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USE OF PHOSPHOLIPID EXCHANGE PROTEIN TO MEASURE INSIDE-OUTSIDE TRANSPOSITION IN PHOSPHATIDYLCHOLINE LIPOSOMES

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SUMMARY

The exchange of phosphatidylcholine between [^{32}P]phosphatidylcholine liposomes and unlabeled mitochondria was catalyzed by a purified phospholipid exchange protein from bovine heart cytosol. The loss of [^{32}P]phosphatidylcholine from the liposomes appeared to proceed in two stages: with 100 units of phospholipid exchange protein per ml the half-time of the initial stage was about 10 min and that of the final stage 4 days or greater. Agarose-gel chromatography of the liposomes showed an elution compatible with a homogeneous pool of small single walled vesicles. Treatment of phosphatidyl [^{14}C]choline liposomes with phospholipase D (phosphatidylcholine phosphatidohydrolase) showed that labeled phospholipid removable during the rapid exchange phase was subject to hydrolysis by the phospholipase, but that the labeled phospholipid left after the rapid exchange was completed could not be hydrolyzed by phospholipase D. It is proposed that the rapidly exchanging phosphatidylcholine constitutes the outer layer of the liposome bilayer. The long half-lives of 4 days or more probably represent the transposition of phosphatidylcholine from the inner to the outer layer of the liposome bilayer.

INTRODUCTION

Several workers have shown that mammalian tissues contain proteins that accelerate the exchange of phospholipids between organelles [1–5]. Further work has shown these proteins to be active with such diverse phospholipid donors and acceptors as mitochondria [4, 6], microsomes [1, 4], chylomicrons [7], lipid emulsions [7], serum lipoproteins [8], liposomes [7, 9, 10] and monolayers [11]. Purified and partially purified phospholipid exchange proteins have been shown to have several types of specificity. A protein specific for phosphatidylcholine has been obtained from

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beef liver [10] while a similar protein from beef heart transfers both phosphatidylcholine and to a lesser degree sphingomyelin [12]. Other exchange proteins have been demonstrated to act on phosphatidylinositol and phosphatidylethanolamine [13]. Specificity for the various fatty acid substituents of natural phosphatidylcholine seems to be lacking in rat liver phosphatidylcholine exchange protein [14].

To date, no report has appeared as to the effects of phospholipid exchange proteins at high concentrations or for extended times. In the following work, highly active purified phosphatidylcholine exchange protein is incubated with radio-labeled liposomes and unlabeled mitochondria. The results are interpreted in relation to phospholipid disposition in the liposomes.

MATERIALS AND METHODS

[^{14}C]Triolein (Applied Science Laboratories, State College, Pa.) and [^{3}H]triolein (Amersham/Searle, Arlington Heights, Ill.) were purified by thin-layer chromatography on silica gel H with the solvent system light petroleum (b.p. 67 °C)–diethyl ether–acetic acid (60 : 40 : 1, by vol.). They were eluted and stored in chloroform. [^{14}C]Choline chloride and [^{32}P]phosphate were obtained from New England Nuclear (Boston, Mass.) and were not further purified before injection into animals. Phospholipase D or phosphatidylcholine phosphatidohydrolase (EC 3.1.4.4) from Savoy cabbage was obtained from Calbiochem (San Diego, Calif.). Radioactivity was determined in a Packard scintillation counter in the medium of Gordon and Wolfe [15].

