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Transbilayer Exchange of Phosphatidylethanolamine for Phosphatidylcholine and *N*-Acetimidoylphosphatidylethanolamine in Single-Walled Bilayer Vesicles†

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ABSTRACT: A preparation of small single-walled liposome vesicles containing a 9:1 mole ratio of phosphatidylcholine to phosphatidylethanolamine was subjected to reaction with the membrane-impermeable reagent, isethionyl acetimidate hydrochloride. This reagent converted 90% of the external phosphatidylethanolamine groups to the amidine derivative, leaving the mole ratio of unreacted phosphatidylethanolamine to phosphatidylcholine on the outside surface of the vesicle much lower than that on the inside surface. Equilibration of phosphatidylethanolamine across the bilayer was then measured as a function of time by monitoring the appearance of phosphatidylethanolamine on the outside surface utilizing the reaction of the amino groups with 2,4,6-trinitrobenzenesulfonic acid. The results show that no new phosphatidylethanolamine appeared on the external surface of the vesicles over a period of 12 days at 22°. A conservative estimate of the precision of the measurements is ±10%. On this basis, the estimated half-time for the equilibration of phosphatidylethanolamine across the bilayer of these vesicles must be at least 80 days at 22°.

Marked transmembrane compositional asymmetries of lipid components have been described recently in erythrocytes (Verkleij et al., 1973), rod outer segment discs (Smith and Litman, 1974), and animal cell cytoplasmic membranes (Wisnieski et al., 1974). Similar compositional asymmetries have also been reported in multicomponent single-walled phospholipid vesicles of very small radius of curvature (Michaelson et al., 1973; Litman, 1973; Huang et al., 1974; Thompson et al., 1974). The stability of such large compositional differences in the opposing faces of biological membranes and in simple bilayer systems has focussed attention on the kinetics of transbilayer movement of component lipid molecules. This so-called flip-flop motion was first examined by Kornberg and McConnell (1971) using electron spin resonance techniques, in a preparation of single-walled phospholipid vesicles. The results of these experiments suggested that the half-time for transbilayer equilibration of a spin-label phospholipid derivative was as short as 6.5 hr at 30°. Similar experiments carried out on vesicles derived from the electroplax of *Electrophorus electricus* (McNamee and McConnell, 1973) gave equilibration half-times of the order of minutes at 15°. However, recent experiments utilizing small single-walled vesicles in which a transbilayer compositional asymmetry of isotopically labeled phospholipid was established with a phospholipid exchange protein suggest that flip-flop half-times for self-exchange are much longer (Johnson et al., 1975; Rothman and Dawidowicz, 1975).

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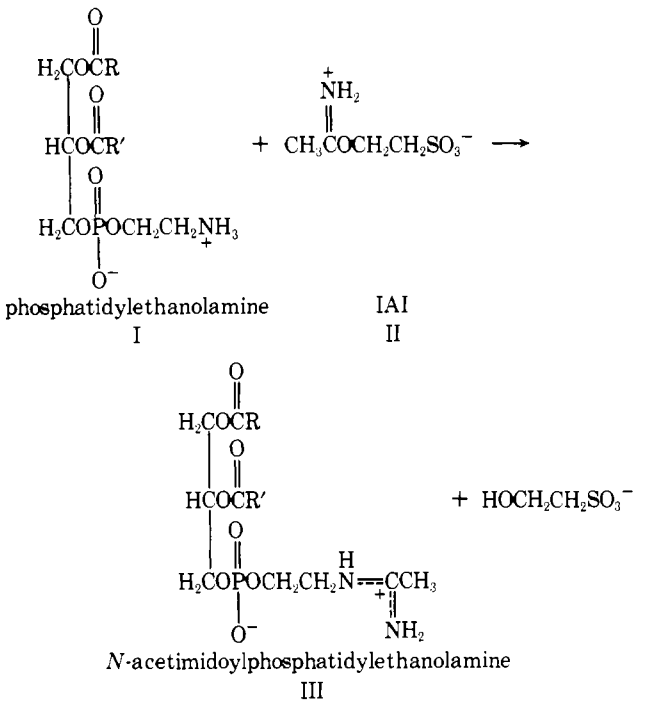
Scheme I



