# Isolation and identification of HDL particles of low molecular weight

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**Abstract** Small particles of high density lipoproteins (HDL) were isolated from fresh, fasting human plasma and from the ultracentrifugally isolated high density lipoprotein fraction by means of ultrafiltration through membranes of molecular weight cutoff of 70,000. These particles were found to contain cholesterol, phospholipids, and apolipoproteins A-I and A-II; moreover, they floated at a density of 1.21 kg/l. They contained 67.5% of their mass as protein and the rest as lipid. Two populations of small HDL particles were identified: one containing apolipoprotein A-I alone [(A-I)HDL] and the other containing both apolipoproteins A-I and A-II [A-I + A-II)HDL]. The molar ratio of apoA-I to apoA-II in the latter subclass isolated from plasma or HDL was 1:1. The molecular weights of these subpopulations were determined by nondenaturing gradient polyacrylamide gel electrophoresis and found to be 70,000; 1.5% of the plasma apoA-I was recovered in the plasma ultrafiltrate.—**Atmeh, R.** Isolation and identification of HDL particles of low molecular weight. *J. Lipid Res.* 1990. **31:** 1771–1780.

**Supplementary key words** gradient polyacrylamide gel electrophoresis • ultrafiltration • high density lipoproteins • apoA-I • apoA-II

High density lipoproteins (HDL) are a group of lipoproteins comprising multiple particle subpopulations differing in size, chemical composition, and function. The structural heterogeneity of human HDL in healthy and diseased states has been confirmed by many workers using different techniques. Zonal (1) and analytical (2) ultracentrifugation have been used to separate HDL into subclasses that differ in their flotation densities. Nondenaturing gradient polyacrylamide gel electrophoresis (3, 4) has been used to separate HDL into several subclasses according to their molecular weights. Recently, immunoaffinity chromatography (5–7) has been used for the isolation of two immunochemically distinct subclasses differing in their apolipoprotein content: one subclass contained apoA-I as the major apolipoprotein [(A-I)HDL] and the other contained both apoA-I and apoA-II [(A-I + A-II) HDL)] at a fixed molar ratio (5, 7). These two subclasses have been shown to have minimal exchange of their apoA-I both in vitro and in vivo, and to be metabolically distinct in that nicotinic acid or probucol therapies exerted different effects on them (5).

The well-known inverse relationship between HDL level and coronary heart disease can be explained by the role of HDL in the reverse cholesterol transport process, which may be confined to one or more of the HDL subclasses (8). Growing evidence suggests that an HDL particle containing apoA-I unassociated with other major apolipoproteins is the best activator of the enzyme lecithin:cholesterol acyltransferase (LCAT) (9) and greatly facilitates the movement of cholesterol from cells into plasma (9, 10). This particle is expected to be a small sphere (11) and poor in free cholesterol content (7, 11–13) so as to be able to initiate the process of its enrichment with cholesteryl ester via the LCAT reaction. On the other hand, most of the cholesteryl ester transfer protein (CET) is associated with the (A-I + A-II)HDL subclass which has been found to enhance the transfer of cholesteryl ester from HDL to VLDL (9, 10). Small HDL subclasses have been identified from plasma of patients with LCAT deficiency (14), Tangier disease (15), and abetalipoproteinemia (ABL) (11); but they have not been isolated, so far, from plasma of healthy individuals. Recently, small particles with a diameter of 7.1 nm were detected in HDL preparations from healthy subjects but not in their fresh plasma (3).

The aim of this study was to isolate small HDL subclasses from fresh human plasma with minimal manipulations by using direct ultrafiltration of the plasma. The results described here indicate the presence of two HDL subclasses with molecular weights of 70,000; one subclass contains apoA-I alone and the other contains both apoA-I and apoA-II at a fixed molar ratio.

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Abbreviations: apo, apolipoprotein; ABL, abetalipoproteinemia; (A-I)HDL, high density lipoproteins containing apoA-I and no apoA-II; (A-I + A-II)HDL, high density lipoproteins containing both apoA-I and apoA-II; CE, cholesteryl ester; CH, cholesterol; gPAGE, gradient polyacrylamide gel electrophoresis; $HDL_2$, high density lipoproteins of d 1.063–1.125 kg/l; $HDL_3$, high density lipoproteins of d 1.125–1.21 kg/l; HDL, high density lipoproteins; HDLF, high density lipoprotein filtrate; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; PF, plasma filtrate; TG, triglycerides; UPF, uncentrifuged plasma filtrate.

## METHODS

### Subjects

Blood samples were obtained from six male and four female healthy normolipidemic adults after a 12–14 h fast. The details of these subjects are summarized in **Table 1**. None of the subjects was on medication. Venous blood was drawn into tubes containing EDTA (1.5 mg/ml). The plasma was separated immediately at low speed centrifugation at 4°C, and 0.1 mg/ml sodium azide, 0.08 mg/ml chloramphenicol, and 0.08 mg/ml gentamycin were promptly added (16). Plasma portions were used for subsequent isolation steps within half an hour. The design of the experiments is represented by a flow diagram in **Fig. 1**.

