The Hepatic Uptake of Rat High-Density Lipoprotein Cholesteryl Ester is Delayed After Treatment with Cholesteryl Ester Transfer Protein (44341)

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Abstract. **The effects of cholesteryl ester transfer protein (CETP) on the direct uptake of HDL cholesteryl ester by the liver was investigated using the rat *in vivo* and the isolated perfused rat liver as experimental models. Rat plasma was incubated with [^{3}H]cholesterol in the presence or absence of partially purified human CETP for 18 hr and [^{3}H]cholesteryl ester-labeled HDL was then isolated by ultracentrifugation. The CETP-treated as compared to untreated HDL showed a small shift toward a lower density in the peak of lipoprotein cholesterol, suggesting that the HDL particle size was increased. After injection of the labeled HDL into rats *in vivo,* more radioactivity remained in the plasma after 60 min when the CETP-treated preparation was used, but the amounts found in the liver and secreted in the bile were not significantly different from those obtained with the untreated HDL. The distribution of the label remaining in the plasma after 60 min between different density fractions corresponding to HDL subclasses suggested that the uptake of HDL_2 and HDL_3 was delayed by CETP treatment. Radioactivity from CETP-treated HDL was also removed from the perfusate of isolated perfused rat livers more slowly than that from untreated HDL, and in this case the amount found in the liver after 60 min was significantly lower. These findings indicate that treatment with CETP has a direct inhibitory effect on the clearance of rat HDL cholesteryl ester from the blood and its uptake by the liver.** [P.S.E.B.M. 1999, Vol 220]

High-density lipoprotein (HDL) plays an important role in reverse cholesterol transport, the process by which cholesterol from the peripheral tissues is returned to the liver for elimination from the body, and this is believed to contribute to its protective effect in the development of atherosclerosis and related cardiovascular diseases (1). Plasma contains various subclasses of HDL of differing size and density, which are interconverted by the action of a number of proteins, including cholesteryl ester transfer protein (CETP), lecithin:cholesterol acyltransferase (LCAT), and hepatic lipase (2, 3).

Cholesterol is transferred from the tissues to discoidal nascent HDL and esterified by LCAT, causing the particles to become the spherical HDL_3, and eventually to increase in size to form cholesteryl ester-rich HDL_2. At this stage, CETP mediates the transfer of cholesteryl ester from HDL to triglyceride-rich lipoproteins, such as very low density lipoproteins (VLDL), in exchange for triacylglycerol, and larger triacylglycerol-enriched HDL particles, designated HDL_{2b}, are produced. The cholesteryl ester removed from HDL_2 is eventually cleared from the blood *via* the low-density receptor (LDL) pathway, whereas HDL_{2b} is converted back to HDL_3 through lipolysis of the triacylglycerol by hepatic lipase and lipoprotein lipase (1–4). Therefore, CETP plays a central role in the process of reverse cholesterol transport.

The impact of CETP on atherosclerosis is open to ques-

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tion. Its action in transferring cholesteryl ester from the antiatherogenic HDL to the atherogenic apoB-containing lipoproteins suggests that it may promote the development of the disease, and there is some evidence to support this. In CETP-deficient humans, HDL cholesterol levels are increased, and plasma CETP activity is raised in many dyslipidemias associated with accelerated atherosclerosis (4, 5). Furthermore, expression of human or simian CETP in transgenic mice has been found to reduce HDL cholesterol concentrations and increase the incidence of atherosclerotic lesions (6, 7). However, more recently, Hayek *et al.* (8) reported that the development of early atherosclerotic lesions is inhibited in hypertriglyceridemic transgenic mice overexpressing CETP and apoCIII, and CETP deficiency has been shown to be associated with coronary artery disease in hyperalphalipoproteinemic patients (9, 10). Therefore, it is possible that CETP may have an antiatherogenic effect in some physiological conditions.

After uptake by HDL and esterification by LCAT, cholesterol from the peripheral tissues has two possible fates; it may be transferred to apolipoprotein B (apoB)-containing lipoproteins, or it may remain with HDL until it is taken up by the liver. It is clear that the activity of CETP present in the plasma determines the amount of cholesteryl ester metabolized by each route (11, 12), but it is also possible that the changes in HDL composition brought about by the transfer protein influence the rate of removal of HDL cholesterol from the blood, and this may be a factor in the effects of CETP on atherosclerosis. Inhibition of CETP has been reported to delay the clearance of [^{3}H]cholesteryl ether, a nonmetabolizable analog of cholesteryl ester, from HDL in rabbits (13) although Rinniger and Pittman (14) did not find any direct effect of CETP on the uptake of HDL cholesteryl ester in the human hepatoma cell line HepG2.

