Distribution and localization of lecithin:cholesterol acyltransferase and cholesteryl ester transfer activity in A-I-containing lipoproteins

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Abstract Two types of A-I-containing lipoproteins are found in human high density lipoproteins (HDL): particles with A-II (Lp(A-I with A-II)) and particles without A-II (Lp(A-I without A-II)). We have studied the distribution of lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer (CET) activities in these particles. Lp(A-I with A-II) and Lp(A-I without A-II) particles were isolated from ten normolipidemic subjects by anti-A-I and anti-A-II immunosorbents. Most plasma LCAT mass (70 ± 15%), LCAT (69 ± 16%), and CET (81 ± 15%) activites were detected in Lp(A-I without A-II). Some LCAT (mass: 16 ± 7%, activity: 17 ± 8%) and CET activities (7 ± 8%) were detected in Lp(A-I with A-II). To determine the size subspecies that contain LCAT and CET activities, isolated Lp(A-I with A-II) and Lp(A-I without A-II) particles of six subjects were further fractionated by gel filtration column chromatography. In Lp(A-I without A-II), most LCAT and CET activities were associated with different size particles, with the majority of the LCAT and CET activities located in particles with hydrated Stokes diameters of 11.6 ± 0.4 nm and 10.0 ± 0.6 nm, respectively. In Lp(A-I with A-II), most of the LCAT and CET activities were located in particles similar in size: 11.1 ± 0.4 nm and 10.6 ± 0.3 nm, respectively. Ultracentrifugation of A-I-containing lipoproteins resulted in dissociation of both LCAT and CET activities from the particles. Furthermore, essentially all CET and LCAT activities were recovered in the non-B-containing plasma obtained by anti-LDL immunoaffinity chromatography. This report, therefore, provides direct evidence for the association of LCAT and CET protein with A-I-containing lipoproteins. Our conclusions pertain to fasting normolipidemic subjects and may not be applicable to hyperlipidemic or nonfasting subjects.—**Cheung, M. C., A. C. Wolf, K. D. Lum, J. H. Tollefson, and J. J. Albers.** Distribution and localization of lecithin:cholesterol acyltransferase and cholesteryl ester transferase activity in A-I-containing lipoproteins. *J. Lipid Res.* 1986. **27:** 1135–1144.

Supplementary key words cholesteryl ester transfer protein • immunoaffinity chromatography • apoA-I • apoD

The high density lipoproteins (HDL) are an integral part of the human plasma lipid transport system. Numerous epidemiological studies have associated HDL with an inverse risk for coronary artery disease. The mechanism behind this inverse relationship is still not clear. However, it has been postulated that HDL may facilitate "reverse cholesterol transport" by promoting cholesterol efflux from peripheral cells (1, 2). The free cholesterol effluxed from the cell is then converted to cholesteryl ester by the enzyme lecithin:cholesterol acyltransferase (LCAT), and transferred from HDL to the lower density lipoproteins by cholesteryl ester transfer protein(s) (CETP) for hepatic catabolism.

Although LCAT and CETP are usually purified from the d > 1.21 g/ml lipoprotein-deficient plasma fraction when ultracentrifugation is used as the initial purification step, observations from our laboratory as well as others suggest that most LCAT and CETP may exist bound to HDL in human plasma but dissociate from the d < 1.21 g/ml lipoprotein fraction during ultracentrifugation (3–6). Recently, we have developed an immunoaffinity chromatography technique for isolating lipoproteins without using ultracentrifugation (7). With this technique, we have isolated two types of A-I-containing lipoprotein particles from human plasma: particles containing both A-I and A-II (Lp(A-I with A-II)), and particles containing A-I but no A-II (Lp(A-I without A-II)). Since the majority of both types of particles demonstrate similar physiochemical properties such as electrophoretic mobility, size, and density of HDL, we have proceeded to investigate whether LCAT and CETP are associated with these particles. In this report we show that most of the plasma LCAT and cholesteryl ester transfer (CET) activities are located in these particles. We characterize the distribution of these activities in A-I-containing lipoprotein

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; gPAGE, gradient polyacrylamide gel electrophoresis; HAc, acetic acid; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; CETA, cholesteryl ester transfer activity; CE, cholesteryl ester; LTP, lipid transfer protein.

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subspecies. We further demonstrate that ultracentrifugation of lipoprotein particles isolated by immunoaffinity chromatography results in dissociation of LCAT and CET activities from the particles.

MATERIALS AND METHODS

Isolation of lipoproteins by immunoaffinity chromatography

Blood samples used in this study were obtained from ten normolipidemic adult volunteers after a 12–14-hr overnight fast. Venous blood was drawn into Vacutainer tubes (Becton-Dickinson) containing disodium EDTA (1.5 mg/dl). Upon separation of plasma at 4°C by low speed centrifugation (1000 *g*), sodium azide, chloramphenicol, and gentamycin (final concentration 0.5, 0.01, 0.005 g/L, respectively), were promptly added, and aliquots of the plasma were immediately subjected to immunosorbents for isolation of lipoproteins. The anti-A-I and anti-A-II immunosorbents used for lipoprotein isolation were prepared by conjugating affinity-isolated goat anti-A-I and anti-A-II antibodies to CNBr-activated Sepharose 4B (Pharmacia Inc., Piscataway, NJ) as described (7). These antibodies were tested to be specific for either A-I or A-II by the immunoprecipitation and/or immunoblotting technique. Immunoprecipitation was performed with ^{125}I-labeled A-I, A-II, B, C-I, C-II, C-III, E, and LCAT as described (7). Immunoblotting was performed according to the method of Towbin, Staehelin, and Gordon (8) using apoHDL, purified A-I, A-II, and LCAT as the testing antigens. The anti-A-I and A-II antibodies used in this study had affinity constants in the order of 10^7 M^{-1} at 4°C at pH 7.4 in 0.01 M Tris-HCl, 0.15 M NaCl buffer.