### Preparation of HDL

An aliquot of plasma (5.0 ml) was used to prepare HDL (d 1.063–1.21 kg/l) by sequential ultracentrifugation at 10°C according to Havel, Eder, and Bragdon (17) in a Beckman model L8-70M ultracentrifuge using a Beckman 50.3 Ti rotor (Beckman, Palo Alto, CA). All density fractions were dialyzed against saline containing 0.01% EDTA, pH 7.0.

### Isolation of HDL subclasses by ultrafiltration

*From plasma.* Five ml of fresh plasma was mixed with 45 ml of a diluent (0.15 M NaCl, 0.01% EDTA, 0.1 mg/ml sodium azide, 0.08 mg/ml chloramphenicol, and 0.08 mg/ml gentamycin), pH 7.0, and placed in an ultrafiltration unit (Schleicher & Schuell, GmbH, Dassel, West Germany) fitted with a cellulose acetate membrane (Schleicher & Schuell standard type, wet, AC 63) of a molecular weight cutoff of 70,000. A pressure of 4 psi was applied from a nitrogen cylinder and the filtrate was collected. When the volume of the diluted plasma in the filtration unit reached about 20 ml, another 30 ml of the diluent was added and filtration was continued. The last two steps were repeated one time and filtration was continued until the volume of the retentate was about 5.0 ml. The volume of the combined filtrates was measured and placed in another ultrafiltration unit fitted with a cellulose acetate membrane of molecular weight cutoff of 20,000 (Schleicher & Schuell standard type, wet, AC 62) and concentrated to a volume of about 5.0 ml to give the plasma filtrate (PF).

A portion of the PF (about 4.0 ml) was adjusted to a density of 1.21 kg/l with solid KBr, overlayered with a KBr solution of density 1.21 kg/l, and subjected to ultracentrifugation at 10°C for 24 h in a Beckman 50.3 Ti rotor. The top third of the tube was collected and dialyzed to give the ultracentrifuged plasma filtrate (UPF).

*From HDL.* About 2.0 ml of the prepared HDL was mixed with 25 ml of the diluent and treated in a similar way to that of plasma. The concentrated filtrate was collected to give the HDL filtrate (HDLF).

*Recovery experiment.* UPF was subjected to refiltration through a membrane of 70,000 cutoff (AC 63), as described above for plasma, and washed twice with the diluent. The apoA-I content of UPF before filtration and that of the filtrate were measured by electroimmunoassay, as described below.

### Electroimmunoassay of proteins (EIA)

*Total apolipoproteins and albumin.* Total apoA-I and apoA-II, apoB, and albumin were assayed by EIA at 10°C according to Laurell (18) using rabbit IgG fraction of polyclonal specific antibodies. Antibody against human albumin was purchased from Pharmacia (Sweden); antibodies against apoA-I, apoA-II, and apoB were purchased from Behringwerke (AG, Marburg, West Germany).

TABLE 1. Plasma lipid and apolipoprotein concentrations in the subjects

| | | | Plasma | | | | | HDL |
|---|---|---|---|---|---|---|---|---|
| Subject | Sex | Age | CH | TG | ApoA-I | ApoA-II | ApoA-I in (A-I)HDL | CH |
| | | *yr* | | | *mg/dl* | | | *mg/dl* |
| 1 | F | 34 | 181 | 59 | 106 | 42 | 40 | 42 |
| 2 | M | 42 | 153 | 57 | 110 | 46 | 33 | 80 |
| 3 | M | 27 | 143 | 88 | 128 | 57 | 39 | NA |
| 4 | M | 26 | 156 | 79 | 114 | 49 | 42 | 68 |
| 5 | M | 42 | 203 | 97 | 126 | 61 | 39 | 71 |
| 6 | F | 23 | 110 | 26 | 120 | 45 | 48 | 59 |
| 7 | M | 43 | 198 | 57 | 142 | 61 | 46 | 59 |
| 8 | F | 24 | 143 | 34 | 111 | 52 | 36 | 47 |
| 9 | M | 30 | 101 | 52 | 98 | 44 | 25 | 33 |
| 10 | F | 20 | 127 | 45 | 121 | 50 | 39 | 58 |
| Mean ± SEM | | | 152 ± 10 | 59 ± 7 | 118 ± 4 | 51 ± 2 | 39 ± 2 | 57 ± 4 |

CH, cholesterol; TG, triglycerides; NA, not analyzed.

