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SEPARATION AND CHARACTERIZATION OF HIGH-DENSITY LIPOPROTEIN SUBPOPULATIONS BY GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

High-density lipoproteins (HDL) contain at least five distinct subpopulations when analyzed by gradient gel electrophoresis. This report represents the first description of a simple technique for isolating these subpopulations of HDL in quantities sufficient to enable characterization in terms of particle size, apolipoprotein AI and apolipoprotein AII content and chemical composition. Lipoproteins were separated and subfractionated on a column of Superose 6B using a fast protein liquid chromatography system. Five normal subjects were studied: HDL_{2b} and HDL_{3a} were isolated as essentially single subpopulations from all subjects, while HDL_{2a} could be isolated from only three of the subjects. HDL_{3b} was isolated in a relatively impure form (70%) from all subjects. Identical subpopulations were identified in each subject by gradient gel electrophoresis of unseparated HDL.

INTRODUCTION

The high-density lipoproteins (HDL) in human plasma comprise a number of apparently discrete subpopulations that are separable on the basis of particle density using ultracentrifugation [1] or particle size using gradient gel electrophoresis [2]. Subpopulations have also been separated on the basis of apolipoprotein (Apo) composition using immunoaffinity chromatography [3].

Ultracentrifugation readily isolates two major subfractions of HDL, designated HDL_2 (density 1.063–1.125 g/ml) and HDL_3 (1.125–1.21 g/ml) [1]. The techniques of rate zonal and density gradient ultracentrifugation have been able to subfractionate each of the major subfractions into two subpopulations [4–6]. The existence of multiple subpopulations of HDL has been confirmed by the use of non-denaturing gradient gel electrophoresis. Thus, HDL_2 has been separated into HDL_{2a} and HDL_{2b} of mean particle radii 4.58 and 5.28 nm, respectively;

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HDL_3 has been separated into HDL_{3a} (mean particle radius 4.22 nm), HDL_{3b} (3.98 nm) and HDL_{3c} (3.81 nm) [2].

Immunoaffinity chromatography has also been employed to separate subpopulations of HDL of differing apolipoprotein composition, but this technique does not separate HDL into subpopulations of a single particle size. The subpopulation separated by gradient gel electrophoresis, on the other hand, cannot be recovered in sufficient quantities to allow complete analysis. Although the fractions isolated by rate zonal or density gradient ultracentrifugation can be recovered in amounts sufficient for further analysis, the sensitivity of these techniques is not as great as gradient gel electrophoresis. In any case it has been reported that the high gravitational forces and the high salt concentrations used in ultracentrifugation may modify the lipoproteins during the separation process [7].

Another method which has been used to separate lipoproteins has been gel permeation chromatography. Previous studies with this technique have tended to focus on the separation of the major classes of lipoproteins rather than the subfractions within the major classes [8–11]. High-performance gel permeation chromatography of plasma has been reported to separate HDL into five subfractions [12], analogous to those seen with gradient gel electrophoresis [2]. Holmquist and Carlson [13] have provided complete compositional data on three fractions of HDL separated by gel permeation chromatography.

This issue of the separation and recovery of HDL subpopulations by gel permeation chromatography is addressed further in the present report. It has been found possible not only to separate HDL into most of the subpopulations identified by gradient gel electrophoresis, but in amounts sufficient to enable a complete chemical composition of each to be obtained.

EXPERIMENTAL

Five healthy, normolipidemic subjects aged between 25 and 35 years were bled after an overnight fast. The two female subjects were both taking combination estrogen and progestogen contraceptive pills.

Lipoprotein preparations

A 50-ml volume of blood was collected into tubes containing Na_2 EDTA (final concentration 1 mg/ml) and plasma was separated immediately by low-speed centrifugation. Plasma was adjusted to a density of 1.25 g/ml with solid potassium bromide and the lipoproteins were separated by ultracentrifugation at 250 000 *g* for 28 h in a Ti 55.2 rotor (Beckman). Ultracentrifugation was performed at 4 °C in a Beckman L5-65 or L8-70M ultracentrifuge. The supernatant (total volume 5 ml) was collected by tube slicing and immediately filtered with a 0.22-μm filter (Gelman).