To isolate lipoprotein particles containing both A-I and A-II (Lp(A-I with A-II)), 7 ml of plasma was incubated with anti-A-II immunosorbent (30–35 ml) for 1 hr at 4°C. The immunosorbent was packed in an Econo-Column (Bio-Rad Laboratories, Richmond, CA) of 1.5 cm internal diameter. Proteins that did not bind to the immunosorbent were washed off 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. Lipoproteins bound to the immunosorbent were eluted with 3 M NaSCN in 0.02 M sodium phosphate buffer, pH 7.0, at 60 ml/hr, and immediately dialyzed against three changes of 4 L of Tris buffer.

To isolate lipoprotein particles containing A-I but not A-II (Lp(A-I without A-II)), plasma devoid of A-II (the nonbinding plasma proteins from the above anti-A-II immunosorbent) was incubated with 15 ml of anti-A-I immunosorbent for 1 hr at 4°C. The nonbinding proteins and bound lipoproteins were sequentially eluted from the immunosorbent and processed as described above.

Gel filtration chromatography

After dialysis, the Lp(A-I with A-II) and Lp(A-I without A-II) particles isolated by immunoabsorption were concentrated to 1–3 mg of protein/ml by Micro-Confilt concentrator using protein dialysis membrane of M_r = 10,000 cut off (Biomolecular Dynamics, Beaverton, OR). Two-ml aliquots of the concentrated lipoprotein particles from six subjects were fractionated by ascending gel filtration chromatography (1.6 × 90 cm) on Sephacryl S-300 superfine medium (Pharmacia Inc, Piscataway, NJ) at 4°C using Tris buffer containing 0.3 M NaCl at a flow rate of 8 ml/hr. Fractions were collected at 5-min intervals. Alternate fractions between the void volume and salt volume were assayed directly for apoprotein (apo)A-I, LCAT and CET activities. In some cases, LCAT mass and apoD contents in the fractions were also determined.

Protein and LCAT mass analyses

ApoA-I, A-II, D, and LCAT contents of the isolated A-I-containing lipoprotein particles, plasma, the various nonbinding plasma fractions of the anti-A-I and anti-A-II immunosorbents, and the fractions from the gel filtration columns were analyzed by either radioimmunoassay (RIA) (LCAT) (9), radial immunodiffusion assays (A-I, A-II, D) (10–12), or enzyme-linked immunosorbent assay (ELISA) (D). The D ELISA assay gave values comparable to our previously published radial immunodiffusion assay (12). Total protein in plasma and nonbinding plasma was measured by the method of Lowry et al. (13).

Cholesteryl ester transfer activity (CETA) and LCAT activity assays

CETA and LCAT activity in plasma, nonbinding plasma fractions of the anti-A-I and anti-A-II immunosorbents, isolated A-I-containing lipoprotein particles, and fractions from the gel filtration columns were measured by the cholesteryl ester transfer assay of Albers et al. (14), and the A-I/lecithin/cholesterol proteoliposome common substrate method of Chen and Albers (15), respectively. Briefly, to measure CETA, aliquots (plasma fractions and isolated lipoproteins: 10 μl or 25 μl; Sephacryl S-300 fractions: 100 μl or 200 μl) of each test fraction were mixed with 0.2 ml of a ^{14}C-labeled CE-HDL$_3$ donor d < 1.06 g/ml lipoprotein acceptor mixture (donor:acceptor cholesteryl ester mass ratio equaled 1:8) and incubated at 37°C in a shaking water bath for 18 hr. The reaction was stopped by chilling the tubes in ice. Donor and acceptor lipoproteins were separated by the dextran sulfate–magnesium chloride precipitation procedure (16). CETA was expressed as percentage of ^{14}C-labeled CE-HDL transferred per 18 hr per specified

volume of sample. The CETA was linear with time for 18 hr according to these experimental conditions (**Fig. 1A**) (r = 0.9967).

To assay for LCAT activity, 0.025 ml of 2% HSA and 0.025 ml of an A-I:lecithin:^{14}C-cholesterol liposome were incubated at 37°C for 20 min, and 0.010 ml of 50 mM β-mercaptoethanol was added. Aliquots (3-μl plasma fractions and isolated lipoproteins; 40-μl Sephacryl S-300 fractions) of each test sample were then added to this preincubated substrate and the whole mixture was incubated at 37°C in a shaking water bath for 90 min (plasma fractions and isolated lipoproteins) or 18 hr (gel filtration fractions). The enzyme reaction was stopped by immersing the tubes in an ice bath. Esterified and unesterified cholesterol were separated by digitonin precipitation (17). LCAT activity was expressed as the percentage of cholesterol esterified per 90 min or 18 hr per specified volume of sample. The LCAT activity was linear with time for 90 min according to these experimental conditions (Fig. 1B) (r = 0.9969).