The rat lacks plasma CETP activity (15), so that once accepted by HDL, cholesterol remains with this lipoprotein until it is taken up by the liver. Intravenous injection of human CETP into rats has been shown to result in the transfer of cholesteryl esters from HDL to lower density lipoproteins, and to lead to the disappearance of an apoE-rich HDL subclass from the plasma (16, 17). In the studies of Ha *et al.* (16), the level of CETP in the rat plasma was close to or above that found in human plasma for 24 hr, and in these conditions there was a marked loss of cholesteryl ester from HDL, whereas the triacylglycerol:cholesteryl ester ratio in VLDL was reduced by 50%. Thus, human CETP causes significant transfer of cholesteryl ester between lipoprotein classes in the rat *in vivo,* and this species provides a useful model for the study of the effects of the active protein on HDL metabolism. In the present work, we have investigated the influence of human CETP on the removal from the blood and uptake by the liver of cholesteryl ester from rat HDL. The HDL was prepared from plasma incubated in the presence or absence of partially purified human CETP *in vitro* and labeled with [^{3}H]cholesteryl ester, and the fate of the radioactivity was followed after intravenous injection *in vivo* using an experimental system previously established in our laboratory (18). In addition, hepatic uptake of the label was studied directly using the isolated perfused rat liver (19).

Materials and Methods

Male Wistar rats (Charles River Italia, Calco-Como, Italy) weighing 290–360 g were used in all experiments. The animals were housed under constant day length (12 hr) and allowed free access to food (standard pellet diet) and water. Animal care was in accordance with D.L. 116/92, which is the Italian enforcement of the European Directive 86/609 on the protection of animals used for experimental purposes.

Phenyl-Sepharose CL-6B and DEAE-Sepharose CL-6B were obtained from Pharmacia (Uppsala, Sweden). Heparin, sodium azide, manganese chloride, EDTA, and other chemicals were purchased from Aldrich-Sigma (Milan, Italy). Solvents of analytical grade were from Carlo Erba and enzymatic kits from Boehringer Biochemia Robin (Milan, Italy). [1α-2α ^{3}H]-Cholesterol was supplied by Amersham International (Amersham, Buckinghamshire, U.K.). All ultracentrifugations were performed in an L-70 Beckman ultracentrifuge using a 60Ti rotor, and NaBr was used in all cases to adjust the density of lipoprotein fractions.

Purification of Human CETP. The partial purification of CETP was performed by a combination of two ultracentrifugation steps followed by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B and anion exchange on DEAE-Sepharose CL-6B (20). Briefly, 300 ml of human plasma obtained from a single blood donor subject were adjusted to d = 1.250 g/ml and centrifuged at 40,000 rpm for 40 hr at 4°C. The top half of each tube was harvested, adjusted to d = 1.21 g/ml and recentrifuged for 48 hr as described above. The clear one-third middle zone was then collected and applied to a phenyl-Sepharose CL-4B column (30 × 2 cm) equilibrated with 2.0 *M* NaCl, 10 m*M* Tris, pH 7.4, and eluted with the same buffer (250 ml) at a flow rate of 2.5 ml/min. The column was then washed with buffer (500 ml) containing 0.15 *M* NaCl, 10 m*M* Tris, pH 7.4, and the CETP was finally eluted with distilled water (300 ml). The fractions containing CETP activity were pooled, dialyzed against 25 m*M* NaCl, 10 m*M* Tris, pH 7.4, and applied to a DEAE-Sepharose CL-6B column (1.0 × 15 cm) equilibrated with the same buffer in order to remove lecithin:cholesterol acyltransferase (LCAT) activity. The sample was eluted with a linear NaCl gradient (300 ml, final concentration 250 m*M* NaCl, 10 m*M* Tris) at a flow rate of 40 ml/hr. Fractions containing CETP activity were pooled and dialyzed against 0.15 m*M* NaCl, 10 m*M* Tris/HCL, pH 7.4 (dialysis buffer). The purified enzyme (total volume 46 ml) was immediately frozen at –80°C in small aliquots.

