papers and notes on methodology

A one-step separation of human serum high density lipoproteins 2 and 3 by rate-zonal density gradient ultracentrifugation in a swinging bucket rotor

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Abstract A method was developed for the separation of the high density lipoprotein subclasses HDL_2 and HDL_3 from human serum. Six serum samples are fractionated in a single-step ultracentrifugal procedure using the Beckman (SW-40) swinging bucket rotor. The method is based on a difference in flotation rate of the high density lipoprotein subclasses. Separation of HDL_2 and HDL_3 is accomplished by a discontinuous NaBr density gradient applied on top of 2 ml of serum brought to a density of 1.40 g/ml. After centrifugation, high density lipoprotein subclass profiles were obtained using a specially designed gradient fractionator. Contamination of the isolated high density lipoprotein subclasses by serum albumin or by apolipoprotein B-containing lipoproteins was minimal while only a slight overlap between the HDL_2 and HDL_3 profiles was observed. Chemical and immunochemical analyses of the high density lipoprotein subclasses isolated by the present method were in close agreement with the results obtained by rate-zonal density gradient ultracentrifugation in zonal rotors (Patsch, et al. 1980. *J. Biol. Chem.* **255:** 3178–3185). The major advantage of the method presented in this paper as compared with the zonal rotor method is the possibility to analyze as many as six serum samples simultaneously.—**Groot, P. H. E., L. M. Scheek, L. Havekes, W. L. van Noort, and F. M. van't Hooft.** A one-step separation of human serum high density lipoproteins 2 and 3 by rate-zonal density gradient ultracentrifugation in a swinging bucket rotor. *J. Lipid Res.* 1982. **23:** 1342–1353.

Supplementary key words apolipoprotein A-I • apolipoprotein A-II • radial immunodiffusion

High density lipoproteins (HDL) from human plasma are isolated by ultracentrifugation in the density interval of 1.063 to 1.21 g/ml. Close examination of this density interval has shown that human HDL is heterogeneous in composition. Using the moving boundary method in the analytical ultracentrifuge, de Lalla and Gofman (1) defined two subclasses, HDL_2 and HDL_3, with flotation rates ($F^{\circ}_{1.20}$) of 3.5 to 9 and 0 to 3.5, respectively. The HDL_2 and HDL_3 subclasses in plasma have been isolated on a preparative scale by a variety of techniques, including sequential ultracentrifugation (2), rate-zonal ultracentrifugation (3, 4), density gradient equilibrium centrifugation (5, 6), and polyanion precipitation (7). An excellent separation of HDL_2 and HDL_3 was obtained by rate zonal ultracentrifugation using a zonal rotor (3, 4). By this method plasma is subfractionated on a discontinuous sodium bromide density gradient during a one-step ultracentrifugal procedure and a profile of the HDL subclass distribution is obtained as the rotor is discharged. However, this method also has some drawbacks. As a consequence of the large rotor volume, HDL subclasses are isolated in a rather diluted form. Moreover, the experimental set-up allows the analysis

Abbreviations: VLDL, very low density lipoproteins of $d < 1.006$ g/ml; LDL, low density lipoproteins of $1.006 < d < 1.063$ g/ml; HDL, high density lipoproteins of $1.063 < d < 1.21$ g/ml; HDL_2, HDL of $1.063 < d < 1.125$ g/ml unless otherwise defined; HDL_3, HDL of $1.125 < d < 1.21$ g/ml unless otherwise defined; apoA-I, A-II, B, or C, apolipoprotein A-I, A-II, B, or C; EC, cholesteryl esters; UC, unesterified cholesterol; PL, phospholipids; EDTA, ethylenediaminetetraacetic acid; TMU, tetramethylurea; SDS, sodium dodecyl sulfate; RID, radial immunodiffusion.

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of only one sample during each (24 hr) centrifugation period. Therefore zonal ultracentrifugation is less attractive for studies in which a large number of samples have to be analyzed simultaneously. We have developed a new method for the separation of HDL_2 and HDL_3 in human serum using a one-step ultracentrifugal procedure with the SW-40 swinging bucket rotor. Separation is accomplished by density gradient ultracentrifugation. The underlying principle of our method is rate movement, similar to the zonal rotor method. The HDL subclass profile that can be obtained by this method approaches the resolution of the zonal rotor method, but six serum samples can be analyzed during each (21 hr) centrifugation period. In the present study this new method is evaluated.

MATERIALS AND METHODS

Blood samples

Blood was obtained at 9 AM from fasting healthy normolipidemic subjects, aged 20 to 39 years. The blood was allowed to clot for 3 hr at 0°C. Serum was isolated at 4°C by low speed centrifugation and an EDTA solution (pH 7.4) was added (final concentration 2.5 mM).

Isolation of the HDL_2 and HDL_3 subclasses

The procedure for the lipoprotein isolation was started on the day of the blood collection. Serum was adjusted to the nonprotein solvent density of 1.40 g/ml with solid NaBr. Two-ml aliquots of this solution were pipetted on the bottom of nitrocellulose Spinco SW-40 tubes (Beckman Instruments Inc., Palo Alto, CA) and carefully overlayered with NaBr/1 mM EDTA (pH 7.4) solutions using 5-ml syringes without plungers connected to umbrella-shaped long lumbar puncture needles. The composition of the discontinuous NaBr density gradient that resulted in an optimal separation of HDL_2 and HDL_3 and a minimal contamination of the HDL subclasses by serum albumin or apolipoprotein B-containing lipoproteins was worked out in initial studies. Results of a set of experiments are shown in **Fig. 1.** In all six density gradients, in which the density of the main layer of 7.5 ml was varied between 1.15 and 1.20 g/ml, a bimodal distribution of HDL cholesterol was observed. The best separation was obtained by layering from bottom to top, 2.5, 7.5, and 2.0 ml of NaBr (or H_2O)/1 mM EDTA (pH 7.4) solutions with densities of 1.25, 1.19, and 1.00 g/ml respectively. These conditions were used in all the subsequent experiments. The tubes were centrifuged at 15°C for 21 hr at 40,000 rpm in a Beckman Ti SW-40 swinging bucket rotor using a Kontron TGA-65 ultracentrifuge (MSE Scientific Instruments, Crawley, England). The rotor was stopped without braking. The gradients were fractionated using a specially designed fractionator (**Fig. 2**) connected to a micropump (model 2132) and an Uvicord III U.V. spectrophotometer (both from LKB, Bromma, Sweden) equipped with a quartz micro flow-through cell. Absorbance at 280 nm was measured. The flow rate was 0.56 ml/min and 37 fractions of 0.28 ml were