Gel permeation chromatography

Gel permeation chromatography was performed using 75×1.6 cm column of Superose 6B (Pharmacia, Uppsala, Sweden) attached to a Pharmacia fast protein liquid chromatography (FPLC) system. Equilibration of the column and

elution of lipoproteins was performed with a solution of 0.15 M sodium chloride, 0.001 M Na_2 EDTA and 0.02% sodium azide, at a standard flow-rate of 30 ml/h. A 5-ml volume of the $d < 1.25$ g/ml supernatant, containing all of the lipoproteins from 50 ml of blood, was applied to the column for a single run. All solutions were filtered with a 0.22-μm Gelman filter and degassed prior to use. A continuous absorbance reading of the column eluate was obtained with a UVI cell (Pharmacia). Fractions of 0.5 ml were collected on a Pharmacia FRAC 100 fraction collector. The peak concentration of HDL eluted from the column ranged from 4 to 6 mg/ml.

Polyacrylamide gradient gel electrophoresis of HDL

Polyacrylamide gradient gel electrophoresis of HDL was performed using slab gradient gels (2.5–27% acrylamide, Gradipore, Sydney, Australia). Samples containing 30 μg of HDL protein (separated by gel chromatography) were applied in a volume of 15–200 μl (5–190 μl sample and 10 μl of a solution containing 40% sucrose and 0.01% Bromophenol Blue) and subjected to electrophoresis at 160 V for 17 h in a Tris–borate buffer (pH 8.35). The Stokes' radii of the HDL particles were calculated by reference to coelectrophoresed standards of thyroglobulin (8.50 nm), ferritin (6.10 nm), lactate dehydrogenase (4.08 nm) and bovine serum albumin (3.55 nm) from a high-molecular-weight electrophoresis calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden). Gels were fixed in 10% sulfosalicylic acid for 1 h, stained for 4 h in 0.04% Coomassie G-250 in 3.5% perchloric acid and destained in 5% acetic acid. The gels were then scanned in a laser densitometer (2202 Ultrascan, LKB, Bromma, Sweden) and quantified using a Hewlett-Packard 3390A integrator.

Chemical composition

Total cholesterol and free cholesterol were measured using Boehringer kits (CHOD-PAP method kit Nos. 290319 and 310328, respectively). Triglycerides were measured using Boehringer perodichrom kit No. 701904. Phospholipids were estimated using a Wako kit No. 279 54009 which measures the choline-containing phospholipids. Choline is found in 95–97% of human HDL phospholipids. Protein levels were estimated using the method of Lowry adapted for use on a centrifugal analyzer (Cobas-Bio, Roche). The test was modified by scaling all volumes down to a total volume of 0.4 ml and reducing the total incubation times to 20 min. No loss of linearity or sensitivity was noted within the concentration range 50–1000 μg/ml. Bovine serum albumin was used as a standard.

All assays were performed in duplicate on a Cobas-Bio (Roche) centrifugal analyzer. The coefficients of variation (C.V.) within a single run were: cholesterol (free and total), $< 0.5\%$; triglyceride, $< 1\%$; phospholipid $< 0.5\%$; protein, 4% in the concentration range 0–250 μg/ml and 2% in the range 250–1000 μg/ml. Day-to-day and run-to-run C.V.s were: total cholesterol, $< 0.5\%$; free cholesterol, 5%; triglyceride, 3%; phospholipids, 1%; protein 5% (0–250 μg/ml) and 3% (250–1000 μg/ml). To minimize errors all subfractions from all subjects were assayed within the same run.

Apo AI and Apo AII were estimated by laser turbidimetry on a Cobas-Bio

autoanalyzer using Boehringer antisera. Standards were prepared from a Hyland Omega lipid control serum. The C.V. for both Apo AI and Apo AII within a single run was 6%.

