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# Protein-Catalyzed Exchange of Phosphatidylcholine between Sonicated Liposomes and Multilamellar Vesicles†

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ABSTRACT: Phospholipid exchange protein from beef heart or beef liver does not catalyze the transfer of phosphatidylcholine from multilamellar vesicles of phosphatidylcholine. Certain combinations of phospholipids, however, do yield multilamellar vesicles that will exchange phosphatidylcholine with liposomes in the presence of exchange protein. Multilamellar vesicles of phosphatidylcholine:phosphatidylethanolamine:cardiolipin (70:25:5, mol %) can be used in place of mitochondria or erythrocyte ghosts as an improved acceptor particle in the study of liposome structure with phospholipid exchange proteins. These multilamellar vesicles act as a well-defined reservoir of unlabeled phosphatidylcholine with 7% exchangeable phospholipid. When the distribution of phosphatidylcholine in liposomes is studied by the exchange protein technique, results can be influenced by the choice of phospholipid acceptor particle. With mitochondria as acceptor particle, the percentage of phosphatidylcholine in the outer monolayer of a liposome appears to be 60%, whereas a value of 70% is obtained when multilamellar vesicles are the acceptor. The discrepancy can be explained by a heterogeneity in liposomes prepared by sonication. A size-dependent fusion or adsorption process occurs between liposomes and mitochondria; the very small liposomal vesicles, obtained by gel filtration, combine nearly quantitatively with the natural membrane. This phenomenon is not seen with multilamellar vesicles. Thus by using multilamellar vesicles one obtains a less biased estimate of phospholipid distribution between inner and outer layers of liposomes.

Proteins that catalyze the transfer of phospholipid molecules between membranes have been isolated and characterized from several sources (for review, see Zilversmit and Hughes, 1976). Recently, these phospholipid exchange proteins have been employed in several laboratories as membrane probes in the study of phospholipid distribution and motion in liposomes—sonicated, unilamellar vesicles (Johnson et al., 1975; Rothman and Dawidowicz, 1975) and natural membranes (Bloj and Zilversmit, 1976; Rothman et al., 1976; Rousselet et al., 1976). The value of the technique lies in the high degree of sensitivity of radioisotope monitoring and the apparently minimal perturbation of the bilayer. The transposition (flip-flop) rate of PC[1] in liposomes has been determined with this technique in two laboratories by the use of radioactive PC liposomes as donor particles and mitochondria (Johnson et al., 1975) or erythrocyte ghosts (Rothman and Dawidowicz, 1975) as acceptor particles. The kinetics of catalyzed replacement of donor

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[1] Abbreviations used: PC, phosphatidylcholine; CL, cardiolipin; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid; $P_i$, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

PC with acceptor PC can be analyzed to obtain values for the fraction of PC in the outer monolayer of the liposome, and the rate of PC flip-flop.

Two suppositions underlie the exchange protein technique as a nonperturbing method for studying liposomes. One assumption is that a head group specific, one-for-one, phospholipid exchange process is occurring and that the acceptor particle is an inert reservoir of the necessary phospholipid. In the case of natural membranes, such as the erythrocyte ghost, which contain such freely transferable lipids as lysophospholipids and cholesterol (Bruckdorfer et al., 1968) and potentially transferable molecules as fatty acids and hydrophobic proteins, this assumption may be a dangerous one. The liposome structure under study may be altered to an indeterminable degree by the acquisition of foreign molecules during the incubation with exchange protein. A second drawback to the use of natural membranes as acceptor particles is the apparent physical interaction between them and the liposomes. Upon separation of the donor and acceptor particles by centrifugation, recovery of the liposome in the supernatant is not quantitative. The second supposition that is made, then, is that the liposomes are a homogeneous population such that an aliquot of the recovered liposomes is representative for subsequent analysis.

