The effect of apolipoprotein (apo) composition of high density lipoproteins (HDL) on cholesteryl ester transfer protein (CETP) activity was studied by measuring the rate of radiolabeled cholesteryl esters transferred between low density lipoproteins (LDL) and HDL which contained various proportions of apoAI and apoAII. Ultracentrifugally isolated HDL(A), which contained virtually only apoAI and apoAII in their protein moiety, were progressively enriched with apoAII upon the incubation with increasing amounts of delipidated HDL apolipoproteins. The substitution of apoAII for apoAI in HDL(A) did not induce marked alteration of the lipid composition of the lipoprotein particles. The rates of cholesteryl ester exchanges with LDL in the presence of purified human CETP were significantly reduced with apoAII-enriched HDL(A) as compared with non-enriched homologous particles. Consistent results were obtained by determining the rate of cholesteryl esters transferred either from LDL toward HDL(A), or in the opposite direction, from HDL(A) to LDL. The effect of apoAI and apoAII content of HDL particles on CETP activity was also investigated by measuring the rate of cholesteryl esters transferred from LDL to plasma HDL(A) particles which contained either only apoAI, HDL(A)-apoAI, or both apoAI and apoAII, HDL(A)-apoAI apoAII. HDL(A)-apoAI and HDL(A)-apoAI apoAII particles were isolated from human plasma by a sequential procedure which combined ultracentrifugation and anti-apoAII immunoaffinity chromatography. As observed with HDL(A) artificially enriched with apoAII, cholesteryl ester transfer rates were significantly lower with plasma HDL(A)-apoAII than with plasma HDL(A)-apoAI particles. Kinetic analysis of the interaction of CETP with apoAII-enriched HDL(A) revealed that apoAII could act as an uncompetitive inhibitor of the cholesteryl ester transfer reaction. Since the plasma levels of HDL-AI, HDL-AIAII, and HDL-AII may undergo significant physiological fluctuation, the present study suggests that HDL apoproteins may be important factors in modulating cholesteryl ester transfer rates in vivo.

In human plasma, cholesteryl esters and triglycerides can exchange between high density lipoproteins (HDL), low density lipoproteins (LDL), and very low density lipoproteins (VLDL) through a transfer reaction catalyzed by the cholesteryl ester transfer protein (CETP) (1). In vitro studies have also demonstrated that purified CETP, in the absence of other lipoproteins, can promote the size redistribution, or conversion, of HDL into populations of larger and smaller particles (2, 3). In vivo, CETP facilitates the net mass transfer of cholesteryl esters from HDL toward the atherogenic apoB-containing lipoprotein fractions, VLDL and LDL (1), and plasma CETP activity correlates positively with the relative abundance of small sized HDL particles which have been shown to be associated with an increased risk for coronary artery disease (5, 6). Therefore, CETP may be regarded as a potentially atherogenic factor, and several lines of evidence sustain this concept. Indeed, interspecies comparison of plasma CETP activity, diet-induced changes in plasma CETP levels, expression of human CETP gene in transgenic mice, studies of subjects with inherited CETP deficiency, and variations of plasma CETP activity during alimentary lipemia, in patients with dyslipidemia and vascular disease, led to the same statement, i.e. decreased plasma CETP activity is associated with a low atherogenic potential while, conversely, elevated CETP activity is associated with a high atherogenic potential (1). These observations raised interest in identifying factors which modulate in vivo CETP activity. In human plasma, a number of factors can potentially influence the cholesteryl ester transfer activity, including the concentration, structure, and composition of donor as well as acceptor lipoprotein substrates. For example, variations in cholesteryl ester and triglyceride (7-9), free cholesterol (10, 11), and non-esterified fatty acid (12, 13) contents of lipoprotein particles have been shown to modulate CETP activity. In addition to the lipid composition of lipoprotein substrates, the cholesteryl ester transfer protein activity has also been shown to be regulated by an inhibitory HDL protein which could suppress cholesteryl ester transfer activity by dissociating the binding of CETP to lipoprotein particles (14-18). This inhibitor could not be the only protein able to modulate CETP activity. Indeed several studies using either artificial lipid emulsions or plasma lipoprotein fractions have suggested that HDL apolipoproteins could also participate in the transfer of neutral lipids (15, 19-22) and HDL conversion (23, 24) mediated by CETP. However, results of the effects of the two major HDL apolipoproteins, apoAI and apoAII, on the cholesteryl ester transfer activity are controversial (15, 18-21). For example,
apoAI has been described successively as an inhibitory (15), neutral (18), or activating (19–21) factor of the cholesteryl ester transfer process. The precise effect of apoAI and apoAII on CETP activity is important to establish since these two apolipoproteins define two major populations of lipoproteins which contain either both apoAI and apoAII, designated LpAIAII, or apoAI but no apoAII, designated LpAII (25). In addition, the occurrence in human plasma of significant amounts of lipoproteins containing apoAII but no apoAI, designated LpAII, has been reported (26–28). Recent studies have shown that LpAII and LpAIAII particles have different properties with regard to their role in the reverse cholesterol transport pathway (29). Since CETP is one of the factors implicated in reverse cholesterol transport (1), it was of interest to determine whether CETP varies in its ability to interact with either LpAI or LpAII particles. In the present study, this question was addressed by measuring the rate of radiolabeled cholesteryl ester transfer between LDL and HDL particles which differed in their apolipoprotein AI and AII contents.

