Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma

John H. Tollefson,* Stuart Ravnik,* and John J. Albers[1,*,†]

Departments of Medicine* and Pathology,† University of Washington School of Medicine and Northwest Lipid Research Center, Harborview Medical Center, Seattle, WA 98104

Abstract In this report we have described the purification of a human plasma phospholipid transfer protein, designated LTP-II, which displayed the following characteristics: i) facilitated both the exchange and net mass transfer of lipoprotein phospholipids; ii) did not facilitate the transfer of lipoprotein cholesteryl esters (CE) or triglycerides (TG); iii) was not recognized by antibody to the human cholesteryl ester transfer protein (LTP-I); iv) showed no amino acid sequence homology to the cholesteryl ester transfer protein (LTP-I); v) has an apparent molecular weight (M_r) of 70,000 off Sephacryl S200, and 69,000 off sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE); vi) has an apparent isoelectric point of 5.0 by chromatofocusing; and vii) when added to an incubation mixture of VLDL, HDL_3, and the human plasma cholesteryl ester transfer protein (LTP-I), enhanced the observed transfer of cholesteryl esters from HDL_3 to VLDL, even though LTP-II has no intrinsic cholesteryl ester transfer activity of its own. These results show that this phospholipid transfer protein is unique from the human plasma cholesteryl ester transfer protein, and may play an important role in human lipoprotein lipid metabolism.—**Tollefson, J. H., S. Ravnik, and J. J. Albers.** Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. *J. Lipid Res.* 1988. **29:** 1593–1602.

Supplementary key words lipid transfer proteins • phospholipid • cholesteryl ester • triglyceride • lipoproteins • atherosclerosis • protein purification

The various species of the plasma phospholipids, in particular the phosphatidylcholines, play an important structural role in transporting hydrophobic lipid molecules through an aqueous environment as part of a macromolecular lipoprotein complex (1). This is achieved in part by the amphipathic structure of the surface-bound phospholipids positioning their polar-hydrophilic head towards the aqueous plasma environment, and inserting their nonpolar-hydrophobic fatty acid hydrocarbon tail towards the lipophilic core of the lipoprotein particles.

However, beyond a structural role of the plasma phospholipids, they also serve as substrates for some of the major enzymes of lipoprotein physiology such as lecithin:cholesterol acyltransferase (LCAT), lipoprotein lipase, and hepatic lipase. It has also been shown that there exists in human plasma at least two lipid transfer proteins, the cholesteryl ester transfer protein (CETP, or LTP-I) which has been shown to facilitate the transfer of both neutral lipids (cholesteryl ester and triglycerides) and phospholipids between the plasma lipoproteins (2–5) and LTP-II which only facilitates phospholipid transfer between the plasma lipoproteins (3, 4). In an earlier report (3) we described the isolation and characterization of LTP-I. In that report we observed that approximately one-half of the plasma phospholipid transfer activity bound to heparin-Sepharose, and that this activity (designated LTP-II) was not heat-stable and had little or no ability to facilitate neutral lipid (CE or TG) transfer between the plasma lipoproteins. This report describes the isolation and characterization of LTP-II. A preliminary report of this work has been presented (6).

METHODS

Preparation of labeled lipoprotein substrates

All isotopically labeled lipids were purchased from New England Nuclear (Boston, MA). The [^{14}C]cholesteryl ester (CE)-labeled HDL_3, [^{3}H]triglyceride (TG)-labeled HDL_3, and [^{3}H]phosphatidylcholine (PC)-labeled HDL_3 were prepared as described previously (3). The approximate specific activity of each substrate was: [^{14}C]CE-HDL_3, approximately 2,500 dpm/μg CE, assuming an average molecular weight of 650 for CE; [^{3}H]TG-HDL_3, approximately 18,000 dpm/μg TG; and [^{3}H]PC-HDL_3, approximately 8,000 dpm/μg PL, respectively. The preparation of the lipoprotein substrates was essentially as described (3).

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; LTP, liquid transfer protein; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid; VLDL, very low density lipoprotein; HDL, high density lipoprotein; HSA, human serum albumin; PC, phosphatidylcholine.

[1]To whom to address correspondence at NWLRC Harborview Medical Center, Seattle, WA 98104.

Downloaded from www.jlr.org by guest, on June 18, 2012

Briefly, the [^{14}C]CE-HDL$_3$ was prepared by the endogenous esterification of [^{14}C]cholesterol (which was added in a small aliquot of ethanol) by the lecithin:cholesterol acyltransferase (LCAT) present in freshly isolated and dialyzed d > 1.125 g/ml plasma. Once the percentage of radioactive cholesteryl esters was > 85% of the total counts (free cholesterol plus cholesteryl esters), the [^{14}C]CE-HDL$_3$ was isolated by ultracentrifugation. The [^{3}H]TG-HDL$_3$ and [^{3}H]PC-HDL$_3$ were both prepared by the direct addition of the labeled lipid (dissolved in ethanol) to the isolated HDL$_3$. Although adequate for monitoring the transfer of labeled triglyceride, the [^{3}H]TG may simply be adsorbed on the surface of the lipoprotein, and not in the core of the HDL$_3$.

Radioassays of lipid transfer activity

All of the assays were performed at optimum lipoprotein donor:lipoprotein acceptor lipid ratio for each substrate to yield the maximum activity (percentage of label transferred/incubation) while remaining within the linear range of the assay (normally about 25% transfer/incubation). Each assay included a negative control incubation, labeled donor plus unlabeled acceptor lipoproteins (ordinarily d < 1.063 g/ml lipoproteins) without an added source of lipid transfer activity, and a positive control incubation (a sample with a known level of lipid transfer activity). Activity is expressed as a percentage of labeled donor lipid transferred to unlabeled acceptor lipoprotein/volume or mass of transfer protein assayed/incubation time, and calculated as described previously (3). The 37°C negative control incubation corrects for any nonfacilitated lipid transfer and/or incomplete separation of donor and acceptor lipoprotein (7).