In both biological membranes and simple bilayer systems the disappearance of transbilayer compositional asymmetries requires the exchange of one lipid species for another, not self-exchange. We report here the results of experiments designed to establish the rate of transbilayer exchange of phosphatidylethanolamine for phosphatidylcholine and a closely related amidine derivative of phosphatidylethanolamine, *N*-acetimidoylphosphatidylethanolamine (III), in small single-walled homogeneously sized vesicles (Litman, 1973). This exchange has direct bearing on the preservation of the transmembrane compositional asymmetry found in the red blood cell (Verkleij et al., 1973) in which the choline-containing phospholipids are located primarily on the exterior surface of the plasma membrane, and the primary amine-containing lipids are located primarily on the interior surface. In addition, since phosphatidylethanolamine, *N*-acetimidoylphosphatidylethanolamine, and phosphatidylcholine are all zwitterionic, the exchange of these lipids should occur more rapidly than the exchange of lipids where one or both members bear a net charge. However, the results of these experiments clearly show that the half-time for decay of a tenfold transbilayer gradient of phosphatidylethanolamine in this system is greater than 80 days at 22°.

In order to measure the net exchange of phosphatidylethanolamine in phospholipid vesicles, it is necessary to prepare vesicles having a nonequilibrium distribution of this phospholipid across the bilayer. This was accomplished by reacting a phosphatidylcholine vesicle preparation containing 0.1 mole fraction of phosphatidylethanolamine with the reagent, isethionyl acetimidate hydrochloride (IAI),[1] to which both phospholipid bilayers (H. G. Smith and B. J. Litman, unpublished results) and erythrocyte membranes (Whiteley and Berg, 1974) have been found to be impermeable, according to Scheme I. This reaction converted 90% of the external phosphatidylethanolamine molecules to their amidine derivative, leaving the mole ratio of unreacted phosphatidylethanolamine to phosphatidylcholine on the outside surface of the vesicle much lower than that on the inside surface. Equilibration of phosphatidylethanolamine across the bilayer was then measured as a function of time by monitoring the appearance of phosphatidylethanolamine on the outside surface utilizing the reaction of the amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS) reagent as described by Litman (1973). The amidine derivative of phosphatidylethanolamine does not react with TNBS.

Experimental Section

Materials. Phosphatidylcholine and phosphatidylethanolamine were isolated from hen egg yolks by silicic acid column chromatography (Litman, 1973). Alumina column chromatography (Singleton et al., 1965) was utilized before the final purification step for phosphatidylcholine to ensure complete removal of lysophosphatidylethanolamine, which cochromatographs with phosphatidylcholine on silicic acid. The lipids were stored in $CHCl_3$ under argon at −20°. IAI (Pierce) and TNBS (Sigma) were used without further purification.

Preparation of Vesicles. A lipid mixture containing 300–400 μmol of a 9:1 ratio of phosphatidylcholine to phosphatidylethanolamine was colyophilized from benzene overnight in the dark, then suspended in 4 ml of 0.05 *M* NaCl and subjected to sonic irradiation in a Branson sonifier at 75 W for 25–40 min at 0°. During this, and subsequent operations, care was taken to maintain an inert atmosphere of nitrogen or argon over the lipids; phosphatidylethanolamine is especially susceptible to oxidation. It has recently been found in this laboratory that a homogeneous population of small vesicles can be obtained from a sonicated dispersion by removing the larger particles with prolonged high speed centrifugation. In this particular case, the sonicated lipids were centrifuged at 150000*g* for 4 hr. Phospholipid concentrations in vesicle solutions are expressed in terms of micromoles of lipid phosphorus per milliliter as determined by either the Gomori (1942) or modified Bartlett procedures (Litman, 1973).

Amidination. A solution containing 0.5–1.0 g of IAI in 20 ml of 0.2 *M* $NaHCO_3$ was rapidly adjusted to pH 8.6 with NaOH and immediately added to the vesicle solution recovered from high speed centrifugation. The mixture was allowed to incubate at room temperature under argon for 30 min, then concentrated to approximately 3 ml in a Diaflow ultrafiltration apparatus at 0°, using an XM-50 membrane. The concentrated vesicle solution was subjected to gel filtration at 4° on a Sephadex G-50 column (36 × 1.5 cm) to remove excess reagent and reaction by-products. The vesicles were eluted with 0.05 *M* NaCl, with which the column had been previously equilibrated. The flow rate was 1.6 ml/min. Figure 1 shows the elution profile from the Sephadex column. The fractions were assayed for turbidity at 300 nm, phosphate, and TNBS-reacting material. For this purpose the TNBS procedure indicated as "Labeling of Total Phosphatidylethanolamine" was used. Presumably, the TNBS-reacting material in the included volume fractions results from the ammonia formed by hydrolysis of IAI. Reaction of this material with TNBS is much slower than reaction with phosphatidylethanolamine; therefore, a 3-hr incubation period of this material with TNBS was utilized. As shown in Figure 1, the separation of vesicles from the reaction by-products is excellent. The vesicles, which

[1] Abbreviations used are: IAI, isethionyl acetimidate hydrochloride; TNBS, 2,4,6-trinitrobenzenesulfonate.