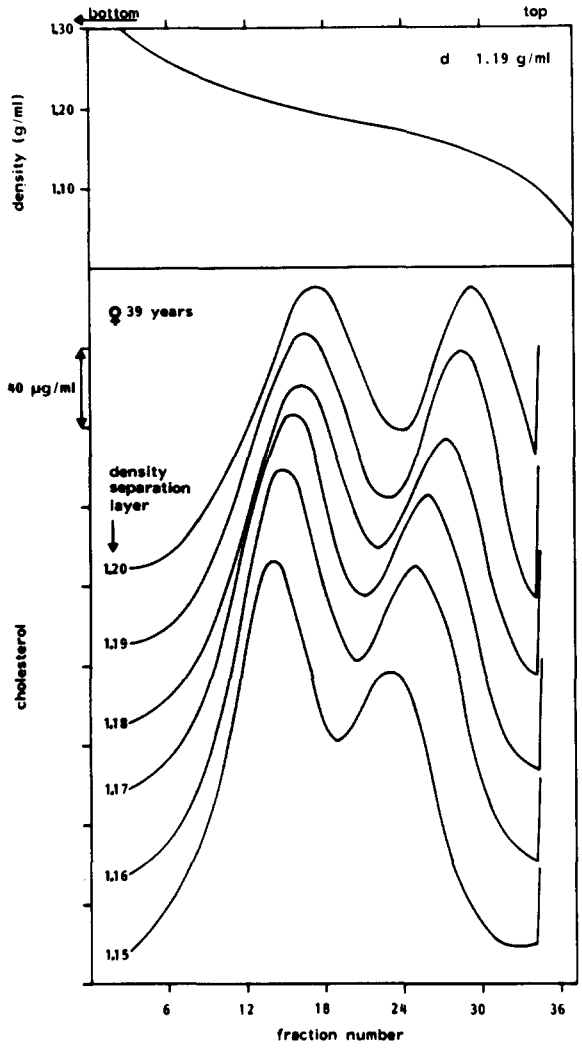

Fig. 1. The effect of NaBr density gradient composition on the separation of human HDL_2 and HDL_3. Two-ml aliquots of serum, brought to a nonprotein solvent density of 1.40 g/ml with solid NaBr, were overlayered with 2.5 ml NaBr–1 mM EDTA (pH 7.4) of d 1.25 g/ml, 7.5 ml NaBr–1 mM EDTA (pH 7.4) of a density as indicated in the figure and 2.0 ml 1 mM EDTA (pH 7.4). The gradients were centrifuged and fractionated as indicated in the text. Cholesterol concentrations were measured in all the 37 fractions collected from each gradient and are shown in the figure. For reasons of clarity the individual data points are omitted. Albumin and apolipoprotein B were detected in fractions 3 to 12 and 34 to 37, respectively (not shown). Albumin concentrations were less than 10 µg/ml in fractions 9 to 12. Note the difference in zero cholesterol concentration which is raised by one unit (40 µg/ml) for each successive profile. The top panel shows the density profile after centrifugation in the gradient with 7.5 ml of NaBr solution of a density of 1.19 g/ml which was used in all subsequent studies.

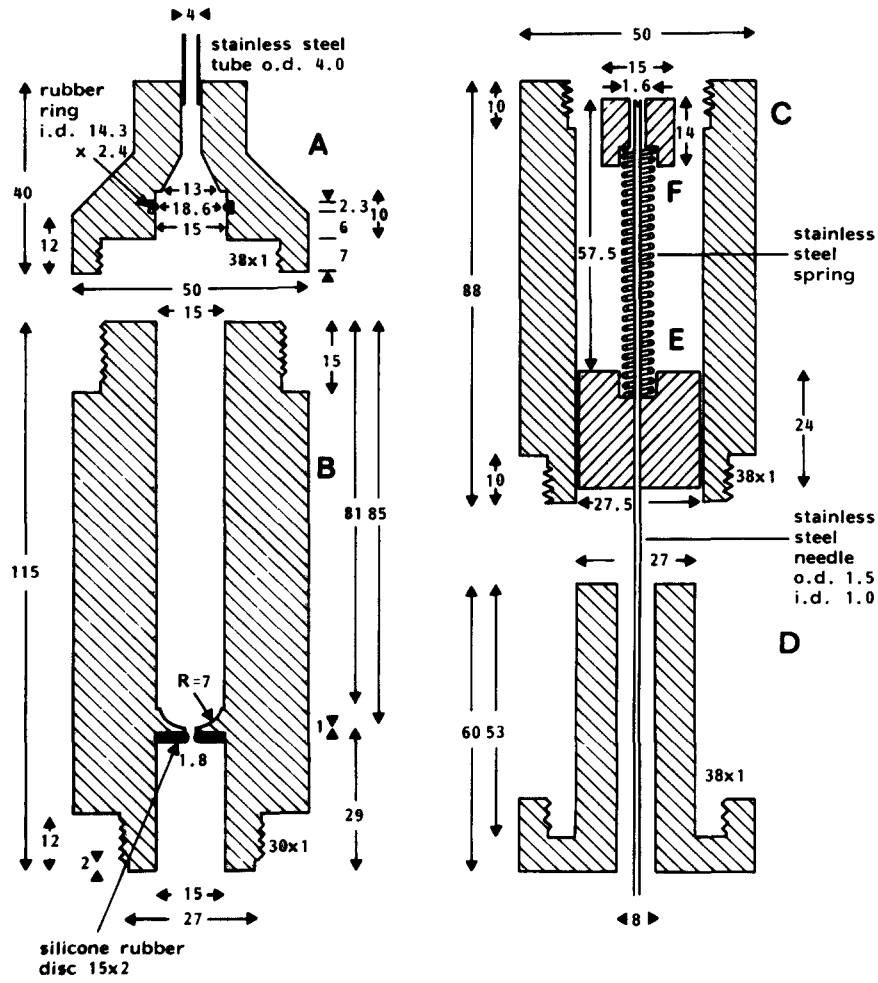

Fig. 2. Apparatus to fractionate density gradients in Spinco SW-40 tubes. The apparatus is constructed out of perspex except for parts E and F which are made of polyvinylchloride. The apparatus was assembled in the following way. Part B is screwed on top of part C and attached to a labstand. Next, the Spinco SW-40 nitrocellulose tube is placed in position in part B and part A is screwed tightly on top of part B. By this action the diameter of the tube increases slightly and the tube is tightly pressed against the inner wall of part B. The air inlet on part A, which is connected to a rubber tubing, is closed with an artery clamp. The silicone rubber disk, slightly greased with vaseline, is placed in position in part B. Parts EF are slipped into part D and the needle and the tubing, which connects the needle outlet with the micropump, are filled with a NaBr solution of d 1.30 g/ml. Parts DEF are now slipped into part C and moved up until the needle hits the bottom of the centrifuge tube. The tube is punctured (leakage is prevented by the pressure of part F on the silicone rubber disk) and part DEF is carefully moved up and tightened by screwing part D on part C. The air inlet on part A is opened and the fractionation of the gradient is started by switching on the micropump.

collected from each tube. The gradient fractionator was designed in such a way that the nonlipoprotein serum proteins (bottom phase) remained in the bottom 3.55 ml of the tube during the fractionation. As a result of this construction the contamination of the collected lipoproteins by these residual serum proteins was found to be minimal. The bottom phase could be recovered after the lipoprotein phase had been removed. The fractionation procedure was performed at room temperature and six tubes, the content of one rotor, could be fractionated in 3 hr. During this time the 280 nm absorbance profile of the effluent was not changed by diffusion.

Determination of the apparent hydrated densities of isolated HDL_2 and HDL_3

The apparent hydrated densities of HDL_2 and HDL_3 were determined by equilibrium density gradient ultracentrifugation. HDL_2 and HDL_3 were isolated from serum using the procedure described in the preceding section. The isolated high density lipoprotein subclasses were subsequently analyzed by equilibrium density gradient ultracentrifugation in a gradient described by Chapman et al. (6), but adapted to a Spinco SW-40 rotor. Tubes were centrifuged at 15°C at 40,000 rpm for 48 or 72 hr and the gradients were fractionated as described in the preceding section.

All the density measurements were performed at 15°C with a digital precision density meter (model DMA 40, Anton Paar, Graz, Austria).

Analytical ultracentrifugation of isolated HDL_2 and HDL_3

Analytical ultracentrifugation was performed in a Beckman model E instrument using the An-D rotor and a Kel F double sector cell. For measurements of flotation rates, either schlieren optics or the photoelectric scanning system (at 280 nm) was used. Measurements were performed at 52,000 rpm, 26°C and at a density of 1.200 g/ml (0.195 M NaCl, 2.762 M NaBr, and 0.1 g/l EDTA, pH 7.4). Photographs or scans were taken every 8 min for a period of 80 min after reaching full speed and $F_{1.20}$ flotation rates were calculated from ln x versus t plots.

Characterization of the lipoprotein fractions

Chemical analysis. Cholesterol was determined by an enzymatic method (8) using cholesterol esterase (cat. no. 161772) and a free cholesterol test combination (cat. no. 310328) both from Boehringer Mannheim GmbH, G.F.R. Unesterified cholesterol was determined with the same method except that cholesterol esterase was omitted from the incubation mixture.