Mass assay of phospholipid transfer activity

Unlabeled isolated very low density lipoprotein (VLDL) and unlabeled isolated high density lipoprotein$_3$ (HDL$_3$) were incubated for 3 hr at 37°C at an 8:1 VLDL–HDL$_3$ phospholipid mass ratio with or without purified LTP-II. Following the incubation, the samples were chilled on ice, and then the VLDL and HDL$_3$ were separated by ultracentrifugation (18 hr, 40,000 rpm, 40.3 rotor). Phospholipid mass was measured on both VLDL and HDL$_3$, following tube slicing and lipid extraction, by the method of Bartlett (8). Results are expressed as μg of VLDL phospholipid transferred to HDL$_3$ per 3-hr incubation.

Purification of the phospholipid transfer protein (LTP-II)

All of the purification steps were performed at 4°C, and all buffers were at pH 7.4 unless otherwise specified. Approximately 1 liter of fresh human plasma was brought to a nonprotein solvent density of 1.21 g/ml with solid KBr, and the clear "1.21 g/ml middle" fraction was isolated by ultracentrifugation as described (3). Approximately 475 ml of the "1.21 g/ml middle" plasma fraction was applied directly (without dialysis) to a 2.6 × 40 cm phenyl-Sepharose CL-4B column, and the transfer activity was eluted with deionized water as described for LTP-I (3). Fractions containing transfer activity were then dialyzed against 20 mM NaCl, 10 mM Tris, pH 7.4, and chromatographed on a 2.5 × 26 cm anion exchange column of DEAE-Sepharose CL-6B, and eluted with a linear salt gradient of 20–350 mM NaCl, flow rate: approximately 45–60 ml/hr. The active fractions off DEAE-Sepharose were pooled and dialyzed against 50 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA. Approximately 55 mg of protein in 140 ml was applied to a 1.5 × 30 cm column of heparin-Sepharose (Pharmacia). After extensive washing with 50 mM NaCl, 10 mM Tris, pH 7.4, buffer to wash out any residual cholesteryl ester transfer protein (LTP-I), the phospholipid transfer protein was eluted from the column with a linear NaCl gradient (50–500 mM) in a 10 mM Tris, pH 7.4, buffer. The active fractions eluted from the heparin-Sepharose column were next pooled and dialyzed against 10 mM sodium acetate buffer, pH 4.5, and rapidly applied to a 1.0 × 10.0 cm column of carboxymethyl cellulose equilibrated against the same buffer. The column was developed with a linear NaCl salt gradient (0–500 mM NaCl, 10 mM sodium acetate, pH 4.5). All fractions were collected into 1.0 ml of 1 M Tris, pH 8.0, then rapidly dialyzed against 0.15 M NaCl, 10 mM Tris, pH 7.4. Fractions with phospholipid transfer activity were pooled, then dialyzed against 0.15 M NaCl, 4 mM phosphate buffer, pH 6.9. After dialysis, approximately 3.5 mg of protein (in approximately 100 ml) was applied to a 0.9 × 8.0 cm column of hydroxyapatite equilibrated against the same buffer. After thorough washing of the column with the equilibration buffer, the column was then developed with a linear (4–60 mM) phosphate gradient.

Molecular sieve chromatography of LTP-II

Approximately 10 ml of the active phospholipid transfer activity that had bound to the heparin-Sepharose column (free of the cholesteryl ester transfer protein) was concentrated by vacuum ultrafiltration to approximately 1.0 ml. The concentrated LTP-II sample was then applied in an ascending manner to a calibrated 1.5 × 100 cm column of Sephacryl S-200 (Pharmacia), as described previously (3).

Chromatofocusing of LTP-II

Fifty ml of the active heparin-Sepharose bound fraction was dialyzed against 25 mM imidazole adjusted to pH 7.4 with HCl, and applied to a column of PBE 94 polybuffer exchanger (Pharmacia) equilibrated against the same buffer. The pH gradient was established with 25 ml of Pharmacia polybuffer PBE 74 diluted 1:8 to a final concentration of 25 mM imidazole, pH 4.0. The column flow rate was approximately 25 ml/hr, collecting 5 ml/fraction. The pH was determined for each fraction, and aliquots were

Downloaded from www.jlr.org by guest, on June 18, 2012

subsequently dialyzed against 0.15 M NaCl, 10 mM Tris, pH 7.4, prior to assay for phospholipid transfer activity.

Thermal stability of LTP-II

To monitor the stability of LTP-II, aliquots of partially purified LTP-II (purified through the heparin-Sepharose step, devoid of any cholesteryl ester transfer/phospholipid transfer activity attributable to LTP-I) were incubated at the following temperatures: −70°C, +4°C, +20°C, +37°C, and +58°C for the time indicated in Fig. 4. In addition, aliquots of the cholesteryl ester transfer protein (LTP-I) were incubated at +58°C for comparison. Following the preincubation at the indicated temperatures, the samples were assayed in triplicate using the same lot of substrates (labeled HDL_3 phospholipid donor and unlabeled acceptor lipoproteins). Interassay variation was less than 10% for this study.

Effect of chemical inhibitors on LTP-I and LTP-II activity

Triplicate aliquots of LTP-I and LTP-II (heparin-Sepharose nonbound and bound fractions) were assayed for phospholipid transfer activity in the absence (control activity) or with incremental concentrations of the following chemical inhibitors: *p*-chloromercuriphenylsulphate (*p*CMPS): 5, 10, 15, 20, and 25 mM; ethylmercurithiosalicylate (Thimerasol): 0.25, 1.0, 10, 20, and 25 mM; dithio-bis-nitrobenzoic acid (DTNB): 0.25, 1.0, 2.5, and 5.0 mM; and iodoacetic acid: 1.0, 10, 20, 30, 40, and 50 mM.

Preparation of polyclonal antibody to LTP-I

LTP-I was purified through ultracentrifugation, and chromatography on phenyl-Sepharose, DEAE-Sepharose, CM-cellulose, heparin-Sepharose, and hydroxyapatite, and demonstrated a single major staining band near 65 kD when electrophoresed on an SDS-PAGE gel. As a final precaution to avoid potential contamination of human serum albumin (HSA), the active material eluted from the hydroxyapatite column was chromatographed on an immunoaffinity column constructed of purified anti-HSA coupled to cyanogen bromide Sepharose CL-4B according to the manufacturers (Pharmacia) recommendations. The purified LTP-I was concentrated by vacuum ultrafiltration, then electrophoresed, localized, excised, and electroeluted out of a preparative polyacrylamide gel. The purity was confirmed by re-electrophoresis of an aliquot on an SDS-PAGE gel. Approximately 200 μg of the electroeluted LTP-I was injected into a goat, followed by two booster injections of the same material (which had been stored at − 20°C) at 2-week intervals. A preimmunization bleeding was taken from the goat, and antisera were harvested 1 week following the final booster injection of antigen. To remove any endogenous goat lipid transfer activity, or any potential nonimmunoglobulin inhibitor(s) of the lipid transfer protein (9, 10, 11), control (preinjection) and immune sera were ultracentrifuged at a nonprotein solvent density of 1.21 g/ml to remove the lipoprotein fraction. The d > 1.21 g/ml plasma fraction was then applied to a phenyl-Sepharose column, and the nonbinding fraction was collected and tested for: i) lack of cholesteryl ester and phospholipid transfer activity; and ii) immunoinhibition of LTP-I lipid transfer activity (see below).