RESULTS

Separation of HDL subpopulations

Gel permeation chromatography on a column of Superose 6B separated human lipoproteins into fractions of very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and HDL (Fig. 1). Under the standard load and flow-rate adopted in these studies the HDL fraction eluted as a single peak which sometimes was asymmetrical in shape. Normally, there was no obvious separation into discrete peaks of HDL_2 and HDL_3. If, however, individual fractions were subjected to gradient gel electrophoresis, it became apparent that separation of HDL subpopulations had occurred.

Fig. 2a shows a sequence of gradient gel electrophoretic profiles of gel permeation fractions of HDL from a male subject. Fraction 6 contained an apparently single subpopulation in which the mean particle radius was 5.2 nm, while fractions 22 and 32 contained essentially single populations of particles of radii 4.6 and 4.1 nm, respectively. In fractions 46 and beyond 70% of the particles were of radius 3.9 nm and smaller but all of these fractions were contaminated by 20% or more of albumin. Albumin could be eliminated by ultracentrifugation at 1.21 g/ml rather than 1.25 g/ml or by a second spin at 1.25 g/ml prior to gel permeation, but both of these procedures led to a significant loss of smaller HDL particles.

Fig. 2b shows duplicate gradient gel electrophoretic profiles of the total HDL fractionated in Fig. 2a. It was apparent that this profile represented the sum of the individual subpopulations shown in Fig. 2a. In some subjects, however, gel permeation chromatography revealed the existence of subpopulations not readily apparent in the unseparated sample. For example, fraction 2 in Fig. 2a contained at least one subpopulation in which the particle radius was greater than 5.2 nm, while fraction 46 clearly shows the presence of a subpopulation of particle radius 3.7 nm.

Fig. 3a shows a similar sequence of gradient gel electrophoretic profiles of fractions of HDL recovered after gel permeation chromatography of lipoproteins from a normal female subject. Duplicate gradient gel electrophoretic profiles of the total HDL from this subject are also shown in Fig. 3b. Analysis of the gel permeation fractions revealed the presence of three major subpopulations with particle radii of 5.2, 4.2 and 3.9 nm. In fraction 52, 80% of the particles were of radius 3.9 nm or less, with two additional subpopulations of particle radii 3.8 and 3.7 nm.

All fractions from a single subject that were homogeneous in size and had a mean particle radius between 5.2 and 5.3 nm were pooled and designated HDL_{2b}. HDL_{2a} (4.5–4.6 nm) and HDL_{3a} (4.1–4.3 nm) were similarly defined. The HDL_{3b} subpopulation was defined by those fractions whose major peak was 3.9–4.0 nm in radius and which contained less than 30% contamination by other fractions. Thus for the subject described in Fig. 2a, fractions 5–7 were pooled to form HDL_{2b},

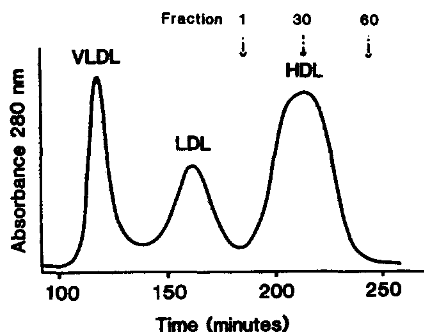

Fig. 1. Separation of plasma lipoproteins by gel permeation chromatography. Lipoproteins in the 1.25 g/ml supernatant of 30 ml plasma were applied to a 75 × 1.6 cm column of Superose 6B. Samples were eluted at a flow-rate of 30 ml/h with a solution of 0.15 *M* sodium chloride, 0.001 *M* Na_2 EDTA and 0.02% sodium azide. The absorbance at 280 nm was monitored continuously and 0.5-ml fractions of HDL were collected.

fraction 21–23 were pooled for form HDL_{2a}, fractions 31–33 were pooled to form HDL_{3a}, while fraction 46, although not homogeneous, was designated HDL_{3b}.