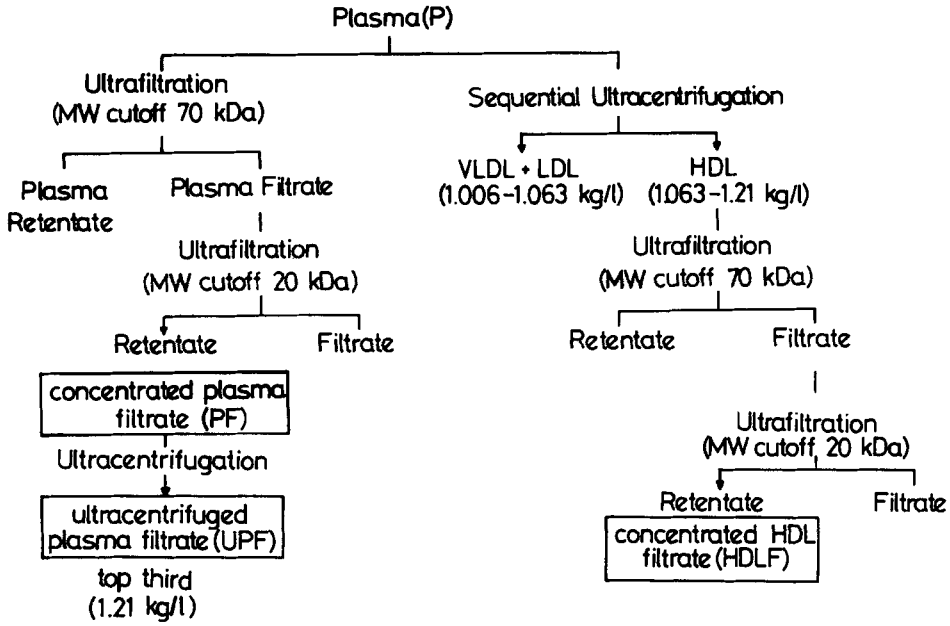


**Fig. 1.** Flow diagram for preparation of HDL subpopulations by ultrafiltration. The boxes represent fractions containing the HDL subpopulations. Plasma, P, was subjected to ultrafiltration through a membrane with molecular weight cutoff of 70,000, and the filtrate was further ultrafiltered through a second membrane with molecular weight cutoff of 20,000; the retentate was designated as the plasma filtrate (PF). An aliquot of PF was ultracentrifuged at d 1.21 kg/l and the top fraction was designated as the ultracentrifuged plasma filtrate (UPF). Another portion of P was used to prepare HDL which was subjected to ultrafiltration in a way similar to that described for plasma; the retentate over the 20 kDa membrane was designated as HDL filtrate (HDLF).

*ApoA-I associated with (A-I)HDL.* ApoA-I content of the (A-I)HDL subpopulation was measured by electroimmunoassay procedure at 10°C as described previously (5). In brief, a two-phase 1% agarose gel plate (1.5 mm thick) was prepared to contain in its lower half the anti-apoA-II (2%) phase and in its upper half the anti-apoA-I (1%) phase, and the two antibody phases were separated by a narrow (ca. 0.5 cm) zone of 1% agarose gel containing no antibodies. Holes were made in the anti-apoA-II portion where the samples were applied. The electric current was passed where the lipoproteins moved towards the anode in the anti-apoA-I section. Two sets of rockets were formed: one set in the anti-apoA-II phase and the other set in the anti-apoA-I phase. Particles containing no apoA-II did not form rockets in the anti-apoA-II phase. The rockets in the anti-apoA-I phase represent the (A-I)HDL particles that were not captured in the anti-apoA-II lower phase, and could be quantitated according to their apoA-I content by measuring the area under each rocket. The height of each rocket and its width at half height were measured under a magnifying lens and used for the area calculation.

*Standardization.* ApoA-I and apoA-II were prepared and purified as described previously (19). Pure albumin (Sigma, St. Louis, MO), pure apoA-II, and LDL fraction (d 1.006–1.063 kg/l) were used as standards for the electroimmunoassay of the corresponding antigen. Pure apoA-I and the (A-I)HDL, eluted from anti-apoA-II–Sepharose column after the application of total HDL (d 1.063–1.21 kg/l) (5), were used as standards for the assay of both total apoA-I and the apoA-I content of (A-I)HDL. The concentration of apoA-I in the (A-I)HDL preparation used as a standard was determined with reference to pure apoA-I and apolipoprotein A-I standard serum (Behringwerke).

### Chemical assay of lipids and proteins

Total and free cholesterol were determined by the cholesterol oxidase/peroxidase method (Boehringer Mannheim GmbH, Mannheim, West Germany); phospholipids were determined by the phospholipase D/choline oxidase method (Boehringer); and triglcyerides were determined by the lipase/glycerol kinase method (Boehringer). Cholesterol in HDL was determined by the heparin–manganese precipitation method (20). Total protein was measured by the method of Lowry et al. (21).

### Gradient polyacrylamide gel electrophoresis (gPAGE)

The molecular weights of the (A-I)HDL and (A-I + A-II)HDL subclasses were estimated by nondenaturing gPAGE using 4–30% gradient gel in 14 mM Tris, 110 mM glycine, 0.1% $NaN_3$, pH 8.3, according to the method of Nichols, Blanche, and Gong (22). The gel was cast (1.5 mm thick) and run in the LKB 2001 vertical electrophoresis system (LKB, Sweden). Electrophoresis was performed at 10°C and 125 V for 24 h. Gels were fixed with 10% sulfosalicylic acid and stained for 90 min in 0.04%

Coomassie Brilliant Blue G-250 in 3.5% perchloric acid and then destained in 5% acetic acid until a clear background was obtained. Carbonic anhydrase (bovine erythrocytes, 29 kDa); bovine serum albumin, 66 kDa for the monomer and 132 kDa for the dimer; and urease (jack beans) 272 kDa for the trimer and 545 kDa for the hexamer (nondenatured protein molecular weight marker kit, Sigma), were used as calibrating proteins.