MATERIALS AND METHODS

Isolation of HDL Particles—Blood from healthy normolipidemic volunteers was collected in tubes containing Na$_2$EDTA (1 mg/ml) and placed immediately on ice. Plasma was separated by centrifugation at 4°C. HDL$_2$ were isolated as the plasma fraction of density 1.13–1.21 g/dl by sequential ultracentrifugation at 55,000 rpm (223,000 x g) in a 70Ti rotor in an L7 ultracentrifuge (Beckman, Palo Alto, CA), with one 24-h spin at the lower density and one 36-h spin at the higher density. The isolated plasma HDL$_2$ fraction was subsequently washed with one 6-h spin at a density of 1.21 g/dl and at a speed of 90,000 rpm (561,000 x g) in a NVT-90 rotor on a Beckman XL-90 ultracentrifuge. The isolated HDL$_2$ fraction was finally dialyzed against a 10 mmol/liter Tris, 150 mmol/liter NaCl, pH 7.4, buffer containing 5 mmol/liter Na$_2$EDTA and 3 mmol/liter sodium azide (TBS buffer).

Anti-apoAI Immunofinity Chromatography—Isolation of anti-apoAI antibodies, preparation of anti-apoAI immunosorbent, and separation of HDL-I containing only apoAI (HDL-AI) from those containing both apoAI and apoAII (HDL-AII-AII) were conducted according to the general procedure previously described by Cheung and Albers (25).

Antiserum to apoAI was purchased from Boehringer Mannheim. Antibodies specific to apoAI were isolated by affinity chromatography on a HDL-Sepharose column as previously described (25). Antibodies to apoAII were purified by chromatography on CNBr-activated Sepharose 4B at a ratio of 8 mg of protein/mg of gel (25). The maximal binding capacity of the resulting anti-apoAI column was approximately 3 mg of apoAI.

To separate HDL-AI from HDL-AII-AI particles, HDL$_2$ were applied at room temperature on the anti-apoAI column at a flow rate of 60 ml/h. Particles which did not bind to the anti-apoAI immunosorbent were washed off with TBS buffer until the absorbance returned to base line. Nonspecifically bound proteins were removed with a 50 mmol/liter Tris, 1 mol/liter NaCl, pH 10.6 buffer. ApoAII-containing particles were subsequently eluted with a 0.1 mol/liter, pH 3.0, acetic acid solution. Lipoprotein particles eluted from the column were immediately neutralized by adding solid Tris. Finally, HDL-AI and HDL-AII-AI fractions were dialyzed overnight against TBS buffer.

Preparation of ApoAII-enriched HDL—ApoAII-enriched HDL$_2$ was prepared according to the general procedure described by Vadiveloo and Pidge (30). This procedure was based on the replacement of apoAI by apoAII upon the incubation of HDL$_2$ in the presence of HDL apoaproteins resulting from the delipidation of HDL$_2$ particles. Under these conditions, apoAI was replaced by apoAII, due to the higher affinity of apoAI for HDL particles (31).

HDL$_2$ particles were prepared from normolipidemic plasma as described above. HDL-apolipoproteins were obtained after delipidation of HDL$_2$ particles by using the method of Cham and Knowles (32). Briefly, 1 volume of HDL$_2$ (protein concentration, 15 mg/ml) was mixed with 2 volumes of butanol-diisopropyl ether 40:60 (v/v). The mixture was successively mixed for 3 min using a Vortex agitator, rotated end-over-end for 1 h at room temperature, mixed again for 3 min on a Vortex agitator, and finally centrifuged for 15 min at 10,000 g. The aqueous solution containing the apoHDL$_2$ was recovered by pipetting and dialyzed overnight against TBS in order to remove any traces of organic solvent. ApoAII-enriched HDL$_2$ was prepared by incubating HDL$_2$ for 2 h at 37°C with apoHDL$_2$ at a HDL$_2$ to apoHDL$_2$ ratio ranging between 1:0 and 1:6. The incubated mixtures were subsequently adjusted to d = 1.21 g/ml with solid KBr, and HDL$_2$ particles were recovered by sequential ultracentrifugation for 15 h at 100,000 g in a Ti-100 ultracentrifuge (Beckman). The resulting apoAII-enriched HDL$_2$ particles were dialyzed overnight against TBS.