Radioactive lipids

Phosphatidylcholine was labeled by injecting rats intraperitoneally 16 h before sacrifice with 800 μCi $^{32}P_i$ or 1 h before sacrifice with 75 μCi [^{14}C]choline chloride in normal saline. Liver was minced and extracted with 20 vol. of chloroform–methanol (2 : 1, by vol.), and the extract was washed by the procedure of Folch et al. [16]. Phosphatidylcholine was isolated by either preparative thin-layer chromatography or by column chromatography. Thin-layer chromatography was done on silica gel H which contained the fluor ultraphor [17] according to the method of Skipski et al. [18]. Phosphatidylcholine was eluted with chloroform–methanol–water (16 : 7 : 1, by vol.). In the column procedure the solvent from the washed liver extract was evaporated under N_2 and the residue was redissolved in chloroform. It was then applied to a water-cooled column (1.8 cm $\times$ 11 cm) containing neutral alumina activity I (EM Laboratories, Elmsford, N.Y.). The column was eluted by a slightly modified procedure of Singleton, et al. [19] with chloroform and then with chloroform–methanol (9 : 1, by vol.). With neutral alumina the 9 : 1 eluent elutes neutral lipids but 0.5 % of concentrated NH_4OH is added to the eluent for satisfactory elution of phosphatidylcholine. Fractions containing only phosphatidylcholine, according to analytical thin-layer chromatography, were pooled and stored under N_2 at 4 °C in the dark.

Liposomes

[^{32}P]Phosphatidylcholine with 10 % (w/w) butylated hydroxytoluene as antioxidant and a trace amount of [^{14}C]triolein was dried from the stock solution described above at 25 °C under a stream of N_2. The dried lipid was resuspended in 5 ml

of diethyl ether (Mallinckrodt AR) and redried under N_2 to form a thin film in a test tube. Sufficient 0.25 M sucrose, 0.001 M EDTA, 0.05 M Tris–HCl buffer (pH 7.4) (Sucrose–EDTA–Tris–HCl buffer) was added to give a lipid suspension of 0.25 mg/ml. This was agitated for 10 min with a Vortex mixer and then allowed to stand for 1 h at room temperature. The sealed test tube containing the lipid suspension under N_2 was sonicated in a Branson HD-50 sonicating water bath for 30 min at 25 °C during which time the turbidity of the lipid decreased markedly.

The phosphatidylcholine subspecies present in these liposomes were investigated by gas–liquid chromatography of their fatty acids. Liposomes were repeatedly dissolved in ethanol and dried under N_2 to remove all water. An internal standard of 17 : 0 fatty acid was added and the sample was transesterified in 2 % H_2SO_4 in methanol [20]. Gas–liquid chromatography was done on an F and M Model 810 apparatus with a 6 ft × 1/4 inch glass column packed with 10 % ethylene glycol adipate on a fire brick base. Column temperature was 180 °C. The fatty acid composition of a typical preparation was palmitic acid 19.7 %, palmitoleic acid 0.8 %, stearic acid 28.0 %, oleic acid 5.0 %, linoleic acid 17.9 % and arachidonic acid 30.0 %.

Purification of phosphatidylcholine exchange protein

A protein which catalyzes the exchange of phosphatidylcholine between membranes was purified from beef heart cytosol. Post-mitochondrial supernatant from beef heart was generously donated by Dr E. Racker and the purified exchange protein was derived from it according to procedures which include isoelectric precipitation, $(NH_4)_2SO_4$ precipitation, Sephadex G-75 chromatography and carboxymethylcellulose chromatography [21]. It was assayed by incubating [^{32}P]phosphatidylcholine liposomes (10 μg phospholipid phosphorus) and mitochondria (12.5 mg protein) in a volume of 4 ml sucrose–EDTA–Tris–HCl buffer in the presence and absence of the exchange protein. The enhancement of [^{32}P]phosphatidylcholine exchange between the labeled liposomes and the unlabeled mitochondria as promoted by the protein served to measure the activity. One unit of activity was defined as the activity required to exchange 1 % of the [^{32}P]phospholipid during a 40 min incubation at 37 °C.

