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CATALYTIC PROPERTIES OF PHOSPHOLIPID EXCHANGE PROTEIN FROM BOVINE HEART

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SUMMARY

A protein which catalyzes the exchange of phosphatidylcholine between membranes has been purified from heart tissue homogenates up to 300-fold by acidic pH precipitation, $(NH_4)_2SO_4$ precipitation, gel filtration, and ion-exchange chromatography. Binding of the protein to phosphatidylcholine liposomes as measured by Sepharose chromatography was nondetectable. However, isoelectric focusing experiments showed that individual molecules of phosphatidylcholine were transferred from liposomes to the soluble, partially purified protein. Exchange of phospholipid between liposomes and mitochondria was not affected by the presence of moderate amounts of cholesterol in liposomes. A search for competitive inhibitors among moieties similar to phosphatidylcholine failed to show strong binding sites in the hydrophilic part of the substrate. High concentrations of Na^+, Ca^{2+} and Mg^{2+} impaired the exchange activity.

INTRODUCTION

The most abundant phospholipid present in many mammalian membranes is phosphatidylcholine. However, the enzymes for its synthesis are not uniformly distributed among cellular membranes [1]. Several workers have shown that a soluble protein in cytoplasm greatly accelerates the exchange of phospholipids between microsomes, which are rich in phospholipid synthesizing enzymes, and mitochondria, which may lack certain key enzymes [1]. A protein that catalyzes phospholipid exchange provides a possible source of phosphatidylcholine for mitochondria in vivo [2–6].This is particularly important in light of the extremely low solubility of phosphatidylcholine in aqueous media [7]. Phospholipid exchange

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proteins have been shown to facilitate phospholipid exchange with membrane fractions [2–6], serum lipoproteins [8], chylomicrons [9], artificial vesicles, liposomes [9], monolayers [10] and phosphorylating vesicles containing mitochondrial proteins [11]. Similar proteins have been found in several tissues including heart [12], liver [2–4], brain [13], cauliflower and potato [6].

In the present work phospholipid exchange protein is purified from bovine heart and some of the phospholipid exchange characteristics are presented.

MATERIALS AND METHODS

Chemicals

Dicetylphosphate, choline chloride and *N*-ethylmaleimide were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio). Phosphorylcholine was purchased from Sigma Chemical Co. (St. Louis, Mo.). All of the foregoing were used without further purification. *sn*-Glycerol-3-phosphorylcholine was obtained as the $CdCl_2$ complex from Pierce Chemical (Rockford, Ill.) Cadmium was removed from the dissolved salt by adjusting the pH to 11, centrifuging the solution and retitrating the supernatant to neutrality.

Radioactive lipids

Labeled phosphatidylcholine was prepared by injecting rats intraperitoneally with 800 μCi $^{32}P_i$ in normal saline 16 h before sacrifice. Their livers were diced and extracted with chloroform–methanol (2:1, by vol.) and the extract was washed by the method of Folch et al. [14]. Phospholipid classes were separated from the extract by silica gel H thin-layer chromatography with chloroform–methanol–acetic acid–water (25:15:4:2, by vol.). The bands were visualized with Ultraphor [15], scraped and eluted with chloroform–methanol–water (80:35:5, by vol.) Larger amounts of labeled phosphatidylcholine were prepared from rat liver extracts with water-cooled alumina columns according to a slightly modified procedure of Singleton et al. [16]. Neutral alumina (activity I, E.M. Laboratories, Elmsford, N. Y.) required chloroform–methanol (9:1, by vol.) plus 0.5 % concentrated NH_4OH for satisfactory elution of phosphatidylcholine. [^{14}C] Triolein was obtained from Applied Science Laboratories (State College, Pa.) and purified by silica gel H thin-layer chromatography with hexane–diethyl ether–acetic acid (60:40:1, by vol.). The triolein band was eluted with chloroform. Radioactivity was determined in a Packard scintillation counter in the medium of Gordon and Wolfe [17].

Liposomes

To [^{32}P] phosphatidylcholine, present in eluates from silica gel or alumina, were added 10 % butylated hydroxytoluene and a trace amount of [^{14}C] triolein. The solvent was evaporated at 40 °C under a stream of N_2. The lipid was redissolved in a test tube with diethyl ether and redried which left a thin lipid film. Buffer, containing 0.25 M sucrose, 1 mM EDTA, and 0.05 M Tris–HCl (pH 7.4) (sucrose–EDTA–Tris–HCl buffer) was added to a final lipid concentration of 1.67 mg/ml. The suspension was mixed vigorously for 10 min and the lipid was allowed to swell for 1 h under a N_2 atmosphere. The test tube containing the suspension was then immersed in a Branson HD-50 sonicating bath and sonicated at 25 °C for 30 min.