The triglycerides in HDL_2 and HDL_3 were determined in pooled gradient fractions. Lipids in 2.5 to 6 ml of the HDL_2 or HDL_3 pool fractions were extracted according to Bligh and Dyer (9) in a chloroform–methanol–water 2:2:1.8 (v/v) system. The chloroform phase of this extraction was transferred to a glass tube and the solvent was evaporated at 50°C under a stream of nitrogen. The lipid residue was solubilized in 5 ml of isopropylether–ethanol 95:5 (v/v) and 0.1 ml of water was added. Just before use, the isopropylether was passed through an Al_2O_3 column, to remove peroxides. Phospholipids in the lipid extract were removed with 0.5 g of SiO_2 and triglyceride glycerol was determined as described by Laurell (10). Triolein standards were treated similarly. The triglyceride concentrations are expressed as triolein.

Total protein was determined by the method of Lowry et al. (11) with bovine serum albumin as standard. Aliquots of the gradient fractions (50 μl) were used without prior delipidation. Turbidity by lipids after the color development could only be detected in the top fractions of the gradients and was removed by extractions with diethylether.

Phospholipid phosphorus concentrations were determined according to Bartlett (12). Prior to this procedure, lipoproteins were precipitated with trichloroacetic acid (final concentration 5%, w/v) and collected by centrifugation. The phospholipid phosphorus content was multiplied by 25 to approximate the original amount of phospholipid.

Immunological methods. Apolipoprotein B concentrations were determined by quantitative immunoelectrophoresis according to Laurell (13) as described previously (14). Human serum albumin concentrations were measured by radial immunodiffusion (RID) using an antiserum raised in rabbits (cat. no. 103113 B, Behring Werke AG, Marburg, G.F.R.). Pure human serum albumin was used as the standard.

Apolipoprotein A-I and A-II concentrations were determined by radial immunodiffusion. Monospecific antisera against pure human apolipoprotein A-I and A-II were raised in goats.

The gel plates for the apolipoprotein A-I assay were prepared as described before (15) and contained 1% (w/v) agarose, 0.01% (w/v) NaN_3, 30 mM sodium barbital buffer (pH 8.6), and 1.18% (v/v) antiserum. The thickness of the antiserum-supporting gel layer was 1.5 mm. Wells (diameter 3 mm) were introduced with a punch. Prior to application to the RID plate, samples were delipidated with tetramethylurea (TMU) (16). Aliquots (50 μl) of the serum standard, gradient fractions, or sera were mixed with redistilled TMU (50 μl), incubated at room temperature for 30 min and diluted with 150 μl of a 10 M urea–20 mM Tris-HCl (pH 8.0) solution. The final dilutions that were applied to the RID plate (serum 1/200–1/25; gradient fractions (1/10–1/15) contained 6 M urea, 10% (v/v) TMU, and 12 mM Tris-HCl (pH 8), and were prepared by appropriate dilution. Aliquots (7 μl) of these samples were applied to the wells, using a microsyringe, and allowed to diffuse into the antiserum-containing gel during a 44- to 64-hr period at 37°C in a humid chamber. The ring-shaped immunoprecipitates, stained as described previously (15), were measured in 0.1-mm units using a measuring projector designed for this purpose (Model VDC 02, Behring Werke AG, Marburg, G.F.R.). For the day to day standardization, a serum pool of known apolipoprotein A-I content was used. Eight dilutions (1/250–1/20) of the standard were applied to each RID plate and the surface area of the immunoprecipitate rings was found to be linearly related to the standard serum concentration ($r > 0.995$). The apolipoprotein A-I concentration in the serum standard was determined by the same procedure using pure apolipoprotein A-I as primary standard. The protein content in the primary standard was determined by the method of Lowry et al. (11) using bovine serum albumin as reference protein.

The gel plates for the apolipoprotein A-II assay contained 1% (w/v) agarose, 5% dextran T-10 (Pharmacia, Uppsala, Sweden), 0.01% (w/v) NaN_3, 30 mM sodium barbital buffer (pH 8.6), and 3.3% (v/v) antiserum.

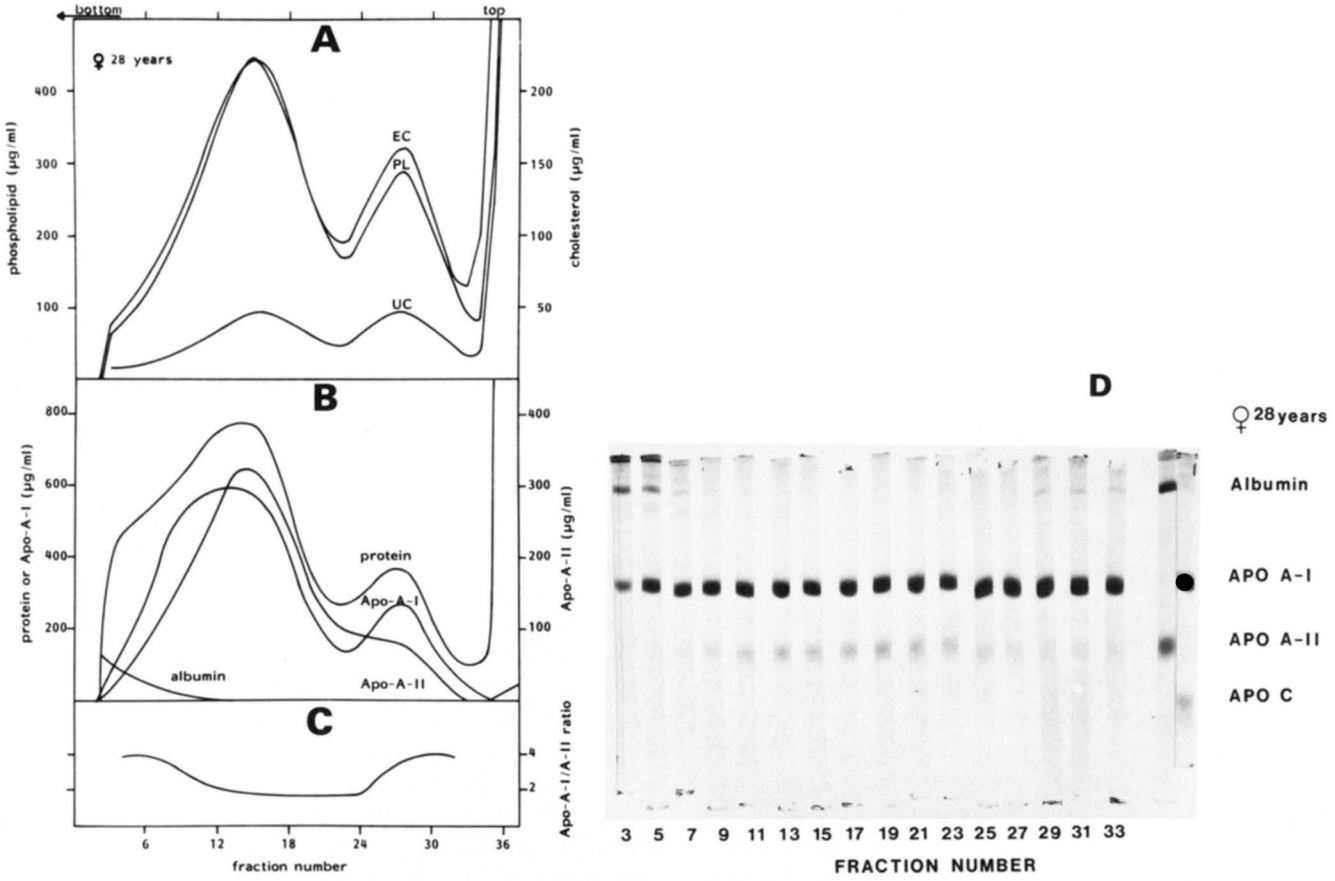

Fig. 3. HDL subclass profiles after rate-zonal ultracentrifugation of serum on density gradients in the SW-40 rotor. Details of the ultracentrifugation are described in Materials and Methods. Serum was obtained from a female (left) and male (right) healthy donor. Corresponding fractions from six identical density gradients were pooled and analyzed for phospholipid (PL), cholesteryl ester (EC), unesterified cholesterol (UC) (Fig. 3A); protein, apolipoprotein A-I (apoA-I), apolipoprotein A-II (apoA-II), and albumin are in Fig. 3B; apolipoprotein B is not shown. Concentrations are expressed in μg/ml gradient fraction (volume 6 × 0.28 ml). Apolipoprotein A-I/A-II ratios (mg/mg protein) are shown in Fig. 3C. For reasons of clarity the individual data points are omitted in this figure. Fig. 3D; protein pattern analyzed by SDS polyacrylamide gel electrophoresis. Aliquots containing 10 μg of protein (left) and 15 μg of protein (right) were applied to each gel. Pure human apoA-I, apoA-II, apoC-III, and bovine serum albumin were applied to the reference gels.

