

## Cholesteryl Ester Transfer Activity in Plasma Measured by Using Solid-Phase-Bound High-Density Lipoprotein

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We studied the ability of lipid-transfer factors in plasma to promote transfer, to endogenous lipoproteins, of [<sup>3</sup>H]cholesteryl ester from high-density lipoprotein (HDL) covalently bound to Sepharose 4B beads. After incubation for 2 h at 37 °C, 12 to 14% of the [<sup>3</sup>H]cholesteryl ester had been transferred to the lipoproteins of the plasma, in the proportions 57% to HDL and 43% to low- and very-low-density lipoproteins. This process was a function of the amount of plasma present and was stimulated by addition of partly purified lipid-transfer protein. Transfer also depended on the concentration of donor HDL but was independent of the amount of acceptor lipoprotein. This simple evaluation of cholesteryl ester transfer does not require removal of lipoproteins from the plasma before incubation.

**Additional Keyphrases:** radioassay · kinetic analysis · lipid transfer protein · triglycerides · role of HDL in anti-atherogenesis · interlaboratory comparisons

A lipid-transfer protein that mediates the net transfer and exchange of triglycerides and cholesteryl esters between lipoproteins has been identified in and purified from human plasma (1, 2). Although this protein is associated with high-density lipoprotein (HDL), it may be recovered in the *d* >1.21 fraction of plasma after the lipoproteins have been removed by ultracentrifugation (2).<sup>3</sup> This protein, designated LTP-I, can facilitate exchanges of cholesteryl ester or triglyceride, or both, between lipoproteins (3, 4). The ability of LTP-I to promote a net flux of cholesterol between HDL and lipoproteins of lower density (5) suggests a central role for this process in removal of cholesterol from peripheral tissues and its return to the liver (reverse cholesterol transport) (6). Thus, this transfer may be involved in the anti-atherogenic role proposed for HDL (5).

To assay lipid-transfer activity in plasma, investigators have measured cholesterol mass transfer (7, 8) or the movement of radiolabeled lipid (2, 9, 10) between different lipoprotein classes. The usefulness of these measurements in plasma has been limited by exogenous lipid-exchange reactions as well as difficulties in separating donor lipids from acceptor molecules. In many assays, delipidated plasmas have served as the source of lipid-transfer activity and isolated lipoproteins as the acceptors and donors (9, 10). These studies have contributed much to our understanding of the roles of the individual components in the transfer process, but large variations in the methods used make very

difficult any interlaboratory comparison of data.

To develop a simple, reliable assay that will give reproducible results in many different laboratories, we have developed the following method for determining lipid-transfer activity in fresh plasma. We measure the transfer of radiolabeled cholesteryl ester from a solid-phase-bound HDL to lipoproteins in plasma. Because the lipoproteins need not be removed from the plasma before incubation, a major source of interlaboratory variation is obviated.

### Materials and Methods

**Materials.** Cholesteryl [<sup>3</sup>H]oleate (specific activity 72 kCi/mol) was obtained from NEN Research Products, Quebec, Canada. Bovine and human serum albumin, CNBr-activated Sepharose-4B, and diisopropyl fluorophosphate were from Sigma Chemical Co., St. Louis, MO. Reagent kits for total and free cholesterol determination were from Boehringer, Mannheim, F.R.G. All other chemicals were analytical grade, from BDH Chemicals Canada Ltd., Vancouver, B.C.

**Isolation of lipoproteins.** Blood from normal male volunteers who had fasted for 16 h was collected into EDTA-containing tubes. Plasma was removed by centrifugation at 1750 × *g* for 10 min. HDL, LDL, and VLDL were isolated by ultracentrifugation at densities 1.210–1.063, 1.063–1.006, and 1.006 g/mL, respectively (11), then recentrifuged at the same density to remove any remaining plasma proteins. The washed lipoproteins were dialyzed four times, for 4 to 12 h each time, against 100-fold greater volumes of NaCl/Tris buffer (per liter: 150 mmol of NaCl, 10 mmol of Tris HCl, 1 mmol of EDTA, and 3 mmol of NaN<sub>3</sub>, pH 7.4), then characterized by electrophoresis on agarose (11) and by gel filtration through a 10 × 300 mm column of Superose 6 (Pharmacia, Uppsala, Sweden) (12). Free and total cholesterol were determined enzymatically (13), with a commercial kit, and protein was determined by the method of Lowry et al. as modified for plasma lipoproteins (11).