Mitochondria

Beef heart mitochondria were prepared according to the procedure of Green et al. [18]. They were subsequently washed twice by centrifugation in sucrose–EDTA–Tris–HCl buffer and stored at −20 °C. Upon thawing, they were rewashed once before use.

Assay method

Exchange activity was determined by measuring the transfer of [^{32}P] phosphatidylcholine from artificial vesicles to mitochondria. Liposomes (10 μg phospholipid phosphorus) were incubated with mitochondria (12.5 mg mitochondrial protein) and an appropriate aliquot of the exchange protein in a total volume of 4 ml of sucrose–EDTA–Tris–HCl buffer. Incubation was carried out for 40 min at 37 °C with constant agitation. At the end of the incubation, exchange was stopped by chilling and sedimenting the mitochondria at 15 000 × *g* for 15 min. Aliquots of the supernatant, which contained liposomes, were counted in the scintillation medium of Gordon and Wolfe [17]. The [^{14}C] triolein trace in the liposomes served as a nonexchangeable reference marker so that the decrease in the ^{32}P/^{14}C ratio during incubation measured the transfer of [^{32}P] phosphatidylcholine. One unit of activity is defined as the transfer of 1 % of the labeled phosphatidylcholine during a 40-min incubation period. The small amount of [^{32}P] phosphatidylcholine which transfers in the absence of exchange protein was subtracted as background.

Purification of phospholipid exchange protein

All manipulations during the isolation were carried out at 4 °C. Beef hearts were homogenized in four volumes of 0.25 M sucrose, 20 mM potassium phosphate and 10 mM EDTA (18). The results are given in Table 1.

Acid pH step. The post-mitochondrial supernatant was adjusted to pH 5.1 with 3 M HCl to flocculate microsomes and other components. After 1 h the precipitate was sedimented by centrifugation (15 min at 10 000 × *g*) and discarded. The pH of the supernatant was readjusted to 7.4 with 18 M NaOH. This fraction was stored at −70 °C for up to 4 months with very little loss of activity.

$(NH_4)_2SO_4$ step. Solid $(NH_4)_2SO_4$ was used to adjust 900 ml of the supernatant to 90 % sat and the mixture was stirred overnight. The precipitate was sedimented by centrifugation and transferred to dialysis tubing with a minimal volume of water. It was dialyzed against 40 vol. of water with at least two changes of medium during 40 h.

TABLE I

PURIFICATION OF PHOSPHOLIPID EXCHANGE PROTEIN

Average from 25 to 30 preparations for Step 1–3; 15 preparations for Step 4.
Specific activity is expressed as units of phospholipid exchange activity per mg of protein.

Step	Volume ml	Protein mg	Spec. act.	Recovery %	Purification Factor
1. pH 5.1 supernatant	900	8416	3.9	100	—
2. $(NH_4)_2SO_4$ precipitation	120	4966	5.7	89	1.5
3. Sephadex G-75 eluate	188	204	44.7	31	11.5
4. CM-cellulose eluate	202	6	818	13	210

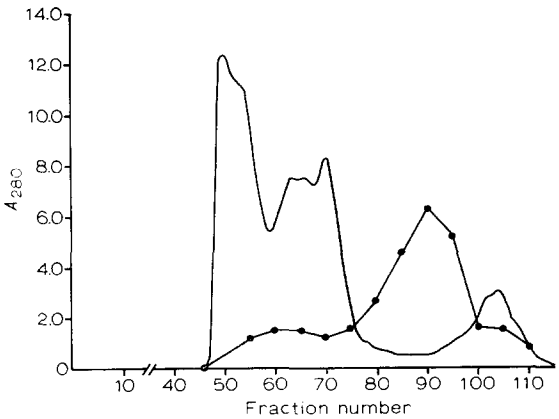

Fig. 1. Elution profile for Sephadex G-75 columns. About 5 g of protein were applied to the first of two columns connected in series. Flow rate was 45 ml/h and 15-ml fractions were collected. , absorbance at 280 nm; ● ··· ●, exchange activity in arbitrary units. Fractions 80–95 were pooled for concentration.

