A quick and large-scale density gradient subfractionation method for low density lipoproteins

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Abstract A quick density gradient-banding subfractionation method has been developed for d < 1.063 g/ml lipoproteins. Up to 324 ml of plasma can be resolved into five distinct layers by a single ultracentrifugation. The separation was achieved with a discontinuous density gradient formed between plasma and a layer of NaCl solution of d 1.080 g/ml in an angle-head rotor during centrifugation at 45,000 rpm for 26 hr at 5°C. VLDL and LDL_1 (layer 1, d < 1.020 g/ml) were at the top. Layer 2 (apparent d 1.025–1.028 g/ml), layer 3 (apparent d 1.032–1.043 g/ml) and layer 4 (apparent d 1.046–1.054 g/ml) were subfractions of normal LDL_2. Layer 5 (d > 1.071 g/ml) contained HDL and plasma proteins. A second step centrifugation separates VLDL from LDL_1. When opaque tubes are used, additional centrifugation is needed to separate layer 4 from layer 5. The subfractionation method was reproducible and was verified by analytical ultracentrifugation, chemical analyses, agarose electrophoresis, and electron microscopy. This method has been applied to plasma of normal males and females of the same age group. The chemical composition of a given subfraction from subjects of the same category was constant. However, compositional differences were found between normal males and females. The triglyceride content was higher in layer 2 and the cholesteryl ester content was lower in layer 4 for normal females than for males. Quantitatively, cholesterol concentration was significantly higher in layer 2 for normal males than for females. Layer 4 and layer 5 were the only fractions containing Lp(a). Applicability of the subfractionation method to studies of dyslipoproteinemia was demonstrated with plasma from patients with type III and type IV hyperlipoproteinemias. Marked differences were found in VLDL and LDL_1, both qualitatively and quantitatively, between the two types of patients and between the type III patient and normal subjects. A primarily quantitative difference was found in VLDL between the type IV patient and normal subjects. This isolation method yields concentrated subfractions that reveal the heterogeneity of LDL_2 in one spin, and offers quick isolation of narrow density ranges of LDL species, thereby providing better defined molecular entities for structural and/or metabolic studies.—**Lee, D. M., and D. Downs.** A quick and large-scale density gradient subfractionation method for low density lipoproteins. *J. Lipid Res.* 1982. **23:** 14–27.

Supplementary key words very low density lipoproteins • narrow density range • hyperlipoproteinemia • Lp(a) • electron microscopy • agarose electrophoresis

Chemical characterization and metabolic studies often necessitate the subfractionation of low density lipoproteins in order to obtain materials relatively homogeneous in particle size, molecular weight, hydrated density, and chemical composition. However, the processing of large quantities of plasma by sequential flotation is time-consuming and the selection of density ranges is arbitrary. Isolation procedures using a swinging bucket rotor and density gradient for equilibrium banding can separate lipoproteins of particular hydrated densities, but the sample size is limited (12–24 ml of plasma per spin) (1–4). The latter method requires the plasma to be exposed initially to high salt concentrations (density 1.2–1.4 g/ml).

We have developed a density gradient ultracentrifugal method for an angle-head rotor which retains the maximum capacity of the centrifuge tubes (12 × 26 ml plasma for 50.2 Ti rotor). By layering a single salt solution beneath plasma, without adjusting the density of the plasma, we have taken advantage of the fact that both plasma and salt solution will redistribute, each forming its own density gradient after high speed centrifugation (5). After a 26-hr centrifugation, we obtained two discontinuous density gradients. Very low and low density lipoproteins (VLDL and LDL) were separated into four distinct layers.

The present report describes the methodology, its verification, and its application to the studies of VLDL and LDL subfractions from normal male and female subjects and also presents examples for its application of type III and type IV hyperlipoproteinemic plasma.

Abbreviations: VLDL, very low density lipoproteins, d < 1.006 g/ml: LDL_1, low density lipoproteins of S_f 12–20 or d 1.006–1.019 g/ml; LDL_2, low density lipoproteins of S_f 0–12 or d 1.019–1.063 g/ml; LDL, low density lipoproteins of S_f 0–20 or d 1.006–1.063 g/ml; HDL, high density lipoproteins; HDL_2, high density liproproteins of d 1.063–1.125 g/ml; Lp(a), lipoprotein (a), a polymorphic form of lipoprotein B; TG, triglycerides; TC, total cholesterol; CE, cholesteryl ester; FC, free cholesterol; S_f, flotation coefficient, measured at solvent density 1.063 g/ml of NaCl; S_f^0, flotation coefficient at infinite dilution; LP-A, lipoprotein A, characterized by the presence of apolipoprotein A; LP-B, lipoprotein B, characterized by the presence of apolipoprotein B.

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METHODS

Plasma samples

Plasma samples were collected by plasmapheresis from normolipidemic male and female subjects between 24 and 45 years old and from two hyperlipoproteinemic patients, after a 12-hr overnight fast (6). The descriptions of the two patients are as follows.

Patient J.B., male, age 37, was identified as having type III hyperlipoproteinemia. His serum lipid levels for eight visits over a year and a half were triglyceride (TG) 195.0 ± 59.9 mg/dl (range 102–300 mg/dl) and total cholesterol (TC) 332.3 ± 65.4 mg/dl (range 222–438 mg/dl). He had been on a low cholesterol and low triglyceride diet for a year before the onset of this study. The plasma for this study was drawn on his fifth visit, when he had a serum TG of 139 mg/dl and a TC of 332 mg/dl. His serum electrophoresis showed a broad β band and contained floating β in VLDL; the ratios of VLDL-TC to plasma TG were 0.61, 0.57, and 0.76 on three different occasions, which greatly exceed the value of 0.3 regarded as diagnostic of type III hyperlipoproteinemia (7). The patient was on clofibrate treatment and was off the drug for 2 months prior to this study.

Patient D.W., male, age 42, was identified as having type IV hyperlipoproteinemia. He was on a low carbohydrate diet. We have followed his lipid profile over a 3-year span. His average serum TG was 196.4 ± 42.2 mg/dl (range 128–260 mg/dl) and average serum TC was 200.6 ± 10.2 mg/dl (range 188–213 mg/dl). Decreased HDL-TC (25.0 ± 2.3 mg/dl), an increased pre-β-band, and a decreased α-band were always characteristic of his lipid and lipoprotein patterns.

Immediately after collection of a plasma sample, a preservative solution mixture A (unless otherwise stated) was injected into the plasmapheresis bag to yield final concentrations of EDTA 0.05%, NaN_3 0.05%, diisopropylfluorophosphate 10 mM, and thimerosal 0.05%. The bag was resealed without exposing the plasma to air. Isolation of lipoprotein was carried out within 2 days of plasma collection. Due to the anticoagulant and dextrose present in plasma bags (6), the resultant plasma density was in the range of 1.025–1.028 g/ml. A dilution factor of about 1.26 was introduced by the anticoagulant.

Density gradient centrifugation

Twenty-six milliliters of plasma at its unadjusted density (d 1.025–1.028 g/ml) was placed in each centrifuge tube (size 1 × 3.5 in) of either cellulose nitrate, (Beckman, discontinued) or polyallomer (Beckman, Part No. 326823, Spinco, Palo Alto, CA). After the tube caps (Beckman, Part No. 331151) were tightly attached, a layer of NaCl solution of d 1.080 g/ml containing 0.05% EDTA, pH 7.0, was introduced through the center hole of each tube cap to fill the remaining 9-ml volume. The addition of NaCl solution was carried out by letting the solution run downward very slowly to the bottom along the tube wall through a filled syringe attached to a 1.5-inch-long 21-gauge needle. The needle was slightly bent so that the syringe could balance its weight on top of the tube cap with its bevel side facing and touching the wall. Thus, several tubes could be filled simultaneously. Both plasma and NaCl solution were prechilled at 5°C and loaded at 5°C (although loading at room temperature did not change the banding patterns). When the tubes were filled, the center set screws (Beckman, Part No. 338864) were screwed in air-tight. Centrifugation was carried out at 45,000 rpm (185,000 *g*) for 26 hr in a 50.2 Ti rotor at 5°C.

