Further resolution of the low density lipoprotein spectrum in normal human plasma: physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifugation

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Abstract The molecular basis of the heterogeneity of plasma low density lipoproteins (LDL, d 1.024–1.050 g/ml) was evaluated in 40 normolipidemic male subjects following fractionation by isopycnic density gradient ultracentrifugation into eight major subspecies. The mass profile of our subjects' LDL uniformly displayed single symmetric or asymmetric peaks as a function of density; the peak occurred most frequently (20 subjects) in subfraction 7 (d 1.0297–1.0327 g/ml). Several physicochemical properties (hydrodynamic behavior, electrophoretic mobility, chemical composition, size and particle heterogeneity, and apolipoprotein heterogeneity) of the LDL subfractions were examined. Hydrodynamic analyses revealed unimodal distributions and distinct peak S_f^0 rates in individual subfractions. Such behavior correlated well with particle size and heterogeneity data, in which LDL subspecies were typically resolved as unique narrow bands by gradient gel electrophoresis. Subspecies with average densities of 1.024 to 1.0409 g/ml ranged from 229 to 214 Å in particle diameter. LDL protein content increased in parallel with density while the proportion of triglyceride diminished; cholesteryl esters predominated, accounting for ~40% or more by weight. Distinct differences in net electric charge were demonstrated by electrophoresis in agarose gel, the subspecies with average density of 1.0314 g/ml displaying the lowest net negative charge. ApoB-100 was the major apoprotein in all subspecies, and constituted the unique protein component over the density interval 1.0271–1.0393 g/ml. ApoE and apo[a] were detected at densities less than 1.0271 and greater than 1.0393 g/ml. While apoE was evenly distributed within these two regions, representing up to 2% of apoLDL, the distribution of apo[a] was skewed towards the denser region, in which it amounted to 3–7% of apoLDL. ApoC-III was detectable as a trace component at densities > 1.0358 g/ml. Calculation of the number of molecules of each chemical component per LDL subspecies showed the presence of one copy of apoB-100 per particle, in association with decreasing amounts of cholesteryl ester, free cholesterol, and phospholipid. These data indicate that a similar overall molecular organization and structure is maintained in a unimodal distribution of LDL particle subspecies over the density range ~1.02 to 1.05 g/ml. In sum, our data may be interpreted to suggest that microheterogeneity in the physicochemical properties of human LDL subspecies reflects dissimilarities in their origins, intravascular metabolism, tissular fate, and possibly in their atherogenicity.—**Chapman, M. J., P. M. Laplaud, G. Luc, P. Forgez, E. Bruckert, S. Goulinet, and D. Lagrange.** Further resolution of the low density lipoprotein spectrum in normal human plasma: physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifugation. *J. Lipid Res.* 1988. **29:** 442–458.

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The low density lipoproteins (LDL) constitute the major vehicle for cholesterol transport in human plasma (1). These quasi-spherical particles are pseudomicellar complexes with diameters in the range of 18 to 26 nm (2), and contain a hydrophobic core of apolar constituents, primarily cholesteryl esters and triglycerides, surrounded by a polar coat of phospholipids, some free cholesterol, and protein (3). The protein moiety consists essentially of apolipoprotein B-100, a high molecular weight protein of predominantly hepatic origin (4), which plays a determining role both in the molecular structure of LDL particles and in their in vivo metabolism (5–7).

In normal subjects, the exclusive origin of circulating LDL appears to lie in hepatic VLDL, from which they are derived by intravascular transformation and remodel-

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; Lp[a], lipoprotein[a]; apo[a], a major apoprotein and characteristic of Lp[a]; apoB-100, the major apoprotein of LDL and a principal component of Lp[a]; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetraacetic acid. The centile nomenclature for the B proteins has been adopted.

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ing (8). Such LDL are distributed as a continuum of particles over the density range 1.006 to 1.063 g/ml (flotation range $S^{\circ}_{f\,1.063}$ 0–20). The less dense region of this distribution (i.e., d 1.006–1.019 g/ml, $S^{\circ}_{f\,1.063}$ 12–20), is usually equated with intermediate density lipoproteins (IDL). The bulk of LDL is, however, typically distributed within the density interval 1.019–1.063 g/ml ($S^{\circ}_{f\,1.063}$ 0–12), and in a symmetrical fashion about a peak with $S^{\circ}_{f\,1.063}$ rate in the range ~5 to 7, hydrated density ~1.030 to 1.040 g/ml and molecular weight ~2.0 to 2.4 × 10^6 (2, 9). Such LDL distributions have been termed "monodisperse," most of the mass of LDL being located over a narrow density region in a single peak (10).

Heterogeneity in physical, chemical, hydrodynamic, and immunological properties is an inherent characteristic of the low density lipoprotein particles that compose the density profile, even in normolipidemic individuals (2, 9, 10–24). Indeed, evidence has been provided for heterogeneity in the hydrated density, molecular weight, molecular size, chemical composition, isoelectric point, surface charge density, and hydrodynamic properties of circulating LDL, as well as in the immunoreactivities of LDL subclasses with monoclonal antibodies directed against LDL-apoB-100 (21).

Despite recent progress in methodological approaches to the preparative fractionation of human low density lipoproteins (2, 17–22), a paucity of information surrounds a number of fundamental aspects of their heterogeneity. Thus, the possibility that one or more discontinuities or transition points may exist in the molecular properties of LDL at a certain point(s) along the density distribution from 1.019 to 1.063 g/ml is indeterminate. Furthermore, little is known of the qualitative and quantitative distribution of apolipoproteins among distinct LDL subspecies.

With these points in mind, we were prompted to develop a fractionation procedure of superior resolutive power, which might facilitate isolation of discrete LDL subspecies. Our initial studies were inspired by the equilibrium density gradient approach of Shen and colleagues (17).

We have presently evaluated the heterogeneity of plasma LDL in 40 normolipidemic Caucasian males by development and application of a new isopycnic density gradient ultracentrifugal procedure of high precision and reproducibility. We describe our methodology and report the physicochemical characteristics of a series of up to 15 discrete LDL subspecies separated by this procedure. These data provide new insight into the microheterogeneity of circulating LDL.

MATERIALS AND METHODS

Blood samples

Subjects were healthy, normolipidemic male volunteers (n = 40; age range 23 to 54 yr) who had normally fasted overnight for 12 to 14 hr. No selection of blood groups was made, although group AB predominated (~30% of donors). None of our volunteers were receiving drugs known to perturb plasma lipoprotein metabolism; subjects were either abstainers or consumed only moderate amounts of alcohol.

Venous blood (250–300 ml) was collected *i*) in bottles containing Na_2 EDTA (final concentration 1 mg/ml), from which plasma (17 subjects) was immediately separated by low speed centrifugation (1000 *g*, 20 min) at 4°C, or *ii*) in empty bottles for subsequent isolation of serum (23 subjects) by low speed centrifugation after clotting for ca. 3 hr at 4°C. Immediately upon collection of plasma and serum samples, solutions of EDTA, sodium azide, and sodium merthiolate (thimerosal) were added to final concentrations of 0.01% (w/v), 0.01% (w/v), and 0.001% (w/v), respectively, in order to inhibit microbial growth and metal cation-catalyzed peroxidative degradation of lipoproteins. Plasma and serum samples were normally taken for lipoprotein separation within 5 hr of blood collection, during which time they were maintained at 4°C.

The concentrations of lipids (total cholesterol and triglyceride), apolipoproteins (apoB, apoA-I, and apoA-II), HDL-cholesterol, and Lp[a] for our subjects are summarized in **Table 1,** and in each case correspond to values typical of a normolipidemic population (25, 26).

Since the physicochemical properties (chemical composition, Stokes diameter, hydrodynamic behavior, and electrophoretic mobility) and apolipoprotein content of native LDL (d 1.024–1.050 g/ml) and of the derived density gradient subfractions (which we presently describe) were not detectably affected by the nature of the biological fluid from which lipoproteins were isolated, be it plasma or serum, our analytical data on plasma and serum LDL and their subspecies have been grouped together and treated as one. The terms plasma and serum may therefore be considered as interchangeable.

TABLE 1. Plasma lipid, apolipoprotein and Lp[a] concentrations in male blood donors

Parameter	mg/100 ml
Total cholesterol[a]	183 ± 37 (40)[b]
Total triglyceride	70 ± 30 (40)
HDL-cholesterol	45 ± 11 (36)
Apolipoprotein B	103 ± 24 (33)
Apolipoprotein A-I	168 ± 53 (20)
Apolipoprotein A-II	38 ± 11 (20)
Lp[a]	25 ± 6 (15)

[a] Methods employed for the quantitation of plasma and lipoprotein lipids, and for the immunological quantitation of apolipoproteins and Lp[a] are detailed in the Analytical Methods section.

[b] Values are means ± SD of the number of individuals given in parentheses.

Isolation of LDL

LDL were isolated from serum or plasma (150–250 ml) in the density interval 1.024–1.050 g/ml by sequential preparative ultracentrifugation (27). The background density of serum or plasma was first adjusted to 1.024 g/ml by addition of solid KBr (28), and subsequently distributed into either Beckman Quick-Seal polyallomer (ref. 342414; vol 39 ml) or thick wall polycarbonate (ref. 336091; vol 22 ml) tubes; the plasma was then overlayered with a salt solution of d 1.024 g/ml (~1 vol:4 vol plasma). Quick-Seal tubes were heat-sealed using metal seal formers (Beckman, ref. 343421) and a tube sealer (Beckman, ref. 343428); polycarbonate tubes were used without caps. Tubes were inserted into either the Beckman Type 50.2 Ti or Type 60 Ti rotor, and in the case of Quick-Seal tubes, spacers (Beckman, Type 342699) were applied. Centrifugation was then performed in either a Beckman L5-50 or L8-55 ultracentrifuge at 10°C for 20 to 22 hr at 45,000 rpm (polycarbonate tubes) or 50,000 rpm (Quick-Seal tubes). Lipoproteins of d < 1.024 g/ml (essentially VLDL and IDL) were removed in the top 3–5 ml, either by tube slicing (Quick-Seal tubes) or with a Pasteur pipette (polycarbonate tubes) as proposed by de Lalla and Gofman (29). The intermediate clear region in each tube (corresponding to about one-third of its total volume) was aspirated off and the infranatants were pooled. The background density of the infranatant was then raised to 1.050 g/ml by addition of solid KBr (28), the infranatant was distributed among polycarbonate tubes (approx. 15 ml/tube; ref. 336091) and overlayered with ~5 ml of a d 1.050 g/ml salt solution. Centrifugation was subsequently performed at 45,000 rpm as detailed above. The supernatant LDL was aspirated off (29), mixed with a d 1.050 g/ml salt solution (1 vol LDL:2 vol salt solution) and washed by a final centrifugation under the same conditions. This latter washing step was not obligatory, since its omission had no detectable effect on the physicochemical properties of the major LDL subfractions isolated by the density gradient procedure described below. LDL of d 1.024–1.045 g/ml was dialyzed in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA, exclusion limit ca. 12,000–14,000) against a solution (d 1.006 g/ml) containing 0.15 M NaCl, 0.01% EDTA, 0.02% sodium azide, and 0.005% sodium merthiolate at pH 7.4 and 4°C.