We report here on a new, well-defined, acceptor particle, multilamellar phospholipid vesicles, the use of which removes the need for the above suppositions. These vesicles have been well characterized in terms of size, structure, and permeability by Bangham and co-workers (for review, see Bangham et al., 1974) and have been referred to in the literature as hydrated liquid crystals (Papahadjopoulos and Miller, 1967), unsonicated vesicles (DeKruijff et al., 1975), and hand-shaken liposomes (Wirtz et al., 1976). These multilamellar vesicles are separable from liposomes by centrifugation at low speed. Experiments showing the advantages of multilamellar vesicles as acceptor particles are presented.

## Materials and Methods

*Lipids.* Highest purity cardiolipin (CL) from beef heart and phosphatidylinositol (PI) and phosphatidylethanolamine (PE) from bovine brain were obtained from Avanti Biochemicals (Birmingham, Ala.) and used without further purification. Nonradioactive soy phosphatidylcholine (PC) from Nattermann (Köln, Germany) was repurified by neutral alumina chromatography according to a slightly modified procedure of Singleton et al. (1965) to remove traces of lysolecithin. Phosphatidic acid (PA) was prepared from the soy PC by the action of phospholipase D according to the procedure of Yang (1969) and purified on silica gel H with the solvent system $CHCl_3$–methanol–acetic acid–water (80:13:8:0.3, v/v/v/v). All phospholipids exhibited a single spot by thin-layer chromatography on silica gel H with the solvent system $CHCl_3$–methanol–acetic acid–water (25:15:4:2, v/v/v/v), and were stored under $N_2$ at −20 °C. $^{32}P$-labeled PC, PE, and CL were isolated from livers of rats injected with 1 mCi/100 g of body weight of $^{32}P_i$ (New England Nuclear, Boston, Mass.) 16 h before sacrifice. A liver lipid extract was prepared and the phospholipids were separated by chromatography on alumina ("neutral", Woelm, Eschwego, West Germany) as described by Luthra and Sheltawy (1972). Total phospholipids from a liver were separated on 25 g of alumina. The PC fractions were further purified on a second neutral alumina column as described above for soy PC. Cardiolipin was further purified by column chromatography on silicic acid (Mallinckrodt, St. Louis, Mo.) by elution with $CHCl_3$–methanol (19:1, v/v). The radiopurity of the phospholipids, checked by thin-layer chromatography on silica gel H with the solvent system $CHCl_3$–methanol–acetic acid–water (25:15:4:2, v/v/v/v), was greater than 98%. The specific activity of the lipids ranged between 0.1 and 0.3 μCi/mg.

Cetyltrimethylammonium bromide was obtained from K & K Laboratories, Inc. (Plainview, N.Y.). Cholesterol from Sigma Chemical Co. (St. Louis, Mo.) was purified as the dibromide derivative, crystallized from methanol, and stored at 4 °C in ethanol. Glycerol 9,10-[$^3$H]trioleate (397 mCi/mmol; [$^3$H]triolein) was obtained from Amersham/Searle (Arlington Heights, Ill.) and purified by silica gel H thin-layer chromatography with hexane–diethyl ether–acetic acid (60:40:1, v/v/v). The triolein band was eluted with chloroform and stored at −20 °C. Lipid phosphorus was determined by the method of Bartlett (1959).

*Phospholipid Exchange Proteins.* Phospholipid exchange protein from beef heart cytosol was purified and assayed according to Johnson and Zilversmit (1975). Beef liver phospholipid exchange protein was isolated according to Kamp et al. (1973). One unit of phospholipid exchange protein activity is defined as 1 nmol of PC transferred/min at 37 °C (Zilversmit and Hughes, 1976).

*Mitochondria.* Beef heart mitochondria were prepared as described previously (Johnson and Zilversmit, 1975) and stored at −20 °C. Upon thawing they were heated for 20 min at 80 °C to destroy lipolytic activity (Zilversmit and Hughes, 1976) and washed with incubation buffer before use. Mitochondrial protein concentration was determined by the biuret reaction (Gornall et al., 1949).

