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Two Nonspecific Phospholipid Exchange Proteins from Beef Liver. 1. Purification and Characterization†

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ABSTRACT: Two proteins have been purified from the postmicrosomal fraction of beef liver homogenate. They accelerate the transfer of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin, phosphatidylglycerol, phosphatidic acid, and cholesterol from unilamellar vesicles to either mitochondria or multilamellar vesicles and are therefore referred to as "nonspecific" exchange proteins. These are the first purified exchange proteins which have been found capable of accelerating the transfer of phosphatidic acid and phosphatidylglycerol. However, the transfer of diphosphatidylglycerol from unilamellar vesicles to multilamellar vesicles and cholesteryl esters from low-density lipoprotein to high-density lipoprotein is not accelerated by these proteins. Characterization of the two exchange proteins has revealed a striking similarity. Both accelerate the transfer of phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine from unilamellar vesicles to multilamellar vesicles with the same relative specificity (2.2:1.7:1). Both have molecular weights of 13 600 as determined by molecular filtration through Sephadex G-50 and 14 500 as determined by electrophoresis on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate. They have isoelectric points of 9.55 and 9.75 as determined on an isoelectric focusing column. Amino acid analyses reveal only two differences. One protein contains neither histidine nor arginine whereas the other contains one residue of each per protein molecule.

Proteins that accelerate the exchange of phospholipids between membranes have been isolated from a number of sources (Wirtz, 1974; Zilversmit & Hughes, 1976) and have been used to study the distribution and transbilayer movement of phosphatidylcholine in artificial and biological membranes (Johnson et al., 1975; Bloj & Zilversmit, 1976; Rothman & Dawidowicz, 1975). Recently, purified proteins from rat liver and rat liver hepatoma have been shown to enhance the exchange of phosphatidylethanolamine and sphingomyelin (Bloj & Zilversmit, 1977a; Dyatlovitskaya et al., 1978). These proteins should be useful in studying the asymmetry and transbilayer movement of phospholipids in mixed lipid vesicles and biological membranes. One of these has been used to

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measure phospholipid transbilayer movement in rat liver microsomes (Zilversmit & Hughes, 1977). One drawback to their use for these types of experiments is the difficulty involved in purifying large quantities of protein from these sources.

In the present paper we report the purification and characterization of two nonspecific exchange proteins from beef liver. These proteins can be prepared in bulk to high purity and have been found to enhance the exchange of most phospholipids tested as well as cholesterol.

Materials and Methods

Lipids. ^{32}P-Labeled phosphatidylcholine and phosphatidylethanolamine were isolated from the liver of a rat injected intraperitoneally with 3–5 mCi of $^{32}P_i$ (New England Nuclear, Boston, MA) per 100 g of body weight 16 h before sacrifice and purified as previously described (Bloj & Zilversmit, 1976). [^{32}P]Diphosphatidylglycerol was isolated from the livers of two rats injected intraperitoneally with 2 mCi of $^{32}P_i$ per 100 g of body weight 72 h before sacrifice and purified by column chromatography on silicic acid (Sweeley, 1969).

[^{3}H]Phosphatidylinositol was prepared by a modification of the base exchange procedure described by Helmkamp et al. (1974). Rat liver microsomes (from one liver) were homogenized in 5 mL of SET buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris, and 0.02% NaN_3, pH 7.4). To this was added 1.2 mL of a solution containing 200 μCi of [^{3}H]inositol in 75 mM Hepes and 8 mM $MnCl_2$, pH 7.4. The flask was flushed with nitrogen and incubated at 37 °C for 90 min. Phospholipid was extracted by addition of 200 mL of chloroform–methanol (2:1 v/v). The extract was filtered and 100 mL of 40 mM $CaCl_2$ containing 0.5 mmol of inositol was added. After the solution was thoroughly mixed, the two phases were separated by centrifugation. The upper phase was discarded. The lower phase was washed twice with 40 mL of the upper phase (methanol–40 mM $CaCl_2$, 2:3 v/v) and once with 40 mL of the upper phase (methanol–water, 2:3 v/v). The solvent was removed in vacuo, the lipids were dissolved in 20 mL of chloroform–methanol (2:1 v/v), and the phospholipids were separated on a 10% alumina column (neutral; Woelm, Eschwege, West Germany) as described by Luthra & Sheltawy (1972). The total lipid extract from the microsomes prepared from one rat liver was applied to 20 g of alumina. The fraction containing phosphatidylinositol was collected and stored at −20 °C.