**Radiolabeling of HDL.** We dried a mixture of 200–500 μL of HDL (5 g/L) and 10–50 μCi of [<sup>3</sup>H]cholesteryl oleate, under nitrogen. After adding 20 to 60 mg of additional HDL, we gently vortex-mixed, then stirred the sample for 4 h at room temperature. After storage overnight at 4 °C, the labeled HDL was re-isolated by ultracentrifugation or filtration through a 0.22-μm (pore size) filter. Gel filtration through a Superose 6 column gave identical elution profiles for [<sup>3</sup>H]HDL that had been re-isolated by ultracentrifugation or by filtration. Labeling efficiencies were usually between 20 and 60%. Labeled HDL was stored at 4 °C in the presence of EDTA (1 mmol/L) and NaN<sub>3</sub> (3 mmol/L).

**Linking of labeled HDL to Sepharose 4B beads.** Radiolabeled HDL was covalently linked to CNBr-activated Sepharose 4B by the manufacturer's suggested procedures (14). After this coupling, any remaining active groups were blocked by incubation in a 1 mol/L ethanolamine solution for 2 h at room temperature. The [<sup>3</sup>H]HDL–Sepharose beads were washed several times with the NaCl/Tris buffer, then with a pH 4 buffer (0.1 mol of acetate and 0.5 mol of NaCl

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<sup>3</sup> Nonstandard abbreviations: HDL, LDL, VLDL, high-, low-, and very-low-density lipoproteins, respectively; LTP-I, lipid transfer protein; LCAT, lecithin-cholesterol acyltransferase (phosphatidyl-sterol acyltransferase, EC 2.3.1.43).

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per liter), then several more times with the NaCl/Tris buffer. About 98% of the total protein was bound to the beads, but the washing procedures removed 20 to 30% of the total radiolabel. The beads were then stored in NaCl/Tris buffer at 4 °C until use. Beads used as long as 2 months after preparation showed no significant differences from freshly prepared beads. Before each assay, we determined their total <sup>3</sup>H, protein, and cholesteryl ester contents.

**Partial purification of LTP-I.** Partly purified LTP-I was prepared by a modified version of the method of Albers et al. (15), as described by Eisenberg (16). VLDL and LDL were precipitated from plasma and the supernate was chromatographed on phenyl-Sepharose and diethylaminoethyl-Sepharose. The partly purified LTP-I was approximately 250-fold more active than in plasma, as determined by the percent transfer of [<sup>3</sup>H]cholesteryl ester from HDL to LDL as measured by the method of Albers et al. (1).

**Cholesteryl ester transfer assay procedure.** To 13 × 100 mm test tubes was added 25–50 μL of [<sup>3</sup>H]HDL-Sepharose beads containing 200 μg of HDL protein. Just before the assay, we aspirated the supernatant buffer from the beads and replaced it with 800 μL of fresh plasma per tube. The mixture was incubated on a horizontal tube-rocker at 37 °C for the times indicated in the figures. After the incubation, we centrifuged the tubes for 2.5 min at 430 × g to pellet the HDL-bound beads and immediately removed 600 μL of plasma from each tube. Using 200 μL of this, we determined the total [<sup>3</sup>H]cholesteryl ester transferred to plasma. We precipitated the VLDL and LDL in the remaining 400 μL by adding 40 μL of heparin/MnCl<sub>2</sub> (per liter: 0.5 mol of MnCl<sub>2</sub>, 38 mmol of NaCl, and 2500 USP units of heparin) and centrifuging at 13 000 × g for 10 min. We took an aliquot of each supernate to use in determining the amount of [<sup>3</sup>H]cholesteryl ester transferred to HDL. Nonprecipitable lipoproteins were considered to be HDL, whereas those precipitated were termed LDL + VLDL. Of the <sup>3</sup>H transferred during the assay procedure, >98% was associated with cholesteryl ester, as shown by lipid extraction and thin-layer chromatography (11).