Since the quick-seal tubes (Beckman, Part No. 342414, Spinco, Palo Alto, CA) have become available, we have adapted this gradient-making method to use them. These tubes have a 39.9-ml capacity compared to 35 ml for regular tubes. In this case, 27 ml of plasma was placed in each centrifuge tube initially. The NaCl solution was delivered to the bottom of the tube through narrow rigid Teflon tubing from a reservoir containing the salt solution. The tubing was removed when the plasma level reached the neck of the centrifuge tube. The tip of the polyallomer tube was then sealed using a metal seal former (Beckman, Part No. 343421) with a tube sealer (Beckman, Part No. 342420). The tubes were placed in a 50.2 Ti rotor with spacers (Beckman, Part No. 342418) placed on top of the tubes. Centrifugation was also carried out at 45,000 rpm for 26 hr at 5°C.

During centrifugation, density gradients formed and the lipoproteins banded at their equilibrium density regions. At the end of the run, five layers were visible through cellulose nitrate tubes for normal lipidemic plasma (**Fig. 1**). Layer 1, within the tube cap, was slightly opaque for normal plasma (visible through a hole in the metal cap). Layer 2, below the tube cap, had an orange tint for normal plasma. Layer 3, below the midpoint of the tube and bright orange-colored, was the major fraction of LDL. Layer 4 was also orange-colored and a minor fraction of LDL. Layer 5, at the bottom of the tube, contained high density lipoproteins (HDL) and the plasma proteins. The tubes were cut with a tube slicer at the clear zone just below the caps. Layer 1 was recovered quantitatively with a hypodermic syringe and was pooled from all tubes from the same subject isolated in one rotor. Layers 2, 3, and 4 were collected individually and quantitatively from the remaining tubes with disposable pipets by siphoning carefully from the surface of the solution along the tube wall. All 12 tubes were marked at the middle of the clear zones between layers and each fraction was collected between the markers.

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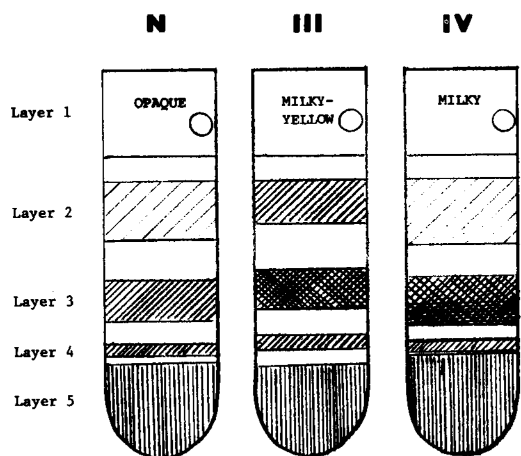

Fig. 1. Banding patterns in cellulose nitrate preparative ultracentrifuge tubes after centrifugation at 45,000 rpm in a 50.2 Ti angle-head rotor for 26 hr at 5°C. Layer 1, within the tube cap, consists of VLDL + LDL_1; layers 2, 3, and 4 are the subfractions of LDL; layer 5 consists of HDL and plasma proteins. N, normal plasma; III, type III hyperlipoproteinemia; IV, type IV hyperlipoproteinemia.

Banding positions were identical among all tubes in the same rotor for plasma from the same subject. For processing two pints of plasma from one donor, two rotors were usually employed simultaneously. No difference was observed in the banding pattern or positions between the two centrifugations. Thus, identical layers were pooled from all tubes. When polyallomer tubes were used, layer 2 was hardly visible for normal subjects and the demarcation between layers 4 and 5 could not be clearly distinguished. In this situation, layer 2 was collected according to the position shown in cellulose nitrate tubes, which was an apparent clear zone in polyallomer tubes between layer 1 and layer 3. Layers 4 and 5 were combined and recentrifuged at 45,000 rpm for 24 hr at d 1.078 g/ml with NaCl to recover layer 4 at the top of the tubes. Redistribution of NaCl during centifugation yielded a solvent density at the top of the tubes of less than d 1.063 g/ml (5).

For construction of density gradient curves, subfractions of layer 5 were siphoned off at 1-cm intervals and pooled from 12 tubes. Where sample volume permitted (130–250 ml), the specific gravity of the fractions was measured at 15°C by a long precision hydrometer (325 mm) (Fisher Scientific Co., Pittsburgh, PA, Cat. No. 11-555), calibrated with water at 15°C by the manufacturer. The density of fractions at 15°C was calculated by multiplying the values for specific gravity by 0.9991, the density of water at 15°C. Alternatively, smaller precision hydrometers (165 mm) (Sargent-Welch Scientific Co., Skokie, IL, Cat. No. S-41885), which were calibrated against the long precision hydrometers with known solvent densities, were employed. These small hydrometers required 25 ml of solution to cover the higher specific gravity readings (i.e., 1.070 or 1.130) and 45 ml to cover the lower specific gravity readings (i.e., 1.000 or 1.060). The density of the fraction obtained at 15°C was corrected to that at 25°C, according to the differences of known concentration of NaCl solutions measured at both 15.0°C and 25.0°C with precision hydrometers. For normal plasma, the density of layer 1 was found to be d 1.0204 ± 0.0016 (n = 7) g/ml and d 1.0210 ± 0.0010 (n = 5) g/ml for males and females, respectively. Thus layer 1 contained both VLDL and LDL_1. For further separation of VLDL from LDL_1, 20 ml of layer 1 was placed in a centrifuge tube and over-layered with 15 ml of NaCl + 0.05% EDTA, pH 7.0, solution of d 1.006 g/ml. Centrifugation under the previously described conditions yielded VLDL on top and LDL_1 (d 1.006–1.020 g/ml) in the lower portion of the tube. The tubes were then cut at the middle of the clear zone. Alternatively, to avoid the dilution caused by the layering, layer 1 was dialyzed against NaCl solution + 0.05% EDTA of d 1.010 g/ml. This slightly elevated density provided room for salt redistribution (5) so that recentrifugation yielded VLDL of d < 1.006 g/ml on top and LDL_1 in the lower portion of the tube. Thus, when cellulose nitrate tubes were used, two centrifugations yielded five well-separated subfractions of lipoproteins of d < 1.063 g/ml.

Layers 1 and 2 were usually devoid of albumin. But layers 3 and 4 contained trace amounts of albumin as judged by immunodiffusion with anti-albumin. For study of chemical composition including protein content, the layers were all washed at higher densities (density increment, Δd 0.030 g/ml). The washing step also served to concentrate the fractions. The lipoproteins were then dialyzed exhaustively against 0.15 M NaCl + 0.05% EDTA, pH 7.0, prior to chemical analyses.