[^{32}P]Phosphatidylglycerol was isolated from *Staphylococcus aureus* grown in the presence of 0.2 mCi of $^{32}P_i$. *S. aureus* was grown in 50 mL of a medium containing 20 g of proteose peptone (Difco Laboratories, Detroit, MI), 2 mL of 10% $MgSO_4$, 5 g of lactic acid, and 10 mL of 10% K_2HPO_4 brought to 1 L with distilled water and adjusted to pH 7.4 with NH_4OH. After 24 h at 37 °C, 0.1 mL was transferred to a flask containing 0.2 mCi of $^{32}P_i$ but no K_2HPO_4 in 50 mL of growth medium, and the incubation was continued for 24 h. The bacteria were sedimented by centrifugation for 10 min at 10000*g*, and the lipid was extracted in 27 mL of chloroform–methanol (2:1 v/v). The lipid was partitioned with 0.2 volume of water, and the lower phase was dried under nitrogen. [^{32}P]Phosphatidylglycerol was purified by thin-layer chromatography on silica gel H with chloroform–acetone–methanol–acetic acid–water (60:80:20:20:10 v/v) and stored in chloroform–methanol (2:1 v/v) at −20 °C under nitrogen.

[^{32}P]Phosphatidic acid was prepared by the action of cabbage phospholipase D (General Biochemicals, Chagrin Falls, OH) suspended in 10 mL of 70 mM sodium acetate and 30 mM $CaCl_2$, pH 5.6 (0.25 unit of phospholipase D per mL), on 30 μmol of [^{32}P]phosphatidylcholine (prepared from rat liver as described above) dissolved in 10 mL of diethyl ether (washed 3 times with 2 volumes of glass-distilled water). After 2 h of mixing at 25 °C, phospholipid was extracted with ether and purified by column chromatography on silicic acid (Sweeley, 1969). [^{32}P]Phosphatidylserine was prepared by transphosphatidylation. [^{32}P]Phosphatidylcholine (30 μmol) was dissolved in 10 mL of diethyl ether (washed 3 times with 2 volumes of glass-distilled water) and incubated for 2 h at 37 °C with 5 units of phospholipase D in 10 mL of 70 mM sodium acetate and 30 mM $CaCl_2$, pH 5.6, containing 25% by weight L-serine (Sigma Chemical Co., St. Louis, MO). Purification was achieved by thin-layer chromatography on silica gel H with chloroform–acetone–methanol–acetic acid–water (60:80:20:20:10 v/v).

Bovine [^{14}C]sphingomyelin (New England Nuclear, Boston, MA) was diluted with unlabeled bovine sphingomyelin (Avanti Biochemicals, Birmingham, AL) in chloroform to a final specific activity of 6.7 μCi/μmol. Glycerol [9,10-^{3}H]- or [^{14}C]trioleate (Applied Science Laboratories, State College, PA) was purified by thin-layer chromatography on silica gel H with hexane–diethyl ether–acetic acid (60:40:1 v/v). The glycerol trioleate was eluted with hexane and stored at −20 °C. [4-^{14}C]Cholesterol (Amersham/Searle, Arlington, IL) was purified by silica gel H thin-layer chromatography with hexane–diethyl ether (50:50 v/v) and stored in chloroform–methanol (2:1 v/v) at −20 °C. Butylated hydroxytoluene (Nutritional Biochemical Corp., Cleveland, OH) was dissolved in chloroform and used without further purification. Unlabeled phosphatidylcholine (egg), phosphatidylethanolamine (egg), phosphatidylglycerol (egg), phosphatidylserine (bovine spinal cord), phosphatidylinositol (wheat germ), phosphatidic acid (egg), and diphosphatidylglycerol (ox heart) (Lipid Products, South Nutfield, England) were diluted in chloroform. The purity of these was determined to be greater than 99% from analysis by thin-layer chromatography. Unlabeled cholesterol (Sigma Chemical Co., St. Louis, MO) was purified as the dibromide derivative, crystallized from methanol, and stored at 4 °C in ethanol.

Lipid Vesicles. Multilamellar vesicles were prepared as described previously (DiCorleto & Zilversmit, 1977). Small sonicated unilamellar vesicles were prepared as described by Johnson & Zilversmit (1975). Butylated hydroxytoluene (0.1% by mass) was used as an antioxidant. A trace of either glycerol [^{3}H]trioleate or glycerol [^{14}C]trioleate was included in small unilamellar vesicle preparations as a nonexchangeable marker.

Mitochondria. Beef heart mitochondria were prepared as previously described (Johnson & Zilversmit, 1975) and stored at −20 °C. After being thawed, they were heated for 30 min at 60 °C and washed 3 times with SET buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris, and 0.02% NaN_3, pH 7.4).