Preparation of Radiolabeled HDL$_2$ and LDL—HDLDL$_2$ were biosynthetically labeled according to the general procedure previously described (17). A d > 1.13 g/ml plasma fraction was delipidated by sequential ultracentrifugation of 20 ml of total normolipidemic plasma, dialyzed against TBS, and incubated with 10 nmol of [1,2,6-3H]cholesterol (specific activity, 46 Ci/mmol; Amersham Corp.) for 24 h at 37°C in a shaking water bath. Subsequently, the 1.019 < d < 1.055 g/ml fraction obtained from 10 ml of total normolipidemic plasma (about 15 mg of LDL cholesterol) was added to the incubated mixtures. The incubation was then prolonged for a 6-h period to allow the exchange of radiolabeled esterified cholesterol between lipoprotein substrates. At the end of the incubation the radiolabeled LDL and HDL$_2$ fractions were recovered by sequential ultracentrifugation as described above. Typically labeled preparations of LDL and HDL$_2$ obtained with this procedure had specific activities of approximately 4,000 and 12,000 cpm/nmol of cholesterol, respectively. As judged by thin-layer chromatography, more than 95% of the total radioactivity of both lipoprotein substrates resided in the cholesteryl ester moiety.

Purification of Cholesteryl Ester Transfer Protein—CETP was purified from human plasma as previously described (33). Briefly, the d > 1.25 g/ml fraction of plasma proteins precipitated with ammonium sulfate between 35 and 55% saturation was subjected successively to hydrophilic interaction chromatography on a phenyl-Sepharose CL-4B column (Pharmacia LKB Biotechnology Inc.), to cathein exchange chromatography on a carboxymethylcellose column (Whatman), and then on anion exchange chromatography on a Mono Q HR 5/5 column (Pharmacia). The chromatographic procedures were performed using a fast protein liquid chromatography system (Pharmacia).

Each fraction recovered from the Mono Q column was assayed for cholesterol ester transfer activity by measuring the transfer of the radiolabeled cholesteryl ester from HDL$_2$ to LDL during a 3-h incubation at 37°C (17). Active fractions eluted from the Mono Q column were pooled, and the preparation of CETP used in the present experiments corresponded to an approximate 5,000-fold purification compared with the original lipoprotein-free plasma and was deficient in activity of lecithin:cholesterol acyltransferase. Active fractions, in which CETP accounted for about 20% of the total protein content, were aliquoted and stored at −80°C.

Measurement of Cholesteryl Ester Transfer Activity—Cholesteryl ester transfer activity was evaluated by measuring the amount of radiolabeled cholesteryl ester transferred to HDL particles. The lipid acceptor for nonradiolabeled donor was unlabeled apolipoprotein B-containing lipoprotein particles. For that purpose, the radiolabeled lipoprotein donor (about 2.5 nmol of cholesterol) was mixed with the unlabeled lipoprotein acceptor (about 10 nmol of radiolabeled cholesterol) in the presence of partially purified CETP (0.5 μg of protein) in a final volume of 50 μl. Duplicate mixtures were then incubated for 8 or 37°C in a shaking incubator. At the end of the incubation, the tubes were immediately placed on ice. A volume of 45 μl of each incubated mixture was added to 1.95 ml of a d = 1.07 g/ml KBr solution in 2-ml Quickseal centrifugation tubes (Beckman). The tubes were then sealed and subjected to ultracentrifugation for 7 h at 50,000 rpm (265,000 x g) in a 50.4 Ti rotor and a L7 ultracentrifuge (Beckman). The plasma fractions of d < 1.068 g/ml (plasma apoB-containing lipoproteins) and of d > 1.068 g/ml (HDL-containing plasma fraction) were recovered in a volume of about 1 ml and transferred into counting vials containing 2 ml of scintillation fluid (Optralnt 1). The radioactivity was counted in a 2,000 μl volume in a Wallac 1410 liquid scintillation counter (Pharmacia). The recovery of total radioactivity in the d < 1.068 and d > 1.068 g/ml fractions was consistently greater than 95%. In non-incubated controls containing radiolabeled HDL$_2$, the radioactivity recovered in the d < 1.068 g/ml fraction did not exceed 4% of the total. In non-incubated controls containing radiolabeled LDL, less than 10% of the radioactivity was recovered in the d > 1.068 g/ml fraction. Cholesteryl ester transfer activity was expressed as the percentage of cholesteryl ester radioactivity transferred from the lipoprotein tracer to the d < 1.068 g/ml or the d > 1.068 g/ml acceptor fraction (12). Blank values of control mixtures kept at 4°C were subtracted.

Electrophoretic Analyses—HDL particle sizes were determined by native polyacrylamide gel electrophoresis in 40–300 g/liter gradient gels according to the procedure previously described (34, 35). The migration
buffer was a 14 mmol/liter Tris, 110 mmol/liter glycine, pH 8.3, solution. After a 1-h preelectrophoresis at 200 V, 10-μl aliquots of each sample (approximately 5–10 μg protein) were applied to the gels in 3-mm large slots. The electrophoresis was performed at 4 °C for 26 h, 2 h at 30 V, 12 h at 50 V, and 12 h at 150 V. At the end of the electrophoresis, gels were fixed, stained with Coomassie Brilliant Blue G, and destained as previously described (34). The distribution profile of lipoprotein subfractions was finally obtained by densitometric scanning of the gels at 633 nm with a 2202 Ultrascans laser densitometer (LKB, Bromma, Sweden) attached to a 2220 integrator (LKB). The apparent diameters of the separated HDL subfractions were determined by comparison with calibrating proteins (Pharmacia high molecular weight protein calibration kit) submitted to electrophoresis together with the samples. The mean apparent diameters of HDL subfractions were determined by comparison with a calibration curve constructed with albumin (7.10 nm), lactate deshydrogenase (8.16 nm), ferritin (12.20 nm), and thyroglobulin (17.00 nm).