For determination of recoveries during this fractionation procedure, analyses of TC and TG were performed directly on the isolated layers without washing or dialysis. It was necessary to substitute 10 mM benzamidine or ε-amino caproic acid for diisopropylfluorophosphate, since the latter interfered with the chemical determinations. The following equations were used for calculation.

$$\% \text{ recovery of TG} = \frac{\sum_{i=1}^{5} ([TG]_i \times \text{Volume}_i)}{\text{plasma TG in mg/ml} \times \text{Volume of plasma used for isolation}} \times 100\%$$

$$\% \text{ recovery of TC} = \frac{\sum_{i=1}^{5} ([TC]_i \times \text{Volume}_i)}{\text{plasma TC in mg/ml} \times \text{Volume of plasma used for isolation}} \times 100\%$$

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where $[TG]_i$ = TG of layer i in mg/ml; $[TC]_i$ = TC of layer i in mg/ml; $Volume_i$ = volume of layer i in ml; and i = layer 1 through 5.

The concentration of each component isolated in each fraction after washing and dialysis was calculated back to 100 ml of the original undiluted plasma volume according to the equation:

$$TG_j \text{ in mg/dl of plasma} = [TG]_j \times \frac{Volume_j \times 100}{\text{Volume of undiluted plasma used}}$$

where $[TG]_j$ = concentration of TG in fraction j in mg/ml after washing and dialysis; $Volume_j$ = volume of fraction j in ml after washing and dialysis; and j = fraction VLDL, LDL_1, layer 2, 3, and 4 after washing and dialysis.

Calculations for TC, CE, free cholesterol (FC), phospholipids (PL), and protein (PR) in mg/dl of plasma were done by the same manner as for TG. Thus the concentration of lipoproteins occurring in each fraction was the summation of all components recovered in mg/dl plasma, i.e.,

$$Lipoproteins_j \text{ in mg/dl plasma} = TG_j + CE_j + FC_j + PL_j + PR_j \text{ in mg/dl plasma.}$$

Chemical analyses

Triglycerides and total cholesterol of lipoproteins and of whole plasma were determined by autoanalysis according to the methods of Kessler and Lederer (8) and of Rush, Leon, and Turrell (9), respectively. Free cholesterol was assayed according to the method of Sperry and Webb (10). Cholesteryl ester was calculated from the difference between total cholesterol and free cholesterol and expressed as cholesteryl linoleate. Phospholipid analyses was carried out according to a micromethod of Gerlach and Deuticke (11). Protein was assayed by the method of Lowry et al. (12). Sodium dodecyl sulfate was included in the reaction mixture to clear the turbidity caused by lipids (13).

Immunochemical analyses

Antibodies to lipoprotein B (LP-B) and antibodies to lipoprotein A (LP-A), which contained antibodies to the protein moieties A-I and A-II, were the same as described previously (14). Antiserum to Lp(a) was a generous gift from Dr. K. Walton of Birmingham, England. Double immunodiffusion was carried out in 1% agarose in barbital buffer (15).

Agarose electrophoresis

A solution of 1% agarose (Standard low-mr. Bio-Rad, Richmond, CA) and 2% bovine albumin in barbital buffer, pH 8.6, ionic strength 0.1, was used to cast the gel. Electrophoresis was carried out at 8 V/cm for 55 min in barbital buffer. The slide was stained with Oil Red O. The lipoprotein fractions were concentrated or diluted to appropriate concentrations to ensure the visibility of bands after staining.

Ultracentrifugal analyses

Sedimentation– and flotation–velocity experiments were performed on lipoprotein subfractions in a Spinco model E analytical ultracentrifuge at 25°C in an An-H rotor using schlieren optics as described previously (5). Lipoproteins were dialyzed in NaCl solutions of two known densities containing preservatives, 0.05% EDTA, 0.05% NaN_3, and 0.05% thimerosal, pH 7.0. At least two different lipoprotein concentrations were used in order to extrapolate the S_f values to infinite dilution. Values for hydrated density were obtained by interpolating the S_η versus ρ data (η = solvent viscosity; ρ = solvent density) to zero sedimentation (5). Densities of salt solutions were measured at 25°C with a long precision hydrometer as described earlier.

Electron microscopy

Morphological characteristics of the subfractions were studied by electron microscopy. The washed and dialyzed lipoprotein layers in 0.85% NaCl + 0.05% EDTA were diluted 2 to 4-fold with 1% ammonium acetate containing 0.05% EDTA and 0.05% NaN_3, pH 7.0. A copper grid was coated with 0.25% Formvar in ethylene dichloride (Ernest F. Fullam, Inc., Schenectady, NY), and dried in vacuo. The coated grid was dipped in a drop of lipoprotein sample, blotted with filter paper, and allowed to air dry. It was then dipped in a drop of staining solution, blotted with filter paper, and allowed to air dry. A negative stain with 1% phosphotungstic acid at pH 7.2 was employed. Microscopy was performed in a Hitachi electron microscope HU11B (Tokyo, Japan) operating at 75 KV. Mean particle size of the LDL fractions was calculated from measurements of 50–100 particles in each fraction.

RESULTS

Subfractionation of lipoproteins

This subfractionation method separated normal lipoproteins into five fractions in one spin (Fig. 1). Layer 1 contained the associated lipoprotein families (two or more apolipoproteins on the same lipoprotein particle), including VLDL + LDL_1. The average density for layer 2 was 1.0272 ± 0.0014 g/ml (n = 7, male) and 1.0278 ± 0.0010 g/ml (n = 5, female), respectively. Layer 3 was characterized by densities of 1.0365 ± 0.0014 g/ml (n = 7, male) and 1.0384 ± 0.0009 g/ml (n = 5, female). Layer 4 was in the range of d 1.046–1.054 g/ml. All four layers gave positive immunoreactions with antibod-

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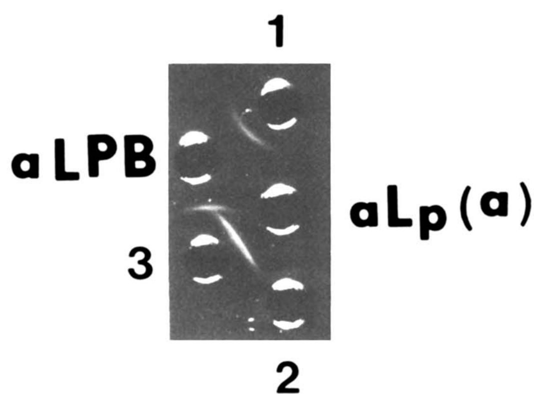

Fig. 2. Immunochemical evidence for the presence of Lp(a) in layer 4. aLp(a): anti-Lp(a), a generous gift from Dr. K. Walton of the University of Birmingham, England; aLPB: anti-LP-B; 1, 2, and 3 are layer 4 from three different subjects. Sample 1 showed undetectable Lp(a), 2 showed weakly detectable Lp(a), and 3 showed strongly positive Lp(a). Note the positive LP-B reactions in layer 4 of subjects 1 and 3, and the partial identity between LP-B and Lp(a) in layer 4 of subject 3.

ies to LP-B. The upper 1 cm of layer 5 had a density of or greater than 1.071 g/ml. Layer 5 reacted with antibodies to LP-A. Thus in normal plasma, layers 2, 3, and 4 are subfractions of LDL_2, whereas layer 5 contains HDL and plasma proteins.

Among the upper four layers, layer 4 was the only fraction that reacted positively with anti-Lp(a). Six out of eleven normal individuals tested showed immunochemically positive Lp(a) in layer 4. They included both males and females. Layer 4 from both the type III and the type IV patients showed an Lp(a) positive reaction. The anti-LP-B reaction of layer 4 showed partial identity with the anti-Lp(a) reaction when Lp(a) was present (**Fig. 2**). This suggests that on some particles both the protein moieties (a) and apoB were present and that LP-B particles not associated with Lp(a) were also present. Therefore, layer 4 was a heterogeneous fraction when Lp(a) was present.

