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Dissociation of lipid-free apolipoprotein A-I from high density lipoproteins

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Abstract Conditions under which apolipoprotein (apo) A-I dissociates from human high density lipoproteins (HDL) during incubation in vitro have been investigated. Dissociation of apoA-I was demonstrated by non-denaturing gradient gel electrophoresis followed by immunoblotting for apoA-I and by size-exclusion chromatography. It was quantitated after ultracentrifugation as the loss of apoA-I from the fraction of d < 1.25 g/ml. ApoA-I did not dissociate from HDL when they were incubated alone at 37°C for up to 24 h. Nor was there dissociation of apoA-I when the HDL were incubated either with the cholesteryl ester transfer protein (CETP) in the absence of other lipoprotein fractions or with other lipoproteins in the absence of CETP. However, when mixtures of HDL and CETP were incubated for 24 h in the presence of physiological concentrations of either very low density lipoproteins (VLDL) or low density lipoproteins (LDL), there was a dissociation of up to 36% of the apoA-I from the HDL fraction that was linear with time. The dissociation of apoA-I coincided with a time-dependent reduction in HDL particle size. The percentage of apoA-I that dissociated from HDL correlated positively with the concentrations of VLDL, LDL, and CETP in the incubation but negatively with the concentration of HDL. When lecithin:cholesterol acyltransferase was added to mixtures at the completion of 24 h of incubation with CETP, the size of the HDL increased and the dissociated apoA-I returned to the fraction of d < 1.25 g/ml. Analysis of the lipoprotein-deficient fraction of d > 1.25 g/ml isolated by ultracentrifugation and of the lower molecular weight fractions recovered after size-exclusion chromatography revealed that the dissociated apoA-I was not associated with significant quantities of either cholesterol, phospholipids, or other apolipoproteins. When the dissociated apoA-I was subjected to agarose gel electrophoresis, it migrated to a prebeta position comparable to that of purified, lipid-free apoA-I. This contrasted with the original HDL that exhibited alpha migration. Thus, CETP-mediated transfers of cholesteryl esters from HDL to VLDL and LDL are accompanied not only by a reduction in HDL size but also by the progressive dissociation from HDL of a pool of prebeta-migrating, essentially lipid-free apoA-I.—**Liang, H-Q., K-A. Rye, and P. J. Barter.** Dissociation of lipid-free apolipoprotein A-I from high density lipoproteins. *J. Lipid Res.* 1994. **35:** 1187–1199.

Supplementary key words cholesteryl ester transfer protein • prebeta apoA-I • very low density lipoproteins • low density lipoproteins

The observation that the concentration of cholesterol in plasma high density lipoproteins (HDL) correlates inversely with the development of premature coronary heart disease (1) has stimulated a sustained interest in the metabolism and regulation of this lipoprotein fraction. Of particular interest has been the remodeling of HDL that takes place during their circulation in plasma (2). Under certain circumstances, the remodelling of HDL results in the formation of a population of small HDL particles (Stokes' diameter 7.6 nm) which have lost a substantial proportion of their lipid and from which a proportion of the main protein constituent, apolipoprotein (apo) A-I, has dissociated (3, 4). This process may have an impact on both the concentration and the metabolic function of this important lipoprotein fraction. While the 7.6-nm HDL particles have previously been partially characterized (5), there is relatively little information regarding the apoA-I that dissociates from larger HDL during their conversion into the smaller 7.6-nm particles.

We recently described the dissociation of apoA-I from HDL in studies in which human plasma was supplemented with exogenous cholesteryl ester transfer protein (CETP), hepatic lipase, and very low density lipoproteins (VLDL) and incubated in vitro at 37°C (3). These incubation conditions led to a reduction in both the core lipid content and the particle size of HDL and the coincident dissociation of a portion of the apoA-I from the HDL fraction. The dissociation of apoA-I was maximal after 2–3 h of incubation. However, with continuation of the incubation beyond 3 h, the dissociated apoA-I progressively returned to the HDL density range, such that after 8 h virtually all of the apoA-I was again recovered in the HDL fraction (4). While the mechanism responsible for this reversibility was not determined, the fact that it occurred made it difficult either to characterize the dissociated apoA-I or to determine how the dissociation is regulated.

Abbreviations: apo, apolipoprotein; CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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In the present study we investigate the dissociation of apoA-I under conditions where it is shed from HDL and is not further metabolized. Under these circumstances the apoA-I is recoverable in a form identical to that in which it dissociates. Specifically, we investigate: *i)* factors that regulate the dissociation of apoA-I from HDL and *ii)* whether the apoA-I dissociates as lipid-free apolipoprotein or as a component of a lipid–protein complex or both.

METHODS

Isolation of lipoproteins and apolipoproteins

Blood from healthy, normolipidemic subjects was collected into tubes containing EDTA-Na_2 (1 mg/ml) and placed immediately on ice. Samples for isolation of VLDL were collected from subjects who had fasted for 14 h. Plasma was separated by centrifugation at 4°C. The fractions of density < 1.006 g/ml (VLDL), 1.019–1.21 g/ml (containing low density lipoproteins (LDL) and HDL), 1.019–1.055 g/ml (LDL), 1.07–1.21 g/ml (HDL), 1.13–1.21 g/ml (HDL_3), and < 1.21 g/ml (containing VLDL, LDL, and HDL) were isolated ultracentrifugally as described previously (5). In each case samples were subjected to two successive spins at the higher density. After their isolation all lipoprotein fractions were dialyzed extensively against Tris-buffered saline, pH 7.4 (TBS) containing 0.01 M Tris, 0.15 M NaCl, 0.006% (w/v) NaN_3, and 0.005% (w/v) EDTA-Na_2.

ApoA-I was purified to homogeneity as described previously (6).

Isolation and assay of the cholesteryl ester transfer protein

CETP was isolated from 2 l of human plasma (Transfusion Service, Royal Adelaide Hospital) as described (5). Briefly, the proteins that precipitated between 35% and 55% saturation with ammonium sulfate were recovered and subjected to ultracentrifugation to obtain the fraction of d > 1.25 g/ml. This fraction was subjected to hydrophobic interaction chromatography on a column of phenyl-Sepharose CL-4B (Pharmacia Biotechnology AB, Uppsala, Sweden), cation-exchange chromatography on CM-52 cellulose (Whatman Biosystems Ltd., Kent, England), and anion-exchange chromatography on a column of Mono Q HR 5/5 (Pharmacia Biotechnology AB, Uppsala, Sweden). Active fractions recovered from the Mono Q column were pooled and stored at −70°C until used. Transfer activity was measured as the transfer of labeled cholesteryl esters from HDL_3 to LDL as described previously (6). This assay was linear so long as the transfer of cholesteryl esters was less than 35%. Activity has been expressed relative to that in a pool of human lipoprotein-deficient plasma that was stored in aliquots at −70°C; activity in the lipoprotein-deficient plasma was arbitrarily set at 1 unit per ml. The CETP used in these studies was purified approximately 5,000-fold relative to lipoprotein-deficient human plasma and appeared as a single band after SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue (see Fig. 9). The CETP preparation contained no activity of lecithin:cholesterol acyltransferase (LCAT) and did not contain detectable amounts of apoA-I or A-II.

