papers and notes on methodology

A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum

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Abstract A density gradient ultracentrifugal procedure is described for the rapid and reproducible isolation of the major lipoprotein classes, VLDL, LDL, HDL_2, and HDL_3, from human serum. A step gradient is constructed from four NaCl/KBr solutions varying in density from 1.006 to 1.24 g/ml and from 3 ml of serum adjusted to d 1.21 g/ml. Separation is achieved after a single ultracentrifugation for some 56×10^7 g_{avg} min at 15°C in a swinging bucket rotor, at which time the lipoproteins band isopycnically and albumin and other serum proteins are sedimented. Densitometric scanning of gradients revealed a lipoprotein mass profile distinguished by four absorption maxima which fell within the hydrated density ranges of VLDL ($d < 1.016$ g/ml), LDL (1.028–1.050 g/ml), HDL_2 (1.066–1.100 g/ml), and HDL_3 (1.100–1.153 g/ml). Fractionation of gradients on the basis of band distribution, followed by chemical, physical, and immunological analyses of the four principal fractions (i.e., bands) provided data on their electrophoretic mobility, chemical composition, morphology and size distribution, immunological reactivity and apolipoprotein content, thereby confirming their identities as VLDL, LDL, HDL_2, and HDL_3. The validity of this separation was supported by the quantitative distribution of apo B and apo A-I as assessed by radial immunodiffusion. Lipoprotein quantitation based on chemical analysis of gradient fractions was compared with that by analytical ultracentrifugation for a group of normolipidemic males; results concorded well, giving a similar HDL_2:HDL_3 ratio (0.35–0.36). Our procedure thus provides a simple and precise manner in which to assess the lipoprotein and apolipoprotein profile of human serum quantitatively and qualitatively.—**Chapman, M. J., S. Goldstein, D. Lagrange, and P. M. Laplaud.** A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J. Lipid Res.* 1981. **22:** 339–358.

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A number of procedures are presently available for the preparative fractionation of serum lipoproteins, among which the most widely employed are precipitation techniques (1, 2), sequential flotation ultracentrifugation (3, 4), density gradient ultracentrifugation (5, 6), gel filtration chromatography (7), and zonal ultracentrifugation (8, 9). In recent years, considerable attention has been given to the ultracentrifugal separation of lipoproteins on density gradients in swinging bucket rotors. The methods pioneered by Lindgren, Jensen and Hatch (5) have typically involved cumulative flotation of discrete lipoprotein fractions and thus have not allowed the simultaneous isolation of the three major lipoprotein classes, i.e., VLDL, LDL, and HDL. Of late however, both Redgrave, Roberts, and West (10) and Foreman

Abbreviations: VLDL, very low density lipoproteins of $d < 1.006$ g/ml unless otherwise defined; IDL, intermediate density lipoproteins of d 1.006–1.019 g/ml unless otherwise defined; LDL, low density lipoproteins, density as defined; HDL, high density lipoproteins, density as defined; HDL_2, HDL of density 1.063–1.125 g/ml; HDL_3, HDL of d 1.125–1.21 g/ml unless otherwise defined; VHDL, very high density lipoproteins, density as defined; apo B, apolipoprotein B; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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et al. (11) have described procedures for the separation of VLDL, LDL, and HDL from human serum by single-spin gradient ultracentrifugation in 24 and 48 hr, respectively. In the former report (10), data on the quantitative recovery of the various fractions was lacking, as were details of their lipid and protein composition; in the latter (11), only small volumes of serum (1 ml) could be fractionated on each gradient, thereby necessitating the use of radioactive lipids as markers for the localization of the lipoprotein peaks and for lipid quantitation. Neither of these gradients (10, 11) was reported to permit subfractionation of the high-density lipoproteins.

Substantial evidence has accumulated for the role of HDL in affording protection against atherogenesis in man (12, 13). It has therefore become important to develop relatively simple and rapid methods which facilitate the isolation and quantification of HDL in high yield, and which avoid some of the structural modifications (especially loss of apolipoproteins) associated with certain procedures (14–16). Furthermore, separation of HDL into its respective subclasses, i.e., HDL_2 and HDL_3, now appears desirable as a preliminary to its detailed study since Anderson et al. (17) have shown that HDL_2 may be a crucial factor in determining the apparent inverse correlation between coronary heart disease and HDL cholesterol levels. We have therefore addressed this problem and presently describe a density-gradient ultracentrifugal procedure for the isolation of lipoprotein fractions with the properties of VLDL, LDL, and HDL_2 and HDL_3 from normolipemic human serum both in high yield and in amounts which allow not only for their quantitation but also for their physical, chemical, and immunological characterization.

MATERIALS AND METHODS

Blood samples

Serum was isolated from the freshly drawn venous blood of nine healthy normolipidemic males 25 to 35 years old, without restriction of blood groups; all donors had fasted overnight for 12–14 hr.

The mean serum lipid concentrations (±S.D.) in these individuals were cholesterol, 174 ± 40 mg/dl and triglyceride, 64 ± 12 mg/dl.

The serum samples, to which sodium azide (0.01% w/v) and sodium merthiolate (0.001% w/v) were added immediately upon separation in order to inhibit any microbial growth, were normally taken for lipoprotein fractionation within 24 hr of their isolation; during this period they were stored at 4°C.

Isolation of serum lipoproteins

Density gradient ultracentrifugation (i)

Construction of the density gradient. The non-protein solvent density of the serum samples was first increased to 1.210 g/ml by addition of solid potassium bromide (0.325 g/ml serum). Each discontinuous density gradient was then constructed at ambient temperature in a cellulose nitrate tube (9/16″ diam × 3 1/2″ length) of the Beckman Sw 41-Ti swinging bucket rotor (capacity six tubes). With the use of a Buchler Auto-Densi-flow II (Buchler Instruments, Searle Analytic Inc., Fort Lee, NJ) coupled to a Gilson Minipuls II peristaltic pump, 2 ml of a sodium chloride–potassium bromide solution of density 1.240 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 Densi-flow: 3 ml of serum at d 1.21 g/ml, 2 ml of NaCl–KBr solution of d 1.063 g/ml, 2.5 ml of d 1.019 g/ml, and 3 ml of NaCl solution of d 1.006 g/ml. During gradient construction, the temperature of these salt solutions was in the range 17–20°C; determination of density (see below) was however performed at the same temperature as for centrifugation, i.e., 15°C.

Gradients constructed for the purpose of determining the density profile contained 3 ml of NaCl–KBr solution of d 1.21 g/ml instead of a serum sample; all salt solutions contained 0.1% sodium azide, 0.04% EDTA, and 0.005% Merthiolate, and their densities were verified with a precision density meter (Anton Paar, Graz, Austria; Model DMA 40) at 15°C.

It is noteworthy that some diffusion of the serum layer into that below it occurred, an effect favored by the similar solution density of the serum–KBr sample (1.23 g/ml) relative to that of the d 1.24 g/ml NaCl–KBr bottom layer. For this reason, gradients were constructed with the minimum of mechanical disturbance and centrifugation commenced as rapidly as possible. Furthermore, as a result of the expansion in volume due to KBr addition, 3 ml of "d 1.21 g/ml serum" contained the equivalent of 2.84 ml serum.

In order to provide sufficient material for subsequent analyses, two gradients were normally constructed from each serum sample (i.e., 6 ml serum was required). Immediately upon completion, the gradients were centrifuged at 40,000 rpm for 48 hr (56.7×10^7 g_{avg} min) at 15°C in a Beckman L5-50 ultracentrifuge; no braking was used at the end of the run.

Fractionation of gradients. Prior to fractionation, gradients were photographed on Kodak Ektachrome 50T film; after development, films were scanned

densitometrically at 435 nm with a Gilford model 250 recording spectrophotometer. Such scans provide, at most, a semi-quantitative estimate of lipoprotein mass distribution. Gradients were fractionated according to one of two regimens, both of which involved removal of successive fractions from the meniscus downwards by aspiration into a narrow-bore Pasteur pipette.

The first series of fractionations *A*) was performed with the aim of illustrating the potential resolutory power of the gradient. Lipoprotein and protein distributions were thus examined over the entire gradient. Successive fractions of 1.0 ml were removed, with the exception of a yellow-orange band (containing LDL) present in the upper half of the tube, which was normally aspirated in a volume of 1.5 ml. Two gradient tubes (i.e., 6 ml of serum) were spun from each of three different sera, and corresponding fractions from each pair of gradients were pooled.

In the second series *B*), fractions were removed on the basis of the disposition of the various lipoprotein bands, these being clearly evident either as a result of their light scattering, or of their color, or both.

Band I was present as a white lactescent layer at the meniscus, and could be removed in 0.5–1.0 ml; Band II, yellow-orange in color, varied in its precise position from individual to individual, but was usually spread between the 2.5th and 4.5th ml, i.e., in a volume of 2 ml. Bands III and IV, yellowish in color, were present in close proximity to each other; on occasion, these bands appeared to the naked eye to be separated by an essentially colorless, narrow zone. This suggestion was not however borne out by densitometric scanning of the gradients (see Fig. 3), which typically showed that the absorbance at 435 nm in this region did not return to baseline levels. Band III was typically removed in the 5.3rd to 6.6th ml and Band IV in the 6.6th to 8.2nd ml; the exact position of these bands, like that of Band II, varied by ~0.2 ml in its position according to the individual. The fractions intermediate between Bands I and II, and between Bands II and III were also recovered, and are denoted respectively IF-a (i.e., intermediate fraction a) and IF-b. Material situated between Band IV and the ultracentrifugal residue in the bottom 2.5 ml was divided into two further intermediate fractions, i.e., IF-c (8.2nd to 8.7th ml) and If-d (8.7th to 10.0th ml). A total of two or three gradients (containing in all 6 or 9 ml of serum), was prepared from each subject's serum; corresponding fractions from the same serum were pooled.

All fractions were exhaustively dialyzed in "Spectrapor" membrane tubing (mol wt cut off 3,500; Spectrum Medical Industries, Los Angeles, CA) at 4°C against a solution containing sodium azide (0.02% w/v), Merthiolate (0.001%), EDTA (0.04%), Tris (5 mM), and NaCl (50 mM) at pH 7.4.

In order to establish the density profile obtained upon completion of ultracentrifugation, gradients containing only NaCl–KBr solutions were fractionated into successive 1-ml aliquots (taken from the meniscus downwards by aspiration), and their densities were determined at 15°C with the precision density meter.

Sequential ultracentrifugation (ii)

For comparative purposes, serum lipoprotein fractions (VLDL, d < 1.006 g/ml; LDL, d 1.006–1.063 and 1.024–1.045 g/ml; HDL, d 1.070–1.21; HDL_2, d 1.063–1.125; and HDL_3, d 1.125–1.210 g/ml) were isolated by sequential ultracentrifugal flotation essentially as outlined by Havel, Eder, and Bragdon (4); the actual procedures (for VLDL and LDL) have been described (19, 20). The final preparations were dialyzed in the same manner as those obtained by density gradient ultracentrifugation.