Assay of CETP. CETP activity was assayed, and the percentage transfer activity was calculated according to Tollefson and Albers (20). To prepare the substrate lipoproteins, human plasma (100 ml) was adjusted to d = 1.060

g/ml and centrifuged at 40,000 rpm for 20 hr at 4°C. The top fraction was harvested, dialyzed against dialysis buffer containing 1 m*M* EDTA, and used as the acceptor lipoprotein in the assay. After a second centrifugation at d = 1.125 g/ml at 40,000 rpm for 40 hr at 4°C, the top fraction was discarded, the bottom fraction was adjusted to d = 1.250 g/ml and centrifuged under the same conditions. The lipoprotein in the top fraction was harvested and dialyzed as above, 1.1 MBq of [^{3}H]cholesterol in 95% ethanolic solution was added with gentle, continuous stirring, and the mixture was incubated for 18 hr at 37°C to allow cholesteryl ester formation by the action of LCAT. HDL_3 was then re-isolated by centrifugation at d = 1.210 g/ml and dialyzed as before.

The transfer of [^{3}H]cholesteryl ester from HDL_3 (d = 1.125–1.210 g/ml) to lipoproteins of d < 1.060 g/ml lipoproteins was measured after 18 hr of incubation at 37°C in 10 m*M* Tris buffer containing 0.15 *M* NaCl, pH 7.4, with or without a source (plasma, column fractions) of CETP activity. The donor and acceptor lipoproteins (present in a ratio of approximately 1:5 in terms of cholesteryl ester mass) were separated by precipitation with heparin (2500 U/ml)-$MnCl_2$ (1 *M*), and the radioactivity remaining in HDL_3 in the supernatant was determined. Nonspecific transfer that occurred in the absence of CETP was subtracted from the values obtained.

Preparation of [^{3}H]Cholesteryl Ester-Labeled Rat HDL Treated with CETP for Use in Whole Animals and Perfused Liver Experiments. Blood was withdrawn in EDTA from 50 rats, and the plasma was separated immediately from red blood cells and divided into 2 equal aliquots. 110 KBq/ml plasma of [^{3}H]cholesterol was slowly added with gentle stirring to both aliquots. Partially purified CETP (93.8 μg in 125 μl/ml plasma, an amount calculated to make the activity of CETP in the incubation similar to that found in the human plasma used to prepare the protein) was then added to one (treated HDL), while an equal volume of dialysis buffer was added to the other (untreated HDL). After incubation for 18 hr at 37°C, the plasma samples were centrifuged (40,000 rpm, 24 hr, 4°C) at d = 1.080 g/ml, the resulting top fraction was discarded, and the bottom fraction was centrifuged (40,000 rpm, 40 hr, 4°C) at d = 1.230 g/ml. The top fraction containing HDL_2 and HDL_3 was collected and dialyzed against 0.15 *M* NaCl.

Analysis of Rat Plasma by Gradient Ultracentrifugation. To evaluate the effect of incubation of rat plasma with purified CETP on the density distribution of cholesterol, samples (3 ml) of plasma incubated with or without CETP as described above were analyzed by discontinuous gradient ultracentrifugation, as previously described (21). Each fraction eluted from the gradient was assayed for protein, triacylglycerol, and unesterified and total cholesterol content.

***In Vivo* Experiments.** Rats were anesthetized with Zoletin 20 (20 mg/kg body weight), the left jugular vein and common bile duct were cannulated (PE50, Clay-Adams, Parsipanny, NJ), and the animals were put in a restraining cage and allowed to recover for one-half hour. [^{3}H]Cholesteryl ester–labeled HDL (100 KBq), treated or untreated with CETP and containing approximately 2.0 μmol total cholesterol, was injected intrajugularly in a volume of 0.5 ml. The animals were sacrificed 60 min after the injection, and the recovery and distribution of radioactivity in the blood, liver, and bile were analyzed (22). Lipoproteins were separated from the plasma after removal of the erythrocytes by ultracentrifugation (40,000 rpm, 20 hr, 4°C) at d = 1.050, 1.085, 1.125 and 1.250 g/ml, and the radioactivity in the density ranges of d = 1.050–1.085, 1.085–1.125, and 1.125–1.250 was calculated from the differences between the values found in the top 1–1.5 cm of the tubes centrifuged at the higher and lower densities of each range.