Assay Procedures. Phospholipid exchange activities were determined by measuring the transfer of labeled phospholipid from sonicated small unilamellar vesicles to either mitochondria or large multilamellar vesicles. In the former case, small unilamellar vesicles (50–60 nmol of total phospholipid) were incubated with mitochondria (580 nmol of total phospholipid) for 90 min at 37 °C in a total volume of 0.5 mL of SET buffer. One percent fatty acid poor BSA (Miles Laboratories, Elkhart, IN) was included. Exchange was terminated by centrifugation at 8000*g* for 2 min in an Eppendorf microcentrifuge, Model 3200 (Brinkman Instruments, Inc., Westbury, NY). Aliquots of the supernatant were counted in Liquiscint scintillation counting solution (National Diag-

nostics, Parsippany, NJ) in a Beckman LS-250 scintillation counter. The labeled glycerol trioleate included in the sonicated small unilamellar vesicles served as a nonexchangeable reference so that the decrease in the labeled phospholipid to nonexchangeable marker ratio of the supernatant after incubation measured the transfer of labeled lipid (Johnson & Zilversmit, 1975). The small amount of transfer in the absence of exchange protein was subtracted as background.

When multilamellar vesicles were used as acceptor particles, the assay was similar; however, mitochondria were replaced by multilamellar vesicles of the same phospholipid composition as the small unilamellar vesicles used as donors. If no other acidic phospholipid was present, 10 mol % diphosphatidylglycerol was included in the multilamellar vesicles. Unilamellar vesicles (50 nmol of total phospholipid) were incubated with multilamellar vesicles (2 μmol of total phospholipid) at 37 °C for 30 min in 1 mL of ET buffer (5 mM EDTA, 50 mM Tris, and 0.02% NaN_3, pH 7.4). Exchange was terminated by centrifugation at 40000*g* for 20 min at 4 °C in a Sorvall RC-2B centrifuge. Exchange of lipid was determined from an aliquot of supernatant as described above.

The exchange of cholesteryl esters between high-density lipoprotein (HDL) and low-density lipoprotein (LDL) was measured as previously described by using lipoprotein fractionated by ultracentrifugation (Pattnaik et al., 1978). The small amount of exchange (less than 1%) observed in the absence of added protein was subtracted as background.

Analytical Procedures. Polyacrylamide gel electrophoresis was performed on 15% acrylamide gels at pH 4.3 as described by Gabriel (1971). Electrophoresis in the presence of sodium dodecyl sulfate was performed on 12% acrylamide gels, according to Weber & Osborn (1975). The protein molecular weight standards were phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400) (Pharmacia Fine Chemicals, Piscataway, NJ). Lipid phosphorus was measured by the method of Bartlett (1959). Protein was determined by the biuret method (Gornall et al., 1949) or by a modification of the method of Sedmark & Grossberg (1977). Isoelectric focusing was carried out on an LKB Model 810 column (Produktor AB, Bromma, Sweden) at 10–12 °C with solutions of 0.2 M H_2SO_4 and 0.2 M NaOH in the anode and cathode compartments, respectively (Vesterberg & Svensson, 1966). Ampholytes of range pH 8–10, 7–9, and 3–10 (Bio-Rad Laboratories, Richmond, CA) in a final concentration of 2% were used in a weight ratio of 45:45:10. Voltage was applied and gradually increased to maintain a power flow of 2–3 W, until a potential of 600 V was reached. After 40 h, the current reached a minimal and stable value, indicating that the focusing was complete. Three-milliliter fractions were collected, the pH was measured with a combined electrode, and the fractions were assayed for phosphatidylethanolamine exchange activity.

Amino acid analyses were performed on purified protein after dialysis against 0.1% $NaDodSO_4$ in distilled water, lyophilization, and hydrolysis in 6 M HCl at 110 °C using a Beckman 119 CL amino acid analyzer. No significant difference was found between hydrolysis for 24 h and hydrolysis for 48 h. Cystine plus cysteine was determined by the method of Hirs (1967). Tryptophan and methionine were determined after hydrolysis for 24 h at 110 °C in 3 N mercaptoethanesulfonic acid.

Purification of Bovine Liver Nonspecific Exchange Protein. (*1*) *pH 5.1 Supernatant.* The liver (4–5.5 kg) of one steer (age 12–18 months) was trimmed of fat and cartilage, cut into 1-in. cubes, and rinsed with 0.25 M sucrose to remove blood and debris. This and all subsequent steps were performed at 0–4 °C unless indicated otherwise. A 35% homogenate of liver was prepared in SET buffer and centrifuged for 30 min at 13000*g*. The pellet was discarded and the supernatant was brought to pH 5.1 with 3 N HCl. After being stirred for 30 min, the solution was centrifuged for 20 min at 13000*g*. The clear supernatant was decanted, adjusted to pH 7.4 with 3 N NaOH, and stored at −20 °C. The exchange activity was stable for at least 6 months. From one beef liver, yields of 7–9 L of supernatant were obtained.