Purity and integrity of LDL and apolipoprotein B-100

The purity and integrity of LDL preparations of d 1.024–1.050 g/ml was established on the basis of criteria described earlier (30, 31) which included size, morphological appearance, immunological reactivity, and electrophoretic mobility. In this way, the potential contamination of LDL with other lipoproteins (VLDL and HDL) was excluded. Trace amounts of albumin and globulins were associated with d 1.024–1.050 g/ml LDL when the ultracentrifugal washing step was omitted; such contaminating protein was removed during density gradient ultracentrifugation (see below). As in our earlier studies, the integrity of LDL apoB-100 was verified by electrophoresis in SDS–polyacrylamide gels (3% monomer) (32–35), and the B-74 and B-26 forms were shown to be typically undetectable.

Density gradient fractionation of LDL

Gradient construction. The nonprotein solvent density of dialyzed LDL samples was first raised to 1.040 g/ml by addition of solid KBr (28). Discontinuous density gradients were then constructed at ambient temperature in Ultraclear tubes (Beckman, ref. 344059; capacity 13.2 ml; 9/16 × 3½ in) in the Beckman SW41 rotor. Using an Auto-Densiflow II (Buchler Instruments, Searle Analytical Inc., Fort Lee, NJ) coupled to a Minipuls II (Gilson Instruments, Villiers-le-Bel, France) peristaltic pump, 4.5 ml of an NaCl–KBr solution of d 1.054 g/ml was pumped into the bottom of the tube at a rate of 1 ml/min. The following solutions were then layered onto the latter with the aid of the Auto-Densiflow II: 3.5 ml of LDL, containing up to 15 mg of protein at d 1.040 g/ml, 2 ml of d 1.024 g/ml, and finally 2 ml of NaCl solution of d 1.019 g/ml. Immediately upon completion, the gradients were centrifuged at 40,000 rpm for 44 hr (47.3×10^7 g_{av} min) at 15°C in a Sorvall OTD-50 or Beckman L8-55 ultracentrifuge, with the former instrument in the ARC-slow/Reograd mode for maximal stabilization of the gradient during acceleration and deceleration. Typically, two or more gradients were normally constructed from each LDL preparation.

All salt solutions, adjusted to pH 7.4, contained 0.02% sodium azide, 0.01% EDTA, and 0.005% sodium merthiolate, and their densities were verified to the fourth decimal place with a precision density meter (Anton Paar, Graz, Austria; Model DMA 40) equilibrated at 15°C. Gradients constructed for the purpose of determining the density profile contained 3.5 ml of NaCl solution of d 1.040 g/ml instead of an LDL sample.

Fractionation of gradients. The gradients were fractionated with a density gradient fractionator (Model 185; ISCO, Lincoln, NE) by puncture of the bottom of the tube and upward displacement of the gradient using Fluorinert FC40 (an organic compound of d 1.85 g/ml and nonmiscible with water; ISCO). The fractionator was coupled to a fraction collector (Model 1200; ISCO). Fifteen successive fractions of 0.8 ml were removed from each gradient tube with the exception of fraction 1 which contained ~0.84 ml. Corresponding gradient fractions derived from the same starting LDL preparation were pooled before exhaustive dialysis at 4°C under the same conditions as above for LDL of d 1.024–1.050 g/ml.

To establish the density profile obtained upon completion of ultracentrifugation, blank gradients containing

only NaCl-KBr solutions were fractionated into 0.5 ml volumes and their densities were determined at 15°C with the precision density meter. For determination of the density intervals of the 15 successive subfractions (0.8 ml) of LDL, densities were read from a plot of density versus volume and are shown in Table 3.

Chemical analysis

Plasma lipids. Total plasma cholesterol and triglyceride concentrations were determined by a modification of the Liebermann-Burchard reaction (36) and by the method of Kessler and Lederer (37) after zeolite extraction, respectively; both methods were adapted to an Autoanalyzer system (Technicon, Tarrytown, NY) and 'Seronorm lipid' (Nyegaard AS, Oslo, Norway) was used as the working standard for both assays. Plasma HDL-cholesterol was estimated by the procedure of Allain et al. (38) using an enzymatic kit (Biotrol, Paris, France); LDL were initially precipitated with phosphotungstate-$MnCl_2$ (39).

Lipoprotein fractions. Chemical analyses of the starting LDL (d 1.024-1.050 g/ml) and of the derived gradient subfractions were performed by the series of procedures described elsewhere (see ref. 40), using the method of Lowry et al. (41) for protein quantitation, that of Roeschlau, Bernt, and Gruber (42) for free and for esterified cholesterol, that of Takayama et al. (43) for phospholipids, and that of Biggs, Erickson, and Moorehead (44) for triglycerides. The reproducibility of our chemical analyses was examined by calculation of the technical errors (40), which were 3.6, 2.8, 3.5, and 2.8% for protein, cholesterol, triglyceride, and phospholipid, respectively.

The mean recovery of LDL, determined by chemical analysis of an aliquot of each LDL preparation placed on the gradient and of the 15 subfractions derived from it, was 87.7 ± 3.9% (n = 15).

Analytical ultracentrifugation

This type of analysis was performed according to Laplaud, Beaubatie, and Maurel (45), on an MSE Centriscan 75 analytical ultracentrifuge operating in the refractometric mode (schlieren analysis), at 550 nm. Flotation coefficients were corrected for concentration dependence using a K value of 0.89×10^{-4} $(mg/dl)^{-1}$, and to standard conditions according to Ewing, Freeman, and Lindgren (46), using stored tables of lnF versus hydrated densities.

Lipoprotein electrophoresis

Agarose gel. Aliquots (2-5 μl) of whole plasma and of native lipoprotein fractions were electrophoresed for 40 min on agarose gel films (Universal electrophoresis film agarose; cat. no. 470100, Corning, Palo Alto, CA) using the Corning ACI system. On completion of electrophoresis, sheets were stained for lipid with Fat Red O. This procedure is essentially as described by Nobel (47).

Polyacrylamide gradient gels. Continuous gradient slab gel electrophoresis of native LDL and gradient subfractions was performed in a Pharmacia electrophoresis apparatus GE-2/4 LS loaded with gels containing a 2-16% gradient (PAA 2/16; Pharmacia Fine Chemicals, Uppsala, Sweden). Approximately 15 μg of lipoprotein protein was applied to each well and electrophoresis was carried out at 125 V for 14 hr at 4°C in a Tris-borate buffer (0.09 M Tris, 0.08 M boric acid, 0.003 M EDTA, pH 8.35) (48). Gels were subsequently stained with 0.7% Amido Black in 7% acetic acid and diffusion destained in 7% acetic acid. A set of standard proteins with known hydrated diameters (thyroglobulin, 170 Å; ferritin, 122 Å; catalase, 104 Å; lactate dehydrogenase, 81 Å; bovine serum albumin, 71 Å; HMW electrophoresis calibration kit, Pharmacia Fine Chemicals) was run in duplicate on each slab as a reference marker. Under these conditions, albumin migrated out of the gel. From the migration distances of the different lipoprotein subfractions and those of the remaining calibration proteins, it was possible to calculate the Stokes diameters of LDL and its subfractions using the Stokes-Einstein equation (48). The correlation coefficient for the regression line of the relationship between the logarithm of the diameter of the calibration proteins and their migration distance was typically > -0.96.

Immunological quantitation of lipoproteins and apolipoproteins

The quantitation of apolipoproteins B, A-I, and A-II, and of lipoprotein Lp[a], in whole plasma, in LDL of d 1.024-1.050 g/ml, and in density gradient subfractions derived from the latter, was performed by laser immunophelometry, using a video nephelometer (Immuno-France SA, Rungis, France). These assays were carried out according to the respective protocols proposed by the manufacturer (Immuno AG, Vienna, Austria), and according to the general principles described by Wider et al. (49). Monospecific rabbit antisera to human apoA-I and to apoA-II and a monospecific goat antiserum to human apoB were used (Immuno AG). The corresponding purified human apolipoproteins (i.e., apoA-I, 100 mg/dl; apoA-II, 35.1 mg/dl; and apoB, 57.2 mg/dl) were used as standards, and were supplied as Immunoneph Reference Standard by Immuno AG. A secondary standard, Immunoneph Norm Control, containing apoA-I, apoA-II, and apoB at concentrations of 71.5, 24.3 and 59.2 mg/dl, respectively, was also assayed in each series of analyses.

The monospecificity of the antisera to apoA-I, apoA-II, and apoB had been verified in earlier studies in our laboratory (50). The assay for apoB was linear over the range 10 to 725 mg/dl, the apoA-I assay was linear over a range of 5 to 650 mg/dl, and that of apoA-II from 5 to 700 mg/dl. Prior to immunoassay, plasma was diluted 1:200 for quantitation of apoB, and 1:160 for quantitation

of both apoA-I and apoA-II. Dilutions of the antisera used were: 1:60 for apoB, 1:20 for apoA-I and 1:30 for apoA-II.

The ranges of plasma levels of apoB, apoA-I, and apoA-II in our normolipidemic males were 52 to 151, 61 to 192, and 18 to 50 mg/dl, respectively.

The immunonephelometric assay of Lp[a] was performed after 1:30 dilution of the monospecific sheep antiserum (Immuno AG) and 1:20 dilutions of both plasma and lipoprotein samples. The reference standard for Lp[a] contained 72.5 mg of Lp[a] per dl, as determined by electroimmunodiffusion (Immuno AG). This immunoassay was linear over the range of 1 to 500 mg/dl.

Dot immunobinding assays

We have employed a dot immunobinding assay for the detection of specific apolipoproteins in LDL of d 1.024–1.050 g/ml and in density gradient subfractions derived from such preparations. Our method is adapted from that described by Hawkes, Niday, and Gordon (51).

Aliquots of each fraction from the same density gradient separation and containing 200 ng–12 μg of LDL protein in a 2-μl volume were spotted onto nitrocellulose sheets (Bio-Rad, Richmond, CA; Trans-Blot Transfer medium, ref. 162-0113). The paper was then blocked by incubation for 1 hr at 40°C with a 3% (w/v) solution of bovine serum albumin (Sigma) in a buffer containing 150 mM NaCl and 10 mM Tris–HCl, pH 7.3 (buffer A). The first antibody (see below), diluted in the same buffer (A) to which 10% pig serum had been added, was then incubated overnight at ambient temperature with the various antigens that had been adsorbed to nitrocellulose. After three washings with buffer A, each of 10 min duration (with the exception that the second washing contained, in addition, 0.025% Tween 20), the nitrocellulose paper was incubated for 4 hr at ambient temperature with the second antibody conjugated to horseradish peroxidase (Dakopatts a/s, Glostrup, Denmark). Following incubation, the sheets were washed three times with buffer A as before, and the blot was developed by exposure to the 4-chloro-1-naphthol-peroxide substrate (51).