Purity of lipoprotein fractions

Lipoprotein samples prepared by sequential ultracentrifugation were examined by several procedures for evaluation of their purity, primarily with the aim of assessing the degree of overlap of lipoprotein species from one fraction to another, as well as any possible contamination with serum proteins. For these reasons, immunological studies were done with an antiserum to human whole serum. VLDL and LDL fractions (densities as above) each showed a single precipitation line in the β-region which stained (separately) for protein, lipid, and carbohydrate; the arc due to VLDL was, however, of lower mobility. HDL, HDL_2, and HDL_3 each gave a single precipitin line in the α-region which stained similarly. Apo B-containing lipoproteins could not therefore be detected in these latter fractions, if indeed such were present (21); our failure to demonstrate them is probably a result of the relatively small amounts of antigen employed (~20 μg protein).

Agarose gel electrophoresis showed each fraction to give a single band of characteristic mobility. Electron microscopic examination (22) of the fractions indicated that, on the basis of the range in particle diameters, there was less than 5% overlap in the particle contents of VLDL and LDL (d 1.024–1.045 g/ml), and 1% or less in the case of LDL and HDL (d 1.070–1.21 g/ml).

These data establish the purity of the lipoproteins prepared by sequential flotation and indicate the

absence of any serum proteins as contaminants. The purity of fractions separated by the density gradient method (i) will be considered in the Results.

Characterization of lipoprotein fractions

Chemical analysis

Lipoprotein fractions prepared in density gradient series A (ultracentrifugal procedure (i)) were analyzed as follows: samples (0.5–2 mg lipoprotein in 0.5–0.7 ml) were extracted in 6.5 ml of a solvent containing heptane–isopropanol–sulfuric acid (40 mM) in the ratio 2:3.5:1 (v/v) (23). Aliquots of the organic phase were then taken for determination of total triglyceride (24), employing purified triolein (99%; Sigma) as standard. A further sample (2 mg lipoprotein) was taken for extraction by the procedure of Folch, Lees, and Sloane Stanley (25), aliquots of the organic phase being assayed for total cholesterol and total phospholipid by the procedures of Abell et al. (26) and Bartlett (27), respectively. The cholesterol used as a standard was 98.5% pure (Merck). Total protein concentrations were normally determined by the method of Lowry et al. (28) on aliquots (5–50 μg) of each (native) lipoprotein prior to delipidation: any turbidity (specifically in VLDL) was removed from the final assay mixtures by extraction with ether, a procedure which did not produce a detectable alteration in the color yield in the assays employed for calibration. Bovine serum albumin (Sigma) was used as the standard.

The lipid and protein moieties of lipoprotein preparations (2–10 mg samples) isolated in gradient series B (ultracentrifugal method (i)) were first separated by solvent extraction at 4°C (29, 30). The various lipid components were then fractionated by silicic acid chromatography, essentially as described by Hirsch and Ahrens (31); duplicate samples of each fraction were fractionated in parallel. The cholesteryl esters (Eluate I) were hydrolyzed and determined as ester cholesterol by the Liebermann-Burchard reaction (26), the same procedure being used to assay unesterified cholesterol in Eluate II; the original amount of cholesteryl esters was estimated as 1.67 × ester cholesterol, this factor representing the ratio of the average molecular weight of cholesteryl ester to free cholesterol. The triglycerides, also eluted in Eluate II, were determined by the method of Biggs, Erickson, and Moorehead (23); the total phospholipids (Eluate III) were assayed for phosphorus by Bartlett's method (27), and phospholipid phosphorus content multiplied by 25 to estimate the original amount of phospholipid. The washed protein precipitates obtained after solvent extraction (29, 30) were solubilized, either in 1 N NaOH, or in a solution containing 1% (w/v) sodium dodecyl sulfate, 1% β-mercaptoethanol, and 0.01 M sodium phosphate (pH 7.0) (solution S), and assayed (28), employing appropriate controls.

Later lipoprotein samples, isolated in gradient series B, were analyzed by a slightly modified series of procedures that took advantage of enzymatic assays for cholesterol and phospholipid. Thus, total cholesterol was estimated according to the procedure of Roeschlau, Bernt, and Gruber (32) using the enzymatic kit of Boehringer/Mannheim GmbH; ester cholesterol was determined as the difference between total and free cholesterol, the latter being assayed by the same technique in the absence of cholesterol esterase. Phospholipid was estimated directly on aliquots of each lipoprotein fraction (containing 30 μg phospholipid) by use of the 'Phospholipids B-Test Wako' (BioLyon, BP 13, 69570 Dardilly, France); this procedure is a direct and specific measure of the choline content of all choline-containing phospholipids present. Such phospholipids typically represent 95% or more of the total phospholipids of human serum VLDL, LDL, and total HDL, HDL_2 and HDL_3 (33). Triglycerides were again assayed by the method of Biggs et al. (23), and protein by that of Lowry et al. (28).

The reproducibility of these analyses was examined by calculation of the technical error, the latter being defined as $\sqrt{d^2/2N}$, where d is the difference between duplicate estimations and N the number of duplicates. The technical errors were: triglyceride 3.8%, cholesterol 1.2%, phospholipid 4.8%, and protein 5.7%.

In order to compare and validate the two series of methods applied to analysis of fractions in gradient series B, lipoprotein samples were analyzed in parallel by the two methodologies. Composition data obtained with the enzymatic (latter) series of procedures could not be distinguished from those obtained with the former (column methodology), although recoveries of total material were some 10% higher by the enzymatic methodology. For these reasons, we have not differentiated between data obtained with the two series of assay techniques in the Results section.

Electron microscopy

Samples of each fraction, at a protein concentration of 0.05–0.2 mg/ml, were negatively stained with 2% potassium phosphotungstate at pH 7.4, essentially by the procedure of Forte, Nichols, and Glaeser (22) as described earlier (20). These preparations were examined at 60 Kv with a Philips EM 300 electron microscope whose magnification had

been calibrated with a germanium-shadowed carbon replica of a ruled diffraction grating bearing 54,864 lines per inch. At least two grids of each fraction were normally examined and photographed; microscope magnifications were 43,500, 55,500, and 70,500. For determination of the frequency distribution of particle diameters, a minimum of 200 freely dispersed (and apparently intact) particles was measured on each of a series of at least three negatives. Sizes were determined with a microcomparator (L'Optique Scientifique, Paris).

Lipoprotein electrophoresis

Whole serum and lipoprotein fractions were electrophoresed on polyacrylamide gel sheets ("Lipofilm", Sebia, Issy-les-Moulineaux, France) constructed to give a discontinuous gradient from 2% (at point of sample application) to 3% (running gel). After prestaining of the lipoproteins with Sudan black, samples (2.5 μl) were electrophoresed for 1 hr at 250 V and 15 mA.

Immunological methods

The lipoprotein fractions employed as immunogens were isolated by sequential ultracentrifugation (method (ii)) under conditions described elsewhere (19, 20). Antisera to human LDL (d 1.024–1.045 g/ml) were prepared in rabbits of the Fauve de Bourgogne strain as previously described (20). As some 95–98% of the protein moiety of this LDL density fraction is represented by apolipoprotein B (20), the antisera obtained were considered as specific for this apolipoprotein. Rabbit antisera to human α-lipoprotein, to human β-lipoprotein, and to human serum albumin were supplied by Behringwerke, A.G., Marburg Lahn, West Germany, and horse antiserum to human whole serum by Institut Pasteur, Paris, France.

For the preparation of antisera to human apolipoprotein A-I, apo A-I was isolated by gel filtration chromatography of apo HDL_3 (15–40 mg) on a column (200 × 1.7 cm) of Sephacryl S-200 Superfine (Pharmacia Fine Chemicals, Sweden); the sample and elution buffer contained 6M guanidine-HCl (Merck, Darmstadt), 0.2 M Tris, and 1% (w/v) Thiodiglycol (Pierce Chemicals) at pH 8.0. The characteristics of the separation profile differed little from those originally reported for the Sephadex G-200/6 M urea system of Scanu et al. (34). After extensive dialysis against double-distilled water followed by lyophilization, samples (up to 200 μg of protein) of the eluted fractions, dissolved in detergent solution (i.e., solution S) or in 0.02 M Tris containing 8 M urea at pH 8.0, were monitored by polyacrylamide gel electrophoresis in the SDS system of Weber and Osborn (35) and in the alkaline urea system (36, 37), respectively; monomer gel concentrations employed in both techniques were either 7.5 or 10%.

Material in the leading edge of the apo A-I peak approached homogeneity, whereas the trailing portion was always contaminated substantially with apo A-II. Fractions corresponding to leading portions of the peak were therefore pooled from separate chromatographic runs, after dialysis and lyophilization. The pooled material was subsequently rechromatographed under the same conditions. The degree of purity of apo A-I was evaluated by disc gel electrophoresis in the two electrophoretic systems indicated above and by amino acid analysis. A single band was seen in both electrophoretic systems and the amino acid composition closely resembled that reported by others (34, 38).

The purified apo A-I (600 μg) was suspended in 0.2 ml of Freund's complete adjuvant and 2 ml of saline, and injected into a rabbit as described earlier (20). Two booster injections, containing the same amount of immunogen, were performed 3 and 5 weeks after the initial injection. Double immunodiffusion and immunoelectrophoresis were carried out by the techniques of Ouchterlony (39) and Scheidegger (40) respectively, under conditions previously reported (20).

The quantitation of apo B and of apo A-I was performed by the antibody overlay technique for single radial immunodiffusion of Baumstark, Lee, and Luby (41). For apo B, the calibration curves were constructed with amounts of LDL protein ranging from 0.25 to 10 μg, each in a volume of 10 μl. As seen in **Fig. 1a**, three different LDL preparations gave a calibration curve that was essentially linear (with the exception of amounts below 1 μg of protein) and highly reproducible.