(*2*) *Ammonium Sulfate Precipitation.* pH 5.1 supernatant (1800 mL) was thawed. Over a period of 1 h, ammonium sulfate was gradually added to 40% saturation. The solution was stirred for 1 h, and the precipitate was removed by centrifugation at 13000*g* for 30 min. Ammonium sulfate was gradually added to the supernatant over a period of 1 h to 90% saturation. Stirring was continued for 1 h, followed by centrifugation at 13000*g* for 30 min. The clear supernatant was discarded, and the pellet was suspended in 100 mL of 25 mM sodium phosphate, 10 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4. This suspension was dialyzed extensively vs. 5 mM sodium phosphate, 5 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4.

(*3*) *CM-cellulose Column Chromatography.* The solution was applied to a CM-cellulose column (CM-52 microgranular, Whatman Ltd., Boonton, NJ) prepared from 250 mL of preswollen packed CM-cellulose preequilibrated in 5 mM sodium phosphate, 5 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4. The column was washed once with 2 column volumes of this buffer and eluted with 2 column volumes of 25 mM sodium phosphate, 45 mM NaCl, 5 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4.

(*4*) *Heat Treatment.* The eluant from the CM-cellulose column (~500 mL) was then adjusted to pH 6.3 with 3 N HCl and heated at 90 °C for 5 min. The pH was readjusted to 7.4 with 3 N NaOH. The precipitated protein was filtered, and the filtrate was concentrated to 50 mL by ultrafiltration through a Diaflo UM 10 membrane (Amicon Corp., Lexington, MA).

(*5*) *Octylagarose Column Chromatography.* At room temperature, the concentrate was applied to a column (1.0 × 30 cm) of 20 mL of octylagarose slurry (Miles Yeda Ltd., Elkhart, IN) which had been equilibrated with 25 mM sodium phosphate, 45 mM NaCl, 5 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4. The column was washed with 50 mL of this buffer and 50 mL of 5 mM sodium phosphate, 5 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4. The exchange activity was eluted with 50 mL of this solution, pH 3.0, and collected in 7-mL fractions. The pH was immediately increased by addition to each fraction of an equal volume of 25 mM sodium phosphate, 45 mM NaCl, 5 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4. The samples were refrigerated at 4 °C and lost no exchange activity during a 2-month period. Samples were further purified on a second CM-cellulose column as described in the legend to Figure 1.

Results

Purification of Proteins Capable of Transferring Phosphatidylethanolamine. Table I summarizes a typical purification of beef liver proteins capable of catalyzing the transfer of phosphatidylethanolamine from unilamellar vesicles (prepared from equal molar amounts of [^{32}P]phosphatidylethanolamine and nonradioactive phosphatidylcholine) to beef heart mitochondria. The small amount of transfer occurring in the absence of protein was subtracted as background. This

Table I: Purification of Phospholipid Exchange Protein from Beef Liver[a]

step	vol (mL)	protein (mg)	act. (μmol/h)[b]	recovery (%)	sp act. [nmol/(h mg)]	purifn factor
(1) pH 5.1 supernatant	1800					
(2) $(NH_4)_2SO_4$ precipitation	570	38400	127	100	3.3	1
(3) CM-cellulose	430	3100	92.5	73	29.8	9
(4) heat treatment	475	56	50.7	40	912	276
(5) concentration	55	58	46.6	37	803	245
(6) octylagarose	75	6.7	28.3	19	4200	1270

[a] Typical purification of nine preparations from three different livers. [b] Assayed by exchange of phosphatidylethanolamine from phosphatidylcholine–phosphatidylethanolamine unilamellar vesicles to heat-treated mitochondria as described under Materials and Methods.

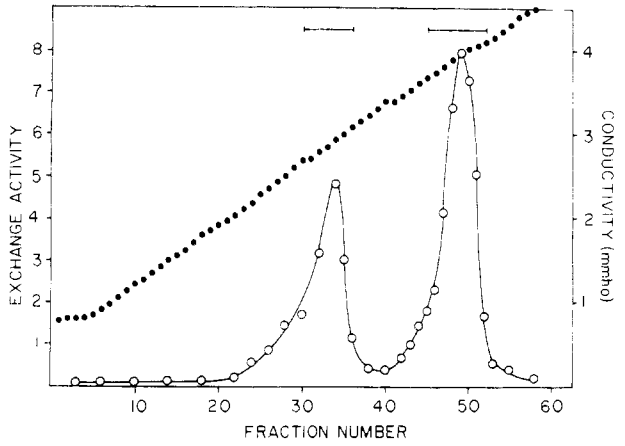

FIGURE 1: Elution profile on CM-cellulose. The pooled fractions from the octylagarose column were dialyzed 3 times against 20 volumes of 5 mM sodium phosphate, 5 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4. The CM-cellulose column (27 × 1.5 cm) was prepared by using 48 mL of packed CM-cellulose which had been equilibrated at 4 °C in the same buffer. The sample (containing about 5 mg of protein) was loaded at a flow rate of 60 mL/h. The column was washed with 50 mL of the 5 mM buffer, and the protein was eluted with a linear gradient formed from 250 mL of the 5 mM buffer and 250 mL of 25 mM sodium phosphate, 50 mM NaCl, 5 mM β-mercaptoethanol, and 0.02% NaN_3, pH 7.4. Samples were collected at 7-min intervals. (○) Phosphatidylethanolamine exchange activity [nmol/(0.1 mL 1.0 h)] from unilamellar vesicles to mitochondria; (●) conductivity at room temperature. Fractions 30–36 (CM I) and 45–52 (CM II) were pooled. This figure is representative of two experiments.

