Differential effect of ultracentrifugation on apolipoprotein A-I-containing lipoprotein subpopulations

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Abstract Two populations of apolipoprotein (apo) A-I-containing lipoprotein particles are found in high density lipoproteins (HDL): those that also contain apo A-II[Lp(A-I w A-II)] and those that do not [Lp(A-I w/o A-II)]. Lp(A-I w/o A-II) comprised two distinct particle sizes with mean hydrated Stokes diameter of 10.5 nm for Lp(A-I w/o A-II)$_1$ and 8.5 nm for Lp(A-I w/o A-II)$_2$. To study the effect of ultracentrifugation on these particles, Lp(A-I w/o A-II) and Lp(A-I w A-II) were isolated from the plasma and the ultracentrifugal HDL (d 1.063–1.21 g/ml fractions) of five normolipidemic and three hyperlipidemic subjects. The size subpopulations of these particles were studied by gradient polyacrylamide gel electrophoresis. Several consistent differences were detected between plasma Lp(A-I w/o A-II) and HDL Lp(A-I w/o A-II). First, in all subjects, the relative proportion of Lp(A-I w/o A-II)$_1$ to Lp(A-I w/o A-II)$_2$ isolated from HDL was reduced. Second, particles larger than Lp(A-I w/o A-II)$_1$ and smaller than Lp(A-I w/o A-II)$_2$ were considerably reduced in HDL. Third, a distinct population of particles with approximate Stokes diameter of 7.1 nm usually absent in plasma was detected in HDL Lp(A-I w/o A-II). Little difference in subpopulation distribution was detected between Lp(A-I w A-II) isolated from the plasma and HDL of the same subject. When plasma Lp(A-I w/o A-II) and Lp(A-I w A-II) were centrifuged, 14% and 4% of A-I were, respectively, recovered in the D > 1.21 g/ml fraction. Only 2% A-II was found in this density fraction. These studies show that the Lp(A-I w/o A-II) particles are less stable than Lp(A-I w A-II) particles upon ultracentrifugation. Among the various Lp(A-I w/o A-II) subpopulations, particles larger than Lp(A-I w/o A-II)$_1$ and smaller than Lp(A-I w/o A-II)$_2$ are most labile.—**Cheung, M. C., and A. C. Wolf.** Differential effect of ultracentrifugation on apolipoprotein A-I-containing lipoprotein subpopulations. *J. Lipid Res.* 1988. **29:** 15–25.

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Two populations of apoA-I-containing lipoprotein particles are found in plasma, those that also contain apoA-II[Lp(A-I w A-II)] and those that do not [Lp(A-I w/o A-II)]. Together, they represent essentially all the particles in high density lipoproteins (HDL). These particles are heterogeneous in density, size, protein and lipid composition, and in their lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) activities (1–3). These observations suggest that Lp(A-I w A-II) and Lp(A-I w/o A-II) or their component subpopulations may have different specific functional roles in lipid metabolism. Our laboratory is interested in elucidating the structure, function, and origin of these two populations of particles. To isolate these particles from plasma requires a two-step immunoaffinity chromatography procedure employing antibodies specific for apoA-I and A-II (1, 2). This isolation scheme is laborious compared to the conventional ultracentrifugation technique used for lipoprotein isolation, and cannot be efficiently applied to studies requiring a large sample size. However, if ultracentrifuged HDL can be used as the starting material, separation of Lp(A-I w A-II) and Lp(A-I w/o A-II) can be accomplished by a single immunoadsorption step using only anti-A-II antibodies. This procedure, if proven valid, would greatly simplify the isolation of Lp(A-I w A-II) and Lp(A-I w/o A-II) particles for chemical, physical, functional, metabolic, and perhaps epidemiological studies. The present experiments were undertaken to evaluate this possibility. Our data show that the subpopulation components of apoA-I-containing lipoproteins, in particular Lp(A-I w/o A-II), isolated from HDL are different from those isolated directly from plasma.

Abbreviations: apo, apolipoprotein; Lp(A-I w A-II), lipoproteins containing both apoA-I and apoA-II; Lp(A-I w/o A-II), lipoproteins containing apoA-I but not apoA-II; HDL, high density lipoproteins (d 1.063–1.21 g/ml); LCAT, lecithin:cholesterol acyltransferase; gPAGE, gradient polyacrylamide gel electrophoresis; FCHL, familial combined hyperlipidemia; CETP, cholesteryl ester transfer protein.

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METHODS

Plasma

Venous blood was drawn from the antecubital vein into EDTA-containing Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) from four male and four female adult volunteers after a 12–14-hr overnight fast. Five of the subjects were normolipidemic, three were hyperlipidemic. Upon separation of plasma at 4°C by low-speed centrifugation, preservatives were promptly added to final concentrations of 0.5 g/l sodium azide, 0.01 g/l chloramphenicol, and 0.005 g/l gentamycin.

Experimental design

A flow diagram of the experimental design is shown in **Fig. 1**. The various lipoprotein preparations labeled 3A-D and 4A-C correspond to those in Figs. 3 and 4, respectively. Details of the immunoaffinity chromatography and ultracentrifugation procedures are described in subsequent sections. Within an hour after each blood sample was drawn, an aliquot of the plasma was immediately used for isolation of Lp(A-I w A-II) and Lp(A-I w/o A-II). The Lp(A-I w A-II) and Lp(A-I w/o A-II) isolated from the plasma of six of the eight subjects were centrifuged to obtain the HDL (d 1.063–1.21 g/ml) fraction. In all subjects, another aliquot of plasma was centrifuged to prepare HDL. The isolated HDL was then chromatographed on an anti-A-II affinity column. Since Lp(A-I w A-II) and Lp(A-I w/o A-II) together constitute all the lipoprotein particles found in HDL, the anti-A-II affinity column alone was sufficient to separate these two populations of particles. The nonadsorbed proteins represented Lp(A-I w/o A-II) and possible albumin contaminants. Lp(A-I w A-II) was recovered as the bound proteins. In four of the eight subjects, however, the nonadsorbed HDL proteins of the anti-A-II column were further adsorbed with anti-A-I affinity column to remove the contaminating albumin and to assess the effect of immunoadsorption and thiocyanate elution on Lp(A-I w/o A-II). This experimental scheme allowed us to directly assess the effect of ultracentrifugation on Lp(A-I w A-II) and Lp(A-I w/o A-II), and to compare the Lp(A-I w A-II) and Lp(A-I w/o A-II) particles isolated from plasma and HDL.