RESULTS

Extensive phospholipid exchange in short periods

Labeled liposomes (2 μg of phospholipid phosphorus) were incubated at 37 °C with an excess of mitochondria (84 μg of phospholipid phosphorus) in 2 ml of a medium containing 200 units of phospholipid exchange protein in sucrose–EDTA–Tris–HCl buffer. The medium was agitated gently to provide mixing of the exchanging particles. Under these conditions labeled phosphatidylcholine from the liposomes was readily exchanged for unlabeled phosphatidylcholine from the mitochondria. As the exchange proceeded, the accumulation of label in mitochondria provided some opportunity for a reverse flow of label, i.e. from mitochondria back to liposomes. This was largely eliminated by replacing the mitochondria as their level of [^{32}P]phosphatidylcholine became significant. Thus, after 12–20 min of phospholipid exchange, the suspension was quickly chilled, and the mitochondria were sedimented at 15 000 × *g* and replaced with fresh mitochondria. The incubation at 37 °C was then resumed

with fresh mitochondria, and finally the exchange was terminated by again chilling and centrifuging the suspension.

In this system the [^{32}P]phosphatidylcholine in the liposomes exchanged with unlabeled phosphatidylcholine from the mitochondria very rapidly for about 10 min, at which time approximately one-half of the labeled phospholipid had been removed from the liposomes. The rapid exchange of label then slowed quite abruptly. The abrupt change could not be attributed to inactivation of the exchange protein; it remained fully active for many hours as evidenced by phospholipid exchanged from newly added liposomes. The abrupt slowing could not be ascribed to approaching isotopic equilibrium since adding fresh mitochondria had only small effects. This suggested that about one-half of the phospholipid in the liposomes belonged to a pool which was not readily available to the exchange protein.

The relative amounts of phosphatidylcholine in the rapidly exchanging pool and the nature of the subsequent slow exchange depended on the preparation of the phosphatidylcholine from which the liposomes were made. Five experiments done with lipid purified by thin-layer chromatography or done with lipid stored beyond 2 weeks in chloroform–methanol (9 : 1, by vol.) or exposed to O_2 as the solvents were evaporated, exchanged 61 ± 0.4 % and showed a half-life of the final slope varying from 1.5 to 7.0 h. Five experiments which were done with freshly prepared lipid from alumina columns showed 65 ± 2 % readily exchanged phospholipid and a half-life for the subsequent removal which was longer than could be determined from these short-term experiments. Eluate from blank silica gel plates and alumina columns when added to phospholipids prepared in different ways did not affect the exchange characteristics of liposomes. Furthermore, a shorter half life for the final slope was observed with alumina-prepared phosphatidylcholine if it was subsequently chromatographed on silica gel thin-layers before preparation of liposomes (Fig. 1). However, phosphatidylcholine prepared by thin-layer chromatography retained its exchange characteristics even after subsequent chromatography on an alumina column. Rechromatog-

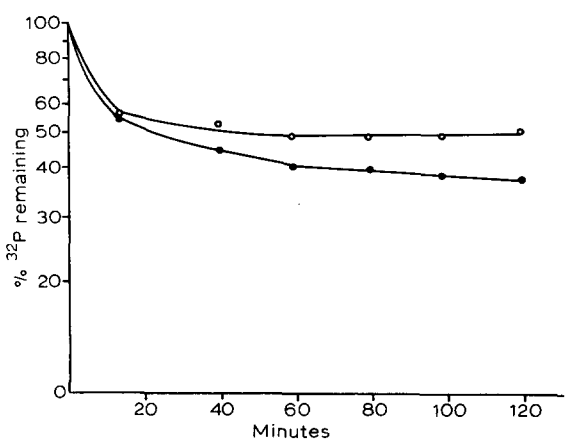

Fig. 1. Exchange of [^{32}P]phosphatidylcholine between liposomes (50 μg phospholipid) and mitochondria (6.25 mg protein) in the presence of 150 units of phospholipid exchange protein ○—○, phosphatidylcholine purified on alumina column; ●—●, the same phosphatidylcholine purified on alumina column followed by thin-layer chromatography on silica gel H.

raphy of phosphatidylcholine isolated from thin-layer plates failed to show the presence of degradation products such as lysophosphatidylcholine.