Isolation and assay of lecithin:cholesterol acyltransferase

LCAT was purified to homogeneity as described (7, 8). Two litres of human plasma (Transfusion Service, Royal Adelaide Hospital) was subjected to precipitation with ammonium sulfate and citric acid. The precipitated proteins were then subjected to ultracentrifugation followed by chromatography on phenyl-Sepharose CL-4B (Pharmacia Biotechnology AB, Uppsala, Sweden), DEAE-Sephacel (Pharmacia Biotechnology AB, Uppsala, Sweden) and hydroxyapatite (Bio-Rad, Hercules, CA). After this procedure, LCAT appeared as a single band after electrophoresis on a 20% homogeneous SDS gel and silver staining. Activity was determined as described by Piran and Morin (9) and expressed in units according to the formula of Mahadevan and Soloff (7).

Experimental conditions

Incubation mixtures were placed in sealed plastic tubes and either kept at 4°C or incubated at 37°C in a shaking water bath. The incubations were terminated by placing the tubes on ice. At the end of the incubations, an aliquot of each mixture was adjusted to d 1.25 g/ml with KBr and subjected to ultracentrifugation at 4°C at 100,000 rpm for 16 h in a Beckman 100.2 rotor in a Beckman TL-100 ultracentrifuge. The fraction of d < 1.25 g/ml was recovered and assayed for lipids and apolipoproteins as described below. HDL particle size distribution in the d < 1.25 g/ml fraction was determined by nondenaturing polyacrylamide gradient gel electrophoresis. In selected experiments the fraction of d > 1.25 g/ml was also recovered, concentrated approximately 10-fold by lyophilization (Heto Lab Equipment, Denmark), and assayed for lipids and apolipoproteins. Aliquots of the d > 1.25 g/ml fraction were also subjected to agarose gel electrophoresis and SDS-polyacrylamide gel electrophoresis. In some experiments an aliquot of the total incubation mixture was subjected directly to nondenaturing polyacrylamide gradient gel electrophoresis and immunoblotted for apoA-I.

Electrophoretic techniques

Gradient gel electrophoresis and immunoblot analysis. Samples were subjected to electrophoresis on 3–40% nondenaturing polyacrylamide gradient gels (Gradipore, Australia). The electrophoresis was performed for 3,000 V-hours after which the gels were either stained and scanned as

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described previously (10, 11) or subjected to immunoblot analysis. In the case of immunoblotting, the separated proteins were electrophoretically transferred from the gradient gel to nitrocellulose membranes (pore size 0.45 μm, Hybond-C extra, Amersham, Netherlands) on a Multiphore II electrophoresis unit (Pharmacia Biotechnology AB, Uppsala, Sweden). The anodic transfer buffer was 0.025 M Tris, 0.2 M glycine, and 20% (v/v) methanol, pH 8.3. The cathodic transfer buffer was 0.025 M Tris, 0.2 M glycine, and 0.003 M SDS, pH 8.3. The transfer was carried out at 90 mA for 8 h at room temperature. After transfer, the membrane was kept at 4°C overnight in 0.2 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.02% (w/v) NaN_3, and 0.01% (w/v) EDTA-Na_2 (phosphate-buffered saline, PBS) containing 0.1% (v/v) Tween 20 (PBS-T) to block unbound membrane sites. The membrane was then incubated for 1.5 h at 37°C with sheep anti-human apoA-I antiserum (Boehringer-Mannheim, Germany) diluted 1:300 with PBS-T. After washing with PBS-T (3 × 10 min), the membrane was incubated for 1 h at 30°C with donkey anti-sheep and goat IgG conjugated to horseradish peroxidase (Silenus Laboratories Pty. Ltd., Hawthorn, Australia) diluted 1:500 with PBS-T. The membrane was then washed with PBS-T (2 × 10 min), and finally in PBS alone for 10 min. Human apoA-I was detected by treating the membrane with 0.1 M sodium acetate, pH 5.0, containing 0.6% (v/v) hydrogen peroxide, 5% (v/v) dimethyl formamide, and 0.1% (w/v) 3-amino-9-ethyl carbazole.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed on the PhastSystem (Pharmacia Biotechnology AB, Uppsala, Sweden) using 20% homogenous polyacrylamide gels. Molecular weights were determined by reference to a calibration kit for low molecular weight proteins (Pharmacia Biotechnology AB, Uppsala, Sweden).

Agarose gel electrophoresis. Agarose gel electrophoresis was carried out in a 0.6% agarose gel, using a 10 mM barbitone, 50 mM sodium barbitone (pH 8.6, ionic strength 0.05) buffer. Electrophoresis was conducted at 100 V for 1 h. After electrophoresis, the gel was fixed for 10 min in ethanol–water–acetic acid 60:30:10, stained with a 0.04% (w/v) Coomassie Blue G 250, 3.5% (v/v) perchloric acid solution for 30 min, and destained with 5% (v/v) acetic acid.

Size-exclusion chromatography

In some experiments, the incubation mixtures were separated by size exclusion chromatography (3) without prior ultracentrifugation of the samples. Chromatography was performed on a column of Superose 6 HR 10/30 (Pharmacia, Biotechnology AB) connected to a P-500 pump (Pharmacia, Biotechnology AB). Lipoproteins were eluted with 0.05 M Tris-HCl (pH 7.4) containing 0.15 M NaCl at a flow rate of 30 ml/h. Individual fractions of 0.6 ml were collected and assayed for lipid and apolipoprotein constituents. Recoveries of all constituents from the column were 85–90%.

Chemical analyses

All assays were performed on a Cobas-Fara centrifugal analyzer (Roche Diagnostics, Basel, Switzerland). Concentrations of total cholesterol, triglycerides, and phospholipid were measured using enzymatic kits (Boehringer-Mannheim, Germany). Concentrations of apoA-I, apoA-II, and apoB were measured immunoturbidimetrically (12) using antisera to human apoA-I, apoA-II, or apoB raised in sheep (Boehringer-Mannheim, Germany). The assay was standardized using appropriate dilutions of apo calibration serum (Boehringer-Mannheim, Germany).

Statistical analyses

All statistical analyses were performed using Student's t test for paired samples. The accepted level of significance was taken to be $P < 0.05$.

RESULTS

Evidence that apoA-I dissociates from HDL

Dissociation of apoA-I from HDL was demonstrated by nondenaturing gradient gel electrophoresis followed by immunoblot analysis for apoA-I (Fig. 1), by size-exclusion chromatography (Fig. 2) and by ultracentrifugation (Table 1).

Mixtures of human LDL and HDL (the plasma fraction of d 1.019–1.21 g/ml) were either kept at 4°C or incubated at 37°C in the absence or presence of CETP. As reported previously (5, 13), incubation in the presence of CETP resulted in a net mass transfer of cholesteryl esters from HDL to LDL and a consequent reduction in the particle size of HDL (result not shown). After the incubation, an aliquot of each mixture was subjected to gradient gel electrophoresis without prior ultracentrifugation. The particle size distribution of apoA-I, as determined by immunoblot analysis, is shown in **Fig. 1.** In the samples kept at 4°C (Fig. 1, track A) or incubated at 37°C in the absence of CETP (Fig. 1, track B), the apoA-I was identified only in particles within the size range of HDL_2 and HDL_3. By contrast, in the samples incubated at 37°C with CETP (Fig. 1, track C), not only was most of the apoA-I now associated with a subpopulation of HDL that was smaller than the original HDL_3 but a proportion of the apoA-I migrated considerably further than albumin (Stokes' diameter = 7.1) to a position identical to that of purified, lipid-free apoA-I (Fig. 1, track D).