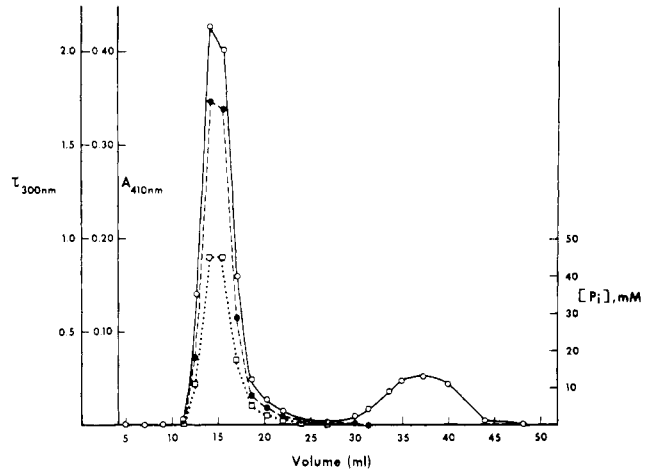


FIGURE 1: Sephadex G-50 chromatography elution profile of IAI-treated vesicles, showing turbidity at 300 nm (—), absorbance at 410 nm after reaction with TNBS (- - -) and 1 m*M* phosphate (• • •).

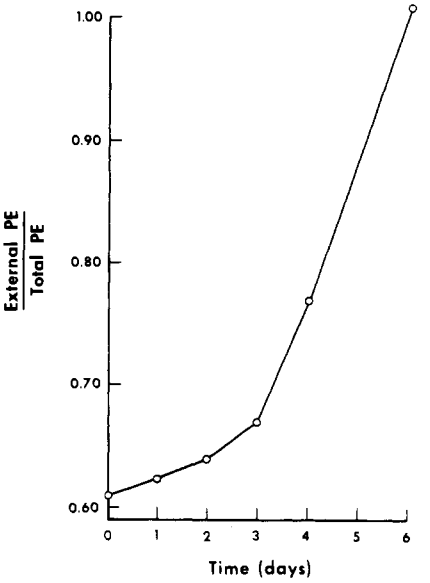


FIGURE 2: Ratio of external to total phosphatidylethanolamine in an equimolar phosphatidylethanolamine-phosphatidylcholine vesicle preparation incubated at 25° in a screw-capped tube under argon. These conditions are adequate for the storage of vesicles at 4°.

were recovered in a volume of 6 ml, were diluted to a volume of 25 ml with 0.05 *M* NaCl and stored under argon. The final concentration of phospholipid was 6.63 μmol/ml.

Assay for Amino Groups. In earlier experiments, the original assay procedure described by Litman (1973) was used to determine the amount of phosphatidylethanolamine in each face of the vesicle bilayer. In this procedure, propanolic solutions are utilized to disrupt the vesicles at appropriate stages of the assay. Recently, a faster, more sensitive assay has been developed by replacing propanol with Triton X-100. However, it was found that the amount of Triton X-100 normally used was not sufficient to clarify effectively the turbid vesicle solution. This is probably due to the rather high concentration of amidinated vesicles which must be used to obtain sufficient levels of phosphatidylethanolamine for the assay. Therefore, the following modified procedure was used.

(i) Labeling of External Phosphatidylethanolamine. To 0.6 ml of vesicle solution containing ca. 0.2 mmol of phosphatidylethanolamine in 0.05 *M* NaCl was added 0.2 ml of 0.8 *M* $NaHCO_3$ (pH 8.5) and 20 μl of 1.5% TNBS in H_2O. The reaction was allowed to proceed in the dark for 30 min, then terminated by addition of 1.2 ml of 1.06% Triton X-100 in 1.5 *N* HCl.