Immunoinhibition of lipid transfer by goat anti-human LTP-I antibody

Triplicate aliquots of partially purified (heparin-Sepharose nonbinding fraction) cholesteryl ester transfer protein (LTP-I) and the plasma phospholipid transfer protein which binds to heparin-Sepharose (LTP-II) were assayed for phospholipid transfer activity after an overnight, 4°C preincubation with increasing amounts of either preimmune goat d > 1.21 g/ml plasma fraction that did not bind to phenyl-Sepharose (an IgG-enriched fraction, devoid of both lipoproteins and lipid transfer activity) or the postimmunization antisera that had been processed in an identical manner. After the 18-hr preincubation, the samples were centrifuged at low speed (3000 rpm, 30 min) to remove any immune complexes. The supernates were then assayed for phospholipid transfer activity as described in Methods.

Stimulation of isolated cholesteryl ester transfer protein (LTP-I) activity by LTP-II

Aliquots of the cholesteryl ester transfer protein (LTP-I) and the plasma phospholipid transfer protein (LTP-II) (purified through the heparin-Sepharose step) were assayed for contaminating LTP-II phospholipid transfer activity in the case of LTP-I, and residual LTP-I cholesteryl ester transfer activity in the case of LTP-II. Our indices for lack of cross-contamination were as follows. i) LTP-I was considered free of LTP-II when both its cholesteryl ester and phospholipid transfer activities were resistant to a 60-min exposure to a 58°C heat treatment (> 95% of CE and PC lipid transfer activity remaining). ii) LTP-II was considered free of LTP-I when *a*) there was no detectable cholesteryl ester transfer activity and *b*) all of the phospholipid transfer activity was destroyed by heat treatment for 1 hr at 58°C. To evaluate the effect of LTP-II phospholipid transfer activity on LTP-I-mediated cholesteryl ester transfer, aliquots of LTP-II (0% CE transfer/200 μl per 1 hr; 25% PC transfer/200 μl per 1 hr) and LTP-I (15% CE transfer/200 μl per 1 hr) were assayed separately or in combination for 0 to 120 min, then processed as described in Methods.

Other methods

Sodium dodecyl sulfated gel electrophoresis was performed as described by Laemmli (12); gels were stained with Coomassie Brilliant Blue G-250 dissolved in 3.5% perchloric acid, and then destained with 7% acetic acid until the

Downloaded from www.jlr.org by guest, on June 18, 2012

background was clear. Western blotting was performed as described by Towbin, Staehelin, and Gordon (13). Protein mass was determined by the method of Lowry et al. (14) using bovine serum albumin as a standard.

RESULTS

The purification scheme developed for the isolation of the plasma phospholipid transfer protein, LTP-II, was patterned after those developed for LCAT (15), and the cholesteryl ester transfer protein (LTP-I, 3). This scheme allows for the recovery of each of these proteins, thus maximizing the yield from a single isolation. Starting from 1 liter of human plasma, we achieved approximately a 15,000-fold purification with an overall recovery of approximately 10% for LTP-II through the following six purification steps: i) ultracentrifugation; ii) hydrophobic-interaction chromatography (phenyl-Sepharose); iii) anion exchange (DEAE-Sepharose) chromatography; iv) affinity chromatography (heparin-Sepharose); v) cation exchange (carboxymethyl cellulose) chromatography; and vi) adsorption chromatography (hydroxyapatite) (**Table 1**). The most efficient step, which yielded the greatest fold purification, was phenyl-Sepharose (approximately 260-fold purification). This is most likely because of the nonhydrophobic nature of most of the plasma proteins, because they have negligible affinity for the hydrophobic matrix of phenyl-Sepharose. However, this is also most likely the explanation for the fact that the fold-purification at the successive steps is nearly two orders of magnitude less than that achieved at phenyl-Sepharose. The proteins eluted from the phenyl-Sepharose are all hydrophobic in character, and thus have similar chromatographic behavior. As with any "enzyme" purification, the values obtained for specific activity (total activity /total protein) and fold-purification can only be approximations, as one cannot correct for loss of activity due to natural deterioration of the phospholipid transfer protein during the purification procedure.

Both the cholesteryl ester transfer protein, LTP-I (3), and the phospholipid transfer protein examined in this study, LTP-II, elute in the same position through the first three purification steps: ultracentrifugation, hydrophobic-interaction (phenyl-Sepharose), and anion exchange chromatography (DEAE-Sepharose). This suggests, but does not prove, that there initially may be some structural association of these two lipid transfer proteins in plasma. However, LTP-I shows little, if any, affinity for heparin-Sepharose (see Fig. 4 and ref. 3), whereas under the conditions described in Methods for the isolation of LTP-II, virtually all of the LTP-II binds, and is eluted with good yield off heparin-Sepharose.