*Preparation of Multilamellar Vesicles.* These vesicles were prepared essentially as described by Bangham et al. (1965). Appropriate aliquots of lipids and antioxidant (0.1 mol % butylated hydroxytoluene, Nutritional Biochemicals, Cleveland, Ohio) in organic solvent were combined in a round-bottomed flask. A trace of [$^3$H]triolein was added as a nontransferable marker where appropriate (Zilversmit and Hughes, 1976). The solvent was removed by rotary evaporation in vacuo. The lipid was redissolved in diethyl ether, and subsequent rotary evaporation yielded a thin lipid film on the walls of the flask. Tris-HCl (pH 7.4; 50 mM)–$Na_2EDTA$ (5 mM) (Tris–EDTA buffer) was added to give a dispersion of 10 mg of lipid/mL of buffer. The flask was swirled by hand until all lipid was freed from the sides of the flask and all large lipid aggregates were dispersed. The milky solution was allowed to stand for 2 h at room temperature before use. The vesicles could be stored under nitrogen for several days at 4 °C with no apparent changes.

*Preparation of Liposomes.* Lipids and butylated hydroxytoluene (0.1 mol %) were mixed in organic solvent in a screw cap test tube. A trace of [$^3$H]triolein (less than 0.1 mol %) was added as a nontransferable marker where appropriate (Zilversmit and Hughes, 1976). The solvent was evaporated at 20 °C under a stream of nitrogen. The lipid was redissolved with diethyl ether and redried leaving a thin lipid film. Tris–EDTA was added to a final lipid concentration of 1–10 mg/mL. The suspension was placed under $N_2$ and mixed vigorously. Sonication was then performed in a bath-type sonifier (Lab Supplies Co., Hicksville, N.Y.) at 10–15 °C for 30 min or until the sample was clear.

*Incubations: Multilamellar Vesicles as Donor Particles.* Multilamellar vesicles containing [$^{32}$P]PC, [$^3$H]triolein, and other additives were prepared as described above. Before incubation the vesicles were centrifuged at 40 000*g* for 15 min at 4 °C and the pellet was gently resuspended. The incubation

consisted of multilamellar vesicles, liposomes, and phospholipid exchange protein in Tris–EDTA buffer at 37 °C. Concentrations are described in figure and table legends. The transfer reaction was halted by placing the mix on ice for 5 min, followed by centrifugation as above. Radioactivity in an aliquot of the supernatant was determined in a Packard scintillation counter in the medium of Gordon and Wolfe (1960). The amount of PC transferred to acceptor liposome particles was determined from the $^{32}P$ cpm in the supernatant corrected for nonsedimentation of the multilamellar vesicles by use of the $^{3}H$ cpm in the supernatant and the original $^{32}P/^{3}H$ ratio of the multilamellar vesicles.

*Multilamellar Vesicles and Mitochondria as Acceptor Particles.* Liposomes containing [$^{32}P$]PC and [$^{3}H$]triolein were prepared as described above. The elution pattern after Sepharose 4B fractionation showed a major peak containing more than 95% of the phospholipid and a minor peak in the void volume. The liposomes were incubated with phospholipid exchange protein and either mitochondria or multilamellar vesicles in Tris–EDTA buffer at 37 °C. The reaction was halted and particles were separated by centrifugation as above. The decrease in the $^{32}P/^{3}H$ ratio of the supernatant measured the transfer of PC to the acceptor particles. Exchange of PC in the absence of exchange protein was less than 10% in a 24-h incubation.