Perfused Liver Experiments. Rats were anesthetized with Thiopental (Farmotal, Grison Pharma, Roma, Italy) (40 mg/kg body weight). The surgical procedure and liver perfusions were performed as described previously (19). After a 5-min washout with Krebs-Ringer bicarbonate buffer (pH 7.4), the livers were placed in the perfusion apparatus (Disa, Milan, Italy) and perfused with 85 ml–90 ml of medium at a flow rate of 1 ml/min/g liver in a recirculating system. The medium contained 33% freshly prepared bovine erythrocytes and 67% Krebs-Ringer bicarbonate buffer (pH 7.4) together with BSA (4% w/v) and glucose (0.1% w/v). After an equilibration period of about 30 min, [^{3}H]cholesteryl ester–labeled HDL (800 KBq), treated or untreated with CETP and containing approximately 16 μmol total cholesterol, was added to the perfusion medium (in the reservoir) in a volume of about 4 ml, and the perfusion was continued for an additional 60 min. The perfusion apparatus was kept at 37°C throughout the experiment. At various times after addition of the labeled lipoprotein, samples (100 μl) of perfusion medium were taken and centrifuged at 500*g* to remove the erythrocytes, and the supernatant was assayed for radioactivity. After 60 min, the radioactivity in liver and perfusate was analyzed as described above for the *in vivo* experiments. The viability of each liver in the course of the experiments was demonstrated by standard parameters (i.e., aspartate amino-transferase level, O_2 utilization, pH values, and degree of hemolysis of the perfusate (19).

Analytical Methods. Radioactivity in samples of plasma, plasma density fractions, erythrocytes, liver, bile, and lipoprotein preparations was determined by liquid scintillation counting. Cholesterol and cholesteryl ester in chloroform:methanol (2:1 v/v) extracts of HDL preparations were separated by thin layer chromatography on silica gel G using hexane:diethyl ether:formic acid (40:10:1 v/v/v). The relevant bands were then scraped into vials, and the radioactivity was determined by liquid scintillation counting (23). Cholesterol (F-CHOL, CHOD-PAP), cholesteryl ester (CHOL, CHOD-PAP), and triacylglycerol (TG, GPO-PAP) mass was determined using commercial enzyme kits (Boehringer Mannheim GmbH). Significance limits were calculated using Student's *t* test.

Results

Activity of Partially Purified Human CETP. When 200 μl (containing 150 μg protein) of the final preparation of CETP was incubated in the presence of [^{3}H]cholesteryl ester-labeled HDL (180 nmol cholesteryl ester) and lipoproteins of d < 1.060 g/ml (900 nmol cholesteryl ester) at 37°C, 59.3 ± 2.4% (mean ± range, n = 2) of the radioactivity was transferred to the lower density lipoproteins after 18 hr. This compared to 42.5 ± 1.5% transfer obtained using 200 μl of the plasma used for preparation of the CETP. Marcel *et al.* (24) have reported that the level of CETP in the human population varies from 1–3.5 μg/ml. However, the activity found in the plasma used here (equivalent to 145 μg cholesteryl ester transferred/ml plasma in 18 hr) is comparable to what has been reported by other researchers (40 μg/ml plasma/6 hr (25); 80 μg/ml plasma/8 hr (26)) for normal human subjects, indicating that it falls within the normal range.

Preparation of CETP-Treated and Untreated HDL. CETP treatment of rat plasma and the labeling of HDL in cholesteryl ester by the action of LCAT on [^{3}H] cholesterol (20) was carried out in a combined incubation. After 18 hr at 37°C in the presence or absence of CETP, 69% and 64% of the added radioactivity, respectively, was associated with the HDL fraction (d = 1.080–1.230 g/ml) isolated, whereas 22% and 28% were found in lipoproteins of d < 1.080 g/ml and 8%–9% was in the d > 1.230 g/ml fraction. Approximately 90% of the radioactivity was in cholesteryl ester with the remainder in cholesterol in both CETP-treated and untreated HDL preparations. The CETP-treated HDL had a slightly lower cholesterol and higher triacylglycerol content than the untreated HDL (Table I).

Effect of CETP Treatment on the Gradient Ultracentrifugation Elution Profile of Rat Plasma. The elution profile of lipoprotein cholesterol separated by gradient ultracentrifugation from CETP-treated or untreated rat plasma is reported in Figure 1. CETP treatment produced a slight shift toward lower density in the peak of lipoprotein total cholesterol and cholesteryl ester, but not unesterified cholesterol, which occurred at d = 1.09 g/ml in the untreated plasma. In addition, the CETP-treated as compared to untreated plasma, there was a decrease in a second, small peak in the concentration of total cholesterol, unesterified cholesterol, and triacylglycerol found at d = 1.20 g/ml.