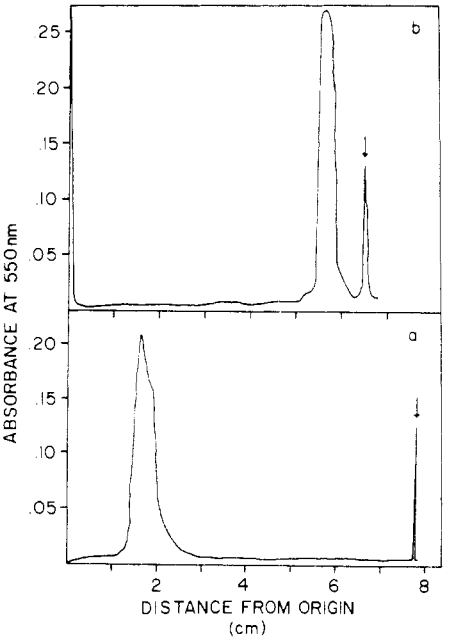

FIGURE 2: Polyacrylamide gels scanned at 550 nm. (a) Electrophoresis on polyacrylamide gels (15%) at pH 4.3 (Gabriel, 1971) of exchange protein after purification by column chromatography on octylagarose. 20 μg of protein was applied. The front of the gel is marked by an arrow. (b) Electrophoresis on polyacrylamide gels (12%) in the presence of 0.1% sodium dodecyl sulfate (Weber & Osborn, 1975) of exchange protein after purification by column chromatography on octylagarose. 20 μg of protein was applied. The front of the gel is marked by an arrow.

was ~15% of total transfer in a typical assay. After step 6, the octylagarose column, the purification compared to the ammonium sulfate precipitate was 1270-fold while a yield of 19% was attained. At this step, greater than 95% of the protein appeared as a single band on disc gel electrophoresis on 15% polyacrylamide gels at pH 4.3 (Figure 2a). Furthermore, electrophoresis on 12% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate revealed only one major component (Figure 2b) with a molecular weight of 14 500 as calculated by this technique (Weber & Osborn, 1975). The molecular weight of the phosphatidylethanolamine exchange activity was also determined on Sephadex G-50 superfine. On this column, ovalbumin (M_r 43 000), chymotrypsinogen A (M_r 25 000), and ribonuclease A (M_r 13 700) were used as standards. The exchange activity eluted as a single peak with a K_{av} of 0.31 which corresponds to a molecular weight of 13 600. This is in good agreement with the data from sodium dodecyl sulfate–polyacrylamide gels and supports our conclusion that the predominant band on gels is responsible for the phosphatidylethanolamine exchange activity. Fractionation of the octylagarose eluant on a second CM-cellulose column revealed two proteins containing exchange activity (Figure 1). Very little additional purification was achieved in this step

Table II: Purification of Phospholipid Exchange Proteins from Beef Liver on CM-cellulose[a]

step	vol (mL)	protein (mg)	act. (μmol/h)[b]	recovery (%)	sp act. [nmol/(h mg)]	purifn factor
(1) octylagarose	25	5.2	4.9	100	950	1
(2) CM-cellulose						
CM I	46	1.2	1.2	24	960	1
CM II	50	1.7	1.9	39	1140	1.2

[a] Typical purification of two preparations. [b] Phosphatidylethanolamine exchange activity.

(Table II), and the two proteins could not be distinguished by electrophoresis on 15% polyacrylamide gels at pH 4.3 or by electrophoresis on 12% polyacrylamide gels at pH 7.0 in the presence of 0.1% sodium dodecyl sulfate. The protein represented by the first peak is referred to as CM I whereas the second is referred to as CM II.