It should be pointed out that under the described centrifugation conditions the apparent densities measured for the layers served as operational references. They reflected density gradient formation and equilibrium banding of the lipoproteins. But these numbers deviated slightly from the exact values of hydrated densities of the banded lipoproteins for the following two reasons. *1)* The densities measured included the densities contributed by the lipoproteins, so the solvent densities excluding lipoproteins would be slightly lower than the numbers measured. *2)* Since the centrifugation was carried out at 5°C rather than 25°C (for the sake of lipoprotein stability), the measured numbers represented the densities of NaCl corrected for its thermal expansivity, which differs from the thermal expansivity of the lipoproteins (16). Thus, a slight deviation of the true hydrated density of lipoproteins from the isopycnic banding density is expected.

A typical density gradient curve is shown in **Fig 3** for normal plasma with NaCl solution layered underneath the plasma. The upper portion of the gradient was formed from the background solvent of the plasma; the lower portion of the gradient was formed from the layered 1.080 g/ml NaCl solution. The two gradients formed a joint at the junction where the slope changed abruptly. For the alternate layering method, i.e., when NaCl solution was delivered through a needle along the tube wall, the resulting density gradient curve was similar.

When this subfractionation method was applied to type III and type IV hyperlipoproteinemic plasma, the lipoproteins banded at similar positions but with remarkably different appearances (Fig. 1). Layer 1 was milky-yellow for type III and milky for type IV. This was a reflection of the high content of TG and TC in the type III sample and the high content of TG in the type IV sample (see Table 1). Layer 2 was bright orange in the type III sample and only slightly orange in the type IV sample. Although the intensity of orange color frequently correlates positively with the content of cholesterol in normals, it may also reflect the dietary content of carotenoids. Layer 3 from the type IV patient tended to be wider than those from other subjects; the majority of the materials were concentrated at the lower portion of the layer. Although the average apparent density of the entire layer 3 from the type IV plasma was 1.036 g/ml, the major portion banded at d 1.042 g/ml. Layer 4 from type IV was not well separated from layer 5 and always required reflotation at d 1.078 g/ml to obtain complete separation from the latter.

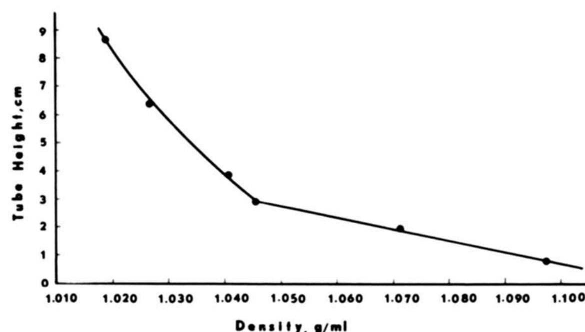

Fig. 3. A typical density gradient formed after ultracentrifugation at 45,000 rpm in a 50.2 Ti angle-head rotor for 26 hr at 5°C. Quick-seal tubes containing 27 ml of plasma were initially layered with 12.9 ml of NaCl solution of d 1.080 g/ml containing 0.05% EDTA. Density was determined at 15°C with a calibrated precision hydrometer (Fisher or Sargent-Welch) after collection of the fractions. See details in Methods.

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Agarose electrophoresis

The electrophoretic mobilities of the isolated subfractions on agarose decreased in the order VLDL, LDL_1, layer 2 then layer 3, then increased again for layer 4 and layer 5 (the lipid staining portion). However, the mobilities also increased with aging of the isolated samples, possibly due to the time-dependent occurrence of lipid peroxidation of the lipoproteins (17, 18). To eliminate this possible interference, we have excluded oxygen throughout the isolation procedure and employed a plasma preservative mixture B which contained an antioxidant when fractionating samples for electrophoresis (17). Preservative mixture B contained final concentrations of penicillin-G, 500 units/ml; streptomycin sulfate, 50 μg/ml; EDTA, 0.1%; glutathione, reduced form, 0.05%; ϵ-amino caproic acid, 0.13%; and chloramphenicol, 0.002%. The d 1.080 g/ml NaCl layering solution also contained the same amount of EDTA, glutathione, and ϵ-amino caproic acid. Nitrogen was used to exclude the oxygen from solutions and from containers (17). The isolated fractions were dialyzed in 0.85% NaCl + 0.1% EDTA + 0.05% glutathione and 0.13% ϵ-amino caproic acid under N_2 for 24 hr at 5°C with three changes of dialysate prior to electrophoresis. The electrophoretic mobilities of lipoproteins prepared with such precautions showed minimal changes attributable to lipid peroxidation (17). As shown in **Fig. 4,** the fractionated lipoproteins from normal subjects had the following mobilities: layer 1 showed a broad pre-β band. This was the combination of a pre-β band at the front, and a "late pre-β band" at the tail, representing VLDL and LDL_1, respectively, as identified with separate VLDL and LDL_1 (not shown in the figure). Layer 3 showed a typical β band; the mobility of layer 2 was slightly faster than the band of layer 3. The mobility of layer 4 varied with individuals. Layer 4 showing immunochemically undetectable Lp(a) migrated similarly to layer 2 with slightly faster than β mobility (sample 4 in Fig. 4); Lp(a) positive layer 4 moved to a "late pre-β" position (sample 6 in Fig. 5), similar to that which Berg, Dahlén, and Frick (19) termed the "pre-β_1" lipoproteins. The mixture of layer 4 and layer 5 for Lp(a) positive subjects showed an α band and a doublet with pre-β and "late pre-β" mobilities (sample 9 in Fig. 4). When this layer 4 was separated from layer 5, the "late pre-β" lipoprotein was recovered in the d < 1.063 g/ml fraction while the pre-β lipoproteins were recovered in the d > 1.063 g/ml (HDL_2) fraction. Both showed immunochemically positive reactions against antiLp(a). Thus for Lp(a) positive subjects, layer 5 showed α as the major and pre-β as the minor band. When Lp(a) was undetectable, layer 5 showed only an α band when lipid stained (sample 5 in Fig. 4). The electrophoretic mobilities of the type IV subfractions were similar to their counterparts in normals. However, layer 1 and layer 2 from the type III patient had β-mobility bands; for layer 1, a faint stain appeared at the pre-β position as well. Both the type III and the type IV patients showed detectable Lp(a), the mobilities of their layer 4 and layer 5 were the same as the normal Lp(a) positive subjects.

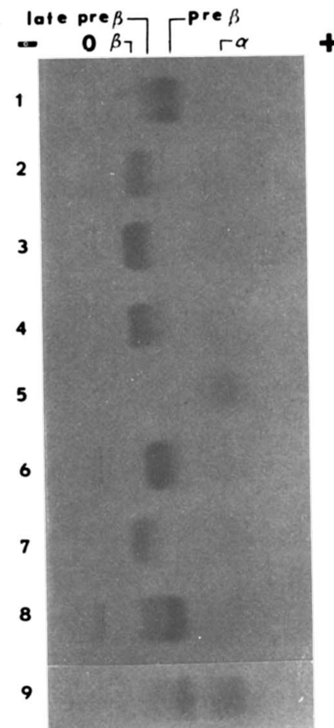

Fig. 4. Agarose electrophoretic mobilities of the separated layers from density gradient ultracentrifugation. 0, origin; 1–5, layer 1, layer 2, layer 3, layer 4, and layer 5, respectively, from a subject with undetectable Lp(a); 6, layer 4 containing immunochemically positive Lp(a); 7, normal whole plasma; 8, layer 1 from a subject with positive Lp(a) in whole plasma but undetectable Lp(a) in this fraction; and 9, a mixture of layer 4 and layer 5 containing positive Lp(a). All samples were isolated under N_2 and in the presence of preservative mixture B (containing antioxidant, see results in text). The dialyzed samples were mixed with equal volume of 1% agarose solution containing 2% bovine albumin in barbital buffer before application to the slots. After solidifying, the samples were further covered with a drop of the agarose–albumin solution. Electrophoresis was carried out at 8 V/cm for 55 min in barbital buffer. The plate was stained with Oil Red O.