Apolipoprotein composition of HDL was determined by SDS-polyacrylamide gradient gel electrophoresis on 8-250 g/liter gradient gels (Phastsystem: Pharmacia). Sample preparation, migration, and staining were conducted as recommended by the manufacturer. Apparent molecular weights of individual protein bands were determined by reference to protein standards (Pharmacia low molecular weight calibration kit).

Protein and Lipid Analyses—All chemical assays were performed on a Cobas-Fara Centrifugal Analyzer (Hoffmann-La Roche). Total cholesterol, unesterified cholesterol, triglyceride, and phospholipid concentrations were measured by enzymatic methods using Boehringer Mannheim reagents. Concentrations of apoA1 and apoAII were determined by immunoturbidimetric assays confirmed that, as previously observed (30), cholesterol content of HDL3 particles was progressively reduced, and the apolipoprotein AI content of HDL3 particles was progressively increased as the native HDL3 content of HDL apolipoproteins (36) with anti-apoAI and anti-apoAII antibodies purchased from Behringwerke AG (Marburg, FRG). ApoAII standard was purchased from Behringwerke AG. ApoAII standard was purchased from Immuno AG (Vienna, Austria).

RESULTS

Preparation and Characterization of ApoAII-enriched HDL3 Particles—In ultracentrifugally isolated HDL3 particles, apolipoprotein AII was progressively substituted for apolipoprotein AI upon the incorporation of native lipoprotein particles in the presence of increasing amounts of HDL apolipoproteins (see “Materials and Methods”). As shown by SDS-polyacrylamide gradient gel electrophoresis, the apolipoprotein AI content of HDL3 particles was progressively reduced, and the apolipoprotein AI content of HDL3 particles was progressively increased as the HDL3 content of HDL apolipoproteins (36) with anti-apoAI and anti-apoAII antibodies increased from 1:0 (profile A) to 1:4 (profile E) (Fig. 1). ApoAII and ApoAII were virtually the only proteins present in the ultracentrifugally isolated HDL3 particles (Fig. 1). This apolipoprotein composition was in contrast with the higher protein heterogeneity of homologous native particles which have been previously isolated from human plasma by selected affinity immunosorption of apoAI (37, 38). These differences have been explained by the dissociation of minor lipoprotein-associated HDL apoproteins during prolonged ultracentrifugation (37, 38).

The quantitation of apolipoproteins AI and AII in HDL3 by immunoturbidimetric assays confirmed that, as previously observed (30), incubation of HDL3 in the presence of HDL apolipoproteins allowed to raise the apoAII/apoAI + apoAII percentage mass from about 30% up to 98% (Table I). Composition analysis of AII-enriched HDL3 particles indicated that the substitution of apoAI for apoAII was not accompanied by substantial changes in the lipid composition of HDL3 particles (Table I). As previously observed (30, 31, 39, 40), the uptake of apoAII by HDL4 was accompanied by a parallel release of apoAII from the HDL4 particles and approximately 2 molecules of apoAII displaced 1 molecule of apoAI from HDL4.

Laser densitometric scanning of native polyacrylamide gradient gels revealed that the mean size of particles increased slightly as apoAII was substituted for apoAI (Fig. 2). The apparent diameter of native HDL4 (apoAI/apoAI + apoAII percentage mass = 28.3%), as determined by comparison with globular protein standards of known Stokes diameter, was 8.6 nm while the apparent diameter of the corresponding AII-enriched particles (apoAI/apoAI + apoAII percentage mass = 93.0%) was 9.0 nm (Fig. 2). The slight increase in the mean apparent diameter of native particles can be explained by the raise in the molecular weight of total HDL proteins, cross-linked with bis(sulfosuccinimidyl)suberate (41, 42), which increased from about 94,500 to 99,500 as the AI1:AI + percentage mass increased from 28.3 to 93.0% (results not shown). These results are in very good agreement with data previously obtained under similar conditions (30).