Since controversial results have been reported for the quantitation of apo A-I as a function of its form (e.g., as in a native lipoprotein or delipidated or denatured material) (42–44), we compared calibration curves obtained with HDL_2, HDL_3, and total HDL (Fig. 1b). These data indicate that minor dissimilarities exist between the two HDL subfractions on the one hand, and between these subfractions and total HDL (d 1.07–1.21 g/ml) on the other. We therefore adopted HDL_3 as the working standard; its content of apo A-I was calculated as suggested by Weech et al. (45) on the basis of the amount of histidine present upon amino acid analysis (30) of delipidated HDL_3 (apo A-I contains 5 moles of histidine per mole and this residue is absent from apo A-II). A high degree of reproducibility was noted in

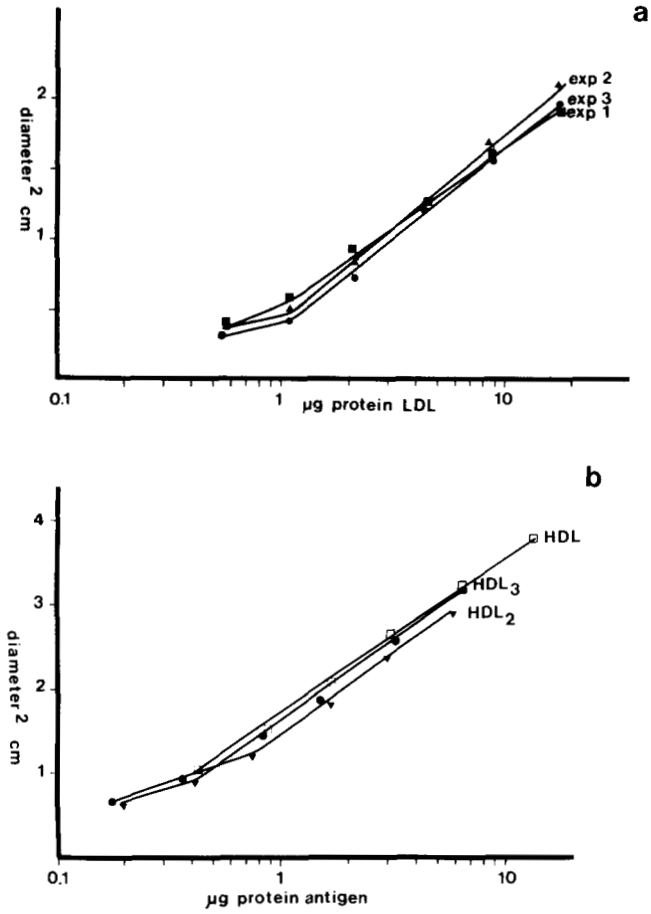

Fig. 1. Calibration curves for apolipoprotein quantitation by radial immunodiffusion assay. The square of the diameter of the immunoprecipitate (ordinate) is plotted against μg protein antigen (abscissa) on a log scale. The means of duplicate determinations at each quantity are shown. a, Standard curve for apo B, employing three separate preparations of LDL of d 1.024–1.045 g/ml as antigen. b, Comparison of standard curves for apolipoprotein A-I, using HDL_2 (d 1.063–1.125 g/ml) (▼), HDL_3 (d 1.125–1.21 g/ml) (●), and total HDL (d 1.070–1.21 g/ml) (□) as antigens.

standard curves derived from the HDL_3 separated from three different individuals.

Electrophoretic evaluation of apolipoprotein contents

Apolipoprotein components of lipoprotein fractions prepared by ultracentrifugal procedures (i) and (ii) were examined by electrophoresis in 7.5% polyacrylamide gels containing 8 M urea at pH 8.91; the procedure employed was Kane's modification (37) of the technique of Davis (36), involving solubilization of the low molecular weight apolipoprotein species (and precipitation of lipids and apolipoprotein B) by tetramethylurea (Merck-Schuchardt, 99% pure by gas–liquid chromatography). Gels were fixed and stained with Coomassie Brilliant Blue R (Sigma) as outlined earlier (30). After destaining (>72 hr at ambient temp.) in 10% trichloroacetic acid, scanning was performed at 550 nm with a Gilford 250 spectrophotometer fitted with a linear scanner. The percentage area of each stained band was determined by gravimetry after the corresponding peak had been cut from the scan. We have previously discussed the limitations of this approach to apolipoprotein quantification (30).

The total apoprotein content of each lipoprotein fraction, prepared by ultracentrifugal methods (i) or (ii), was examined by electrophoresis in SDS-polyacrylamide gels (3.3% and 7.5% acrylamide), essentially according to Weber and Osborn (35). Samples were pretreated at 90°C for 3 min after solubilization in solution S. Protein fixation and staining were carried out as outlined elsewhere (30). Molecular weights were determined from a calibration curve which had been constructed with two series of polymerized molecular weight markers (BDH Biochemicals Ltd., Poole, United Kingdom), from 14,300 to 71,500 and from 53,000 to 265,000.

Determination of lipoprotein concentrations

Lipoprotein levels in each whole serum were assessed in two ways in order to permit comparison of the methodologies: *a*) by chemical analysis of fractions isolated quantitatively with the density gradient procedure (method (ii), Series B) and, *b*), by analytical ultracentrifugation. For the latter, samples were shipped on ice at 4°C to Limoges, and analysis commenced within 48 hr of arrival.

Plasma lipoproteins were first isolated by preparative ultracentrifugation. Conditions similar to those of Ewing, Freeman, and Lindgren (46), i.e., 100,000 *g*, 18 hr, 17°C, were used for VLDL and LDL, thus attaining in the lipoprotein-containing top 1-ml a background density of 1.063 g/ml after completion of the run.

Lipoproteins with hydrated density lower than 1.20 g/ml were obtained by centrifugation in a NaCl–NaBr medium for 36 hr at 100,000 *g* and 17°C; ultracentrifugation was prolonged from the 24–26 hr used by Ewing, Freeman, and Lindgren (46) to 36 hr in order to fully recover HDL. In each type of manipulation, the second 1-ml of each tube was used for the monitoring of actual densities by means of a DMA 46 calculating density meter (A. Paar, Graz, Austria).

Analytical runs were then carried out on a MSE Centriscan 75 analytical ultracentrifuge operating in the refractometric (schlieren) mode at 550 nm. A pair of 10-mm single-sector cells was used, one of the cells being filled with the lipoprotein solution to be studied and the second filled with the NaCl–NaBr

solution corresponding to the background density of the sample. Conditions employed in the runs were 52,000 rpm (196,000 *g*) and 26°C. A wedge-window cell was used for checking the reproducibility of the settings of the optical system, at a constant selected knife-edge angle of 50°.

For the low density runs (i.e., d 1.063 g/ml) scans were taken at 9, 16, and 40 min, respectively, after the beginning of the run, thus allowing analysis of VLDL of S_f 100–400, VLDL of S_f 20–100, and LDL of S_f 0–20. In the high density run, the scan was taken after 60 min. Immediately after each scan, the knife-edge of the optical system was withdrawn from the light beam pathway and another scan taken, giving the position of the corresponding baseline.

Determination of the plasma lipoprotein concentrations from analytical scans involved calibration of the machine by spinning solutions of bovine serum albumin in NaCl (0.15 M) at known concentrations (under the same conditions as outlined above), and measurement of areas inscribed between the curves and baselines. The proportionality between the areas and concentrations of albumin and of the different classes of lipoproteins was then obtained by using the following values for specific refractive increments: 0.00189 Δn/g per 100 ml for albumin, 0.00154 Δn/g per 100 ml for VLDL and LDL, and 0.00149 Δn/g per 100 ml for HDL. These values are those proposed by Lindgren, Freeman, and Ewing (47). Areas were standardized for correction for knife-edge angle influence and radial dilution; flotation coefficients were corrected for the viscosity and density of the medium, and for concentration-dependence using a K value of 0.89×10^{-4} (mg/100 ml)$^{-1}$.

RESULTS

Our final conditions for ultracentrifugation were established after examination of the density profile of control salt gradients spun for 24, 48, 64, and 72 hr. These data showed that equilibrium was attained at 48 hr; the only detectable differences between 48 and 72 hr gradients occurred in the upper 6 ml and were minor (of the order of 0.004 g/ml).

A high degree of reproducibility in density profile was found within gradients in the same ultracentrifugal run; variability between duplicates was ~0.001 g/ml in the first 6 ml and ~0.002 g/ml in the lower 6 ml. Inter-run variation is given in **Table 1**, in which the mean density ± S.D. was calculated from three separate runs, each containing two tubes.

The density profile at 48 hr is plotted as a function of volume in **Fig. 2**; two-thirds of the gradient was linear. The shallow nature of the gradient in the uppermost 2 ml is notable.

TABLE 1. Inter-run reproducibility of density gradient profile

Gradient Fraction[a]	Mean Density ± S.D.
1	1.0155 ± 0.0010
2	1.0195 ± 0.0008
3	1.0270 ± 0.0010
4	1.0384 ± 0.0015
5	1.0528 ± 0.0014
6	1.0710 ± 0.0009
7	1.0933 ± 0.0024
8	1.1181 ± 0.0035
9	1.1451 ± 0.0047
10	1.1730 ± 0.0048
11	1.2031 ± 0.0061
12	1.2283 ± 0.0084

[a] The volume of each fraction, taken from the meniscus downwards, corresponds to 1 ml, with the exception of fraction 12 whose volume was 1.5 ml. Gradients were ultracentrifuged for 48 h at 15°C as described in Methods.

A lipoprotein mass profile obtained by densitometric scanning is shown for a representative male serum in **Fig. 3.** Four absorption maxima were consistently present, and three of these typically fell within the hydrated density ranges characteristic of LDL (1.027–1.059 g/ml), HDL_2 (1.067–1.100 g/ml),

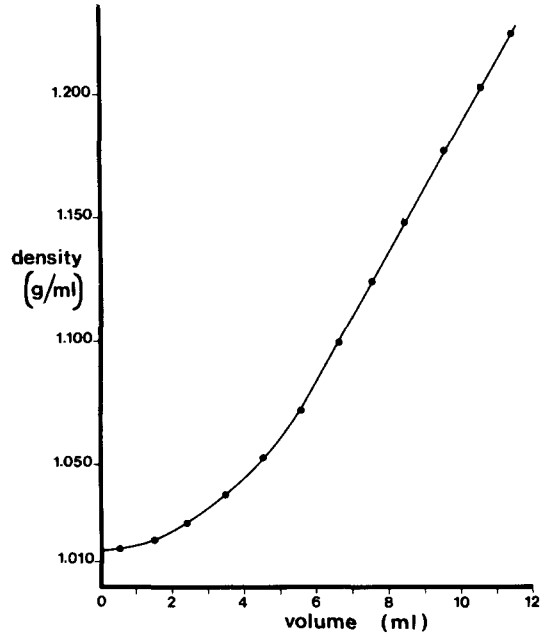

Fig. 2. Density profile obtained in "control" NaCl–KBr gradients after ultracentrifugation for 48 hr. Density (g/ml) is plotted on the ordinate against volume (from meniscus downwards) on the abscissa. Densities were determined at 15°C on successive 1-ml fractions with a digital precision density meter. Points represent the means of determinations on two gradients from each of four ultracentrifugal runs; measurements were made in duplicate or triplicate on individual fractions.

