Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma

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Abstract Lipid transfer activities from human plasma have been characterized to determine whether triglyceride and cholesteryl ester transfer proteins are identical. After sequential purification by phenyl-Sepharose, CM-cellulose, chromatofocusing, and gel filtration, both triglyceride and cholesteryl ester transfer activities were purified ~15,000-fold compared to lipoprotein-deficient plasma, with a 14% recovery of both transfer activities. The gel filtration fraction showed two bands, M_r 58,300 and 66,400, as determined by electrophoresis in sodium dodecyl sulfate. Two samples, each containing predominately one of the two bands, were obtained by selectively combining the eluates from the gel filtration column. The specific activities of triglyceride and cholesteryl ester transfer promoted by the larger protein were within 10% of those for the smaller protein. The relative rates of transfer for cholesteryl ester, triglyceride, retinyl ester, and cholesteryl ether for each fraction were the same. The transfer of triglyceride by either the large or small molecular weight component was almost completely inhibited by mercurial compounds, whereas cholesteryl ester transfer was relatively unaffected. We conclude that triglyceride and cholesteryl ester are transferred by the same plasma protein(s).—**Morton, R. E., and D. B. Zilversmit.** Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* 1982. **23:** 1058–1067.

Supplementary key words cholesteryl ester • triglyceride • substrate specificity • isoelectric points • sulfhydryl reagents • low density lipoprotein • high density lipoprotein

Phospholipid (1–2) and free cholesterol (3), which occupy the surface or coat of plasma lipoproteins, are readily exchangeable or transferable between isolated lipoproteins. In contrast, the lipids comprising the core of these lipoproteins, predominately cholesteryl ester and triglyceride, do not freely exchange or transfer. The transfer of these core components is accomplished, however, by the addition of lipoprotein-deficient plasma to isolated donor and acceptor lipoproteins. Zilversmit, Hughes, and Balmer (4) demonstrated that an acidic protein in lipoprotein-free plasma from hypercholesterolemic rabbits stimulated the transfer of cholesteryl ester between very low density and low density lipoproteins. A similar cholesteryl ester transfer protein was subsequently identified in human lipoprotein-deficient plasma (5), and was described as a large molecular weight, acidic glycoprotein. Studies with the plasmas of other species, such as rat (6, 7) and guinea pig (7), have demonstrated only very low or no detectable cholesteryl ester transfer activity.

Rajaram, White, and Barter (8) established that the transfer of the other major core lipid, triglyceride, is stimulated by a large molecular weight, basic glycoprotein isolated from rabbit lipoprotein-deficient plasma. Triglyceride transfer activity has been observed in human lipoprotein-deficient plasma (9, 10), and, like cholesteryl ester transfer activity, is essentially absent from rat plasma (9).

The function of these transfer activities is not completely understood, although the cholesteryl ester transfer protein may exist in human plasma as a macromolecular complex with LCAT (11, 12) and may play an important role in modulating this enzyme's activity (13). A more complete understanding of lipid transfer activities and their relationship to lipoprotein metabolism will require different interpretations of data or even different approaches if cholesteryl ester and triglyceride transfers are coupled as the function of a single protein or if these transfers are the result of different proteins that could independently affect lipoprotein metabolism.

Current data are ambiguous regarding the identity of the plasma triglyceride and cholesteryl ester transfer proteins. A single plasma transfer protein is suggested by the species distribution of cholesteryl ester and triglyceride transfer activities (7, 9), by the identical inactivation profiles of these activities in human plasma upon proteolytic digestion with trypsin and chymotrypsin, or upon thermal denaturation (14), and by their

Abbreviations: BHT, butylated hydroxytoluene; LCAT, lecithin:cholesterol acyltransferase; EDTA, disodium ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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similar kinetic properties (15). Conversely, the existence of separate triglyceride and cholesteryl ester transfer proteins is supported by evidence showing significantly different physical parameters for the transfer proteins partially purified from rabbit plasma (5, 8), and data demonstrating that triglyceride transfer activity is almost completely inhibited by *p*-chloromercuriphenyl sulfonate whereas cholesteryl ester transfer activity is only slightly diminished (8, 16).

In the present study, we have prepared highly purified lipid transfer protein and assessed its ability to transfer various lipids. Additionally, we have attempted to resolve the apparent discrepancy in the literature concerning the identity of triglyceride and cholesteryl ester transfer proteins.

EXPERIMENTAL PROCEDURE

Materials

Glycerol tri[9,10(n)-^{3}H]oleate (470 mCi/mmol), [4-^{14}C]cholesterol (57.8 mCi/mmol), and [1α,2α(n)-^{3}H]cholesterol were purchased from Amersham Corp. (Arlington Heights, IL). [^{14}C]Cholesteryl oleate was synthesized from [4-^{14}C]cholesterol and oleoyl chloride (Nu-Chek Prep, Inc., Elysian, MN) as described by Pinter, Hamilton, and Muldrey (17). [11,12-^{3}H]Retinyl oleate was prepared from [^{3}H]retinyl acetate (a gift from Hoffmann-LaRoche) and oleoyl chloride according to the procedure of Huang and Goodman (18). The retinyl ester was stored at 4°C in the dark in absolute ethanol containing 50 mg BHT/l. [^{32}P]Phosphatidylcholine was isolated from rat liver and purified as described by Crain and Zilversmit (19). [^{3}H]Hexadecyl cholesteryl ether was a gift from Dr. R. Lumb (Western Carolina University).

The purity of the [^{3}H]retinyl oleate was determined to be >96% by high pressure liquid chromatography on an Ultrasphere ODS column (Altex Scientific, Inc., Berkeley, CA). All other labeled lipids, with an initial radiochemical purity of <98%, were purified by thin-layer chromatography on pre-coated SG 60 plates (E. Merck, Darmstadt, Germany) in a developing system of hexane–diethyl ether 80:20 and were eluted with chloroform.