To establish the isoelectric point of LTP-II, active material eluted from the heparin-Sepharose column, free of any LTP-I lipid transfer activity, was run on a chromatofocusing column as described in Methods. A linear pH gradient from pH 7.0 to pH 4.0 was generated with Pharmacia polybuffer PBE 74. As shown in **Fig. 1A**, LTP-II elutes as a symmetrical peak, with the peak activity eluting in the region of pH 5.0

To establish the approximate molecular weight of LTP-II, pooled active material eluted from the heparin-Sepharose column was dialyzed and concentrated as described in Methods. The concentrated material was then applied in an ascending manner to a 1.5 × 100 cm column of Sephacryl-S200 (molecular sieve chromatography was not part of our purification scheme) previously calibrated with protein standards of known molecular weight. The peak activity eluted in a position just preceding human serum albumin, and we estimate the molecular weight of LTP-II, off Sephacryl-S200, to be approximately 70,000 ± 2,000 (n = 3),

TABLE 1. Purification of a phospholipid transfer protein (LTP-II) from human plasma

Fraction	Total Protein	Total Activity	Transfer Activity (units)[a]		% Recovery of Heat-Labile Activity	Fold-Purification
			Heat-Labile Activity	Specific Activity of Heat-Labile Activity[b]		
	mg					
d > 1.21 g/ml plasma	58,500	64,125	34,627	0.60	100	1
Phenyl-Sepharose	190	51,711	29,992	157	87	261
DEAE-Sepharose	55	42,000	23,520	428	68	713
Heparin-Sepharose	10.4	16,460[c]	16,460	1,583	48	2,638
CM-cellulose	3.3	9,500	9,500	2,878	27	4,800
Hydroxyapatite	0.4	3,500	3,500	8,750	10	14,583

Values are representative of six different isolations.
[a]One unit = 1% transfer/3 hr.
[b]Specific activity = total activity/protein mass.
[c]Total phospholipid transfer activity represents the fraction retained on heparin-Sepharose.

Downloaded from www.jlr.org by guest, on June 18, 2012

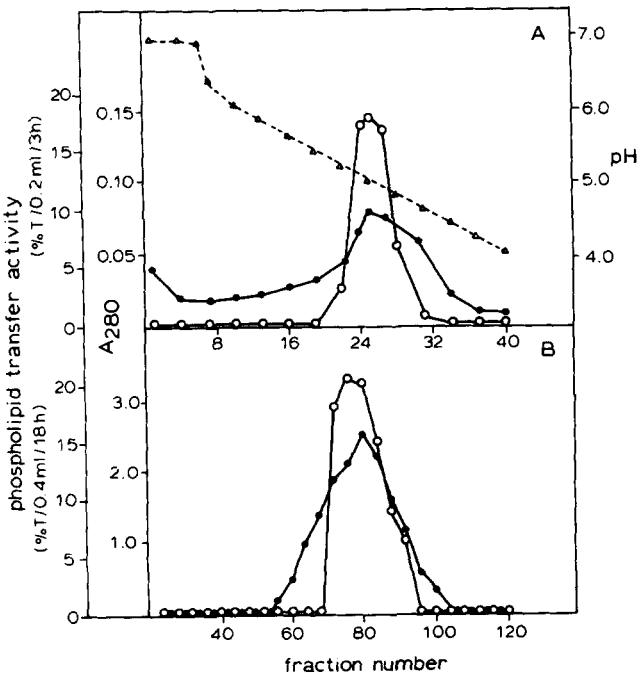


Fig. 1. Chromatofocusing of LTP-II. Partially purified LTP-II (heparin-Sepharose retained fraction) was dialyzed against 25 mM imidazole buffer, pH 7.4, and applied to a 1.5 × 30 cm column as described in Methods. The pH gradient was developed with Pharmacia PBE 74 polybuffer diluted 1:8, pH 7.4. Each fraction was assayed for pH (triangles), A_{280} (solid circles), and phospholipid transfer activity (open circles). B: Molecular-sieve chromatography of LTP-II. Active LTP-II, which was eluted from a heparin-Sepharose column, was concentrated to approximately 1.0 ml by vacuum, then applied in an ascending direction to a calibrated 1.5 × 100 cm column of Sephacryl-S200. Immediately prior to sample application, 5 mg of human serum albumin was added to the concentrated LTP-II sample to serve as an internal standard. Fractions were assayed for phospholipid transfer activity (open circles) and A_{280} (solid circles).

Fig. 1B. This agrees well with the apparent molecular weight of purified LTP-II (the post-hydroxyapatite material, **Fig. 2A**) obtained by SDS polyacrylamide gel electrophoresis: 69,000 ± 2,000 (n = 3), Fig. 2B. This suggests that LTP-II is a monomeric protein, as no larger molecular weight component with phospholipid transfer activity was detected by molecular sieve chromatography, nor were any smaller components observed after SDS polyacrylamide gel electrophoresis with reduction.

We next investigated whether or not partially purified LTP-II (through the heparin-Sepharose step, and devoid of cholesteryl ester or triglyceride transfer activity) could promote the net mass transfer of phospholipid between isolated VLDL and HDL_3. As **Fig. 3** indicates, an increasing amount of LTP-II added to an incubation of VLDL and HDL_3 (8:1 phospholipid mass ratio) resulted in a decrease of VLDL phospholipid mass, with a resultant increase of HDL_3 phospholipid mass.

We next examined the stability of partially purified LTP-II (through the heparin-Sepharose step, **Fig. 4**). Compared to LTP-I, which lost little cholesteryl ester transfer activity at 58°C for up to 60 min, LTP-II lost > 90% of its transfer activity after a 60-min incubation at 58°C, and there was no detectable transfer activity after 2 hr at 58°C with LTP-II. At 37°C for 2 days, LTP-II had lost approximately 90% of its transfer activity (approximately 50% was lost in 6 hr at 37°C). At room temperature, 22°C, it required approximately 4 days for LTP-II to lose 50% of its transfer activity. However, as shown in Fig. 4, LTP-II was very stable at both 4°C, and −70°C, losing only a few percent of its transfer activity after a 3-week storage period.

We next performed a series of experiments investigating the effects of a battery of chemical reagents on both LTP-I and LTP-II-mediated phospholipid transfer. The reagents were: *p*-chloromercuriphenylsulphate, 5–25 mM; ethylmercurithiosalicylate, 0.25–25 mM; dithio-bis-nitrobenzoic acid, 0.25–5 mM; and iodoacetic acid, 1.0–50 mM. Both LTP-I and LTP-II-mediated phospholipid transfer was similarly affected by each of the reagents at the indicated concentrations (no effect with *p*CMPS or iodoacetate, and a slight activation, approximately 25%, was observed with DTNB) except for ethylmercurithiosalicylate, which at 25 mM inhibited LTP-II by 50%, but was without effect on LTP-I.