(ii) Labeling of Total Phosphatidylethanolamine. To 0.6 ml of vesicle solution was added 0.2 ml of 1.6% Triton in 0.8 *M* $NaHCO_3$ (pH 8.5) and 20 μl of 1.5% TNBS in H_2O. The solutions showed a marked increase in turbidity upon addition of the Triton X-100. However, the amino groups became susceptible to trinitrophenylation as indicated by the rapid appearance of yellow color upon addition of TNBS. After 15-min incubation in the dark, an additional 0.4 ml of 1.6% Triton X-100 was added, which caused the solution to become clear. After an additional 15-min incubation, the reaction was terminated by addition of 0.8 ml of 0.4% Triton X-100 in 1.5 *N* HCl. Due to excessive CO_2 evolution at this stage, the acidic Triton X-100 was added in two 0.4-ml portions.

The extent of outside and total labeling was determined from the absorbance of the trinitrophenyl derivative at 410 nm. At this wavelength an absorbance of 1.0 corresponds to 0.368 μmol of phosphatidylethanolamine in the assay mixture. Appropriate blanks were determined for both the outside and total labeling since the absorbance resulting from TNBS hydrolysis is different for each. In order to minimize the problem of residual CO_2 evolution, the solutions containing an acid-washed boiling chip were vigorously agitated with a Vortex mixer prior to reading. Fresh TNBS solution was prepared every 4 days and stored in the dark at 4°.

Incubation of Amidinated Vesicles. In order to minimize oxidation of the acyl and amino groups of phosphatidylethanolamine during prolonged incubations of the amidinated vesicles at 22°, the vesicle solution was stored in a sealed 40-ml serum vial, fitted with a rubber septum. Water-saturated argon was slowly bubbled through the solution for about 1 hr each day, using a 22 gauge syringe needle inserted through the septum and extending to the bottom of the vial. A second larger gauge needle served as a vent. After each argon flush, the needles were stoppered with silicon rubber plugs, and the solution was returned to storage in the dark. Samples were withdrawn for assay with a syringe while allowing argon to displace the volume of sample taken from the vial. Flushing was carried out in the dark.

Results and Discussion

The primary difficulty in designing experiments to measure net chemical exchange of different phospholipids across the bilayer is preparation of vesicles containing an initial transbilayer nonequilibrium distribution of these lipids. A serious secondary problem is the establishment of conditions which preserve vesicle integrity at room temperature for long periods of time. Figure 2 illustrates this point. A preparation of vesicles containing a 1:1 ratio of phosphatidylcholine to phosphatidylethanolamine was incubated at room temperature for several days in a screw-capped test tube under argon. Each day a sample was withdrawn, after which the air space was flushed with argon. Experience has shown that these conditions of storage and sampling are sufficient to preserve the integrity of vesicle preparations at 4°. However, at room temperature, the ratio of external to total phosphatidylethanolamine increased exponentially with time and reached a value of 1 after 6 days; this change was accompanied by a loss of total phosphatidylethanolamine. This result could obtain only if the vesicle bilayer per-

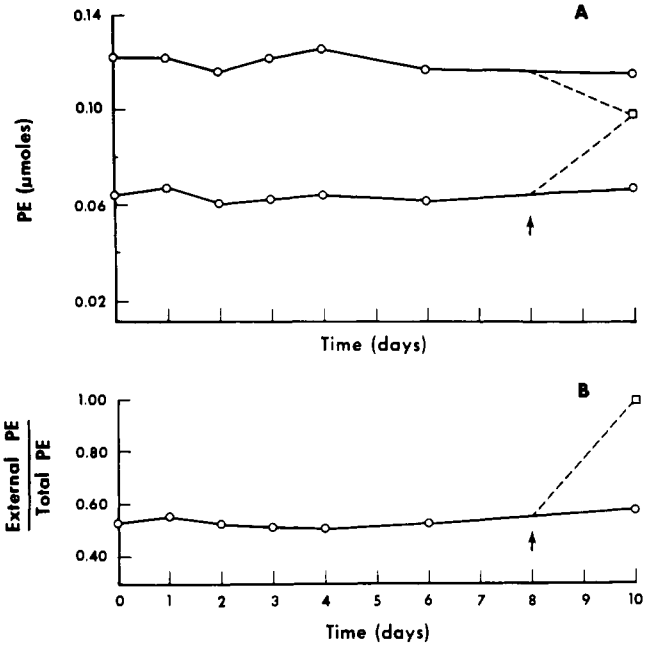


FIGURE 3: (A) External (lower curve) and total (upper curve) phosphatidylethanolamine in an equimolar phosphatidylethanolamine–phosphatidylcholine vesicle preparation, incubated at 22° in a sealed serum vial as described in the Experimental Section. The 10-day points indicated by the symbol shows the results obtained upon aeration of a sample withdrawn after 8 days as indicated by the arrow. (B) The ratio of external to total phosphatidylethanolamine.