An indication of lipid oxidation was obtained after 2 months of storage at 4 °C in chloroform : methanol (9 : 1, by vol.) with phosphatidylcholine from an alumina column. The absorbance at 233 nm, which reflects conjugated diene hydroperoxide, ($\varepsilon = 27\,400\ cm^{-1} \cdot M^{-1}$) showed that up to 2.5 % of the aged phosphatidylcholine might be conjugated diene, whereas freshly prepared phosphatidylcholine contained up to 0.6 % diene by this method [22]. Malondialdehyde levels as measured with thiobarbituric acid [23] were also generally higher in aged or thin-layer phosphatidylcholine than in that from alumina columns.

Since oxidation of fatty acids should degrade mostly polyunsaturated phospholipid, an experiment was performed to test for the loss of unsaturated fatty acids caused by thin-layer chromatography. Analysis by gas–liquid chromatography showed that a sample of alumina lipid contained 26.6 % arachidonate and 11.6 % linoleate and that after thin-layer chromatography it contained 26.0 and 10.0 % of these acids, respectively. Losses of unsaturated fatty acids were apparently small. Nevertheless, for all subsequent work phosphatidylcholine was prepared with alumina columns and used within 4 days.

Long-term exchange of phosphatidylcholine

Since the exchange of labeled phosphatidylcholine from liposomes slowed markedly during short exchange periods, longer periods were utilized to study the slow phase of the exchange process. However, labeled triolein, which was used as a nonexchangeable marker for liposomes, hydrolyzed during lengthy exchange periods and the liberated fatty acids were found with the mitochondria. Other labeled lipids failed to solve the problem. Cholesterol oleate [10] was hydrolyzed and gave two exchangeable residues, cholesterol and oleic acid, while labeled *n*-hexadecane, which does not hydrolyze, was also found to be exchangeable. Therefore, labeled triolein was used as the marker with two modifications in the procedure to reduce hydrolysis. First, the time of association of liposomes with mitochondria was kept to a minimum. Second, a lipase inhibitor, diethyl *p*-nitrophenyl phosphate (5 mM), was added to the mitochondria at a ratio of 0.16 mg of inhibitor per mg of mitochondrial protein. The mitochondria were incubated 2.5 h at 25 °C with the inhibitor, and then washed 7 times in sucrose–EDTA–Tris–HCl buffer. This treatment reduced the lipase activity to one-tenth. No differences in the phospholipid exchange properties of the mitochondria could be found following treatment with inhibitor. One further change in the procedure was the inclusion of 0.02 % NaN_3 to inhibit microbial growth. NaN_3 did not reduce the activity of the exchange protein in 24 h and phosphatidylcholine was unchanged by azide according to thin-layer chromatography.

Liposomes, mitochondria and phospholipid exchange protein were pipetted together in the same proportions as for the shorter exchange experiments. After 40 min of gentle agitation at 37 °C most of the exchangeable labeled phosphatidylcholine had moved from the liposomes to the mitochondria. The mitochondria were then removed and the liposomes were left to stand at 37 °C for up to 23 h. At various intervals during this period aliquots of liposomes were removed and incubated with fresh mitochondria for 40 min to determine how much of the previously nonexchangeable pool had become exchangeable.

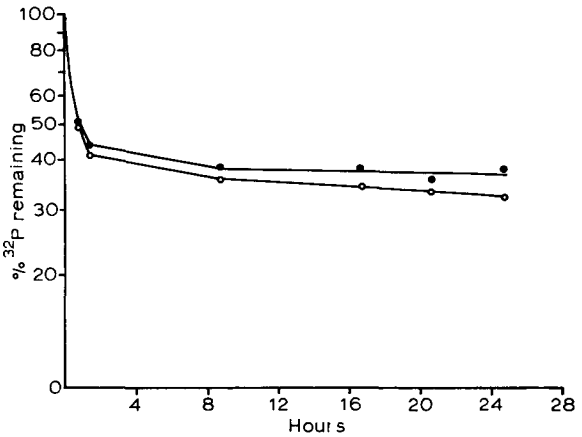

Fig. 2. Removal of [^{32}P]phosphatidylcholine from liposomes resulting from exchange with unlabeled mitochondria. The two curves represent two separate experiments. Lines connecting the last 4 points were fitted by least squares analysis.