Effect of ApoAII Enrichment of HDL3 Particles on Cholesteryl Ester Transfer Activity—The apoAII-enriched HDL3 particles described above were used to investigate the effect of variations in apolipoprotein content of HDL3 particles on cholesteryl ester transfer protein activity. For that purpose, equal amounts of various fractions of apoAII-enriched HDL3 particles were incubated at 37 °C with radiolabeled LDL in the presence of partially purified cholesteryl ester transfer protein. Time course study revealed that the transfer of radiolabeled cholesteryl esters from LDL toward HDL3, containing various proportions of apoAI and apoAII, increased progressively during the first 12 h incubation (Fig. 3). After only 3 h of incubation, differences in the ability of various HDL3 particles to act as substrates in the cholesteryl ester transfer reaction appeared. Indeed, the rate of cholesteryl ester transfer measured with non-enriched HDL3 particles was significantly higher than activities measured with apoAII-enriched homologous particles (21.3 ± 3.2% versus 8.4 ± 4.9% for particles with AI1:AI + percentage mass of 28 and 93%, respectively; p < 0.01 (ANOVA)) (Fig. 3). Enrichment of HDL3 particles with apoAII decreased significantly the cholesteryl ester transfer activity all along the incubation period compared to non-enriched HDL3 particles. After 24 h, cho-
Modulation of CETP Activity by HDL Apolipoproteins

Table I

<table>
<thead>
<tr>
<th>Native HDL:delipidated HDL protein ratio</th>
<th>Free cholesterol</th>
<th>Phospholipids</th>
<th>Cholesteryl esters</th>
<th>Triglycerides</th>
<th>ApoAI</th>
<th>ApoAII</th>
<th>AII:AII+AII%</th>
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FIG. 2. Particle size distribution of human HDL3 and apoAII-enriched HDL3 particles. HDL3 particles containing various proportions of apoAI and apoAII (16 μg of HDL protein) were applied on a native 40-300 g/liter polyacrylamide gel in a 10-μl volume and were analyzed by laser densitometric scanning, as described under "Materials and Methods." These results are representative of five similar experiments.

Cholesteryl ester transfer rates measured in the presence of the same amounts of HDL3 particles with AII:AI+AII percentage mass of 28, 70, and 93% were 58.9 ± 1.5, 49.6 ± 2.1, and 37.8 ± 2.1%, respectively. Differences between the three groups were highly significant (p < 0.001; ANOVA).

In a second set of experiments we determined whether the apoprotein composition of HDL3 particles could also influence the rate of cholesteryl ester transfer in the opposite direction, from HDL3 toward LDL. For that purpose, mixtures containing either HDL3 particles with radiolabeled LDL or radiolabeled HDL3 particles with LDL were incubated for 3 h at 37 °C in the presence of CETP. As shown in Fig. 4, increasing the AII:AI+AII percentage mass of HDL3 particles progressively inhibited the rate of cholesteryl ester transfer as measured either from LDL to HDL3 or from HDL3 to LDL. Transfer of radiolabeled cholesteryl esters from LDL to HDL3 was significantly reduced from 21.3 ± 3.2% to 5.6 ± 0.7% as the AII:AI+AII percentage mass in HDL3 particles increased from 28 to 93%, respectively (p < 0.001; ANOVA). Similarly, transfer activity in the opposite direction, from radiolabeled HDL3 toward LDL, decreased significantly from 18.1 ± 1.6% to 9.5 ± 0.7% as the AII:AI+AII ratio in HDL3 increased from 24.2 to 91.5%, respectively (p < 0.01; ANOVA).

Experiments described above have been conducted with ultracentrifugally isolated HDL3 which corresponded in fact to a mixture of two types of particles, HDL3-AI and HDL3-AII, which in human plasma represent approximately 70 and 30% of the total HDL3 fraction, respectively (25). Consequently, variations in CETP activity as those we observed could relate in part to variations in the ability of the two types of HDL3 particles to acquire additional apoAII. To clarify this point, HDL3-AI were separated from HDL3-AII by anti-apoAII immunoaffinity chromatography and were progressively enriched with apoAII as described above. As shown in Table II, the substitution of apoAII for apoAI in immunoadfinity-purified HDL3-AI particles induced some alterations in the lipid content of the particles. These changes were explained mainly by the relative increase in total protein content of HDL3 rather than by significant alterations in the relative proportions of various lipid components (Table II). As shown in Fig. 5, increasing the AII:AI+AII percentage mass in HDL3-AI from 0 to 67.6% progressively reduced the cholesteryl ester transfer activity.

Comparative Effect of HDL3-AI and HDL3-AII Particles Isolated from Plasma on Cholesteryl Ester Transfer Activity—HDL3-AI and HDL3-AII particles were isolated from human plasma by a sequential procedure which combined ultracentrifugation and anti-apoAII immunoaffinity chromatography (see "Materials and Methods"). As observed above (Fig. 1), SDS-polyacrylamide gradient gel electrophoresis revealed that, under these conditions, apolipoproteins AI and AII were virtually the only protein constituents associated with the ultracentrifugated HDL3 particles (Fig. 6). Only trace amounts of proteins in the 45,000–90,000 molecular weight region were visible in HDL3 and HDL3-AI particles (Fig. 6).