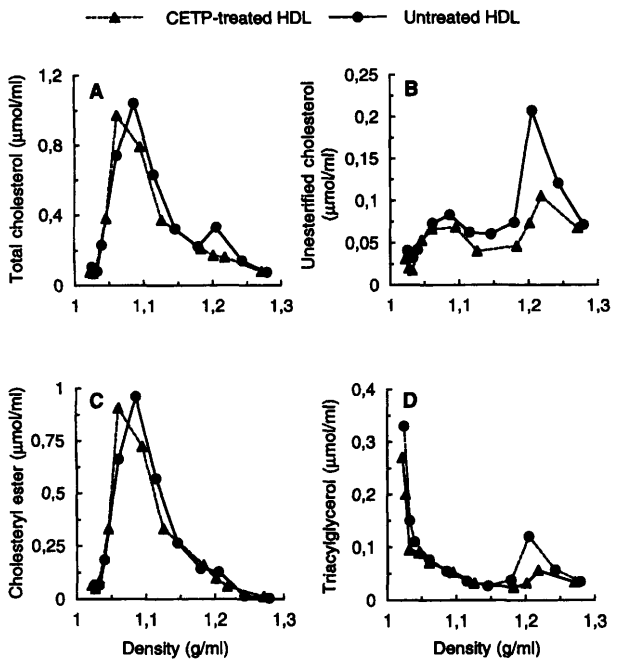

Figure 1. Elution profile of lipoprotein cholesterol and triacylglycerol after separation from CETP-treated or untreated rat plasma by gradient ultracentrifugation. (A) Total cholesterol; (B) unesterified cholesterol; (C) cholesteryl ester; (D) triacylglycerol.

Effect of CETP Treatment on the Removal from the Blood and Uptake by the Liver of Rat HDL *In Vivo*. The proportion of the administered radioactivity remaining in the plasma 60 min after intravenous injection of [^{3}H]cholesteryl ester-labeled rat HDL into rats *in vivo* was significantly higher when the CETP-treated preparation was used (Table II). However, the percentage of label found in the liver or secreted in the bile was not changed. Only very small amounts of radioactivity were transferred to erythrocytes during the experiments.

Table I. The Composition of CETP-Treated and Untreated HDL

Component	Untreated HDL	CETP-treated HDL
Total cholesterol (μmol/ml)	4.64	3.97
Unesterified cholesterol (μmol/ml)	0.33	0.26
Cholesteryl ester (μmol/ml)	4.31	3.71
Triacylglycerol (μmol/ml)	0.30	0.33
Phospholipid (μmol/ml)	1.57	1.82
Protein (mg/ml)	4.92	4.64

Note. Rat plasma was incubated in the presence or absence of partially purified human CETP. HDL was then isolated by ultracentrifugation and the cholesterol, triacylglycerol, phospholipid, and protein contents of the two preparations were determined.

Table II. The Effect of Treatment of [^{3}H]Cholesteryl Ester-Labeled Rat HDL with CETP on the Recovery of Radioactivity in Blood, Liver, and Bile after Intravenous Injection into Rats *in vivo*

Tissue	Untreated HDL	CETP-treated HDL
Plasma	45.8 ± 5.6	57.9 ± 8.1*
Erythrocytes	2.0 ± 0.6	1.8 ± 0.8
Liver	15.2 ± 1.1	14.1 ± 3.1
Bile	0.29 ± 0.08	0.32 ± 0.02

Note. [^{3}H]cholesteryl ester-labeled HDL prepared from rat plasma treated or untreated with partially purified human CETP was injected into rats intrajugularly. After 60 min, the animals were sacrificed, and the radioactivity associated with the plasma, erythrocytes, liver, and bile collected *via* a cannula in the common bile duct during the experiment was determined. The data are expressed as a percentage of the injected radioactive dose and are the mean ± SD from three (CETP-treated HDL) or four (untreated HDL) rats. Significance limits: *P < 0.05 versus untreated HDL.

The distribution of radioactivity between plasma density fractions 60 min after injection is shown in Figure 2. A greater percentage of the injected dose was recovered in HDL_2 (d = 1.085–1.125 g/ml) and HDL_3 (d = 1.125–1.250 g/ml) in the CETP-treated HDL group (Fig. 2A), whereas the proportion of the radioactivity remaining in the plasma found in HDL_1 (d = 1.050–1.085 g/ml) was decreased in this group (Fig. 2B). In comparison to the distribution of radioactivity between the density fractions in the injected dose (i.e., at Time 0), the proportion in HDL_3 was decreased, and the proportion in HDL_2 was increased after 60 min in both experimental groups.