Isoelectric Focusing. An isoelectric focusing column performed after the heat treatment of the CM-cellulose eluant

Table III: Amino Acid Composition of Purified Phospholipid Exchange Proteins[a]

amino acid	beef liver CM I		beef liver CM II		rat liver[b] CM II (nearest integer/ mol of protein)
	mol %	nearest integer/ mol of protein	mol %	nearest integer/ mol of protein	
aspartic acid plus asparagine	12.6 ± 0.08	16	12.2 ± 0.2	16	12
threonine	4.0 ± 0.5	5	3.8 ± 0.5	5	4
serine	3.7 ± 0.4	5	3.8 ± 0.5	5	7
glutamic acid plus glutamine	10.6 ± 0.2	13	10.2 ± 0.2	13	13
proline	3.1 ± 0.2	4	3.1 ± 0.6	4	4
glycine	11.8 ± 0.3	15	12.1 ± 0.2	15	14
alanine	7.3 ± 0.5	9	7.4 ± 0.4	9	11
valine	6.5 ± 0.2	8	6.3 ± 0.2	8	5
methionine[c]	4.0	5	3.9	5	5
isoleucine	4.1 ± 0.3	5	3.8 ± 0.2	5	5
leucine	9.0 ± 0.3	11	8.9 ± 0.3	11	10
tyrosine	0.04 ± 0.04	0	0.2 ± 0.2	0	0
phenylalanine	5.7 ± 0.2	7	5.5 ± 0.2	7	6
histidine	0.08 ± 0.08	0	0.7 ± 0.2	1	0
lysine	13.6 ± 0.08	17	13.2 ± 0.2	17	19
arginine	0.15 ± 0.15	0	1.1 ± 0.1	1	0
tryptophan[c]	0.8	1	0.8	1	0
half-cystine plus cysteine[d]	2.9	4	3.2	4	2

[a] Determined as described under Materials and Methods; mean ± standard deviation of three analyses, each done in duplicate. [b] Taken from Bloj et al. (1978). [c] Determined after hydrolysis for 24 h at 110 °C in 3 N mercaptoethanesulfonic acid; average of duplicate determinations. [d] Determined by the method of Hirs (1967); average of duplicate determinations.

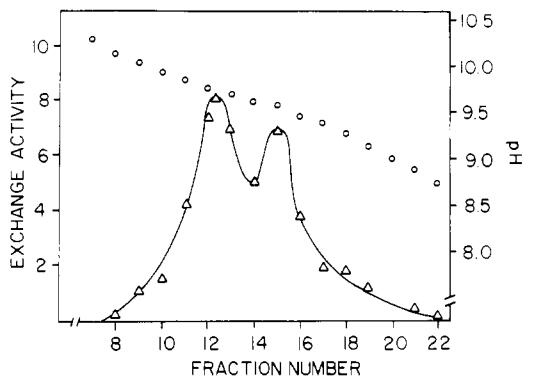

FIGURE 3: Isoelectric focusing of the beef liver exchange proteins. Exchange protein after heat treatment (10 mg) was applied to an isoelectric focusing column and focused at 10 °C as described under Materials and Methods. (○) pH at 10 °C; (△) phosphatidylethanolamine exchange activity [nmol/(50 μL 60 min)] measured from unilamellar vesicles to mitochondria. This figure is representative of two experiments.

(step 4) also revealed two peaks of exchange activity of slightly different charge characteristics (Figure 3). One protein (CM I) was found to have an isoelectric point of 9.55 while the second protein (CM II) had an isoelectric point of 9.75.

Amino Acid Analysis. Amino acid analysis on the two pure proteins isolated from the CM-cellulose column revealed two significant differences (Table III). CM I was found to contain no histidine or arginine whereas CM II contained one residue of each per molecule of protein. The amino acid composition of both is marked by a high proportion of lysine, glycine, and aspartate (including asparagine) and little or no tyrosine, tryptophan, histidine, and arginine. Such has also been observed for the composition of the nonspecific exchange protein from rat liver (Table III) (Bloj et al., 1978).

Specificity. The relative transfer of lipids between sonicated unilamellar vesicles and multilamellar vesicles was measured with vesicles of different compositions. Unilamellar vesicles were prepared from labeled phospholipids and a trace of labeled glycerol trioleate (included as a nonexchangeable marker). When possible, the relative phospholipid transfers were determined with unilamellar vesicles composed of phospholipid mixtures labeled with different isotopes. When two ^{32}P-labeled phospholipids were compared, the relative transfer was calculated by comparing the transfer from unilamellar vesicles composed of ^{32}P-labeled phospholipid A and unlabeled phospholipid B to vesicles composed of unlabeled phospholipid A and ^{32}P-labeled phospholipid B. Identical results were obtained with unilamellar vesicles composed of ^{32}P-labeled lipids A and B which were separated by thin-layer chromatography after the exchange experiment. The lipid composition of the nonradioactive acceptor multilamellar vesicles was made identical with that of the unilamellar vesicles. However, we found that little phospholipid transfer occurred when multilamellar vesicles contained no acidic phospholipid. This is in agreement with data of DiCorleto & Zilversmit (1977) on the transfer of phosphatidylcholine from multilamellar vesicles catalyzed by beef heart exchange protein or beef liver phosphatidylcholine exchange protein. Therefore, 10 mol % diphosphatidylglycerol was added to multilamellar vesicles that contained no other acidic phospholipid.