The antibodies used in the first series of immunobinding reactions were as follows. In the case of apoB-100 detection, a monospecific polyclonal sheep antiserum (dilution 1:200) prepared by CEA-ORIS, Marcoule, France (Dr. A. Dedieu) to an antigen (human serum LDL of d 1.024–1.050 g/ml, containing only apoB-100 after purification by gel filtration chromatography on Sepharose 4B) separated in our laboratory. For apoE detection, a mouse monoclonal antibody, 13.7. C4, was used at a dilution of 1:100 after ammonium sulfate precipitation from murine ascites fluid. This antibody specifically blocks the binding of apoE to the cellular apoB,E receptor (Dr. T. L. Innerarity, personal communication). In the second series of immunobinding reactions, the peroxidase-conjugated antibody to sheep IgG and the peroxidase-conjugated antibody to murine IgG were each used at dilutions of 1:200 (Dakopatts a/s).

For Lp[a] detection, a monospecific polyclonal sheep antibody was used in the first immunoblotting reaction at a dilution of 1:100; this antibody was supplied by Immuno-France. The second antibody, directed against sheep IgG, was the same as that used in the equivalent step above for apoB detection.

In order to quantitate the apolipoprotein content of individual blots from LDL subfractions, calibration strips were established by the above methods for apoE, the standard being a purified preparation of apoE3 (500 ng/μl, a kind gift from Dr. K. Weisgraber). Successive dilutions of 1:1 of this standard were made, with the maximal dilution (1:512) corresponding to a blot containing 0.98 ng/μl. In a similar fashion, calibration strips were established for the Lp[a] standard (725 μg/μl: Immuno-France), with the dot at maximal detectable dilution (1:4096) containing 0.44 ng/μl. Dilution was with a Gilson dilutor (Model 401 Dilutor, Gilson Electronics S.A., France), using buffer A. The intensity of each violet-colored dot allowed clear differentiation of successive dots differing by one-half of their content of the apolipoprotein standard on the respective calibration strip. The amounts of apoE and of Lp[a] in dots from each LDL subfraction were determined by visual comparison of color intensity with dots on the corresponding calibration strip.

Electrophoretic analysis of apolipoprotein content

The protein moieties of LDL of d 1.024–1.050 g/ml and of the derived LDL subspecies were examined by two electrophoretic procedures chosen to provide data on the isoelectric points and on the molecular weights of the constituent proteins.

To determine the content of low molecular weight apolipoproteins (M_r < 100,000), analytical isoelectric focusing was performed in glass tubes in a Hoefer electrophoresis unit as outlined elsewhere (52, 53). Apolipoproteins and their isoforms were identified by their pI values, the latter being assessed on the basis of a calibration curve constructed from the pH values of aqueous eluates of slices (0.5-cm thick) cut from unstained reference gels.

To obtain data on the qualitative and quantitative aspects of the B protein content in the subfractions, we electrophoresed 20 to 100 μg of LDL protein in SDS-polyacrylamide gels by the method of Weber and Osborn (32), as modified by Stephens (54) and Weisgraber et al. (33); both disc and slab gel systems were used. Slab gels were made up at 3% monomer acrylamide concentration and were 14 cm in length and 15 mm in thickness; a vertical slab gel electrophoresis cell (Model SE 600, Hoefer Scientific, San Francisco, CA) was used. Gels were stained with Coomassie Brilliant Blue R250 (55). Molecular weights were determined as described earlier (53).

Apoprotein samples studied by both electrophoretic methods were prepared as follows. Aliquots of LDL and its subspecies were first lyophilized and then delipidated immediately with a mixture of ethanol–diethyl ether (peroxide-free) 3:1 (v/v) (30). For isoelectric focusing, the final dried apoprotein residues were extracted in 100 μl of a solution containing 6 M urea, 20 mM N-ethyl morpholine (SDS, Vitry-sur-Seine, France) and 20% (w/v) sucrose, and applied directly to the gel. For SDS gels, solubilization of the total apoprotein residues was performed with 100 μl of a solution containing 2.5 mM Tris–glycine and 0.3% SDS, pH 8.3. Immediately before electrophoresis, samples were incubated at 37°C for 2 hr (32).

RESULTS

Density gradient profile

Our final conditions for discontinuous gradient ultracentrifugal fractionation of LDL provided a density profile that took advantage of the radial path length of the SW41 tube, involving the spreading and equilibrium banding of the d 1.024–1.050 g/ml LDL fraction over almost the entire tube length.

The gradient attained equilibrium after 36 to 40 hr; continued ultracentrifugation to 64 hr led to only minor modification in the densities of fractions in the upper 8.5 ml (0.001 g/ml or less; **Table 2**), differences becoming progressively larger with increase in density (increasing from 0.0015 g/ml at 9.5 ml to 0.0055 g/ml in the bottom fraction; Table 2). A high degree of reproducibility in density profile was observed within gradients in the same ultracentrifugal run; variability between duplicates was 0.001 g/ml or less in the first 8 ml and 0.002 g/ml or less thereafter. Inter-run variation is shown in Table 2, in which the mean density ± SD of each fraction represents measurements on four separate runs, each containing two tubes.

The density profile at 44 hr was curvilinear as a function of volume (not shown), but essentially linear (r = 0.9909) in the volume interval corresponding to salt fractions 3 to 19, i.e., from d 1.020 to d 1.051 g/ml (Table 2). The overall density range was from ~1.0172 g/ml at the meniscus to ~1.081 g/ml at the bottom of the tube (Tables 2 and 3).

The average densities of successive LDL subfractions, removed automatically in volumes of 0.8 ml, are shown in **Table 3**, with the corresponding density intervals. The increments in average densities between successive subfractions ranged from 0.0016 to 0.0029 g/ml for fractions 1 to 9 inclusive (d < 1.0393 g/ml), from 0.0037 to 0.0049 g/ml for fractions 9 to 12 (d 1.0393–1.0483 g/ml), and by 0.008 g/ml or less in the range beyond 1.0483 g/ml, excluding the bottom fraction (no. 15).

TABLE 2. Inter-run reproducibility of density gradient profile[a]

Gradient Fraction No.	Volume	Mean Density (g/ml) Ultracentrifugal Run Time 44 hr[b]	64 hr[c]
1	0.5	1.0177 ± 0.001	1.0175
2	1.0	1.0185 ± 0.001	1.0185
3	1.5	1.0195 ± 0.001	1.0194
4	2.0	1.0206 ± 0.001	1.0205
5	2.5	1.0219 ± 0.001	1.0217
6	3.0	1.0232 ± 0.001	1.0229
7	3.5	1.0248 ± 0.001	1.0243
8	4.0	1.0262 ± 0.001	1.0259
9	4.5	1.0279 ± 0.001	1.0275
10	5.0	1.0296 ± 0.001	1.0292
11	5.5	1.0315 ± 0.001	1.0309
12	6.0	1.0333 ± 0.001	1.0329
13	6.5	1.0352 ± 0.001	1.0351
14	7.0	1.0374 ± 0.001	1.0375
15	7.5	1.0400 ± 0.001	1.0399
16	8.0	1.0420 ± 0.001	1.0427
17	8.5	1.0447 ± 0.001	1.0454
18	9.0	1.0477 ± 0.001	1.0486
19	9.5	1.0511 ± 0.001	1.0525
20	10.0	1.0550 ± 0.001	1.0571
21	10.5	1.0594 ± 0.001	1.0627
22	11.0	1.0644 ± 0.002	1.0686
23	11.5	1.0702 ± 0.001	1.0747
24	12.0	1.0760 ± 0.001	1.0815

[a] Gradients were constructed and ultracentrifuged as described in Methods, but fractionated with a Pasteur pipette (29).
[b] Values are means ± SD from determinations on fractions from paired gradients in each of four separate runs.
[c] Values are means from two separate determinations.

Physicochemical characterization of LDL subspecies

Analysis of hydrodynamic properties by analytical ultracentrifugation. Analytical ultracentrifugal analysis of plasma LDL of d 1.006–1.063 g/ml, of LDL of d 1.024–1.050 g/ml, and of density gradient subfractions derived from the latter were performed in parallel from each of six subjects. Amounts of LDL in certain gradient subfractions, and notably fractions 1 to 4 and 11 to 15, were however insufficient to allow reliable detection by the analytical instrument.

The LDL fractions of d 1.006–1.063 g/ml and 1.024–1.050 g/ml from all subjects displayed a unimodal distribution, with peak S_f° rate in the range 6.9 to 7.9 (**Fig. 1**). In all subjects examined, isolation of d 1.024–1.050 g/ml LDL resulted in the elimination of components at each extreme of the distribution, thereby creating new lower and upper limits of the density spectrum, at approximately S_f° 3.3–3.6 and 12.0–12.5, according to the individual considered (see Fig. 1). In this way, the contribution of triglyceride- and apoE-rich IDL and of Lp[a] particles occurring in the density ranges 1.006–1.024 g/ml (8) and 1.050–1.063 g/ml (19), respectively, were reduced.

Data obtained upon analysis of individual density gradient subfractions of d 1.024–1.050 g/ml LDL are sum-

TABLE 3. Average densities and density intervals of LDL gradient subfractions

Gradient Fraction No.	Average Density[a]	Density Interval[b]
	g/ml	*g/ml*
1	1.0180 ± 0.001	1.0172–1.0187
2	1.0196 ± 0.001	1.0187–1.0202
3	1.0215 ± 0.001	1.0202–1.0222
4	1.0234 ± 0.001	1.0222–1.0244
5	1.0260 ± 0.001	1.0244–1.0271
6	1.0286 ± 0.001	1.0271–1.0297
7	1.0314 ± 0.001	1.0297–1.0327
8	1.0343 ± 0.001	1.0327–1.0358
9	1.0372 ± 0.001	1.0358–1.0393
10	1.0409 ± 0.001	1.0393–1.0435
11	1.0451 ± 0.001	1.0435–1.0483
12	1.0502 ± 0.002	1.0483–1.0538
13	1.0580 ± 0.004	1.0538–1.0610
14	1.0660 ± 0.004	1.0610–1.0690
15	1.0745 ± 0.005	1.0690–1.080

[a]Values are the means ± SD obtained from successive 0.8-ml fractions removed from NaCl-KBr gradients centrifuged as outlined in Methods; the means were derived from density measurements on salt fractions from four separate runs, each containing paired gradient tubes.