Effect of CETP Treatment on the Uptake of Rat HDL by the Perfused Rat Liver. Because the volume of perfusate used (85–90 ml) was much larger than the volume of blood circulating in the rat, the amount of [^{3}H]cholesteryl ester-labeled HDL added to the perfusate in these experiments was increased compared to that injected *in vivo* in order to maintain a similar concentration of HDL cholesterol. Figure 3 shows the time course of the clearance of radioactivity from the whole perfusate (buffer + erythrocytes) during the experiments. [^{3}H]Cholesteryl ester from CETP-treated HDL was cleared less rapidly than from the untreated preparation, and this difference was significant after 60 min. In addition, the amount of radioactivity remaining in the buffer medium (minus the erythrocytes) at the end of the experiment was higher in the experiments with CETP-treated HDL, whereas the amount recovered in the liver was lower (Table III). As in the experiments *in vivo*, there was very little transfer of radioactivity to erythrocytes during the perfusions.

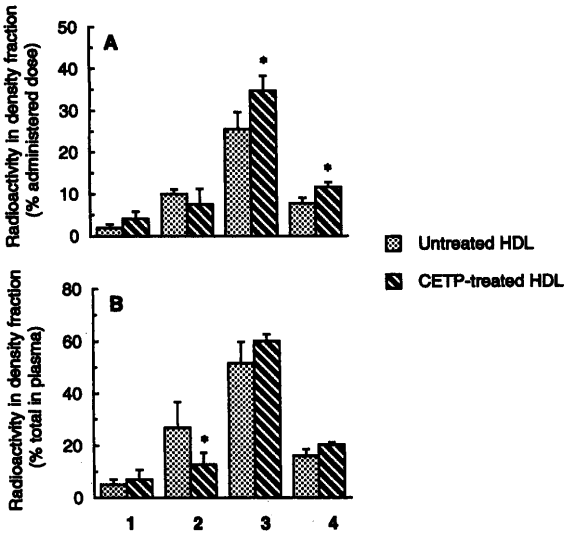

Figure 2. The effect of treatment of [^{3}H]cholesteryl ester-labeled rat HDL with CETP on the distribution of radioactivity in plasma density fractions after intravenous injection into rats *in vivo*. [^{3}H]cholesteryl ester-labeled HDL prepared from rat plasma treated or untreated with partially purified human CETP was injected into rats intrajugularly. After 60 min, the animals were sacrificed, and density fractions of (1) d < 1.050 g/ml; (2) d = 1.050–1.085 g/ml; (3) d = 1.085–1.125 g/ml; (4) d = 1.125–1.250 g/ml were isolated from the plasma by ultracentrifugation. Data are expressed as a percentage of (A) the administered radioactive dose and of (B) the total radioactivity remaining in the plasma after 60 min. Each point is the mean from three (CETP-treated HDL) or four (untreated HDL) rats. Error bars show the SD. *P < 0.05 versus untreated HDL. The distribution of radioactivity in the injected preparations (i.e., at zero time) in fractions 1, 2, 3, and 4, respectively was: untreated HDL, 5.3, 26.0, 38.8, and 29.9%; treated HDL, 2.9, 24.5, 43.9, and 28.8%.

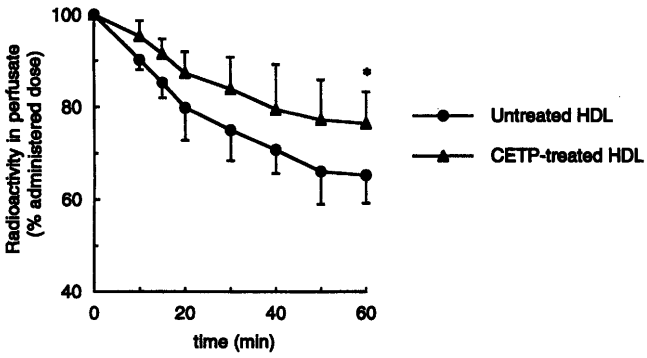

Figure 3. The effect of treatment of [^{3}H]cholesteryl ester-labeled rat HDL with CETP on the disappearance of radioactivity from the perfusate in the isolated perfused rat liver. [^{3}H]cholesteryl ester-labeled HDL prepared from rat plasma treated or untreated with partially purified human CETP was added to the perfusate of isolated perfused rat livers. Samples of perfusate were taken at the times indicated, and the radioactivity remaining was determined. Each point is the mean from four rats, and error bars show the SD. *P < 0.05 versus untreated HDL.

Analysis of the distribution of the label in the perfusate between the various density fractions (Fig. 4) after 60 min showed that the percentage of the added dose found in HDL_3 was higher when CETP-treated rather than untreated HDL was used (Fig. 4A). However, the proportion of the amount of radioactivity remaining in the perfusate in each fraction was not significantly different either between the two experimental groups or in comparison to the proportions found in the added dose (Time 0).