In other experiments, mixtures of HDL and LDL were incubated for 24 h at 37°C in the absence or presence of CETP and then separated by size exclusion chromatography. **Figure 2** shows the elution profiles of apoA-I, apoA-

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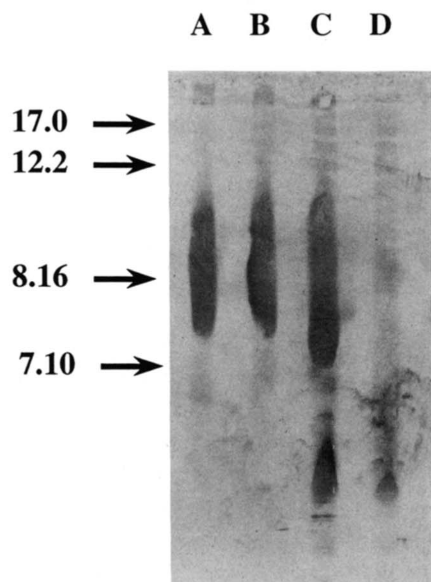

Fig. 1. Photograph of an immunoblot showing the size distribution of apoA-I. The human plasma fraction of d 1.019–1.21 g/ml was incubated under various conditions before being subjected (without prior ultracentrifugation) to gradient gel electrophoresis on 3–40% polyacrylamide gels as described under Methods. The size distribution of apoA-I was determined by immunoblot analysis as described. Track A shows a sample kept at 4°C. Track B shows the effects of incubating the sample (895 nmol total cholesterol and 114 μg apoA-I in a volume of 123 μl) at 37°C for 24 h. Track C shows the effects of incubation in the presence of CETP at a final concentration of 4.4 units/ml. Track D shows a sample of purified apoA-I. Numbers refer to the Stokes' diameters of high-molecular-weight protein standards.

II, phospholipid, and cholesterol in a representative experiment. In non-incubated samples (results not shown) and in samples incubated in the absence of CETP (Fig. 2, Control), apoA-I co-eluted from the column with cholesterol, phospholipid, and apoA-II. The highest concentration of each constituent was in fraction 27. After incubation with CETP, a substantial proportion of the HDL cholesterol (mainly cholesteryl esters) and a lesser proportion of the HDL phospholipids were transferred to the LDL fraction (results not shown). Furthermore, after incubation with CETP, the elution of all HDL constituents was delayed, consistent with the reduction in HDL size as shown by gradient gel electrophoresis (Fig. 1). Whereas the phospholipid, cholesterol, and apoA-II still co-eluted from the column (peak in fraction 28), the elution of apoA-I was delayed even further and did not peak until fraction 30. A significant proportion of the apoA-I was recovered in fractions 31 and 32, which were essentially free of lipids and apoA-II. As a consequence, the molar ratios of phospholipid–apoA-I and of cholesterol–apoA-I were close to zero in fractions 31 and 32. (Note that albumin elutes from this column with a peak between fractions 29 and 30.) A comparable dissociation of essentially lipid-free apoA-I from HDL was also observed in other experiments in which mixtures of HDL and VLDL were incubated with CETP before being fractionated by size exclusion chromatography (results not shown).

To quantitate how much apoA-I had dissociated from

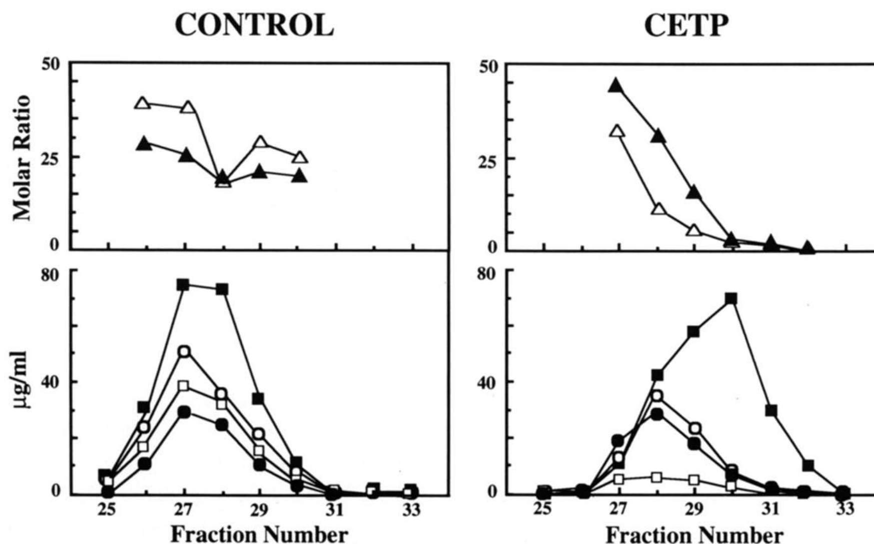

Fig. 2. Fractionation of HDL by size-exclusion chromatography. The plasma fraction of d 1.019–1.21 g/ml (993 nmol total cholesterol and 170 μg apoA-I in a final volume of 0.6 ml) was incubated for 24 h at 37°C in the absence (Control) or presence (CETP) of CETP (final concentration 4.8 units/ml). Incubations were stopped by placing samples on ice. Mixtures were then subjected to size-exclusion chromatography on a Superose 6 HR 10/30 column. The lipoproteins were eluted with TBS at a flow rate of 30 ml/h and fractions of 0.6 ml were collected and assayed for apoA-I, apoA-II, total cholesterol, and phospholipids. As apoA-I and apoA-II were measurable only in fractions 25–32 and as the recoveries of each apolipoprotein from the column were 85–90%, only fractions 24–33 are shown. The lower panels show the distribution of apoA-I (–■–), apoA-II (–●–), total cholesterol (–□–), and phospholipids (–○–). The upper panels show the molar ratios of phospholipid:apoA-I (–▲–), and cholesterol: apoA-I (–△–) in the eluted fractions.

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TABLE 1. Concentrations of constituents in the fraction of d < 1.25 g/ml

Additions	ApoA-I	ApoA-II	Cholesterol
	μg/ml	*μg/ml*	*nmol/ml*
None	372 ± 195	150 ± 67	2324 ± 1140
CETP	284 ± 145[a]	141 ± 60[a]	2360 ± 1151[ns]

Values represent mean ± SD of seven experiments. The human plasma fraction of d 1.019–1.21 g/ml (containing a mixture of LDL and HDL) was incubated at 37°C for 24 h in the absence or presence of CETP (mean concentration 4.6 units/ml). After incubation, the mixture was subjected to ultracentrifugation to recover the fraction of d<1.25 g/ml as described in the Methods section. Concentrations are expressed per ml incubation mixture; ns, not significant.

[a]Significance of difference from sample incubated in the absence of CETP: $P < 0.05$.

HDL, the incubation mixtures were subjected to ultracentrifugation to separate the lipoprotein fraction (d< 1.25 g/ml) from the lipoprotein-deficient fraction (d>1.25 g/ml). Recovery of constituents in the fraction of d<1.25 g/ml is shown in **Table 1.** Compared with incubating HDL and LDL in the absence of CETP, incubation of HDL and LDL in the presence of CETP resulted in a mean loss of 23% of the apoA-I from the fraction of d<1.25 g/ml ($P < 0.05$). This loss of apoA-I from the d<1.25 g/ml fraction was accompanied by a small but significant ($P < 0.05$) loss of apoA-II but no loss of cholesterol from the 1.25 g/ml supernatant (Table 1). The results shown in Table 1 are from incubations performed in the absence of albumin. In four of the experiments, replicate samples were incubated in the presence of 1% BSA; the extent of dissociation of apoA-I was identical to that in the corresponding sample incubated in the absence of albumin (result not shown).