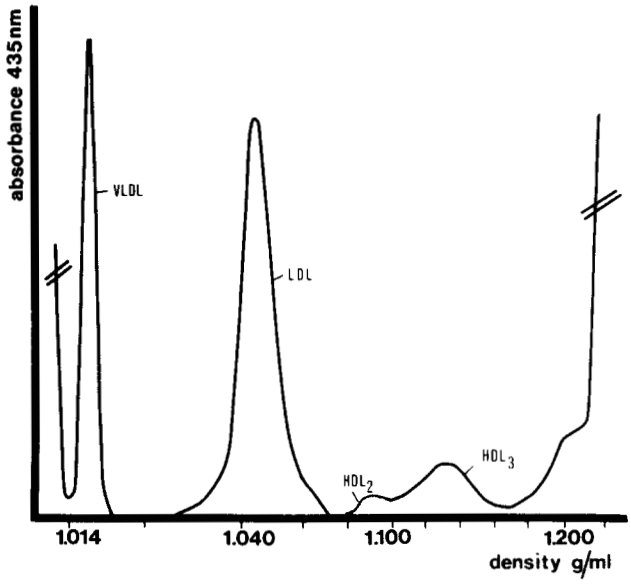

Fig. 3. Lipoprotein mass profile in the density gradient after a 48 hr centrifugal separation of a normolipidemic serum from a 33-yr-old healthy male (serum cholesterol and triglyceride 160 and 53 mg/dl, respectively). After photography on "Kodachrome" or "Ektachrome 50T", the positive was scanned at 435 nm. Absorbance is plotted against density (g/ml), the latter being derived from Fig. 2 as a function of tube length. Each absorption maximum is labeled according to its subsequent identification by chemical, physical, and immunological analyses.

and HDL_3 (1.100–1.150 g/ml) (5, 49, 50). The fourth maximum corresponded to the lightest fraction (apparently VLDL) and was present at the meniscus; its hydrated density was equal to or less than that of the solvent at this point, i.e., 1.015 g/ml. A fifth broad peak present at the bottom of the gradient corresponded to two colored layers, the upper was yellow (possibly due to bilirubin, bilirubin conjugates, retinol and very high density lipoproteins), the lower appearing reddish (probably due to transferrin and small amounts of hemoglobin).

Scanning of two or more gradients containing the same serum sample and spun in the same run revealed mass profiles that were indistinguishable. Similarly, the profiles obtained from gradients of the same serum centrifuged on different occasions within an 8-day period were essentially identical. These data attest to both the intra- and inter-run reproducibility of the fractionation.

The above findings, considered together with the observation that lipoprotein mass profile was essentially unchanged when centrifugation was prolonged to 72 hr, are consistent with the interpretation that the various lipoprotein species had attained an equilibrium distribution along the gradient after 48 hr. This would suggest that their banding densities closely approximate their hydrated densities.

Density gradient series A

In order to evaluate the distribution of lipoproteins, apolipoproteins, and serum proteins over the entire density gradient, the results of gradients fractionated into successive 1-ml portions will first be considered, i.e., gradient series A. The chemical composition of the fractions is shown in **Table 2**; the density range of each fraction was taken from the plot of density against volume (Fig. 2). Fraction 1 (density < 1.017 g/ml) exhibited the chemical properties typical of VLDL. The composition of fraction 2 is

TABLE 2. Percent weight chemical composition of human serum lipoprotein fractions isolated over the entire density gradient

	Gradient Fraction Number[a]											
	1	2	3	4	5	6	7	8	9	10	11	12
Density limits (g/ml)[b]	<1.017	1.017–1.023	1.023–1.043	1.043–1.061	1.061–1.070	1.070–1.092	1.092–1.115	1.115–1.145	1.145–1.173	1.173–1.199	1.199–1.225	1.225–1.253
						%						
Component[c]												
Total cholesterol[d]	17.2	32.0	39.4	42.5	29.5	21.1	16.5	16.4	13.2	12.8	0.1	0
Triglyceride	52.8	19.9	10.4	5.3	8.5	5.2	3.5	3.5	1.0	0.3	0	0
Phospholipid	16.2	24.1	24.1	24.0	24.8	25.9	27.4	26.7	22.1	13.1	0.4	0.3
Protein	13.8	24.1	26.1	28.1	37.2	47.7	52.5	53.4	63.7	73.7	99.5	99.7
Percent of total lipoprotein[e]	8.5	2.3	28.1	10.8	4.2	10.1	18.0	7.7	5.9	4.0		

[a] All fractions were of 1-ml volume, with the exception of fractions 2, 3, and 5 which were removed in volumes of 1.25, 1.75, and 0.5 ml, respectively.

[b] Density limits are taken from Fig. 1 as a function of volume.

[c] Values are average data from gradients of three different normolipidemic sera; each fraction was analyzed in duplicate.

[d] Values for cholesterol content represent those of free sterol and ester cholesterol; no correction for the contribution of the cholesteryl ester fatty acids has been made.

[e] Percent of total lipoprotein excludes that material present in fractions 11 and 12 that contained protein almost exclusively.

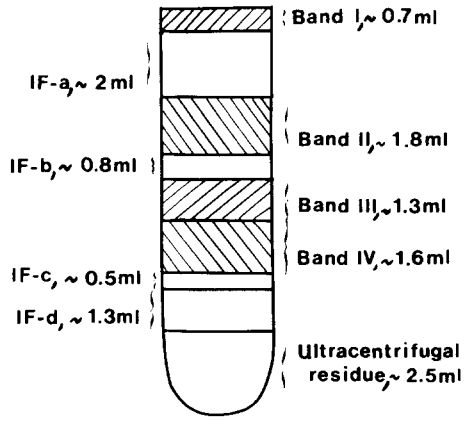

Fig. 4. Schematic representation of the distribution of lipoprotein bands I-IV (hatched areas) and intermediate fractions (IF-a–IF-d) in the density gradient after a 48-hr ultracentrifugation.

similar to that of an intermediate density lipoprotein (d 1.009–1.019 g/ml (48)). Fractions 3 and 4 exhibit compositions typical of low density lipoproteins and correspond to LDL in their overall hydrated density range, i.e., d 1.023–1.061 g/ml; together they accounted for about 39% of the lipoproteins of d < 1.199 g/ml. In the representative serum fractionation presented (Table 2), the major LDL species, (accounting for about 28% of the total lipoproteins) was that of d 1.023–1.043; it is noteworthy however that this species varied in its hydrated density (and thus relative position) from individual to individual. Fraction 5 (d 1.061–1.070 g/ml) displayed a composition intermediary between those considered typical of LDL and HDL (33).

Both fractions 6 and 7 exhibit the chemical characteristics of high density lipoproteins; they accounted for about 10 and 18%, respectively, of the total lipoproteins of d < 1.199 g/ml. Substances having the composition anticipated for HDL were also detected in small amounts (<10% of the total) in fraction 8 (d 1.115–1.145 g/ml). Comparison of fraction 9 with $VHDL_1$ of hydrated density 1.16 g/ml, that exhibits a protein:lipid ratio of 65:35 (49), suggests it to be a form of very high density lipoprotein; fraction 10 may also be a VHDL species. In contrast, fractions 11 and 12 (density range 1.199–1.253 g/ml) contain 99% protein and 1% or less lipid and do not appear to contain lipoproteins; $VHDL_2$ apparently contains 3% lipid (49).

Comparison by gradient gel electrophoresis of the lipoproteins in whole serum with those from the gradient confirmed the tentative identifications made above. Thus, material exhibiting the mobility of VLDL was present in fraction 1, while fraction 2, present in minor amounts, was seen as a faint band with mobility intermediate between VLDL and LDL of whole serum; this latter band, of weak intensity, was also evident in fraction 1. Fraction 3 showed a strong LDL band, a fainter one being present in fraction 4. Very faint bands of both LDL and HDL mobility were detected in fraction 5. Strongly staining bands corresponding to HDL were seen in fractions 6 and 7, smaller amounts being present in both fractions 8 and 9. Essentially no lipid staining material could be detected in fractions 10–12.

Immunological techniques were also employed in the study of gradient fractions 1–12, and the subsequent results represent a summary of those obtained from examination of five separate sera.

Upon immunoelectrophoresis with an antiserum to human whole serum, lipoproteins displaying reactivity typical of β-lipoproteins were predominantly found in fractions 1, 3, and 4, whereas those reacting as

TABLE 3. Comparison of the % weight chemical compositions of gradient band I and intermediate fraction-a with VLDL and IDL from normolipidemic human sera

	Density Gradient	Flotation[f]	Column Chromatography	Density Gradient	Flotation[f]	Flotation[g] Precipitation
Density fraction (g/ml)	Band I[a] <1.016	VLDL[b] <1.006	VLDL[c]	IF-a[d] 1.016–1.028	LDL-II[e] 1.009–1.019	LP-B-II[e] 1.009–1.019
Component						
Cholesteryl ester	11.7 ± 4.6	14.9	12.6	28.1 ± 4.2	29.1	25.0
Free cholesterol	6.6 ± 1.2	6.4	5.4	10.4 ± 2.1	8.4	6.8
Triglyceride	52.3 ± 4.3	49.9	41.8	17.1 ± 5.4	20.2	17.4
Phospholipid	16.5 ± 3.9	18.6	21.5	22.3 ± 2.4	23.3	27.0
Protein	12.9 ± 4.3	7.7	11.6	22.1 ± 1.2	19.0	23.6

[a] Values are the means ± S.D. of fractions isolated from the sera of seven males.
[b] Data from Mills and Taylaur (53).
[c] Data from Rudel et al. (54).
[d] Values are means ± S.D. of fractions from five (male) sera.
[e] Data from Lee and Alaupovic (48).
[f] Flotation indicates isolation by sequential ultracentrifugation (see ref. 4).
[g] Apo A-containing lipoproteins removed from LDL-II by immunoprecipitation (48).