Discussion

CETP is believed to play a major part in determining HDL lipid composition and particle size (3, 4). Inhibition of CETP activity using appropriate antibodies has been shown to raise cholesterol and lower triacylglycerol levels in HDL and to increase the diameter of the particles in rabbits (27)

Table III. Effect of Treatment of [^{3}H]Cholesteryl Ester-Labeled Rat HDL with CETP on its Uptake by the Isolated Perfused Rat Liver

Compartment	Untreated HDL	CETP-treated HDL
Medium	65.2 ± 6.0	76.4 ± 6.8*
Erythrocytes	1.0 ± 0.2	0.6 ± 0.1
Liver	44.8 ± 2.3	23.0 ± 11.2*

Note. [^{3}H]Cholesteryl ester-labeled HDL prepared from rat plasma treated or untreated with partially purified human CETP was added to the perfusate of isolated perfused rat livers. The experiments were terminated after 60 min, and the radioactivity associated with the perfusion medium, erythrocytes, and liver was determined. The data are expressed as a percentage of the added radioactive dose and are the mean ± SD from four rats. Significance limits: *P < 0.05 versus untreated HDL.

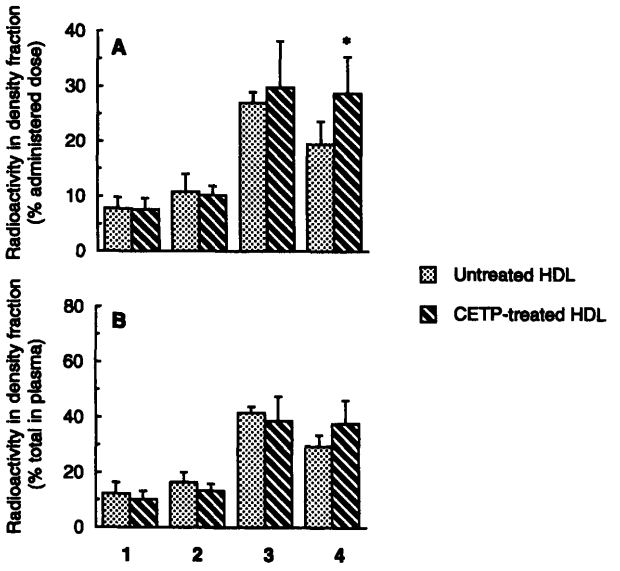

Figure 4. The effect of treatment of [^{3}H]cholesteryl ester-labeled rat HDL with CETP on the distribution of radioactivity in density fractions after its addition to the perfusate of isolated perfused rat livers. [^{3}H]Cholesteryl ester-labeled HDL prepared from rat plasma treated or untreated with partially purified human CETP was used. After 60 min of perfusion, the experiments were terminated, and density fractions of (1) d < 1.050 g/ml; (2) d = 1.050–1.085 g/ml; (3) d = 1.085–1.125 g/ml; (4) d = 1.125–1.250 g/ml were isolated from the plasma by ultracentrifugation. Data are expressed as a percentage of (A) the administered radioactive dose and of (B) the total radioactivity remaining in the plasma after 60 min. Each point is the mean from four rats, and error bars show the SD. *P < 0.05 versus untreated HDL. The distribution of radioactivity in the injected preparations (i.e., at Time 0) in fractions 1, 2, 3, and 4 was as shown for Figure 2.

and hamsters (28). HDL particle size has also been found to be increased in CETP-deficient humans (29). Furthermore, the larger HDL_1 particles have been reported to disappear from rat plasma when human CETP is infused (16). These findings indicate that the presence of active CETP leads to the production of smaller HDL particles *in vivo*. However, in our experiments, incubation of rat plasma with CETP was associated with a slight shift of the peak in cholesterol concentration to a lower density, suggesting that the size of the HDL particles was increased (Fig. 1). This result was consistent with the work of Zechner *et al.* (30), who showed that incubation of HDL_3 with VLDL, LCAT, and CETP *in vitro* caused a change of the HDL_3 into a larger, less dense HDL_{2b}-like fraction. The apparent discrepancy between the results obtained *in vivo* and *in vitro* can be explained by the absence in the *in vitro* incubations of hepatic lipase or lipoprotein lipase activity, which would be present *in vivo*, and which is necessary to convert HDL_{2b} to the smaller HDL_3 by lipolysis of triacylglycerol (1–4, 31). The lipases are active when bound to vascular surfaces, and little activity is found circulating in plasma in most species, including the rat, unless the enzymes are released by injection of heparin (32). Thus, the incubations used for the treatment of rat plasma with CETP in our experiments contained triacylglycerol-rich lipoproteins and LCAT, but very little lipase activity, and an increase in HDL particle size was the expected result.