## Results

*Interaction of Phospholipid Exchange Protein with Multilamellar Phospholipid Vesicles.* Liposomes prepared from pure PC serve as a useful substrate for the assay of phospholipid exchange activity (Zilversmit, 1971). It is therefore surprising that the exchange protein will not interact with multilamellar PC vesicles. Transfer of radioactive PC from multilamellar PC vesicles to either liposomes or mitochondria occurs at a negligible rate in the presence or absence of beef heart or beef liver exchange protein. Furthermore, addition of multilamellar vesicles did not inhibit the transfer of PC from liposomes to mitochondria. Subjecting the vesicles to osmotic pressure, with 20 mM sucrose inside and 1 mM sucrose outside, causes swelling of these vesicles (Bangham et al., 1967). This does not improve their interaction with the exchange protein. The addition of lipids that have served as "spacer" molecules in monolayer studies (Demel et al., 1975) also proved unsuccessful in altering the exchangeability of PC in multilamellar vesicles: when cholesterol (30 mol %), cetyltrimethylammonium bromide (30 mol %), or lysolecithin (10 mol %) was individually added to PC multilamellar vesicles, no catalyzed [$^{32}P$]PC transfer from these particles to liposomes of identical composition was observed in the presence of beef heart exchange protein. However, a combination of [$^{32}P$]PC and soy asolectin yields a multilamellar structure that exhibits an exchange protein dependent [$^{32}P$]PC transfer to PC liposomes (Table I).

Asolectin is an ill-defined mixture of phospholipids, glycolipids, proteolipids, and neutral lipids. The use of PC–asolectin vesicles would have many of the disadvantages of a natural membrane. Combinations of purified phospholipids were thus employed in the preparation of multilamellar vesicles of varying, but defined, composition. As shown in Table I, certain molar ratios of purified phospholipids yield vesicles that transfer PC in the presence of beef heart or beef liver exchange protein. Negligible transfer was observed in every case in the absence of protein. The catalyzed transfer can not be accounted for by contamination of the multilamellar vesicles with liposomes since 98–99% of the donor particles sedimented as judged by the recovery of [$^{3}H$]triolein. A correction was made for the small percentage of multilamellar vesicles remaining in the supernatant. The lack of transfer of PC from pure PC multilamellar vesicles does not appear to be a result of low substrate availability since a tenfold increase in the concentration of these vesicles in the incubation still showed virtually no transfer of PC to liposomes. Acidic phospholipids appear to play a permissive role in the interaction of exchange protein with multilamellar phospholipid vesicles.

TABLE I: Transfer of Radioactive PC from Multilamellar Vesicles to PC Liposomes.[a]

| Composition of Multilamellar Vesicles | PC transferred (nmol)/min: Beef heart exchange protein | PC transferred (nmol)/min: Beef liver exchange protein |
|---|---|---|
| PC | 0.02 | 0.1 |
| PC:asolectin (50:50)[b] | 1.42 | 1.85 |
| PC:PE (70:30)[c] | 0.23 | 0.53 |
| PC:PI (70:30)[c] | 0.79 | 1.55 |
| PC:CL (95:5)[c] | 0.93 | 1.60 |
| PC:PE:PI (70:20:10)[c] | 0.84 | 1.73 |
| PC:PE:PA (70:20:10)[c] | 0.89 | 2.21 |
| PC:PE:CL (70:25:5)[c] | 1.26 | 1.79 |
| PC:PI:CL (70:25:5)[c] | 1.73 | 1.94 |

[a] Multilamellar vesicles (1.4 μmol of PC/mL), containing [$^{32}P$]PC and [$^{3}H$]triolein, and PC liposomes (1.6 μmol of PC/mL) were incubated for 10 min at 37 °C in Tris–EDTA buffer in the presence of beef heart phospholipid exchange protein (5.0 units/mL) or beef liver phospholipid exchange protein (8.0 units/mL). The transfer of PC was determined as described in Materials and Methods. No transfer was observed in the absence of protein. [b] Weight %. [c] Mol %.