Chemical composition of HDL subpopulations

Table I shows the composition of each of the discrete HDL subpopulations isolated from five normal subjects. Subpopulations of HDL_{2b} (particle radius 5.2–5.3 nm), HDL_{3a} (4.1–4.3 nm) and HDL_{3b} (3.9–4.0 nm) were recoverable from all subjects. However, the HDL_{3b} subpopulation was contaminated with 10–30% of the HDL_{3a} subpopulation. A subpopulation of HDL_{2a} (4.5–4.6 nm) was only found in the three male subjects.

In the small HDL of particle radius 3.9–4.0 nm the percentage protein was very consistent from one subject to another. As the particle size increased, however, the percentage protein in particles of a given size began to vary between subjects. For particles of radius 5.2–5.3 nm, for example, the percentage protein ranged from 35.0 to 43.3%. Much of the increase in protein content was accounted for by an increase in Apo AII. There were also variations in triglyceride and free cholesterol between individuals in all subpopulations. The phospholipid and cholesteryl ester content of a given subpopulation, on the other hand, varied very little between subjects. In all individuals a decreasing particle size was associated with a progressive decrease in the content of phospholipid, cholesteryl ester and free cholesterol and with a progressive increase in the content of protein. There appeared to be no significant discontinuities and each subpopulation had a composition similar to those nearest to it in particle size.

The composition of a given subfraction from an individual was very reproducible (C.V. $<5\%$) both from one separation to another and for repeated plasma samples taken up to two months apart. Subject 1 was tested on three different occasions with at least two chromatography runs on each sample, while subject 2 was tested twice.