Our finding of a small peak in the concentration of triacylglycerol at a density of about 1.2 g/ml in untreated plasma (Fig. 1) was in agreement with a previous similar study using plasma from male Wistar rats (19). However, in CETP-treated plasma, this peak was markedly reduced, suggesting that the size of the particles was increased. The results from the gradient ultracentrifugation profiles, therefore, indicated that the CETP treatment of rat plasma caused significant changes in the density distribution of HDL lipids in the present work, and the effects of these changes on the clearance of HDL from the blood and its uptake by the liver were investigated in the experiments *in vivo* and with the perfused liver.

Few previous studies have investigated the direct effects of CETP on the removal of HDL cholesteryl ester from the blood. Whitlock *et al.* (13) found that inhibition of CETP activity by about 50% using a monoclonal antibody delayed the clearance of [^{3}H]cholesteryl ester-labeled HDL in rabbits *in vivo*. However, as CETP activity was present in the plasma in these experiments, this difference could be explained by decreased transfer to apoB-containing lipoproteins prior to uptake, as well as by a direct effect on HDL clearance. Indeed, studies with the human hepatoma cell line HepG2 showing that CETP enhanced the uptake of HDL cholesteryl ester only in the presence of LDL (33), and that blocking the LDL receptor abolished the effect (14), suggest that an influence on transfer between lipoprotein classes may be the predominant effect. Rat plasma lacks CETP activity (15); thus, in our experiments any differences observed in the removal of CETP-treated or untreated HDL cholesteryl ester from the blood can be attributed to a direct effect on HDL uptake. Prior treatment of HDL with CETP led to a decreased rate of clearance of the [^{3}H]cholesteryl ester label both *in vivo* (Table II) and in the perfused liver (Fig. 3, Table II). Furthermore, the amount of radioactivity found in the liver after 60 min was lower when the CETP-treated preparation was used. These findings indicate that CETP-treatment has a direct inhibitory effect on the uptake of HDL cholesteryl ester by the liver.

After injection of the labeled HDL into the whole animal, there was a marked redistribution of the radioactivity from HDL_3 to HDL_2. This is in agreement with our previous work (18), and can be explained by the action of LCAT in increasing the size of the particles, as this enzyme was not present in the perfused liver experiments, and no similar redistribution occurred (1–3). The differences in the distribution of the label between the HDL subclasses found with the CETP-treated and untreated HDL (Figs. 2, 4) may be due to selective changes in their uptake, or simply to differences in the remodeling of the particles by LCAT and lipases. As all the enzymes are present *in vivo*, and hepatic lipase is found in the perfused liver, we can assume that some remodeling will take place. If this were the only change, then the redistribution of label between the subclasses would determine the relative proportion of the administered radioactivity found in each fraction, and the pat-

tern of the differences between the CETP-treated and untreated HDL observed when the data were expressed as a percentage of the administered dose and as a percentage of the total radioactivity remaining in the plasma would be the same. In fact, 60 min after injection of the label *in vivo,* the percentage of the administered dose remaining in the plasma in HDL_2 and HDL_3 was greater when the CETP-treated HDL was used (Fig. 2A), whereas the percentage of the total plasma radioactivity in these fractions was not significantly changed (Fig. 2B), suggesting that the uptake of HDL_2 and HDL_3 was retarded. In addition, the proportion of the total radioactivity in the plasma at 60 min found in HDL_1 was decreased, although the percentage of the administered dose was unchanged. Therefore, the predominant effect on HDL_1 may be redistribution of the label by the action of lipases. As the perfusate did not contain any plasma, LCAT was absent from the perfused liver experiments, and although lipoprotein lipase is found in the liver (34), the added HDL would be exposed to much less activity than the HDL used for the *in vivo* work. Thus, the remodeling of the lipoprotein in the perfused liver is likely to be different from the process *in vivo;* therefore, it is not surprising that different patterns of results were obtained with the two sets of experiments. The data suggest, however, that HDL_3 was also taken up more slowly from the CETP-treated HDL by the isolated perfused livers (Fig. 4).

The findings reported here show that treatment of rat HDL with human CETP alters the density distribution of the lipids in HDL and demonstrate for the first time that the action of the transfer protein has a direct inhibitory effect on the clearance of HDL cholesteryl ester from the blood and its uptake by the liver. In addition they provide evidence to suggest that the removal of cholesteryl ester in the HDL_2 and HDL_3 subclasses is delayed by CETP treatment.

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