Factors influencing the dissociation of apoA-I from HDL

*Duration of incubation (**Fig. 3**).* Mixtures of LDL plus HDL (the plasma fraction of d 1.019–1.21 g/ml) and CETP were incubated at 37°C for varying periods. The loss of apoA-I from the fraction of d<1.25 g/ml was approximately linear with time up to 24 h (Fig. 3B) and coincided with the progressive conversion of HDL_{3a} (diameter 8.8 nm) into HDL_{3c} (diameter 7.6 nm) (Fig. 3A, profiles I–IV). Incubation for 24 h in the absence of CETP had no effect on HDL size (Fig. 3A, profile V) and was not associated with a loss of apoA-I from the d 1.25 g/ml supernatant (result not shown).

*Concentration of CETP (**Fig. 4**).* Mixtures of LDL and HDL (the plasma fraction of d 1.019–1.21 g/ml) were incubated at 37°C for 24 h in the presence of varying concentrations of CETP. Incubation in the absence of CETP (Fig. 4A; profile I) had no effect on HDL particle size distribution and did not promote dissociation of apoA-I as

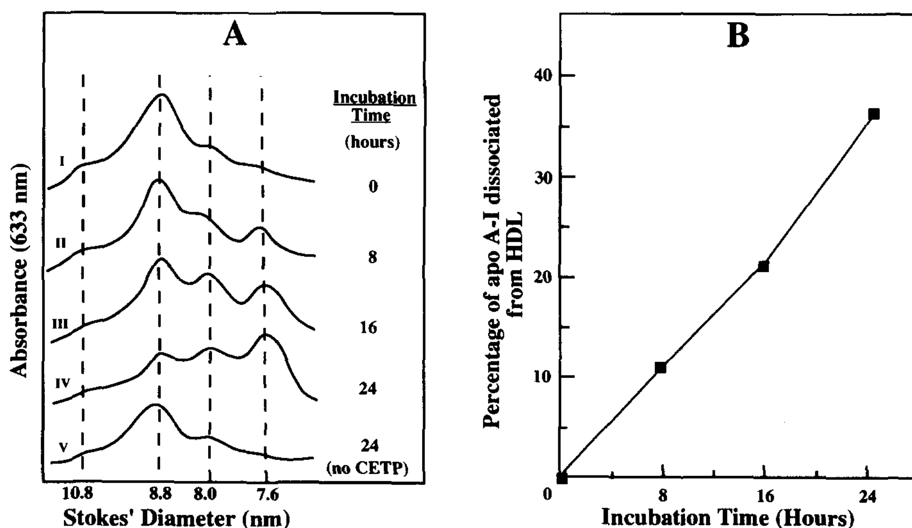

Fig. 3. Effect of incubation time on changes to HDL particle size and dissociation of apoA-I. The d 1.019–1.21 g/ml fraction of plasma (20 μl containing 600 nmol of total cholesterol and 80 μg of apoA-I) was either kept at 4°C in the presence of CETP (final concentration 4.6 units/ml) (panel A, profile I), incubated at 37°C in the absence of CETP (profile V), or supplemented with CETP (4.6 units/ml) and incubated at 37°C for 8, 16, and 24 h (profiles II–IV). The final incubation volume was 0.12 ml. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and were electrophoresed on a 3–30% nondenaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apoA-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apoA-I recovered in the d 1.25 g/ml supernatant of the experimental and control (incubated for 24 h in the absence of CETP) incubations. The dissociation is expressed as a percentage of the apoA-I in the 1.25 g/ml supernatant of the control sample.

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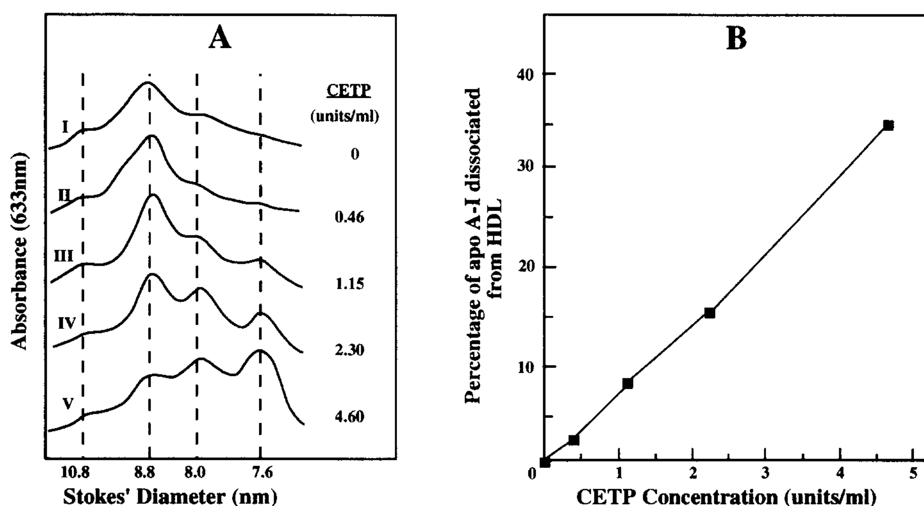

Fig. 4. Effect of CETP concentration on changes to HDL particle size and dissociation of apoA-I. The d 1.019–1.21 g/ml fraction of plasma (40 μl containing 1200 nmol of total cholesterol and 160 μg of apoA-I) was incubated at 37°C for 24 h in the absence of CETP (panel A, profile I) or in the presence of CETP (20, 50, 100, and 200 μl containing respectively 0.11, 0.28, 0.55, and 1.10 units of CETP) (profiles II-V). The final incubation volume was adjusted to 0.24 ml with TBS. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and were electrophoresed on a 3–30% nondenaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apoA-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apoA-I recovered in the d 1.25 g/ml supernatants of the experimental and control (containing no CETP) incubations. The dissociation is expressed as a percentage of the apoA-I in the d 1.25 g/ml supernatant of the control sample.

judged by comparison with a sample kept at 4°C (result not shown). However, the presence of CETP in the incubation was accompanied by a progressive conversion of HDL_{3a} to HDL_{3c} (Fig. 4A, profiles II-V) and a loss of apoA-I from the d 1.25 g/ml supernatant that was dependent on the concentration of CETP (Fig. 4B).

*Concentration of VLDL (**Fig. 5**).* Mixtures of HDL_3 and CETP were incubated at 37°C for 24 h in the presence of varying concentrations of VLDL. Taking the incubation of HDL_3 in the absence of both CETP and VLDL