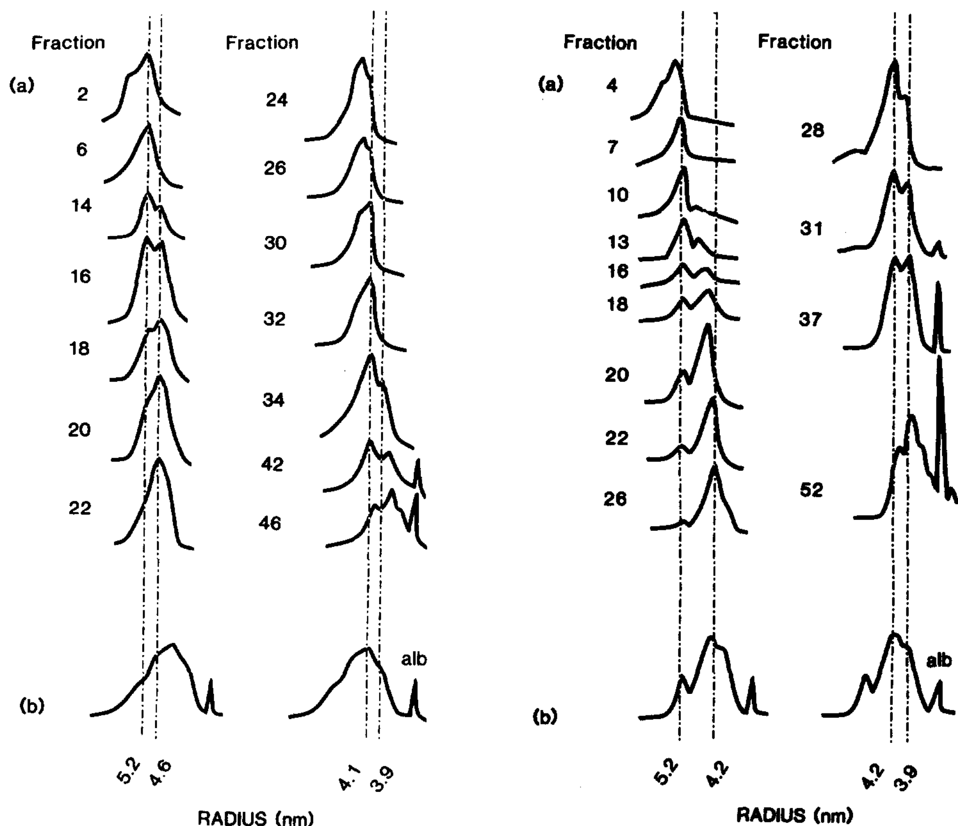

Fig. 2. HDL particle size distributions as assessed by gradient gel electrophoresis. (a) HDL fractions from subject I (male) were isolated by gel permeation chromatography as in Fig. 1. Aliquots containing 30 μg protein (5–200 μl) from alternate fractions were subjected to polyacrylamide gradient gel electrophoresis at 160 V for 17 h. Profiles were obtained by scanning the stained gels with a laser densitometer; particle radii were calculated by reference to coelectrophoresed standards of known size (see Experimental). (b) A 5-μl volume of the 1.25 g/ml supernatant from subject I was subjected to polyacrylamide gradient gel electrophoresis as described above. The two profiles are duplicates.

Fig. 3. HDL particle size distribution in subject 5 (female). (a) HDL fractions isolated by gel permeation chromatography were assessed by polyacrylamide gradient gel electrophoresis as described in the legend to Fig. 2. (b) The 1.25 g/ml supernatant from subject 5 was subjected to polyacrylamide gradient gel electrophoresis as described in the legend to Fig. 2. The two profiles shown are duplicates.

Apo AI and Apo AII composition of HDL subpopulations

The mass ratio of Apo AI to Apo AII for each subpopulation is shown in Table I. The ratios are very similar in the HDL_{3a} and HDL_{3b} subfractions but in the HDL_{2b} subfraction there are considerable differences between individuals.

When the ratio of Apo AI to Apo AII was determined in all the gel permeation fractions containing HDL and plotted against the radius of the predominant particle in that fraction two patterns emerged (Fig. 4). In the three male subjects whose HDL contained an identifiable HDL_{2a} subpopulation, the curve was trimodal, with peaks of the ratio coinciding with particle radii of 6.3, 4.3 and 3.7 nm

TABLE I

COMPOSITION OF HDL SUBFRACTIONS (PERCENTAGE WEIGHT)

CE = cholesteryl ester; FC = free cholesterol; PL = phospholipid; TG = triglyceride; AI = apolipoprotein AI; AII = apolipoprotein AII.

Subfraction	Subject	Protein	CE	FC	PL	TG	AI/AII
HDL_{2b}	1	38.5	25.8	6.1	27.1	2.6	7.2
(5.2-5.3 nm)	2	40.6	23.6	4.9	27.1	3.8	5.0
	3	43.3	24.9	3.9	24.5	4.3	4.7
	4	39.1	22.4	5.4	27.4	6.1	6.7
	5	35.0	22.3	6.4	29.9	6.4	9.4
Mean		39.3	23.8	5.3	27.2	4.6	6.6
HDL_{2a}	1	44.9	23.7	4.4	25.0	2.0	4.6
(4.5-4.6 nm)	2	47.8	21.6	3.1	24.6	3.1	2.3
	3	48.3	21.7	2.3	25.1	2.6	2.3
	4	–	–	–	–	–	–
	5	–	–	–	–	–	–
Mean		47.0	22.3	3.3	24.9	2.6	3.1
HDL_{3a}	1	45.3	23.8	4.0	25.1	1.8	3.1
(4.1-4.3 nm)	2	48.7	21.6	2.9	23.8	2.9	2.4
	3	49.8	21.4	2.1	23.9	2.4	2.7
	4	47.4	19.6	2.9	25.4	4.8	2.7
	5	44.5	18.3	3.5	28.8	4.5	3.4
Mean		47.1	20.9	3.1	25.4	3.3	2.9
HDL_{3b}	1	51.2	22.0	3.0	22.1	1.6	3.3
(3.9-4.0 nm)	2	51.5	20.6	2.5	22.1	2.9	2.8
	3	51.1	19.9	2.0	23.8	2.4	3.0
	4	50.8	18.3	2.4	23.5	4.5	3.4
	5	50.5	17.3	2.8	25.1	4.3	4.0
Mean		51.0	19.6	2.5	23.3	3.1	3.3