Chemical analyses

When analyses of TG and TC were performed on layer 1 through layer 5 without washing or dialysis, the recovery of lipids from fractions was 95–97% based on either TG or TC values. These recoveries were calculated on fractions from two normal plasmas. These results suggest that there was very little loss of lipoproteins due to the fractionation method. After washing and di-

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TABLE 1. Concentration of lipoproteins in subfractions of fasting plasma

Subfraction		Normal Female	Normal Male	Type III (1)	Type IV (1 × 4)
			mg/dl of plasma		
Layer 1	VLDL	25.7[a] ± 15.2 (3)	56.3 ± 28.5 (5)	336.6	171.1 ± 49.0
	LDL_1	10.8[a] ± 5.8 (3)	18.3 ± 13.3 (5)	103.6	18.9 ± 4.9
Layer 2		53.6[a] ± 22.8 (5)	88.4 ± 56.9 (5)	126.4	108.2 ± 46.4
Layer 3		175.3[a] ± 83.9 (5)	157.2 ± 35.0 (5)	203.0	304.0 ± 42.5
Layer 4		28.1[a] ± 4.1 (3)	30.7 ± 6.0 (4)	24.9	19.5 ± 4.5

[a] Not significant; statistical evaluation between normal females and normal males for the significance of differences.

Values were based on actual recoveries of lipoproteins after washing and dialysis and were expressed as mean ± SD; numbers in parentheses represent the number of subjects; (1 × 4) means the same subject was studied four times over a 2-year span.

alysis of the lipoproteins, the recovery decreased to 76–85%. Losses of lipoproteins to the infranatant fractions after washing accounted for 10–15%. The other major loss was during dialysis, when small amounts of precipitation occurred, possibly due to oxidation and aggregation of lipoproteins. Since all samples were treated equally, it is believed that a quantitative comparison between fractions or among subjects is valid. Concentration of lipoproteins in subfractions of fasting plasma are shown in **Table 1.** Results of statistical evaluation between normal females and normal males showed no significant difference in any fraction. It was interesting to note that the concentration ratio between layer 2 and layer 3 was about 1:2 in normal males but 1:3 in normal females (Table 1).

The expected high levels of VLDL for type III and type IV patients (20–23) were observed in this study. In addition, the elevated LDL_1 in the type III patient (20, 23–25) was also confirmed by this fractionation method (Table 1).

The concentrations of TG and TC for each washed and dialyzed subfraction are shown in **Table 2.** No significant difference in the distribution of TG among layers between normal males and females was observed. The expected higher contents in VLDL-TG for type III and type IV patients (20, 22) were observed in the present study. The type IV patient also appeared to have higher TG in layer 3 than the normal males.

The distribution of TC in subfractions was similar between normal males and females except in layer 2 (Table 2). The TC content in layer 2 was significantly different ($P < 0.05$) between the two normal groups; the TC in males was twice as high as in females.

The type IV patient appeared to have mildly elevated TC in VLDL when compared to normal males (Table 2). The elevation of TC in the type III patient was markedly pronounced both in VLDL and in LDL_1 (Table 2).

The chemical composition of VLDL was similar among normal males, females, and the type IV patient (**Table 3**). A striking difference was observed in the composition of VLDL in the type III patient, whose percent CE was higher, while TG and proteins were lower, than normal subjects. These were reflected in the extremely low TG/CE ratio.

The composition of LDL_1 (**Table 4**) was amazingly

TABLE 2. Concentrations of triglyceride and total cholesterol in subfractions of fasting plasma

	Triglyceride				Cholesterol			
Subfraction	N ♀ (3)	N ♂ (5)	III (1)	IV (1 × 4)	N ♀ (3)	N ♂ (5)	III (1)	IV (1 × 4)
		mg/dl				*mg/dl*		
VLDL	13.4[a] ± 7.8	27.7 ± 11.5	101.3	100.2 ± 30.0	3.0[a] ± 1.7	7.5 ± 4.8	102.9	17.5 ± 4.6
LDL_1	3.5[a] ± 1.7	5.9 ± 3.4	10.0	6.6 ± 1.7	2.2[a] ± 1.3	2.6 ± 1.4	37.2	3.6 ± 0.9
Layer 2	6.2[a] ± 1.8	8.3 ± 7.2	8.5	12.7 ± 3.4	16.8[b] ± 7.9	35.3 ± 9.3	45.1	33.5 ± 13.4
Layer 3	8.4[a] ± 4.5	6.5 ± 1.4	9.2	15.9 ± 2.3	57.5[a] ± 25.1	52.0 ± 14.0	68.8	84.2 ± 30.6
Layer 4	1.7[a] ± 0.2	1.6 ± 0.4	0.9	1.3 ± 0.4	7.8[a] ± 1.0	9.8 ± 2.2	8.3	5.7 ± 1.1
Plasma	55.8 ± 16.4	79.8 ± 26.0	139.0	198.0 ± 54.8	182.0 ± 42.5	184.0 ± 9.3	332.0	210.8 ± 18.0

[a] Not significant.
[b] $P < 0.05$; statistical evaluation between normal females and normal males for the significance of differences.

N, normal; III, type III hyperlipoproteinemia; IV, type IV hyperlipoproteinemia; numbers in parentheses represent the number of subjects studied; (1 × 4), four studies were performed on the same subject over a 2-year span; results were based on the actual recoveries of subfractions after washings and dialyses and were expressed as mean ± SD.

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TABLE 3. Chemical composition of VLDL

Subjects (n)	Proteins	Phospholipids	Free Cholesterol	Cholesteryl Esters	Triglycerides	TG/CE	EC%
			%				
N ♀ (5)	12.08[a] ± 1.96	18.65[a] ± 2.99	5.08[a] ± 0.77	10.45[a] ± 0.60	53.92[a] ± 2.47	5.16	55.5
N ♂ (7)	11.53 ± 0.82	19.04 ± 3.09	5.49 ± 0.89	12.95 ± 4.55	50.98 ± 5.40	3.93	58.3
III ♂ (1)	7.09	17.16	8.42	37.23	30.10	0.81	72.5
IV ♂ (1 × 4)	10.09 ± 1.06	17.14 ± 0.41	3.79 ± 0.54	10.59 ± 1.24	58.38 ± 1.74	5.5	61.3
N ♂ (7)[b]	9.37 ± 0.96	18.03 ± 2.60	5.61 ± 0.90	12.80 ± 3.46	55.35 ± 5.76	4.32	57.6

[a] Not significant; Statistical evaluation between normal females and normal males for the significance of differences.
[b] Data taken from Lee et al. (31) for VLDL isolated by sequential flotation from normal male subjects.
N, normal; III and IV, types III and IV hyperlipoproteinemia; number in parentheses is number of subjects studied; (1 × 4), same subject was studied four times within a 2-year span; percent chemical composition was expressed as mean ± SD; TG, triglyceride; CE, cholesteryl ester; EC%, percent of esterified cholesterol out of total cholesterol.

similar among normal males, females, and the type IV patient. The LDL_1 composition of the type III patient stood out with its increased FC and CE combined with a decreased TG, resulting in the extremely low TG/CE ratio.