In both the LCAT and CET activity assays, conditions for measuring plasma, anti-A-I, and anti-A-II nonbinding plasma, and the isolated Lp(A-I with A-II) and Lp(A-I without A-II) particles were those under which percent esterification (LCAT) and percent cholesteryl ester transferred (CETA) were directly proportional to the amount of the test samples. The linearity of the CETA and LCAT activity assays over 18 hr and 90 min, respectively, allowed us to perform quantitative comparison among the various plasma and lipoprotein fractions from an individual. However, assay conditions used for measuring CET and LCAT activities in the gel filtration column fractions did not necessarily meet these criteria due to limited availability of samples.

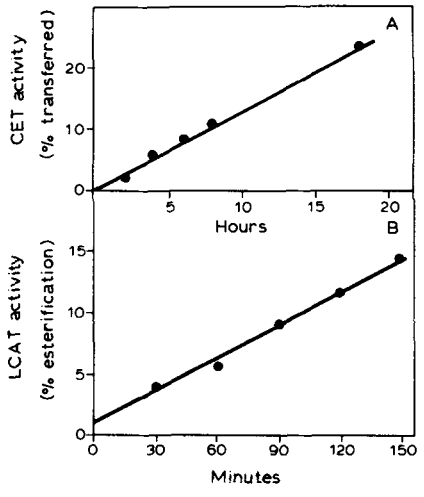

Fig. 1. Time course of CETP (A) and LCAT (B) activity assays. Fifty μl of a partially purified preparation of CETP (phenyl Sepharose pool of ref. 14) (A) and 3 μl of plasma (B) were assayed as described in Methods. The reactions were stopped at various time intervals as indicated. Each point represents the mean of duplicate or triplicate determinations. The lines are the least square best fit regression lines with correlation coefficient of 0.997 for both.

Molecular size determination

To estimate the mean molecular weight of lipoprotein particles associated with LCAT and CET activities, the elution volume of fractions from each column containing peak LCAT and CET activities was used as the basis for calculation. Furthermore, gradient polyacrylamide gel electrophoresis (gPAGE) was performed on these fractions using precast PAA 4/30 gels (Pharmacia), and apparent Stokes diameters were estimated as described (18, 19). The proteins and their molecular weights and Stokes diameters used for electrophoresis and/or initial gel filtration column calibrations were: thyroglobulin (669,000, 17.0 nm), apoferritin (440,000, 12.2 nm), catalase (232,000, 10.4 nm), lactate dehydrogenase (140,000, 8.2 nm), bovine albumin (67,000, 7.5 nm), cytochrome C (12,400, 3.4 nm). In later studies, we used the premixed Gel Filtration Standard from Bio-Rad Laboratories that contained thyroglobulin, gamma globulin (158,000, 10.6 nm), ovalbumin (44,000, 5.5 nm), myoglobulin (17,000), and cyanocobalamin (vitamin B-12) (1350).

RESULTS

Isolation of A-I-containing lipoproteins by immunoaffinity chromatography

We have previously reported the immunochemical isolation of A-I-containing lipoproteins using 0.1 M HAc, pH 3.0, to desorb the lipoproteins from anti-A-I or anti-A-II immunosorbent (7). Under such conditions, recoveries of A-I, A-II, and D from the immunosorbents were better than 92%, however recovery of LCAT mass was less than 15%. In order to perform the present study, we re-evaluated the desorption conditions in order to obtain the maximal LCAT and CET activities. After performing a series of preliminary experiments, we concluded that in order to quantitatively recover LCAT mass from our immunosorbents, it was necessary to use either 0.5 M HAc with 0.5 M NaCl, pH 3.0, or buffer containing 3 M sodium thiocyanate as the desorbing agent. However, while CETA could be detected in lipoproteins eluted with 3 M thiocyanate, no CETA could be detected in the particles eluted with acetic acid. To assess the affect of 0.5 M HAc, 0.5 M NaCl, pH 3.0, and 3 M thiocyanate on LCAT and CETP activities, partially purified LCAT and CETP (LTP-I) (14) were added to 1-ml aliquots of d 1.063–1.21 g/ml HDL at plasma concentration. Each aliquot was brought to 0.5 M HAc, 0.5 M NaCl, pH 3.0, by glacial acetic acid and solid NaCl or to 3 M thiocyanate with

solid sodium thiocyanate and allowed to sit at 4°C for 1 or 2 hr. At the end of the first and second hour, the acetic acid and thiocyanate were removed by rapid dialysis in two changes of 4 L of Tris buffer and assayed for LCAT and CET activities. Under these conditions, recovery of LCAT activity and CETA were 66% and 10%, respectively, for HAc-treated samples, and 51% and 63%, respectively, for thiocyanate-treated samples. There was no significant difference between 1- and 2-hr contact. Recovery was calculated by comparing percent cholesterol esterified (LCAT activity) or cholesteryl ester transferred (CETA) per unit protein mass per unit time before and after acetic acid or thiocyanate treatment. The conditions of the LCAT and CETA assays used in this study were those giving activities at the linear rate range. In this same experiment, the recoveries of LCAT mass as measured by our RIA were 82% and 72%, respectively, for the 1- and 2-hr incubation in thiocyanate. This suggested that after 1–2 hr of exposure to thiocyanate, 20–30% of apparent LCAT mass was lost as determined by competitive RIA assay. Hence, while acetic acid at pH 3.0 inactivated 90% CETA, by minimizing the thiocyanate contact time to under 2 hr, we could recover 50–60% active LCAT and CETP. Consequently, in this study, we limited our immunosorbent column volume to under 35 ml and used an elution flow rate of 60 ml/hr to desorb lipoproteins from the immunosorbent.