[b]Density intervals correspond to the upper and lower limits of successive fractions of 0.8 ml, and were determined from a calibration curve of density (ordinate) plotted against cumulative fraction volume (abscissa).

marized in **Table 4,** while the corresponding refractometric patterns from a representative male subject are depicted in **Fig. 2.** Thus, it is clearly evident that each LDL gradient subfraction displayed a unimodal distribution and possessed a distinct peak flotation rate, despite the acute narrowness of the density intervals of individual subfractions; this latter characteristic did, however, result in some overlapping between the analytical ultracentrifugal profiles of successive subspecies (Fig. 2). Overlap between individual gradient fractions was also noted by Shen et al. (17), in which the interval between the average densities of each of the six fractions varied from 0.004 to 0.010 g/ml, these increments being substantially greater than those described herein. The density intervals in the aforementioned study were, therefore, rather wider than our own, an observation that is also consistent with the larger increments between the peak S_f° rates of the respective LDL subfractions (1.1 to 2.5 S_f° units in males) as compared to those described presently (0.7 to 0.9 S_f° units). The S_f° rates of LDL subfractions 5 to 10 ranged from 9.3 to 5.2 (Table 4); their hydrated densities (σ-densities, taken from the data of Kahlon et al. (2), who determined them from the ϱ-intercept in studies of ηF° versus ϱ), ranged from 1.0196 to 1.0343 g/ml and their 1-g

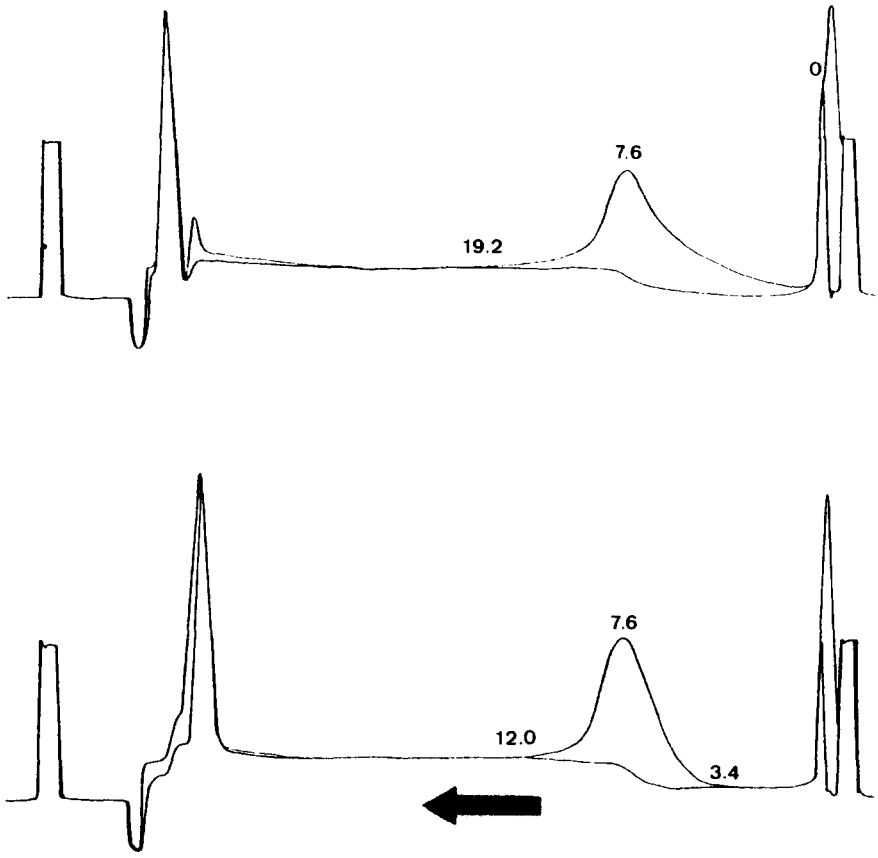

Fig. 1. Analytical ultracentrifugal patterns of LDL of d 1.006–1.063 g/ml (top) and of d 1.024–1.050 g/ml (bottom) from a representative subject. Direction of migration is from right to left. Flotation coefficients corresponding, respectively, to the beginning, the peak, and the end of the distribution, are indicated on the profiles.

TABLE 4. Average densities and peak flotation rates of LDL subfractions isolated by density gradient ultracentrifugation

Density Gradient Subfraction No.[a]	Number of Samples Examined	1-g Density[b]	σ-Density[c]	S°_f
		g/ml		*mean ± SD*
5	3	1.0260	1.0196	9.3 ± 0.6
6	5	1.0286	1.0224	8.5 ± 0.3
7	6	1.0314	1.0256	7.6 ± 0.4
8	6	1.0343	1.0289	6.7 ± 0.3
9	5	1.0372	1.0318	5.9 ± 0.4
10	1	1.0409	1.0343	5.2

[a] Limitations imposed by the small amounts of lipoprotein available in subfractions nos. 1–4 and 11–14 precluded analytical ultracentrifugal analysis.

[b] These data originate from the measurement of the mean densities of the corresponding fractions, obtained from identical gradients with no lipoprotein added.

[c] Values calculated from the data of Kahlon et al. (2).

densities from 1.0260 to 1.0409 g/ml (Table 4). As reported earlier (17), these two methods for calculation of lipoprotein densities lead to substantially different values. Such discrepancies may be attributed, at least in part, to the existence of a compression gradient during the preparative ultracentrifugal step. Moreover, the σ-densities, being derived from data extrapolated to zero migration, may be subject to some inaccuracy.

Evaluation of particle size and heterogeneity by gradient gel electrophoresis. Preparation of LDL of d 1.024–1.050 g/ml from 17 male subjects, together with the corresponding density gradient subfractions, were examined by electrophoresis in 2 to 16% polyacrylamide gradient gels (**Fig. 3**); as a consequence of the limited amounts of LDL available in certain subfractions, this type of analysis was limited to fractions 4 to 10 (or 11).

The parent LDL fraction (d 1.024–1.050 g/ml) typically displayed a single, intensely stained band, whose width corresponded to particles with diameters in the range of ~215 to 225 Å (Fig. 3). However, in about half of our subjects this band was preceded by a second, very faintly stained component, whose migration corresponded to LDL particles some 10 Å smaller in diameter than the former, predominating component(s).

Density gradient fractionation provided a series of LDL subspecies which resolved, in the vast majority of our subjects' subfractions, as unique, narrow, intensely stained bands. The electrophoretic mobility of individual bands increased slightly, but significantly, with increase in the hydrated density of the respective subfractions, their Stokes diameters decreasing progressively from 229 ± 3 Å in subfraction 4 (d 1.022–1.0244 g/ml) to 214 ± 4 Å in subfraction 10 (d 1.0393–1.0435 g/ml) (Fig. 3 and **Table 5**). This range in particle size (i.e., 214–229 Å) within the density range 1.0222–1.0435 g/ml, which is superimposable on that of the parent LDL of d 1.024–1.050 g/ml (~215–225 Å), is entirely compatible with that which we determined earlier by negative stain electron microscopy on human LDL of d 1.024–1.045 g/ml (30, 31). In analyses of subfraction 11 (d 1.0435–1.0483 g/ml) from five of eight density gradients in which lipoprotein concentration was suitable for gradient gel electrophoresis, two bands were detected corresponding to particles with Stokes diameters of approximately 225 to 235 and 210 to 215 Å, respectively; on a densitometric basis, these bands were comparable quantitatively. This separate series of five gradient

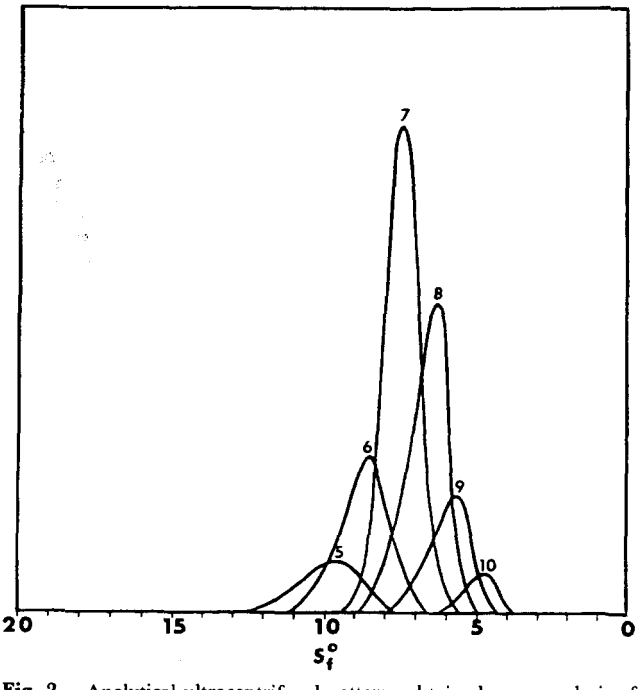

Fig. 2. Analytical ultracentrifugal patterns obtained upon analysis of six density gradient LDL subfractions (fractions 5 to 10) from a representative subject.

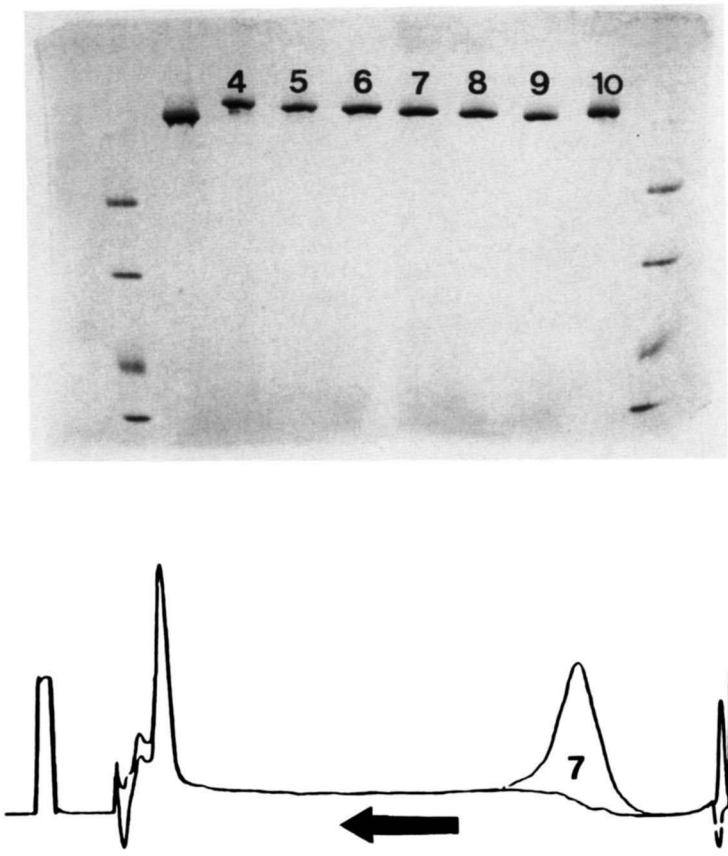

Fig. 3. Typical example of results obtained when examining density gradient LDL subfractions by polyacrylamide gradient gel electrophoresis on a 2–16% gradient and by analytical ultracentrifugation at a solvent density of 1.063 g/ml. Upper section of the figure and from left to right on the photograph: electrophoresis patterns obtained from LDL of d 1.024–1.050 g/ml, and from density gradient fractions 4 to 10. A set of marker proteins (from top to bottom, identity and Stokes diameter: thyroglobulin, 170 Å; ferritin, 122 Å; catalase, 104 Å; and lactate dehydrogenase, 81 Å) were applied to the right and left outer lanes on the electrophoretic slabs. Lower section: analytical ultracentrifugal scan of fraction 7; the direction of flotation is from right to left.

subfractions was, however, otherwise indistinguishable from those described above.