Aliquots of the serum standard, gradient fractions, or sera were applied to the RID plate without prior delipidation (17, 18) after appropriate dilution (serum 1/100–1/10; gradient fractions, 1/5–1/8) with 30 mM sodium barbital buffer (pH 8.6). The gel plates were treated as described above. The apolipoprotein A-II concentration in the serum standard was determined after delipidation as described for the apoA-I assay. Pure apolipoprotein A-II was used as primary standard. The interassay coefficients of variation were 4% and 6% for apoA-I and apoA-II analysis, respectively.

Electrophoretic methods. The protein composition of the density gradient fractions was analyzed by SDS polyacrylamide gel electrophoresis on 12% (w/v) gels according to Weber and Osborne (19). Samples were extensively dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4) in Spectrapor membrane tubing (cut-off mol wt 3500, Spectrum Medical Industries, Los Angeles, CA) and delipidated at 4°C with 20 vol ethanol–diethylether 3:1 (v/v) according to Brown, Levy, and Fredrikson (20). The apolipoproteins were solubilized in 100 mM sodium phosphate buffer (pH 7.0)/2% (w/v) SDS. A slight turbidity was removed by centrifugation and the protein content of the supernatant was determined by the method of Lowry et al. (11). Gels were loaded with 10 or 15 μg of protein.

RESULTS

Separation of HDL_2 and HDL_3

Ultracentrifugation of human serum in discontinuous sodium bromide density gradients resulted in a bimodal distribution of the high density lipoproteins that is based on a difference in flotation rate of HDL subclasses (Fig. 1). To investigate the nature of these

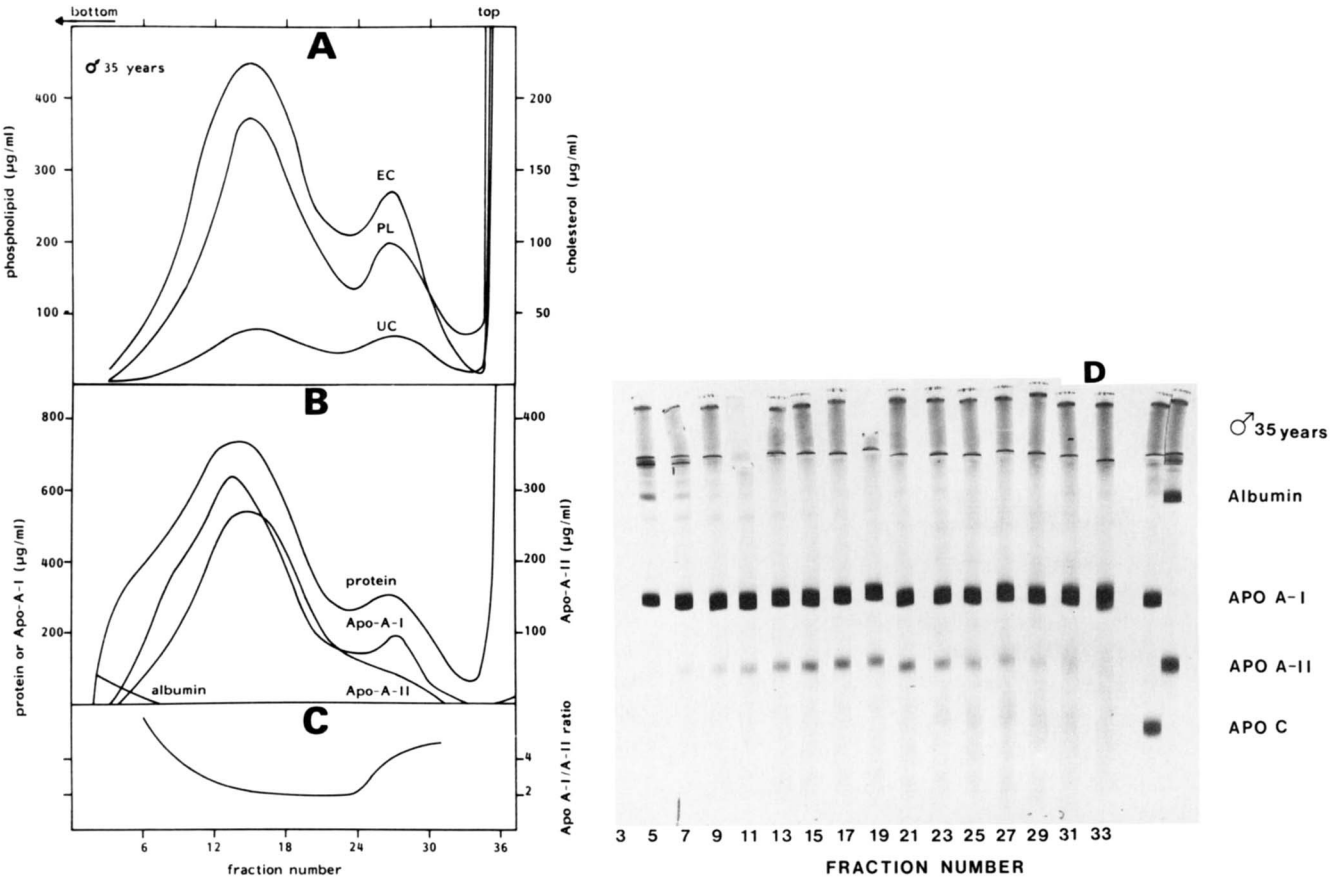

Fig. 3. Continued

HDL subclasses, serums from a normolipidemic female and male were centrifuged and the lipid and protein profiles were analyzed (**Fig. 3**). There was clearly a bimodal distribution of all the constituents of HDL. For both sexes the tube numbers, in which the two major populations of HDL were recovered from the gradient, were rather similar. From the data in Fig. 3A and 3B, we calculated the mean chemical composition of the HDL subclass with low (fractions 7 to 22) and high flotation rate (fractions 24 to 32). The compositions of the low flotation rate subclass [54.9%, 56.9% (w/w) protein; 27.7%, 24.2% (w/w) phospholipid; 14.2%, 16.2% (w/w) cholesteryl esters; and 2.9%, 2.7% (w/w) unesterified cholesterol for the female and male serum, respectively] and the high flotation rate subclass [41.5%, 46.1% (w/w) protein; 33.9%, 29% (w/w) phospholipid; 18.9%, 18.7% (w/w) cholesteryl esters; and 5.7%, 6.2% (w/w) unesterified cholesterol for the female and male serum, respectively] are comparable with the compositions of HDL_3 and HDL_2, respectively, isolated by rate-zonal ultracentrifugation in the zonal rotor (4, 21). Therefore we have tentatively designated these two HDL subclasses as HDL_2 and HDL_3.

Measurements of the apolipoprotein A-I and A-II profiles by RID indicated that the two major HDL apolipoproteins were distributed differently. Most of the apoA-I and apoA-II was associated with HDL_3 (fractions 3 to 22). However, the apolipoprotein A-I/A-II ratio was higher in the "heavy" HDL_3 region (fractions 3 to 12) as compared with the bulk of the HDL_3 peak (Fig. 3C), which suggests that HDL_3 is heterogeneous in composition.