Unlabeled oleyl cholesteryl ether was synthesized by adding 20 mg of cholesterol, 200 μl of oleylmethane sulfonate (Nu-Chek), and several drops of a KH suspension (Aldrich Chemical Co., Milwaukee, WI) to 0.5 ml of dry benzene. After the mixture was heated overnight at 75°C, the cholesteryl ether was partitioned into hexane following the procedure of Thompson et al. (20). The hexane phase was applied to an 8-g column of partially inactivated (5% water) neutral aluminum oxide (Woelm Pharma, ICN-Nutritional Biochem., Cleveland, OH) in hexane. The cholesteryl ether was eluted with hexane–benzene 9:1. Analysis, and additional purification if necessary, was carried out by thin-layer chromatography on pre-coated silica gel plates with hexane as the developing solvent.

Bovine serum albumin (fraction V), dithiothreitol, *p*-chloromercuriphenyl sulfonate, *p*-hydroxymercuribenzoate, 5,5′-dithiobis-(2-nitrobenzoate), cholesteryl oleate, and trioleoyl glycerol were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium ethyl mercurithiosalicylate and BHT were purchased from Nutritional Biochemical Co. (Cleveland, OH); egg phosphatidylcholine was purchased from Lipid Products (South Nutfield, England); dextran sulfate, IEF-Sephadex, phenyl-Sepharose CL-4B, and protein molecular weight standards were obtained from Pharmacia Fine Chemicals (Piscataway, NJ); and diethyl *p*-nitrophenyl phosphate was purchased from Aldrich. Cholesterol was purified as the dibromide derivative and crystallized from methanol.

METHODS

Purification of lipid transfer protein

Through all procedures, the recovery and fold-purification of both triglyceride and cholesteryl ester transfer activities were monitored. These activities are collectively referred to as lipid transfer activity. Citrated human plasma (24–48-hr old) was obtained from the Red Cross blood bank and kept at 4°C. Lipoprotein-deficient plasma was prepared by a modification (21) of the dextran sulfate-Mn^{2+} precipitation procedure of Burstein, Scholnick, and Morfin (22). Lipoprotein-deficient plasma was adjusted to 4 M NaCl, applied to phenyl-Sepharose CL-4B (23). The column was washed with 50 mM Tris–HCl, 150 mM NaCl, 0.02% NaN_3, pH 7.4 (Tris/NaCl buffer) until the eluant absorbance at 280 nm was 0.3. More extensive washing of the column significantly lowered the recovery of transfer activity. After elution of lipid transfer activity with H_2O, phenyl-Sepharose was recycled for subsequent use by extensive washing with 95% ethanol before re-equilibration with 4 M NaCl. Lipid transfer fractions from the phenyl-Sepharose column were pooled and subsequently applied to a CM-cellulose column (CM-52, Whatman) and eluted with 150 mM NaCl at pH 4.5 (23). Active fractions were pooled and dialyzed against Tris/NaCl buffer. Immediate dialysis to the higher pH was essential in order to stabilize lipid transfer activity. Dialyzed fractions (0.5–1.0 mg of protein/ml) retained >90% of their lipid transfer activity upon storage for 1 month at 4°C.

Lipid transfer activity was further purified by chromatofocusing. A column (1 × 19 cm) of PBE-94 chromatofocusing gel (Pharmacia) was packed and equilibrated at room temperature in 25 mM histidine base titrated to pH 5.7 with HCl. An aliquot of the CM-cellulose pool of lipid transfer activity, 7–10 mg of protein (15–20 ml), was dialyzed at room temperature against 25 mM Tris–HCl, pH 7.4, and applied to the equilibrated chromatofocusing column. After sample application, a 5-ml cushion of 25 mM histidine-HCl buffer was applied prior to starting the developing buffer. The pH gradient of the column was developed with 25 ml of Polybuffer 74 (Pharmacia) diluted to 200 ml and adjusted to pH 3.5 with HCl. The elution rate was 30 ml/hr.

Pooled fractions from the chromatofocusing column were adjusted to 150 mM NaCl with the solid salt. Three hundred–400 μg of protein (15–20 ml) of the adjusted pool was applied to a Bio-Gel A-0.5 m (100–200 mesh) column (2.5 × 89 cm) equilibrated in Tris/NaCl buffer. The sample was eluted at a linear flow rate of 7.4 cm/hr. Lipid transfer activity tends to adsorb strongly to freshly prepared Bio-Gel columns. Pretreatment of the columns with other proteins, such as calibration standards, greatly improved recoveries.

Unlike the dialyzed CM-cellulose fractions, pooled fractions from both the chromatofocusing and the Bio-Gel columns lost >60% of their lipid transfer activity within 2 days. Thus, to maximize recovery of activity, chromatofocusing and gel filtration had to be carried out in rapid succession. When it was compatible with subsequent analyses, the lipid transfer activity in the pooled fractions from the Bio-Gel column could be stabilized (>65% of the transfer activity remained after 2 weeks) by adding 0.5% bovine serum albumin.

All procedures, except the chromatofocusing step, were performed at 4°C.