When the mean Stokes diameter of each LDL subfraction was compared with that of its nearest neighbor by the nonparametric test of Mann and Whitney (56), particles in subfractions 5 and 6, 6 and 7, and 8 and 9 displayed significantly different sizes ($P < 0.01$), as indeed did those in subfractions 7 and 8 ($P < 0.05$). Significant differences were also detected between alternate subfractions, i.e., between nos. 4 and 6 ($P < 0.05$), nos. 6 and 8 ($P < 0.01$), and nos. 8 and 10 ($P < 0.01$).

Similar findings were made when this same test was applied to our hydrodynamic data. Thus, the peak S_f° values of subfractions 5 and 6, 6 and 7, and of 8 and 9 were significantly different at the $P < 0.05$ level, while subfractions 7 and 8 differed at the $P < 0.01$ level.

The concordance between data obtained on LDL gradient subspecies by gradient gel electrophoresis and by analytical ultracentrifugation is also illustrated in Fig. 3.

TABLE 5. Stokes diameters of parent LDL of d 1.024–1.050 g/ml and of LDL subfractions derived by density gradient ultracentrifugation

Fraction	Density Interval	Stokes Diameter
	g/ml	*Å*
Parent LDL	1.024–1.050	221 ± 3 (10)[a]
Gradient LDL subfraction		
4	1.0222–1.0244	229 ± 3 (5)
5	1.0244–1.0271	227 ± 2 (14)
6	1.0271–1.0297	224 ± 2 (15)
7	1.0297–1.0327	222 ± 3 (15)
8	1.0327–1.0358	219 ± 3 (15)
9	1.0358–1.0393	216 ± 4 (15)
10	1.0393–1.0435	214 ± 4 (15)
	Average Δ:	2.5 ± 0.5

[a] Values are the means ± SD of the number of separate preparations of each fraction given in parentheses. Diameters were determined by electrophoresis on polyacrylamide gradient gels as described in the Materials and Methods section. Average Δ indicates the average difference in diameter between successive subspecies.

Thus the detection of a single and unique band in a given subfraction upon gradient gel electrophoresis was always correlated with a unimodal profile upon refractometric examination of the same subspecies.

Evaluation of net electrical charge by agarose gel electrophoresis. The net negative electric charge of individual LDL density gradient subspecies was examined semiquantitatively by electrophoresis of identical amounts of lipoprotein protein on agarose gel films. Fat Red O staining revealed the presence of a single band in each case (**Fig. 4**), of β-mobility. Analysis of the major subspecies (subfractions 5 to 11) from a series of eight different LDL gradient fractionations consistently revealed that the mobilities of certain subspecies, and thus their net negative charge, were distinct (Fig. 4). Indeed, subfraction 7, from the center of the distribution and of d 1.0297–1.0327 g/ml, typically displayed a mobility from 5 to 15% less than that of both the native LDL, from which it was derived, and of the subspecies immediately adjacent to it (subfractions 5, 6, 8, and 9). By contrast, minor LDL subfractions 10 and 11, of d 1.0393–1.0483 g/ml, were of slightly elevated mobility (10 to 20%) as compared to both the native LDL and subfractions 5 to 9. The mobilities of subfractions 5, 6, 8, and 9 closely resembled that of the corresponding native LDL; indeed, when an aliquot of a mixture of subfractions 5 to 11 was electrophoresed adjacent to the native LDL at the same concentration, their mobilities were indistinguishable.

Chemical composition and mass distribution. The mean weight % chemical compositions of native LDL of d 1.024–1.050 g/ml and of the density gradient subfractions isolated from such preparations are presented in **Table 6;** note that insufficient material was available for accurate analyses of subfractions 1, 2, 3, and 15, which were present as minor components (see Fig. 5; mass distribution among LDL subfractions).

All of the subfractions shared certain common compositional features: cholesteryl ester was the principal lipid ester (38.3–42.8%), triglycerides were a minor component representing only ~3 to 5% in the major subfractions (i.e., nos. 5–12). Free cholesterol accounted for a relatively constant proportion of the subfractions (8.5–11.6%), but tended to diminish with increase in density.

The protein content of LDL subspecies increased progressively with elevation in density (from 21.3% in subfractions 4 and 5 of d 1.0222–1.0271 g/ml to ~30% in subfractions of d > 1.0483 g/ml); indeed, the proportion of protein was the only parameter to evolve in parallel with density. The second hydrophilic component, phospholipid, tended to diminish with increase in density, i.e., from ~20–21% in subfractions of d < 1.0358 g/ml to 18.5–19.9% in fractions of d > 1.0358 g/ml. The proportion of cholesteryl ester initially rose from ~41% at d 1.022–1.030 g/ml to a peak (42.8%) in fraction 8 (d 1.0327–1.0358 g/ml), and then fell to about 39% in fractions of d > 1.0483 g/ml).

The average composition of the parent native LDL (d 1.024–1.050 g/ml) most closely resembled that of subfractions 7 to 8, a finding entirely consistent with our observation that these subfractions were typically the most abundant (**Fig. 5**).

Examination of the distribution of lipoprotein mass between the density gradient fractions showed that approximately 70% of the native LDL of d 1.024–1.050 g/ml was recovered in subfractions 7, 8, and 9 (1.0297–1.0393 g/ml) (Fig. 5). Furthermore, in the 40 different LDL preparations examined, the gradient subfraction that occurred most frequently at highest concentration was no. 7 (d 1.0297–1.0327 g/ml; 50% of all LDLs examined), with lower frequencies in fractions 8 (d 1.0327–1.0358 g/ml; 32.5%), 6 (d 1.0271–1.0297 g/ml; 15%), and 9 (d 1.0352–1.0393 g/ml; 2.5%). Subfractions of d < 1.0244 g/ml contained only ~0.8% of the total LDL recovered, and subfractions of d > 1.0483 g/ml about 2.5–3.0%. The minor increase in material in fraction 15 (d > 1.069

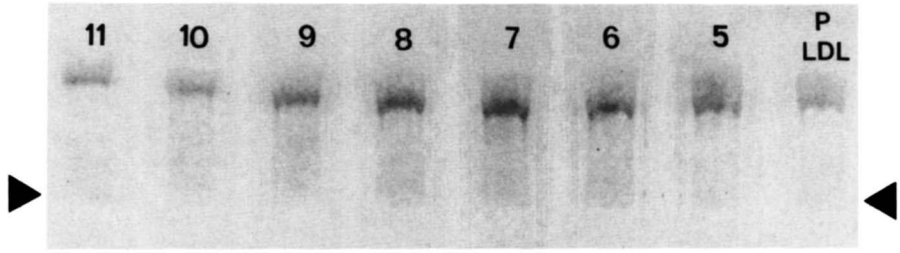

Fig. 4. Electrophoretic analysis of LDL density gradient subfractions on agarose gel films. Electrophoresis was performed in 1% agarose gel essentially by Noble's procedure (47); films were stained for lipid with Fat Red O. Samples of individual subfractions (no. 5 to 11) were initially diluted to the same concentration as that subfraction of lowest protein concentration (typically no. 5 or 11), and an aliquot containing ~1 μg of protein was applied to the appropriate well. An equivalent aliquot of the parent LDL (P-LDL), d 1.024–1.050 g/ml, from which the subfractions were derived is shown at right. Arrows mark the point of application.

TABLE 6. Percent chemical composition of native plasma human LDL and of the derived density (g/ml) gradient subfractions

Component	Native LDL 1.024–1.050	Subfraction No.[a] (n = 10) 4[b] 1.0222–1.0244	5 1.0244–1.0271	6 1.0271–1.0297	7 1.0297–1.0327	8 1.0327–1.0358	9 1.0358–1.0393	10 1.0393–1.0435	11 1.0435–1.0483	12[b] 1.0483–1.0538	13[b] 1.0538–1.0610	14[b] 1.0610–1.0690
Cholesteryl ester	42.8 ± 1.4	40.8	41.1 ± 1.3	40.8 ± 2.8	42.3 ± 0.9	42.8 ± 2.5	42.5 ± 3.6	42.0 ± 2.5	39.8 ± 0.8	38.3 ± 2.6	38.8 ± 2.1	39.6 ± 1.9
Free cholesterol	10.1 ± 2.0	10.8	11.4 ± 1.7	10.5 ± 1.5	11.6 ± 0.8	11.0 ± 0.6	10.7 ± 1.0	9.5 ± 0.5	9.4 ± 0.5	8.5 ± 2.1	9.1 ± 0.8	8.9 ± 1.6
Triglyceride	3.6 ± 0.9	6.2	5.4 ± 1.4	4.8 ± 2.5	3.7 ± 0.2	3.5 ± 0.5	3.7 ± 0.9	3.8 ± 0.8	3.2 ± 2.8	3.0 ± 2.9	2.2 ± 2.1	2.5 ± 2.8
Phospholipid[c]	21.0 ± 1.7	20.8	20.7 ± 1.4	21.1 ± 1.9	20.2 ± 0.8	20.3 ± 0.4	19.0 ± 0.8	19.4 ± 1.2	18.6 ± 0.8	19.9 ± 4.1	19.2 ± 2.2	18.5 ± 0.9
Protein	22.3 ± 1.6	21.3	21.3 ± 1.2	22.6 ± 2.2	22.2 ± 0.6	22.3 ± 1.5	24.1 ± 2.1	25.3 ± 0.5	28.8 ± 1.6	30.2 ± 2.7	30.6 ± 2.1	30.3 ± 1.8

[a] Values are means ± SD of duplicate determination of each component. The amounts of lipoprotein lipid and protein in subfractions 1, 2, 3, and 15 were insufficient (<200 μg lipoprotein/fraction) for analytical purposes. Analyses were performed as described in Materials and Methods; native LDL and their gradient subfractions were isolated from the normolipidemic plasma of male subjects.
[b] Present in trace amounts (<1% of total LDL mass).
[c] Only choline-containing phospholipids were estimated.

g/ml) as compared to subfractions 12–14 may reflect a small degree of lipoprotein denaturation, or degradation, or both.

The quantitative distribution of LDL subclasses isolated from the same starting LDL preparation in separate ultracentrifugal runs was indistinguishable, as indeed were the respective physicochemical properties of corresponding subfractions isolated from such runs (data not shown).

Apolipoprotein content

Analysis of the apolipoprotein contents of the protein moieties of the native LDL preparations of d 1.024–1.050 g/ml and of the respective density gradient subfractions by electrophoresis in 3% SDS-polyacrylamide gel slabs consistently revealed a single high molecular weight band in each case (**Fig. 6**), whose mean M_r ± SD in a representative series of 15 subfractions was 565,000 ± 8950 (range 556,000–581,000). Such behavior is typical of human apolipoprotein B-100 (M_r 549,000 (4)), and indeed the identity of this band was confirmed by its specific immunoblotting with a polyclonal antibody to apoB-100 as well as by the dot immunobinding assay (see below). Clearly then, apoB-100 predominated as the major protein component in all LDL gradient subfractions, a result again confirmed in 10% SDS gels in which the high M_r apoB-100 band was unique (data not shown).