A second peak in the apoA-I profile coincided with HDL_2 (fractions 24 to 32), while the apoA-II profile only showed a shoulder in this region of the gradient. The apolipoprotein A-I/A-II ratio was higher in HDL_2 than in the bulk of HDL_3 (Fig. 3C). ApoA-I was also found in the bottom phase (10.8% and 9.4% of the serum apoA-I concentration) and to a very small extent in association with the apolipoprotein B-containing lipoproteins (fractions 36 and 37; approximately 0.5%). ApoA-II could not be detected in these fractions. The recoveries of apolipoproteins after the fractionation procedure were 94% and 88.9% (apoA-I) and 98.3% and 77% (apoA-II) for the female and male, respectively.

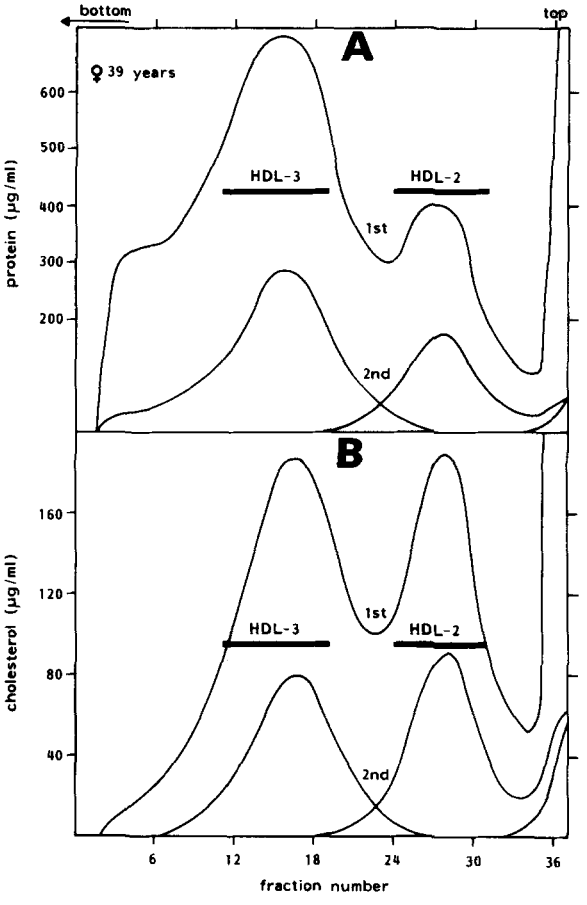

Fig. 4. The effect of recentrifugation of HDL_2 and HDL_3 isolated by rate-zonal ultracentrifugation on density gradients in the SW-40 rotor. The protein and cholesterol profiles of HDL_2 and HDL_3, isolated from serum of a female donor as described in Materials and Methods, were determined (Fig. 4A and 4B, first centrifugation). Fractions were pooled as indicated by the black bars and the densities of the HDL_2 and HDL_3 pooled fractions were determined. Part of the HDL_3 pool (1.80 ml) and the HDL_2 pool (1.75 ml) were adjusted to a nonprotein solvent density of 1.40 g/ml using solid NaBr (expanded volumes were 2 ml) and recentrifuged under identical conditions as applied in the first centrifugation. Protein and cholesterol profiles after the second centrifugation are shown (Fig. 4A and 4B, 2nd centrifugation). For reasons of clarity the individual data points are omitted in this figure.

The gradient fractions were also analyzed for albumin and apolipoprotein B. Albumin was detected in the first 12 fractions and a slight contamination with nonlipoprotein serum proteins probably explains the shoulder in the protein profile in this region of the gradient Fig. 3B). ApoB was associated with the top fractions of the gradient (fractions 34 to 37). VLDL and LDL were not separated from each other by this procedure. All the observations based on specific immunoassays were confirmed by SDS-polyacrylamide electrophoresis using delipidated gradient fractions (Fig. 3D).

Recentrifugation of isolated HDL_2 and HDL_3

We recentrifuged isolated HDL_2 and HDL_3 in order to test whether these HDL subclasses, isolated by the present method, were stable particle populations. The results are shown in **Fig. 4.** The protein and cholesterol profiles of HDL_2 and HDL_3 after the second centrifugation were similar to the original profiles. This indicates that stable particle populations had been isolated.

Determination of the apparent density of isolated HDL_2 and HDL_3

To obtain information about the physical properties of the HDL_2 and HDL_3 subpopulations, isolated by the present method, we determined the apparent hydrated densities by equilibrium density gradient centrifugation. Isolated HDL_2 or HDL_3 (fraction number 23–32 and 7–22, Fig. 3) from a female and male donor was recentrifuged in KBr density gradients until flotation equilibrium was reached. The gradients were fractionated using the fractionator described in Fig. 2 and the density and protein profile of the gradients were determined. The HDL_2 and HDL_3 were recovered in single bands with somewhat overlapping solvent densities. The top of the HDL_2 protein profile coincided with a density of 1.089 g/ml (female) and 1.093 g/ml (male) while HDL_3 was found at a density of 1.129 g/ml (female) and 1.130 g/ml (male). Prolonged centrifugation (72 hr instead of 48 hr) did not change the HDL density distribution, which indicates that the observed densities can be considered as apparent hydrated densities of HDL_2 and HDL_3.

Flotation characteristics of isolated HDL_2 and HDL_3 determined by analytical ultracentrifugation

The flotation rates of the HDL_2 and HDL_3 subpopulations were determined by analytical ultracentrifugation at a density of 1.200 g/ml and 26°C and compared to those of a total HDL fraction isolated from the same serum by conventional sequential ultracentrifugation ($1.063 < d < 1.21$ g/ml density fraction). Schlieren photographs of this study are shown in **Fig. 5.** From these data it is evident that the flotation rates in the HDL_2 and HDL_3 subpopulations, isolated by the present method, were different and overlapped only slightly. A close agreement in flotation rates of isolated HDL_2 and HDL_3 subpopulations with those in total HDL is also seen in this figure. Peak $F_{1.200}$ flotation rates were calculated from runs at a low protein concentration (0.75 mg/ml) using the photoelectric scanning equipment and were found to be 5.18 S (HDL_2) and 2.34 S (HDL_3).

The HDL_2 and HDL_3 concentration and composition in serum of normolipidemic subjects

To study the subject variability and sex-related differences in the serum HDL subclass distribution, we analyzed sera from 12 normolipidemic individuals (6 males and 6 non-pregnant females). The cholesterol