Substrate preparation

Lipoproteins. Lipoproteins labeled with [^{3}H]triglyceride and [^{14}C]cholesteryl ester were prepared by the lipid dispersion procedure described by Morton and Zilversmit (23). Briefly, fresh human plasma was incubated at 37°C with a sonicated lipid dispersion of egg phosphatidylcholine containing [^{3}H]triglyceride and [^{14}C]cholesteryl ester (a total of 20 mol% with respect to phosphatidylcholine) in the presence of diethyl *p*-nitrophenyl phosphate for 16–18 hr at 37°C. Lipoproteins were isolated from the incubation mixture and from control plasma by sequential ultracentrifugation at the appropriate solvent densities in a 60 Ti rotor (Beckman Instruments Inc., Palo Alto, CA) at 4°C (24). Centrifugations were 18 hr for very low density lipoprotein and low density lipoprotein (2.76×10^8 g × min) and 26 hr for high density lipoprotein (3.99×10^8 g × min). Isolated lipoproteins were dialyzed at 4°C against 0.9% NaCl, 0.01% EDTA, and 0.02% NaN_3, then passed through a 0.45 μm Millex-HA filter (Millipore Corp., Bedford, MA). Lipoproteins were stored at 4°C in the presence of 0.5% bovine serum albumin.

Lipoproteins were also labeled with [^{32}P]-phosphatidylcholine or [^{3}H]cholesterol by a similar approach. For [^{32}P]phosphatidylcholine labeling, lipid dispersions were prepared as described above, except that rat [^{32}P]phosphatidylcholine was substituted for egg phosphatidylcholine. Radiolabeled free cholesterol was incorporated by substituting a trace quantity of [^{3}H]cholesterol for the [^{3}H]triglyceride used in the previous dispersions.

Liposomes. Phosphatidylcholine-cholesterol liposomes were prepared by a modification of the cholate procedure described by Brunner, Skrabal, and Hauser (25). Egg phosphatidylcholine (20 μmol), cholesterol (5 μmol), and 40 nmol of either [^{14}C]cholesteryl ester (5×10^5 dpm), [^{3}H]triglyceride (7×10^5 dpm), [^{3}H]retinyl ester (11×10^5 dpm), or [^{3}H]cholesteryl ether (2×10^5 dpm), were added to 0.2 ml of 227 μM BHT in $CHCl_3$, 0.5 ml of methanol, and 80 μl of 1 M sodium cholate. The solvent was removed under a stream of N_2. Residual solvent was removed under reduced pressure on a rotary evaporator for 10 min. Two ml of 10 mM Tris–HCl, 100 mM NaCl, 0.02% NaN_3, pH 7.4, were added, and the lipids were dispersed by vortex mixing. The cholate-lipid solution was then dialyzed against the same buffer (three changes of 2 liters each, the first two changes for 2 hr and the final change overnight). The resulting liposomes were centrifuged for 30 min at 35,900 *g* to remove multilamellar vesicles, and were then used immediately.

Lipid transfer assay

Lipoprotein donor. The transfer of triglyceride and cholesteryl ester was measured as previously described (5, 21) except that human low density lipoprotein and high density lipoprotein (10 μg of total cholesterol in each lipoprotein) were used as donor and acceptor particles, respectively. This amount of low density lipoprotein typically contained 1×10^4 dpm of [^{3}H]triglyceride and 3.4×10^3 dpm of [^{14}C]cholesteryl ester. Samples were incubated at 37°C, in a nonshaking water bath, for 1.5–4.5 hr. Assays were terminated by the precipitation of low density lipoprotein (21). An aliquot of the supernatant (0.5 ml) was added to 5 ml of ACS scintillation mixture (Amersham) and the radioactivity was measured in a Beckman LS 8100 counter. Lipid transfer was calculated as described by Pattnaik et al. (5) and

expressed as 100 times kt, where k is the fraction of lipid transferred per unit of time and t is the assay time.

Low density lipoproteins labeled with [^{32}P]-phosphatidylcholine and [^{14}C]cholesteryl ester or [^{3}H]cholesterol and [^{14}C]cholesteryl ester were also utilized for lipid transfer studies. These assays were usually the same as those described above. However, it was occasionally necessary to use higher lipoprotein concentrations due to the decay of ^{32}P. In these instances, both donor and acceptor lipoproteins were increased by the same amount. Up to 100 μg total cholesterol of each lipoprotein have been used without significantly decreasing the precipitation of low density lipoprotein at termination of the assay (normally >97%).

Liposome donor. The transfer of various lipids was also studied with phosphatidylcholine-cholesterol liposomes and human low density lipoprotein as donor and acceptor, respectively. The assay of lipid transfer from liposomes (500 nmol phosphatidylcholine) to low density lipoprotein (10 μg of total cholesterol) was the same as that described above. Lipid transfer was terminated by the precipitation of low density lipoprotein with concanavalin A-Sepharose (Pharmacia). Concanavalin A-Sepharose was diluted with water and 300 μl (150 μl settled gel volume) was added to each assay. After the samples were mixed for 1 hr at room temperature and centrifuged, an aliquot of the supernatant was counted as before. More than 98% of the low density lipoprotein was precipitated by this technique, and the recovery of liposomes in the supernatant was nearly quantitative. The percentage of lipid transfer was calculated as 100 minus the percentage of total label remaining in the supernatant.

Isoelectric focusing

Isoelectric focusing was performed in thin-layer granular gels by a modification of the procedure of Radola (26). IEF-Sephadex (4 g) was added to 58 ml of a solution containing 1.45 ml Ampholine 3.5–10 (LKB Instruments Inc., Rockville, MD) and 0.725 ml of Ampholine 9–11, either with or without 13.93 g urea. The slurry was allowed to stand overnight at room temperature and was then spread on a glass plate to yield a 230 × 230 × 1 mm gel bed. Samples were dialyzed as indicated in the figure legends and applied to the gel. Anode and cathode wicks, containing 1 M H_3PO_4 and NaOH, respectively, were positioned to give an electrode distance of 21 cm. The sample was focused at 8 watts for 12–15 hr at 8–10°C. After focusing, the gel was divided into 21 1-cm sections and each section was eluted with two 3-ml portions of water. Eluates were cooled to the running temperature before measuring the pH. Fractions were dialyzed against Tris/NaCl buffer before assay.