Sephadex step. The dialyzed protein (about 5 g) was loaded onto a Sephadex G-75 column (5 cm × 85 cm) which was connected in series with a second Sephadex G-75 column (5 cm × 47 cm) to increase the resolution. The columns were eluted at 2 ml/cm^2/h with 0.042 M Tris–acetate buffer, (pH 7.4), containing 5 mM β-mercaptoethanol, the same buffer used to pre-equilibrate the columns. A typical elution profile is shown in Fig. 1. The fractions containing exchange activity were pooled and concentrated to 15 ml with an Amicon ultrafiltration cell and a PM-10 membrane.

Carboxymethyl cellulose step. The concentrated protein was titrated to pH 6.0 with dilute acetic acid and applied to a 3.8 cm × 45 cm column which contained

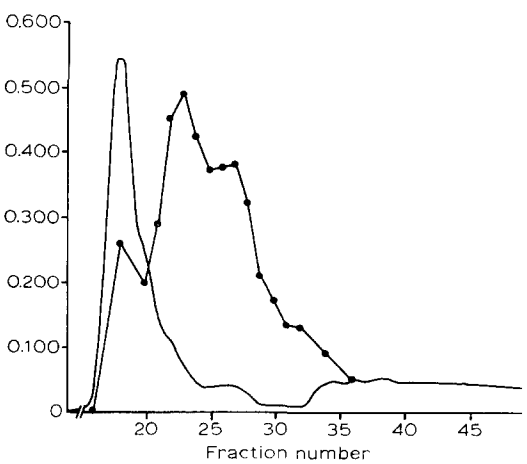

Fig. 2. Elution profile for carboxymethylcellulose column. The concentrated protein from Sephadex G-75 was applied to a column containing carboxymethylcellulose equilibrated with 0.042 Tris titrated to pH 6.0 with acetic acid. It was eluted with the same buffer at a flow rate of 45 ml/h and 15-ml fractions were collected. —, absorbance at 280 nm; ● — ●, exchange activity in arbitrary units. Fractions 23–36 were pooled for concentration.

carboxymethylcellulose (CM-52 microgranular, Whatman) in 0.042 M Tris acetate (pH 6.0), 5 mM β-mercaptoethanol. The column was eluted with the same buffer and the active fractions were pooled and concentrated by ultrafiltration on a PM-10 membrane. A typical elution profile is given in Fig. 2. The pooled protein solution showed a purification of about 300-fold over the post-microsomal supernatant. It contained two active protein fractions after isoelectric focusing. The two active proteins were found to be distributed equally in different portions of the eluate from the carboxymethylcellulose column.

RESULTS

Binding

Binding of the protein to membrane was investigated by gel filtration. Phospholipid exchange protein was chromatographed with liposomes on Sepharose 6B. This gel largely excludes sonicated liposomes but includes small proteins, which elute near its total volume. The exchange protein fraction used for this particular experiment contained at least six additional extraneous protein species as determined by polyacrylamide-gel electrophoresis. These proteins had been eluted in a single peak from Sephadex G-75 and were therefore of a size similar to that of the active proteins. Therefore during chromatography on Sepharose 6 B a shift of phospholipid exchange activity away from these extraneous proteins in the direction of the liposomes should indicate affinity of exchange protein for liposomes with a low degree of reversibility.

Sepharose 6B was packed in a 1.3 cm × 87 cm column in 0.15 M potassium phosphate buffer (pH 7.4), and the bed volume was stabilized over 24 h with a constant flow rate of 4.8 ml/h. The lipid binding sites in the Sepharose were saturated by passing 2.5 ml of a 1.5 mg/ml labeled liposome suspension through the column during the stabilization period.

A second aliquot of the same liposomes was added to phospholipid exchange protein in phosphate buffer. The mixture containing 3.7 mg of liposomal phospholipid and 940 units of phospholipid exchange protein (6 mg protein) was chromatographed at 4.8 ml/h on the Sepharose 6B column, and 2-ml fractions were collected. Radioactivity and the absorbance at 280 nm were determined in the eluted samples. The exchange activity of the fractions was measured by incubating aliquots containing 75 μg of liposomal phospholipid with mitochondria (12.5 mg protein) for 40 min in 4 ml of phosphate buffer. Eluted fractions which contained no lipid were assayed by the same procedure with added liposomes.