Each of the isolated lipid transfer proteins (the heparin-Sepharose non-binding, LTP-I, and retained fractions, LTP-II) was tested with antibody developed to purified LTP-I as described in Methods. As shown in **Fig. 5**, all of the phospholipid transfer activity attributable to LTP-I was completely suppressed by the goat anti-human LTP-I antibody. However, none of the LTP-II-mediated phospholipid transfer activity was inhibited by the antibody. Furthermore, as shown in the inset of Fig. 5, all of the whole plasma neutral lipid transfer activity, cholesteryl ester and triglyceride, was totally suppressed by anti-LTP-I, whereas only one-half of the whole plasma phospholipid transfer activity was immunoprecipitable by anti-LTP-I. In addition, Western blotting of LTP-I and LTP-II with the goat anti-human LTP-I antibody revealed a major staining LTP-I band near 65 kD, with no staining material visable in the LTP-II lane, **Fig. 6**.

As shown in **Fig. 7**, incubation of LTP-II (free of residual LTP-I cholesteryl ester transfer activity) with a fixed quantity of the cholesteryl ester transfer protein (LTP-I) resulted in a marked enhancement of cholesteryl ester transfer during the course of a 2-hr incubation. The enhancement of cholesteryl ester transfer activity was most pronounced during the first hour of the incubation, and reached a maximum at 60 min, but little or no further enhancement of cholesteryl ester transfer occurred during the second hour.

DISCUSSION

This report extends our earlier observation (3) that there exists in human plasma a phospholipid transfer protein distinct from the phospholipid transfer activity expressed by

Downloaded from www.jlr.org by guest, on June 18, 2012

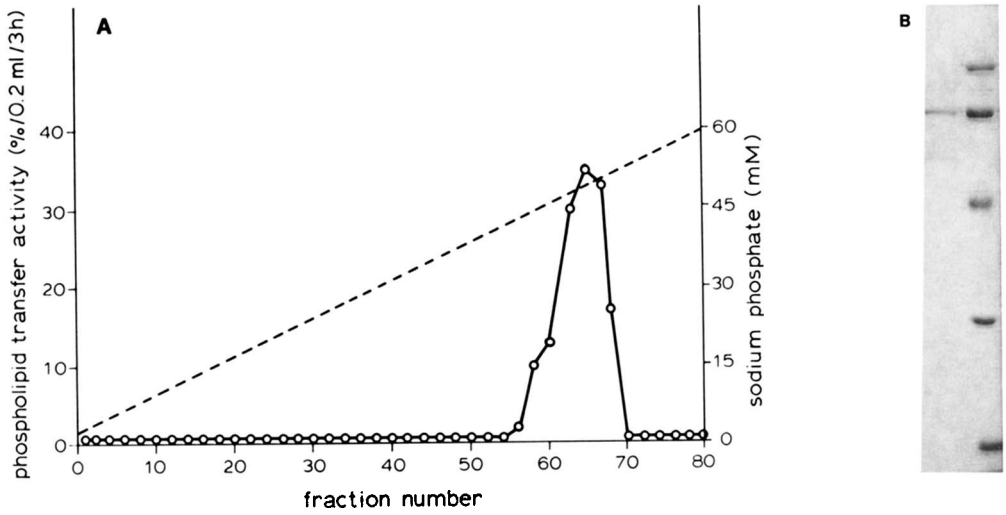
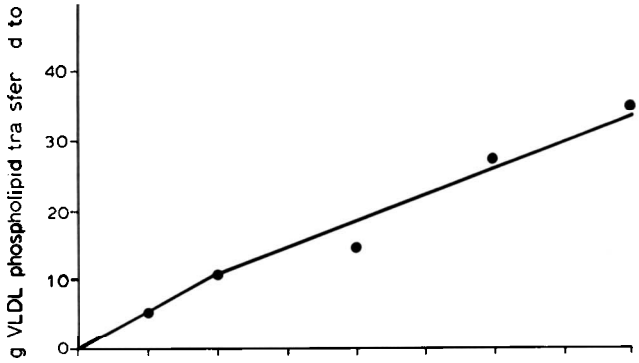


Fig. 2. A: Elution profile of LTP-II off hydroxyapatite. Approximately 800 μg of active LTP-II eluted from the carboxymethyl-cellulose column was immediately dialyzed against 0.15 M NaCl, 4 mM sodium phosphate, pH 6.9, buffer, then applied to a 0.9 × 8 cm column of hydroxyapatite equilibrated against the same buffer at a flow rate of approximately 5 ml/hr. The column was developed with a 200-ml linear gradient of 4–60 mM phosphate buffer, pH 6.9, in 0.15 M NaCl; 2.5-ml fractions were collected. Every other fraction was assayed in duplicate for phospholipid transfer activity (open circles). B: SDS polyacrylamide gel electrophoresis of purified LTP-II. Approximately 5 μg of the active post-hydroxyapatite LTP-II (from Fig. 2A) was applied to a 12% polyacrylamide gel with reduction as described by Laemmli (12), and stained and destained as described in Methods. The molecular weight markers are (from top to bottom): 92.5 kDa (phosphorylase b), 66.2 kDa (bovine serum albumin), 45 kDa (ovalbumin), 31.5 kDa (carbonic anhydrase), 21.5 kDa (soybean trypsin inhibitor), and 14.4 kDa (lysozyme).

the cholesteryl ester transfer protein first reported (16) and purified by Zilversmit's group (2, 17) and subsequently by others (18, 19). There has recently been a number of reports examining the role of facilitated phospholipid transfer in human plasma (5, 20, 21). Each of these studies warrants examination in order to clarify possible confusion as to the protein(s) responsible for the observed facilitated phospholipid transfer, and to note the observed similarities and differences in the protein species studied and, in particular, the type of assay used to monitor facilitated phospholipid transfer.