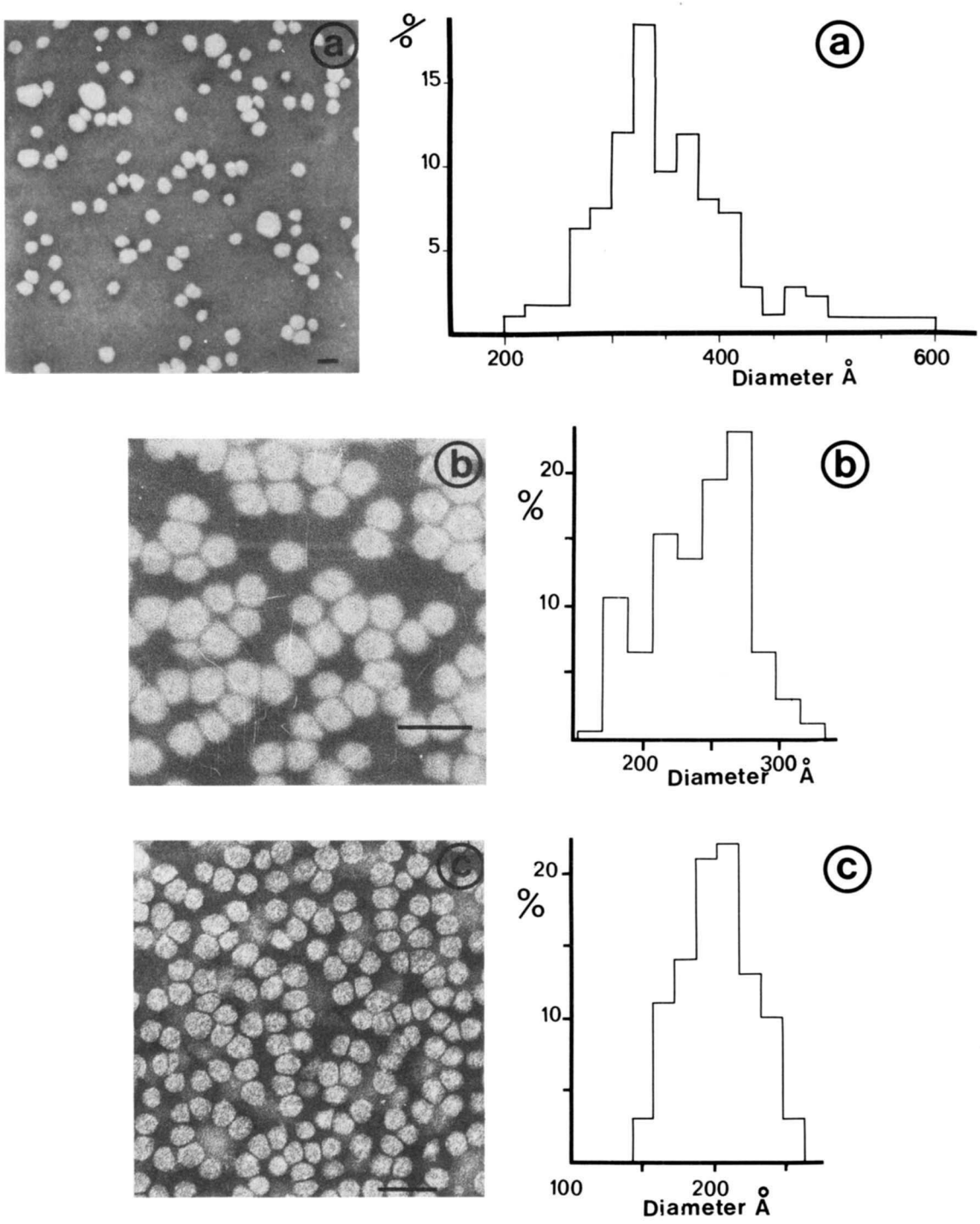

Fig. 5. Electron photomicrographs of negatively stained human serum lipoproteins isolated by density gradient ultracentrifugation (gradient series B) and the frequency distribution of particle diameters in each fraction. Samples are: (a) Band I, d < 1.016 g/ml; (b) IF-a, d 1.016–1.028 g/ml; (c) Band II, d 1.028–1.050 g/ml; (d) Band III, d 1.066–1.100 g/ml; and (e) Band IV, d 1.100–1.140 g/ml. At left is shown a representative electron micrograph of each fraction: each bar represents 500 Å. At right is shown the frequency distribution of lipoprotein particles, which excludes up to 3% of the occasional particles detected at either extreme of the ranges. Note low magnification in (a).

α-lipoproteins were primarily present in fractions 6 to 8. Some β-lipoprotein was detected in fraction 2, while some α-lipoprotein was found in fraction 9, fraction 5 containing a mixture of β- and α-lipoproteins in trace amounts.

Additional traces of β-lipoproteins were occasionally found in fraction 6. Minor amounts of lipid-staining material were found in fraction 10. These results were confirmed upon immunoelectrophoresis and immunodiffusion with antisera to LDL, HDL, and

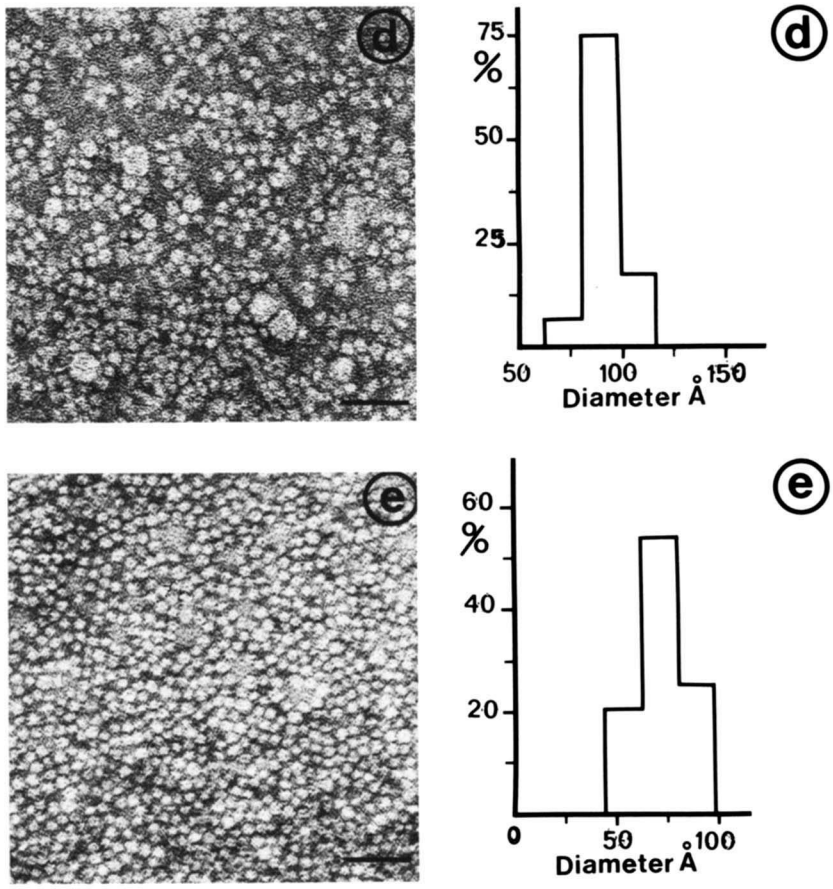

Fig. 5. *(Continued)*

serum albumin. Albumin, together with other serum proteins, was typically detected in fractions 10–12; minor quantities (<10% of total serum albumin) were found in fraction 10, the remainder being almost equally distributed between the denser fractions 11 and 12 (quantitative estimates obtained by radial immunodiffusion assay using an antiserum to human serum albumin). Recovery of lipoprotein lipid from the gradient, based on serum lipid content, was essentially complete for triglyceride and total cholesterol (98.3 ± 2.5% and 97.7 ± 1.5% in fractions from three different sera, respectively); that of phospholipid was lower and more variable (85.4 ± 7.5%).

The above results indicated that lipoproteins with the physical, chemical, and immunological properties of VLDL were present in gradient fraction 1, and that, on a similar basis, substances with the properties of IDL were present in fractions 1 and 2; LDL was in fractions 3 and 4, while material isolated in fractions 6, 7, and 8 corresponded in its general properties to high density lipoproteins.

Fraction 5, (d 1.061–1.070 g/ml) which consistently represented less than 5% of the total material of d < 1.199 g/ml, displayed both apo A- and apo B-containing lipoproteins. The trace amounts of apo B-containing material detected immunologically in fraction 6 (1.070–1.092 g/ml) probably correspond to Lp-B of hydrated density 1.084 g/ml, characterized from this density region by Kostner (21). Finally, it is noteworthy that on a quantitative basis, fraction 6, corresponding in hydrated density to HDL_2 (1.09 g/ml) (49), accounted for some 28% of the total high density material (i.e., fractions 6–8, hydrated density range 1.070–1.145 g/ml). This finding is in good agreement with published estimates of the proportion of total HDL represented by HDL_2 in adult males (15–30%) (50–52) and was confirmed in gradient series B.

Density gradient series B

In this series, we sought to remove lipoprotein fractions from the gradient as a function of their isopycnic banding, inasmuch as the bands were visible to the eye and readily detectable upon mass profiling (Fig. 3). The correspondence of the four bands (I–IV, **Fig. 4**) to VLDL, LDL, HDL_2, and HDL_3, as classically defined by sequential ultracentrifugation (33), was then assessed on the basis of chemical, physical, and immunological analyses. We also ex-

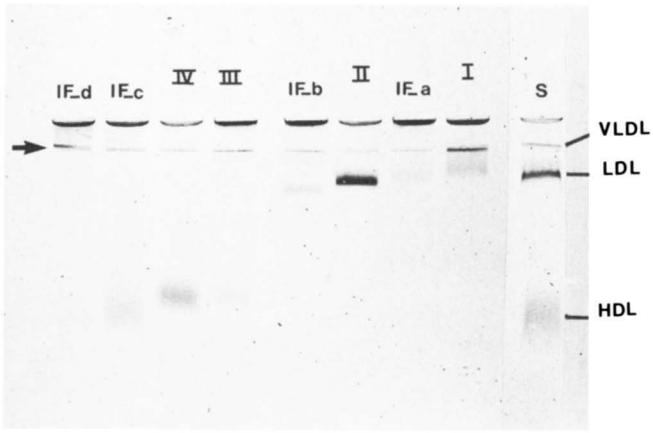

Fig. 6. Polyacrylamide gel film ('Lipofilm') electrophoresis of lipoprotein fractions from gradient series B. Samples of successive fractions, removed as indicated in Fig. 4, were prestained with Sudan black, applied in order from right to left, and electrophoresed in parallel. For comparative purposes, a sample of whole serum (S) is shown at right. The arrow indicates the junction of the 2% and 3% gels.

amined the utility of this approach as a means to determine quantitatively apolipoprotein and lipoprotein profile, and compared the latter with the classical procedure of analytical ultracentrifugation (52).

Band I from each gradient (pooled in the case of duplicate or triplicate tubes of the same serum and equivalent to fraction 1 of gradient series A) corresponded largely to VLDL in its chemical composition (**Table 3**), particle size, and morphological appearance (**Fig. 5a**), mobility on polyacrylamide gel film (**Fig. 6**), immunological reactivity, and apolipoprotein content (**Fig. 7**).