*Multilamellar Vesicles of PC:PE:CL (70:25:5 mol %) as Acceptor Particles.* A single type of multilamellar vesicle composed of PC, PE, and CL (70:25:5 mol %) was chosen for further characterization and use as a PC acceptor particle in subsequent studies. Separation of these vesicles from liposomes is rapidly achieved by centrifugation at 40 000*g* for 15 min. The liposomes are recovered quantitatively in the supernatant, while nearly complete pelleting of the acceptor particles is achieved. The transfer of [$^{32}P$]PC from liposomes to PC:PE:CL vesicles exhibits similar kinetics to those obtained for mitochondria as acceptor (Zilversmit and Hughes, 1976). The assay system is linear with respect to protein concentration. These multilamellar vesicles contain two other phospholipids potentially capable of transfer—PE and CL. Multilamellar vesicles prepared with radioactive CL or PE exhibited less than 0.1% transfer of label to liposomes in the presence or absence of beef heart exchange protein. Under these conditions, multilamellar vesicles appear to serve as a well-defined reservoir of PC.

In the study of liposome structure with exchange protein, the acceptor particle should be maintained in large excess such that the entire available pool of radioactive liposomal PC may be replaced with unlabeled PC without significant back flow of label. It is therefore useful to know the amount of PC that is available for exchange from the acceptor particle. This value was obtained by incubating [$^{32}P$]PC multilamellar vesicles, PC:PE:CL (70:25:5), with a large excess of PC liposomes and a large amount of exchange protein. As shown in Table II, approximately 7% of the total PC of the vesicle is available for exchange. This value is reasonable for the outermost monolayer of a large multilamellar structure. It is in excellent agreement

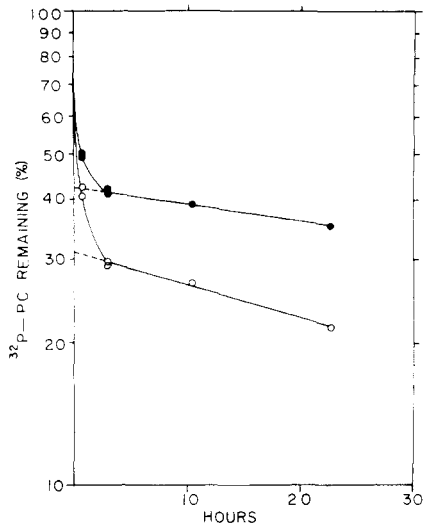


FIGURE 1: Transfer of radioactive PC from PC liposomes to mitochondria (●) or PC:PE:CL (70:25:5) multilamellar vesicles (○). Liposomes of [$^{32}$P]PC (32 nmol of PC/mL) and a trace of [$^{3}$H]triolein were incubated with PC:PE:CL (70:25:5) multilamellar vesicles (2.6 μmol of phospholipid/mL) or mitochondria (5 mg of protein/mL) and beef heart exchange protein (5.0 units/mL) in Tris–EDTA buffer at 37 °C. After 40 min, the acceptor particle was completely removed by centrifugation and replaced by fresh acceptor in order to prevent reverse flow of labeled phospholipid during subsequent incubation. For further details, see Materials and Methods.

TABLE II: Transfer of [$^{32}$P]PC from PC:PE:CL (70:25:5) Multilamellar Vesicles.[a]

| Time (min) | % [$^{32}$P]PC transferred |
|---|---|
| 20 | 3.3 |
| 60 | 5.3 |
| 90 | 6.1 |
| 150 | 6.9 |
| 210 | 6.9 |

[a] Multilamellar vesicles containing [$^{32}$P]PC:PE:CL and a trace of [$^{3}$H]triolein were incubated with PC liposomes and beef heart protein under the conditions of Table I; however, the concentration of protein was increased to 7.5 units/mL.

with a value of 8% for PC multilamellar vesicles that was obtained by Bangham et al. (1967) by $UO_2^{2+}$ titration.

*Mitochondria vs. Multilamellar Vesicles as Acceptor Particles.* Johnson et al. (1975) have employed beef heart exchange protein to examine [$^{32}$P]PC liposome structure. They incubated these vesicles with an excess of mitochondria and exchange protein and found that only about 60% of the label in liposomes was rapidly transferred to the mitochondria; the other 40% being transferred with a half-life of days. They concluded that the rapidly exchanging pool was the PC of the outer monolayer of the liposome, and that the very slow exchange of the remaining 40% represented the transposition of PC across the bilayer.