## Results

**Cholesteryl ester transfer.** The amount of [<sup>3</sup>H]cholesteryl ester transferred from solid-phase-bound HDL to acceptor lipoproteins in normal plasmas increased with the duration of incubation and appeared to be complete within 2 h at 37 °C (Figure 1). By this time, between 12 and 14% of the total donor radiolabel had been transferred to the plasma. As calculated from the specific activity of the donor, this saturation point was equivalent to a total transfer of 7 to 9 μg of [<sup>3</sup>H]cholesteryl ester to acceptor lipoproteins. Transfer rates were greatest from 0 to 10 min (12 μg/h), but were much slower between 10 and 120 min (3–7 μg/h).

The total amount of [<sup>3</sup>H]cholesteryl ester transferred depended on the amount of plasma present in the incubation mixtures but became saturated above 1.3 mL (Figure 2). This protein dependence may be related to a requirement for lipid transfer factors, because addition of partly pure LTP-I increased the rate of transfer (Figure 3). For example, adding 820 μg of partly purified LTP-I increased total transfer by 22%.

**Effects of donor and acceptor lipoprotein concentrations.** Figure 4 shows that [<sup>3</sup>H]cholesteryl ester was transferred to both the HDL and the LDL + VLDL fractions of plasma, with 57% of the total amount transferred to plasma being recovered in HDL. Cholesteryl ester transferred to these

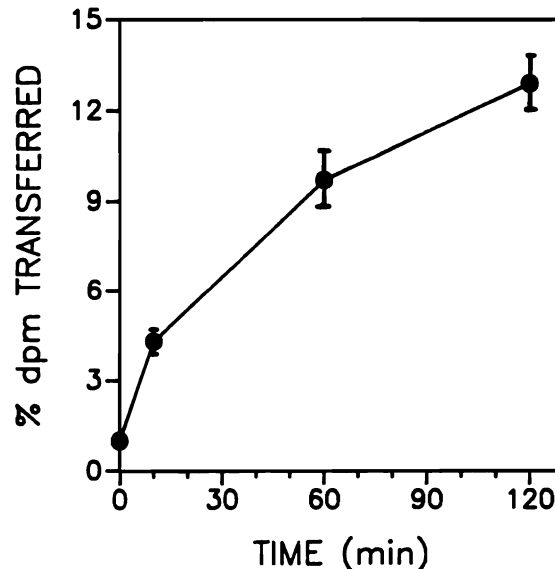


Fig. 1. Total cholesteryl ester transferred from solid-phase-bound HDL to plasma

We incubated 800 μL of plasma with 200 μg of solid-phase-bound HDL protein at 37 °C for 0–120 min. The Sepharose-bound-HDL was removed by centrifugation and the radioactivity in a 200-μL aliquot of supernate was determined. The percentage of the initial [<sup>3</sup>H]cholesteryl ester radioactivity that was transferred to the plasma is shown as the mean ± SD for seven normolipidemic individuals