### Immunoblotting

Fresh plasma, UPF, HDL, and HDLF were run on 4–30% nondenaturing gPAGE as described above, transferred onto nitrocellulose (0.45 μm, Bio-Rad) in the electrophoresis buffer where two layers of nitrocellulose sheets were used (23). The transferred bands on the nitrocellulose were immunoblotted for anti-apoA-I and anti-albumin according to the method of Towbin, Staehelin, and Gordon (24). Goat anti-rabbit IgG conjugated to horseradish peroxidase and 4-chloro-1-naphthol substrate (Bio-Rad) were used to visualize the bands on the nitrocellulose membrane.

### Statistical analysis

Student's *t*-test was used. All results were expressed as mean ± SEM.

## RESULTS

### Plasma lipid and apolipoprotein levels

The plasma lipid and apolipoprotein levels in the subjects are summarized in Table 1. Their plasma levels of triglycerides, total and HDL cholesterol, apoA-I, and apoA-II were within the reference ranges of healthy adults.

### Plasma ultrafiltration

To exclude the possibility of membrane leakage during the ultrafiltration process, apoB was used as a marker because it is present on large size lipoproteins. Its concentration in plasma and plasma ultrafiltrate was measured and no significant difference was observed (mean ± SEM, 70 ± 13.5 and 69 ± 14.3 mg/dl, respectively). On the other hand, albumin concentration dropped significantly (29%) after plasma ultrafiltration (3.7 ± 0.2 and 2.6 ± 0.2 g/dl, respectively, $P < 0.001$). When UPF was re-filtered through a membrane of 70 kDa cutoff, 63 ± 1.5% of the apoA-I content was recovered in the filtrate.

### Apolipoprotein composition of plasma, HDL, and their ultrafiltrates

About 1.5% and 6% of plasma apoA-I were detected in the fractions of densities <1.063 and >1.21 kg/l, respectively, and traces of apoA-II (less than 1%) could be detected in these fractions. The apoA-I content of PF was found to be 1.5 ± 0.2% of the total plasma apoA-I (**Table 2**). Using the two-phase agarose gel plates for immunoelectrophoresis, two sets of rockets were obtained for each of the fractions PF, UPF, and HDLF. One set in the anti-apoA-I phase represented HDL particles containing apoA-I but no apoA-II [(A-I)HDL], and the second set of rockets obtained in the anti-apoA-II phase represented the HDL particles containing both apoA-I and apoA-II [(A-I + A-II)HDL]. Table 2, **Table 3**, and **Table 4** summarize the distribution of apoA-I and apo-II in the different fractions. Approximately one-quarter of the apoA-I content of PF (4.4 ± 0.7 μg/ml), UPF (2.3 ± 0.3 μg/ml), HDL (2.4 ± 1.9 mg/dl), and HDLF (9.5 ± 1.9 μg/ml) was associated with the (A-I)HDL subclass, while one-third of the plasma apoA-I content (39 ± 2 mg/dl) was associated

TABLE 2. Total apoA-I concentration in plasma, PF, and HDLF

| | Total apoA-I | | | Percentage[a] | |
|---|---|---|---|---|---|
| Subject | Plasma | PF | HDLF | PF | HDLF |
| | | μg/ml | | % | |
| 1 | 1060 | 14.4 | 33.0 | 1.4 | 3.1 |
| 2 | 1100 | 19.4 | 86.0 | 1.7 | 7.8 |
| 3 | 1280 | 34.5 | 12.0 | 2.7 | 0.9 |
| 4 | 1140 | 27.1 | 84.0 | 2.4 | 7.4 |
| 5 | 1260 | 22.4 | NA | 1.8 | NA |
| 6 | 1200 | 2.1 | NA | 0.2 | NA |
| 7 | 1420 | 15.5 | 12.0 | 1.1 | 0.9 |
| 8 | 1110 | 15.8 | NA | 1.4 | NA |
| 9 | 980 | 9.4 | 18.0 | 1.0 | 1.8 |
| 10 | 1210 | 10.3 | NA | 0.9 | NA |
| Mean ± SEM | 1176 ± 38 | 17.0 ± 2.8 | 41.0 ± 13.0 | 1.5 ± 0.2 | 3.7 ± 1.2 |
| *P* | | | | NS | |

[a]The percentage of apoA-I content of PF or HDLF is relative to the total plasma apoA-I. Plasma and HDL were ultrafiltered through a membrane with molecular weight cutoff of 70,000 and concentrated; PF and HDLF, plasma and HDL fitrates, respectively; NA, not analyzed; NS, not significant.