This experiment was repeated with either mitochondria or PC:PE:CL multilamellar vesicles as acceptor particles. The results are shown in Figure 1. The flip-flop rates are similarly slow: in five experiments, mean half-lives ± SD of 3.0 ± 1.7 and 5.8 ± 4.5 days are obtained with mitochondria or multilamellar vesicles, respectively. However, the exchangeable pool size is significantly ($P < 0.001$) different: 72 ± 2.2% with

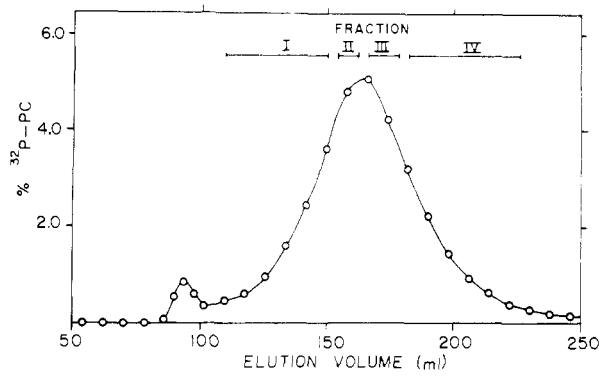


FIGURE 2: Size fractionation of liposomes on Sepharose 4B. Liposomes of [$^{32}$P]PC (10 mg/1.5 mL Tris-EDTA) and a trace of [$^{3}$H]triolein were eluted from a column (60 cm × 2.5 cm) of Sepharose 4B that had been preequilibrated with soy PC liposomes (100 mg). Flow rate was 16 mL/h. Tubes (4 mL) were pooled as shown. Recovery of label was 98%. The $^{32}$P/$^{3}$H ratio was constant throughout the peak. The ratios of the pooled fractions are given in the text.

mitochondria and 61 ± 2.2% with multilamellar vesicles. A second difference in the use of the two acceptors was found in the recovery of liposomes in the supernatant after the separation of liposomes and multilamellar vesicles or mitochondria by centrifugation. In the experiment with the mitochondria (Figure 1), approximately 60% of the liposomes were recovered in the supernatant, whereas 90% were recovered when multilamellar vesicles were employed.

The fact that multilamellar vesicles allow for the transfer of a larger percentage of liposomal radioactivity than do mitochondria might be attributed to a greater efficiency of exchange. However, when labeled liposomes that had been extensively incubated in the presence of exchange protein with mitochondria were then isolated and incubated with multilamellar vesicles, no further transfer of [$^{32}$P]PC was observed. In the converse experiment, liposomes first extensively incubated with multilamellar vesicles in the presence of exchange protein and then with mitochondria showed an increase in the ratio of [$^{32}$P]PC to [$^{3}$H]triolein of the liposome, and a drop in recovery of [$^{3}$H]triolein from 90 to 40%. The latter experiment indicates the removal by mitochondria of a subpopulation of vesicles with a low $^{32}$P/$^{3}$H ratio, as would be true for small vesicles after the $^{32}$P in the outer monolayer had been exchanged. The discrepancy in the apparent exchangeable pool sizes of liposomes with the two acceptor particles may therefore be explained by a preferential "sticking" of small liposomes to the mitochondria.

To test the above hypothesis, liposomes were fractionated according to size by Sepharose 4B chromatography (Huang, 1969; Andrews et al., 1975). Multilamellar structures are eluted with the void volume; whereas single bilayer vesicles are retained in the gel. An elution profile for PC liposomes on a preloaded Sepharose 4B column is shown in Figure 2. The multilamellar vesicles can be seen to comprise less than 5% of the total phospholipid mass. The liposome peak was divided into quarters according to mass and the tubes were combined into fractions I through IV as indicated. The $^{32}$P/$^{3}$H ratio of these fractions was 0.47, 0.48, 0.46, and 0.46 for I through IV, respectively. The fractions were individually incubated under the conditions of Figure 1 with excess mitochondria or excess multilamellar vesicles—PC:PE:CL (70:25:5). The recovery of the liposomes in the supernatant after pelleting of the acceptor particles is shown in Table III. It is clear that there is a size-dependent interaction of the liposomes with mitochon-