Isolation of A-I-containing lipoproteins

Apo A-I-containing lipoproteins with and without A-II were isolated from plasma or HDL by a two-step immunoaffinity chromatography procedure (1, 2). To isolate Lp(A-I w A-II), plasma or HDL from each subject was incubated with anti-A-II immunoadsorbent for 1 hr at 4°C. The immunoadsorbent was packed in 1.5 × 30 cm Econo-column (Bio-Rad Laboratories, Richmond, CA), and extensively washed free of nonadsorbed proteins with 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA, and 0.05% sodium azide (Tris buffer) at 30 ml/hr until the A_{280} of the eluate was below 0.02 units. The bound lipoproteins were eluted with 3 M NaSCN in 0.02 M sodium phosphate buffer, pH 7.0, at 60 ml/hr, im-

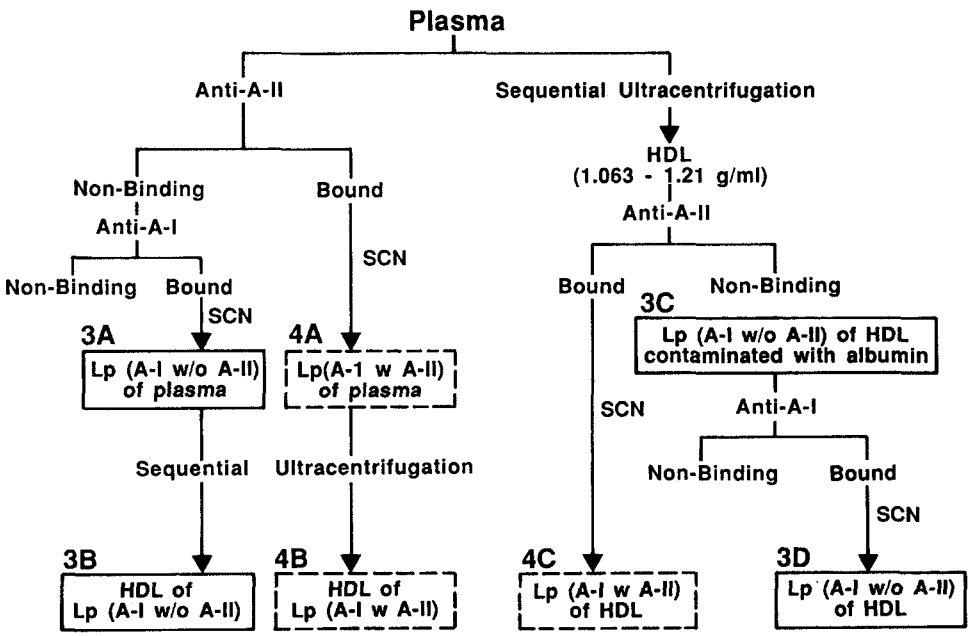

Fig. 1. Flow diagram of the experimental design. Details of the ultracentrifugation and immunoaffinity chromatography procedures are described in Methods. Lipoprotein particles enclosed by ▭ and ⬚ labeled 3A–D and 4A–C are those shown in Figs. 3 and 4, respectively.

mediately filtered through a column packed with Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) to remove the thiocyanate, and concentrated by Micro-Confilt concentrator (Biomolecular Dynamics, Beaverton, OR) for further processing.

To isolate Lp(A-I w/o A-II), plasma or HDL devoid of A-II (the nonadsorbed proteins from the anti-A-II immunoadsorbent) was incubated with anti-A-I immunoadsorbent for 1 hr at 4°C. The nonadsorbed proteins and bound lipoproteins were sequentially eluted from the immunoadsorbent and processed as described above.

Preparation of HDL

The d 1.063–1.21 g/ml (HDL) fraction was isolated from plasma and from Lp(A-I w A-II) and Lp(A-I w/o A-II) by sequential ultracentrifugation using a Beckman 40.3 Ti rotor. Two 4-ml aliquots of plasma and a 4-ml aliquot each of Lp(A-I w A-II) and Lp(A-I w/o A-II) were adjusted to the nonprotein solvent density of d 1.063 g/ml by solid KBr, overlayered with KBr solution containing NaN_3 and EDTA (pH 7.4), and centrifuged at 35K rpm for 24 hr. The top 2.5 ml containing the d < 1.063 g/ml fraction was carefully aspirated with a fine-tipped Pasteur pipet. The bottom 3.5 ml containing the d < 1.063 g/ml fraction was adjusted to 1.21 g/ml by solid KBr and overlayered with KBr solution of 1.21 g/ml and centrifuged at 35K rpm for 44 hr. The top 2.5 ml containing the d 1.063 g/ml fraction was carefully aspirated. All density fractions were dialyzed against Tris buffer for further studies. Centrifugation was carried out at 10°C with samples from subjects 5 and 8, and at 4°C with all other samples.