Distribution of LCAT and CET activities in plasma

To study the distribution of LCAT and CET activities in plasma, the Lp(A-I with A-II), Lp(A-I without A-II), as well as the A-I-free and A-II-free plasma of each subject were assayed for LCAT mass and exogenous LCAT and CET activities as described in Methods. To account for differences in protein concentration among samples, the A-I-free and A-II-free plasma LCAT and CET activities, as well as LCAT mass, were normalized to plasma total protein concentration while LCAT mass, LCAT and CET activities in Lp(A-I with A-II) and Lp(A-I without A-II) were normalized to their plasma A-I concentrations. Since thiocyanate inactivated some LCAT and CETA, the actual LCAT mass (78 ± 13%), LCAT (64 ± 21%) and CET (70 ± 23%) activities recovered in the two types of particles were used for calculating distribution between these particles. Details for calculating distribution between Lp(A-I with A-II) and Lp(A-I without A-II) based on plasma and nonbinding plasma fractions have been described (7). **Table 1** and **Table 2** show the distribution of LCAT and CET activity, respectively, in the ten subjects studied using activity values measured directly in the two types of particles. As a group, 69 ± 16% (mean ± SD) of plasma LCAT activity and 81 ± 15% of CETA were associated with Lp(A-I without A-II) particles. The LCAT mass associated with this type of particles was 70 ± 15%, comparable to LCAT activity. One of the subjects had greater than 90% of the plasma LCAT activity (subject 1) and three subjects (subjects 5, 9, and 10) had greater than 95% of the plasma CETA in this type of particle. Seventeen ± 8% of LCAT activity, 16 ± 7% LCAT mass, and 7 ± 8% of CETA were found in particles containing both A-I and A-II. In some subjects, however, little or no LCAT or CET activity could be detected in these particles. The remaining 14 ± 13% LCAT activity, 14 ± 10% LCAT mass and 12 ± 10% CETA were found in plasma free of A-I. Several subjects had little or no LCAT or CET activity in plasma after all A-I was removed. Hence, most plasma LCAT and CETA were detected in lipoprotein particles containing A-I but no A-II.

The distribution of LCAT and CETA between Lp(A-I with A-II) and Lp(A-I without A-II) calculated from measurement of LCAT and CETA in plasma, A-I-free plasma, and A-II-free plasma according to the previously described method (7) compared well with the values shown in Tables 1 and 2. The LCAT mass, LCAT activity,

TABLE 1. Distribution of LCAT activity in plasma

Subject	Sex	% Plasma Activity in		% Activity Not Associated with A-I
		Lp(A-I without A-II)	Lp(A-I with A-II)	
1	F	94	ND[a]	6
2	M	44	22	34
3	F	78	22	ND
4	F	54	27	19
5	M	68	22	3
6	M	84	12	4
7	M	65	9	26
8	F	84	16	ND
9	M	51	17	32
10	F	64	23	13
Mean ± SD		69 ± 16	17 ± 8	14 ± 13

[a] ND, not detectable (less than 1% plasma LCAT activity); this is considered as zero percent in calculating the mean.

TABLE 2. Distribution of CET activity in plasma

Subject	Sex	% Plasma Activity in Lp(A-I without A-II)	% Plasma Activity in Lp(A-I with A-II)	% Activity Not Associated with A-I
1	F	75	7	18
2	M	81	6	13
3	F	82	14	4
4	F	58	24	18
5	M	100	ND[a]	ND
6	M	88	1	11
7	M	60	5	35
8	F	73	12	15
9	M	97	ND	3
10	F	97	ND	3
Mean ± SD		81 ± 15	7 ± 8	12 ± 10

[a]ND, not detectable (less than 1% plasma CET activity); this is considered as zero percent in calculating the mean.

and CETA in Lp(A-I without A-II) were 66 ± 10%, 67 ± 19%, and 74 ± 18%, respectively, and in Lp(A-I with A-II) were 20 ± 12%, 19 ± 10%, and 14 ± 15%, respectively.

Localization of LCAT and CETP activities

In order to determine the approximate size(s) of the particles associated with LCAT and CETP, the Lp(A-I with A-II) and Lp(A-I without A-II) particles of six subjects isolated by anti-A-II and anti-A-I immunosorbents were further fractionated by Sephacryl S-300 gel filtration chromatography. Aliquots from alternate fractions between the void volume and the salt volume of the column were assayed for LCAT and CET activities. Because of sample dilution, the incubation time in both assays was 18 hr. In four of the six cases, LCAT mass was also measured. **Fig. 2, A and B** shows representative LCAT and CET activities as well as LCAT mass profiles of Lp(A-I without A-II) and Lp(A-I and A-II) of one of the subjects after Sephacryl S-300 gel filtration chromatography. Several observa-