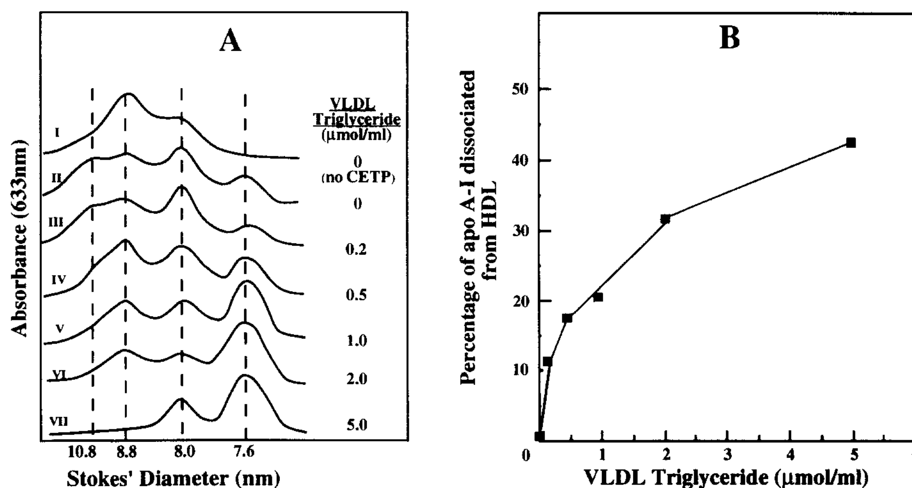

Fig. 5. Effect of VLDL concentration on changes to HDL size and dissociation of apoA-I. The plasma fraction of d 1.13–1.21 g/ml (HDL_3) was incubated alone at 37°C for 24 h (panel A, profile I) or after addition of CETP (profile II) or CETP plus VLDL (profiles III-VII). All incubations included 26 μl of HDL_3 (containing 81 nmol total cholesterol, 114 μg apoA-I). CETP was present at a final concentration 2.7 units/ml and VLDL at triglyceride concentrations of 0, 0.2, 0.5, 1.0, 2.0, and 5.0 mmol/l. The final incubation volume was adjusted to 0.2 ml with TBS. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and electrophoresed on a 3–30% nondenaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apoA-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apoA-I recovered in the d 1.25 g/ml supernatants of the experimental and control (containing equivalent concentrations of VLDL but no CETP) incubations. The dissociation is expressed as a percentage of the apoA-I in the d 1.25 g/ml supernatant of the control.

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as the reference point (Fig. 5A, profile I), incubation in the presence of CETP but in the absence of VLDL (Fig. 5A, profile II) resulted in the conversion of a proportion of the HDL_{3a} (diameter 8.8 nm) into both smaller HDL_{3c} particles (diameter 8.0 and 7.6 nm) and larger HDL_2 particles (diameter 10.8 nm) as reported previously (5); under these conditions there was no loss of apoA-I from the d 1.25 g/ml supernatant. The addition of VLDL to the incubation mixture resulted in a progressive decrease in the conversion to larger HDL_2 particles, a progressive increase in the conversion to smaller HDL_{3c} particles (Fig. 5A, profiles III–VII), and a loss of apoA-I from the d 1.25 g/ml supernatant that was dependent on the concentration of VLDL (Fig. 5B). As reported previously (14), the reduction in HDL particle size that occurred during incubation with CETP and VLDL was associated with (and was probably secondary to) a CETP-mediated net reduction in the core lipid content of HDL (result not shown).

*Concentration of LDL (**Fig. 6**).* Mixtures of HDL_3 (d 1.13–1.21 g/ml) and CETP were incubated in the presence of varying concentrations of LDL (d 1.019–1.055 g/ml). By comparison with the control incubation of HDL_3 which was carried out with no additions (Fig. 6A, profile I), incubation in the presence of CETP but no LDL (Fig. 6A; profile II) resulted in the conversion of the HDL_{3a} into both larger and smaller particles as outlined above. Under these conditions there was minimal loss of apoA-I from the d 1.25 g/ml supernatant. However, when LDL was added to the incubation mixture, as was observed with the addition of VLDL, there was a progressive reduction in the conversion to larger, HDL_2-like particles, a progressive increase in conversion to smaller HDL_{3c} particles (Fig. 6A, profiles III–VII), and a concentration-dependent loss of apoA-I from the d 1.25 g/ml supernatant (Fig. 6B).

*Concentration of HDL (**Fig. 7**).* To determine whether varying the concentration of HDL affected the dissociation of apoA-I, mixtures containing fixed concentrations of LDL and CETP were incubated in the presence of varying concentrations of HDL_3. For each concentration of HDL_3 there was also a control incubation that contained HDL_3 and LDL but no CETP. In these control incubations there were no changes to the particle size distribution of HDL and no loss of apoA-I from the d 1.25 g/ml supernatant compared with a sample kept at 4°C (result not shown). The losses of apoA-I from the d 1.25 g/ml supernatant shown in Fig. 7B have been calculated as the difference between the samples that were incubated with CETP and the control samples that were incubated in the absence of CETP.

When HDL_3 were incubated with LDL and CETP, both the reduction in HDL size and the dissociation of apoA-I from HDL were inversely related to the concentra-

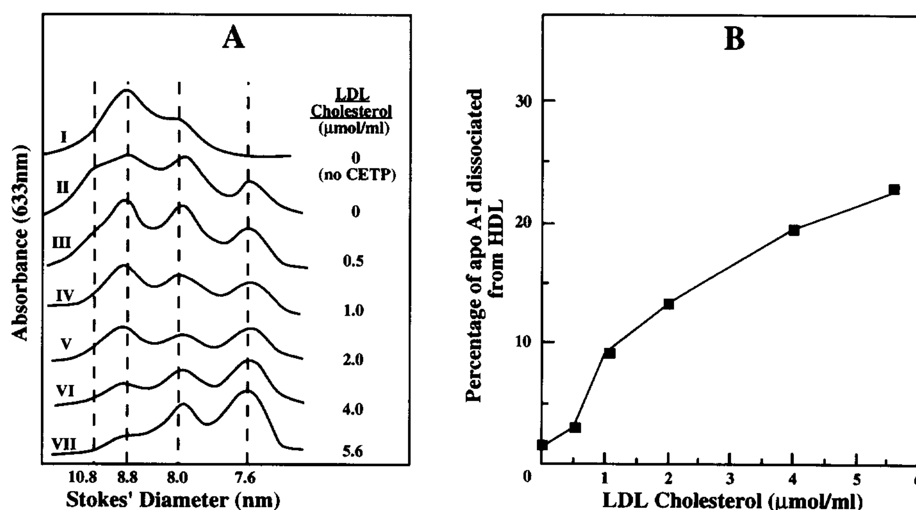

Fig. 6. Effect of LDL concentration on changes to HDL size and dissociation of apoA-I. The plasma fraction of d 1.13–1.21 g/ml (HDL_3) was incubated alone at 37°C for 24 h (panel A, profile I) or with CETP (profile II) or CETP plus LDL (profiles III–VII). All incubations included 52 μl of HDL_3 (containing 162 nmol total cholesterol, 228 μg apoA-I). CETP was present at a final concentration of 2.7 units/ml and LDL at cholesterol concentrations of 0, 0.5, 1.0, 2.0, 4.0, and 5.6 mmol/l. The final incubation volume was adjusted to 0.4 ml with TBS. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and electrophoresed on a 3–30% nondenaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apoA-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apoA-I recovered in the d 1.25 g/ml supernatants of the experimental and control (containing equivalent concentrations of LDL but no CETP) incubations. The dissociation is expressed as a percentage of the apoA-I in the d 1.25 g/ml supernatant of the control samples.