SDS electrophoresis

One-mm thick slabs of 5% polyacrylamide (27) were prepared on glass plates (115 × 230 mm) pretreated with Silane A-174 (Pharmacia). Samples were heated for 5 min at 100°C under reducing conditions (27) and 70-μl aliquots were applied to wells cut into the slab. Electrophoresis was performed at 40 ma for 10 hr (electrode distance of 8 cm). Proteins were fixed in 50% methanol and stained with ammoniacal silver nitrate (28).

Other analyses

Protein was quantitated by the dye binding assay procedure described by Sedmak and Grossberg (29) as modified by Crain and Zilversmit (30) with bovine serum albumin as standard. This procedure was additionally modified in order to measure the very low protein concentration (1–3 μg/ml) of the highly purified lipid transfer protein. Ten–20 ml of sample was dialyzed against 2 l of 25 mM Tris–HCl, pH 7.4 (two changes, 1 hr each). Aliquots of the dialyzed samples and protein standards were adjusted to a final volume of 10 ml with 25 mM Tris–HCl. A slurry containing 0.1 ml settled volume of chromatofocusing gel (PBE-94, Pharmacia) in 25 mM Tris–HCl was added, and the sample was mixed for 30 min. Low speed centrifugation pelleted the protein-gel complex. For purified lipid transfer protein fraction, >96% of the lipid transfer activity was removed from the solution after treatment with the PBE-94 gel. The supernatant was partially removed to yield a final volume of 1.5 ml. Standard Coomassie Blue dye solution (1.5 ml) was added (30) and the sample was mixed for 5 min. The samples were immediately centrifuged to pellet the gel and the supernatant was removed. After 15 min, the A_{600} of the supernatant was determined. To minimize variations caused by the binding of dye to the gel matrix, it was essential that samples were processed rapidly after addition of the dye. A linear response was observed with up to 30 μg of protein. For nine different lipid transfer fractions from CM-cellulose and chromatofocusing, the protein concentrations determined by this method were within 10% of those determined by the standard assay.

Lipids were extracted from lipoproteins by the method of Thompson et al. (20). When quantitative recovery of phospholipid was required, lipid was extracted by the procedure of Folch, Lees, and Sloane Stanley (31). Cholesterol was determined by the ferric chloride procedure (32) after saponification according to Abell et al. (33). Triglyceride was measured by the

TABLE 1. Purification of lipid transfer protein from human plasma[a]

Fraction	Total Protein	Specific Activity		% Recovery		Fold Purification	
		TG[b]	CE[b]	TG	CE	TG	CE
	mg	*kt × 100/mg protein*					
Lipoprotein-deficient plasma	29,824	1.16	1.75	100	100		
Phenyl-Sepharose	126.4	303	425	110.7	102.9	261	243
CM-Cellulose	27.8	1,117	1,563	89.8	83.3	963	893
Chromatofocusing	1.3	8.455	11,665	31.8	29.1	7,289	6,666
Bio-Gel A-0.5 m	0.3	18,071	24,456	15.7	14.1	15,578	13,975

[a] Values are averages obtained from three separate preparations. The starting point for each preparation was 800 ml of citrated plasma (i.e., ~575 ml undiluted plasma), which yields 1 liter of lipoprotein-deficient plasma upon delipidation.
[b] TG, triglyceride; CE, cholesteryl ester.

method of Sardesai and Manning (34) after isolation by thin-layer chromatography (see Materials). Lipid phosphorus was measured by the method of Bartlett (35).

RESULTS

The removal of plasma lipoproteins by the dextran sulfate-Mn^{2+} precipitation procedure was preferred over the ultracentrifugation step used previously (5). Both techniques were comparable in removing lipoproteins, but the former technique is faster and more adaptable to large scale work. With lipoprotein-deficient plasma as a starting material, a protocol for the purification of lipid transfer protein(s) to apparent homogeneity was developed (**Table 1**). The recovery and purification of both triglyceride and cholesteryl ester transfer activities were nearly the same at each step. Details of this purification are given below.

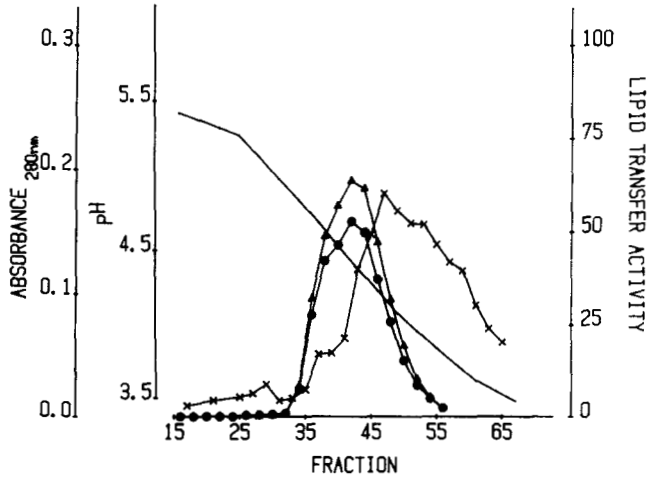

Fig. 1. Chromatofocusing of the CM-cellulose fraction of lipid transfer activity. A 1 × 19 cm (15 ml) column of PBE-94 chromatofocusing gel was eluted with 25 mM histidine buffer until both the eluant pH and conductivity values indicated equilibration. Twenty-one ml (8.9 mg protein) of the CM-cellulose pool of lipid transfer activity was dialyzed against Tris–HCl and applied to the column. Lipid transfer activity was eluted with a linear pH gradient at a flow rate of 30 ml/hr and 2.7-ml fractions were collected. The eluate absorbance at 280 nm (x) and pH (——) were measured and 100-μl aliquots were assayed for cholesteryl ester (▲) and triglyceride (●) transfer activity (t = 1.5 hr).