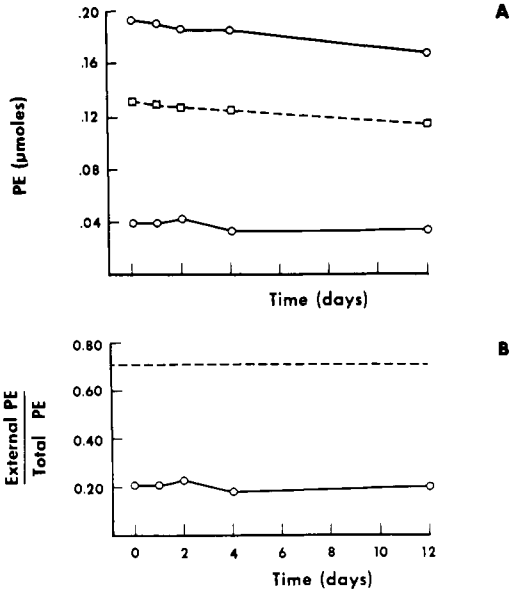


FIGURE 4: (A) External (lower curve) and total (upper curve) phosphatidylethanolamine in amidinated vesicles incubated at 22°. The initial phosphatidylcholine–phosphatidylethanolamine mole ratio was 9:1. The predicted external phosphatidylethanolamine concentration, if equilibrium were achieved (middle curve). (B) Observed ratio of external to total phosphatidylethanolamine (—). Predicted ratio of external to total phosphatidylethanolamine if equilibrium were achieved (- - -).

meability barrier to TNBS was lost during that period.

Since oxidation of the lipids is the most likely cause of the loss of vesicle integrity, future preparations were stored in serum vials in which argon was bubbled through the solution itself and samples were withdrawn without exposure to air. Details of this procedure are given in the Experimental Section.

Figure 3 shows the results obtained with a standard vesicle preparation containing a 1:1 ratio of phosphatidylcholine to phosphatidylethanolamine stored under these conditions. Virtually no change is observed in the external and total phosphatidylethanolamine over a period of 10 days. Ultraviolet spectral measurements made daily indicated that essentially no oxidation of the lipid had occurred under these conditions. In order to examine the effects of oxidation, a sample was withdrawn after 8 days, shaken with air, and stored in a screw-capped test tube. After 2 days, the ratio of external to total phosphatidylethanolamine in the oxygenated sample had increased to 1 and a significant loss of total phosphatidylethanolamine was observed. These results show that the storage technique adopted preserves vesicle integrity for at least 10 days, and suggest that oxidation is a major factor leading to the loss of vesicle integrity.

Figure 4 shows the results obtained with the amidinated vesicles. The initial ratio of external to total phosphatidylethanolamine was approximately 0.2. This value is compared to the equilibrium ratio of 0.71 in vesicles containing 0.1 mole fraction of phosphatidylethanolamine. Calculation shows that 90% of the external phosphatidylethanolamine groups were amidinated. Figure 4 clearly shows that no change in external to total phosphatidylethanolamine is observed over a period of 12 days.

The process we would have liked to measure is the rate at which opposing transbilayer chemical gradients of phosphatidylethanolamine and phosphatidylcholine decay to equilibrium in a vesicle system containing only these phospholipids. Since we have not yet been able to prepare vesicles of this kind, we have had to create nonequilibrium distribution of phospholipids by amidination of the external phosphatidylethanolamine groups in a vesicle which initially contained an equilibrium distribution of phosphatidylethanolamine and phosphatidylcholine across the bilayer. It is therefore necessary to describe the chemical gradients which exist in the resulting three-component system.

The ratios of the concentrations in the opposite faces of the bilayer of each lipid species, both before and after amidination, can be calculated as follows. If the ratio of the amount of a specific lipid in the outer bilayer face to the total amount of that lipid in the vesicle is x, then the ratio of the amount of that lipid in the outer face to the amount in the inner face is $x/(1-x)$. The ratio of the concentrations of that lipid in the two faces is than obtained by multiplying $x/(1-x)$ by R_i^2/R_o^2 where R_i and R_o are the radii of the inner and outer bilayer faces, respectively. The outer radius of the vesicle determined by autocorrelation light scattering is 112 Å (J. Goll, . F. Carlson, and B. Litman, manuscript in preparation). Assuming that the bilayer thickness is 40 Å (Thompson et al., 1974) then $R_i^2/R_o^2 = 0.41$. Before amidination $x = 0.71$ for phosphatidylethanolamine (although a value of $x = 0.61$ was obtained in one experiment). Employing the value $x = 0.71$, the ratio of the concentrations of phosphatidylethanolamine in the outer to that in the inner bilayer surface is 1.01. Although the ratio of the amounts of phosphatidylcholine in the two faces of the bilayer has not been determined, if equal packing densities in both surfaces is assumed, then the ratio of the concentrations of phosphatidylcholine in the outer to that in the inner face is 0.99. Thus, in the initial configuration of the system before amidination there are essentially no chemical concentration gradients of lipid species existing across the bilayer. However, this situation is altered by amidination of 90% of the external phosphatidylethanolamine. Now $x =$