Several investigators (5, 20, 21) have used a pure phospholipid liposome donor:high density lipoprotein (HDL) acceptor assay to monitor the facilitated transfer by factors isolated from plasma. Tall, Abreu, and Shuman (5) were able to identify two different types of phospholipid transfer promoted by specific protein factors isolated from human plasma, depending upon the type of assay used, either the above-mentioned phospholipid vesicle donor:HDL acceptor assay, or a more physiological lipoprotein (LDL) donor:lipoprotein acceptor (HDL) assay. The phospholipid transfer promoted by the "cholesteryl ester transfer protein" (LTP-I) did not promote any phospholipid transfer in the PC-vesicle donor:HDL acceptor assay, nor did the distinct 41,000 dalton PC transfer protein reported by Tall et. al. (5) promote the transfer of phospholipid between lipoproteins. Although we have not used the PC-vesicle donor:HDL acceptor assay, we have clearly demonstrated that there are at least two proteins in human plasma that facilitate phos-



Downloaded from www.jlr.org by guest, on June 18, 2012

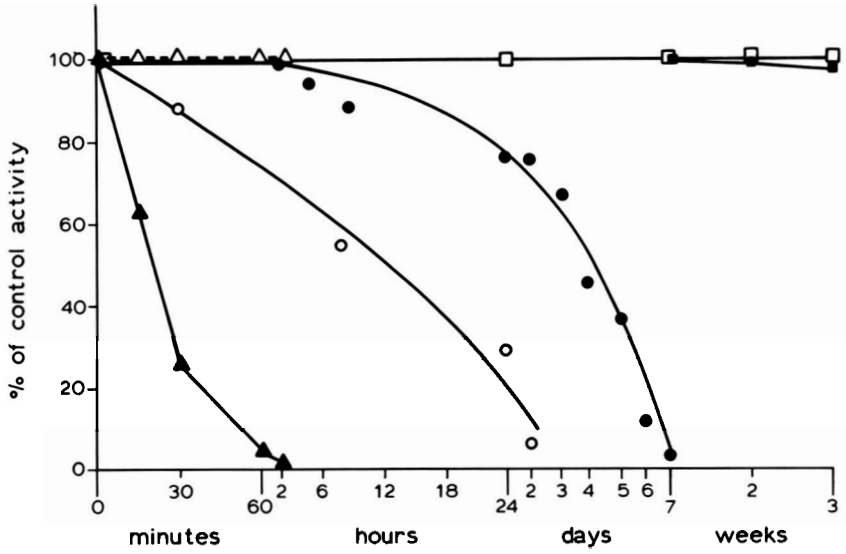


Fig. 4. Temperature/stability profile of LTP-II. Aliquots of LTP-II (purified through the heparin-Sepharose step, Table 1) were stored in Tris-saline buffer, pH 7.4, at the following temperatures: – 70°C (open square), + 4°C (solid square), + 20°C (solid circle), + 37°C (open circle), and + 58°C (solid triangle) for the indicated times. For comparison, aliquots of the cholesteryl ester transfer protein, LTP-I, were also incubated at + 58°C. The samples were assayed in triplicate using the same lot of substrates and each assay included an internal standard which did not vary by more than 10% between assays. Activity (approximately 25% transfer/200 μl per 3-hr incubation) is expressed as % of the original (time zero), + 4°C, LTP-II starting activity.

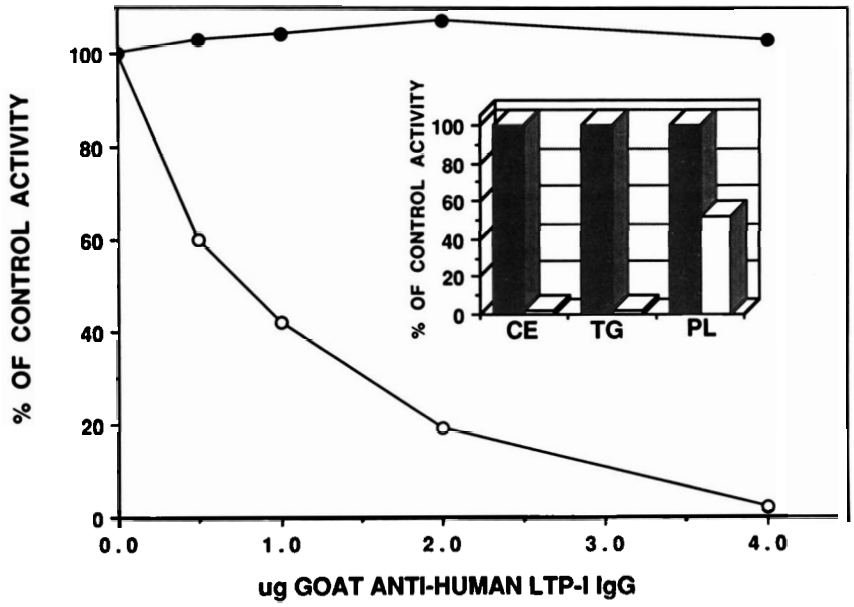


Fig. 5. Immunoprecipitation of LTP-I and LTP-II. Triplicate aliquots of partially purified (heparin-Sepharose nonbinding) LTP-I (open circles) and partially purified (heparin-Sepharose-retained) LTP-II (solid circles) were incubated with increasing amounts of partially purified goat anti-human LTP-I antibody fraction (see Methods). Each mixture was incubated for 30 min at + 37°C to facilitate antibody-antigen complex formation, then overnight at + 4°C. Immune complexes were then removed by low speed centrifugation (3000 rpm for 30 min). Each supernate was then assayed for facilitated phospholipid transfer activity. The results are expressed as % of the starting activity remaining. The absolute phospholipid transfer activities were: LTP-I, 35% transfer/200 μl per 3-hr incubation; LTP-II, 25% transfer/200 μl per 3-hr incubation. Occasionally we observed an increase (always < 10%) in LTP-II PC transfer activity upon incubation with the goat anti-human LTP-I; there was no effect of nonimmune goat serum processed exactly as the immune serum on either transfer protein (data not shown). Inset: Immunoprecipitation of whole plasma CE, TG, and PC lipid transfer activity with goat anti-human LTP-I. Aliquots (0.5 ml) of freshly isolated whole plasma from a normolipidemic donor were incubated with 1.0 ml of either partially purified goat anti-human LTP-I or preimmune control goat serum as described above. Triplicate aliquots (50 μl) were taken for the assay of cholesteryl ester, triglyceride, and phospholipid transfer activity. The control goat serum was without effect on any of the assays. Each activity is expressed as a % of the lipid transfer activity observed after incubation with the control goat serum, which is expressed as 100%.