TABLE III: Recovery of Fractionated Liposomes after Incubation with Acceptor Particles.[a]

| Liposome | Acceptor Particle: Mitochondria[b] | Acceptor Particle: Multilamellar Vesicles[b] |
|---|---|---|
| Fraction I | 92 | 98 |
| Fraction II | 48 | 86 |
| Fraction III | 21 | 86 |
| Fraction IV | 16 | 83 |

[a] Fractionated liposomes of [$^{32}$P]PC and a trace of [$^{3}$H]triolein were incubated with mitochondria or PC:PE:CL multilamellar vesicles for 80 min under the conditions of Figure 1. [b] Percent [$^{3}$H]TO in supernatant.

dria. A sixfold higher recovery of liposomes is obtained with fraction I over fraction IV; thus the smaller vesicles appear to "stick" to mitochondria to a much greater extent. This size dependency is much less pronounced when the fractions are incubated with multilamellar vesicles. The recovery of fraction IV compared with that of fraction I is 85%.

The fractionation of vesicles according to size should be reflected in a shift in exchangeable PC pool sizes. Large liposomes (fraction I) would be expected to have a lower percentage of the total PC in the outer monolayer than the smaller liposomes. This prediction was confirmed by extensive exchange experiments performed with each of the fractions, and PC:PE:CL multilamellar vesicles as acceptor particles (Figure 3). The outer monolayer pool size of the original, unfractionated, sample can be quantitatively accounted for by the curves obtained for the four fractionated samples. The average size of the vesicles in each fraction may be calculated from the outer pool size by assuming equal packing of phospholipids in the two monolayers and a bilayer thickness of 35 Å. The average diameter of fractions I thru IV are 350, 200, 170, and 160 Å, respectively. The average diameter of the unfractionated vesicles is 210 Å.

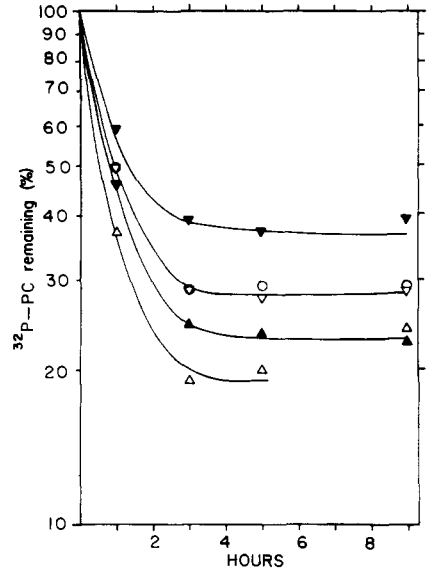


FIGURE 3: Transfer of [$^{32}$P]PC from fractionated liposomes to multilamellar vesicles. The fractions from the Sepharose to 4B column and an unfractionated sample were incubated individually with PC:PE:CL multilamellar vesicles and beef heart exchange protein under the conditions of Figure 1. Fraction I (▼), II (▽), III (▲), IV (△), unfractionated (○).

## Discussion

Structural comparisons between multilamellar vesicles and liposomes have been made in studies with such varied techniques as nuclear magnetic resonance (Sheetz and Chan, 1972), differential scanning calorimetry (DeKruijff et al., 1975), and hydrolysis by phospholipases (Dawson and Hauser, 1967). It has been proposed that the multilamellar vesicles possess a more rigid, tightly packed, phospholipid structure than sonicated vesicles (Lichtenberg et al., 1975). Our experiments point out another difference between these model membrane systems—the exchangeability of their phospholipids. Multilamellar vesicles of pure phosphatidylcholine will not transfer PC in the presence of phospholipid exchange protein from beef heart (Johnson and Zilversmit, 1975) or beef liver (Kamp et al., 1973). This may indicate that, in these vesicles, interaction between PC molecules is much stronger than in PC liposomes. This tight structure is apparently altered by the presence of certain acidic phospholipids such as cardiolipin, but not by a zwitterionic lipid such as PE or lysolecithin. The negatively charged lipids may perturb the PC bilayer structure due to electrostatic repulsion, thus causing a greater spacing between phospholipid head groups. It is possible that the exchange protein is stimulated by the presence of a negatively charged surface, although Wirtz et al. (1976) have reported inhibition of beef liver exchange protein by negative charge. Factors such as the availability of substrate or the sensitivity of the assay method have been eliminated.