The elution profiles of cholesteryl ester and triglyceride transfer activities from phenyl-Sepharose and CM-cellulose have been published elsewhere (23) and are essentially the same as the profiles shown by Pattnaik et al. (5). The recovery of lipid transfer activity from the phenyl-Sepharose column appeared quantitative. However, this high recovery reflects, in part, the separation of lipid transfer activity from an inhibitor protein during phenyl-Sepharose chromatography (23).

An aliquot of the CM-cellulose pool was dialyzed against 25 mM Tris–HCl, pH 7.4, and applied to a chromatofocusing column equilibrated in histidine buffer, pH 5.7 (**Fig. 1**). Dialysis against Tris buffer was preferable to histidine since the recovery of lipid transfer activity after dialysis was greater. Under the starting conditions, all detectable lipid transfer activity was bound to the chromatofocusing column. Lipid transfer activity was eluted by a linear pH gradient. Cholesteryl ester and triglyceride transfer activities co-eluted as a single peak at a buffer pH of 4.54 ± 0.07 (mean ± S.D., n = 4). The recovery of applied triglyceride and cholesteryl ester transfer activity was 60%. Since the lipid transfer activity peak and the major peak of contaminating protein overlapped, pooled fractions from the leading half of the lipid transfer activity peak had a specific activity 4–5 times greater than that of pooled fractions from the tailing half. Therefore, only the leading half of the lipid transfer activity peak (~50% of the recovered activity) was taken for further purification.

Pooled fractions from the chromatofocusing column were adjusted to 150 mM NaCl and applied to a Bio-Gel A-0.5 m column. Unlike the results seen with less pure fractions (21), a single peak of lipid transfer activity was observed (**Fig. 2**). Triglyceride and cholesteryl ester transfer activities co-eluted in an elution volume corresponding to an apparent molecular weight of 64,400 ± 1,800 (n = 8, see inset Fig. 2). The overall recovery of triglyceride and cholesteryl ester transfer activities

from lipoprotein-deficient plasma was >14% with a 15,000-fold purification (Table 1).

The purity of the pooled fractions from the Bio-Gel column was assessed by SDS-electrophoresis. Two bands were observed: one of 58,300 ± 500 apparent molecular weight and another of 66,400 ± 400 (n = 6). These two components were partially separated by the Bio-Gel column. When the fractions constituting the leading and tailing halves of the Bio-Gel lipid transfer activity peak were pooled separately, we found that the leading half consisted predominately of the larger molecular weight protein whereas the tailing half consisted mostly of the smaller protein (**Fig. 3**, lanes A and B). The relative concentration of these bands varied between preparations (cf. lanes A–B to lane C). At a sample concentration sufficient to visualize contaminants of 2–3%, no other bands were observed (Fig. 3, lane C). As discussed later, the data suggest that these two proteins are different molecular forms of the same transfer protein.

The ability of the purified protein to promote the transfer of various lipids was determined with lipoprotein and with liposome substrates. The results with lipoprotein substrates are shown in **Table 2**. Unlike the results for cholesteryl ester and triglyceride, the transfer of both free cholesterol and phosphatidylcholine was rapid in the absence of transfer protein. In the presence of purified transfer protein, the transfer of cholesteryl ester, triglyceride, and phosphatidylcholine was stimulated. The protein-mediated transfer of cholesteryl ester and phosphatidylcholine was 1.6:1 (mol/mol), which is higher than the 1:1 ratio reported by Ihm et al. (2) for transfer in the opposite direction (i.e., high- to low-density lipoproteins). The transfer of free cholesterol by purified lipid transfer protein could not be measured under standard assay conditions since free cholesterol reached isotopic equilibrium in ~1.5 hr. In an experiment with a shorter assay time (30 min), and with a high concentration of partially purified lipid transfer protein (CM-cellulose fraction), we were still unable to demonstrate significant stimulation of free cholesterol transfer, although 42% of the cholesteryl ester was transferred.

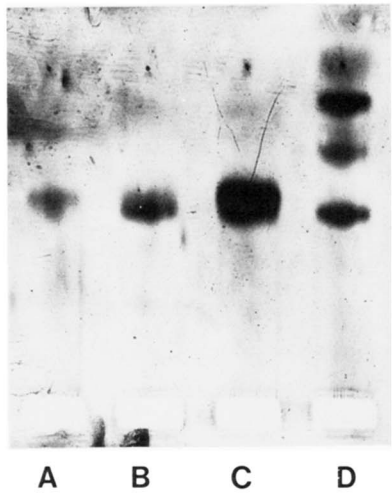

Fig. 3. SDS-electrophoresis of Bio-Gel fractions. Samples and standards were treated with SDS under reducing conditions and then electrophoresed on 5% polyacrylamide gels. Samples migrated from the bottom to the top of the gel. Proteins were visualized by silver staining. Samples: A), pooled fractions from the tailing half of a Bio-Gel transfer activity peak; B), pooled fractions from the leading half of a Bio-Gel transfer activity peak; C), pooled and concentrated fractions from the entire Bio-Gel peak (a different preparation from that shown in lanes A and B); D), molecular weight standards, bovine serum albumin, ovalbumin, and carbonic anhydrase (bottom to top).