Downloaded from www.jlr.org by guest, on June 18, 2012

Fig. 6. Western blotting of LTP-I and LTP-II. Aliquots of partially purified LTP-I (DEAE pool, approximately 1000-fold, ref. 3) and purified LTP-II (hydroxyapatite pool, Table 1) were electrophoresed (12) and transferred to nitrocellulose sheets as described (13), then reacted with goat anti-human LTP-I antibody that had been affinity-purified by passage over an affinity column constructed of LTP-I (CM-pool, ref. 3) coupled to CNBr-Sepharose as per the manufacturer's instructions. Left lane: LTP-I, middle lane: human serum albumin, right lane: LTP-II.

the ability of their PC transfer protein to transfer phospholipids between the plasma lipoproteins.

In another study of facilitated phospholipid transfer in human plasma, Sweeny and Jonas (21) demonstrated, using phospholipid vesicles of varying sizes as PC donors, that an increase in vesicle size and a decrease in bilayer curvature reduced the observed facilitated phospholipid transfer activity. As determined by SDS-polyacrylamide gel electrophoresis, their purified PC transfer factor which displayed the highest specific activity revealed two major bands with the approximate molecular weights of 58,000 ± 3,000 and 38,000. This fraction showed very little affinity or ability to transfer cholesteryl esters between LDL and HDL.

The most purified preparation in the present study displayed the following characteristics (**Table 2**). i) It was heat-labile (Fig. 4); ii) it had an apparent molecular weight of 69,000 ± 3,000 as revealed by SDS-polyacrylamide gel electrophoresis (n = 3), Fig. 2B, and approximately 70,000 ± 3,000 for the functional active component as determined by molecular sieve chromatography, Fig. 1B. This fraction displayed no affinity for cholesteryl ester or triglyceride transfer, and was not recognized by antibody to the cholesteryl ester transfer protein (LTP-I), as iii) no functional LTP-II phospholipid transfer activity was inhibited by anti-LTP-I (Fig. 5) nor iv) was any LTP-II recognized by anti-LTP-I by Western blotting (Fig. 6). Furthermore, the cDNA of the cholesteryl ester transfer protein (CETP) has been cloned and sequenced (22), and the cDNA sequence of CETP agreed with 13 of 17 residues of purified LTP-I protein from our laboratory; thus CETP is identical to LTP-I. In addition, we have obtained preliminary amino acid sequence of LTP-II, and only 2 of 18 residues thus far obtained agree with the published sequence of the cholesteryl ester transfer protein, LTP-I. This further substantiates our immunoinhibition and Western blotting experiments shown in Figs. 5 and 6.

It is possible that these two lipid transfer proteins, LTP-I and LTP-II, are not part of the same macromolecular complex in unfractionated whole plasma, inasmuch as immunoprecipitation of LTP-I from whole plasma removed the whole of cholesteryl ester and triglyceride transfer activity, but only approximately one-half of the observed whole plasma phospholipid transfer activity, and approximately 50% of the phospholipid transfer activity recovered in d > 1.21 g/ml plasma fraction is heat-labile (Table 1). These observations, coupled with the immunoinhibition and Western blotting data (Figs. 5 and 6), strongly support the concept that human plasma contains at least two phospholipid transfer proteins, LTP-II and LTP-I.

Intraplasmic equilibration of the different molecular species of phospholipids may be an important physiological process, and may be mediated by the phospholipid transfer proteins present in human plasma, since Massey et al. (23) have demonstrated that spontaneous or nonfacilitated

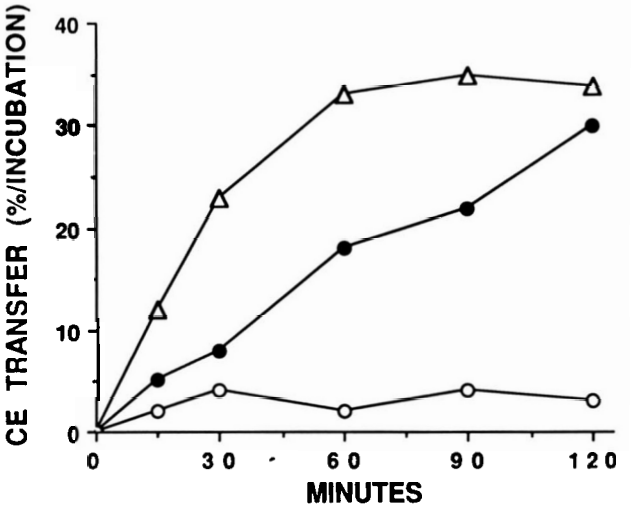


Fig. 7. Enhancement of cholesteryl ester transfer by LTP-II. Aliquots of partially purified (heparin-Sepharose, nonbinding, and heparin-Sepharose, retained) LTP-I and LTP-II were incubated separately (solid circle, LTP-I; open circle, LTP-II) or in combination (open triangle) with [^{14}C]cholesteryl ester-HDL$_3$ donor, and d < 1.006 g/ml lipoprotein (VLDL) acceptor as described. Aliquots were taken at 0, 15, 30, 60, 90, and 120 min and processed as described. Activity is expressed as the % of the [^{14}C]cholesteryl ester-HDL$_3$ label transferred to VLDL, per incubation.

Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 2. Comparison of LTP-I and LTP-II

Property	LTP-I	LTP-II
Molecular weight ($\times 10^{-3}$)		
Gel filtration	64	70
SDS-gel electrophoresis	65	69
Isoelectric point	4.8	5.0
Heat-stable	yes	no
Binds heparin	no	yes
Ppt by anti-LTP-I	yes	no
Transfers CE, TG	yes	no
Transfers PC	yes	yes

transfer of naturally occurring phospholipids between plasma lipoproteins proceeds too slowly to allow for the equilibration of molecular species of phospholipids during their residence time in plasma.