Gradient polyacrylamide gel electrophoresis (gPAGE)

The size subpopulations of the Lp(A-I w A-II) and Lp(A-I w/o A-II) particles isolated from plasma and HDL were separated by nondenaturing gPAGE using precast 4–30% gradient gels (Pharmacia). Routinely, lipoprotein particles containing approximately 20 μg of A-I were applied to each sample well. Electrophoresis was carried out at 10°C at 125 V for 24 hr according to Nichols, Blanche, and Gong (4). Gels were stained for protein overnight with 0.04% Coomassie Brilliant Blue G-250 in 3.5% perchloric acid (5), destained in 5% acetic acid until background was clear, and scanned by a densitometer (Helena Instrument, Beaumont, TX). Particle size of the various subpopulations was determined in reference to the calibration proteins (HMW calibration kit, Pharmacia). The hydrated Stokes diameters of the calibration proteins were: thyroglobulin (17 nm), apoferritin (12.2 nm), lactate dehydrogenase (8.2 nm), and bovine albumin (7.1 nm). To calculate the percentage distribution of the subpopulations, areas under the peaks were integrated by dropping perpendicular lines from the lowest position on each side of the peak to the baseline of the scan. The total integrated area of each densitometric scan between the size interval of 7.1 nm and 17 nm was considered to be 100%. The ratio of the area under each peak to the total area represents the relative proportion of each size subpopulation in Lp(A-I w/o A-II) and Lp(A-I w A-II). All numbers in Results, where appropriate, are expressed as mean ± SD.

Lipid and protein analysis

Phospholipid, cholesterol, and triglyceride were analyzed by enzymic methods as previously described (6). All lipid determinations were performed on an ABA bichromatic analyzer (Abbott Laboratories, Irvine, TX) with proper adjustment of specimen to reagent volume to optimize measurements on HDL particles. Cholesterol in HDL, HDL_2, and HDL_3 was determined by a two-step dextran sulfate–magnesium precipitation method (7). ApoA-I, A-II, E, and LCAT were quantitated by specific immunoassays (8–11). Total protein in the various density fractions was measured by the method of Lowry et al. (12). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of the various lipoprotein fractions was performed on 5–18% polyacrylamide gels according to the method of Laemmli (13). Protein bands were visualized by staining in 0.03% Coomassie Blue R250 dissolved in a mixture of isopropanol, acetic acid, and water (25:10:65) and destained in the same solvent.

RESULTS

Plasma lipid and apoprotein profile

The general lipid and apoA-I and A-II profiles of the subjects in this study are shown in **Table 1**. Subjects 1–5 were apparently healthy normolipidemic adults. Subjects 6 and 7 are familial combined hyperlipidemic (FCHL) individuals according to the criteria described (14). Subject 8 had adult onset (Type II) diabetes. Together, they represent a spectrum of individuals with plasma cholesterol and triglyceride levels ranging from less than the 10th percentile (subject 1) to over the 90th percentile (subject 6) values reported for their age and sex (15). All except subject 8 had normal HDL cholesterol levels. Their apoA-I and A-II levels were either normal or elevated when compared to previously established population means using the same immunoassays (8, 9).

Distribution of apoA-I, A-II, E, and LCAT in density fractions

When the plasma of these subjects was centrifuged as described under Methods, approximately 85% of A-I and 93% of A-II were recovered in the HDL fraction **(Table 2 and Table 3)**. An average of 2 to 3% of A-I and A-II was found in the d < 1.063 g/ml fraction. Whereas only

TABLE 1. Lipid and apoA profile of the subjects

Subject	Age	Sex	Plasma CH	Plasma TG	Plasma A-I	Plasma A-II	Cholesterol LDL	Cholesterol HDL	Cholesterol HDL_2	Cholesterol HDL_3
	yr						*mg/dl*			
1	32	F	135	44	113	24	86	40	11	29
2	29	F	159	58	149	29	94	53	17	36
3	70	M	211	59	166	30	144	55	19	36
4	55	M	187	42	168	28	124	55	17	38
5	26	M	149	72	126	31	83	52	14	38
6	38	F	281	609	235	38	117	42	7	35
7	57	F	254	262	251	46	123	79	15	64
8	29	M	194	306	95	27	108	25	1	24

Abbreviations: CH, cholesterol; TG, triglyceride; LDL, low density lipoproteins; HDL, high density lipoproteins.

4% of A-II was located in the d > 1.21 g/ml fraction, 13% of total plasma A-I was found in this fraction.

To investigate the source of the A-I in the d > 1.21 g/ml fraction, Lp(A-I w/o A-II) and Lp(A-I w A-II) isolated directly from plasma by immunoaffinity chromatography were likewise centrifuged. While 95–97% of the A-I and A-II in Lp(A-I w A-II) was recovered in HDL (Tables 2 and 3), only 83% of A-I in Lp(A-I w/o A-II) was in this density fraction. An average of 14% of A-I in Lp(A-I w/o A-II) was located in the d > 1.21 g/ml fraction (Table 2). Hence, most of the A-I in the lipoprotein-deficient plasma fraction was either Lp(A-I w/o A-II) particles or was dissociated from these particles upon ultracentrifugation.

The distribution of apoE in the various density fractions of plasma, Lp(A-I w/o A-II), and Lp(A-I w A-II) is shown in **Table 4**. In the four normolipidemic subjects (nos. 1, 3, 4, 5), the relative proportion of plasma apoE recovered in HDL (38 ± 7%) was not significantly different from that in the d < 1.063 g/ml fraction (44 ± 13%). However, in the three hyperlipidemic subjects (Nos. 6–8), the majority of plasma apoE (71 ± 9%) was found in the d < 1.063 g/ml fraction. As a group, an average of 16% apoE was located in the d > 1.21 g/ml fractions (Table 4).