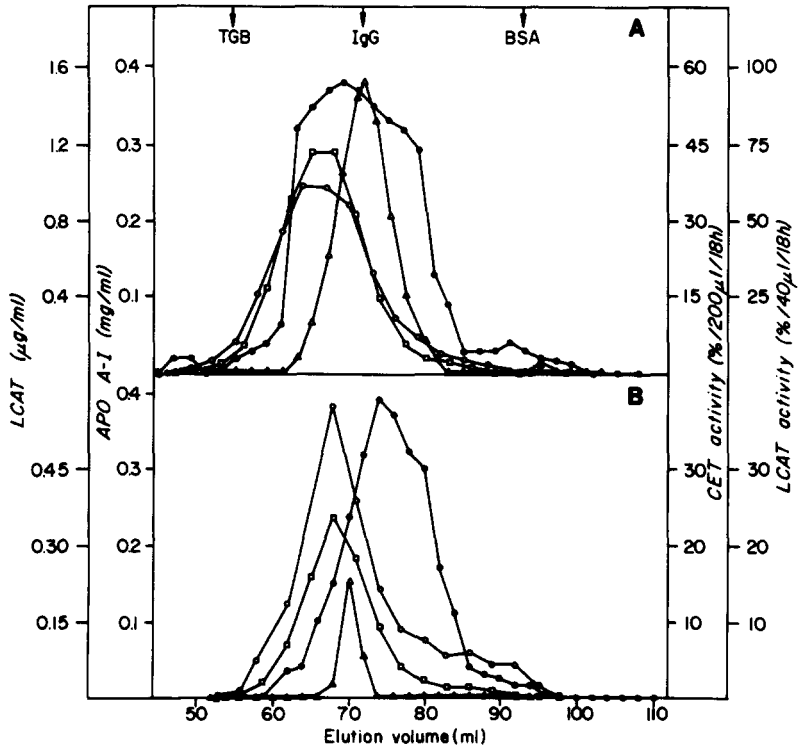

Fig. 2. Sephacryl S-300 chromatography of Lp(A-I without A-II) (A) and Lp(A-I with A-II) (B) of subject 2. The profiles are: LCAT activity (○—○), LCAT mass (□—□), CET activity (△—△), and apoA-I (●—●).

tions could be made; in all cases, the LCAT activity profile paralleled the LCAT mass profile. In Lp(A-I without A-II) particles, most of the LCAT activity preceded the CETA, suggesting that most LCAT and CET activities were associated with different-sized Lp(A-I without A-II) particles. Furthermore, LCAT was detected over a larger number of fractions than CET activity. In Lp(A-I with A-II) particles, the LCAT and CETP activity profiles overlap considerably with the peak of LCAT activity preceding the peak of CETA by no more than two to four fractions, suggesting that they may be found in particles quite similar in size. In all cases, peak LCAT and CET activities preceded the bulk of Lp(A-I with A-II) particles.

To estimate the apparent Stokes diameter of lipoprotein particles associated with LCAT and CETA, the elution volumes of fractions from each column containing peak LCAT and CETP activities were used as the basis for calculation. Furthermore, gradient polyacrylamide gel electrophoresis (gPAGE) was performed on these fractions and the Stokes diameters of the darkest protein bands were calculated. The results are shown in **Table 3** and **Table 4.** Apparent diameters calculated from gel filtration chromatography were consistently higher than those calculated from gPAGE. Combining the data from these two methods of size determination shows that in Lp(A-I without A-II) most LCAT was located in the larger (Stokes diameter = 11.6 ± 0.4 nm) while CETA was located in the smaller (Stokes diameter = 10.0 ± 0.6 nm) subpopulation of particles. Due to the broadness of the LCAT profile, it appeared that some LCAT was also associated with the smaller subpopulation of Lp(A-I without A-II) particles overlapping in size with particles containing CETA. In Lp(A-I with A-II), LCAT and CET activities were located in subpopulations with apparent Stokes diameter of 11.1 ± 0.4 nm and 10.6 ± 0.3 nm, respectively. Hence in Lp(A-I with A-II), CETA was associated with a relatively larger-sized subpopulation.

Distribution of apoD in Lp(A-I with A-II) and Lp(A-I without A-II)

Since it has been suggested that apoD is involved in cholesteryl ester transport in plasma (20), the apoD content of Lp(A-I without A-II) and Lp(A-I with A-II) of four subjects after fractionation by Sephacryl S-300 gel filtration chromatography was measured by a specific D ELISA. **Fig. 3, A and B** shows representative distributions in these two types of particles. (The distribution of A-I has been included for comparison.) In all subjects, apoD was found to be associated with particles of all sizes, having profiles with multiple discernible peaks or shoulders. Trace quantities of A-I and D were consistently found in fractions eluted around the void volume. No consistent relationship could be detected between the various apoD peaks and position of maximal CETA in the four subjects studied.

Effect of ultracentrifugation on the distribution of LCAT and CETP in A-I-containing lipoproteins

Most LCAT and CETP were usually found in the d > 1.21 g/ml fraction after prolonged ultracentrifugation of plasma. However, by using an isolation technique (immunoaffinity chromatography) that did not involve ultracentrifugation, we were able to locate the majority of LCAT mass and a considerable amount of plasma LCAT and CET activities in A-I-containing lipoprotein particles of HDL size. To investigate whether the LCAT and CETP normally found in the d > 1.21 g/ml plasma fraction were dissociated from A-I-containing lipoproteins during ultracentrifugation, distribution of LCAT and CET activities in the plasma of a subject (subject 4) was studied. Two ml of fresh plasma from this subject was fractionated directly on a gel filtration column packed with Sephacryl S-300. A-I-containing lipoproteins were isolated from 8 ml of fresh plasma by direct absorption with anti-A-I immunoabsorbent using conditions described in Methods.