The observed enhancement of LTP-I-mediated cholesteryl ester transfer by LTP-II (Fig. 7) is of particular importance when considering the potential role of the cholesteryl ester transfer protein in lipid metabolism in vivo. It has been proposed (3, 24, 25) that the cholesteryl ester transfer protein may relieve or prevent cholesteryl ester accumulation on the high density lipoproteins, the site of cholesterol esterification by LCAT. Thus, by maintaining the LCAT esterification rate and cholesteryl ester transfer, these reactions create a chemical gradient favoring cholesterol transfer from cells to lipoproteins. It has been shown in vitro that increased delivery of phospholipid to an LCAT substrate enhances its observed activity (26); thus at times, such as after a meal, there will be an increased influx of phospholipid into the plasma compartment, primarily associated with the increase in the lipid-rich plasma chylomicrons. LTP-II has been shown to promote the net mass transfer of apoB lipoprotein phospholipid (VLDL, Fig. 3) to HDL. This in turn may stimulate LCAT such that cholesterol esterification is increased when there is both increased number and mass of cholesteryl ester acceptors (chylomicrons and chylomicron remnants) to accommodate the increased cholesteryl ester synthesized and transferred. Thus, although LTP-II may not directly facilitate the transfer of cholesteryl esters, it may play a very important role in postprandial lipid metabolism by i) directly facilitating phospholipid transfer from apoB lipoproteins towards HDL (Fig. 3); ii) indirectly enhancing cholesteryl ester transfer between the plasma lipoproteins (Fig. 7); and iii) enhancing plasma LCAT esterification by a) providing phospholipid substrate to the site of the reaction (HDL) and b) preventing accumulation of product cholesteryl esters by stimulating LTP-I-mediated cholesteryl ester transfer (Fig. 7).

Although we have established an approximate molecular weight for LTP-II of 68,000–70,000 by two different methodologies, SDS-polyacrylamide gel electrophoresis (Fig. 2B) and molecular sieve chromatography (Fig. 1B), a more sensitive and definitive approach will come from Western blotting of LTP-II with monospecific antibody to purified LTP-II, and from the cloning and sequencing of the LTP-II cDNA. These experiments are currently in progress in our laboratory.

The authors gratefully acknowledge the excellent technical contributions of Kyle D. Kern. This work was supported by NIH Program Project Grant HL-30068.

Manuscript received 30 October 1987 and in revised form 11 July 1988.

REFERENCES

1. Skipski, V. P. 1972. Lipid composition of lipoproteins in normal and diseased states. *In* Blood Lipids and Lipoproteins. G. C. Nelson, editor. Wiley Interscience, New York. 471–583.
2. Morton, R. E., and D. B. Zilversmit. 1982. Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* **23:** 1058–1067.
3. Albers, J. J., J. H. Tollefson, C. H. Chen, and A. Steinmetz. 1984. Isolation and characterization of human plasma lipid transfer proteins. *Arteriosclerosis.* **4:** 49–58.
4. Tollefson, J. H., and J. J. Albers. 1986. Isolation, characterization, and assay of plasma lipid transfer proteins. *Methods Enzymol.* **129:** 797–816.
5. Tall, A. R., E. Abreu, and J. Shuman. 1983. Separation of a plasma phospholipid transfer protein from cholesterol ester/phospholipid exchange protein. *J. Biol. Chem.* **258:** 2174–2180.
6. Tollefson, J. H., and J. J. Albers. 1986. Isolation and characterization of a human plasma phospholipid transfer protein. *Arteriosclerosis.* **6:** 520a (abstract).
7. Bachorik, P. S., and J. J. Albers. 1986. Precipitation methods for quantification of lipoproteins. *Methods Enzymol.* **129:** 78–100.
8. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
9. Morton, R. E., and D. B. Zilversmit. 1981. A plasma inhibitor of triglyceride and cholesteryl ester transfer activities. *J. Biol. Chem.* **256:** 11992–11995.
10. Tollefson, J. H., and J. J. Albers. 1985. Identification of a human plasma protein that inhibits LCAT and human plasma lipid transfer proteins. *Arteriosclerosis.* **5:** 534a (abstract).
11. Son, Y. S. C., and D. B. Zilversmit. 1984. Purification and characterization of human plasma proteins that inhibit lipid transfer activities. *Biochim. Biophys. Acta.* **795:** 473–480.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
13. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
15. Chen, C. H., and J. J. Albers. 1981. Further characterization of purified lecithin:cholesterol acyltransferase from human plasma. *Biochem. Med.* **25:** 215–226.

Downloaded from www.jlr.org by guest, on June 18, 2012

16. Zilversmit, D. B., L. B. Hughes, and J. Balmer. 1975. Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma. *Biochim. Biophys. Acta.* **409:** 393–398.
17. Pattnaik, N. M., A. Montes, L. B. Hughes, and D. B. Zilversmit. 1978. Cholesteryl ester exchange protein in human plasma isolation and characterization. *Biochim. Biophys. Acta.* **530:** 428–438.
18. Ihm, J., J. L. Ellsworth, B. Chataing, and J. A. K. Harmony. 1982. Plasma protein facilitated exchange of phosphatidylcholine and cholesteryl ester in the absence of cholesterol esterification. *J. Biol. Chem.* **257:** 4818–4827.
19. Hesler, C. B., T. L. Swenson, and A. R. Tall. 1987. Purification of human plasma cholesteryl ester transfer protein by binding to synthetic lipid emulsion. *J. Biol. Chem.* **262:** 2275–2282.
20. Damen, J., J. Regts, and G. Scherphof. 1982. Transfer of [^{14}C]phosphatidylcholine between liposomes and human plasma high density lipoprotein. Partial purification of a transfer-stimulating plasma factor using a rapid transfer assay. *Biochim. Biophys. Acta.* **712:** 444–452.
21. Sweeny, S. A., and A. Jonas. 1985. Substrate specificity of human plasma phospholipid transfer protein. *Biochim. Biophys. Acta.* **835:** 279–290.
22. Drayna, D., A. Jarnagin, J. McLean, W. Henzel, W. Kohr, C. Fielding, and R. Lawn. 1987. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature.* **327:** 632–634.
23. Massey, J. B., D. Hickson, H. S. She, J. T. Sparrow, D. P. Via, A. M. Gotto, and H. J. Pownall. 1984. Measurement and prediction of the rates of spontaneous transfer of phospholipids between plasma lipoproteins. *Biochim. Biophys. Acta.* **794:** 274–280.
24. Fielding, C. J. 1987. Factors affecting the rate of catalyzed transfer of cholesteryl esters in plasma. *Am. Heart J.* **113:** 532–537.
25. Chajek, T., L. Aron, and C. J. Fielding. 1980. Interaction of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in the transport of cholesteryl ester into sphingomyelin liposomes. *Biochemistry.* **19:** 3673–3677.
26. Nichols, A. V., and E. L. Gong. 1971. Use of sonicated dispersions of mixtures of cholesterol with lecithin as substrates of lecithin:cholesterol acyltransferase. *Biochim. Biophys. Acta.* **231:** 175–184.

Downloaded from www.jlr.org by guest, on June 18, 2012