0.2 for phosphatidylethanolamine and hence the ratio of concentrations of this component in the outer face to that in the inner face is 0.10. The corresponding concentration ratio for the amidine derivative is infinite while the transbilayer concentration ratio of phosphatidylcholine remains 1.0. Assuming that the total number of lipid molecules in each bilayer face remains constant, two exchange processes could result in the appearance of external phosphatidylethanolamine. (i) The exchange of internal phosphatidylethanolamine for the amidinated derivative. This process would cause the decay of the gradients of both of these species and hence would be expected to occur spontaneously. (ii) The exchange of internal phosphatidylethanolamine for external phosphatidylcholine. In this case decay of the transbilayer gradient of phosphatidylethanolamine would necessarily be coupled to the generation of a transbilayer gradient of phosphatidylcholine. Nonetheless, this process would also be spontaneous since, in order to achieve equilibrium, the relative concentrations of all exchangeable phospholipids must reach the same value on each side of the bilayer. Since the data presented in Figure 4 clearly show that no new phosphatidylethanolamine appears on the external surface of the vesicle over a period of 12 days, it can be concluded that neither of these processes occurred. These results, of course, say nothing about self-exchange or the exchange of phosphatidylcholine for the amidine derivative. A conservative estimate of the precision of the measurements is ±10%. On this basis, the estimated half-time for phosphatidylethanolamine exchange by either process in small vesicles must be greater than 80 days at 22°.

It is important to note that if an increase in the ratio had been observed, it would have been difficult to attribute this change unambiguously to phospholipid exchange. As indicated earlier, it is always possible that changes in the ratio and total values are caused by oxidation or hydrolysis of the component lipids resulting in the loss of vesicle integrity possibly followed by fusion.

On the other hand, it is possible that the absence of observed exchange under the conditions utilized is in fact due to alteration in the structure of the bilayer caused by the amidine derivative. This seems unlikely, however, since the initial low concentration of phosphatidylethanolamine was specifically chosen so that the total mole fraction of amidine in the external vesicle wall would be small. In addition, the chemical structure and charge configuration of the amidine derivative are quite similar to both phosphatidylcholine and phosphatidylethanolamine.

There is considerable evidence to suggest that the structure of the bilayer wall is unusual in the very small unilamellar vesicles employed in this study (Thompson et al., 1974). It is therefore possible that the absence of transbilayer exchange of phosphatidylethanolamine for phosphatidylcholine reported herein or the absence of self-exchange reported previously (Rothman and Dawidowicz, 1975; Johnson et al., 1975) is peculiar to this type of vesicle. One possible explanation is that a small vesicle cannot readily accommodate the temporary mass imbalance that must result from the transfer of a single phospholipid molecule from one side of the bilayer to the other, since the spherical geometry of the vesicle sets rather close limits on the number of molecules in each bilayer face. It therefore may be that the flip-flop of a molecule from one face to the other is closely coupled in time to the motion of a molecule in the opposite direction. If the two events were in fact concerted, the probability of the exchange would be the product of the probabilities for each unidirectional event. It seems possible, however, in planar bilayers of large area in which the coupling of opposing unidirectional flip-flop might be relaxed, that both transbilayer self-exchange and the exchange of different chemical species may be more rapid. The resolution of this question requires further experimentation.

Assuming, however, that the long half-time of transbilayer exchange of phosphatidylethanolamine for phosphatidylcholine or the amidine derivative observed in small vesicles obtains in all bilayer systems, maintenance of the very large transmembrane compositional asymmetries seen in certain biological membranes could be largely accounted for. Thus, it is not surprising that the localization of the choline lipids in the outer surface and the primary amine lipids in the inner surface of the erythrocyte membrane is stable for the lifetime of this biosynthetically incompetent cell.

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