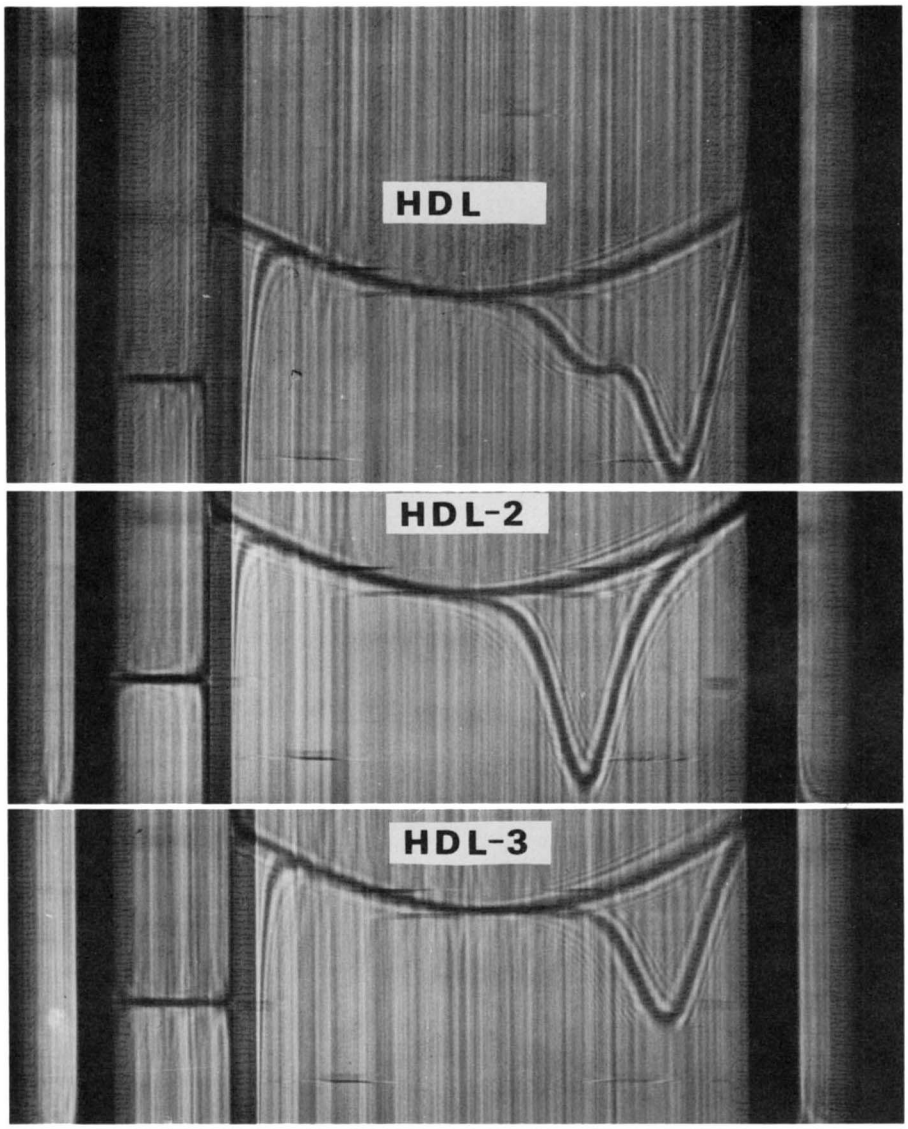

Fig. 5. Schlieren photographs of total HDL isolated by sequential ultracentrifugation ($1.063 < d < 1.21$ g/ml), HDL_2 (fractions 23 to 32, compare Figs. 3 and 6) and HDL_3 (fractions 7 to 22) of a female donor. (The HDL cholesterol profile of this subject is shown in Fig. 6 and indicated with *.) Prior to analytical ultracentrifugation, HDL_2 and HDL_3 were concentrated by flotation at d 1.24 g/ml and all HDL fractions were extensively dialyzed against 0.195 M NaCl, 2.762 M NaBr, 0.1 g/l EDTA (pH 7.4) ($d^{26°}$ 1.200 g/ml). HDL protein concentrations were 5.45 mg/ml (HDL_2 and HDL_3) or 11 mg/ml (total HDL). Cell contained 0.42 ml (HDL_2 and total HDL) or 0.40 ml (HDL_3) lipoprotein solution. All photographs were taken 64 min after reaching full speed (52,000 rpm). Direction of flotation: from right to left.

profiles are shown in **Fig. 6.** The position of the peak of the cholesterol profile of HDL_3 was identical in males and females. However, the cholesterol profiles in the HDL_2 region suggested a slight sex difference. The HDL_2 in females as compared with males was shifted about one fraction in the direction of the top of the gradient.

Based on the cholesterol analysis, gradient fractions were pooled as indicated in Fig. 6 and analyzed chemically. The serum concentrations of HDL_2 and HDL_3, calculated from these analyses, are shown in **Table 1.** The concentration (Table 1) and cholesterol profiles (Fig. 6) of HDL_2 showed a greater subject variability as compared with HDL_3. No statistically significant differences in serum HDL lipid and protein concentrations were observed between the male and female group, but the number of sera tested was small and the subject variability was large. A statistically higher concentration of HDL_2 in serum of females as compared to males has been well established (21) and we do not want to conclude from the present data that such a sex difference has to be denied. Interestingly, in two subjects, one male

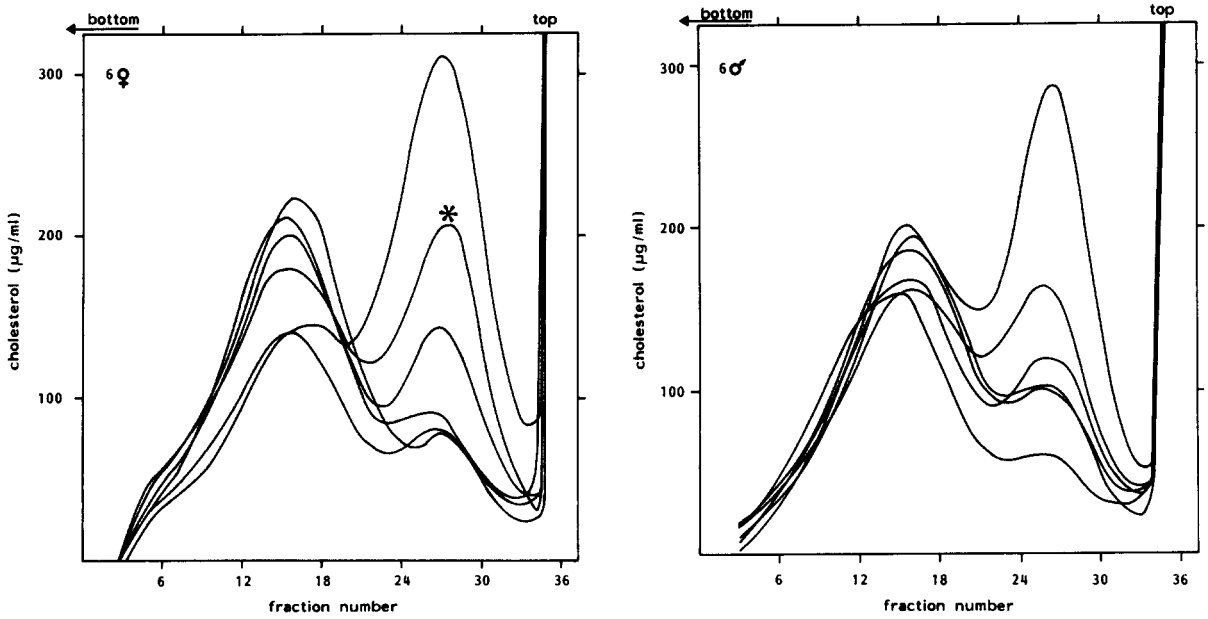

Fig. 6. The HDL cholesterol profiles of 12 normolipidemic subjects determined after rate-zonal ultracentrifugation of serum on density gradients in the SW-40 rotor. Details of the ultracentrifugation are described in Materials and Methods. Left: cholesterol profiles in sera of six females. Right: cholesterol profiles of six males. For reasons of clarity the individual data points are omitted in this figure. Fraction numbers 7 to 22 (females, HDL_3), 7 to 21 (males, HDL_3), 23 to 32 (females, HDL_2) and 22 to 32 (males, HDL_2) were pooled for each individual donor and analyzed chemically (Tables 1 and 2) and immunochemically (Table 3). The mean serum cholesterol concentration averaged 179 ± 27 (SD) mg/ml (n = 12). After centrifugation, 96 ± 6% was recovered in the isolated fractions including the bottom phases.

and one female, the serum concentrations of HDL_2 exceeded HDL_3. The serum HDL cholesterol concentration in both subjects was 72 mg/100 ml.

We calculated the chemical composition of the HDL subclasses (**Table 2**). The subject variability in HDL subclass composition was small. The protein content of HDL_2 and HDL_3 was slightly higher in males than in females. A small sex difference was also observed in the unesterified cholesterol content of HDL_2. The chemical composition of both HDL subclasses is in good agreement with previously published data from studies using other isolation procedures (3, 4, 6, 21–23). We also measured the concentration of apolipoproteins A-I and A-II in the isolated HDL subfractions using specific immunoassays (**Table 3**). No statistically significant differences were observed in serum and HDL subclasses apolipoprotein concentrations between the male and female group. Approximately 13% of the total serum apoA-I was recovered in the bottom phase of our gradient. No apoA-II could be detected in this fraction. The apolipoprotein A-I/A-II ratio in HDL_2 was slightly higher than in the HDL_3 subclass but this difference was only statistically significant in the group of six males (Table 3).