The liposomes eluted in a double peak due to the fact that the liposome sizes spanned the exclusion limits for Sepharose 6 B (Fig. 3). The double character disappears in gels of larger pore size such as Sepharose 4B, but proteins and liposomes did not separate well in that gel. No exchange activity was found associated with the liposomal fractions. The total protein and the exchange activity peaks were very nearly coincident, indicating that binding to the liposome membranes was weak or absent. It is unlikely that exchange protein was bound to liposomes in an inactive form since 102% of the phospholipid exchange activity was recovered in the protein peak*. Although individual molecules of phospholipid may have eluted with

* The assays for phospholipid exchange activity were done with 75 μg of liposomal phospholipid rather than with 250 μg as in the standard assay. We used an experimentally determined factor of 0.85 to convert phospholipid exchange activity to standard units.

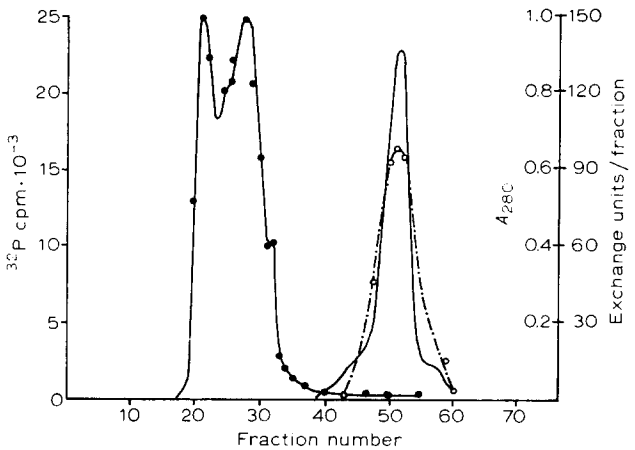

Fig. 3. Binding of liposomes and phospholipid exchange protein studied with agarose chromatography. [^{32}P]Phosphatidylcholine liposomes were chromatographed with phospholipid exchange protein at a flow rate of 4.8 ml/h and 2-ml fractions were collected. ●—●, ^{32}P cpm; ○- - -○, exchange activity; —, absorbance at 280 nm.

the exchange protein, these were not detectable with the amounts of phospholipid exchange protein and specific activity of phospholipid employed.

The possibility that individual molecules of phospholipid bind to the exchange protein was investigated with isoelectric focusing because it was technically feasible to handle very small amounts of highly radioactive lipid in this system. High specific activity [^{32}P] phosphatidylcholine was prepared by injecting a rat with six times the usual dose of $^{32}P_i$. Sonicated liposomes were prepared from liver phosphatidylcholine according to the procedure in Materials and Methods. However, 1 mole of dicetylphosphate per 10 moles of phospholipid was included and the trace of labeled triolein was increased 6-fold. The inclusion of dicetylphosphate in the otherwise neutral liposomes resulted in an acidic isoelectric point for the particles and allowed them to be focused. These liposomes (75 μg of phospholipid) plus 4800 units of active exchange protein (5mg protein) were included in the light density solution used in preparing a sucrose gradient for isoelectric focusing. The 110-ml column (LKB Produktur, Stockholm) contained 2 ml of 40 % pH 4–6 ampholine and 0.5 ml of 40 % pH 5–8 ampholine. After 40 h of focusing (400 V and 15 °C) the column was drained and 2-ml fractions were collected. Aliquots were assayed for phospholipid exchange protein activity and ^{32}P and ^{14}C radioactivities in each fraction were determined.

The acidic liposomes focused near the anode at the ampholine–H_2SO_4 interface (Fig. 4). Since the peak of radioactivity still had a tail toward the less acidic fractions, the liposomes may not have focused completely. The phospholipid exchange protein focused in two peaks at pH 4.5–4.7 and 4.8–5.1. These values are slightly lower than those reported by Ehnholm and Zilversmit [19] for this protein in the absence of added phospholipid. Two peaks of [^{32}P] phospholipid coincided precisely with the two peaks of phospholipid exchange protein. Since there was no [^{14}C] triolein associated with these peaks, they represented molecular phospholipid derived from liposomes rather than intact liposomes. The peak representing the larger exchange activity (pH 4.5–4.7) contained the greatest amount of phosphatidylcholine

