,190 \pm\ \ \ 978$
	b	349	$1,850 \pm\ \ \ 323$	$2,010 \pm\ \ \ 966$	$2,560 \pm\ \ \ 861$
14	a	120	$1,720 \pm\ \ \ 270$	$1,610 \pm\ \ \ 712$	$2,210 \pm\ \ \ 673$
	b	339	$1,640 \pm\ \ \ 315$	$1,450 \pm\ \ \ 828$	$2,030 \pm\ \ \ 778$
15	a	150	$1,610 \pm\ \ \ 174$	$1,260 \pm\ \ \ 395$	$1,900 \pm\ \ \ 407$
	b	120	$1,270 \pm\ \ \ 194$	$650 \pm\ \ \ 293$	$1,190 \pm\ \ \ 367$
16	a	150	$1,160 \pm\ \ \ 208$	$504 \pm\ \ \ 280$	$997 \pm\ \ \ 365$
	b	150	$1,160 \pm\ \ \ 250$	$523 \pm\ \ \ 313$	$1,010 \pm\ \ \ 425$
17	a	249	$1,150 \pm\ \ \ 215$	$489 \pm\ \ \ 266$	$975 \pm\ \ \ 364$
	b	235	$1,132 \pm\ \ \ 214$	$472 \pm\ \ \ 267$	$950 \pm\ \ \ 365$

* a and b represent duplicate electron microscopic determinations.
† Means ± SD.

microscope by using the usual techniques of osmium fixation.

The calculated recovery of subfractions from the density gradient flotation experiment and the recovery determined experimentally from electron micrographs are shown in Fig. 5. From particle-size measurements obtained from this density gradient separation by electron microscopy, the average particle size, molecular weight, and S_f rate for the 17 fractions were calculated by means of the computer program (Table 1). The particle-diameter distributions obtained for the 3rd, 7th, 11th, and 15th fractions are given in Fig. 6. Figure 7 shows a typical electron micrograph for fraction 13.

The comparison between particle sizes obtained experimentally by means of electron microscopy and those obtained by calculation from ultracentrifugal flotation was made with the simplifying assumptions that a continuous size distribution of lipoprotein particles of constant hydrated density (0.93 g/ml) existed and that each of the 17 regions of the gradient was a homogeneous medium. Although there is considerable overlap in size distribution for adjacent fractions, and somewhat smaller particle sizes were obtained experimentally, the results are consistent with anticipated recovery. These results suggest that preparative centrifugation does not significantly alter the particle-size distribution.

DISCUSSION

The particle-size distributions of electrophoretically and ultracentrifugally fractionated very low density plasma lipoproteins from subjects fed corn oil have been determined using the electron microscope. Results obtained on four secondary-particle fractions and three primary-
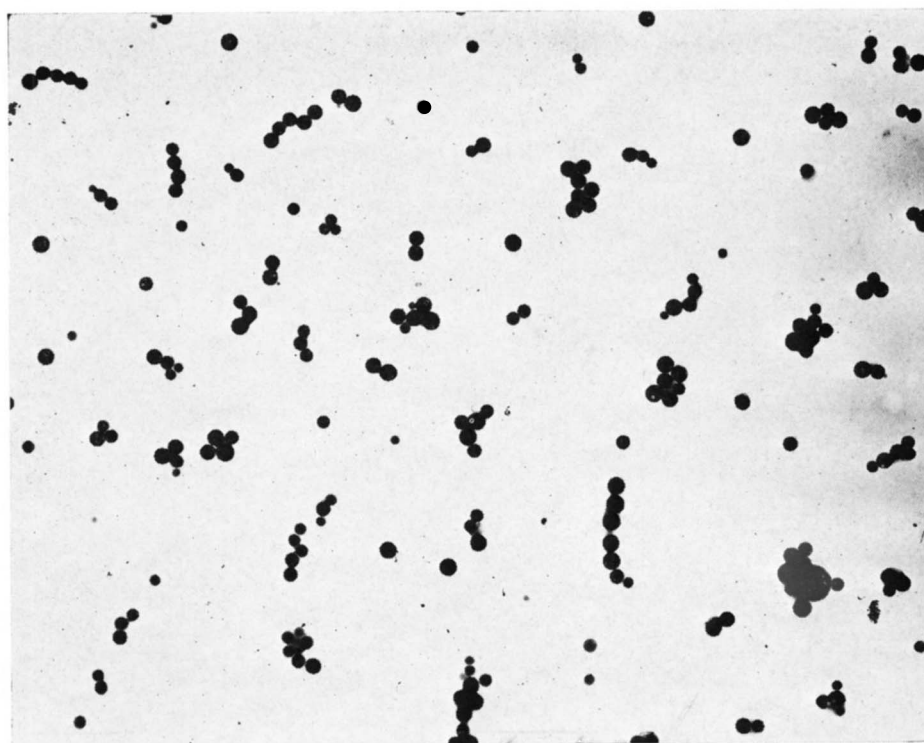
FIG. 7.   Electron micrograph of ultracentrifugal subfraction 13 (Table 1).   ×11,400.

particle fractions indicate a considerably broader parti-cle-size distribution than was previously estimated from centrifugal behavior (9). Clumping of particles during preparative centrifugation at low temperature could yield operational estimates of size differing from those observed with the electron microscope. In addition, these results suggest that both primary and secondary particles have a size distribution that extends over most of the known plasma lipoprotein spectrum above S_f 400. If the cubic relationship of volume to diameter is con-sidered, approximately two-thirds of the particle mass (and hence, triglyceride) may be found in the diameter range of 1500–4000 A. Further, from the fact that the particle-size distributions were similar in primary and secondary particles from any one plasma sample, it is apparent that significantly different properties may exist among lipoproteins in the same size range.

Using only the measurement of diameter, we have calculated molecular-weight distribution and flotation-rate distributions of each lipoprotein fraction and sub-fraction studied with the electron microscope. In these calculations it has been assumed that no change in particle size occurs as a result of osmium fixation. Since at 30 min the fixation reaction (16) with very low den-sity lipoproteins requires approximately 1–2 atoms of osmium per double bond, the lipoproteins might be ex-pected to increase their size by an amount approximately equal to the added volume of osmium. Because of the very small partial specific volume of osmium, it is estimated

that the fixed lipoproteins are about 5% larger than the native unfixed lipoproteins.

The degree of lipid unsaturation is important in the osmium tetroxide–lipoprotein reaction (16, 17), and the failure of osmium to fix the more saturated butter fat-produced particles may reflect this phenomenon. Alter-natively, since chylomicron triglyceride produced by feeding butter fat is semisolid at room temperature and separates into an oil phase, after being cooled to 4° and then rewarmed, more readily than corn oil-produced chylomicron triglyceride (18), it is possible that differences in the physical state of lipids in lipoproteins also influence the fixation reaction. In contrast, Jones, Thomas, and Scott (6) did observe osmiophilic masses in the chyle of rats fed butter fat.

Further studies are needed to determine the effect of diet and several hyperlipemic states on the particle-size distribution of these very low density plasma lipo-proteins.

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