DISCUSSION

Rate-zonal ultracentrifugation of human serum on density gradients in zonal rotors has proved to be a

TABLE 1. The concentration of HDL_2 and HDL_3 in sera of 12 normolipidemic individuals

	Protein	Phospholipids	Cholesteryl Esters	Unesterified Cholesterol	Triglycerides	Total
			mg/l			
HDL_3						
Males (n = 6)	1363 ± 146	623 ± 84	400 ± 42	64 ± 7.7	35 ± 14.4	2484 ± 263
Females (n = 6)	1249 ± 216	643 ± 139	414 ± 61	74 ± 10.5	32 ± 6.3	2411 ± 419
HDL_2						
Males (n = 6)	413 ± 165	300 ± 152	205 ± 105	52 ± 24.6	22 ± 10.9	985 ± 457
Females (n = 6)	349 ± 175	293 ± 143	204 ± 110	53 ± 28.8	20 ± 10.4	919 ± 468

For details of the lipoprotein separation see Fig. 6. The concentrations are expressed in mg of lipid or protein per liter serum. For the calculations it is assumed that the molecular weights of phospholipids, cholesteryl esters, and triglycerides are 775, 651, and 885, respectively. All values are mean ± SD.

TABLE 2. The composition of HDL_2 and HDL_3 in sera of 12 normolipidemic individuals

	Protein	Phospholipids	Cholesteryl Esters	Unesterified Cholesterol	Triglycerides
			% by weight		
HDL_3					
Males (n = 6)	54.9 ± 1.5 [a]	25.0 ± 1.7	16.1 ± 1.1	2.58 ± 0.13 [a]	1.40 ± 0.58
Females (n =6)	51.8 ± 1.0 [a]	26.6 ± 1.6	17.3 ± 1.1	3.10 ± 0.2 [a]	1.38 ± 0.20
HDL_2					
Males (n = 6)	42.6 ± 2.1 [a]	30.1 ± 1.6	20.3 ± 1.7	5.2 ± 0.46	2.25 ± 0.80
Females (n = 6)	38.2 ± 0.9 [a]	32.0 ± 0.8	21.9 ± 1.0	5.7 ± 0.4	2.23 ± 0.43

[a] Statistically different at $P = 0.01$ level. All values are mean ± SD. For details see Fig. 6 and Table 1.

useful method to monitor the HDL population distribution and to separate HDL_2 and HDL_3 (3, 4, 24). However, this method also has some drawbacks: *a*) zonal rotors permit the analysis of only one serum sample at a time *b*) as a consequence of the large rotor volume dilute solutions of lipoproteins are obtained; and *c*) proper operation of a zonal rotor requires expertise. The aim of the present study was to develop a simple method to characterize the subclass distribution of the high density lipoproteins in human serum. We searched for a one-step ultracentrifugal procedure in which several small serum samples could be analyzed simultaneously. We based our method on the same separation principle as in the zonal rotor method, i.e., rate movement in a density gradient.

The results obtained with the present method are in accordance with the published data for the zonal rotor method. First of all both methods result in a bimodal distribution of the serum HDL population. Secondly, the chemical compositions of the slow and fast flotation rate subclass of HDL, observed in the present study, are in close agreement with the compositions of HDL_3 and HDL_2, respectively, isolated by the zonal rotor method (4, 21). In addition, the distribution of the major HDL peptides, apolipoproteins A-I and A-II, is similar in both methods (compare Fig. 3 with (4)). Furthermore, the apparent hydrated densities of the slow and fast flotation rate subclass of HDL, determined by equilibrium density ultracentrifugation at 15°C (1.13 and 1.091 g/ml, respectively) as compared with those of HDL_3 and HDL_2 obtained by Patsch et al. (4) in the zonal rotor at 20°C (1.14 and 1.096 g/ml, respectively) show only minor differences. Finally, the flotation rates of both HDL subpopulations determined by analytical ultracentrifugation at d 1.200 g/ml and 26°C (5.18 S and 2.34 S for the peak in the fast and slow flotation rate subclass) are comparable to those obtained by Patsch et al. (4) determined at d 1.21 g/ml (5.9 S and 2.9 S for HDL_2 and HDL_3, respectively). Therefore we propose that the two HDL subclasses isolated in the present study represent HDL_2 and HDL_3.

Although the subfractionation of HDL in the present method is based on rate movement and not simply on specific gravity, we observed a gradual decrease in pro-

TABLE 3. The concentration of apolipoproteins A-I and A-II in serum, HDL_2, and HDL_3 of 12 normolipidemic individuals

	Serum	HDL_2	HDL_3	Bottom phase
		mg/l		
Males (n = 6)				
A-I	1600 ± 186	300 ± 127	877 ± 90	206 ± 17
A-II	638 ± 46	95 ± 20	427 ± 40	0
A-I/A-II		3.12 ± 0.83	2.05 ± 0.14	
		$P < 0.01$		
Females (n = 6)				
A-I	1668 ± 189	249 ± 123	871 ± 191	234 ± 41
A-II	619 ± 103	89 ± 31	385 ± 90	0
A-I/A-II		2.73 ± 0.54	2.27 ± 0.27	
		N.S.		

For details of the lipoprotein separation see Fig. 6. All values are expressed in mg of protein per liter serum, and are the mean ± SD.

tein and increase in lipid content of HDL from bottom to top of the gradient (Fig. 3). In accordance with Patsch et al. (4), we observed that the apolipoprotein A-I/A-II ratio in HDL_3 is not uniform but higher in the protein-rich, "heavy" HDL_3 region (Fig. 3). This phenomenon has been discussed by Patsch et al. (4) and the presence of two subpopulations of HDL_3 particles, HDL_{3L} (light) and HDL_{3D} (dense), has been proposed. These observations seem to be in line with earlier studies of Cheung and Albers (25) using equilibrium density ultracentrifugation for HDL subfractionation in which a 2- to 3-times higher apolipoprotein A-I/A-II ratio was found in dense HDL_3 ($d > 1.15$ g/ml) as compared with the bulk of HDL_3.

The HDL_2 isolated by the present method is also enriched in apolipoprotein A-I as illustrated by the higher apolipoprotein A-I/A-II ratio (Fig. 3, Table 3). However, a more pronounced difference in this ratio has been found between HDL_2 and HDL_3 isolated by the zonal rotor method (in HDL_2 this ratio was 1.8- to 4-times higher than in HDL_3 (4, 21)). One explanation for this difference could be a selective loss of apolipoprotein A-I from HDL_2 during the isolation procedure. It has been found that apolipoprotein A-I is lost from HDL during ultracentrifugation (26). The g_{max} forces to which HDL is exposed in the present method (284,000 *g*) are higher than in the zonal rotor method (125,000 *g*). However, the fraction of serum apolipoprotein A-I recovered in the bottom phase in the present method (10–13%, Fig. 3 and Table 3) is only slightly higher than that found in the zonal rotor method (6–8%, ref. 4).

In this study serum instead of plasma was used for HDL subfractionation. After gradient ultracentrifugation of plasma, in spite of the presence of a surplus of EDTA to prevent clotting, a thin, floating pellicle in the bottom phase was observed. This pellicle caused disturbances in the gradient as the needle of the gradient fractionator was brought in position, thereby influencing the separation of HDL_3 from the bulk of the serum proteins.