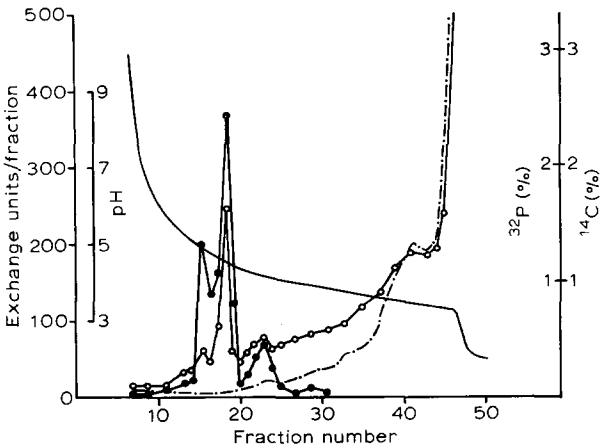

Fig. 4. Isoelectric focusing of exchange protein with radioactive lipids. Liposomes containing dicetylphosphate, [^{32}P]phosphatidylcholine and [^{14}C]triolein were added to phospholipid exchange protein and focused for 40 h. ● — ●, exchange activity; ○ — ○, ^{32}P cpm as percent of total; - . - ., ^{14}C cpm as percent of total; —, pH.

(3.7 p moles of phosphatide/unit of exchange activity). An additional exchange peak (pH 4.2–4.5) followed the two main peaks, and phospholipid was also associated with it. Apparently, one or more molecules of phosphatidylcholine were transferred from the liposomes to the protein and thereby held in solution.

Liposomes with cholesterol

Fatty acid moieties and polar groups of phosphatidylcholine interact intimately with cholesterol resulting in a tightened or condensed membrane. Therefore we have examined possible effects of membrane cholesterol on the catalyzed exchange of phosphatidylcholine.

Liposomes were made as outlined in Materials and Methods except that cholesterol was added in proportions to give liposomes with different mole ratios of cholesterol to phosphatidylcholine : 0.2:1, 0.4:1, 1:1 and a control without cholesterol. Exchange of [^{32}P] phosphatidylcholine was measured in incubations of these liposomes, (10 μg of phospholipid phosphorus) with mitochondria (12.5 mg mitochondrial protein) in the presence of phospholipid exchange protein (20 units) for different periods of time. The exchange was calculated from the decrease in [^{32}P] phosphatidylcholine to [^{14}C] triolein ratio of the liposomes. The exchange was independent of membrane cholesterol content up to cholesterol–phosphatidylcholine ratios of 0.4:1.0 (Fig. 5). At a ratio of 1:1, some decrease in exchange activity was noted.

Ionic interactions

Many enzymes which act on phosphodiesters interact with cations, particularly divalent metal ions.

The effects of three salts, NaCl, $MgCl_2$ and $CaCl_2$ on the exchange reaction were tested. A heavy metal ion, Cd^{2+}, gave irreversibly inactive complexes or precipitates.

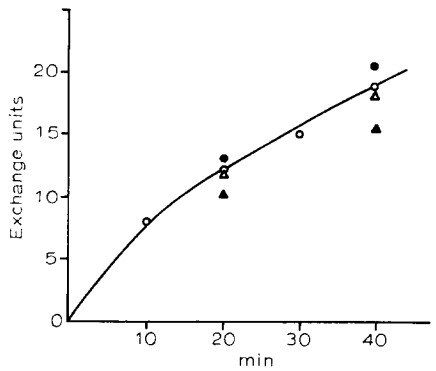

Fig. 5. Effect of membrane cholesterol on phospholipid exchange. Phospholipid exchange protein (20 units) was used to catalyze exchange between mitochondria (12.5 mg protein) and ^{32}P-labeled liposomes (10 μg phospholipid phosphorus) which contained the following amounts of cholesterol to phosphatidylcholine molar ratios: 0 : 1 (○), 0.2 : 1 (●), 0.4 : 1 (△) and 1 : 1 (▲). Each point is the average of two results: mean % difference between duplicates = 3.7 ± 3.3 (S.D.).

Phosphatidylcholine liposomes (10 μg phospholipid phosphorus) were incubated with mitochondria (12.5 mg mitochondrial protein) in the presence of exchange protein (pH 5.1 supernatant, Materials and Methods) in sucrose–Tris–HCl buffer but without EDTA. Increasing concentrations of NaCl, $MgCl_2$ or $CaCl_2$ were added to individual samples at the beginning of the incubation period. No special effects of divalent cations relative to monovalent cations were observed (Fig. 6). Small concentrations of each cation were without effect while large changes in ionic strength of the medium reduced the phosphatidylcholine exchange rate. It would appear that ionic interactions between the protein and its substrate do occur since "ionic strength shielding" reduces the phospholipid exchange activity.