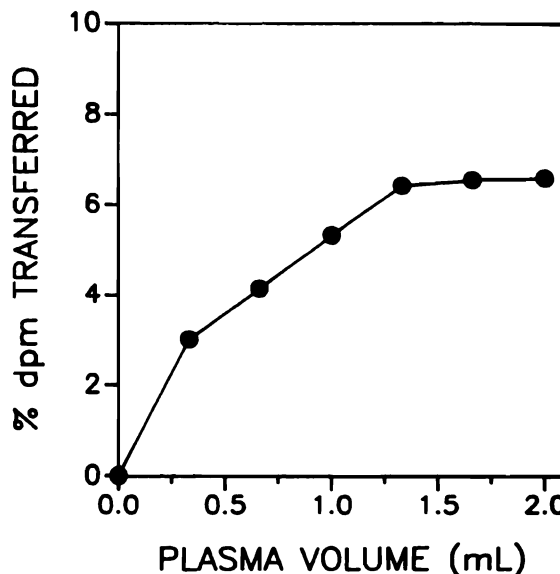


Fig. 2. Effect of volume of plasma on cholesteryl ester transfer

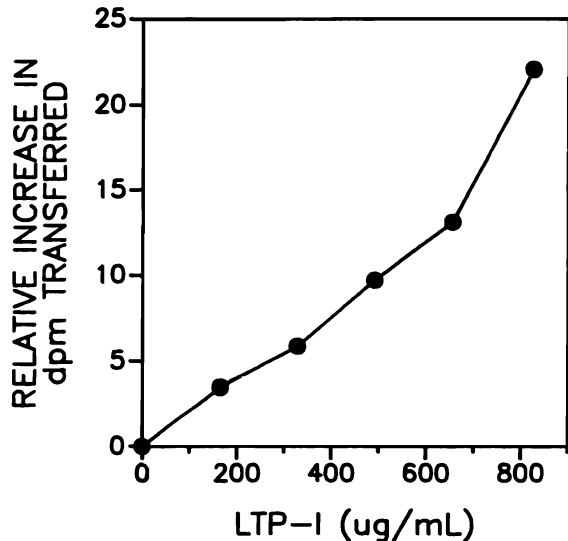
We incubated 200 μg of solid-phase-bound HDL at 37 °C for 60 min with various volumes of plasma and NaCl/Tris buffer (total volume, 2 mL). Transfer of [<sup>3</sup>H]cholesteryl ester was determined as in Fig. 1; values are the average of duplicate determinations

fractions did not differ significantly and ranged between 4 and 5% of the total <sup>3</sup>H in the donor per milliliter of plasma per hour. Titrating exogenous HDL (up to 0.6 mg of HDL protein per milliliter) into plasma produced no substantial changes in transfer rates, nor did addition of either VLDL or LDL to plasma to give concentrations as great as 1.55 mg of exogenous VLDL-triglyceride or 1.20 mg of exogenous LDL-cholesterol per milliliter.

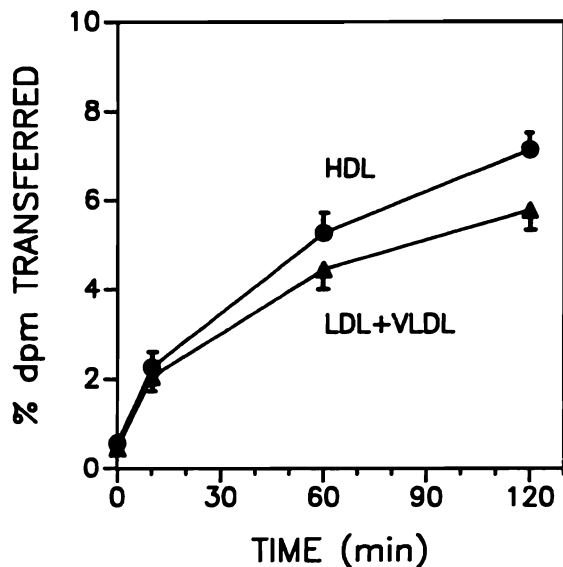
When the concentration of donor HDL in plasma was increased from 125 μg/mL to 750 μg/mL, the rates of cholesteryl ester transfer to both HDL and LDL + VLDL increased linearly (Figure 5). At 750 μg of HDL protein, the total rate of transfer of cholesteryl ester exceeded 10 μg/mL

per hour, 58% of this being associated with transfer to HDL.