Composition analysis of HDL3 particles confirmed that HDL3-AI particles contained only apoAII while HDL3-AII contained apoAI and apoAII in similar proportions (Table III). The AI:AII molar ratio in HDL3-AI particles was clearly lower than that reported in HDL3 subfractions isolated by immunoaffinity chromatography directly from total human plasma (approximately 0.6 versus 2.0, respectively) (25). However, our results resembled those obtained previously by determining the AI:AII molar ratio in HDL3-AI particles isolated by combining both ultracentrifugation and immunoaffinity chromatography (43). The apparent discrepancies between various studies reflect the higher dissociability of apoAI from the HDL surfaces upon ultracentrifugation as compared with apoAII (44, 45). Accordingly, the relative proportions of the
Mixtures containing represents the mean incubated for up to than those reported for HDL isolated by ultracentrifugation particles isolated by using strictly non-denaturing procedures total protein contents (AI + AII) of HDL3-AI and HDL3-AII fractions (42.6 and 46.4%, respectively) were in the same range than those reported for HDL isolated by ultracentrifugation (46) but were lower than those reported in LpAI and LpAII particles isolated by using strictly non-denaturing procedures (approximately 55 and 60%, respectively) (47).

HDL3-AI and HDL3-AII particles, isolated from normolipidemic human plasma by combining ultracentrifugation and anti-apoAII immunoaffinity chromatography, showed significant variations in their ability to act as substrates in the cholesteryl ester transfer reaction (Fig. 7). Indeed, as observed with HDL3 particles artificially enriched with apoAII, the rate of radiolabeled cholesteryl ester transferred from LDL toward the HDL3 fraction was significantly lower with HDL3-AII than with HDL3-AI particles. Consistent results were observed by using two different concentrations of cholesteryl ester transfer protein (Fig. 7).

Kinetic Analysis of the Effect of ApoAII Enrichment of HDL3 on Cholesteryl Ester Transfer Activity—Radiolabeled LDL and CETP were incubated for 3 h at 37°C in the presence of different concentrations of HDL3 which varied only in their apoAI and apoAII contents. At the end of the incubation, LDL and HDL fractions were separated and CETP activity was calculated as described above. As shown in Fig. 8, as HDL3 concentrations increased, cholesteryl ester transfer activity increased progressively until a maximal value was reached. However, significant differences in the shape of the curves obtained with different HDL3 populations were observed, depending on their relative content in apoAI and apoAII. At low HDL3 concentrations (12 and 24 mg/liter of HDL protein), cholesteryl ester transfer rates and AII:AI + AII mass ratios of HDL3 increased parallelly. By contrast, at HDL3 protein concentrations greater than 80 mg/liter, the rate of radiolabeled cholesteryl ester transfer was lower with apoAII-enriched HDL3 than with non-enriched homologous particles (Fig. 8). A substrate inhibition of the reaction, similar to that observed in previous studies (9, 48), appeared for the highest HDL3 protein concentrations (greater than 150 mg/liter) (Fig. 8).

In recent investigations of the CETP reaction, apparent Michaelis-Menten kinetics have been observed, allowing the determination of apparent Vmax (Vmax(app)) and apparent Km (Km(app)) values (9, 49). However, since CETP is not an enzyme and lipoprotein particles cannot be considered as enzyme substrates, Vmax(app) and Km(app) do not have the common meaning found in simple enzyme kinetics. Nevertheless, Vmax(app), corresponding to the maximal transfer rates obtained with each of the HDL3 fractions, and Km(app), corresponding to the HDL3 concentration required to obtained 50% of the maximal transfer rate, constitute a convenient mean to evaluate the ability of different HDL3 particles to interact with CETP. Both Vmax(app) and Km(app) values, determined from direct plots presented in Fig. 8, related inversely with the HDL3-AII:AI + AII ratio (Fig. 9), suggesting that apoAII could act as an uncompetitive in-
Materials and Methods. Each point represents the mean incubated for ultracentrifugation at a density gradient gel electrophoresis. Labeled LDL mediated cholesteryl ester transfer. 

cate determinations. 

A reciprocal plots (50). 

hibitor of the cholesteryl ester transfer reaction. Consistent conclusions were obtained from direct linear plots and double reciprocal plots (50).

FIG. 5. Effect of enrichment of HDL-Al particles on CETP-mediated cholesteryl ester transfer. HDL (12 pg of protein), radio-labeled LDL (1.5 pg of cholesterol), and CETP (2.5 pg of protein) were incubated for 3 h at 37 °C in a final incubation volume of 50 pl. At the end of the incubation, LDL and HDL, fractions were separated by ultracentrifugation at a density of 1.068 g/ml as described under "Materials and Methods." Each point represents the mean ± S.D. of triplicate determinations.

![Graph of Effect of enrichment of HDL-Al particles on CETP-mediated cholesteryl ester transfer.](image)

**FIG. 6.** Densitometric scans of total HDL, (A), HDL-Al (B), and HDL-AII (C) proteins as separated by SDS-polyacrylamide gradient gel electrophoresis. HDL proteins (500 ng) were applied in a 1-μl volume on a 80-250 g/liter polyacrylamide SDS-Phastgel (Pharmacia). Apparent molecular weight of each protein was determined by comparison with globular protein standards (D).