Inhibitors

Phospholipid exchange proteins show specificity based on the composition of

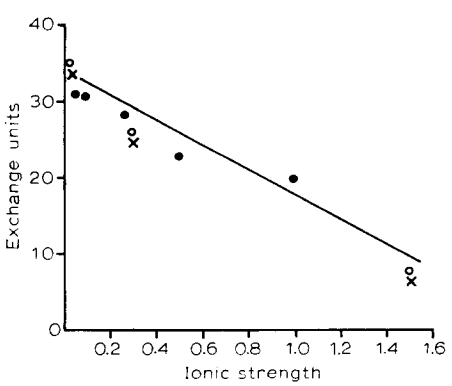

Fig. 6. Effect of salts on phospholipid exchange. Labeled liposomes were incubated with mitochondria and phospholipid exchange protein in the presence of NaCl (●), $CaCl_2$ (○) or $MgCl_2$ ( ) at various concentrations. Concentrations are expressed as ionic strength.

the polar end of the phosphatides but not on the apolar end [20]. Polar interactions of substrate and protein were studied by the use of substrate analogues as potential competitive inhibitors.

The substrate analogues used, such as choline chloride, phosphorylcholine, phosphate, *sn*-glycerol-3-phosphorylcholine, and lysophosphatidylcholine, were related to phosphatidylcholine and were essentially hydrolytic fragments of the parent molecule. In each case liposomes were incubated with mitochondria in the presence of purified protein under standard assay conditions. The substrate analogues were included in increasing amounts to give concentrations up to 50 mM (600 times that of [^{32}P] phosphatidylcholine). In the case of lysophosphatidylcholine, which dissolved phospholipid membranes at high concentrations, concentrations up to 39 μM were tested. In addition, phosphate buffer (0.15 M) was used in place of Tris–HCl in some experiments. None of these analogues produced a significant reduction of phosphatidylcholine exchange.

It was possible to inhibit the protein with *N*-ethylmaleimide, as has been found for liver phosphatidylcholine exchange protein [8]. The phospholipid exchange activity of purified beef heart exchange protein was almost completely inhibited, i.e. from 11.8 units of exchange activity to 0.1 units, by 2.5 mM *N*-ethylmaleimide. The more crude protein preparations were not so strongly inhibited, i.e. from 14.5 to 1.5 units.

DISCUSSION

The finding that the active protein binds to phosphatidylcholine liposomes weakly, if at all, suggests that it is probably not concentrated in the vicinity of membranes during phospholipid exchange. Instead, the finding that the exchange protein solubilizes individual molecules of phosphatidylcholine from liposomes is compatible with a carrier mechanism for the exchange reaction. Kamp et al. [21] found that pure phosphatidylcholine could be extracted from purified phospholipid exchange protein derived from liver. Workers in that laboratory further found that the mole ratio of lipid to purified liver protein was 1:1 and that the protein could remove lipid from and insert it into monolayers [10].

Studies of Ehnholm and Zilversmit [19] and of Kagawa et al. [11] have shown that this protein is capable of a net transfer of phospholipid from one type of particle to another, as well as a one-for-one exchange between membranes. If this net transfer is carried out efficiently by the soluble lipid-protein complexes, the binding of phospholipid to exchange protein should be of the same order of magnitude as that of phospholipid to membrane.

Cholesterol is thought to decrease the mobility of fatty acid chains in membranes orienting them toward the perpendicular [22], and having a condensing or tightening effect on the membrane [23, 24]. Such interactions might be expected to decrease the ease of removal or insertion of phosphatidylcholine into the membrane by phospholipid exchange proteins. In the present study such effects were observed only at high cholesterol to phospholipid ratios (1:1), but not at ratios of 0.4:1 or less. One cannot ascribe the decreased exchange at high cholesterol/phospholipid ratios to a condensing effect of cholesterol on the phospholipid bilayer unless one can eliminate possible effects of cholesterol on the formation of multilayered vesicles in

which a portion of the phospholipid might be unavailable for exchange. Furthermore, one has to ascertain that at lower cholesterol to phospholipid ratios, when no effect on exchange was observed, the cholesterol does not leave the vesicle in favor of the mitochondria. Phospholipid exchange protein does not catalyze the transfer of cholesterol, but cholesterol exchanges rapidly without added protein [19]. In an experiment in which cholesterol was determined chemically, no evidence for net transfer of cholesterol from liposomes to mitochondria was observed.