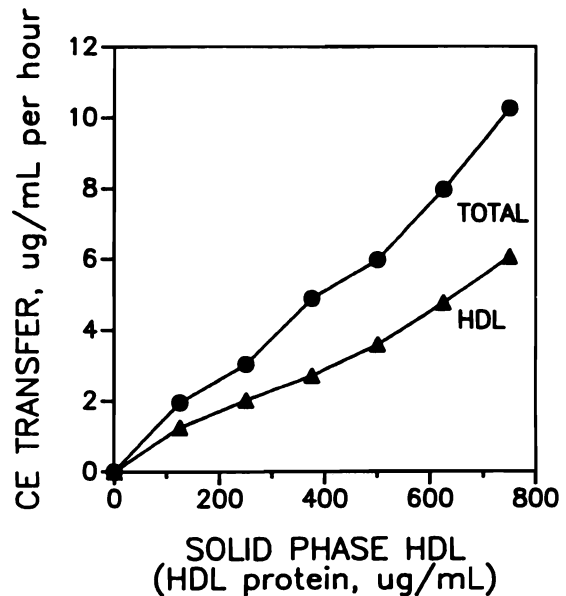
**Transfer of cholesteryl ester to purified lipoproteins in the absence of plasma.** Purified VLDL, LDL, and HDL were recombined, in the presence of 10 mg of human serum albumin per milliliter, at concentrations similar to those usually found in normal plasma (0.1, 1.0, and 1.8 mg of lipoprotein protein per milliliter, respectively) and transfer activity was measured as described above. Total transfer to the lipoprotein mixture was less than half that observed for normal plasma (Figure 6), but this decrease was not equally distributed between the rates of transfer to HDL and LDL + VLDL. The rate of transfer to HDL in the lipoprotein mixture was 75% less than to HDL in plasma, whereas transfer to LDL + VLDL was decreased by only 25%.



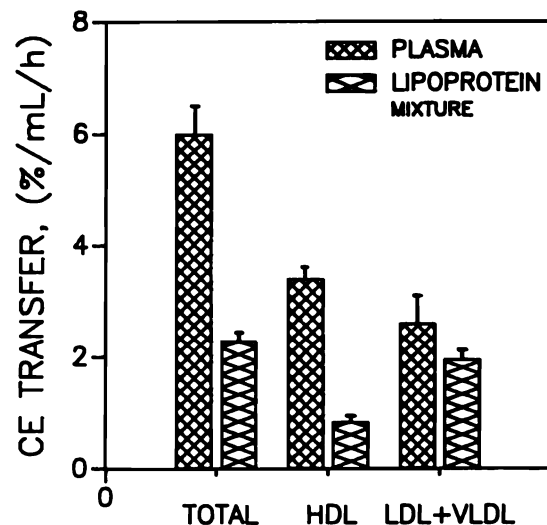
**Fig. 3.** Effect of partly purified LTP-I on cholesteryl ester transfer. We incubated 500  $\mu$ L of plasma with 200  $\mu$ g of solid-phase-bound HDL protein and 0–820  $\mu$ g of 250-fold purified LTP-I for 60 min at 37  $^{\circ}$ C. Transfer rates were determined as described in Fig. 1. Values are the average of duplicate determinations.



**Fig. 4.** Proportion of cholesteryl ester transferred from solid-phase-bound HDL to plasma lipoproteins. Aliquots (400  $\mu$ L) of the supernates from the incubation mixtures described in Fig. 1 were incubated with heparin/ $MnCl_2$ , then centrifuged to pellet the precipitated LDL + VLDL. The radioactivity in 200  $\mu$ L from each supernate was counted to determine the percent of the [ $^3H$ ]cholesteryl ester that was transferred to HDL. Transfer to LDL + VLDL was determined by subtracting the value for HDL from the total percentage transferred. Results shown are mean  $\pm$  SD (n = 7).



**Fig. 5.** Effect of donor concentration on cholesteryl ester transfer rates. We incubated 800  $\mu$ L of plasma with solid-phase-bound HDL at 37  $^{\circ}$ C for 0–120 min. Mass transfer rates, calculated from the initial specific radioactivity of the donor HDL, are expressed as the average of duplicate determinations.



**Fig. 6.** Transfer of cholesteryl ester to plasma and recombined lipoproteins. The rate of cholesteryl ester transfer from solid-phase-bound HDL to a mixture of VLDL, LDL, and HDL (per milliliter, 0.1, 1.0, and 1.8 mg of the respective lipoprotein proteins) in human serum albumin, 10 mg/mL, was compared with the rate of transfer to normal plasma (n = 7). Values for the lipoprotein mixtures are the mean  $\pm$  SD of quadruplicate determinations.

**Effect of LCAT inactivation on transfer activity.** The activity of LCAT was 98% inhibited by the addition to plasma of diisopropyl fluorophosphate, 1 mmol/L, as previously reported (17). Under these conditions, transfer rates after LCAT inactivation did not differ substantially from the rates in normal plasma (Figure 7).