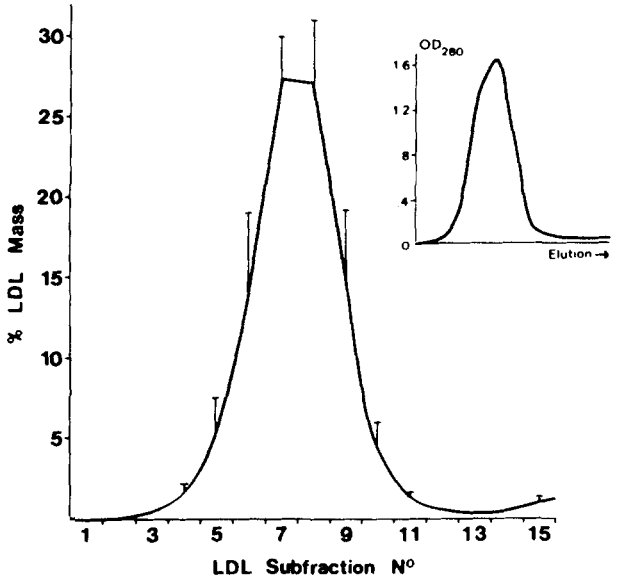

Fig. 5. Distribution of LDL mass between density gradient subfractions. The % of lipoprotein mass in individual LDL subfractions is plotted on the ordinate against subfraction number on the abscissa. Each point represents the mean from 23 separate LDL subfractionations, while the vertical bars correspond to SD/2 for each point. The inset to the figure shows a representative absorbance profile at OD_{280}, obtained by continuous monitoring of the gradient tube eluate during automated fractionation.

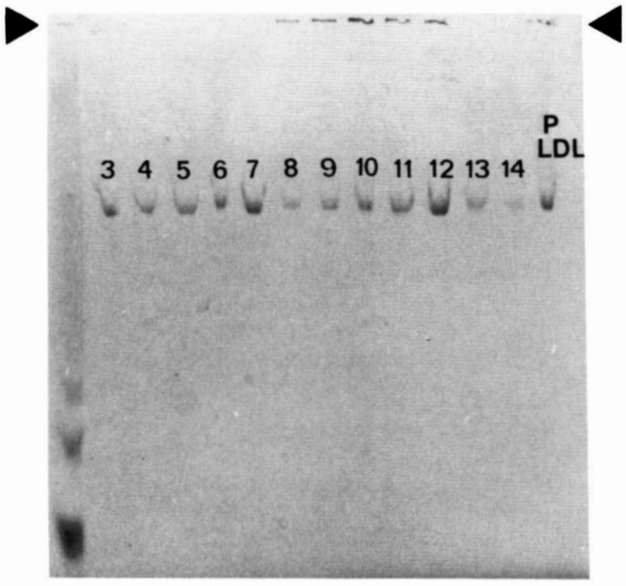

Fig. 6. Electrophoresis of the protein moieties of density gradient subfractions of human LDL in 3% SDS-polyacrylamide gel slabs; ca. 20–30 μg of LDL protein was applied to each well. Gels were stained with Coomassie Brilliant Blue R250. Individual gradient subfractions are numbered in wells from left to right. The parent LDL preparation (P-LDL) of d 1.024–1.050 g/ml, from which the subfractions were derived, is shown at right. Arrowheads mark the origin.

Apolipoproteins of M_r < 100,000 were undetectable in SDS gels of both 3 and 10% monomer. Nonetheless, we earlier documented the presence of up to 2 to 3% of low molecular weight apoproteins in the protein moiety of human LDL of d 1.024–1.045 g/ml (30, 31, 34, 35). To this end, urea extracts of the dried apoprotein (apoLDL) residues were examined by isoelectric focusing in the pH range 4 to 6.5. No apolipoprotein bands with isoelectric point in this range could be detected in LDL gradient subfractions 2 to 8 (d 1.0187–1.0358 g/ml). Trace amounts of apolipoproteins with pI in the range 5.7–6.1 were however visualized in LDL subfractions 10 to 15 (d > 1.0393 g/ml), a pH range characteristic of apoE isoforms (57). Trace amounts of polypeptides with pI in the range 4.68 to 4.76 were also detectable in subfractions 10 to 12 (d 1.0393–1.0538 g/ml), and corresponded to apoC-III isoforms identified in apoVLDL (not shown). Such trace quantities corresponded to the limit of detection by this procedure, which is about 0.5 μg of protein or less. Similar apolipoprotein bands were identified in the "total" LDL preparations from which the subfractions were derived.

For more precise estimation of the contribution of minor low molecular weight apolipoproteins to the protein moieties of LDL subfractions, immunological techniques were applied. Using monospecific antisera to apoA-I and apoA-II, our nephelometric assay showed these two apolipoproteins to be absent in both the total LDL preparations and in the gradient subfractions derived from them. By contrast, apoB-100 was present in all subfractions, both by the use of a polyclonal antibody in the nephelometric assay and by dot immunobinding with monoclonal antibody 1.8.C4. Dot immunobinding also permitted estimation of the contents of apoE and of Lp[a] in LDL gradient subspecies. In this series of gradient subfractions examined from five subjects, both apoE and the apo[a] antigen were absent from subfractions 6, 7, 8, and 9, with the exception of two individuals in which Lp[a] represented 0.2% of the protein moiety of subfraction 9. The contents of apoE in subfractions at each extreme of the density distribution were higher than those in the peak region (i.e., subfractions 6 to 9, Fig. 5). They varied as follows: subfraction 1, 1.0 ± 1.0%; 2, 1.3 ± 1.5%; 3, 0.9 ± 1.7%; 4, 1.1 ± 0.7%; 5, 0.1 ± 0.1%; 10, 0.2 ± 0.1%; 11, 0.6 ± 0.1%; 12, 0.8 ± 0.2%; 13, 0.8 ± 0.2%; 14, 1.9 ± 2.7%; and 15, 1.9 ± 2.7%. It is noteworthy that apoE amounted to significantly less than 1% of the protein moiety in subfractions 5, 10, 11, 12, and 13.

By contrast, Lp[a] represented from 2.0 to 6.7% of apoLDL protein in the same series of gradient subfractions. Absolute values varied as follows: subfraction 1, 2.4 ± 2.8%; 2, 3.3 ± 2.3%; 3, 3.5 ± 1.7%; 4, 3.3 ± 1.9%; 5, 2.0 ± 1.6%; 10, 3.4 ± 2.8%; 11, 6.7 ± 3.0%; 12, 4.5 ± 3.8%; 13, 5.1 ± 1.9%; 14, 3.4 ± 1.5%; and 15, 4.9 ± 2.3%. In further contrast to the density distribution of apoE in these subfractions, the quantitative distribution of Lp[a] across the density gradient was relatively uniform, with a tendency to be higher in the denser subfractions (subfractions 11–15, average 4.9%; 1–5, average 2.9%).

The contribution of apoC-III to the protein content of subfractions 9 to 12 was estimated to be less than 0.5%, and of the order of 0.2–0.3% (see focusing data).

Molecular weights, molar compositions and molecular diameters

The molecular weights and molecular diameters of individual LDL subspecies are shown in **Table 7,** together with the average number of molecules of each chemical component per particle subspecies as calculated from the respective molecular weights (see legend to Table 7). Molecular weight and diameter decreased progressively with increase in density, as indeed did the number of molecules of cholesteryl ester and phospholipid. With the exception of subfraction 7, free cholesterol showed a similar trend. The content of triglyceride molecules diminished by one-third from subfractions 5 to 7, and then stabilized at ~100 molecules/particle. Protein content was stable slightly in excess of 1 molecule of apoB-100 per particle.

The molar ratios of the various components (**Table 8**) reveal a minor increase in the number of cholesteryl ester molecules relative to those of both free cholesterol and phospholipid with increase in density, whereas the ratio

TABLE 7. Average molecular weights, molecular compositions, and molecular diameters of human LDL particle subspecies isolated by density gradient ultracentrifugation

Subfraction No.	Density[a]	Mol. wt.[b] × 10^{-6}	Average Number of Molecules of Each Component per Particle of Each LDL Subspecies[c]					Molecular[b] Diameter
			Cholesteryl Ester	Free Cholesterol	Triglyceride	Phospholipid	Protein	
	g/ml							*Å*
5	1.0260	2.96	1872	872	188	791	1.2	209.6
6	1.0286	2.86	1795	776	161	779	1.2	207.2
7	1.0314	2.75	1790	824	120	717	1.1	204.1
8	1.0343	2.62	1725	745	108	686	1.1	200.7
9	1.0372	2.48	1621	686	108	608	1.1	196.9
10	1.0409	2.33	1505	572	104	583	1.1	192.7

[a] Densities are taken from Table 3.
[b] Values for the average molecular weights and molecular diameters were calculated from hydrodynamic data (Table 4) by application of the basic equations described by Lindgren et al. (9). A Stokes frictional factor of 1.11 was uniformly used in these calculations (58).
[c] The molecular weights of individual components were: cholesteryl ester, 650; free cholesterol, 387; triglyceride, 850; and phospholipid, 755 (17). In light of the findings of Yang et al. (59) and Kane et al. (4), a molecular weight of 550,000 was taken for apoB-100, and includes the carbohydrate moiety.

free cholesterol:phospholipid remained constant. By contrast, the ratio phospholipid:protein was relatively stable in subfractions 5 to 7, and then fell by ~20% across subfractions 8 to 10. The ratio cholesteryl ester:triglyceride tended to stabilize in subfractions 7 through 10.

DISCUSSION

Of the 40 normolipidemic French males whose LDL was subfractionated, none displayed more than a single, well-defined symmetric or asymmetric peak in their gradient (Fig. 5) and analytical ultracentrifugal profiles (Fig. 1 and Table 4). Such findings are entirely consistent with the observations of Fisher (10) in 86 normolipidemic subjects, in which about 91% typically displayed a single major LDL peak by analytical ultracentrifugation. By contrast, Nelson and Morris (15) found a markedly higher frequency (46%) of "polydisperse" or "heterogeneous" LDL with the same methodology in 41 subjects whose plasma cholesterol levels varied over a wide range.

Examination of the hydrodynamic properties of LDL from normal subjects has frequently revealed differences between individuals in the flotation rate (S_f°), or hydrated density of the major component (10, 15, 18), or both, these two parameters being interdependent (9). In a similar fashion, peak S_f° rates varied within a narrow range from 6.9 to 7.9 in six of our subjects, while median peak hydrated densities determined on the isopycnic gradient varied from 1.0286 to 1.0314, to 1.0343, and to 1.0372 g/ml, with frequencies of 15%, 50%, 32.5%, and 2.5%, respectively, in the whole group.