Although the TC content of layer 2 in normal males was significantly higher ($P < 0.05$) than that in normal females, the percent contents of CE and FC showed no significant differences between normal males and females (**Table 5**). The percent content of TG was significantly higher ($P < 0.05$) in normal females than in normal males. The chemical compositions of layer 2 in the type III and the type IV patients were similar to those of normal subjects except that the percent TG in type III resembled that of normal males while the percent TG in type IV resembled that of normal females.

Table 6 shows the percent chemical composition of layer 3, the major fraction of LDL in fasting plasma. Close resemblance was found among normal males and females and the two patients studied, suggesting that this major fraction of LDL was probably near normal in these patients as far as the known lipid components and apolipoproteins were concerned. Differences between males and females appeared again in the chemical composition of layer 4 (**Table 7**), in which the CE was significantly higher ($P < 0.01$) in normal males than in females. The layer 4 composition of the male type III patient resembled that of normal males. The composition of the type IV patient resembled that of normal females except that the percent PL content was lower in the former.

Physical-chemical characteristics of the subfractions

To ensure that the subfractionation had reached completion after 26 hr of centrifugation under the described conditions and to ascertain that the isolated fractions represented molecular species of particular physical-chemical characteristics, aliquots of some of the isolated subfractions were studied in the analytical ultracentrifuge. Flotation coefficients at infinite dilution, S_f^0, were determined for subfractions from a normal male subject, the type III hyperlipoproteinemic, and the type IV hyperlipoproteinemic subject. As shown in **Table 8,** the S_f^0 of the different layers isolated from the same subjects were indeed different. We were particularly concerned about layer 2 since it is the least visible fraction in normal subjects. To ensure that layer 2 was not due to incomplete separation of layer 1 and layer 3, we prolonged the centrifugal isolation time to 30 hr. The amount of layer 2 was unchanged. We also validated the existence of a lipoprotein with physical-chemical characteristics of layer 2 by isolating an LDL_2 at solvent density d 1.019–

TABLE 4. Chemical composition of LDL_1

Subjects (n)	Proteins	Phospholipids	Free Cholesterol	Cholesteryl Esters	Triglycerides	TG/CE	EC%
			%				
N ♀ (5)	15.62[a] ± 1.58	22.17[a] ± 2.63	6.09[a] ± 1.11	22.00[a] ± 2.09	33.35[a] ± 3.03	1.52	68.3
N ♂ (5)	17.85 ± 2.75	21.73 ± 2.62	6.54 ± 2.34	22.50 ± 5.25	31.44 ± 3.83	1.40	67.2
III ♂ (1)	15.08	21.63	10.12	43.48	9.69	0.22	71.9
IV ♂ (1 × 4)	14.53 ± 1.55	21.71 ± 0.91	5.48 ± 0.88	23.13 ± 4.86	35.15 ± 4.85	1.52	71.5
N ♂ (5)[b]	17.97 ± 3.91	24.24 ± 1.23	5.76 ± 1.77	22.71 ± 5.01	29.49 ± 3.97	1.30	68.4

[a] Not significant (male vs female).
[b] Data taken from Lee et al. (31) for LDL_1 isolated by sequential flotation from normal male subjects.
The symbols and abbreviations used are the same as for Table 3.

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TABLE 5. Chemical composition of layer 2 of LDL

Subjects (n)	Proteins	Phosphpolipids	Free Cholesterol	Cholesteryl Esters	Triglycerides	TG/CE	EC%
			%				
N ♀ (5)	19.49[a] ± 0.71	21.96[a] ± 1.33	8.23[a] ± 0.94	37.91[a] ± 2.79	12.39[b] ± 3.39	0.33	73.3
N ♂ (7)	21.32 ± 2.39	23.02 ± 1.27	8.05 ± 0.52	39.80 ± 2.12	7.93 ± 1.59	0.20	74.6
III ♂ (1)	18.10	21.24	8.83	45.13	6.70	0.15	72.4
IV ♂ (1 × 5)	18.17 ± 2.52	22.64 ± 1.32	7.98 ± 1.14	39.00 ± 2.92	11.05 ± 3.09	0.28	74.4
N ♂ (3)[c]	22.2	23.6	9.5	34.2	9.5	0.28	68.2

[a] Not significant (male vs female).
[b] $P < 0.05$.
[c] Data taken from Lee and Alaupovic (29) for LDL-III (d 1.019–1.030 g/ml), a subfraction isolated by sequential flotation from normal male subjects.
The symbols and abbreviations used are the same as for Tables 2 and 3.

1.076 g/ml from the type III plasma by conventional sequential flotation. This LDL_2 showed three distinct floating peaks on the schlieren pattern, with S_f values of 6.8 (major), 12.3 (minor), and 4.5 (minor). We have also isolated an LDL_2 from the same patient at solvent density d 1.019–1.050 g/ml by sequential flotation. This LDL_2 showed two peaks with S_f values of 6.8 and 12.3. Although the S_f^0 12.3 peak was at the border for LDL_2 defined as S_f 0–12, the major LDL_1 peak had a value of S_f^0 20 for this type III patient. These results strongly suggest that layer 2, layer 3, and layer 4 represent discrete molecular species existing within LDL and can be separated by the described methodology.

Differences in S_f^0 values were also observed for the same layers among the normal male and subjects with type III and type IV hyperlipoproteinemia (Table 8). These differences are in agreement with the differences observed in chemical composition. The presence of only the higher S_f^0 peak (53.3) in type IV VLDL compared to the predominance of the low S_f^0 25.6 peak in type III VLDL was in accord with the high TG/CE ratio in the former. These S_f^0 values were in excellent agreement with the earlier observations that the major peak of type III VLDL had a mean S_f^0 of 25 and the major peak of type IV VLDL had a mean S_f^0 of 55 (26).

In some fractions, such as layer 3, the chemical compositions were similar among all normals and patients, yet differences were still found in flotation coefficients. This may suggest that the differences observed were due to factors other than protein and lipid components. Factors such as water of hydration or carbohydrate content may affect the density and the flotation coefficient of lipoproteins.

We have further determined the hydrated densities, $\bar{d}$, of the subfractions from the type III and the type IV patients. The results are shown in Table 8. Indeed, each layer had its characteristic hydrated density, and differences between the two patients were observed. The wide, diffuse appearance of layer 3 in preparative ultracentrifuge tubes as isolated from the type IV patient was reflected in the heterogeneity of its hydrated densities and flotation coefficients. From the hydrated density distribution patterns, it is clear why the subpopulations of lipoproteins distributed in the preparative ultracentrifuge tubes as layers.

Electron microscopy

Fig. 5 shows representative electron micrographs of three of the four LDL subfractions. The particles in all fractions appeared to be round and relatively uniform in size except layer 4, which appeared to have two major

TABLE 6. Chemical composition of layer 3 of LDL

Subjects (n)	Proteins	Phospholipids	Free Cholesterol	Cholesteryl Esters	Triglycerides	TG/CE	EC%
			%				
N ♀ (5)	23.22[a] ± 3.27	21.50[a] ± 2.44	8.61[a] ± 0.89	41.79[a] ± 4.96	4.85[a] ± 1.06	0.12	74.3
N ♂ (7)	23.56 ± 0.77	22.10 ± 1.86	7.80 ± 0.95	41.43 ± 2.73	4.55 ± 1.30	0.11	76.0
III ♂ (1)	23.33	21.18	8.81	42.14	4.54	0.11	74.0
IV ♂ (1 × 5)	24.11 ± 2.18	21.16 ± 1.49	6.94 ± 1.08	42.31 ± 3.20	5.47 ± 0.62	0.13	78.4
N ♂ (3)[b]	25.1	23.5	9.1	37.8	4.5	0.12	71.2

[a] Not significant (male vs female).
[b] Data taken from Lee and Alaupovic (29) for LDL-V (d 1.040–1.053 g/ml), a subfraction isolated by sequential flotation from normal male subjects.
The symbols and abbreviations used are the same as for Tables 2 and 3.