TABLE 3. Molecular size of Sephacryl S-300 fractions containing peak LCAT activity

	Lp(A-I without A-II)		Lp(A-I with A-II)	
Subject	Gel Filtration Diameter[a]	gPAGE Diameter	Gel Filtration Diameter	gPAGE Diameter
		nm		
1	13.8	10.6	ND[a]	ND
2	12.5	10.4	12.1	9.8
5	12.4	10.1	13.4	9.4
6	13.4	10.2	12.8	9.4
7	13.4	10.2	12.1	9.4
8	11.9	10.0	12.8	9.6
Mean ± SD	12.9 ± 0.7	10.3 ± 0.2	12.6 ± 0.6	9.5 ± 0.2

[a] Hydrated Stokes diameter.
[b] ND, not detectable.

TABLE 4. Molecular size of Sephacryl S-300 fractions containing peak CETA

Subject	Lp(A-I without A-II)		Lp(A-I with A-II)	
	Gel Filtration Diameter[a]	gPAGE Diameter	Gel Filtration Diameter	gPAGE Diameter
	nm			
1	11.4	8.6	12.0	9.7
2	11.2	8.5	12.0	8.9
5	11.2	8.5	ND[b]	ND
6	12.4	8.6	12.1	9.0
7	11.4	8.6	11.4	9.3
8	10.4	8.6	12.1	9.2
Mean ± SD	11.3 ± 0.6	8.6 ± 0.05	11.9 ± 0.3	9.2 ± 0.3

[a] Hydrated Stokes diameter.
[b] ND, not detectable.

A 2-ml aliquot of the isolated A-I-containing lipoproteins was fractionated on the Sephacryl S-300 gel filtration column. Another 2-ml aliquot was brought to d 1.21 g/ml by solid KBr, overlayered with d 1.21 g/ml KBr solution containing 1 mM EDTA, pH 7.4, and centrifuged at 35,000 rpm at 4°C for 48 hr in a Beckman 40.3 Ti rotor. At the end of the centrifugation, the top 2.5 ml and bottom 4.0 ml were removed by tube slicing and a 2-ml aliquot of each fraction was separately fractionated on the same gel-filtration column. Fractions between the void volume and the salt volume were assayed for LCAT and CET activities as previously described. As shown in **Fig. 4, A and B,** the LCAT and CET activity profiles of plasma and A-I-containing lipoprotein particles fractionated by gel filtration were comparable. This suggested that immunoaffinity isolation of lipoproteins from plasma had not significantly altered the association of LCAT and CETA with lipoproteins. When A-I-containing lipoproteins were centrifuged at d 1.21 g/ml at 35,000 rpm for 48 hr, LCAT and CET activities werer detected in both top

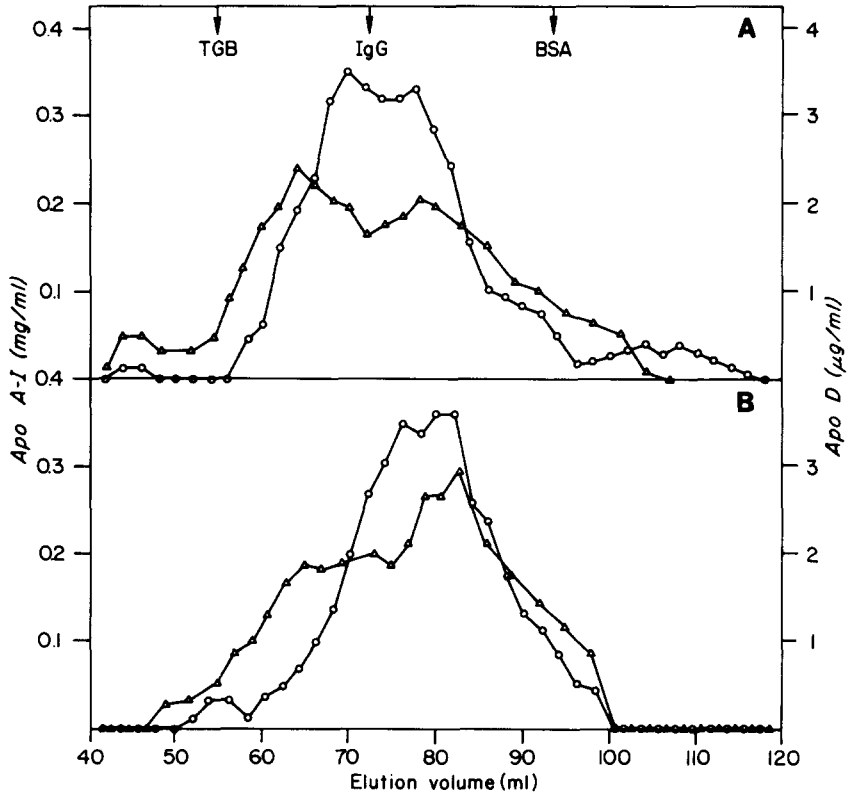

Fig. 3. Distribution of apoA-I (○—○) and D (△ — △) in Lp(A-I without A-II) (A) and Lp(A-I with A-II) (B) of subject 1 after fractionation by Sephacryl S-300 chromatography.