Lp(A-I w/o A-II) and Lp(A-I w A-II) isolated from plasma contained 15 ± 7% and 52 ± 6% of plasma apoE, respectively. Thus 33 ± 8% of plasma apoE was not associated with apoA-I or A-II. Centrifugation of the A-I-containing particles showed that only 25 ± 10% of apoE in Lp(A-I w/o A-II) was found in HDL (Table 4). This was not the case with Lp(A-I w A-II) where (with the exception of subject 6 who had elevated triglyceride and cholesterol) an average of 68% of apoE was recovered in HDL. Furthermore, 14 ± 4% and 4 ± 5 of apoE was detected in the d > 1.21 g/ml fraction of Lp(A-I w/o A-II) and Lp(A-I w A-II), respectively.

When the density fractions from plasma, Lp(A-I w/o A-II), and Lp(A-I w A-II) were assayed for LCAT mass, trace quantities (1 ± 3%) (range: 0–10%) of LCAT were detected in the d < 1.063 g/ml fraction; 65 ± 12% (range: 46–86%) in HDL, and 34 ± 12% (range: 14–54%) in the d > 1.21 g/ml fraction.

Lipid and protein composition of Lp(A-I w/o A-II) and Lp(A-I w A-II)

Besides apoA-I, A-II, E, and LCAT, proteins in the molecular mass region smaller than 20 kDa could be de-

TABLE 2. Distribution (%) of apoA-I in ultracentrifugal fractions

Subject	Plasma d < 1.063	Plasma HDL	Plasma d > 1.21	Lp(A-I w/o A-II) d < 1.063	Lp(A-I w/o A-II) HDL	Lp(A-I w/o A-II) d > 1.21	Lp(A-I w A-II) d < 1.063	Lp(A-I w A-II) HDL	Lp(A-I w A-II) d > 1.21
1	4	83	13	3	82	15	ND	90	10
2	2	84	14	2	78	20	2	92	6
3	5	85	10	3	84	13	2	95	3
4	3	81	16	3	84	13	2	96	2
5	2	71	27	NA	NA	NA	NA	NA	NA
6	2	91	7	3	84	13	1	99	1
7	1	94	6	3	88	9	1	99	1
8	1	89	10	NA	NA	NA	NA	NA	NA
Mean ± SD	2 ± 1	85 ± 7	13 ± 7	3 ± 0.4	83 ± 3	14 ± 4	1 ± 0.9	95 ± 4	4 ± 4

ND, not detectable; NA, not analyzed.

TABLE 3. Distribution (%) of apoA-II in ultracentrifugal fractions

	Plasma			Lp(A-I w A-II)		
Subject	d < 1.063	HDL	d > 1.21	d < 1.063	HDL	d > 1.21
1	2	93	5	0	99	1
2	1	94	5	0	99	1
3	3	97	ND	2	96	2
4	3	90	7	2	96	2
5	3	94	3	NA	NA	NA
6	4	92	4	2	96	2
7	4	92	4	2	97	1
8	ND	88	12	NA	NA	NA
Mean ± SD	3 ± 1	93 ± 3	4 ± 2	1 ± 1	97 ± 2	2 ± 0.5

ND, not detectable; NA, not analyzed.

tected in both types of particles isolated directly from plasma (**Fig. 2**, lanes 3 and 4). These low molecular weight proteins were more prominent in Lp(A-I w A-II) than in Lp(A-I w/o A-II). These protein bands most probably represent the various C peptides. Conversely, relatively more proteins with molecular weights between 30–200 kDa were seen in Lp(A-I w/o A-II) (Fig. 2, lane 4). When plasma Lp(A-I w/o A-II) and Lp(A-I w A-II) were centrifuged (see Fig. 1), considerable amounts of the high molecular weight proteins, along with some A-I and the C peptides, were not recovered in HDL (Fig. 2, lanes 5 and 6) but in the d > 1.21 g/ml fraction (Fig. 2, lanes 7 and 8). Consistent with this observation, other than albumin, ultracentrifugal HDL (Fig. 2, lane 2) also contain minimal proteins with molecular weights larger than A-I.

The lipid and protein composition of the HDL fraction of plasma Lp(A-I w/o A-II) and Lp(A-I w A-II) is shown in **Table 5**. In the four normolipidemic subjects (Nos. 1–4), Lp(A-I w/o A-II) particles contained relatively more lipid than Lp(A-I w A-II) particles (mean: 51% vs. 42%). However, the proportion of phospholipid, cholesterol, and triglyceride was comparable in the two types of particles. In the two FCHL subjects, the lipid and protein composition of Lp(A-I w/o A-II) and Lp(A-I w A-II) was equivalent. In subject 6 whose plasma triglyceride level was 609 mg/dl, both types of particles were enriched with triglyceride. This may explain the low level of HDL cholesterol in this subject despite her very high plasma A-I level (Table 1).

gPAGE analysis of Lp(A-I w/o A-II) and Lp(A-I w A-II)

When Lp(A-I w/o A-II) and Lp(A-I w A-II) were separated by gPAGE and stained for protein, distinct size subpopulations could be seen. Densitometric scans depicting the various size subpopulations of Lp(A-I w/o A-II) are shown in **Fig. 3**. In the five normolipidemic subjects, Lp(A-I w/o A-II) isolated directly from plasma comprised mostly two discrete size subpopulations with mean hydrated Stokes diameters of approximately 10.5 nm for Lp(A-I w/o A-II)$_1$ (42 ± 6%) and 8.5 nm for Lp(A-I w/o A-II)$_2$ (37 ± 6%) (Fig. 3A, 1–5). Particles larger than Lp(A-I w/o A-II)$_1$ and smaller than Lp(A-I w/o A-II)$_2$ were also present in varying quantities. Together, they averaged no more than 20% of the total Lp(A-I w/o A-II) particles. This was not the case in the three hyperlipidemic subjects. In these subjects, Lp(A-I w/o A-II)$_1$ and

TABLE 4. Distribution (%) of apoE in ultracentrifugal fractions

	Plasma			Lp(A-I w/o A-II)			Lp(A-I w A-II)		
Subject	d < 1.063	HDL	d > 1.21	d < 1.063	HDL	d > 1.21	d < 1.063	HDL	d > 1.21
1	24	48	28	59	25	16	25	73	2
2	NA	NA	NA	51	42	7	23	75	2
3	50	30	20	31	19	16	21	78	1
4	49	37	14	57	31	12	33	65	2
5	51	37	12	NA	NA	NA	NA	NA	NA
6	78	14	8	72	16	12	68	29	3
7	74	16	10	32	15	19	38	47	15
8	61	20	19	NA	NA	NA	NA	NA	NA
Mean ± SD	55 ± 18	29 ± 13	16 ± 7	50 ± 16	25 ± 10	14 ± 4	35 ± 18	61 ± 19	4 ± 5

NA, not analyzed.