Each major LDL subfraction displayed a unique peak flotation rate (Fig. 2 and Table 4) ranging from 9.3 (subfraction 5) to 5.2 (subfraction 10), with some overlap between successive subfractions. Such S_f° rates were determined at 26°C on subfractions that had been isolated from gradients centrifuged at 15°C and formed from LDL initially separated at 10°C. In view of the differential thermal expansivity of LDL subfractions and of the various salt solutions used in their isolation (60), a strict comparison between the present hydrodynamic data and those of other authors determined on LDL subfractions under different conditions of temperature (i.e., LDL isolation at 17°C, gradient fractionation at 22–24°C, and analytical ultracentrifugation at 26°C) (17, 18), cannot be made.

The bulk of LDL (ca. 80%) was typically distributed among subfractions 7, 8, and 9 (d 1.0297–1.0393 g/ml), with the greatest quantitative variations occurring in subfractions 6, 7, and 10 (Fig. 5 and text). Despite such variations among individuals, we nonetheless observed a high degree of consistency in the physicochemical properties of corresponding subfractions from different subjects. This aspect is well illustrated by the small standard deviations in the mean values for chemical compositions (Table 6) and particle sizes (Table 5). As previously reported (2, 10,

TABLE 8. Molar ratios of chemical components per particle of each LDL subspecies[a]

Subfraction No.	Molar Ratio				
	CE/FC[b]	CE/TG	CE/PL	FC/PL	PL/PRN
5	2.2	10.0	2.4	1.1	659
6	2.3	11.1	2.3	1.0	649
7	2.2	14.9	2.5	1.1	652
8	2.3	16.0	2.5	1.1	624
9	2.4	15.0	2.7	1.1	553
10	2.6	14.5	2.6	1.0	530

[a] Molar ratios calculated from the data in Table 7.
[b] Abbreviations are: CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride; PL, phospholipid; PRN, protein.

17, 18, 23), our data confirm that the particle size and peak flotation rate of LDL subspecies diminish progressively with increase in hydrated density and protein content.

Our present data are distinguished by the presence of an essentially unique and homogeneous LDL subspecies in each of the major density gradient fractions (nos. 4–10), at least as judged by particle size criteria (Fig. 3 and Table 5). These subspecies were distinct when size data were analyzed statistically, despite average increments in particle diameter of only 2.4 Å. Notwithstanding, a 1% difference in radius is equivalent to a 3% increment in particle volume. Similar statistical treatment of our hydrodynamic data (Fig. 2 and Table 4) led to the same conclusion, again suggesting each subspecies to be distinct and homogeneous. Clearly then, our procedure appears to avoid the size heterogeneity seen earlier in the major LDL subgroups (18), and in addition we have not detected a density heterogeneity involving particles of similar size (18).

The particle diameters of LDL subspecies calculated from our hydrodynamic and molecular weight data were consistent with those determined by gradient gel electrophoresis (Tables 4 and 7). Thus the mean decrement in diameter between subspecies of increasing density was similar by both methods of analysis (3.4 Å and 2.5 Å by the ultracentrifugal and electrophoretic methodologies, respectively). However particle diameters derived from hydrodynamic measurements were up to 10% smaller than corresponding values estimated by gradient gel electrophoresis, but closely resembled the major size species seen in d 1.028–1.050 g/ml LDL by negative stain electron microscopy (40). Such differences undoubtedly reflect the distinct physicochemical environments of lipoprotein particles when studied by each methodology and of criteria employed for size calibration in each case.

No marked discontinuities in particle size or chemical composition were detectable in neighboring LDL subspecies, although minor inflexions occurred in the particle content of triglyceride molecules (Table 7) and in the molar ratio of cholesteryl ester/triglyceride between subfractions 6 and 7 (Table 8). Otherwise, the number of molecules of cholesteryl ester, free cholesterol, and phospholipid diminished progressively with increase in density; in contrast, one molecule of apoB-100 was present per particle in each subspecies. These data indicate then that a similar overall molecular organization and structure maintains in a unimodal distribution of LDL particle subspecies over the density range of ~1.02 to 1.05 g/ml, but equally that the presence of a single copy of apoB-100 per particle may stabilize variable proportions of both surface and core lipids. A single molecule of apoB per LDL particle was suggested earlier on the basis of stoichiometric binding studies with monoclonal antibodies to LDL (61).

We have also documented distinct and consistent differences in the net electrical charge of individual LDL subspecies at neutral pH (7.4), a finding that may be of relevance to both the structure and metabolism of LDL; Ghosh, Basu, and Schweppe (12) originally provided evidence for charge heterogeneity in the LDL of hypercholesterolemic patients. We consider that the observed heterogeneity may arise either from dissimilarities in the relative proportions of various charged phospholipids between subspecies, or from differences in the degrees of glycosylation or of sialylation of their protein moieties, or from a combination of these; chemical modification of certain subspecies as a consequence of cellular interaction cannot, however, be excluded (62).

We attach special interest to the heterogeneous distribution of apoE, apo[a], and apoC-III among our LDL subspecies. Thus, the major LDL subspecies (fractions 6–9, d 1.0286–1.0393 g/ml) were deficient in these apolipoproteins. By contrast, apoE constituted 0.1–1.3% and 0.2–1.9% of apoLDL in subspecies of lesser (nos. 1–5) and of greater (nos. 10–15) density, respectively, than those in the peak region, and apo[a] from 2–3.5% and 3.4–6.7% of apoLDL in the corresponding subfractions, respectively. The gradient density distribution of apoE was not skewed towards the higher densities as might have been expected from the known ultracentrifugally induced changes in lipoprotein apoE content (63). We cannot exclude the possibility that the starting d 1.024–1.050 g/ml LDL were already partially apoE-depleted, however.

The question as to the presence of apoE on apoB-rich LDL particles is of considerable relevance to LDL metabolism (64), since even minor amounts of apoE may significantly increase the affinity of an LDL particle for the cellular apoB,E receptor, given the 20-fold higher receptor binding affinity of apoE as compared to that of apoB-100 (57, 64). Our calculations reveal that, in LDL subspecies adjacent to the peak region (e.g., subfraction 5), the molar ratio of apoE:apoB-100 is ~1:60, suggesting that one in every 60 LDL particles may carry one copy of apoE. This frequency increases to a maximum in the denser subspecies, in which the molar ratio is as low as 1:8, the latter subfraction (no. 12) containing almost 8-fold more apoE than fraction 5. Clearly then, the apoE content of our LDL subspecies is sufficient to warrant further evaluation on a metabolic basis, and indeed our recent findings suggest that these subspecies may differ in their relative binding affinities for the apoB,E receptor (65).

The quantitative distribution of the apo[a] antigen was distinctly skewed towards the higher densities, occurring at an average molar ratio relative to apoB-100 of ~1:17 in subfractions 10 to 15, and of ~1:29 in subfractions 1 to 5; an M_r value of 500,000 was taken for apo[a] (66). This antigen is known to display density heterogeneity (66, 67); in addition, the detection of apo[a] at densities as low as 1.018–1.027 g/ml may be of relevance to its presence in

postprandial VLDL (67). Only in subfraction 11 (d 1.043–1.048 g/ml) did a second size species of 225–235 Å appear, thereby resembling Lp[a] (67). The apparent absence of size species across the density range with diameters typical of Lp[a] may be related to the size heterogeneity of these particles, whose diameters may on occasion resemble those of LDL (66).

Only a minor degree of variability was seen in the qualitative and quantitative aspects of the apolipoprotein content of LDL subspecies from different individuals. That these findings are related in some way to our methodological approach cannot at present be entirely discounted, and conclusive evidence must await the isolation of similar LDL subspecies by a procedure independent of ultracentrifugation. It is, however, relevant that LDL subfractions obtained by gel filtration chromatography after initial ultracentrifugal isolation of d < 1.070 g/ml lipoproteins correspond in several of their physicochemical properties to subspecies separated by our gradient methodology from the same "parent" LDL (E. Bruckert, G. Luc and M. J. Chapman, unpublished findings).

In conclusion, the present investigations have permitted new insight into the microheterogeneity of LDL particles in normolipidemic subjects, and suggest that the LDL spectrum is constituted of numerous, discrete particle species, each of which possesses several distinct physicochemical characteristics rather than of a series of discrete "subclasses." These particle species appear to fluctuate about a mode whose precise features (hydrated density, peak flotation rate, etc.) are characteristic of a given individual. In addition, our studies raise important questions as to the intravascular metabolism and tissular fate of the LDL subspecies described herein. Ultimately this approach may permit identification of molecular species of LDL of elevated atherogenic potential.

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REFERENCES

1. Nichols, A. V. 1967. Human serum lipoproteins and their interrelationships. *Adv. Biol. Med. Phys.* **11:** 109–158.
2. Kahlon, T. S., G. L. Adamson, M. S. S. Shen, and F. T. Lindgren. 1982. Sedimentation equilibrium of human low density lipoprotein subfractions. *Lipids.* **17:** 323–330.
3. Deckelbaum, R. J., G. G. Shipley, and D. M. Small. 1977. Structure and interactions of lipids in human plasma low density lipoproteins. *J. Biol. Chem.* **252:** 744–754.
4. Kane, J. P., D. A. Hardman, and H. E. Paulus. 1980. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. *Proc. Natl. Acad. Sci. USA* **77:** 2465–2469.
5. Chen, G. C., M. J. Chapman, and J. P. Kane. 1983. Secondary structure and thermal behaviour of trypsin-treated low density lipoproteins from human serum, studied by circular dichroism. *Biochim. Biophys. Acta.* **754:** 51–56.
6. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptors and cholesterol homeostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
7. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1981. Regulation of plasma cholesterol by lipoprotein receptors. *Science.* **212:** 628–635.
8. Havel, R. J. 1984. The formation of LDL: mechanisms and regulation. *J. Lipid Res.* **25:** 1570–1576.
9. Lindgren, F. T., L. C. Jensen, R. D. Wills, and N. K. Freeman. 1969. Flotation rates, molecular weight and hydrated densities of the low density lipoproteins. *Lipids.* **4:** 337–344.
10. Fisher, W. R. 1983. Heterogeneity of plasma low density lipoproteins. Manifestations of the physiologic phenomenon in man. *Metabolism.* **32:** 283–291.
11. Adams, G. H., and V. N. Schumaker. 1970. Equilibrium banding of low-density lipoproteins. III. Studies on normal individuals and the effects of diet and heparin-induced lipase. *Biochim. Biophys. Acta.* **210:** 462–472.
12. Ghosh, S., M. K. Basu, and J. S. Schweppe. 1973. Charge heterogeneity of human low density lipoprotein (LDL). *Proc. Soc. Exp. Biol. Med.* **142:** 1322–1325.
13. Lee, D. M. 1976. Isolation and characterization of low density lipoproteins. *In* Low Density Lipoproteins. C. E. Day and R. S. Levy, editors. Plenum Press, New York. 3–45.
14. Mills, G. L., C. E. Taylaur, and M. J. Chapman. 1976. Low-density lipoproteins in patients homozygous for familial hyperbetalipoproteinemia. *Clin. Sci. Mol. Med.* **51:** 221–231.
15. Nelson, C. A., and M. D. Morris. 1977. The ultracentrifugal heterogeneity of serum low density lipoproteins in normal humans. *Biochem. Med.* **18:** 1–9.
16. Rubinstein, B. 1978. Heterogeneity of human plasma low density lipoprotein. *Can. J. Biochem.* **56:** 977–980.
17. Shen, M. M. S., R. M. Krauss, F. T. Lindgren, and T. M. Forte. 1981. Heterogeneity of serum low density lipoproteins in normal human subjects. *J. Lipid Res.* **22:** 236–244.
18. Krauss, R. M., and D. J. Burke. 1982. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *J. Lipid Res.* **23:** 97–104.
19. Lee, D. M., and D. Downs. 1982. A quick and large-scale density gradient subfractionation method for low density lipoproteins. *J. Lipid Res.* **23:** 14–27.
20. Teng, B., G. R. Thompson, A. D. Sniderman, T. M. Forte, R. M. Krauss, and P. O. Kwiterovich. 1983. Composition and distribution of low density lipoprotein fractions in