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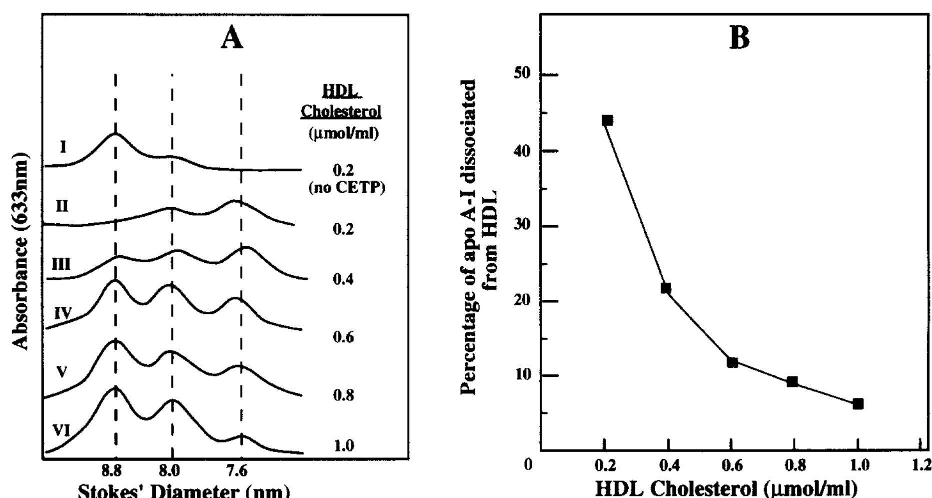

Fig. 7. Effect of HDL concentration on changes to HDL size and dissociation of apoA-I. A preparation of LDL was supplemented with varying amounts of HDL_3 and incubated at 37°C for 24 h in the absence (panel A, profile I) or presence (profiles II–VI) of CETP. Incubation mixtures included 37 µl LDL (containing 814 nmol total cholesterol), 60 µl CETP (final concentration 2.7 units/ml) and HDL_3 at cholesterol concentration of 0.2, 0.4, 0.6, 0.8, and 1.0 mmol/l. The final incubation volume was adjusted to 0.2 ml with TBS. After incubation, lipoproteins were isolated as the d 1.25 g/ml supernatant and electrophoresed on a 3–30% nondenaturing gradient gel (panel A). The distribution profiles of HDL were obtained by laser densitometric scanning of the gel. Panel B shows the dissociation of apoA-I from HDL. The magnitude of the dissociation was calculated as the difference between the amounts of apoA-I recovered in the d 1.25 g/ml supernatants of the experimental and control (containing equivalent concentrations of HDL but no CETP) incubations. The dissociation is expressed as a percentage of the apoA-I in the d 1.25 g/ml supernatant of the control samples.

tion of the HDL. At an HDL_3 cholesterol concentration of 0.2 mM there was a quantitative conversion of HDL_{3a} to HDL_{3c} (Fig. 7A, profile II). This was accompanied by a loss of 44% of the apoA-I from the d 1.25 g/ml supernatant (Fig. 7B). As the concentration of HDL_3 increased, there was a progressive reduction in the conversion to HDL_{3c} (Fig. 7A, profiles III–VI) and a progressive decrease in the percentage of apoA-I lost from the d 1.25 g/ml supernatant (Fig. 7B). At an HDL_3 cholesterol concentration of 1.0 mM, only 6% of the apoA-I was lost.

Return of the dissociated apoA-I to the lipoprotein fraction after incubation with LCAT (Fig. 8)

Having shown that the dissociation of apoA-I from HDL coincides with a reduction in particle size of HDL, experiments were conducted to determine whether subsequent increases in HDL size are accompanied by a return of the dissociated apoA-I to the lipoproteins in the d 1.25 g/ml supernatant. To this end, a mixture of HDL, LDL, and VLDL (the plasma fraction of $d<1.21$ g/ml) was incubated for 24 h in the presence of CETP. As expected, there was a marked reduction in HDL particle size, with an appearance of a substantial population of HDL_{3c} (Fig. 8A, profile II). There was also a loss of 33% of the apoA-I from the d 1.25 g/ml supernatant (Fig. 8B). When the mixture was subsequently supplemented with bovine serum albumin and LCAT in an amount sufficient to esterify 30% of the free cholesterol in the mixture during 12 h of incubation, the HDL size distribution (Fig. 8A, profile III) returned towards that in a nonincubated sample (profile I). Coincident with this increase in HDL size, most of the dissociated apoA-I returned to the d 1.25 g/ml supernatant (Fig. 8B). The presence of albumin but no LCAT in the second 12-h incubation had no effect on the HDL particle size and did not return the dissociated apoA-I to the d 1.25 g/ml supernatant (result not shown).

Characterization of the dissociated apoA-I

Mixtures of HDL and LDL were incubated at 37°C for 24 h in the presence and absence of CETP. After incubation, the fraction of $d>1.25$ g/ml was recovered by ultracentrifugation and concentrated 10-fold by lyophilization. The recovery of constituents in the d 1.25 g/ml infranatant of two experiments (A&B) is shown in **Table 2.** After incubation of mixtures of HDL and LDL in the absence of CETP there was minimal recovery of either apolipoproteins or lipids in the fraction of $d>1.25$ g/ml. The amount of apoA-I recovered represented less than 1% of that in the total incubation mixture. In contrast, after incubation of HDL in the presence of both LDL and CETP, there was a substantial recovery of apoA-I in the $d>1.25$ g/ml fraction. Even in the presence of CETP, however,

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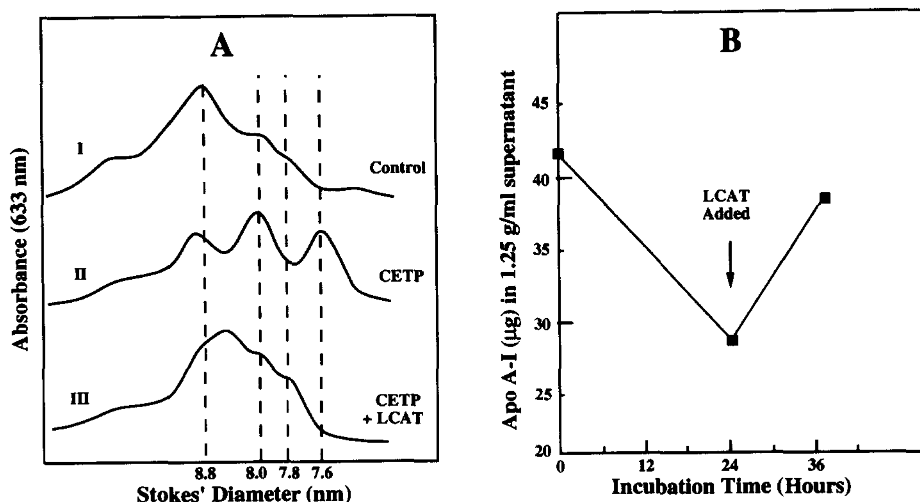

Fig. 8. Reversal of the reduction in HDL particle size and return of the dissociated apoA-I to the lipoprotein fraction during incubation with LCAT. A mixture of HDL, LDL, and VLDL (20 µl of the plasma fraction of d < 1.21 g/ml containing 482 nmol cholesterol and 155 nmol triglyceride) was supplemented with CETP (final concentration 3.1 units/ml) in a final volume of 180 µl and incubated at 37°C for 24 h. After this incubation, one aliquot was placed on ice, while another was supplemented with bovine serum albumin (final concentration 20 mg/ml) and LCAT to give a final mixture volume of 580 µl and incubated for an additional 12 h. The amount of LCAT added was sufficient to esterify 30% of the unesterified cholesterol present in the incubation mixture. After incubation, samples were processed as outlined in the legend to Fig. 3. Panel A presents the particle size distribution of HDL. Profile I shows a sample kept at 4°C. Profile II shows the results of 24 h of incubation with CETP. Profile III shows the results of 24 h of incubation with CETP followed by a further 12 h of incubation with LCAT. Panel B shows the recovery of apoA-I in the d 1.25 g/ml supernatant under the various conditions.

there was minimal apoA-II, phospholipid, or cholesterol in the d 1.25 g/ml infranatant. Expressing the recoveries of constituents in molar terms, it is apparent in both experiments that each molecule of apoA-I is associated with, at most, only one molecule of phospholipid and one molecule of cholesterol. In other words, the apoA-I recovered in the d 1.25 g/ml infranatant is comparable to that recovered in the size-exclusion chromatography fractions 31 and 32 (Fig. 2) in that it was essentially lipid-free.