(solid line). In the two subjects without a subpopulation of HDL_{2a}, on the other hand, the curve was bimodal with the ratio peaking at particle radii of 6.3 nm and 3.7 nm (broken line). In no HDL fraction was the ratio greater than 10:1, even in the particles of radius > 5.4 nm. Indeed, in particles of radius > 6.3 nm the Apo AI/Apo AII ratio began to fall again. The Apo AI/Apo AII ratio and HDL particle size plot was almost identical when whole plasma rather than HDL was separated on the column.

DISCUSSION

Gel permeation chromatography on a column of Superose 6B resulted, as has been reported previously [11], in a clear separation of plasma lipoproteins into the three major classes. The HDL fraction eluted as a single, although sometimes asymmetrical peak (Fig. 1). However, by collecting multiple fractions of HDL it was possible to recover a series of essentially single populations of particles that

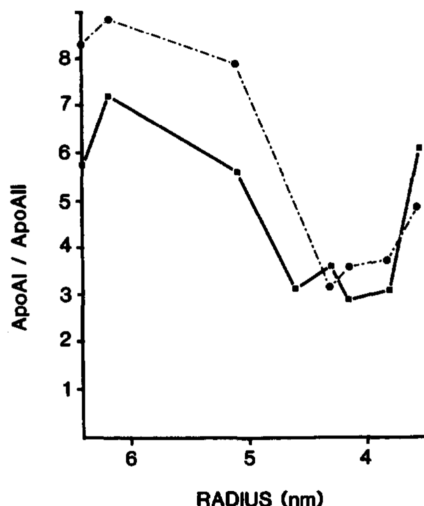

Fig. 4. Apo AI/Apo AII mass ratio as a function of particle size. Apo AI and Apo AII levels in alternate HDL fractions were estimated by laser turbidimetry using specific antisera. Particle size was determined by polyacrylamide gradient gel electrophoresis of the HDL subfractions as described in the legend to Fig. 2. The solid line shows the mean Apo AI/Apo AII ratio as a function of particle size for the three male subjects who had a clear HDL_{2a} subfraction. The broken line shows the Apo AI/Apo AII ratio for the two subjects without an HDL_{2a} subfraction.

coincided with the subpopulations identified by gradient gel electrophoresis of the total HDL fraction.

Gradient gel electrophoresis of the total HDL in these studies revealed a subpopulation distribution comparable to that reported elsewhere [2]. Not every subpopulation was present in each subject. If, however, a given subpopulation was apparent as an identifiable peak (or in some cases as a shoulder on a peak) after gradient gel electrophoresis, it could subsequently be recovered as an essentially single population of particles in one or more of the fractions collected after chromatography on a column of Superose 6B. It was impossible, however, even with rechromatography, to obtain single populations of very small particles. Furthermore, these subpopulations were recovered after only 4 h of chromatography and in amounts sufficient to allow their further characterization. Thus, this report provides the first description of the chemical composition and of the molar Apo AI/Apo AII ratios of the HDL subfractions defined in Nichols' laboratory by gradient gel electrophoresis: HDL_{2a}, HDL_{2b}, HDL_{3a} and HDL_{3b}. It was not possible to obtain comparable data for HDL_{3c} (except Apo AI/Apo AII ratio) which was present in these subjects at too low a concentration to enable its recovery as a single population.

Subpopulations of HDL_{2b} (particle radius 5.2–5.3 nm), HDL_{3a} (4.2–4.3 nm) and HDL_{3b} (3.9–4.0 nm) were recoverable as essentially single populations in all five subjects and were found to have quite distinctive chemical compositions. The HDL_{2a} subpopulation was recoverable only in the three male subjects in whom it appeared to have a chemical composition very similar to that of HDL_{3a}. Overall,

as the particle size increased the percentage protein decreased and the percentage lipid increased.