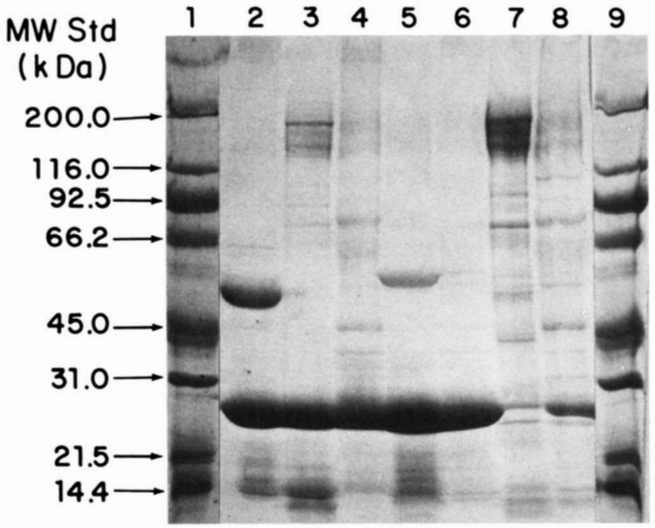

Fig. 2. Sodium dodecyl sulfate gel electrophoresis of plasma Lp(A-I w/o A-II) and Lp(A-I w A-II) and their density subfractions isolated from subject 3. Total proteins applied were between 40 and 80 μg. Lanes 1 and 9: combined low and high molecular weight standards from Bio-Rad Laboratories. Molecular weights (kilodaltons) of the standards are those provided by Bio-Rad; lane 2: HDL; lanes 3 and 4: plasma Lp(A-I w A-II) and Lp(A-I w/o A-II), respectively; lanes 5 and 6: the HDL fraction of plasma Lp(A-I w A-II) and Lp(A-I w/o A-II), respectively; lanes 7 and 8: the d > 1.21 g/ml fraction of Lp(A-I w A-II) and Lp(A-I w/o A-II), respectively.

Lp(A-I w/o A-II)$_2$ represented an average of only 52% of the total subpopulations. Particles smaller than Lp(A-I w/o A-II)$_2$ were substantially increased (34 ± 9%) (Fig. 3A, 6–8).

When Lp(A-I w/o A-II) isolated from the plasma of six subjects were ultracentrifuged (see Fig. 1), the relative proportion of Lp(A-I w/o A-II)$_1$ and Lp(A-I w/o A-II)$_2$ recovered in the HDL fraction was slightly lower than the parent Lp(A-I w/o A-II) prior to centrifugation (1.15 ± 0.39 vs. 0.9 ± 0.34) (Fig. 3, B vs. A). The relative proportion of particles larger than Lp(A-I w/o A-II)$_1$ and smaller than Lp(A-I w/o A-II)$_2$ was reduced from an average of 20% to 11% in the four normolipidemic subjects and from 39% to 30% in the two FCHL subjects. As described in Methods, the lipoproteins in the anti-A-II nonadsorbed HDL fractions were Lp(A-I w/o A-II) (see Fig. 1). They contained size subpopulations comparable to the Lp(A-I w/o A-II) isolated from plasma (Fig. 3C). When these scans were compared to those from plasma (Fig. 3A), several consistent differences were detected. First, in all subjects, the relative proportion of Lp(A-I w/o A-II)$_1$ to Lp(A-I w/o A-II)$_2$ was reduced (1.02 ± 0.45 vs. 0.57 ± 0.33). Second, particles larger than Lp(A-I w/o A-II)$_1$ were barely detected. Third, particles smaller than Lp(A-I w/o A-II)$_2$ were considerably reduced. Fourth, in all cases, a large peak was detected in the position of bovine albumin, our smallest calibration protein. This peak probably contained mostly the albumin usually found in "unwashed" HDL preparations. In four of the subjects, these anti-A-II nonadsorbed proteins were further chromatographed on anti-A-I columns to remove non-A-I materials (Fig. 1). Densitometric scans of the isolated Lp(A-I w/o A-II) showed that, although the protein peak at the albumin position was greatly reduced, a substantial distinct peak was still observed in each case (Fig. 3, 5–8, D). This suggested an increase of Lp(A-I w/o A-II) particles of approximately 7.1 nm diameter in HDL. Particles of this size were either not detected or were found only in trace quantities in plasma Lp(A-I w/o A-II) (Fig. 3A).

The Lp(A-I w/o A-II) population contains mostly particles in the size interval of 7.7 to 9.6 nm. In most normolipidemic subjects studied to date in our laboratory, three peaks or shoulders were observed in this interval. These were interpreted to represent three subpopulations. The mean particle sizes and relative proportions of these subpopulations in the subjects in this study were 9.6 nm (17 ± 11%) for Lp(A-I w A-II)$_1$, 8.9 nm (46 ± 18%) for Lp(A-I w A-II)$_2$, and 8.0 nm (30 ± 11%) for Lp(A-I w A-II)$_3$ **(Fig. 4)**. In subjects 1, 6, and 8, little or no Lp(A-I w A-II)$_1$ was detected. In subjects 2 to 4, additional particles larger than Lp(A-I w A-II)$_1$ were clearly present.