The time courses for two experiments are shown in Fig. 2. The slope of the slow phase of the exchange reaction was determined by least squares analysis of the data points. Between 1 and 8 h the curves exhibit an apparent $t\frac{1}{2}$ of 1.3 and 1.5 days. The slopes between 8 and 24 h had a $t\frac{1}{2}$ of 4 and 25 days. In 2 additional experiments, the values found were 15 and 16 days. The apparent change of rate after 8 h could not be explained on the basis of isotopic equilibration.

Gel chromatography

Two pools of phospholipid with different exchange characteristics could result from the existence of two types of liposomes: small, closed, single-walled vesicles, and larger multilayer structures [24]. Since these classes separate readily on Sepharose 4B [24] we have used Sepharose chromatography to test whether two classes of liposomes were present. Sepharose 4B was equilibrated against 0.05 M Tris–HCl buffer (pH 7.4) and packed into a 1.1 cm $\times$ 80 cm column. Buffer was pumped through it at 4.8 ml/h for several days with a Harvard peristaltic pump. To reduce losses of phospholipid on the column during chromatography, labeled liposomes identical to those to be chromatographed (3.3 mg phospholipid in 2 ml buffer) were loaded onto the column during this preparatory pumping. About 2 mg of this lipid adsorbed to the column. The sample of radiolabeled liposomes to be investigated (1.67 mg of phosphatidylcholine in 2.0 ml) was loaded on the column and eluted at 4.8 ml/h using the same buffer. Radioactivity in the eluted fractions was found in one symmetrical peak (Fig. 3). This indicated that only small single walled vesicles were present. On smaller pore gel, Sepharose 6B, some liposomes did elute in the void volume giving a bimodal appearance to the profile. Apparently the size distribution of liposomes straddled the exclusion limit of this gel. To eliminate the possibility that the two-pool exchange properties were due to the existence of multi and single layer vesicles, an exchange experiment was carried out with the smallest liposomes eluted from Sepharose 6B. The resulting exchange was not significantly different from that

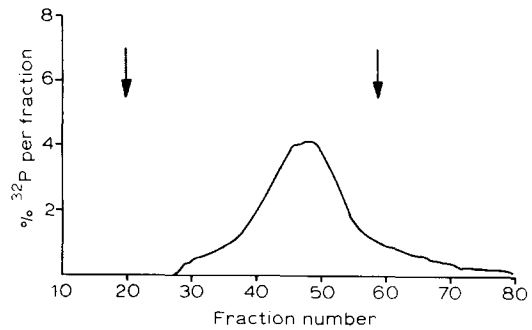

Fig. 3. Agarose chromatography of liposomes. [^{32}P]Phosphatidylcholine liposomes (1.67 mg of phospholipid) were chromatographed on a 1.1 cm × 80 cm column of Sepharose 4B and 2-ml fractions were monitored by scintillation counting. The arrows denote void and total volumes and indicate a small retention even after the total volume.

already described with unfractionated liposomes. The exchange was very rapid until about one-half of the label had been removed, and then slowed abruptly.