Band I was triglyceride-rich (Table 3), thereby resembling VLDL. Comparison of VLDL compositions (Table 3, ref. 11 and 33) revealed marked dissimilarities in the proportions of the various components, which may be a function of both the method of isolation (see for example Ref. 55) as well as the variation which may occur in the particle population constituting a VLDL fraction (56, 57). Band I also behaved as VLDL in remaining at the origin upon slab gel electrophoresis (Fig. 6), and by giving a precipitin arc in the β-region upon immunoelectrophoresis against anti-human whole serum (data not shown). Upon examination by negative stain electron microscopy (Fig. 5a), the morphology of the Band I particles strongly resembled that typical of VLDL (58), i.e., essentially spherical particles which deformed readily upon contact. No subunit structure was evident, although on occasion particles exhibited an electron translucent 'halo' around their periphery. Some (59) consider this a true structural manifestation whereas others (60) regard it an artifact of the technique.

In one representative preparation, the mean particle size ± S.E.M. was 349 ± 61 Å. The overall range was 200–615 Å, and the modal diameter was 330 Å (Fig. 5a). In this individual, 1% of the total VLDL particles was in the range 160–200 Å, and 6% in the range 160–260 Å; in the six fractions examined, the maximal proportion of particles in the former interval was ~10% and ~20% in the latter. Particles less than 260 Å in diameter may thus display hydrated densities approximating those of VLDL, a finding consistent with that of Groszek and Grundy (60), who reported that up to 40% of the total number of VLDL particles of $d < 1.006$ g/ml from fasting plasma may be in the size range 170–250 Å, and up to 33% in the range 100–170 Å. Under the present conditions, the presence of 160–260 Å particles in Band I (hydrated densities < 1.016 g/ml) may be at least partially due to a content of IDL (normally isolated within the density range 1.006–1.019 g/ml). This proposal is entirely compatible with the hydrated density of Band I, with its protein content (mean ~ 13%), and with the presence of an intermediate band, (additional to that of VLDL), on slab gel electrophoresis.

Fraction IF-a, intermediate between Bands I and II and of hydrated density 1.016–1.028 g/ml (Fig. 4), migrated as an IDL (Fig. 6) and exhibited a chemical composition closely resembling that of the LDL-II and LP-B-II subfractions (d 1.009–1.019 g/ml) of Lee and Alaupovic (48) (Table 3). Similarly, the morphology and particle size (mean ± SEM and modal diameters 242 ± 52 Å and 270 Å, respectively) (Fig. 5b) of

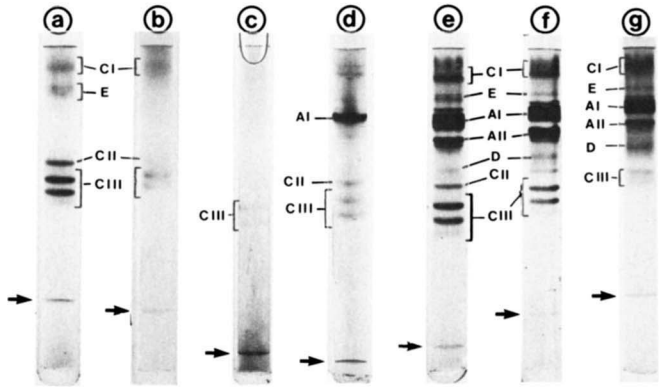

Fig. 7. Electrophoretic patterns of tetramethylurea-soluble apolipoproteins of lipoproteins separated in density gradient series B; fractions were isolated according to the scheme shown in Fig. 4. Samples (approx. 100 μg total protein) are: (a) Band I, d < 1.016 g/ml; (b) IF-a, d 1.016–1.028 g/ml; (c) Band II, d 1.028–1.050 g/ml; (d) IF-b, d 1.050–1.066 g/ml; (e) Band III, d 1.066–1.100 g/ml; (f) Band IV, d 1.100–1.140 g/ml; and (g) IF-c, d 1.140–1.153 g/ml. Electrophoresis was performed in 7.5% polyacrylamide gels containing 8 M urea at pH 8.91; gels were stained with Coomassie Brilliant Blue. Apolipoprotein nomenclature is according to Alaupovic (62). The arrows indicate the dye front in each gel.

TABLE 4. Percent weight chemical composition of gradient Band II, intermediate fraction-b and LDL fractions isolated from normolipidemic human sera

	Density Gradient[a]		Density Gradient[b]	Sequential[c] Ultracentrifugation			Column[d] Chromatography
Density fraction (g/ml)	Band II 1.028–1.050	IF-g 1.050–1.066	1.026–1.060	LDL-IV 1.030–1.040	LDL-V 1.040–1.053	LDL-VI 1.053–1.063	
Component							
Cholesteryl ester	36.8 ± 1.8	31.7 ± 1.3	38.9	39.4	37.8	38.2	39.9
Free cholesterol	9.4 ± 0.9	9.6 ± 0.5		9.7	9.1	6.7	9.4
Triglyceride	4.2 ± 0.9	7.0 ± 0.7	3.2	4.9	4.5	5.5	5.4
Phospholipid	25.8 ± 0.7	23.1 ± 2.3	24.8	22.7	23.5	22.0	22.6
Protein	23.8 ± 1.0	28.6 ± 2.1	29.1	23.3	25.1	27.6	21.6

[a] Values are the means ± S.D. of duplicate determinations on each of seven preparations isolated from the sera of males.
[b] Data taken from Foreman et al. (11).
[c] Data from Lee and Alaupovic (48).
[d] Data from Rudel et al. (54).

material in IF-a corresponded to that described for IDL by others (61) (ref. 60: range of means 231–264 Å). The overall range in diameter in all individuals was 150–360 Å; typically, less than 5% of particles were distributed below 180 Å, and no more than 10% between 300 and 360 Å.

Electrophoresis in 3.3% SDS-polyacrylamide gel showed that the major protein component of both Band I and IF-a migrated as a diffuse band of high molecular weight (>240,000); such behavior is typical of apolipoprotein B in our hands (19, 20). Smaller components (~8,000–15,000 and 27,000–31,000) were also visible in both fractions; these polypeptides were subsequently identified as apo C-I, apo C-II, apo C-III, and apo E on the basis of their characteristic migration (62, 63) in alkaline-urea polyacrylamide gel (Fig. 7b). Intermediate fraction-a was distinct in lacking apo E.

As predicted by analysis of gradient series A, Band II corresponded precisely to LDL in its physical, chemical, and immunological properties. Thus, this material was rich in cholesteryl ester (37%) and triglyceride-poor (4.2%), and closely resembled the composition of LDL isolated by alternative procedures (**Table 4**). The typical hydrated density range of Band II was 1.028–1.050 g/ml but, as noted earlier, its position varied slightly according to the individual (e.g., 1.030–1.054 and 1.026–1.049 g/ml).

Despite such minor variations in density, remarkably little difference in overall composition could be detected, as indicated by the small S.D. values (Table 4). On polyacrylamide gel slabs (Fig. 6), Band II was a single strong band that migrated to the same position as that of human serum LDL isolated by sequential ultracentrifugation. Upon immunoelectrophoresis, either against antiserum to human LDL or to human whole serum, Band II gave a single precipitin line in the β-position that stained separately for lipid, protein, and carbohydrate (data not shown). Electron microscopic examination of Band II revealed particles whose morphology was identical to that of LDL isolated by sequential ultracentrifugation (20), i.e., essentially spherical particles which deformed slightly in packed fields to give polygonal forms: no subunit structure could be discerned (Fig. 5c). In the representative preparation shown (Fig. 5c), the mean particle diameter ± S.E.M. was 211 ± 12 Å, the modal diameter was 210 Å, and the range was 160–250 Å. The fraction of d 1.028–1.050 g/ml, thus resembled that isolated at d 1.024–1.045 g/ml by the sequential procedure in its mean particle size and distribution (211 Å and 180–270 Å, respectively, (64)).

Band II (Fig. 7c), as is typical of LDL isolated in a relatively narrow density interval (20, 64), displayed only trace amounts of components with the mobilities of C-peptides, (and specifically apo C-III-1 and C-III-2); the faint diffuse staining at the top of the gel is probably indicative of the presence of apo C-I. Electrophoresis in SDS-polyacrylamide gel (not shown) subsequently revealed apolipoprotein B to be the major component of the protein moiety of Band II.

Comparison of particle diameters in Band II and IF-a indicated a significant overlap over the entire range of size seen in the former fraction (Fig. 5b, 5c). We therefore interpret our data to indicate that in this portion of the human serum lipoprotein spectrum, particles exist that exhibit distinct hydrated density and physicochemical properties but are of similar dimension.

Fraction IF-b, typically of density 1.050–1.066 g/ml, occurred immediately below the LDL band and above that of Band III (Fig. 4); it primarily contained particles with the characteristics of LDL, migrating to a similar position as Band II on slab electrophoresis (Fig. 6), and giving a strong precipitin arc in the β-position upon immunoelectrophoresis against antiserum to human LDL. The chemical composition of this fraction is comparable to the LDL-VI sub-

TABLE 5. Percent chemical composition of gradient bands III and IV, of intermediate fractions-c and -d, and of HDL subfractions from human serum

	Ultracentrifugal Method										
	Density Gradient[a]				Rate Zonal[b]		Sequential[c]		Sequential[d]		
Fraction	Band III	Band IV	IF-c	IF-d	HDL_2	HDL_3	HDL_2	HDL_3	HDL_{2b}	HDL_{2a}	HDL_3
Density (g/ml)	1.066–1.100	1.100–1.140	1.140–1.153	1.153–1.185	1.145	1.193	1.063–1.125	1.125–1.21	1.063–1.100	1.100–1.125	1.125–1.200
Component											
Cholesteryl ester	22.2 ± 1.9	19.2 ± 2.2	16.9 ± 2.1	8.7 ± 1.9	23.3	16.1	16.2	11.7	14.6	11.4	17.9
Free cholesterol	5.5 ± 1.0	2.6 ± 0.3	2.1 ± 0.7	1.0 ± 1.2			5.4	2.9	6.8	3.2	3.0
Triglyceride	4.7 ± 1.3	3.3 ± 1.1	2.5 ± 1.5	3.0 ± 1.3	6.6	3.9	5.7	6.1	2.0	1.1	1.6
Phospholipid	29.3 ± 2.7	27.1 ± 2.4	21.3 ± 2.5	9.5 ± 3.0	29.9	27.4	29.5	22.5	39.6	36.6	23.0
Protein	38.0 ± 2.5	47.8 ± 1.3	57.2 ± 3.3	77.8 ± 4.4	40.1	52.5	41	55	37.0	47.7	54.5

[a] Values are the means ± S.D. of determinations on eight separate preparations, each isolated from the serum of an adult male.
[b] Data taken from Patsch et al. (51); densities correspond to the peak in the zonal optical density profile for the respective fraction.
[c] From Scanu and Kruski (50) and adapted by them from the data of Skipski (33): unidentified lipids omitted.
[d] Data from Anderson et al. (17).

fraction of d 1.053–1.063 g/ml (48) (Table 4). While the presence of apo A-containing lipoproteins was not suspected by electrophoresis (Fig. 6), a very weak precipitin line was usually detectable upon immunoelectrophoresis of IF-b against antiserum to human HDL. This finding was confirmed by electrophoresis of the tetramethylurea-soluble apolipoproteins of IF-b, which consistently revealed the presence of apo A-I, in addition to apo C-I, apo C-II, and apo C-III; IF-b was notably deficient in apo A-II (Fig. 7d). The relative proportion of apo A-containing particles in IF-b varied according to the individual; lipoproteins containing apo B or apo A, or both, thus overlap in hydrated density in this interval (i.e., 1.050–1.066 g/ml), an observation concordant with that of Lee and Alaupovic (48).