TABLE 3. Apolipoprotein composition of (A-I)HDL and (A-I + A-II)HDL

| Subject | Plasma Filtrate | | | Ultracentrifuged Plasma Filtrate | | | | HDL | | | | HDL Filtrate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (A-I)HDL | (A-I + A-II)HDL | | (A-I)HDL | | (A-I + A-II)HDL | | (A-I)HDL | | (A-I + A-II)HDL | | (A-I)HDL | | (A-I + A-II)HDL | |
| | ApoA-I | ApoA-I | ApoA-II | ApoA-I | | ApoA-I | ApoA-II | ApoA-I | | ApoA-I | ApoA-II | ApoA-I | | ApoA-I | ApoA-II |
| | | | | μg/ml | | | | | | mg/dl | | | | μg/ml | |
| 1 | 4.3 | 10.1 | 6.2 | 2.6 | | 4.6 | 3.3 | 28 | | 64 | 41 | 11 | | 22 | 14 |
| 2 | 4.7 | 14.7 | 8.7 | 2.6 | | 7.4 | 4.8 | 20 | | 67 | 40 | 21 | | 65 | 44 |
| 3 | 6.5 | 28.0 | 18.8 | 4.3 | | 16.4 | 10.7 | 23 | | 89 | 56 | 4 | | 8 | 4 |
| 4 | 7.8 | 19.3 | 14.7 | 2.6 | | 9.4 | 5.6 | 25 | | 75 | 53 | 11 | | 73 | 49 |
| 5 | 6.4 | 16.0 | 11.4 | 2.5 | | 6.6 | 4.4 | 24 | | 91 | 56 | NA | | NA | NA |
| 6 | 0.7 | 1.4 | 0.9 | 0.2 | | 0.7 | 0.5 | 29 | | 60 | 38 | NA | | NA | NA |
| 7 | 3.1 | 12.4 | 6.5 | 2.0 | | 5.8 | 3.6 | 26 | | 73 | 42 | 4 | | 8 | 6 |
| 8 | 4.3 | 11.5 | 6.5 | 3.2 | | 7.4 | 4.1 | 21 | | 50 | 29 | NA | | NA | NA |
| 9 | 1.7 | 7.7 | 4.4 | 1.2 | | 3.3 | 2.2 | 11 | | 45 | 24 | 6 | | 12 | 7 |
| 10 | 4.1 | 6.2 | 4.1 | 2.0 | | 3.7 | 2.7 | 35 | | 66 | 37 | NA | | NA | NA |
| Mean ± SEM | 4.4 ± 0.7 | 12.7 ± 2.2 | 8.2 ± 1.6 | 2.3 ± 0.3 | | 6.5 ± 1.3 | 4.2 ± 0.8 | 24 ± 1.9 | | 68 ± 4.5 | 42 ± 3.2 | 9.5 ± 1.9 | | 31.3 ± 8.6 | 20.7 ± 5.9 |

Concentration is expressed as μg/ml or mg/dl of plasma. Plasma and HDL were filtered through a membrane of molecular weight cutoff of 70,000 and concentrated. The concentrate was ultracentrifuged at d 1.21 kg/l; NA, not analyzed.

with this subclass (Tables 1, 3, and 4). The difference in the plasma apoA-I associated with (A-I)HDL between males and females (31 ± 1.3% and 36 ± 1.8%, respectively) was not statistically significant. A statistically significant difference ($P < 0.01$) in the percentage of apoA-I associated with (A-I)HDL was observed between plasma and HDL (33 ± 1.3% and 27 ± 1.6%, respectively) and between plasma and its ultrafiltrate (33 ± 1.3% and 27 ± 2.1%, respectively); but no significant difference was observed between HDL and PF. A nonsignificantly higher proportion of the plasma apoA-I associated with (A-I)HDL was found in the HDLF (3.7 ± 1.2%) than in the PF (1.5 ± 0.2%) (Table 2), while 28% of the HDLF content of apoA-I was associated with the (A-I)HDL subclass and 27% of PF content of apoA-I was associated with this subclass (Table 4). When PF was ultracentrifuged at d 1.21 kg/l , the top fraction (UPF) contained the two HDL subclasses, (A-I)HDL and (A-I + A-II)HDL, and one-fourth (2.3 ± 0.3 μg/ml) of their total apoA-I (8.85 ± 1.6 μg/ml) was found associated with the (A-I)HDL subclass (Table 4).

### Molar ratio of apoA-I/apoA-II in (A-I + A-II)HDL subclass

The mole/mole ratio of apoA-I/apoA-II in the (A-I + A-II)HDL subclass was found to be approximately 1:1 in plasma, PF, UPF, HDL, and HDLF (**Table 5**). The molecular masses of apoA-I and apoA-II used for the calculations were 28 and 17.4 kDa, respectively.

### Lipid and protein composition of filtered HDL particles

**Table 6** summarizes the composition of HDL particles from UPF and HDLF. The percentage contents of phospholipids and triglycerides in fractions UPF and HDLF were similar, while a statistically insignificant difference was seen in the percentage contents of protein, free cholesterol, and esterified cholesterol. The percentage of free cholesterol relative to the total cholesterol in the two fractions, UPF and HDLF, was similar (15.9 ± 0.9% and 16.7 ± 0.5%, respectively).

### Molecular weight of filtered HDL particles

When the UPF and HDLF were applied to gPAGE and stained with Coomassie Blue, a band was detected for each fraction at a molecular weight slightly higher than that of bovine serum albumin (figure not shown); the estimated molecular weight of the bands was 70,000.