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TABLE 7. Chemical composition of layer 4 of LDL

Subjects (n)	Proteins	Phospholipids	Free Cholesterol	Cholesteryl Esters	Triglycerides	TG/CE	EC%
			%				
N ♀ (3)	25.29[a] ± 1.05	26.44[a] ± 2.73	7.07[a] ± 0.58	35.13[b] ± 2.22	6.04[a] ± 0.16	0.17	74.7
N ♂ (3)	23.63 ± 1.96	22.14 ± 2.43	7.18 ± 0.35	42.23 ± 1.19	4.82 ± 1.61	0.11	77.8
III ♂ (1)	22.73	21.37	8.54	43.70	3.65	0.08	75.3
IV ♂ (1 × 4)	26.96 ± 3.05	21.74 ± 2.31	7.03 ± 1.21	37.65 ± 0.97	6.38 ± 0.80	0.17	76.1
N ♂ (3)[c]	27.6	22.0	6.7	38.2	5.5	0.14	77.2

[a] Not significant (male vs female).
[b] $P < 0.01$.
[c] Data taken from Lee and Alaupovic (29) for LDL-VI (d 1.053–1.063 g/ml), a subfraction isolated by sequential flotation from normal male subjects.
The symbols and abbreviations used are the same as for Tables 2 and 3.

populations of different sizes. The particle size decreased in the order of LDL_1, layer 2, and layer 3. As shown in Fig. 5, the mean diameter for normal layer 2 was 22.0 ± 1.8 nm which was in excellent agreement with the value of 22.7 nm measured by analytical ultracentrifugation on a corresponding subfraction LDL-III (d 1.019–1.030 g/ml) isolated by sequential flotation (5). The mean diameter of normal layer 3 was 19.8 ± 1.4 nm which was also in excellent agreement with the average mean diameter of 19.5 nm determined by hydrodynamic parameters on a corresponding sequentially floated subfraction LDL-V (d 1.040–1.053 g/ml). Normal layer 4 with immunochemically detectable Lp(a) showed two populations of particles with diameters of 32.0 ± 2.0 nm and 21.0 ± 1.4 nm. The larger diameter was probably due to Lp(a) particles, as Lp(a) has been reported to have a larger molecular weight than LP-B (27, 28). The smaller diameter was most likely due to LP-B particles not associated with Lp(a), as this value is close to the hydrodynamically determined 19.8 nm diameter of LDL-VI (d 1.053–1.063 g/ml), a sequentially isolated LDL subfraction (5). Not shown in the figure, layer 4 of the type III patient, being Lp(a) positive, also showed two populations of particles of 32.7 ± 4.5 nm and 23.5 ± 1.6 nm in diameter. These size ranges were similar to those of the normal layer 4 containing Lp(a).

DISCUSSION

A quick single-spin large scale fractionation of lipoproteins into five layers has been achieved by the use of a discontinuous density gradient with an angle-head ultracentrifuge rotor. This method has revealed the heterogeneity present in the major fraction LDL_2 (d 1.019–1.063 g/ml) in normal male and female subjects. Normal LDL was separated into four subfractions, corresponding to four major subpopulations of lipoproteins, differing in their chemical compositions, flotation coefficients, hydrated densities, electrophoretic mobilities, and particle sizes. The methodology was tested with type III and type IV hyperlipoproteinemic plasma. Separation of hy-

TABLE 8. Physical chemical properties of lipoprotein subfractions

Subfraction	Flotation Coefficient, S_f^0			Hydrated Density, $\bar{d}$, g/ml	
	Normal Male	Type III HL	Type IV HL	Type III HL	Type IV HL
VLDL		52.5m 25.6	53.5	0.996	0.965
LDL_1	14.3	20.0	16.0	1.007	1.000
Layer 2	11.4[a] 8.3[b]	12.3[a] 8.2[b]	13.2	1.015 N.D.	1.017
Layer 3	6.0	6.7	6.1m 6.3	1.030	1.033m 1.038
Layer 4	5.3	4.5	4.5	1.045	N.D.

[a] When isolated layer 2 was washed at 1.030 g/ml, only peak a was refloated.
[b] Peak b was recovered as a minor component when the infranate of wash was refloated at 1.050 g/ml. The weight ratio for a:b ≃ 10:1.
Hydrated density was determined by analytical ultracentrifugation by interpolating the solvent density to zero sedimentation. See Methods. HL, hyperlipoproteinemia; m, minor component; N.D., not determined.

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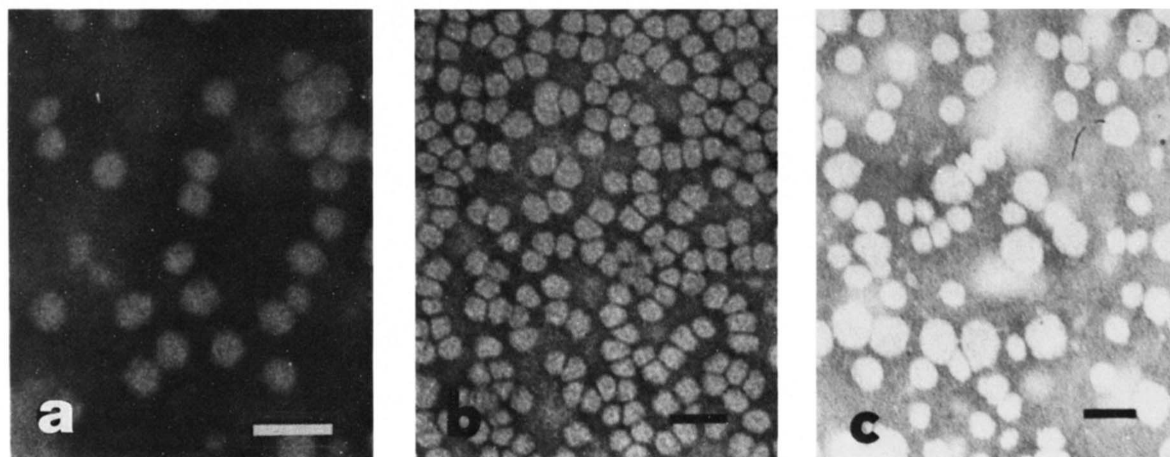

Fig. 5. Electron micrographs of negatively stained subfractions of LDL. (a) Layer 2, 22.0 ± 1.8 nm SD diameter; (b) layer 3, 19.8 ± 1.4 nm SD diameter, both layer 2 and layer 3 were isolated from the same normal female subject; (c) layer 4 from a normal male showing immunochemically positive Lp(a) and LP-B with partial identity between each other. The two populations in size are 32.0 ± 2.0 nm and 21.0 ± 1.4 nm SD diameters. Bar markers represent 50 nm in each micrograph. The original mangification factors for (a), (b), and (c) were 166,925×, 117,425× and 96,250×, respectively.

perlipoproteinemic lipoproteins into five layers was also achieved, though the layers were not necessarily identical to the corresponding normal ones due to deviation in quantity and in quality in certain subfractions.