Phospholipase D hydrolysis

The two pools of phospholipid in the liposomes have been further characterized by the use of phospholipase D. Liposomes prepared from phosphatidyl[^{14}C]choline were incubated with phospholipase D. Fig. 4 (insert) shows the course of the reaction

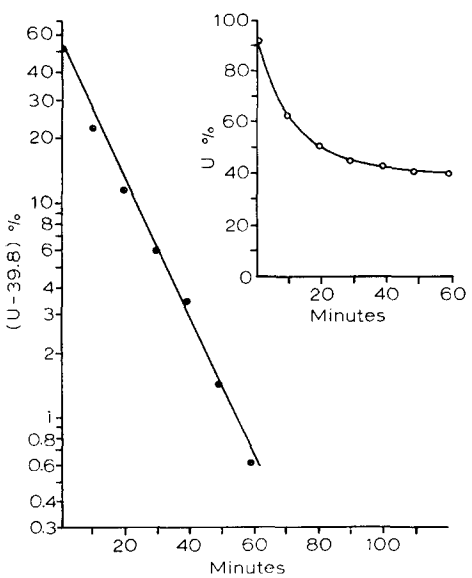

Fig. 4. Hydrolysis of liposomes by phospholipase D. Phosphatidyl[^{14}C]choline liposomes (2 μg phospholipid phosphorus) were hydrolyzed in a total volume of 2 ml sucrose–EDTA–Tris–HCl–buffer, containing 0.5 mg phospholipase D, 1.76 mg $CaCl_2 \cdot 2H_2O$ and 1.0 mg bovine serum albumin. The reaction was stopped after various times with 13 ml of methanol followed by 26 ml of chloroform. Phosphatidyl[^{14}C]choline was determined in the lower phase after addition of 10 ml of water. ○—○, unhydrolyzed phosphatidyl[^{14}C]choline (= U); ●—●, (U—39.8) log scale.

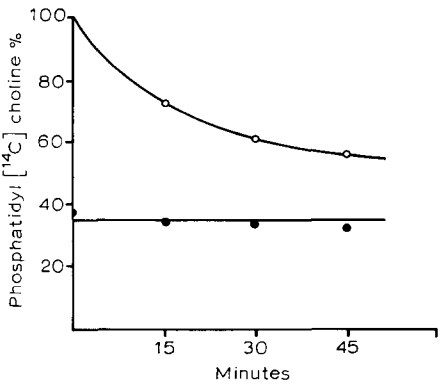

Fig. 5. Exchange of phospholipid followed by phospholipase D hydrolysis. Liposomes (2 μg phospholipid phosphorus), exchange protein (50 units) and mitochondria (6.25 mg protein) were incubated in 2 ml of sucrose–EDTA–Tris–HCl buffer at 37 °C. After sedimentation of mitochondria liposomes were treated with phospholipase D as described in the legend of Fig. 3. ○—○, remaining liposomal phosphatidyl[^{14}C]choline after exchange with unlabeled mitochondria; ●—●, remaining liposomal ^{14}C after exchange followed by treatment with phospholipase D.

in a typical experiment. Apparently about 40 % of the labeled phospholipid was not hydrolyzable. This residual phospholipid (R) was estimated from a semilogarithmic plot of phosphatidyl[^{14}C]choline minus R. By iteration the value of R giving a log linear line was 39.8 % (Fig. 4) indicating that this amount of phosphatidylcholine was not accessible to the enzyme.

It is also apparent (from Fig. 4) that in 60 min the hydrolysis by phospholipase D is more than 99 % complete. In other experiments liposomes were made as described before from phosphatidyl[^{14}C]choline, butylated hydroxytoluene and a trace of [^{3}H]triolein. After varying periods of phospholipid exchange, mitochondria were sedimented. One aliquot of the liposomes was used to assay the extent of exchange, whereas another aliquot was treated with phospholipase D for measurement of the remaining accessible phospholipid. During the exchange phase of the experiment liposomal phosphatidyl[^{14}C]choline decreased over a 45-min period down to 52 % of the original amount (Fig. 5). Hydrolysis of these liposomes with phospholipase D at different stages of exchange removed more ^{14}C down to a residual level of about 37 % of the original label regardless of the amount of label previously removed by exchange (Fig. 5). It thus appears that the exchangeable fraction and the fraction subject to phospholipase D hydrolysis are the same, indicative that surface layer availability may be the limiting factor in each case.