By electron microscopy, a variable proportion (50–90%) of particles (size range 125–200 Å) in IF-b overlapped the lower portion of the recognized size range attributable to LDL (180–270 Å); the modal diameter of such particles was 180 Å. Some 25% of the latter were 150–170 Å in diameter, and may correspond to the fraction defined as HDL_1 (S_f 0–3), separated in the density interval 1.04–1.06 g/ml and displaying a mean diameter of 155 Å (65). A second, minor population of smaller particles showed a modal diameter of 90 Å and a range of 55–110 Å, a size compatible with HDL_2 (see data on Band III).

The positions of Bands III and IV varied slightly (Fig. 4) (see Methods); they could generally be removed in volumes corresponding to density intervals of 1.066–1.100 and 1.100–1.140 g/ml, respectively. Gradient series A (Table 2) indicated the presence of high density lipoproteins predominantly in the range 1.070–1.145 g/ml, thereby implying that their hydrated densities lie within or close to this range. Such findings are consistent with those of others (49, 50) who have reported the hydrated densities of HDL_2 and HDL_3 to be 1.09 and 1.12–1.15 g/ml, respectively.

The chemical composition of Band III was typical of HDL_2 subfractions (**Table 5**); its relative proportion of protein:lipid (38.0:61.7) resembled that reported for HDL_2 by Scanu (41:58.3) (49) and by Patsch et al. (40.1:59.8) (51), but tended to be distinct from that originally found by Skipski (52.3:47.7) (33). Further comparison of Band III revealed that its protein content approximated that of the HDL_{2b} subfraction (Table 5, (17)). The proportion of phospholipid in Band III was essentially identical to that in HDL_2 described by others (50, 51) and separated by both rate zonal and sequential procedures. Rather variable contents of triglyceride are evident (Table 5). The proportion of free cholesterol in Band III was within the range typically seen in the literature (3–7%, Table 5). A similar comment is applicable to its cholesteryl ester content, since examination of reported values reveals wide variation; indeed, quoted levels vary from a minimum of some 11% (HDL_{2b}; (17)) to as high as 25–27% (HDL_2 as LP-B (66); HDL_2 (67); Table 5).

If the hydrated density of particles characteristic of HDL_3 is accepted as being in the range 1.12–1.15 g/ml (49, 50), then both Band IV and IF-c (Fig. 4 and Table 5) must be considered as corresponding to this criterion. As would be anticipated, both the latter fractions were richer in protein (and poorer in lipid, particularly cholesterol) than Band III.

When the composition of these two fractions was calculated on the basis of the sum of the weights of the individual components, then the mean weight %

(±S.D.) of each constituent was: cholesteryl ester 17.7 ± 0.9, free cholesterol 2.6 ± 0.3, triglyceride 2.9 ± 1.0, phospholipid 27.8 ± 1.2, and protein 48.8 ± 1.2. A marked resemblance in composition between the latter and HDL_3 isolated by rate zonal centrifugation by Patsch et al. (51) (Table 5) and by Shepherd et al. (67) is notable. HDL_3 isolated by flotation (Table 5) and Band IV+ IF-c are less alike, although that analyzed by Anderson et al. (17) exhibits similar proportions of neutral lipids and an overall content of polar components (protein + phospholipid, 77.5%) that is indistinguishable from that of Band IV + IF-c (76.6%).

In chemical properties, Band III and Band IV + IF-c appear to correspond to HDL_2 and HDL_3, respectively; their physical and immunological properties were also consistent with this conclusion. Upon slab gel electrophoresis (Fig. 6), Bands III and IV migrated as single bands in the range of mobility of serum HDL; material in IF-c moved slightly further but again migrated as a single (diffuse) band to the typical position of HDL in the 3% gel. Furthermore, immunoelectrophoresis of Band III, Band IV, and IF-c against antiserum to human HDL resulted in production of a precipitin arc in each instance, albeit faint in the case of IF-c; each arc stained (separately) for lipid and protein. Reaction against antiserum to whole human serum and to human albumin consistently failed to detect serum albumin in any of the three fractions. Band III tended to be distinct from Band IV and IF-c, however, since it usually gave an additional (weak) precipitin arc in the β-region upon reaction against antiserum to whole serum; the contribution of apo-B-containing particles was not however quantitated.

Electron microscopic examination of the lipoproteins of Bands III and IV indicated that they displayed the morphological appearance and particle size anticipated for the HDL subclasses HDL_2 and HDL_3, respectively (Fig. 5d,e (58)). Thus, the particles in both Bands III and IV were essentially spherical when free standing, and tended to deform slightly upon close packing to assume polygonal forms, although this tendency was not marked. We were unable to discern any defined subunit structure in either Band III or Band IV HDL. In the representative sample shown (Fig. 5d), the mean diameter ± S.D. of particles in Band III was 96 ± 8 Å, the modal diameter was 90 Å and the range was 72–108 Å; these particles therefore are essentially identical to those isolated as HDL_2 by Forte and Nichols (58) and having an average diameter of 95–100 Å. Furthermore, the particle size and shape of the Band IV particles suggested that they were indistinguishable from the HDL_3 fraction examined by the latter investigators (58) in which the average diameter was 70–75 Å; in the preparation shown the mean diameter was 73 ± 12 Å, the modal diameter 72 Å and the range 54–90 Å (Fig. 7e). It is noteworthy that in the five additional preparations of Band III and of Band IV studied, particle dimensions were rather constant, being in the range 85–91 Å in mean diameter in Band III and 69–78 Å in Band IV, respectively.

Particles in IF-c could not be differentiated from those in Band IV either in size or morphology; their mean diameter was of the order of 70 Å. Examination of the apolipoprotein contents of Bands III and IV and of IF-c gave the patterns characteristic of HDL_2 (Band III) and of HDL_3 (Band IV and IF-c). Thus, in 7.5% SDS-polyacrylamide gel, the major band in both Band III and IV exhibited an average molecular weight of approximately 29,000 (range 28,000–33,000); this species probably corresponds to apo A-I. Also detectable in small amounts were components with molecular weights 40,000–48,000, 17,000–20,000, and 8,000–14,000.

Qualitatively, the tetramethylurea-soluble apoprotein patterns given by Bands III and IV were essentially indistinguishable in the six different preparations of each examined (Fig. 7e and 7f). However, marked differences in the relative proportions of the various polypeptide species were evident upon densitometric scanning of the stained gels (**Table 6**).

TABLE 6. Percent distribution of tetramethylurea-soluble apoproteins in gradient bands III and IV from normolipidemic human serum

	Percent Densitometric Area[a]	
	Band III 1.066–1.100 g/ml	Band IV 1.100–1.140 g/ml
Apolipoprotein[b]		
C-I[c]	9.3 ± 2.2	5.0 ± 2.0
E	5.3 ± 1.8	3.5 ± 1.0
Unidentified	2.3 ± 1.0	3.2 ± 2.1
A-I	46.8 ± 9.2	48.8 ± 10.4
A-II	18.8 ± 0.8	26.8 ± 1.5
D	2.8 ± 0.4	4.2 ± 2.1
C-II	2.7 ± 0.5	1.4 ± 0.1
C-III[d]	8.1 ± 2.5	4.3 ± 1.6

[a] Values are the means ± S.D. of the densitometric area of each apoprotein band in 7.5% polyacrylamide gels stained with Coomassie Brilliant blue. Three separate preparations of band III and of band IV were examined, each in duplicate gels upon which 100 μg protein was electrophoresed.

[b] Apolipoprotein nomenclature is according to Alaupovic (62); tentative identification of apo D is based on the data of McConathy and Alaupovic (68).

[c] Apo C-I was typically resolved as a diffusely stained band with some trailing; the densitometric area due to such trailing was not included in that attributed to apo C-I.

[d] C-III species present in all gels were C-III-0, C-III-1, C-III-2, and C-III-3 (traces).

TABLE 7. Lipoprotein quantitation by density gradient and analytical ultracentrifugal procedures

Fraction	Density Gradient[a]	Analytical Ultracentrifugation
	mg/dl serum ± S.E.M.	
VLDL	56 ± 9.6 (6)[b]	77 ± 11.1 (6)
LDL	281 ± 31.9 (6)	276 ± 17.9 (6)
HDL_2	71 ± 8.7 (4)	86 ± 6.0 (4)
HDL_3	216 ± 20.2 (5)	254 ± 19.7 (5)
Ratio HDL_2/HDL_3[c]	0.35 ± 0.07 (4)	0.36 ± 0.04 (4)

[a] Density gradient fractions were Band I ($d < 1.016$ g/ml), VLDL; IF-a, Band II, and IF-b (hydrated density range 1.016–1.066 g/ml) were taken together as LDL; Band III, HDL_2 (1.066–1.100 g/ml); and Band IV and IF-c (hydrated density range 1.100–1.153 g/ml) as HDL_3. The concentration of each lipoprotein class represents the sum of the individual components determined chemically.

[b] Number of samples is given in parentheses.

[c] Values for the HDL_2/HDL_3 ratio were calculated from the respective ratio in each individual and are means ± S.D.

Data obtained from the sera of normolipidemic males aged from 24 to 35 years.

Thus, the ratio of apo A-I to apo A-II in Band III was consistently greater than that seen in Band IV (mean 2.5:1 and 1.8:1, respectively); this difference was statistically significant by the paired *t*-test for sample means, $P = <0.02$ (69). The proportion of A-I in Bands III and IV was however rather similar (range 40.4–57.3% and 41.3–60.7%, respectively), while the percentage of A-II was accordingly lower in Band III than in Band IV (ranges 18.3–19.7% and 25.1–28.0%, respectively). The proportion of apo C peptides (i.e., C-I, C-II, and C-III species) was typically higher (about twofold greater) in Band III than in Band IV apoprotein; the major components were apo C-I and C-III species (Table 6). The content of the polypeptide(s) with migration typical of apo E was similarly greater in Band III than IV; the gel band tentatively identified as apo D (68) accounted for similar proportions of the soluble apoproteins from both gradient Bands III and IV. The unidentified band noted immediately above A-I in patterns from both gradient fractions accounted for <5% of the total in all cases. A further (faint) peptide band could occasionally be seen below that denoted as apo D; it amounted to 1–2% or less of the total of both Band III and IV apoproteins and was therefore omitted from Table 6. A band migrating as apo D was also detected in IF-c (Fig. 7g), but in larger amounts (10–12%); IF-c contained still less of certain C-peptides than Band IV, being deficient in apo C-III (3% or less) and occasionally lacking apo C-II entirely. The proportion of apo C-I (20–25%) in IF-c was however elevated. The ratio apo A-I:apo A-II in this fraction was slightly lower (1.4:1) than in Band IV (1.8:1).