![Densitometric scans of total HDL, HDL-Al, and HDL-AII proteins](image)

**TABLE II** Composition (mass percent) of HDL after enrichment of HDL-Al with apoAII

<table>
<thead>
<tr>
<th>Native HDL/delipidated HDL protein ratio</th>
<th>Free cholesterol</th>
<th>Phospholipids</th>
<th>Cholesteryl esters</th>
<th>Triglycerides</th>
<th>ApoAII</th>
<th>ApoAII</th>
<th>All AI+Al%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>3.6</td>
<td>27.6</td>
<td>28.2</td>
<td>7.2</td>
<td>33.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1:2</td>
<td>2.7</td>
<td>22.5</td>
<td>24.2</td>
<td>7.2</td>
<td>26.1</td>
<td>17.4</td>
<td>40.0</td>
</tr>
<tr>
<td>1:4</td>
<td>3.0</td>
<td>22.3</td>
<td>22.1</td>
<td>7.1</td>
<td>19.0</td>
<td>26.5</td>
<td>59.2</td>
</tr>
<tr>
<td>1:6</td>
<td>2.6</td>
<td>20.5</td>
<td>21.5</td>
<td>9.0</td>
<td>15.1</td>
<td>31.5</td>
<td>67.6</td>
</tr>
</tbody>
</table>

hDL3 are modulated by the apoAI and apoAII content of HDL, independently of the lipid composition of these lipoprotein particles. We observed that the rate of cholesteryl esters transferred either from LDL toward HDL, or, in the opposite direction, from HDL toward LDL, was considerably reduced as the apoAII content of HDL particles was increased. Consistent results were obtained by using successively apoAII-enriched HDL particles, apoAII-enriched HDL-Al particles, or directly by comparing HDL-Al and HDL-AlII particles isolated from normolipidemic plasmas.

Whereas the influence of the lipid composition of lipoprotein substrates on the cholesteryl ester transfer process has been well studied (51), consequences of variations in their apolipoprotein content remain unclear. In particular, the effect of variations in the apoAI and apoAII content of human HDL particles, independently of other proteins or lipids, is unknown. This question is important to address since HDL play an important role in the lipid transfer reaction. Moreover, several lines of evidence indicated that in vivo particles containing only apoAI, apoLpAI, and proteins containing both apoAI and apoAII, LpAI, represent distinct metabolic entities (52-54).

However, comparison of LpAI and LpAIII particles is made difficult since in plasma native HDL particles do not differ only in their apoAI and apoAII content but also in their lipid composition (38) which is known to influence the cholesteryl ester transfer reaction. In addition, apoAI-containing particles isolated from human plasma by immunosorption techniques have been shown to contain several additional minor apolipoproteins (37) which might also influence independently CETP activity. In an attempt to control the relative proportion of HDL components, we chose first to study the influence of apoAI and apoAII on the cholesteryl ester transfer reaction by replacing progressively apoAI by apoAII in ultracentrifugally isolated HDL particles which contained virtually no additional proteins other than apoAI and apoAII. In accordance with previous studies (30, 31), the substitution of apoAI for apoAI did not induce marked alterations in lipid composition of HDL particles and was accompanied only by a slight increase in HDL particle size. In the present study, the use of HDL particles which presented high apoAII/apoAI ratios was justified by recent observations which indicated that in human plasma significant proportions of total apoAI can be found in LpAI particles (26-28).

The substitution of apoAI for apoAI progressively reduced the rate of radiolabeled cholesteryl ester transfer, as measured either from HDL to LDL or from LDL to HDL. These results indicated therefore that the apoAI to apoAI ratio in HDL particles is an important parameter in determining their ability to serve as donor or acceptor lipoprotein substrates in the cholesteryl ester transfer reaction. In fact, kinetic analysis revealed that apoAI significantly reduced the maximal ability of HDL particles to accept cholesteryl esters but did not reduce the interaction of CETP with low concentrations of HDL. In fact, apoAII could act as an uncompetitive inhibitor of the cholesteryl ester transfer reaction. This observation contrasts with

DISCUSSION

Results from the present study demonstrated that CETP-mediated cholesteryl ester transfers between human HDL3 and