hyperapobetalipoproteinemia, normolipidemia and familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **80:** 6662–6666.
21. Teng, B., A. Sniderman, R. M. Krauss, P. O. Kwiterovich, R. W. Milne, and Y. L. Marcel. 1985. Modulation of apolipoprotein B antigen determinants in human low density lipoprotein subclasses. *J. Biol. Chem.* **260:** 5067–5072.
22. Patsch, W., R. Ostlund, I. Kuisk, R. Levy, and G. Schonfeld. 1982. Characterization of lipoprotein in a kindred with familial hypercholesterolemia. *J. Lipid Res.* **23:** 1196–1205.
23. Lee, D. M., and P. Alaupovic. 1970. Studies of the composition and structure of plasma lipoproteins. Isolation, composition and immunochemical characterization of low density lipoprotein subfractions of human plasma. *Biochemistry.* **9:** 2244–2252.
24. Rubenstein, B., and G. Steiner. 1976. Fractionation of human low density lipoprotein by column chromatography. *Can. J. Biochem.* **22:** 1023–1028.
25. Karlin, J. B., and A. H. Rubenstein. 1979. Serum lipoprotein quantitation by immunochemical methods. *In* The Biochemistry of Atherosclerosis. Vol. 7. The Biochemistry of Disease. A. M. Scanu, R. W. Wissler, and G. S. Getz, editors. Marcel Dekker Inc., New York. 189–227.
26. Desager, J. P., M. Rosseneu, W. F. Riesen, and C. Harvengt. 1984. Limitations of the predictive value for coronary vascular disease of the plasma lipids and apoproteins AI, AII, B levels as measured before coronarography in 317 patients. *In* Latent Dyslipoproteinemias and Atherosclerosis. J. L. De Gennes, J. Polonovski, and R. Paoletti, editors. Raven Press, New York. 165–174.
27. 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.
28. Radding, C. M., and D. Steinberg. 1960. Studies on the synthesis and secretion of serum lipoproteins by rat liver slices. *J. Clin. Invest.* **39:** 1560–1569.
29. De Lalla, O. F., and J. W. Gofman. 1954. Ultracentrifugal analysis of serum lipoproteins. *Methods Biochem. Anal.* **1:** 459–478.
30. Chapman, M. J., and S. Goldstein. 1976. Comparison of the serum low density lipoprotein and of its apoprotein in the pig, rhesus monkey and baboon, and that in man. *Atherosclerosis.* **25:** 267–291.
31. Chapman, M. J., S. Goldstein, and G. L. Mills. 1978. Limited tryptic digestion of human serum low density lipoprotein: isolation and characterization of the protein-deficient particle and of its apoprotein. *Eur. J. Biochem.* **87:** 475–488.
32. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
33. Weisgraber, K. H., T. P. Bersot, R. W. Mahley, G. Franceschini, and C. R. Sirtori. 1980. A-I Milano apoprotein. Isolation and characterization of a cysteine-containing variant of the AI apoprotein from human high density lipoprotein. *J. Clin. Invest.* **66:** 901–907.
34. Chapman, M. J., A. Millet, D. Lagrange, S. Goldstein, Y. Blouquit, C. E. Taylaur, and G. L. Mills. 1982. The surface-exposed, trypsin-accessible segments of apolipoprotein B in the low density lipoprotein of human serum: fractionation and characterization of the liberated peptides. *Eur. J. Biochem.* **125:** 479–489.
35. Forgez, P., M. J. Chapman, and G. L. Mills. 1984. Isolation, characterization and comparative aspects of the major serum apolipoproteins B-100 and AI in the common marmoset, *Callithrix jacchus*. *Biochim. Biophys. Acta.* **754:** 321–333.
36. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195:** 357–362.
37. Kessler, G., and H. Lederer. 1966. Fluorometric measurement of triglycerides. *In* Automation in Analytical Chemistry. Technicon Symposia 1965. L. T. Skeggs, Jr., editor. Mediad Inc., New York. 341–344.
38. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470–475.
39. Lopes-Virella, M. F. L., P. Stone, S. Ellis, and J. A. Colwell. 1977. Cholesterol determination in high density lipoprotein separated by three different methods. *Clin Chem.* **23:** 882–884.
40. Chapman, M. J., S. Goldstein, D. Lagrange, and P. M. Laplaud. 1981. A density gradient ultracentrifugal procedure for isolation of the major lipoprotein classes from human serum. *J. Lipid Res.* **22:** 339–358.
41. 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.
42. Roeschlau, P., E. Bernt, and W. Gruber. 1974. Enzymatische Bestimmung des Gesamt Cholesterins im Serum. *Z. Klin. Chem. Klin. Biochem.* **12:** 403–407.
43. Takayama, M., S. Itoh, T. Nagasaki, and I. Tanimizu. 1977. A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chim. Acta.* **79:** 93–98.
44. Biggs, H. C., J. M. Erickson, and R. Moorehead. 1975. A manual colorimetric assay of triglycerides in serum. *Clin. Chem.* **21:** 437–450.
45. Laplaud, P. M., L. Beaubatie, and D. Maurel. 1980. A spontaneously seasonal hypercholesterolemic animal: plasma lipids and lipoproteins in the European badger (*Meles meles* L.). *J. Lipid Res.* **21:** 724–738.
46. Ewing, A. M., N. K. Freeman, and F. T. Lindgren. 1965. The analysis of human serum lipoprotein distributions. *Adv. Lipid Res.* **3:** 25–61.
47. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
48. Anderson, D. W., A. V. Nichols, T. M. Forte, and F. T. Lindgren. 1977. Particle distribution of human serum high density lipoproteins. *Biochim. Biophys. Acta.* **493:** 55–68.
49. Wider, G., E. Kulnigg, E. Molinari, H. Hotschek, and P. M. Bayer. 1982. Evaluation of a monitor guided nephelometric system. *J. Clin. Chem. Clin. Biochem.* **20:** 1–8.
50. Duhamel, G., B. Nalpas, S. Goldstein, P. M. Laplaud, P. Berthelot, and M. J. Chapman. 1984. Plasma lipoprotein and apolipoprotein profile in alcoholic patients with and without liver disease: on the relative roles of alcohol and liver injury. *Hepatology.* **4:** 577–585.
51. Hawkes, R., E. Niday, and J. Gordon. 1982. A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* **119:** 142–147.
52. Pagnan, A., R. J. Havel, J. P. Kane, and L. Kotite. 1977. Characterization of human very low density lipoproteins containing two electrophoretic populations: double prebeta lipoproteinemia and primary dysbetalipoproteinemia. *J. Lipid Res.* **18:** 613–622.
53. Camus, M. C., M. J. Chapman, P. Forgez, and P. M. Laplaud. 1983. Distribution and characterization of the

serum lipoproteins and their apoproteins in the mouse, *Mus musculus*. *J. Lipid Res.* **24:** 1210–1228.
54. Stephens, R. E. 1975. High resolution SDS-polyacrylamide gel electrophoresis: fluorescent visualization and electrophoretic elution-concentration of protein bands. *Anal. Biochem.* **65:** 369–379.
55. Karlson, C., H. Dories, J. Ohman, and U. B. Anderson. 1973. LKB application note no. 75.
56. Mann, H. B., and D. R. Whitney. 1947. On a test of whether one or two random variables is stochastically larger than the other. *Ann. Math. Stat.* **18:** 50–60.
57. Mahley, R. W., T. L. Innerarity, S. C. Rall, and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.* **25:** 1277–1294.
58. Fisher, W. R., M. E. Granade, and J. Mauldin. 1971. Hydrodynamic studies of human low density lipoproteins. Evaluation of the diffusion coefficient and the preferential hydration. *Biochemistry.* **10:** 1622–1629.
59. Yang, C-Y., F-S. Lee, L. Chan, D. A. Sparrow, J. T. Sparrow, and A. M. Gotto. 1986. Determination of the molecular mass of apolipoprotein B-100. *Biochem. J.* **239:** 777–780.
60. Mills, G. L. 1977. The thermal expansion of human low density lipoproteins. *FEBS Lett.* **84:** 171–173.
61. Wiklund, O., C. A. Dyer, B. P. Tsao, and L. K. Curtiss. 1985. Stoichiometric binding of apolipoprotein B-specific monoclonal antibodies to low density lipoproteins. *J. Biol. Chem.* **260:** 10956–10960.
62. Steinberg, D. 1986. Arterial lipoprotein metabolism in relation to the pathogenesis of atherosclerosis. Atherosclerosis VII. N. H. Fidge and P. J. Nestel, editors. Elsevier, 345–353.
63. Mahley, R. W., and K. S. Holcombe. 1977. Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat. *J. Lipid Res.* **18:** 314–324.
64. Gabelli, C., R. E. Gregg, L. A. Zech, E. Manzato, and H. B. Brewer. 1986. Abnormal low density lipoprotein metabolism in apolipoprotein E deficiency. *J. Lipid Res.* **27:** 326–333.
65. Chapman, M. J., H. Cadman, and P. M. Laplaud. 1984. Heterogeneity in apo-B,E receptor binding of human LDL subspecies. *Circulation.* **70: II:137** (Abstract).
66. Fless, G. M., C. A. Rolih, and A. M. Scanu. 1984. Heterogeneity of plasma lipoprotein[a]. *J. Biol. Chem.* **259:** 11470–11478.
66. Duvic, C. R., G. Smith, W. E. Sledge, L. T. Lee, M. D. Murray, P. S. Roheim, W. R. Gallaher, and J. J. Thompson. 1985. Identification of a mouse monoclonal antibody, LHLP-1, specific for human Lp[a]. *J. Lipid Res.* **26:** 540–548.
67. Bersot, T. P., T. L. Innerarity, R. E. Pitas, S. C. Rall, K. H. Weisgraber, and R. W. Mahley. 1986. Fat feeding in humans induces lipoproteins of density less than 1.006 that are enriched in apolipoprotein [a] and that cause lipid accumulation in macrophages. *J. Clin. Invest.* **77:** 622–630.