There are a number of reports of the subfractionation of LDL by sequential flotation (5, 6, 29) and by density gradient centrifugation in swinging-bucket rotors (1–3, 20), including the use of the zonal rotor (25, 30). However, a usual 10-fold dilution of the isolated fraction may be expected by the latter method and this renders the minor fractions of lipoproteins undetectable. Thus LDL_1, layer 2, and layer 4 from normal subjects usually escape detection by zonal ultracentrifugation (30). The present subfractionation method has several advantages over previously reported methods: a short centrifugation time, simple gradient preparation, a larger maximum sample volume (26–27 ml/tube × 12 tubes/rotor = 312–324 ml/run), a concentration effect after isolation (thus rendering minor fractions or minor components more likely to be detectable), non-arbitrary cuts of density ranges, non-exposure of lipoproteins to high salt concentrations, and revelation of the density heterogeneity of LDL. The disadvantages of this method are: additional centrifugation is needed to separate VLDL from LDL_1, and to separate layer 4 from layer 5 when polyallomer tubes are used; washings for layer 3 and layer 4 are needed to remove trace amounts of albumin if chemical composition is the subject of interest; and washing of layer 2 is recommended for concentration purposes. However, quantitation of cholesterol and triglyceride, and electroimmunoassay with monospecific antibodies to apolipoproteins can be performed directly on the layers without washing or dialysis, and may yield concentration distribution patterns with nearly total recovery of each component. Washing and dialysis are the major sources of loss of lipoproteins; up to 15–25% losses of lipoproteins were observed in the present study. Since the same treatment was performed on all samples, the comparison in distribution patterns throughout density ranges between normal males and females should be valid. In general, the chemical composition of narrow density range subfractions is independent of the recovery. Comparison in composition between different categories is thus valid.

The chemical composition of VLDL of normal males from this study was in excellent agreement with that isolated by conventional methodology (sequential flotation) (31) (see Table 3). The composition of LDL_1 isolated by this method was also in close agreement with that isolated by sequential flotation (31) (see Table 4). The composition of layer 2 was comparable to that of LDL-III (d 1.019–1.030 g/ml) (29) isolated by sequential ultracentrifugation (see Table 5). The composition of layer 3 was in good agreement with that of LDL-V (d 1.040–1.053 g/ml), a sequentially isolated subfraction (29) (see Table 6). The chemical composition of layer 4 was comparable to that of LDL-VI (d 1.053–1.063 g/ml), a subfraction isolated by sequential ultracentrifugation (29) (see Table 7). These similarities between subfractions isolated by this density gradient method and subfractions isolated by conventional sequential ultracentrifugation support the validity of this new method. The concentration of total cholesterol in layer 2 was significantly different between normal males and females. Significant differences were also observed between normal males and females in the chemical composition of layer 2 and layer 4. Normal females had higher TG in layer 2 and lower CE in layer 4 than normal males.

Considerable variation in subfraction concentrations has been observed among subjects of the same category, or even from the same subject at different time periods

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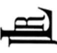

(as for the type IV hyperlipoproteinemic patient). But the composition of each subfraction derived from subjects of the same category was very close, suggesting structural similarity of the lipoprotein species within a given subfraction for all subjects of that category. The variations in concentration were probably dependent on the dietary regimen and metabolic states of each individual at the time of study. The plasma TG is particularly sensitive to dietary management in type III patients (32). Change in subpopulation concentrations of LDL in type III hyperlipoproteinemia due to diet and drug effects has been previously observed (21). The low plasma TG in our type III patient and the different lipoprotein distribution in his LDL, in contrast to that reported in the literature (22, 23), was probably the result of his long-term low cholesterol and low triglyceride diet. Other investigators have previously isolated VLDL and subfractioned LDL from normal, type III, and type IV hyperlipoproteinemic subjects into three subfractions (20, 33). Since the density cuts were different from the present studies, it is difficult to compare their distribution patterns and the compositions of their subfractions with our results. The general observation that the compositions of VLDL and LDL subfractions of type IV plasma were similar to those of normal, whereas VLDL from type III patients deviated from normals due to enriched CE and depleted TG, was in good agreement with our results.

The finding of Lp(a) in layer 4 was consistent with the earlier report that the subfraction d 1.050–1.060 g/ml was heterogeneous, and that LP-B, Lp(a), and LP-A were separated from the fraction (34). The particle size measured by electron microscopy attributed to Lp(a) in this study was larger than the average 25.5 nm reported for Lp(a) isolated by agarose chromatography of d 1.05–1.12 g/ml (27). If the diameter of the two populations of the particles in layer 4 were averaged, 26.0 nm would have been the average diameter, which is much closer to the reported value. Another possible reason for the deviation in the measured size is that their Lp(a) included that isolated from HDL_2 whereas ours was limited to d < 1.063 g/ml. It is possible that the Lp(a) from HDL_2 is smaller than that in LDL. The fact that the anodic mobility of Lp(a) was faster in HDL_2 than that in layer 4 suggests that there may be two different molecular species of Lp(a).

It should be pointed out that the hydrated densities of layers 2, 3, and 4 obtained by analytical ultracentrifugation were all lower than the corresponding isopycnic densities of the bands of NaCl solutions. As explained earlier, the differences in thermal expansivity between NaCl solutions and lipoproteins were the main contributing factors. The banding process occurred at 5°C; when a sample was warmed up to 25°C, the salt solution and lipoprotein each expanded according to its own characteristics and ended up at different densities. This phenomenon was also observed previously with conventional sequential flotation (5, 16). Mills (16) demonstrated that when differences in thermal expansivity between salt and lipoproteins were corrected, the densities of the isolated lipoproteins fell into the expected ranges. The position of layer 1 was not due to equilibrium banding but due to flotation, so the hydrated densities of lipoproteins within layer 1 were considerably lower than the solvent densities of layer 1.

This density gradient banding method serves to concentrate the isolated subfractions. In general, the lipoproteins were concentrated from plasma 4 to 5-fold for layer 1, 1.7 to 2-fold for layer 2, and 3.5 to 5.5-fold for layer 3. When washing was carried out, layer 2 could be further concentrated to 7 to 16-fold and layer 3 to 6 to 10-fold over their original plasma concentrations. Thus, minor components undetectable in the wide density range of LDL_2 isolated by other methods have become detectable by the present method.

It is conceivable that fractionation of severely hypertriglyceridemic plasma (i.e., plasma TG above 1000 mg/dl) may yield TG-rich lipoproteins as a cake along the outer wall of the tube and may contaminate other fractions. We suggest that chylomicrons should be removed initially from the plasma. If the TG in chylomicron-free plasma is still too high, the sample may be diluted with d 1.006 g/ml NaCl solution containing preservatives before being subjected to fractionation.

Heterogeneity of normal LDL_2 has previously been observed by equilibrium banding in a high-salt density gradient during analytical ultracentrifugation. Adams and Schumaker (35) found evidence for three density classes ranging from 1.0252 to 1.0390 g/ml in normal males. Hammond and Fisher (1) separated discrete species from LDL of type IV patients. Most recently, Shen et al. (36) separated the isolated LDL_2 from normal subjects into six subfractions by density gradient ultracentrifugation in a swinging-bucket rotor and found significant differences in chemical composition, flotation coefficient, and particle size among some subfractions. They did not find significant differences in composition of subfractions between the sexes. Their finding that the ρ-intercepts of lipoprotein subfractions (or $\bar{d}$) measured by analytical ultracentrifugation were lower than the isopycnic banding densities were in accord with our observations. These reports strongly substantiate our findings of heterogeneity within LDL of greater complexity than the usually recognized categories of LDL_1 and LDL_2. Our present communication offers a simple and quick separation method for the subpopulations of LDL and has potential for characterizing LDL subfractions in diseased states.

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