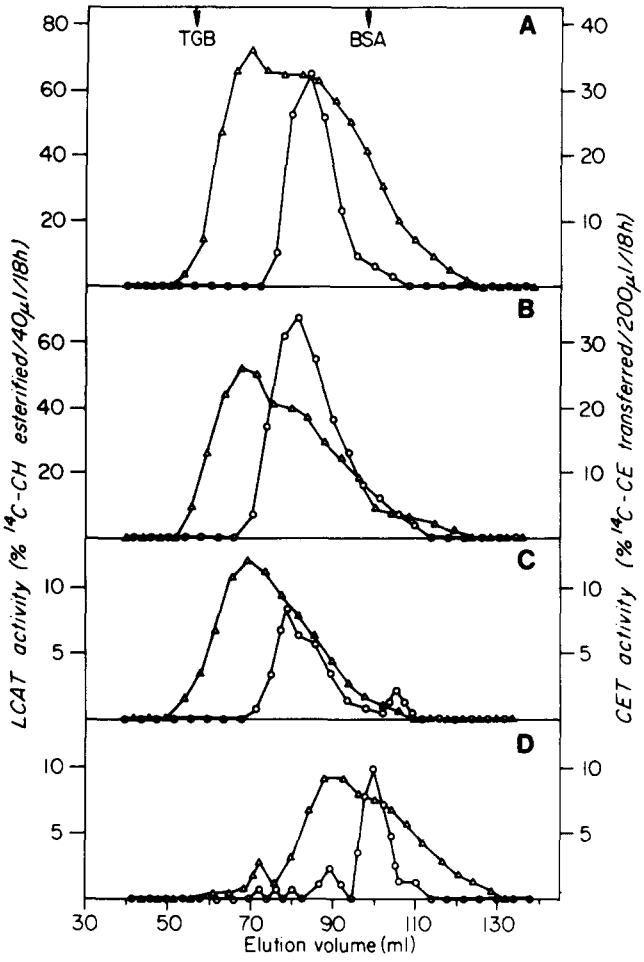

Fig. 4. Sephacryl S-300 chromatography of plasma (A), A-I-containing lipoproteins (B), the d < 1.21 g/ml fraction (C), and the d > 1.21 g/ml fraction (D) of A-I-containing lipoproteins of subject 4. The profiles are LCAT (△ — △) and CET (○—○) activities.

and bottom fractions. In general, the LCAT and CETA profiles of the d < 1.21 g/ml fraction after gel filtration chromatography (Fig. 4, C) resembled those of plasma and the A-I-containing lipoproteins prior to centrifugation (Fig. 4, A and B). However, separation of the d > 1.21 g/ml fraction by gel filtration chromatography showed that, while LCAT activity was found in a broad size range of particles between molecular weights of approximately 60,000–180,000, most of the CETA was located in fractions of particles with approximate mean M_r of 65,000, indicating the dissociation of LCAT and CETA from lipoprotein particles upon ultracentrifugation.

DISCUSSION

We have isolated Lp(A-I with A-II) and Lp(A-I without A-II) particles from ten normolipidemic subjects (five men and five women) by immunoabsorption and fractionated the particles from six of the ten subjects by gel filtration chromatography to localize LCAT and CETP in these particles. The results presented in this report demonstrated that both LCAT (mass and detected activity) and CETA were found in these two types of particles with most of the LCAT and CETA in Lp(A-I without A-II) particles. LCAT and CETP activity profiles in the two types of A-I-containing lipoprotein particles after gel filtration chromatography were consistently different. First, the peak of the LCAT activity profile preceded the peak of CETA profile suggesting that most of the LCAT-containing particles there were larger than CETA-containing particles. This difference in the profiles was particularly evident in Lp(A-I without A-II) particles. Second, while LCAT activity could be detected over a large number of fractions, most CETA was found within 10-20 fractions (Fig. 2). This suggests that while most of the LCAT-containing particles were larger sized HDL particles, some LCAT was also associated with A-I-containing lipoprotein particles in the smaller size range. However, CETA was associated with a narrow size range of particles: relatively larger-sized particles in Lp(A-I with A-II) and smaller-sized particles in Lp(A-I without A-II).

It has been shown that the proteoliposome assay used to measure LCAT activity in this study is not significantly affected by the lipid content of the test sample (15). The CETA assay, however, may be affected by excess exogenous lipoproteins in the test samples. Exogenous d < 1.063 g/ml lipoproteins, which result in increasing the acceptor-to-donor ratio in the assay, enhance observed percent transfer while exogenous HDL reduce observed transfer. Hence, it is possible that the amounts of CETA detected in the A-I-free plasma that contained only the d < 1.063 lipoproteins was slightly overestimated (Table 2). On the other hand, the magnitudes of CETA detected in A-I-containing lipoproteins (Table 2 and Fig. 2) were slightly underestimated. Furthermore, the suppressive effect of HDL on "observed" CETA may have contributed partly to the low calculated CETA recovery (70%) of the A-I-containing particles in this study.