Aliquots of the d 1.25 g/ml infranatant recovered after the incubation of HDL, LDL, and CETP were subjected to gradient gel electrophoresis and immunoblot analysis as shown for the total incubation mixture in Fig. 1. All of the apoA-I migrated further than albumin to a position identical to that of purified, lipid-free apoA-I (result not shown). Aliquots of the d 1.25 g/ml infranatants were also subjected to SDS-polyacrylamide gel electrophoresis (**Fig. 9**). Only two bands were apparent (Fig. 9, profile A): one with a molecular weight of 28,000 was identical to purified apoA-I (Fig. 9, profile B), while the other, with a molecular weight of 71,000, was identical to purified CETP (Fig. 9, profile C).

TABLE 2. Recovery of apolipoproteins and lipids in the lipoprotein-depleted fraction of d > 1.25 g/ml

Experiment	ApoA-I		ApoA-II		Phospholipid		Cholesterol	
	(– CETP)	(+ CETP)	(– CETP)	(+ CETP)	(– CETP)	(+ CETP)	(– CETP)	(+ CETP)
	µg		*µg*		*µg*		*nmol*	
A	10.0	150.0	0	3.8	1.2	4.6	0.8	6.3
B	5.4	42.3	0	0.9	0.5	1.1	0.2	1.4

The human plasma fraction of d 1.019–1.21 g/ml (containing a mixture of LDL and HDL) was incubated at 37°C for 24 h in the absence of CETP or in the presence of CETP. In experiment A, the volume of the incubation was 3.6 ml, which included 0.6 ml of the d 1.019–1.21 g/ml fraction (11 µmol cholesterol and 2.6 mg apoA-I) and either 3 ml of a preparation of CETP to provide a final CETP concentration of 4.6 units/ml or 3 ml TBS. In experiment B, the volume of the incubation was 2.1 ml, which included 0.3 ml of the d 1.019–1.21 g/ml fraction (4 µmol cholesterol and 0.5 mg apoA-I) and either 1.8 ml of a CETP preparation (final incubation concentration of CETP 4 units/ml) or 1.8 ml TBS. After incubation, the mixtures were subjected to ultracentrifugation as described in the Methods section. The fraction of d > 1.25 g/ml (the lipoprotein-depleted fraction) was recovered, concentrated 10-fold, and assayed for apolipoproteins and lipids. Values represent the total mass of each constituent in the lipoprotein-depleted fraction. Analysis of the lipoprotein fraction of d < 1.25 g/ml (results not shown) indicated that in the incubations containing CETP, the loss of apoA-I was 22% and 19%, respectively, in experiments A and B when compared to the incubations that were conducted in the absence of CETP.

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The d 1.25 g/ml infranatant was also subjected to agarose gel electrophoresis (**Fig. 10**). Two bands with beta and prebeta mobilities were apparent (Fig. 10, profile A). These equated with the prebeta mobility of purified apoA-I (Fig. 10, profile B) and the beta mobility of CETP (Fig. 10, profile C). The respective beta and alpha mobilities of LDL and HDL in the plasma fraction of d 1.019–1.25 g/ml (Fig. 10, profile D) and the prebeta mobility of isolated VLDL (Fig. 10, profile E) are also shown.

DISCUSSION

The present study provides the first demonstration that a CETP-mediated reduction in HDL particle size is accompanied by the dissociation of essentially lipid-free apoA-I from HDL. In the earlier studies in which apoA-I was shown to dissociate from HDL, the incubations contained all constituents of human plasma plus additional CETP, hepatic lipase, and VLDL (3, 4). Under those conditions the appearance of lipoprotein-unassociated apoA-I was transient, with a return of the apoA-I to the HDL density range coinciding with the appearance of discoidal HDL in the incubation mixture (4). In these studies, it was postulated that the initial dissociation of apoA-I from HDL was a simple consequence of the shedding of redundant surface constituents from particles of reduced size. The subsequent return of the apoA-I to the HDL density range was explained as the formation of complexes between the dissociated apoA-I and phospholipids and unesterified cholesterol that had been released from the surface of VLDL during lipolysis of VLDL triglyceride by hepatic lipase. However, regardless of the mechanism, the simple fact that the apoA-I did return to the HDL fraction made it difficult either to quantitate the dissociation or to characterize the dissociated apoA-I.

This problem was circumvented in the present study by using incubation conditions in which further metabolic processing of the dissociated apolipoprotein was unlikely. We took advantage of earlier observations that the core lipid content and the particle size of HDL are reduced during incubation with CETP plus either VLDL or LDL (5). We have now shown that under such conditions there is also a dissociation of apoA-I from the HDL fraction. Both the reduction in HDL size and the dissociation of apoA-I from HDL were slower than when the incubation also contained hepatic lipase (3, 4) but they were other-

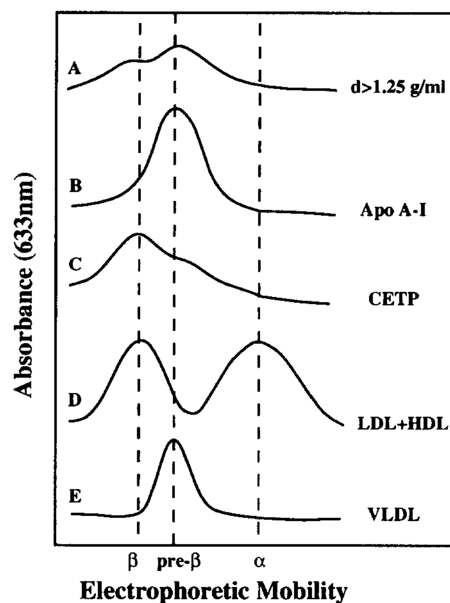

Fig. 10. Determination of mobility of proteins in the d > 1.25 g/ml fraction by agarose gel electrophoresis. The human plasma fraction of d 1.019–1.21 g/ml was incubated at 37°C for 24 h in the presence of CETP as described in the legend to Fig. 9. The lipoprotein-deficient fraction of d > 1.25 g/ml was recovered, concentrated 10-fold by lyophilization, and subjected to agarose gel electrophoresis (profile A). Electrophoresis of purified apoA-I (profile B), purified CETP (profile C), LDL plus HDL in the fraction of d 1.019–1.21 g/ml (profile D), and a preparation of VLDL (profile E) was also performed on the same gel. The profiles were obtained by laser densitometric scanning of the stained gels.