Holmquist and Carlson [13] using TSK-G 3000 SW columns separated HDL into fifteen subfractions and characterized each with chemical composition and Apo AI and Apo AII content. Although the fractions were not completely assessed in terms of particle size and homogeneity, from the data provided it appears that some of the fractions corresponded to HDL_{2b} and HDL_{3a}. This paper extends their observations by providing information on single populations of particles of HDL_{2a}, HDL_{2b} and HDL_{3a} size and on a mixed population of very small particles. In addition the method described is essentially a preparative one and enables a twenty-fold increase in the amount of HDL separated on the column, thus providing workable quantities of small HDL particles. As the column also separates the major lipoprotein classes, prior separation of HDL is not required.

Anderson et al. [4] used density gradient ultracentrifugation to separate and characterize three distinct subpopulations of HDL. Two of them, designated as being within particle size range I and size range II, appear to equate roughly in composition with the HDL_{2b} and HDL_{2a}, respectively, isolated in the present study. The subpopulation designated as size range III was equivalent to a composite of HDL_{3a} and HDL_{3b}. Patsch et al. [6] separated HDL from two normal subjects into eight subfractions by rate zonal ultracentrifugation. The particle size of each subfraction was not determined. It was concluded that HDL_2 was relatively homogeneous,while HDL_3 appeared to comprise at least two subpopulations, neither of which equate closely with either of the two subpopulations of HDL_3 isolated in the present study.

The HDL subpopulations recovered in the present study were distinct not only in chemical composition but also in terms of their apolipoprotein content. The Apo AI/Apo AII ratio was much higher in HDL_{2b} than in any of the other subpopulations. When this Apo AI/Apo AII ratio was examined in multiple fractions across the whole HDL spectrum, a consistent pattern emerged. In the three subjects in whom there was an identifiable subpopulation of HDL_{2a}, the plot of the Apo AI/Apo AII ratio peaked at three points, corresponding to particles of radii 6.3, 4.3 and 3.7 nm. In the two subjects who lacked an identifiable subpopulation of HDL_{2a}, the Apo AI/Apo AII ratio peaked at only two points which coincided with particles of radii 6.3 and 3.7 nm. This result is consistent with the observations of Cheung and Albers [15] using cesium chloride equilibrium gradient ultracentrifugation and of Patsch et al. [6] using rate zonal ultracentrifugation, who detected peaks in the Apo AI/Apo AII ratio in the very light and the very dense HDL fractions.

These peaks in the Apo AI/Apo AII ratio may reflect the presence of an HDL particle containing Apo AI only, whose existence has been postulated by several investigators [16–18]. Using immunoaffinity chromatography, Cheung and Albers [3] provided evidence that Apo AI only particles are contained in two subpopulations of mean particle radii 5.4 and 4.3 nm. Our results demonstrating a peak in the Apo AI/Apo AII ratio in gel permeation fractions containing particles of 6.3 nm radius are not inconsistent with their findings as our HDL_{2b} fraction contains a mixture of Apo AI only particles and Apo AI and Apo AII particles. The

fractions containing particles of 6.3 nm radius presumably contain less Apo AI and Apo AII particles. Whether or not the peak coinciding with particles of radius 3.9 nm represents another population of Apo AI only particles of this size will have to await further investigation; however, Kunitake et al. [19] have identified a subpopulation of very small Apo AI only containing lipoproteins of MW 80 000, while Cheung and Albers [3] have identified minor HDL subpopulations containing Apo AI only < 3.9 nm in radius.

In conclusion, a technique of gel permeation chromatography has been described whereby the subpopulations of HDL identified by gradient gel electrophoresis have been isolated in amounts sufficient to both permit their characterization in terms of chemical and apolipoprotein composition, and the use of this technique should prove to be a valuable adjunct to other methods currently available for metabolic studies.

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