### Discussion

We have shown that [ $^3H$ ]cholesteryl ester may be transferred from immobilized HDL to plasma lipoproteins, with approximately equal distribution to HDL and VLDL + LDL (Figure 1). This transfer is independent of exogenous acceptor lipoproteins; adding VLDL, LDL, or HDL to the plasma before incubation with the immobilized HDL did not affect

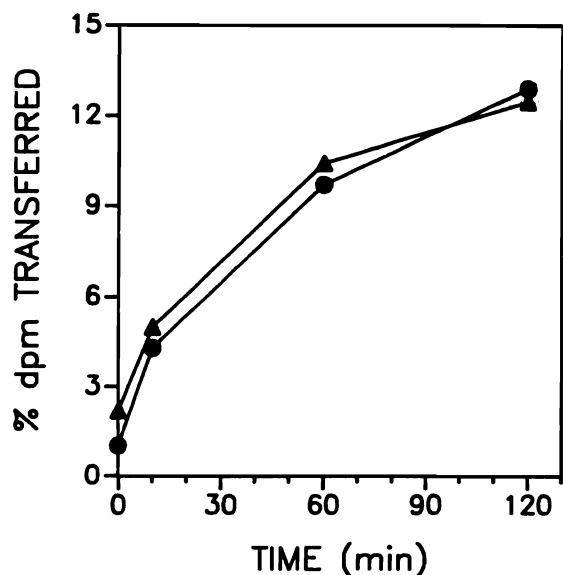


Fig. 7. Effect of LCAT inactivation on transfer activity. Transfer rates shown, determined as described for Fig. 1, for 800  $\mu$ L of plasma in the presence (●) or absence (▲) of diisopropyl fluorophosphate, 1 mmol/L. Values shown are the average of duplicate determinations.

the total amount of label transferred or the amount recovered in the different lipoprotein classes. This suggests that the acceptor particle is not rate limiting in the transfer we measured; some other factor in plasma may regulate this process.

However, transfer activity was dependent on the amount of plasma present (Figure 2), and it may therefore have been associated with increases in the amount of lipid-transfer proteins in the incubations. In fact, adding partly pure LTP-I to plasma before the assays increased transfer rates (Figure 3). This suggests that LTP-I was responsible for a substantial amount of transfer of cholesteryl ester in our system.

The analytical recovery of considerable amounts of label in the HDL fraction indicates that cholesteryl ester may be transferred to this lipoprotein with the same efficiency as to the lower-density lipoproteins (Figure 4). This conclusion is supported by the work of Morton (4), who demonstrated that binding of LTP-I to lipoproteins is required for lipid transfer and that, although all lipoproteins bind LTP-I with the same high affinity, the complex formed with HDL is much more stable than that with other lipoprotein classes. Our observations may reflect the potential for cholesteryl ester to be transferred from the HDL subclass in which it is synthesized to other HDL subclasses before subsequent transfer to VLDL or LDL (5).

When we substituted a mixture of VLDL, LDL, and HDL for whole plasma as acceptor, the proportion of label recovered in HDL was greatly decreased (Figure 6). Thus, the lipid transfer proteins may transfer lipids to HDL in preference to the lower-density lipoproteins. Although Figure 6 indicates that half the total transfer may occur in the absence of the lipid transfer protein, the presence of contaminating transfer proteins in one or more of the purified lipoproteins could have led to an overestimation of non-protein-mediated transfer.

Presumably, the absolute rates of transfer of [ $^3$ H]cholesteryl ester to the plasma lipoproteins could be affected by changes in the specific radioactivity of the donor cholesteryl

ester pool, for example, by endogenous synthesis of cholesteryl ester by LCAT. However, carrying out the incubations in the presence of diisopropyl fluorophosphate indicated that inhibiting this enzyme had no effect on the rate of transfer from the HDL-Sepharose to plasma HDL or VLDL + LDL (Figure 7).

In summary: we have described a rapid, reproducible procedure for measuring the transfer of [ $^3$ H]cholesteryl ester from immobilized HDL to the plasma lipoproteins. We are investigating using this method to estimate cholesteryl ester transport in patients' samples of whole plasma without the need to first remove the lipoproteins by ultracentrifugation.

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