Intermediate fraction-d (Fig. 4), of density 1.153–1.185 g/ml, approximated the $VHDL_1$ subclass in hydrated density (~1.16 g/ml) (70), but was intermediate in protein content (Table 5) (78%) between the latter (62–65% protein) and $VHDL_2$ (97–99% protein) isolated as lipoproteins of $d > 1.250$ g/ml (49, 50, 70). IF-d migrated to a similar position as IF-c on slab gel electrophoresis (Fig. 6). Moreover, immunoelectrophoresis of IF-d against antiserum to whole human serum and to human HDL resulted, in each case, in production of a faint precipitin arc corresponding to α-lipoproteins; in addition, the former antiserum revealed the presence of albumin. As in IF-c, the major polypeptides in IF-d migrated as apo A-I and apo A-II; apo C-I, C-II, C-III, apo D, and apo E were also detectable. Due to a consistently high background stain, scanning was not performed. The apoprotein content of the ultracentrifugal residue (Fig. 4) was not evaluated qualitatively.

Finally, it is noteworthy that the results of densitometric scanning provide only semi-quantitative data on the quantitative distribution of protein bands in Coomassie blue-stained gels, particularly since we have been unable to apply correction factors for the varying chromogenicities of the apolipoproteins as previously discussed (30).

Nonetheless, quantitation of apo A-I and apo A-II by radial immunodiffusion has been found to be comparable to that by polyacrylamide gel electrophoresis and quantitative densitometry (43).

Although not presented, the apoprotein patterns given in urea-polyacrylamide gels by VLDL, LDL (d 1.024–1.045 g/ml), HDL_2, and HDL_3 fractions isolated by sequential flotational ultracentrifugation were not detectably different from those of the corresponding gradient fractions, (i.e., Band I, Band II, Band III, and Band IV+ IF-c, respectively) depicted above (with the exception of the occasional absence of apo C-II in IF-c).

Lipoprotein and apolipoprotein quantitation

A comparison of lipoprotein quantitation by the density gradient and analytical ultracentrifugal procedures is shown in **Table 7** and reveals good agreement; when examined by the Wilcoxon paired rank test (71), estimation of VLDL by the two technologies was not significantly different at the 5% level. A similar finding was made for LDL, HDL_2, and HDL_3.

However, the density gradient typically gave values for VLDL some 25% lower than those measured by analytical ultracentrifugation on the same samples. A ready explanation of this discrepancy is not at hand, since quantitation of VLDL by the gradient procedure is an overestimate, representing VLDL of $d < 1.016$ g/ml. The accuracy of the analytical ultra-

centrifugal estimation of the small quantities of VLDL present in our normolipidemic males remains to be assessed. In regard to measurement of LDL and HDL_2 by the density gradient, it is relevant that IF-b, (which contained primarily LDL with small amounts of apo A-containing particles), was considered as a constituent of LDL. This may have contributed to the slight elevation in LDL levels over those seen by analytical ultracentrifugation, and at the same time, to the slight diminution in HDL_2. The mean ratio of the HDL_2:HDL_3 concentrations was evaluated in four males and found to be indistinguishable by the two techniques.

Apolipoproteins were quantitated by radial immuno-diffusion in the same group of individuals as in Table 7. Apo B was primarily present in Band II as LDL (mean concentration 43.4 ± 18 mg/dl, range 32–74), accounting for 58 ± 14% of total serum apo B (73 ± 13 mg/dl, range 56–92); such serum apo B levels are comparable to those reported by others (mean 93 mg/dl, range 58–139 in normolipidemic males) (72). Smaller amounts of apo B were detected in VLDL (3.4 ± 0.5 mg/dl), IF-a (equivalent to IDL) (mean 8 mg/dl), and IF-b (mean 6.5 mg/dl). Recovery of apo B was 80–100% in fractions of hydrated density < 1.066 g/ml: levels of apo B in lipoproteins of greater density were not determined.

Serum levels of apo A-I (mean 133 ± 0.3 mg/dl, range 98–176) paralleled those previously reported in normolipidemic males (130 mg/dl, range 95–165; (73)). Apo A-I was principally present as HDL_3, i.e., in Band IV and IF-c, where it amounted to 61 ± 9 mg/dl (range 50–72) and represented 42–63% of total serum apo A-I. Lower levels of apo A-I were found in HDL_2 (Band III), amounting to 15 ± 4 mg/dl and representing 10–15% of total apo A-I. Significant quantities of apo A-I were also found in IF-d (9–15 mg/dl; 5–15% of total) and the ultracentrifugal residue (~10 mg/dl; 5–10% of total). Recovery of apo A-I was in the range 70–90%; apo A-I was not quantitated in fractions of d < 1.066 g/ml.

On the basis of the data in Tables 5, 6, and 7, one may calculate that the mean theoretical amount of apo A-I present in HDL_2 and in HDL_3 is 12.6 and 51.4 mg/dl, respectively; such values agree well with those determined experimentally, i.e., 15 and 61 mg apo A-I/dl in HDL_2 and HDL_3, respectively.

DISCUSSION

The present report describes the design and construction of a discontinuous density gradient which, following ultracentrifugation, permits the fractionation and essentially quantitative recovery of the major lipoprotein classes (VLDL, LDL, HDL_2, and HDL_3) of normolipidemic serum in a highly purified state, i.e., lacking albumin and other serum proteins as contaminants. The principal features of this gradient are highly reproducible, such reproducibility extending to the quantitative distribution of lipoproteins along the gradient. Furthermore, the initial discontinuous gradient itself is rapid to construct, allows separation of relatively large volumes of serum (3 ml/tube), but requires of the order of $56 \times 10^7 g_{avg}$ min for the isopycnic banding of the major lipoprotein classes and sedimentation of albumin.

The position of Band II-LDL varied slightly in each subject, thus indicating differences in the density distribution and particle population of LDL between normolipidemic males of similar age; such variations have been detected by others using more sophisticated techniques (74). The gradient method therefore provides the possibility either of isolating LDL as a function of its 'peak' distribution, or of ignoring this aspect and adhering strictly to certain density limits, as is of necessity the case in separations based on the procedure of Havel, Eder, and Bragdon (4). A further possibility is to apply the optimal density limits ascertained by gradient centrifugation to the conventional preparative procedure.

Such considerations are equally applicable to the separation of high density lipoproteins, which are probably more heterogeneous than LDL and contain several subclasses (17, 49, 66, 75, 76). In this respect, we have noted that the major HDL species, (accounting for 80–90% of the total), range in hydrated density from ca. 1.070 to 1.155 g/ml; this range is slightly broader than that (1.069–1.120 g/ml) over which Foreman et al. (11) recovered HDL after gradient subfractionation, but narrower than the total (hydrated) density range (1.063–1.200 g/ml) attributed to HDL_2 and HDL_3 particles on the basis of equilibrium density gradient centrifugation by Anderson et al. (76). Indeed some discrepancy exists between our data and that of the latter workers (76), since we consistently found that particles with hydrated densities in the range 1.150–1.200 g/ml (fractions 9 and 10, Table 2 and IF-d, Table 5) tended to resemble VHDL rather than HDL species. Despite such differences, the denser HDL subfractions isolated in gradient series B, i.e., Band IV + IF-c of density 1.100–1.153 g/ml, exhibited the chemical and physical characteristics typical of HDL_3 (d 1.125–1.21 g/ml) (for details, see Results section).

On the basis of similar analyses, the less dense HDL subfraction, i.e., Band III of density 1.066–1.100 g/ml, corresponded to HDL_2 (d 1.063–1.125 g/ml). Further evidence for the equivalence between Band III and HDL_2 on the one hand and between

Band IV + IF-c and HDL_3 on the other was afforded by analysis of their apolipoprotein contents. Thus, the mean ratio of apo A-I:apo A-II in Band III (2.5:1) was significantly higher than that in Band IV (1.8:1) and IF-c (1.4:1). These findings are consistent with the original observation of Kostner et al. (77) and with the more recent report of Cheung and Albers (78), who found that the lighter HDL subfraction (d 1.063–1.092 g/ml) had a (molar) A-I/A-II ratio twice to thrice that of the heavier d 1.106–1.200 g/ml fraction.

In addition to permitting isolation of fractions with the chemical and physical characteristics of VLDL, IDL, LDL, HDL_2, and HDL_3, the gradient possesses the potential to separate at least two subfractions of very high density lipoproteins (fractions 9 and 10, d 1.145–1.199 g/ml, Table 2, and IF-d, Table 5). Essentially no lipoprotein material was detected at densities greater than 1.200 g/ml, in contrast to other density gradient techniques (10).

Clearly, the subfractionation capability of our gradient is a function of the regimen employed in the removal of fractions, i.e., either in successive fractions of 1 ml or less, or according to the detection and positioning of the various lipoprotein bands. For routine application to human sera, we would recommend the fractionation scheme depicted in Fig. 4 and described as series B. We have found such a scheme adequate not only for normolipidemic but also for hypercholesterolemic and hypertriglyceridemic sera. In the latter case, no provision is made for chylomicron separation and these must be removed prior to application of serum to the gradient; other gradient procedures (10, 11) are subject to the same limitation.

Should it be desired to assess the lipoprotein profile quantitatively, then comparison of lipoprotein quantitation by the gradient technique (combined with chemical analysis) with that by analytical ultracentrifugation (Table 7) attests to the ready and reliable application of the procedure for this purpose. Thus values for HDL_2 and HDL_3 concentrations (71 and 216 mg/dl, respectively) are entirely consistent with previously observed levels in healthy adult males (50, 51, 67, 79). Similarly, apolipoprotein profile may be assessed by use of a simple procedure, in this case radial immunodiffusion; thus, values for apo B levels in LDL (mean 43 mg/dl) and for apo A-I in HDL_2 and HDL_3 (15 and 61 mg/dl, respectively) are within the range of published estimates (14, 43, 44, 67).

The present procedure is admirably suited to evaluation of lipoprotein density distribution in animal species; we have recently described its successful application to the serum lipoprotein spectrum in the common marmoset (*Callithrix jacchus*) (80). Finally, in view of its reproducibility and power to resolve related molecular species, our procedure should find use in studies of lipoprotein metabolism as well as in clinical and dietary investigations when a complete and quantitative assessment of lipoprotein spectrum is needed.

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