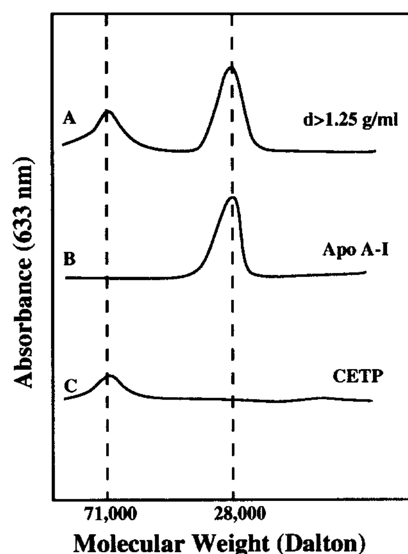

Fig. 9. Determination of the molecular weights of proteins in the d > 1.25 g/ml fraction by SDS-polyacrylamide gel electrophoresis. The human plasma fraction of d 1.019–1.21 g/ml was incubated at 37°C for 24 h in the presence of CETP. Final volume of the incubation was 3.6 ml, which included 0.6 ml of the d 1.019–1.21 g/ml fraction (containing 11 μmol cholesterol and 2.6 mg apoA-I) and 3 ml CETP (final concentration 4.6 units/ml). After incubation, lipoproteins were separated from the lipoprotein-deficient fraction by ultracentrifugation at a density of 1.25 g/ml. The lipoprotein-deficient fraction (d > 1.25 g/ml) was concentrated 10-fold by lyophilization and electrophoresed on a 20% homogenous SDS gel. The profiles were obtained by laser densitometric scanning of stained gels. The molecular weights of proteins in the fraction of d > 1.25 g/ml (profile A) were determined by reference to low molecular weight standards. Profiles B and C represent samples of purified human apoA-I and CETP, respectively.

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wise comparable. The major advantage of this approach was that the dissociated apoA-I did not subsequently return to the HDL fraction and was therefore available for quantification and characterization. Under these conditions, the dissociation of apoA-I was linear with time up to 24 h. It increased with increasing incubation concentrations of CETP, VLDL, and LDL but decreased as the concentration of HDL increased.

The dissociation of apoA-I in the present studies was demonstrated by three independent techniques and could not be dismissed as an artefact of ultracentrifugation. Furthermore, it was totally dependent on the presence of CETP in the incubation mixture. However, CETP alone was not sufficient. As reported previously (5), when HDL are incubated with CETP in the absence of other lipoprotein fractions there is a conversion of the original particles into new populations of both larger and smaller particles. While there may have been a redistribution of apoA-I between different HDL particles, there was no evidence in the present studies of a loss of apoA-I from the total HDL fraction. However, when either VLDL or LDL were added to the mixture of HDL and CETP, formation of the larger conversion products was suppressed while conversion to the smaller HDL particles was enhanced in a concentration-dependent fashion. This enhancement of the conversion of the original HDL to smaller particles coincided with a concentration-dependent dissociation of apoA-I from the HDL fraction. By contrast, increasing the concentration of HDL decreased both the reduction in HDL particle size and the dissociation of apoA-I. Thus, in all experiments, the dissociation of apoA-I from HDL coincided with a reduction in HDL size. Furthermore, when the size of HDL particles was increased by the addition of LCAT, there was a return of the dissociated apoA-I to the lipoprotein fraction.

The dissociated apoA-I recovered both by ultracentrifugation in the fraction of d>1.25 g/ml (Table 2) and by size-exclusion chromatography (Fig. 2) was essentially free of lipid. However, the possibility that each molecule of apoA-I was associated with one or possibly two molecules of either phospholipid or cholesterol cannot be ruled out. This result was similar to that observed previously when size-exclusion chromatography was used to fractionate mixtures of HDL and LDL that had been incubated with CETP in the presence of nonesterified fatty acids (13). As is the case with purified apoA-I, the dissociated apoA-I had a prebeta mobility on agarose gel electrophoresis (Fig. 10), an observation that raises questions regarding its relationship to the prebeta-migrating apoA-I known to exist in human plasma (15).

It has been reported by several workers that up to 10% of the apoA-I in human plasma exists in prebeta-migrating particles rather than in the alpha-migrating bulk HDL (15–18). Activity of CETP (19) and of lipases (20, 21) have been implicated in the release of prebeta apoA-I from HDL, while LCAT has been shown to reconvert prebeta apoA-I to alpha-migrating particles (20, 22). LCAT has also been implicated in the dissociation of apoA-I from HDL in a process that may involve fusion of HDL particles (23). ApoA-I (possibly lipid-free) is displaced from HDL by the addition of apoA-II in vitro (24) and also by the dilution of HDL in a solution of 0.15 M NaCl (25). A proportion of the prebeta-migrating apoA-I found in unmodified human plasma exists in particles which are smaller and denser than the bulk HDL and, in this sense, may be classified as lipoprotein-unassociated apoA-I. While there have been very few studies of the composition of lipoprotein-unassociated, prebeta-migrating apoA-I, at least one report has indicated that such apoA-I is a component of particles that are demonstrably not lipid-free (15). It should be noted, however, that prebeta mobility of apoA-I indicates no more than the fact that it resides in particles that are less negatively charged than those in the bulk HDL fraction. For example, purified, lipid-free apoA-I has prebeta mobility, as do reconstituted discoidal HDL (rHDL) which contain significant amounts of lipid (26). Thus, at some point during the conversion of discoidal HDL to spheroidal HDL there must be a change in the conformation of apoA-I that exposes negatively charged amino acid residues and consequently changes the mobility of the particles. It follows that there exists a spectrum of prebeta particles in human plasma, ranging from lipid-free apoA-I at one end to complex particles in a stage of transition between discoidal and spheroidal HDL at the other. Indeed, several distinct populations of prebeta-migrating HDL have been identified in human plasma (27).

The fact that lipid-free apoA-I has not been identified in human plasma is not surprising in view of the high affinity of this apolipoprotein for phospholipids and the ready availability of phospholipids in plasma. For example, phospholipids are continually being released from VLDL and chylomicrons that are undergoing lipolysis. Even in the absence of lipolysis, it is possible that plasma factors such as the phospholipid transfer protein are capable of delivering phospholipids from a range of lipoproteins to lipid-free apoA-I. This may explain why the smallest of the prebeta-migrating, apoA-I-containing particles so far identified in fresh human plasma (designated prebeta-1 HDL) contain a single molecule of apoA-I combined with substantial amounts of phospholipids (27).

The origin of prebeta-1 HDL is unknown. This is not a trivial issue, as such particles have been implicated as the initial acceptors of cell cholesterol in the pathway of reverse cholesterol transport (27). It is possible that prebeta-1 HDL are formed as a consequence of the binding of phospholipids to the lipid-free apoA-I which dissociates from HDL in the CETP-mediated process described in the present study. If this is so, it follows that the process of apoA-I dissociation may be of importance in

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the pathway of reverse cholesterol transport. However, it is possible that the major significance of the dissociation relates to its role in the regulation of apoA-I concentration in plasma.

Subjects with elevated concentrations of triglyceride-rich lipoproteins are known to have decreased concentrations of plasma apoA-I (28-30). They are also known to have increased plasma concentrations of lipoprotein-unassociated apoA-I (16, 31). It has been suggested that the lipoprotein-unassociated apoA-I in such subjects represents a pool of the apolipoprotein that is especially susceptible to dissociation from HDL and that its subsequent rapid clearance by the kidney explains why the plasma apoA-I concentration is low in some hypertriglyceridemic patients (32). Such a conclusion is supported by the observation in the present study that the dissociation of apoA-I increased as the concentration of VLDL increased. It is interesting to note that the relationship between lipoprotein concentration and the dissociation of apoA-I from HDL in vitro in the present studies (a positive relationship for VLDL and LDL and a negative relationship for HDL) is the same as the relationship between lipoprotein concentrations and the development of coronary heart disease. The implications of this observation are unknown, although it is tempting to speculate that a dissociation of apoA-I from HDL may in some way predispose to the development of atherosclerosis.

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