As seen in the electrofocusing experiment, the nonexchangeable triglyceride remains with the liposomes, whereas phosphatidylcholine is transferred to phospholipid exchange protein. Hence, the exchange protein appears to remove individual phospholipid molecules from the liposomes rather than large fragments of bilayer membrane.

It was somewhat surprising that none of the hydrolytic fragments of phosphatidylcholine inhibited the exchange protein. This protein shows specificity for the hydrophilic part of the phospholipid molecule, since it transfers phosphatidylcholine and sphingomyelin, but not phosphatidylethanolamine [11, 19]. Apparently the choline moiety is essential for recognition, but free choline, phosphorylcholine or *sn*-glycerol-3-phosphorylcholine do not appear to compete for presumed sites that normally interact with covalently bound choline of phosphatides.

We have extended the finding of Illingworth and Portman [8] that *N*-ethylmaleimide inactivates liver proteins that promote phospholipid exchange between serum lipoproteins. This sulfhydril reagent also inactivates purified phospholipid exchange protein from bovine heart tissue. The possible involvement of sulfhydril groups also fits the observation that the phospholipid exchange reaction is sensitive to Cd^{2+}.

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REFERENCES

1 Wilgram, G. F. and Kennedy, E. P. (1963) J. Biol. Chem. 238, 2615–2619
2 Wirtz, K. W. A. and Zilversmit, D. B. (1968) J. Biol. Chem. 243, 3596–3602
3 McMurray, W. C. and Dawson, R. M. C. (1969) Biochem. J. 112, 91–108
4 Akiyama, M. and Sakagami, T. (1969) Biochim. Biophys. Acta 187, 105–112
5 Wojtczak, L., Baranska, J., Zborowski, J. and Drahota, Z. (1971) Biochim. Biophys. Acta 249, 41–52
6 Abdelkader, A. B. and Mazliak, P. (1970) Eur. J. Biochem. 15, 250–262
7 Smith, R. and Tanford, C. (1972) J. Mol. Biol. 67, 75–83
8 Illingworth, D. R. and Portman, O. W. (1972) Biochim. Biophys. Acta 280, 281–289
9 Zilversmit, D. B. (1971) J. Biol. Chem. 246, 2645–2649

10 Demel, R. A., Wirtz, K. W. A., Kamp, H. H., Geurts van Kessel, W. S. M. and van Deenen, L. L. M. (1973) Nat. New Biol. 246, 102–105
11 Kagawa, Y., Johnson, L. W. and Racker, E. (1973) Biochem. Biophys. Res. Commun. 50, 245–251
12 Wirtz, K. W. A. and Zilversmit, D. B. (1970) FEBS Lett. 7, 44–46
13 Miller, E. K. and Dawson, R. M. C. (1972) Biochem. J. 126, 823–835
14 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509
15 Copius Peereboom, J. W. (1960) J. Chromatog. 4, 323–328
16 Singleton, W. S., Gray, M. S., Brown, M. L. and White, J. L. (1965) J. Am. Oil Chem. Soc. 42, 52–56
17 Gordon, C. F. and Wolfe, A. L. (1960) Anal. Chem. 32, 574
18 Green, D. E., Lester, R. L. and Ziegler, D. M. (1957) Biochim. Biophys. Acta 23, 516–524
19 Ehnholm, C. and Zilversmit, D. B. (1973) J. Biol. Chem. 248, 1719–1724
20 Wirtz, K. W. A., van Golde, L. M. G. and van Deenen, L. L. M. (1970) Biochim. Biophys. Acta 218, 176–179
21 Kamp, H. H., Wirtz, K. W. A. and van Deenen, L. L. M. (1973) Biochim. Biophys. Acta 318, 313–325
22 Lapper, R. D., Paterson, S. J. and Smith, I. C. P. (1972) Can. J. Biochem. 50, 969–981
23 Chapman, D., Owens, N. F., Phillips, M. C. and Walker, D. A. (1969) Biochim. Biophys. Acta 183, 458–465
24 Demel, R. A., Geurts van Kessel, W. S. M. and van Deenen, L. L. M. (1972) Biochim. Biophys. Acta 266, 26–40