We measured the relative transfer of phospholipids from unilamellar vesicles, composed of phosphatidylcholine and phosphatidylethanolamine (50:50 mol %), to multilamellar vesicles containing 45 mol % of each of these phospholipids plus 10 mol % diphosphatidylglycerol. The extent of phospholipid accessibility was usually determined from complete exchange experiments between unilamellar vesicles and several changes of multilamellar vesicles. The kinetic constant (Zilversmit & Hughes, 1976) for exchange of phosphatidylcholine was 1.5 times that for phosphatidylethanolamine transfer for the CM I, CM II, and octylagarose protein fractions (Table IV). Similarly, in experiments on unilamellar vesicles composed of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol, no significant difference was found in the relative specifications of the CM I, CM II, and octylagarose protein fractions for these phospholipids

Table IV: Relative Transfer of Phospholipids and Cholesterol from Sonicated Unilamellar Vesicles to Multilamellar Vesicles[a]

donor and acceptor composn[b]	protein fraction[c]	kinetic constant ratio[d]
PC:PE:PI		
45:45:10	octylagarose	PC/PE = 1.7
45:45:10	octylagarose	PI/PE = 2.1
80:10:10	octylagarose	PI/PE = 2.5
80:10:10	CM I	PI/PE = 2.3
80:10:10	CM II	PI/PE = 2.3
10:80:10	octylagarose	PI/PC = 1.2
10:80:10	CM II	PI/PC = 1.3
PC:PE		
50:50[e]	octylagarose	PC/PE = 1.5
50:50	CM I	PC/PE = 1.5
50:50	CM II	PC/PE = 1.5
PC:SM (50:50)[e,f]	octylagarose	SM/PC = 0.33
PC:PA (90:10)	octylagarose	PA/PC = 1.2
PC:PG (90:10)	octylagarose	PG/PC = 1.0
PC:PS (90:10)[f]	octylagarose	PS/PC = 1.0
PC:Chol (80:20)[e,g]	octylagarose	Chol/PC = 1.1
PC:diPG (90:10)	octylagarose	diPG/PC $\leq$ 0.01

[a] Transfer activity was measured as described under Materials and Methods by using multilamellar vesicles as acceptors. [b] PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; Chol, cholesterol; diPG, diphosphatidylglycerol. Compositions are expressed as mole percent. [c] Protein fractions as described in Tables I and II. [d] Kinetic constants were calculated after 30 min as described by Zilversmit & Hughes (1976, p 222). [e] The acceptor multilamellar vesicles contained 10 mol % diphosphatidylglycerol. [f] Accessibility of lipid from Berden et al. (1975). [g] Accessibility of cholesterol from Bloj & Zilversmit (1977b).

(Table IV). The similarity of phospholipid specificity of the CM I, CM II, and octylagarose fractions indicates that the small difference in amino acid composition between CM I and CM II, which gives rise to their slightly different charge characteristics, does not affect their specificity.

Specificity was also determined for unilamellar vesicles composed of phosphatidylcholine in combination with a number of individual lipids: phosphatidylglycerol, phosphatidylserine, sphingomyelin, phosphatidic acid, cholesterol, and diphosphatidylglycerol (Table IV). Only diphosphatidylglycerol transfer was not accelerated by these proteins. All other acidic phospholipids and cholesterol were transferred at about the same rate as phosphatidylcholine, while sphingomyelin exchanged at one-third this rate. Lastly, no acceleration in exchange of cholesteryl esters was found between low-density lipoprotein and high-density lipoprotein, and no exchange of glycerol trioleate occurred between unilamellar vesicles and either mitochondria or multilamellar vesicles.

Discussion

The primary purpose of the present investigation was the purification of a nonspecific protein which could be prepared in large quantities. The purification of a nonspecific protein from rat liver has been described (Bloj & Zilversmit, 1977a) but suffers from the difficulty involved in isolating it in large amounts. It was found in assaying a number of sources that the pH 5.1 supernatant from beef liver contained an appreciable amount of phosphatidylethanolamine exchange activity. By analogy to the rat liver protein, this activity may be due to a nonspecific exchange protein. This activity was stable for more than 6 months at −20 °C, and it was therefore convenient to freeze the pH 5.1 supernatant from one beef liver which was thawed and exchange protein purified as needed. The phosphatidylethanolamine exchange activity in the pH 5.1 supernatant was found to bind to CM-cellulose at low ionic strength and could be eluted by increasing the ionic strength of the buffer. This is consistent with a basic isoelectric point as found for the nonspecific exchange proteins from rat liver (Bloj & Zilversmit, 1977a). The exchange activity eluted from the CM-cellulose column was also stable to heat treatment as was found for that of the rat liver proteins. It can be further purified on an octylagarose column by application in high ionic strength buffer and elution in low ionic strength buffer at pH 3. Though the specific activity after this step was found to vary by as much as a factor of 3–4, in different preparations, it appeared as a single band by electrophoresis on polyacrylamide gels in the presence or absence of sodium dodecyl sulfate. One explanation for a variation in specific activity of a pure protein is that a partial loss in activity occurs due to partial denaturation or aggregation while no loss of protein occurs. This is supported by our finding that preparations of pure protein varying by a factor of 2–4 in specific activity have identical amino acid compositions. The exchange activity found in the pooled octylagarose fractions is stable for greater than 2 months. However, some activity is lost on dialysis against low ionic strength buffer (5 mM phosphate buffer), and the protein stored in low ionic strength buffer at 4 °C loses half of its exchange activity in about 4 weeks.