On the basis that the vesicles seem to be of the small single walled type and that the exchangeable pool of phosphatidylcholine also seems to be the surface-hydrolyzable pool, we suggest that the two pools are the inner and outer layers of bilayer vesicles.

DISCUSSION

From the nature of the kinetics for phosphatidylcholine exchange it is quite apparent that a certain pool of molecules is not readily available for exchange by the

phospholipid exchange protein. In previous work on such systems this fact has not been observed, because it requires large amounts of phospholipid exchange protein, a high specific activity of the donor membrane, and a long period of exchange. When membrane vesicles were given sufficient time, the pool which was apparently inaccessible to the exchange protein equilibrated very slowly with the accessible pool.

Multilamellar structures would have much buried lipid which would probably not be accessible to the phospholipid exchange protein and would create pools other than simple inner and outer layers. Since the smaller liposomes from Sepharose columns, which do not contain multibilayer particles [24], exchanged phospholipid just like the unfractionated sonicated liposomes, we conclude that the sonicated preparations contained little or no large multilamellar particles. Since the vesicles appear to be of the small, single walled type, and since the molecules that are subject to rapid exchange are also the molecules which are attacked by phospholipase D, we suggest that the inaccessible pool of phospholipid constitutes the inner layer and the accessible pool is the outer layer of bilayer vesicles.

Both phosphatidylcholine exchange and hydrolysis by phospholipase D indicate that the inner layer of liposomes contains 37–40 % of the total phosphatidylcholine. These amounts agree with the values reported by Michaelson et al. [25] who measured choline methyl proton resonance in similar vesicles. These workers found that when Eu^{3+} interacted with the outer monolayer of phosphatidylcholine liposomes approx. 63 % of the choline signal was broadened and 37 % was unaffected. Hauser and Barratt [26] reported 70 and 30 % for similar experiments using Nd^{3+} as the interacting ion, while Finer et al. [27] reported 72 and 28 % when Mn^{2+} was the added ion. Kornberg and McConnell [28] reported a ratio of 65–35 % for a distribution of spin labels in similar liposomes. The relative sizes of the two pools in the present work depended upon the preparation and storage conditions of the phospholipid which in this case was from fresh liver, whereas most similar work has been done with egg lecithin.

The inside–outside transposition in phosphatidylcholine vesicles has previously been measured by Kornberg and McConnell [28]. These authors used egg phosphatidylcholine liposomes which also contained distearoylphosphatidylcholine bearing a spin label attached to the polar "head" group. They introduced ascorbate to reduce the spin label groups of the outer monolayer and hence abolish their paramagnetism. The rate at which the inner reporter groups migrated to the outer surface for reduction by ascorbate was used to measure the inside–outside exchange or phospholipid "flip–flop". At 30 °C the half time for signal disappearance was 6.5 h. At 40 °C their rates were several fold faster, as might be expected. A reason for the differences between these half times and the much longer ones reported in this work could be due to the spin label, a nine carbon heterocycle introduced in place of one of the choline methyl groups. With a similar spin label system the half times for transposition of phospholipid in excitable vesicles of electroplax membrane were 3.8–7 min [29] and in *Acholeplasma laidlawii* less than 1 min [30].

The higher transposition rate which seems to occur in liposomes before the 8-h point (Fig. 2) is an area for further investigation. It is tempting to speculate that the vesicles formed during sonication may be subject to stresses which would allow more rapid transposition of phospholipid molecules between the inner and outer layers of the liposomes. If such stresses were relieved during the first 8 h after prepa-

ration the transposition times would be lengthened accordingly.

We conclude that the phospholipids which form artificial bilayers in aqueous environments may consist of two relatively isolated layers in spite of known molecular freedom in each one. The equilibration of phospholipids between these layers can be monitored by maintaining a rapid exchange of labeled phospholipids in the outer monolayers with large external pools of unlabeled phospholipids.

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