TABLE 5. Protein and lipid composition of Lp(A-I w/o A-II) and Lp(A-I w A-II)

	Lp(A-I w/o A-II)				Lp(A-I w A-II)			
Subjects	Protein	PL	CH	TG	Protein	PL	CH	TG
	wt %				*wt %*			
1	48	31	17	4	58	24	15	3
2	52	27	16	4	57	26	15	2
3	43	32	19	6	60	27	11	2
4	55	27	16	2	58	26	14	1
6	56	25	9	10	55	28	9	8
7	55	29	13	3	55	29	13	3

Abbreviations: PL, phospholipid; CH, cholesterol; TG, triglyceride.

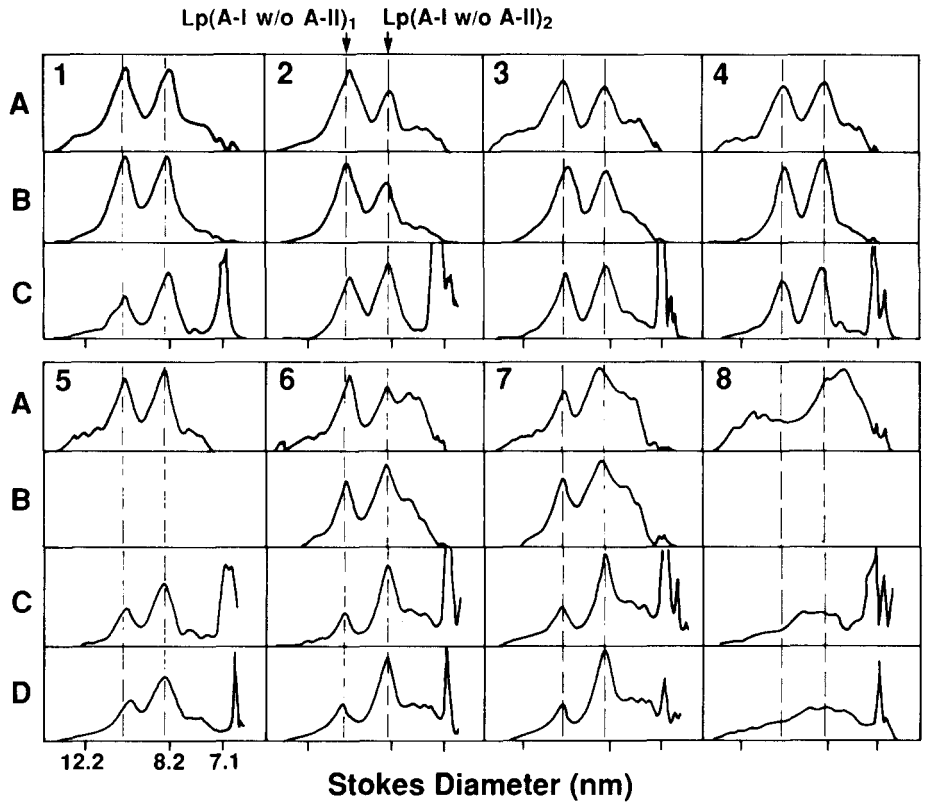

Fig. 3. Densitometric scans of gPAGE of Lp(A-I w/o A-II) of subjects 1-8. A: Particles isolated directly from plasma; B: the HDL fraction of particles isolated from plasma; C and D, respectively, were particles in ultracentrifuged HDL before and after anti-A-I immunoadsorption and thiocyanate elution (see Fig. 1).

When Lp(A-I w A-II) isolated from the plasma of six subjects were centrifuged, particles in the size interval of 7.7 to 9.6 nm recovered in HDL were essentially identical to the parent Lp(A-I w A-II) prior to centrifugation in all but subject 1 (Fig. 4, B vs. A). In this subject, there was a broadening of the Lp(A-I w A-II)$_2$ peak. When particles isolated from HDL (Fig. 4C) were compared to plasma Lp(A-I w A-II) (Fig. 4A), a reduction in the relative proportion of Lp(A-I w A-II)$_3$ was found in subjects 1 to 4 (mean: 29% vs. 15%). This, however, was not evident in the remaining four subjects. Overall, the differences between plasma and HDL Lp(A-I w A-II) subpopulations studied by gPAGE (Fig. 4, A vs. C) were less defined and were not consistent among subjects.

Effect of thiocyanate on A-I-containing lipoprotein subpopulations

A possible explanation for the differences in subpopulation distribution observed between Lp(A-I w/o A-II) isolated from plasma and HDL could be a differential effect of sodium thiocyanate on plasma and isolated HDL. To address this, two studies were performed. In the first study, a preparation of HDL washed free of albumin isolated from another subject was chromatographed on an anti-A-I column which adsorbed both Lp(A-I w/o A-II) and Lp(A-I w A-II) (i.e., all HDL particles). Lipoproteins were eluted from the immunoadsorbent with thiocyanate, and their subpopulation distribution in gPAGE was compared to the original HDL prior to immunoadsorption and thiocyanate elution. **Fig. 5** shows that the subpopulation components of A-I-containing lipoproteins eluted from the anti-A-I column were essentially identical to the pre-chromatographed HDL. In the second study, after the HDL of subjects 5 to 8 were chromatographed on anti-A-II immunoadsorbent, an aliquot of the nonadsorbed proteins which contained essentially Lp(A-I w/o A-II) was further chromatographed on the anti-A-I column to remove non-A-I materials (see Fig. 1). As shown in Fig. 3C, large peaks in the vicinity of bovine albumin were found in the HDL proteins that did not bind to anti-A-II immunoadsorbent. This appeared to be the only difference between these proteins and the isolated Lp(A-I w/o A-II) particles of these subjects (Fig. 3, 5-8, C vs. D). These data, therefore, demonstrate that all the subpopulations observed in immunoaffinity-isolated A-I-containing lipoproteins, in particular, Lp(A-I w/o A-II), were