Recent epidemiological studies have shown that susceptibility to coronary artery disease is negatively correlated with the serum high density lipoprotein cholesterol concentration (27–30). By analysis of the HDL subclass distribution, it has been observed that the subject variation in serum HDL concentrations is mainly localized in HDL_2, while serum HDL_3 concentrations showed relatively less variation (31, 32). This was confirmed in the present study. Thus, measurements of HDL_2 in population studies and in disease may allow a more sensitive discrimination than measurements of total HDL (31).

The interrelationship between HDL_2 and HDL_3 is not completely understood. It has been proposed that HDL_3 is converted into HDL_2 by uptake of a surface remnant from triglyceride-rich lipoproteins as their triglyceride moiety is hydrolyzed by lipoprotein lipase (33). The reverse process, conversion of HDL_2 into HDL_3, may be initiated by degradation of the phosphatidylcholine surface film of HDL_2, a process in which a central role of the endothelial liver lipase has been proposed (23, 34, 35). We feel that the present method for HDL subclass separation and profile analysis is a valuable tool in studies concerning the metabolic interrelationship between HDL_2 and HDL_3.

The authors wish to thank Drs. W. C. Hülsmann and A. van Tol for their valuable criticism and for reading the manuscript. Mr. A. J. J. M. Klerks is thanked for the construction of the gradient fractionator and Miss A. C. Hanson for her secretarial assistance. This work was financed in part by the Dutch Heart Foundation.

Manuscript received 14 March 1982 and in revised form 14 July 1982.

REFERENCES

1. De Lalla, O. F., and J. W. Gofman. 1954. Ultracentrifugal analysis of serum lipoproteins. *Methods Biochem. Anal.* **1:** 459–478.
2. 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.
3. Patsch, J. R., S. Sailer, G. Kostner, F. Sandhofer, A. Holasek, and H. Braunsteiner. 1974. Separation of the main lipoprotein density classes from human plasma by rate-zonal ultracentrifugation. *J. Lipid Res.* **15:** 356–366.
4. Patsch, W., G. Schonfeld, A. M. Gotto, and J. R. Patsch. 1980. Characterization of human high density lipoproteins by zonal ultracentrifugation. *J. Biol. Chem.* **255:** 3178–3185.
5. 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.
6. Chapman, M. J., S. Goldstein, D. Lagrange, and P. M. Laplaud. 1981. A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J. Lipid Res.* **22:** 339–358.
7. Gidez, L. I., G. J. Miller, M. Burstein, S. Slagle, and H. A. Eder. 1982. Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure. *J. Lipid Res.* **23:** 1206–1223.
8. Röschlau, P., E. Bernt, and W. Gruber. 1974. Enzymatische Bestimmung des Gesamtcholesteryls in Serum. *Z. Klin. Chem. Klin. Biochem.* **12:** 226.
9. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
10. Laurell, S. 1966. A method for routine determination of plasma triglycerides. *Scand. J. Clin. Lab. Invest.* **18:** 668–672.

11. 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.
12. Bartlett, C. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
13. Laurell, C. B. 1972. Electroimmunoassay. *Scand. J. Clin. Lab. Invest.*, Suppl. **124:** 21–37.
14. Brussaard, J. H., G. Dallinga-Thie, P. H. E. Groot, and M. B. Katan. 1980. Effects of amount and type of dietary fat on serum lipids, lipoproteins and apolipoproteins in man. A controlled 8-week trial. *Atherosclerosis.* **36:** 515–527.
15. Curry, M. D., P. Alaupovic, and C. A. Suenram. 1976. Determination of apolipoprotein A and its constitutive A-I and A-II polypeptides by separate electroimmunoassays. *Clin. Chem.* **22:** 315–322.
16. Albers, J. J., and M. C. Cheung. 1979. Radial immunodiffusion assay of lipoproteins and apoproteins: application to high density lipoproteins. *In* Report of the High Density Lipoprotein Methodology Workshop. K. Lippel, editor. National Institute of Health (NIH) Publication No. 79-1661. 251–264.
17. Schonfeld, G., J. Chen, W. F. McDonnell, and I. Jeng. 1977. Apolipoprotein A-II content of human plasma high density lipoproteins measured by radioimmunoassay. *J. Lipid Res.* **18:** 645–655.
18. Mao, S. J. T., A. M. Gotto, and R. L. Jackson. 1975. Immunochemistry of human plasma high density lipoproteins. Radioimmunoassay of apolipoprotein A-II. *Biochemistry.* **14:** 4127–4131.
19. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulphate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
20. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. *J. Biol. Chem.* **244:** 5687–5694.
21. Shepherd, J., C. J. Packard, J. M. Stewart, B. D. Vallance, T. D. V. Lawrie, and H. G. Morgan. 1980. The relationship between the cholesterol content and subfraction distribution of plasma high density lipoproteins. *Clin. Chim. Acta.* **101:** 57–62.
22. Shen, B. W., A. M. Scanu, and F. J. Kezdy. 1977. Structure of human serum lipoproteins inferred from compositional analysis. *Proc. Natl. Acad. Sci. USA.* **74:** 837–841.
23. Groot, P. H. E., H. Jansen, and A. van Tol. 1981. Selective degradation of the high density lipoprotein-2 subfraction by heparin-releasable liver lipase. *FEBS Lett.* **129:** 269–272.
24. Patsch, J. R., and A. M. Gotto. 1979. Separation and analysis of HDL subclasses by zonal ultracentrifugation. *In* Report of the High Density Lipoprotein Methodology Workshop. K. Lippel, editor. National Institute of Health (NIH) Publication No. 79-1661. 310–324.
25. Cheung, M. C., and J. J. Albers. 1979. Distribution of cholesterol and apolipoprotein A-I and A-II in human high density lipoprotein subfractions separated by CsCl equilibrium gradient centrifugation: evidence for HDL subpopulations with differing A-I/A-II molar ratios. *J. Lipid Res.* **20:** 200–207.
26. Fainaru, M., M. C. Glangeaud, and S. Eisenberg. 1975. Radioimmunoassay of human high density lipoprotein apo A-I. *Biochim. Biophys. Acta.* **386:** 432–443.
27. Miller, G. J., and N. E. Miller. 1975. Plasma high density lipoprotein concentration and development of ischaemic heart disease. *Lancet.* **i:** 16–19.
28. Gordon, T., W. P. Castelli, M. C. Hjortland, W. B. Kannel, and T. R. Dawber. 1977. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am. J. Med.* **62:** 707–714.
29. Castelli, W. P., J. T. Doyle, T. Gordon, C. G. Haines, M. C. Hjortland, S. B. Hulley, A. Kagan, and W. J. Zukel. 1977. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation.* **55:** 767–772.
30. Miller, N. E., O. J. Førde, D. S. Thelle, and O. D. Mjøs. 1977. The Tromsø Heart Study. High density lipoprotein and coronary heart disease: a prospective case-control study. *Lancet.* **i:** 965–968.
31. Anderson, D. W., A. V. Nichols, and H. B. Brewer. 1979. Ultracentrifugal characterization of the human plasma high density lipoprotein distribution. *In* Report of the High Density Lipoprotein Methodology Workshop. K. Lippel, editor. National Institute of Health (NIH) Publication No. 79-1661. 290–300.
32. Anderson, D. W., A. V. Nichols, S. S. Pan, and F. L. Lindgren. 1978. High density lipoprotein distribution. Resolution and determination of three major components in a normal population sample. *Atherosclerosis.* **29:** 161–179.
33. Patsch, J. R., A. M. Gotto, T. Olivecrona, and S. Eisenberg. 1978. Formation of high density lipoprotein$_2$-like particles during lipolysis of very low density lipoproteins in vitro. *Proc. Natl. Acad. Sci. USA.* **75:** 4519–4523.
34. Jansen, H., A. van Tol, and W. C. Hülsmann. 1980. On the metabolic function of heparin-releasable liver lipase. *Biochem. Biophys. Res. Commun.* **92:** 53–59.
35. Van Tol, A., T. van Gent, and H. Jansen. 1980. Degradation of high density lipoprotein by heparin-releasable liver lipase. *Biochem. Biophys. Res. Commun.* **94:** 101–108.