The pooled octylagarose fractions can be further separated into two proteins by CM-cellulose chromatography (Figure 1), without a significant change in the specific activity (Table II). The two proteins show identical mobilities on sodium dodecyl sulfate–polyacrylamide gels and cannot be clearly separated on cationic gels. Amino acid analysis reveals a striking similarity between these two proteins (Table III). They are also remarkably similar to the CM II nonspecific protein from rat liver (Bloj et al., 1978). The CM I fraction is stable for more than 2 months at 4 °C while the CM II fraction loses half of its activity in 1 month. Dialysis of these dilute protein solutions against low ionic strength buffer results in complete loss of exchange activity and protein. Loss of protein can be prevented by including 0.1% $NaDodSO_4$ in the dialysate.

Isoelectric focusing reveals that these two proteins have slightly different isoelectric points, 9.55 for CM I and 9.75 for CM II. These values are consistent with the amino acid composition, since CM II contains an arginine which is absent in CM I. At pH 9.55 this could contribute a positive charge and could therefore result in a slightly higher isoelectric point (about 0.2 unit assuming a pK_a of 10.5 for the 17 lysines and a pK_a of 9.5 for the N-terminal amino acid). The presence of two proteins of slightly different charge characteristics has been noted before for the exchange proteins isolated from beef brain (Helmkamp et al., 1974), beef heart (DiCorleto et al., 1979), and rat liver (Bloj & Zilversmit, 1977a). In the former two cases the amino acid composition was also shown to be quite similar for the two proteins. The possible physiological significance of this is unknown.

The molecular weight of the two nonspecific proteins from beef liver determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis is 14 500, while the molecular weight determined by gel filtration is 13 600. Both of these values are in good agreement with the molecular weights of 13 900 and 14 200 determined for CM I and CM II, respectively, based on their amino acid compositions. These values are quite similar to the molecular weight of 13 000 for the nonspecific exchange protein from rat liver (Bloj & Zilversmit, 1977a). Phosphate analysis of a chloroform–methanol extract of the protein (30 nmol) after purification by octylagarose column

chromatography revealed no phospholipid (limits of detection 5 nmol) bound to the purified protein. In contrast, purified phosphatidylcholine exchange protein from beef liver has been found to contain 1 mol of phosphatidylcholine per mol of protein (Wirtz, 1974). Staining polyacrylamide gels by the periodic acid–Schiff technique (Glossman & Neville, 1971) revealed no bands, suggesting that these proteins do not contain a substantial amount of carbohydrate.

The transfer of phospholipid was normally measured in low ionic strength buffer (either SET buffer or ET buffer). It was found that increasing concentrations of NaCl had an inhibitory effect on phosphatidylcholine transfer from unilamellar vesicles to multilamellar vesicles. A linear effect of ionic strength (between 0.05 and 0.20) was found. At an ionic strength of 0.20, one-fourth as much phosphatidylcholine was transferred after 60 min compared to that transferred at an ionic strength of 0.05. This is in contrast to the characteristics of the beef heart exchange protein (Johnson & Zilversmit, 1975) which was found to be inhibited only at very high ionic strength.

The relative transfer of phospholipids and cholesterol was measured between unilamellar vesicles and multilamellar vesicles of the same composition. This transfer represents the average for the molecular species of a phospholipid. The use of multilamellar vesicles of like compositions as acceptor vesicles decreases the possibility of differences in specificity being caused by a change in the composition of the donor vesicle. The relative transfer of phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine was found to be the same for the CM I, CM II, and octylagarose protein fractions, indicating no difference in specificity between CM I and CM II. Specificity was examined in vesicles of a variety of compositions. These exchange proteins were found to exhibit the broadest specificity yet demonstrated in a purified preparation (Wirtz, 1974; Zilversmit & Hughes, 1976). They are the first purified proteins which have shown exchange activity for phosphatidic acid and phosphatidylglycerol. They were also capable of accelerating the transfer of phosphatidylserine, sphingomyelin, and cholesterol from unilamellar vesicles to multilamellar vesicles. The exchange of diphosphatidylglycerol between unilamellar vesicles and multilamellar vesicles and of cholesteryl esters between high-density lipoprotein and low-density lipoprotein failed to be stimulated by addition of protein.

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