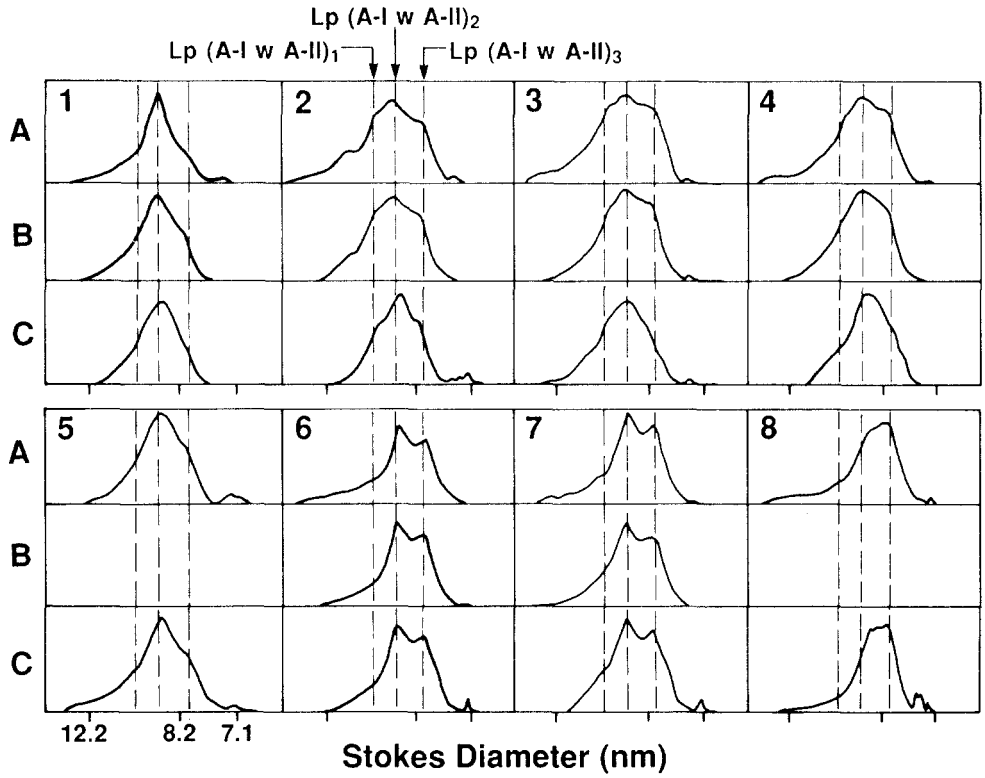

Fig. 4. Densitometric scans of gPAGE of Lp(A-I w A-II) of subjects 1-8. A: Particles isolated from plasma; B: the HDL fraction of particles isolated from plasma; C: particles isolated from ultracentrifuged HDL.

present in HDL prior to their contact with antibodies and thiocyanate. Hence, the observed differences between plasma and HDL in the subpopulation distribution of A-I-containing lipoproteins within the size interval of 7.1 to 17 nm (Fig. 3, A vs. C) were not artifacts of our immunoaffinity chromatography procedure.

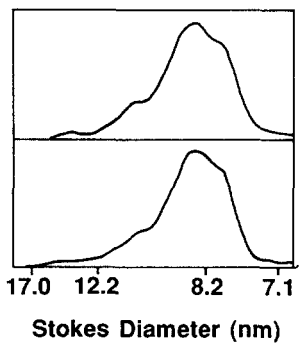

Fig. 5. Densitometric scans of gPAGE of HDL before (top) and after (bottom) anti-A-I affinity chromatography and thiocyanate elution.

DISCUSSION

The distributions of apoA-I, A-II, and E, among the various density fractions observed in this study when plasma was centrifuged, were comparable to earlier reports (9, 11, 16-19). The 34% LCAT detected in the $d > 1.21$ g/ml fraction was considerably less than the 75% reported in an earlier study (20). This may be due to the shorter centrifugal time and lower centrifugal force used in the present study. By combining immunoaffinity chromatography with ultracentrifugation, we were able to gain further insights into the source of these proteins in the $d > 1.21$ g/ml fraction. Centrifugation of Lp(A-I w/o A-II) and Lp(A-I w A-II) showed that 25-54% of the LCAT in both types of particles was recovered in this fraction. Based on our earlier studies, this LCAT was dissociated from larger, more buoyant lipoprotein particles as a consequence of ultracentrifugation (3). No more than 2-4% of apoA-I, A-II, and E in Lp(A-I w A-II) were in this density fraction. This probably reflects the stability of these particles in the ultracentrifuge, due possibly to the strong lipid-binding property of A-II (21, 22), and is in agreement with the observed minimal difference in subpopulation distribution of this type of particle before and