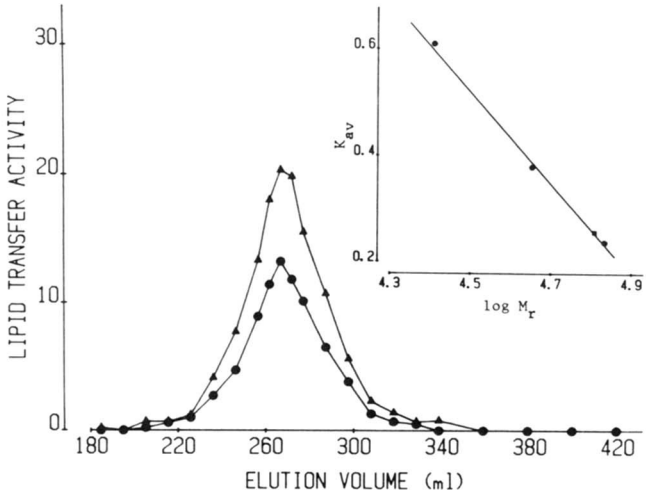

Fig. 2. Gel filtration of the chromatofocusing pool of lipid transfer activity. Sixteen ml (465 μg of protein) of the chromatofocusing pool, adjusted to 150 mM NaCl (see Methods), was applied to a 2.5 × 89 cm column of Bio-Gel A-0.5 m equilibrated in Tris/NaCl buffer. The sample was eluted at a buffer flow rate of 37 ml/hr (7.5 cm/hr) and 5-ml fractions were collected. The cholesteryl ester (▲) and triglyceride (●) transfer activity in 200-μl aliquots was determined under standard assay conditions (t = 3 hr). There was no detectable absorbance at 280 nm. Inset: molecular weight standard calibration curve. The data points (●) shown are the average of two runs each for chymotrypsinogen A (M_r 25,700), ovalbumin (M_r 45,000), and bovine serum albumin (M_r 68,000). (■) denotes the location of lipid transfer protein. V_t = 437 ml, V_0 = 194 ml.

Liposome substrates containing 0.2 mol % of the lipid to be studied were useful in measuring the specificity of highly purified lipid transfer protein. As indicated by the data in **Table 3**, the lipid transfer protein appears to have a broad substrate specificity. The transfer rate for triglyceride and cholesteryl ether was 0.7 of that for cholesteryl ester. Retinyl ester was also transferred by the purified lipid transfer protein, but at a rate one-fifth that for cholesteryl ester.

The transfer of cholesteryl ester and triglyceride by a common protein contradicts the conclusion reached by two other investigators that separate proteins were responsible for these transfers (8, 16). The evidence cited for their conclusion was: *1*) the differences in the inhibition of cholesteryl ester and triglyceride transfer

TABLE 2. Transfer of major lipoprotein lipid components by lipid transfer protein

Transfer Protein	Lipid Measured[a]			
	CE	TG	FC	PC
−	0.2	0.2	55.3	25.2
+	7.0	4.9	56.0	41.8

[a] CE, cholesteryl ester; TG, triglyceride; FC, free (unesterified) cholesterol; PC, phosphatidylcholine.

Doubly-labeled low density lipoprotein (see Methods) and high density lipoprotein acceptor (50 μg of total cholesterol in each) were incubated for 3 hr in the presence or absence of 350–560 ng of lipid transfer protein (Bio-Gel fraction). Transfer activities are expressed as kt × 100. The values presented are the average of two experiments.

by chemical agents, and *2*) the different isoelectric points of triglyceride and cholesteryl ester transfer activities. The lipid transfer protein isolated in this study also demonstrated a differential effect of chemical agents on triglyceride and cholesteryl ester transfer (**Table 4**). Like that previously noted, triglyceride transfer was almost completely abolished by *p*-chloromercuriphenyl sulfonate whereas cholesteryl ester transfer was only slightly decreased. Similar to the effect seen for cholesteryl ester, retinyl ester and phosphatidylcholine transfers were only slightly diminished by this agent (data not shown). Pretreatment of the various components of the lipid transfer assay system with *p*-chloromercuriphenyl sulfonate, followed by dialysis to remove the mercurial compound, showed that the inhibition of triglyceride transfer was not due to an effect on the substrates but to a direct effect on the transfer protein. Triglyceride transfer was also inhibited by the two other mercurials tested, whereas cholesteryl ester transfer was either not affected or increased. Another sulfhydryl reagent, 5,5′-dithiobis-(2-nitrobenzoate), was ineffective in suppressing triglyceride transfer even when used at a concentration of 1 mM. Diethyl-*p*-nitrophenyl phosphate, a serine reactive agent, slightly stimulated both cholesteryl ester and triglyceride transfer.

The isoelectric point observed for lipid transfer protein was dependent on the purity of the fraction analyzed. Isoelectric focusing of lipoprotein-deficient plasma gave a single form of pI = 4.8 for both triglyceride and cholesteryl ester transfer. When the CM-cellulose fraction of lipid transfer protein was electrofocused (data not shown), two greatly different isoelectric forms were found: an acidic form focusing at pI ~5 (30% of total activity) and a basic form with a pI ~9 (70% of activity). The ratio of cholesteryl ester and triglyceride transfer was the same in both forms. In contrast, when the CM fraction was focused in the presence of 4 M urea, only an acidic form was observed, with a pI identical to that seen in lipoprotein-deficient plasma (**Fig. 4**). When the fractions constituting the acidic form of lipid transfer protein in Fig. 4 were pooled, dialyzed to remove urea, and refocused in the absence of urea, the isoelectric point shifted to pI = 9.1 (**Fig. 5**). This shift could not be reversed by focusing the pI = 9.1 form in the presence of urea. Collectively these data indicate that the basic component of partially purified lipid transfer protein is formed during the isoelectric focusing procedure and that the formation can be prevented, but not reversed, by urea.