**Molecular Weight**

![Molecular Weight](image)
TABLE III  Composition (mass percent) of HDL3-AI and HDL3-AII particles  
HDL3-AI and HDL3-AII particles were separated by anti-apoAI immunofluor affinity chromatography as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Particle</th>
<th>ApoAI</th>
<th>ApoAII</th>
<th>Free cholesterol</th>
<th>Phospholipids</th>
<th>Cholesteryl esters</th>
<th>Triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL3-AI</td>
<td>42.6</td>
<td>0</td>
<td>4.4</td>
<td>28.0</td>
<td>21.5</td>
<td>3.4</td>
</tr>
<tr>
<td>HDL3-AII</td>
<td>23.2</td>
<td>23.2</td>
<td>3.9</td>
<td>27.3</td>
<td>19.8</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Further evidence for a role of apoAI and apoAII in the cholesteryl ester transfer process was brought by measuring the rate of cholesteryl ester transfers in the presence of HDL3-AI and HDL3-AII particles which were separated by anti-apoAII immunofluor affinity chromatography of the plasma HDL3 fraction. Indeed, cholesteryl ester transfer activity was found significantly lower with HDL3-AII than with HDL3-AI particles. Although in human plasma LpAI and LpAII do not differ only in their apolipoprotein content but also in their density, size, and lipid composition (25), the results of the present study suggest that modulation of CETP activity by the apoAI and apoAII content of HDL particles may be of potential physiological interest. Indeed, since the LpAI/LpAII ratio (55) as well as LpAII/(LpAI + LpAII) ratio (26) have been shown to undergo significant physiological fluctuation, it is possible that HDL apolipoproteins may influence cholesteryl ester transfer rates in vivo.

Since the present study compared cholesteryl ester transfer activity as measured with plasma HDL particles, which differed mainly in their apoAI and apoAII content but not in their lipid content, the observed variations related likely to differences of structure and physicochemical properties between the two apolipoproteins. Indeed, apoAI and apoAII have been shown to differ in their structure, hydrophobicity, charge, and surface affinity (31, 56, 57). However, the precise mechanism by which the apolipoprotein content of HDL particles can affect the cholesteryl ester transfer protein activity remains to be established, and the elucidation of the process probably will have to wait until the mechanism of action of CETP itself has been clearly elucidated. Two different models have been proposed to explain the activity of CETP, and, to date, it is still unknown whether CETP acts by shuttling cholesteryl ester molecules between HDL and other lipoproteins (58) or.

...
whether it mediates the formation of a ternary collision complex involving one donor and one acceptor lipoprotein substrate (48). In spite of this uncertainty, several hypotheses appear, in attempts to connect the ability of apoAII to inhibit CETP activity, in conjunction with observations from previous studies. For instance, it has been demonstrated that binding of CETP to lipoprotein substrates is the initial event of the cholesteryl ester transfer reaction (16, 59), and lipid transfer activity has been shown to correlate with binding of CETP to lipoproteins (16). Recently, apoE has been shown to enhance cholesteryl ester and triglyceride transfers between VLDL and HDL by enhancing the affinity of CETP for VLDL (22). It is tempting therefore to speculate that, similarly, modulation of CETP activity by apolipoproteins AI and AII may relate to variations they induce in the ability of CETP to bind to HDL subfractions. This hypothesis is sustained by the fact that, in human plasma, about 80% of total cholesteryl ester transfer activity is localized in HDL particles containing only apoAI, HDL-AI, whereas less than 10% were detected in HDL particles containing both apolipoproteins AI and AII, HDL-AI+AI II (60). Recent studies provided evidence that lipid binding to CETP may produce some conformational changes of the CETP molecule which could modulate its ability to transfer lipids between lipoprotein substrates (59). Therefore, inhibition of cholesteryl ester transfer activity by apoAII could also relate to variations in conformational changes that CETP undergoes upon its interaction with HDL4 particles. Physicochemical properties of apoAI and apoAII differ markedly, and the discriminant value Z, which accounts for the global hydrophobicity of protein molecules, is clearly higher for apoAII (0.299) than for apoAI (0.057) (61). Since several lines of evidence indicate that the actual substrates for CETP are the small amounts of neutral lipids that are solubilized in the phospholipid coat of lipoproteins (62), differences in hydrophobicity of the two major HDL apolipoproteins might affect the cholesteryl ester transfer reaction by altering the availability of cholesteryl esters at the lipoprotein surface.

Recent studies in human CETP-transgenic mice have demonstrated the central role of CETP in plasma lipoprotein metabolism (63, 64). However, it appears that the effect of human CETP on mouse lipoprotein profile is strongly influenced by the simultaneous expression of human apoA-I. Indeed, in double transgenic animals, expressing both human apoAI and CETP, CETP has been shown to reduce more markedly plasma HDL cholesterol levels as compared with mice expressing only human CETP (64). These results have been interpreted in terms of an activation of CETP by human apoAI in plasma from transgenic mice which may lack an inhibitory factor, i.e. apoAII (64). It is tempting therefore to speculate that variations of CETP activity observed in transgenic animals may illustrate the differences we observed in the ability of human apoAI and apoAII to modulate the activity of cholesteryl ester transfer protein.

In conclusion, it appears that the relative proportions of apoAI and apoAII present on the HDL surface can modulate the activity of CETP. These observations can be combined with previous data which demonstrated that the apoAI and apoAII content of HDL particles can also affect the activity of other lipoprotein modifying enzymes, among them lecithin: cholesterol acyltransferase (65, 66) and hepatic lipase (67).

Acknowledgment—We greatly acknowledge the assistance of Carolee Rousseau in the preparation of the manuscript.

REFERENCES