Demel et al. (1975) have recently demonstrated that certain phospholipases, such as phospholipase C from *B. cereus,* will not act on high-pressure phospholipid films (monolayers of greater than 30 dynes/cm initial surface pressure). This enzyme will, however, degrade phospholipids above this pressure if "spacer" molecules such as ceramide, diglyceride, or phosphatidic acid are added to the film. It is possible that the structure or packing of phospholipids in a multilamellar vesicle resembles that of a high-pressure film. The addition of acidic phospholipids may cause an increased spacing of head groups without affecting the surface pressure. One could postulate then that the exchange protein exhibits a dependency on surface pressure similar to that of the phospholipases. However, Demel et al. (1973) has reported that beef liver protein-catalyzed transfer of PC from monolayer to liposome is independent of surface pressure over the range of 20 to 40 dynes/cm. If this mechanism were to hold, the multilamellar vesicles would thus have to possess a surface pressure greater than the collapse pressure of a phospholipid monolayer.

Multilamellar vesicles are an excellent choice for acceptor particles in liposome-exchange protein incubations for several reasons. First, the particle is well defined in composition and, through the use of radioactively labeled lipids, one can determine the degree of transfer of each component of the system. Thus, the membrane structure under study is not perturbed by the introduction of unknown foreign molecules such as cholesterol from erythrocyte ghosts. Secondly, the composition of the multilamellar vesicle is easily altered to approximate the composition of the donor particle. Third, the amount of phospholipid in the vesicle available for exchange with the liposome is easily determined. Reverse flow of label can therefore be accounted for quantitatively. Fourth, the physical interaction of these vesicles with liposomes is minimal, so that a quantitative recovery of liposome in the supernatant after separation is achieved. This aspect is particularly important

in studies of negatively charged liposomes which extensively cosediment with such natural membranes as mitochondria or red blood cell ghosts (unpublished observations).

Liposomes that have been used in permeability, fusion, and membrane reconstitution studies are often referred to as a homogeneous population of single-walled vesicles (Bangham et al., 1974). Others have demonstrated by physical methods that the descending portion of the elution profile from Sepharose 4B columns represents a homogeneous population of vesicles (Thompson et al., 1974). Our data indicate a definite functional heterogeneity in liposomes of pure PC with respect to their interaction with mitochondria. This heterogeneity is observed as a size dependent "sticking" to mitochondria. The mechanism of the "sticking" is not clearly understood; however, we have indications that it is a fusion process. The "sticking" is nonsaturable, irreversible, and is greatly enhanced by the presence of acidic PL (unpublished observations). The liposomes employed in these studies were prepared in a bath-type sonicator. Thermodynamically unstable particles could be generated by the high-energy input of the ultrasonifier which may produce some liposomes with extreme degrees of curvature (Sheetz and Chan, 1972). Fusion with the mitochondrial membrane might then result in a more thermodynamically stable structure.

The heterogeneity of liposomes which we have observed may not be universal. Liposomes prepared by sonication with a probe sonifier or by dilution of organic solvent might differ structurally and in their interaction with mitochondria. Also, the high degree of unsaturation of PC from rat liver, used in our studies, may play some role in the formation of these metastable vesicles. The extent to which metastable vesicles are present in a liposome population may well determine their reactivity in fusion or in membrane reconstitution experiments.

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