The highly purified lipid transfer protein (Bio-Gel fraction, Table 1) was focused under conditions similar to those described in Fig. 4, except that the pH interval was 4–6. Under these conditions, the pI could be determined with an accuracy of ± 0.03 pH units. We observed that triglyceride and cholesteryl ester transfer activities co-focused at pI = 5.18, and that lipid transfer activity from the leading and tailing halves of the Bio-Gel peak (see Fig. 3) had the same isoelectric point. Upon digestion with neuraminidase (*Clostridium perfringens*), the isoelectric point of lipid transfer activity quantitatively shifted to a pI of 5.74, indicating that lipid transfer protein contains sialic acid. In the fractions focusing at 5.18 and 5.74 (i.e., before and after neuraminidase treatment), the ratios of triglyceride to cholesteryl ester transfer were within 10% of the ratio observed in the original Bio-Gel fraction.

DISCUSSION

The protocol presented in Table 1 led to the co-purification of triglyceride and cholesteryl ester transfer activities. SDS electrophoresis of the Bio-Gel fraction of lipid transfer activity demonstrated two proteins of apparent molecular weights 58,300 and 66,400. These proteins were partially resolved during gel filtration. Fractions from this column were combined in such a manner as to give two pools, each containing predominately one protein (Fig. 3, lanes A and B). Despite their obvious compositional differences, both pools appeared almost identical in several respects: *1*) the specific activities of triglyceride and cholesteryl ester transfer in the

TABLE 3. Specificity of lipid transfer protein

	Lipid Measured			
	Cholesteryl Ester	Triglyceride	Retinyl Ester	Cholesteryl Ether
% Transfer	34.9	24.4	6.6	23.0
Relative transfer rate	1	0.70	0.19	0.66

The specificity of lipid transfer protein was assessed in a liposome-low density lipoprotein assay system. Highly purified lipid transfer protein (Bio-Gel fraction, 255–405 ng of protein) was incubated for 3 hr with phosphatidylcholine-cholesterol liposomes, containing 0.2 mol % of the lipid to be studied, and human low density lipoprotein as acceptor. The values presented are the average of three experiments.

TABLE 4. Effect of different reagents on triglyceride and cholesteryl ester transfer activities

Addition	Assay Conc.	TG[a]	CE[a]	% Activity Remaining TG	% Activity Remaining CE
	mM	*kt* × *100*			
None	—	10.7	16.2	100	100
p-Chloromercuriphenyl sulfonate	2.0	1.3	13.0	12.5	80.4
p-Hydroxymercuribenzoate	1.0	1.8	17.1	16.5	105.5
Ethyl mercurithiosalicylate	0.19	0.9	25.0	8.1	154.5
5,5′-Dithiobis-(2-nitrobenzoate)	0.28	11.8	18.4	110.6	113.7
Diethyl-*p*-nitrophenyl phosphate	1.0	13.1	21.7	122.6	134.1

[a] TG, triglyceride; CE, cholesteryl ester.

The effect of different reagents on lipid transfer activity was determined with the indicated assay concentrations of each compound. Amounts (340–400 ng) of lipid transfer protein (Bio-Gel fraction) were incubated under standard assay conditions for 4.5 hr. The values presented are the average of two closely agreeing experiments.

two pools differed by less than 10%; *2*) the relative rates of cholesteryl ester, triglyceride, retinyl ester, and cholesterol ether transfer in the pools were almost identical; and *3*) the effect of various chemical agents on lipid transfer activity in each pool was the same. Collectively, these results indicate that, despite their apparently different molecular weights, these proteins are equivalent with respect to lipid transfer activity. Since lipid transfer protein is a glycoprotein (2, 5, 8), we speculate that the loss of carbohydrate residues, thereby altering the electrophoretic mobility on SDS gels (36) and possibly modifying the interaction of transfer protein with the gel matrix (37), may lead to a change in the apparent molecular weight of the transfer protein during isolation and storage. However, the possibility of two distinct transfer proteins, both transferring cholesteryl ester and triglyceride, cannot be presently excluded.

The isoelectric point for lipid transfer protein determined under dissociating conditions (pI = 5.18) is similar to that previously reported for partially purified cholesteryl ester transfer protein isolated from human and rabbit plasma (2, 4, 5). Rajaram et al. (8) demonstrated the affinity of the triglyceride transfer activity in rabbit plasma for CM-cellulose at pH 4.5, suggesting a low isoelectric point for this protein; yet, upon isoelectric focusing, these investigators observed a high isoelectric point. Based on the data shown in Figs. 4 and 5, the basic isoelectric point of triglyceride transfer protein reported by Rajaram et al. (8) most likely results from protein-protein or ampholine-protein interactions during the focusing procedure.

Of the two lines of evidence supporting separate triglyceride and cholesteryl ester transfer proteins (see Results section), only the differential effect of mercurial compounds on triglyceride and cholesteryl ester transfer activities was confirmed by this study (Table 4). However, this differential effect could be observed if these lipids were transferred by separate proteins or by a single protein containing different sites for the transfer of triglyceride and cholesteryl ester. Based on the findings of this report, the latter possibility appears most likely. We conclude that, within the limits of detection, triglyceride and cholesteryl ester transfers are promoted by the same plasma protein(s).