### gPAGE and immunoblotting of plasma and HDL fractions

When the fresh plasma, UPF, HDL, and HDLF were electrophoresed in gPAGE and transferred onto nitrocellulose followed by immunoblotting for anti-apoA-I and

TABLE 4. ApoA-I content of (A-I)HDL particles as a percentage of total apoA-I in the corresponding fractions

| Subject | Plasma | PF | UPF | HDL | HDLF |
|---|---|---|---|---|---|
| | | | % | | |
| 1 | 38 | 30 | 36 | 30 | 33 |
| 2 | 30 | 24 | 26 | 23 | 24 |
| 3 | 31 | 19 | 21 | 21 | 33 |
| 4 | 37 | 29 | 22 | 25 | 13 |
| 5 | 31 | 29 | 28 | 21 | NA |
| 6 | 40 | 33 | 22 | 36 | NA |
| 7 | 32 | 20 | 26 | 26 | 33 |
| 8 | 32 | 27 | 30 | 30 | NA |
| 9 | 26 | 18 | 27 | 20 | 33 |
| 10 | 32 | 40 | 35 | 35 | NA |
| Mean ± SEM | 33 ± 1.3 | 27 ± 2.1 | 27 ± 1.6 | 27 ± 1.6 | 28 ± 2.4 |
| *P* | | <0.01 | | <0.01 | |

Plasma and HDL were ultrafiltered through a membrane with molecular weight cutoff of 70,000 and concentrated. The concentrate was ultracentrifuged at d 1.21 kg/l; PF and HDLF, plasma and HDL filtrates, respectively; UPF, ultracentrifuged plasma filtrate; NA, not analyzed.

visualization, a major band in the range of 132–545 kDa was seen in the case of plasma and HDL in addition to a minor band in the region of the 66 kDa standard. In the case of UPF and HDLF, one single band was seen for each in the region of the minor band detected in plasma and HDL (**Fig. 2**). When the second copy of the nitrocellulose was treated with anti-albumin followed by visualization, no bands were detected in the case of HDL and UPF, while plasma showed a band at the 66 kDa region (figure not shown).

## DISCUSSION

HDL is expected to have an important role in reverse cholesterol transport and the major activity of the enzyme LCAT is associated with this class of lipoprotein (8). At present, HDL can be subclassified to comprise two main groups of particles, i.e., (A-I)HDL and (A-I + A-II)HDL (5). These groups are heterogeneous in terms of their size; each group comprises two or more subclasses (22), and they differ in their cholesterol content as reported by Ohta et al. (7) who found that the (A-I)HDL group contained a significantly lower ratio of cholesteryl ester to total cholesterol than the (A-I + A-II)HDL group. They suggested that the former group could be a carrier of free cholesterol. It seems that each subclass may have a different biological function; whereas the former group is the best activator of LCAT (9), the latter group enhances the CE transfer from HDL to VLDL (9, 10). Moreover, the two groups were affected differently on nicotinic acid or probucol therapies (5). Discoidal HDL recombinants

TABLE 5. Molar ratio of apoA-I to apoA-II in HDL particles containing both apoA-I and apoA-II [(A-I + A-II)HDL] in different fractions

| Subject | Plasma | PF | UPF | HDL | HDLF |
|---|---|---|---|---|---|
| | | | *mole/mole*[a] | | |
| 1 | 0.98 | 1.01 | 0.86 | 0.97 | 0.98 |
| 2 | 1.04 | 1.05 | 0.96 | 1.04 | 0.92 |
| 3 | 0.97 | 0.93 | 0.95 | 0.99 | 1.24 |
| 4 | 0.91 | 0.82 | 1.04 | 0.88 | 0.93 |
| 5 | 0.89 | 0.87 | 0.93 | 1.01 | NA |
| 6 | 1.00 | 0.97 | 0.87 | 0.98 | NA |
| 7 | 0.98 | 1.19 | 1.00 | 1.08 | 0.83 |
| 8 | 0.90 | 1.10 | 1.12 | 1.07 | NA |
| 9 | 1.03 | 1.09 | 0.93 | 1.16 | 1.06 |
| 10 | 1.02 | 0.94 | 0.85 | 1.11 | NA |
| Mean ± SEM | 0.97 ± 0.02 | 1.0 ± 0.03 | 0.95 ± 0.03 | 1.03 ± 0.02 | 0.99 ± 0.04 |

Plasma and HDL were ultrafiltered through a membrane with molecular weight cutoff of 70,000 and concentrated. The concentrate was ultracentrifuged at d 1.21 kg/l; PF and HDLF, plasma and HDL filtrates, respectively; UPF, ultracentrifuged plasma filtrate; NA, not analyzed.

[a] $M_r$: apoA-I, 28 kDa; apoA-II, 17.4 kDa.

TABLE 6. Composition of HDL subfractions

| Fraction | Composition (% by Weight) | | | | |
|---|---|---|---|---|---|
| | Protein | FC | EC | PL | TG |
| UPF | 67.5 ± 1.7 | 2.7 ± 0.2 | 14.2 ± 0.8 | 12.7 ± 1.4 | 3.0 ± 0.1 |
| HDLF | 71.1 ± 4.3 | 1.9 ± 0.3 | 9.2 ± 2.2 | 13.8 ± 2.2 | 3.5 ± 0.3 |
| *P* | NS | NS | NS | NS | NS |

Results are expressed as mean ± SEM; UPF, ultracentrifuged plasma filtrate, as described in the legend of Table 4, (n = 9); HDLF, HDL filtrate (n = 4); FC and EC, free and esterified cholesterol, respectively; PL, phospholipids; TG, triglycerides; NS, not significant.