after centrifugation. Approximately 10–20% of A-I and E in Lp(A-I w/o A-II) were located in this fraction. Sodium dodecyl sulfate gel electrophoresis also revealed the presence of C peptides and several proteins with molecular masses between 30 and 200 kDa. We do not know the exact nature of all the proteins. However, based on data available in the literature, it is likely that besides apoE and LCAT, apoA-IV and CETP were two of these proteins (3, 23). Other plasma proteins known to be associated with HDL or A-I include acid phosphatases and human plasma platelet-activating factor acetylhydrolase (24, 25). Some of the protein bands between 116 and 200 kD may represent immunoglobulins. Using the technique of immunoblotting with commercial enzyme-linked antibodies, we have not been able to determine conclusively whether the immunoglobulins were of human or goat origin or both. A distinct protein band between 45 and 66 kDa was found in the HDL fraction of plasma Lp(A-I w A-II) (Fig. 2, lane 5). This band was not obvious in the particles before ultracentrifugation (Fig. 2, lane 3). We have no explanation for the existence of that protein band except that it was an artifact of ultracentrifugation or sample handling. Lipid analysis of the $d > 1.21$ g/ml fraction showed that about 70% of the lipid (by weight) was phospholipid, and 30% was cholesterol. Comparison of the subpopulation profile of plasma Lp(A-I w/o A-II) before and after centrifugation suggests that the proteins and lipids in the d 1.21 g/ml infranatant fraction might have been dissociated from particles larger than Lp(A-I w/o A-II)$_1$ and smaller than Lp(A-I w/o A-II)$_2$. It is possible that dissociation of lipid and proteins from lipoproteins during ultracentrifugation altered the structure of lipoprotein particles resulting in the changes in subpopulation profile observed in gPAGE. Very dense, small apoA-I-containing lipoproteins have been reported to exist in plasma (16, 26, 27). It is likely that such particles account for some of the A-I found in the $d > 1.21$ g/ml fraction of Lp(A-I w/o A-II).

The conditions of ultracentrifugation used in this study were those commonly used for isolating small quantities of HDL. As others have demonstrated, it is possible that when greater centrifugal force and longer time were employed in large scale HDL preparation, or when HDL were repeatedly centrifuged to remove non-lipoprotein contaminants, considerably more A-I and LCAT could be removed from HDL particles (20, 28). The preferential loss of A-I from Lp(A-I w/o A-II) provides an explanation for the considerably smaller proportion of this type of particle detected in HDL prepared by prolonged ultracentrifugation (29).

The immunoaffinity chromatography procedure we originally developed utilized mild acetic acid at pH 3.0 as the dissociation reagent because it had little adverse effect on LCAT (1). We have since discovered that this condition destroyed greater than 90% of CETP activity in plasma (3). We now routinely use thiocyanate to dissociate lipoproteins from the antibodies. To minimize any possible adverse effect of thiocyanate on lipoproteins, we limited all column volumes to under 50 ml, and used a dissociation rate of 60 ml/hr. Furthermore, the thiocyanate was immediately removed either by dialysis or desalting through a Sephadex G-25 column. As described under results, this procedure had no apparent quantitative or qualitative effect on HDL subpopulations studied by gPAGE (Fig. 3, C vs. D and Fig. 5). Under these conditions, recovery of apoA-I, A-II, E, and LCAT (mass) from the immunosorbents was 95, 90, 80, and 80%, respectively. Lp(A-I w/o A-II) and Lp(A-I w A-II) together represented 3–4% of total plasma proteins. Upon centrifugation, only 65 and 85% of the proteins were recovered in the HDL fraction of Lp(A-I w/o A-II) and Lp(A-I w A-II), respectively. Between 6 and 10% of the proteins were found in the $d < 1.063$ fraction. Thus, approximately 25 and 10% of the proteins in Lp(A-I w/o A-II) and Lp(A-I w A-II) were in the d 1.21 g/ml infranatant fraction.

Centrifugation of Lp(A-I w/o A-II) isolated from plasma (Fig. 3A) showed that particles larger than Lp(A-I w/o A-II)$_1$ and smaller than Lp(A-I w/o A-II)$_2$ were not quantitatively recovered in the HDL fraction (Fig. 3B). Although we cannot exclude the possibility that the densities of those particles were outside the HDL range, we do not believe that this was entirely the case because it had been shown that HDL contain particles between 7.2 and 15.0 nm (4). The relative proportion of Lp(A-I w/o A-II)$_1$ and Lp(A-I w/o A-II)$_2$, the two major subpopulations usually found in normolipidemic individuals, was only slightly reduced upon ultracentrifugation (Fig. 3, B vs. A). This either reflects that these subpopulations were less affected by ultracentrifugation, or the extent of perturbation was comparable. However, when plasma was centrifuged to prepare HDL prior to immunoaffinity chromatography, the Lp(A-I w/o A-II) in HDL was distinctly different from that in plasma (Fig. 3, C vs. A). Besides the loss of particles described above, the relative proportion of Lp(A-I w/o A-II)$_1$ and Lp(A-I w/o A-II)$_2$ was reduced in each case, although to different extents in the subjects. We do not know the reason behind this observation. Data presented in Figs. 3 and 5 provided evidence that this differential loss could not be attributed to antibody adsorption and thiocyanate elution. Since similar centrifugal procedures were used in preparing the HDL fraction from plasma and from Lp(A-I w/o A-II), centrifugation alone could not be the explanation. We speculate that this may be a combined effect of ultracentrifugation and the interaction of various enzymes or biologically active proteins on HDL and/or the lower density lipoproteins during the long course of centrifugation. These enzymes and lower density lipoproteins were present in substantial quantities in the plasma but were either absent or present in much smaller quantities in isolated Lp(A-I

w/o A-II) during ultracentrifugation. This effect was not abolished even when the centrifugation temperature was lowered from 10°C (subjects 5 and 8) to 4°C (all other subjects). The recommended temperature for isolation of lipoproteins by ultracentrifugation has been 15–20°C. In view of the known effect of LCAT and CETP on HDL size and density (30–33), we have chosen to perform ultracentrifugation at 4°C and 10°C. Although we cannot exclude the possibility that these low temperatures may have affected the properties of core lipids and thus led to changes in particle density and size, the minimal changes observed in plasma Lp(A-I w A-II) before and after centrifugation (Fig. 4, A vs. B) suggest that this may not be the case. In conclusion, our present study shows that the Lp(A-I w/o A-II) particles are less stable than Lp(A-I w A-II) particles upon ultracentrifugation. Among the numerous Lp(A-I w/o A-II) subpopulations, particles larger than Lp(A-I w/o A-II)$_1$ and smaller than Lp(A-I w/o A-II)$_2$ were most labile. These observations must be taken into consideration in studying HDL subpopulations.

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