As stated under Results, the 3 M thiocyanate we used to desorb lipoproteins from the immunosorbent inactivated some LCAT and CET activities. In view of this, we cannot be sure that the LCAT and CET activity profiles observed in Fig. 2 represent the true LCAT and CETP distribution of A-I-containing particles in plasma. However, the following circumstantial evidence supports the contention that the distribution reported here does reflect the size distribution of LCAT and CETA in plasma. If there were A-I-bound LCAT or CETP species that either resisted desorption or were selectively inactivated by thiocyanate, they had not significantly affected the distribution pro-

files. First, as shown in fig. 4, A and B, the LCAT and CET activity profiles of plasma and A-I-containing lipoprotein particles fractionated by gel filtration chromatography were comparable. Second, although recovery of LCAT activity in the thiocyanate eluted A-I-containing particles was 64%, close to 80% immunoassayable LCAT mass was recovered from the immunosorbent. In all cases when LCAT mass was measured the LCAT activity distribution paralleled the LCAT mass distribution (Fig. 2). Third, as shown under Results, the distribution of LCAT and CETA between Lp(A-I with A-II) and Lp(A-I without A-II) recovered in the thiocyanate eluted particles was comparable to that calculated for these particles from the measurement of LCAT and CETA in plasma, A-I-free plasma and A-II-free plasma.

In this study, the Lp(A-I with A-II) and Lp(A-I without A-II) particles used for studying LCAT and CETA were isoalted by immunoaffinity chromatography. A possible caveat in this methodology is that the binding of apoA-I and A-II to their antibodies can potentially induce conformational changes in A-I and A-II which may in turn affect the association of LCAT and CETP for A-I-containing lipoprotein particles. Our finding that about 85% of plasma LCAT and 90% of plasma CETA were associated with A-I-containing lipoproteins suggests that binding of A-I to anti-A-I does not significantly alter the binding of these two proteins to the lipoprotein particles. However, we cannot completely rule out the possibility that the small amount of LCAT (15%) and CETA (10%) found in the A-I-free plasma resulted from the binding of A-I-containing lipoproteins to the antibodies.

Several laboratories including ours have reported the isolation of plasma protein(s) with cholesteryl ester transfer activity. The reported molecular weight of the protein(s) ranged beteen 58,000 and 69,000 (14, 21–24). The identification of specific proteins in plasma that promote lipid transfer between the various classes of plasma lipoproteins has enhanced our understanding of lipid metabolism and transport. There are at least two lipid transfer proteins present in human plasma: one facilitates both neutral lipid (cholesteryl esters and triglycerides) and phospholipid transfer among the plasma lipoproteins, the other has little or no affinity for neutral lipids (CE and TG) but promotes phospholipid transfer between the plasma lipoproteins (14, 24). The cholesteryl ester transfer activity observed in this study is most probably due to the neutral lipid transfer protein (LTP-I) (24), as polyclonal antibody to purified LTP-I removes all detectable cholesteryl ester transfer activity from human plasma (Albers, J. J., and J. H. Tollefson, unpublished observation). Furthermore, when our immunoaffinity-isolated A-I-containing lipoproteins were centrifuged, almost all CETA recovered in the d > 1.21 g/ml bottom fraction was detected in gel filtration fractions with apparent mean M_r of about 65,000 suggesting that some, if not all of the CETA measured in this study was due to a protein with apparent M_r of about 65,000, comparable to the M_r of purified LTP-I (14).

In conclusion, this report provides direct evidence for the association of LCAT and CETA with A-I-containing lipoprotein particles. According to the current concept of reverse cholesterol transport, A-I, LCAT, and CETP are three key components required for an efficient means of promoting removal of cholesterol from cells. ApoA-I has been reported to be a major ligand for the binding of HDL particle to cellular receptor (25, 26) and is the primary activator of LCAT. Unesterified cholesterol derived from cells is converted to cholesteryl ester by LCAT thus creating a gradient favoring the efflux of cholesterol from cells (27). The cholesteryl ester transfer protein (LTP-I) may function to transfer cholesteryl ester out of HDL to lipoproteins of lower density and thus prevent end-product (cholesteryl ester) inhibition of the LCAT enzyme (28). It is interesting that most of the LCAT and CETA were associated with Lp(A-I without A-II) particles. Furthermore, while CETA was found to be associated with particles of a narrow size range, LCAT appeared to be associated with particles of a broad size range (although the majority of LCAT was found in the larger-sized particles). The preferential association of LCAT and CETA with Lp(A-I without A-II) may reflect the differential affinity of Lp(A-I with A-II) and Lp(A-I without A-II) particles for LCAT and CETP due to surface properties of these two types of particles. In some subjects, LCAT and CETA were also detected in A-I-free plasma. We do not know whether the non-A-I-associated LCAT and CETA were lipoprotein-associated or not.

While this manuscript was under preparation, Morton (29) reported that lipid transfer protein (LTP) could bind to very low density lipoproteins and low density lipoproteins as well as to HDL. However, VLDL- and LDL-LTP complexes were labile and almost completely dissociated in 90 min (29). The labile nature of VLDL- and LDL-LTP complexes may explain our failure in detecting these complexes in our study. We have performed preliminary studies using affinity purified anti-LDL antibody. In accordance with the present report, we were able to recover essentially all CET and LCAT activity in the non-B-containing plasma (> 99% apoB was removed).

The authors thank Janet Adolphson for performing the LCAT mass assay and Jan Ambrose and Carol Glasser for preparing the manuscript. This work was supported in part by National Institutes of Health grant HL-30086, and by the Council for Tobacco Research, USA, Inc.

Manuscript received 6 January 1986.

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