containing apoA-I were found to have a considerable capacity to acquire cholesterol (12). Recently, Yui et al. (25) reported a new function for apoA-I-containing particles and apoA-I recombinants as being stabilizing factors for prostacyclin, a property that was not observed for other apolipoprotein recombinants such as apolipoproteins A-II, C-I, C-II, C-III, D, and E, or LDL and VLDL. Another reported function of HDL is the binding of plasma acid phosphatase to its apoA-I (26). Fielding and Fielding (9) have shown that the sterol efflux from cultured fibroblasts was highly (80%) dependent upon a minor lipoprotein fraction containing apoA-I unassociated with other apolipoproteins, and this lipoprotein fraction catalyzed the first step of the LCAT-linked sterol transport from cells. It seems that apoA-I-containing particles, and, in particular, a small subpopulation of this family, are the principal factors in reverse cholesterol transport. It is quite reasonable to assume (8, 11) the presence of a small, newly formed HDL particle as being the starting point in the reverse cholesterol transport, because such a small particle would be expected to be relatively poor in cholesterol (11–13) and able to reach the interstitial fluid easily where it interacts with the peripheral cells. Barter and Connor (27) concluded, from in vivo kinetic studies, that all plasma esterified cholesterol is produced in a small, rapidly turning over subfraction of HDL. Detection of small HDL particles was reported by several workers in normal human plasma (3, 28–31), in plasma from patients with ABL (11), Tangier disease (15), hyperlipidemia (31), LCAT-deficiency (31–33), and A-I Milano variant (34), and from dog peripheral lymph (35). However, such particles have not yet been isolated from plasma of healthy individuals. Recently, McCall, Forte, and Shore (13) have reported the isolation of smalll spherical HDL particles (Stokes' diameter of 7.4 nm) from in vitro secretions by the hepatoma-derived cell line, HepG2, that are similar to those detected by Vezina et al. (4).

In order to understand the initial events associated with reverse cholesterol transport, different HDL subclasses should be identified and isolated by methods that are not expected to change their composition or structure. In this study small HDL particles were isolated from fresh plasma and HDL of healthy subjects using an ultrafiltration technique. This technique was chosen in order to minimize plasma manipulations and because it is mild, fast, and nondestructive method routinely used for the concentration of different lipoprotein preparations. Moreover, it produces a homogeneous preparation of small HDL, in terms of mean particle size, directly from plasma. The particles from both plasma and HDL isolated by the ultrafiltration technique were found to contain apoA-I, and apoA-II, cholesterol, triglycerides, and phospholipids. Moreover, when the plasma ultrafiltrate was ultracentrifuged at a density of 1.21 kg/l, the upper third of the tube (UPF) contained apoA-I, apoA-II, cholesterol, triglycerides, and phospholipids (Tables 3 and 6). This indicates that these particles are lipoproteins having the properties of HDL, and their chemical composition from both UPF and HDLF was similar with no statistically significant difference (Table 6). This composition is close to that reported for the small HDL particles isolated from

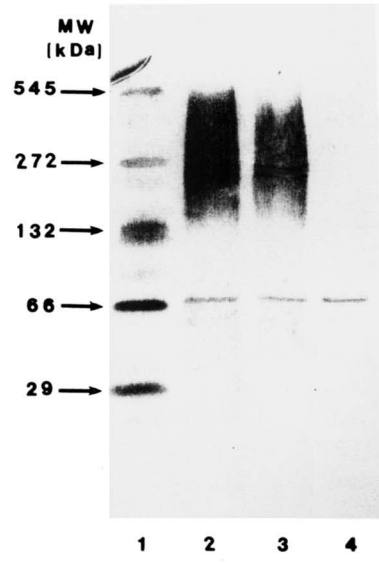


**Fig. 2.** Immunoblots for apoA-I of nondenaturing 4–30% gradient polyacrylamide gel electrophoresis of fresh plasma (5 μl, lane 2), HDL (3 μg protein, lane 3), and UPF (1 μg protein, lane 4) from subject number 4. Lane 1 contains the following molecular weight standards from top to bottom: urease, hexamer (545 kDa); urease, trimer (272 kDa); bovine albumin, dimer (132 kDa); bovine albumin, monomer (66 kDa); and carbonic anhydrase (bovine erythrocytes) (29 kDa).

the plasma of patients with ABL (11) where the mass percentages of proteins, cholesterol, phospholipids, and triglycerides were 63.2, 18.5, 14.7, and 3.5, respectively. Since these small HDL particles in UPF and HDLF were filtered through a membrane of molecular weight cutoff of 70,000, it is expected that they have molecular weights of approximately 70,000. This was confirmed by using gPAGE where a single band was seen at 70 kDa which is very close to the value of 67–75 kDa reported by Ishida, Frolich, and Fielding (31) for similar particles. When HDL was run on the same gPAGE, the major band of HDL was seen in addition to a minor band at 70 kDa which has the same migration as the single band obtained in case of UPF and HDLF. On the other hand, the immunoblotting technique confirmed the presence of these HDL particles where fresh plasma, HDL, and UPF showed a band at 70 kDa when they were blotted for anti-apoA-I (Fig. 2). Immunoblotting for anti-albumin gave a band in the case of plasma, while no bands were detected in the case of HDL and UPF. The detection of an HDL species with a molecular weight of 70 kDa in fresh plasma similar to that detected in the UPF provides evidence that such HDL species is actually present in fresh plasma and is not an artifact of the ultrafiltration technique used. On the average, 1.5 ± 0.2% of the total plasma apoA-I was recovered in the PF, while 3.7 ± 1.2% was recovered in the HDLF (Table 2). This difference was not statistically significant and may be due to the incomplete filtration of the small HDL from plasma. The recovery of albumin from filtered plasma (29%) was lower than that of small HDL (63%). This difference is expected because albumin carries a higher negative charge at neutral pH (36) than HDL, and this charge increases the rejection of albumin by the cellulose acetate filtration membrane. Thus, albumin is drawn away from the membrane into the bulk of the solution (36) more than HDL. Moreover, the spherical nature (37) of the HDL molecules enhances their filtration more than the nonspherical albumin molecules.