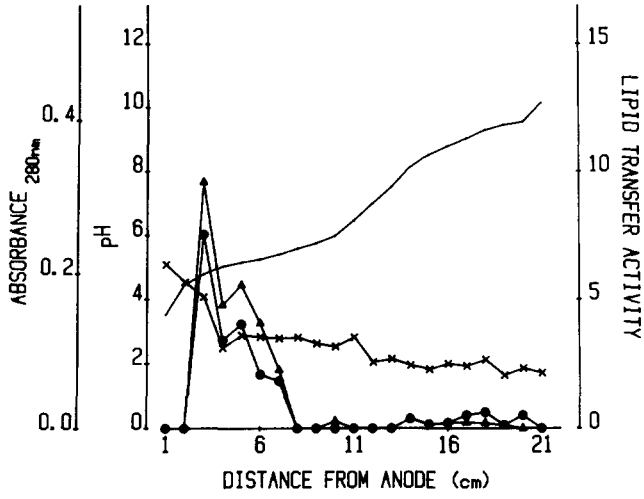

Fig. 4. Isoelectric focusing of lipid transfer protein in the presence of 4 M urea. An aliquot (3.5 mg of protein) of the CM-cellulose pool of lipid transfer activity was dialyzed overnight against 1% glycine, 4 M urea. A 1-cm section was removed from the gel bed at a location 13–14 cm from the anode, mixed with the sample, and reapplied in the same location. The sample was focused for 15.5 hr at 8 watts. Initial V = 600, ma = 54. Final V = 1700, ma = 6. Coolant temperature was 10.5°C. After focusing, the bed was divided into 21 1-cm sections and each section was eluted with H_2O. The pH (——) of the eluates was measured at the coolant temperature. After dialysis against Tris/NaCl buffer, the absorbance at 280 nm (x) was measured, and 300-μl aliquots were assayed for cholesteryl ester (▲) and triglyceride (●) transfer activity (t = 3 hr). The recovery of applied triglyceride and cholesteryl ester transfer activities was 24 and 23%, respectively.

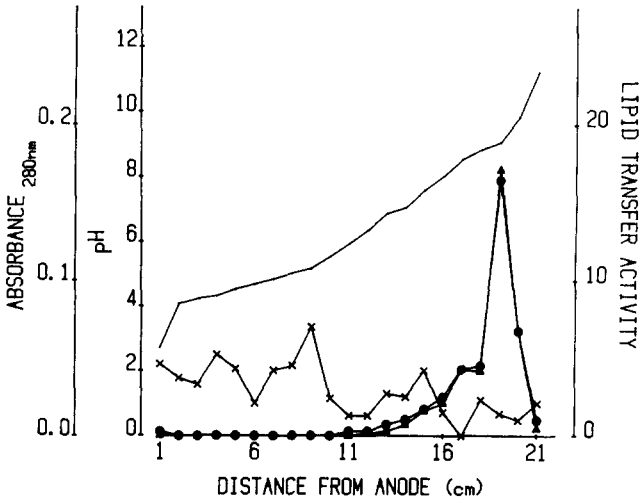

Fig. 5. Isoelectric focusing of the acidic form of lipid transfer activity in the absence of urea. Fractions 3–6 from the isoelectric focusing profile described in Fig. 4 were pooled and dialyzed against H_2O. An IEF-Sephadex slurry was prepared as described in the Methods section, except that the total aqueous volume was decreased by 20 ml. After standing overnight, the gel slurry was mixed with 20 ml of the dialyzed sample and the final slurry was allowed to equilibrate for 8 hr at 4°C. The gel slurry was spread and focused as described in the Methods. Initial V = 650, ma = 24. Final V = 1590, ma = 7. (x), absorbance at 280 nm; (——), pH; (▲), cholesteryl ester, and (●), triglyceride transfer activities in 400-μl aliquots (t = 6 hr). The recovery of applied triglyceride and cholesteryl ester transfer activities was 64 and 57%, respectively.

With the established protocol (Table 1), lipid transfer protein was purified 15,000-fold with respect to lipoprotein-deficient plasma and accounted for the only detectable triglyceride or cholesteryl ester transfer activities in the starting fraction. Based on the protein and percent recovery values in Table 1, the maximum plasma concentration of lipid transfer protein is 60 nM. However, due to the lability of transfer activity in highly purified fractions, this value may be overestimated by a factor of two. By comparison, the plasma concentration of LCAT is 80 nM (38). The physical parameters and chromatographic behavior of the lipid transfer protein differ from that of previously characterized proteins such as the A, B, C, D, E, and F classes of apolipoproteins (39–41) and from LCAT (5).

Cholesteryl ester transfer protein is associated with other plasma components as a lipid-protein complex (11, 12). This complex appears to have been fully disrupted by the purification protocol since the apparent molecular weights determined for purified lipid transfer protein under both denaturing (SDS electrophoresis) and non-denaturing (gel filtration) conditions were similar. This contrasts with the considerably larger apparent molecular weight (150,000) determined by gel filtration for partially purified cholesteryl ester transfer protein (2), which most likely reflects the molecular weight of a fragment of the cholesteryl ester transfer complex (11).

Lipid transfer protein was capable of promoting the transfer of molecules that were chemically diverse (Tables 2 and 3). With lipoprotein substrates, the purified lipid transfer protein stimulated the transfer of cholesteryl ester, triglyceride, and phosphatidylcholine but not of free cholesterol. Among the four lipids incorporated into phosphatidylcholine liposomes, considerable specificity for transfer was observed. Cholesteryl ester was the preferred substrate followed in order by triglyceride, cholesteryl ether, and retinyl ester. The preferential transfer of cholesteryl ester over that of cholesteryl ether and retinyl ester has also been reported for lipoprotein substrates (42, 43). At the very low concentration of labeled lipid incorporated into these liposomes (0.2 mol%) it seems unlikely that heterogeneity in lipid distribution within the bilayer could account for the observed specificity of the transfer protein.

Other workers have noted the co-elution of cholesteryl ester and phospholipid transfer activities during early purification steps (2, 16). Since we have not quantitated the recovery of phospholipid transfer activity from lipoprotein-deficient plasma, the transfer of phospholipid by other plasma proteins can not be excluded. It seems probable that such proteins exist, since rat plasma promotes phospholipid transfer at a rate quantitatively similar to that observed in human plasma (2) even though rat plasma has no measurable cholesteryl ester transfer activity (6).

The technical assistance of Kathleen Feiock is gratefully acknowledged. This research was supported in part by Public Health Service Research Grants HL 10933 and HL 06099A from the National Heart, Lung, and Blood Institute of the U.S. Public Health Service.

Manuscript received 4 January 1982 and in revised form 11 May 1982

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