Two types of particles, i.e., (A-I)HDL and (A-I + A-II)HDL, were identified in PF, UPF, and HDLF by using two-phase agarose gel plates. The distribution of apoA-I among these particle types was about one-quarter in the (A-I)HDL type and the rest was in the (A-I + A-II)HDL; moreover, similar proportions were found in all the fractions, i.e., PF, and UPF, and HDLF (Tables 3 and 4). This indicates that those small HDL particle types are not formed as a result of ultracentrifugation since the PF was not subjected to it, while those that were subjected to ultracentrifugation, i.e., UPF and HDLF, had the same proportion of apoA-I associated with (A-I)HDL. On the other hand, the proportion of apoA-I associated with (A-I)HDL was higher in plasma than in HDL and the difference was statistically significant (Table 4). This difference may indicate the presence of some apoA-I-containing particles in plasma having larger size and lower density than 1.063 kg/l and others having smaller size and higher density than 1.21 kg/l that could not be separated with the HDL fraction. In this study, 1.5% and 6% of the plasma apoA-I were detected in the fractions of $d < 1.063$ and $d > 1.21$ kg/l, respectively, while traces (less than 1%) of apoA-II were detected in these fractions. This is in agreement with the results reported by Schonfeld, Baily, and Steelman (29) and Cheung and Wolf (3). The apoA-I associated with (A-I)HDL subclass in plasma was found to be 33% of the total plasma apoA-I in the group of six males and four females (Table 1) which is close to the value of 45% reported in a previous study in a group of eight females (5). Similar results were reported by Cheung and Albers (6) in a group of four males and six females (38%) and by Ohta et al. (7) who reported 37% in a group of ten males and 44% in a group of ten females, a statistically significant difference. Although the difference between the value of 31% for males and 36% for females in the present study is not statistically significant, which may be due to the small number of the subjects studied, it may be expected that females have a higher proportion of their plasma apoA-I associated with (A-I)HDL than in males, as reported by Ohta et al. (7).

The molar ratio of apoA-I to apoA-II in the (A-I + A-II)HDL subclass isolated from PF, UPF, and HDLF was about 1:1, which is similar to that obtained for this subclass in native plasma and HDL (Table 5). This agrees with the ratio of 1.1:1–1.2:1 reported in a previous study (5) for this subclass in plasma, $HDL_2$, and $HDL_3$. It also agrees with the ratio of 1.3:1 reported by Norfeldt et al. (38), but differs from that reported by Cheung and Albers (6) and Ohta et al. (7) as 2:1 and 1.6:1, respectively. This difference may be due to differences in immunoassay methodology. Nevertheless, the molecular weight of the (A-I + A-II)HDL subclass isolated by ultrafiltration from plasma or HDL was found to be 70,000, and 67.5% of its mass was protein and the rest was lipid (Table 6). This means that the molar protein mass is around 48 kDa, which is very close to the mass (45.4 kDa) obtained from the sum of the masses of one molecule of apoA-I and one molecule of apoA-II, i.e., 28 plus 17.4 kDa. This is in favor of a molar ratio of apoA-I to apoA-II of 1:1; but if we assume a molar ratio of 2:1, this will give a molar protein mass of 73.4 kDa which, alone, is higher than the measured molecular weight of the particle. A similar calculation for the (A-I)HDL particle suggests the presence of two molecules of apoA-I per particle. This is in good agreement with the reported results for apoA-I recombinant small HDL particles, with Stoke's diameter less than 9.0 nm, that they contain two molecules of apoA-I per particle (39–41). A similar value was obtained by Chen et al. (33) for HDL particles from patients with LCAT-deficiency where the spherical small particles with molecular masses of 83–87 kDa comprise two molecules of apoA-I per particle.

At present, evidence is in favor of the hypothesis that an initial small HDL should be present containing mostly apoA-I without apoA-II and is directly secreted into plasma (4, 11). Vezina et al. (4) observed that newly secreted hepatic apoA-I was found mainly in small lipoproteins and that HepG2 cells secreted mostly (A-I)HDL particles. Thus isolation of small HDL subpopulations, by the simple method described in this work, from fresh plasma followed by a single ultracentrafugation at d 1.21 kg/l offers a suitable method for the study of the early processes affecting the metabolism of HDL subpopulations.

The main advantages of this method are its simplicity, rapidity, and freedom from the shortcomings of the prolonged ultracentrifugation and column chromatography techniques. The former method may strip apolipoproteins from lipoproteins and the latter may affect the apolipoprotein–lipoproteins interactions by dilution or by selective adsorption and retardation of eluted proteins (29).

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