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Asymmetric Exchange of Vesicle Phospholipids Catalyzed by the Phosphatidylcholine Exchange Protein. Measurement of Inside–Outside Transitions†

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ABSTRACT: Purified phosphatidylcholine exchange protein was used to exchange phosphatidylcholine between homogeneous single-walled phosphatidylcholine vesicles and human erythrocyte ghosts. When excess ghosts were present, it was found that only 70% of the vesicle phosphatidylcholine was available for exchange. This fraction corresponds closely to the amount of phosphatidylcholine in the outer monolayer of these vesicles, indicating that only the outer surface of the vesicle is accessible to the exchange protein. Also, it was found that all phosphatidylcholine introduced into vesicles by the exchange protein was available for subsequent exchange. Using the exchange protein, asymmetrical vesicles were prepared in which the outer monolayer was either enriched or depleted in radioactive phosphatidylcholine as compared to the inner monolayer. Reequilibration of the radioactivity between the two surfaces of the vesicle (flip-flop) could not be detected, even after 5 days at 37°. It is estimated that the half-time for flip-flop is in excess of 11 days at 37°. These results indicate that the properties of the exchange protein can be exploited to measure phosphatidylcholine flip-flop rates and possible phosphatidylcholine asymmetry in biological and model membranes, without altering the structure of the membrane.

The exchange of phospholipids between membranes is catalyzed by soluble proteins which occur in several tissues (Wirtz and Zilversmit, 1968, 1969; Zilversmit, 1971; Wirtz et al., 1972; Enholm and Zilversmit, 1973). Of these, a phosphatidylcholine exchange protein from liver has been studied in the most detail. This protein, which has been purified to homogeneity (Kamp et al., 1973), is capable of exchanging PC[1] between a variety of different PC containing membranes (Wirtz and Zilversmit, 1968; Zilversmit, 1971; Wirtz et al., 1972) with absolute specificity (Wirtz et al., 1972; Kamp et al., 1973; Harvey et al., 1973) and with no net transfer of PC (Wirtz and Zilversmit, 1968). It acts by equilibrating the PC among the substrate membranes (Demel et al., 1973). In order to investigate whether this equilibrium involves all the PC in the membrane, or merely that fraction present on the same side of the membrane as the exchange protein, we have undertaken a detailed investigation of the kinetics of the exchange reaction using a model membrane of known structure as a substrate. In the process, we have employed the properties of the exchange protein to measure the rate of transbilayer movement of PC in vesicles, the so-called "flip-flop" process. A preliminary report of this work has been published elsewhere (Dawidowicz and Rothman, 1975).

Materials and Methods

Isotopes. [*methyl*-^{3}H]Choline chloride (2.34 Ci/mmol),

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[1] Abbreviations used are: PC, phosphatidylcholine; DOPC, 1,2-dioleoylphosphatidylcholine; KCl-Tris buffer, 0.1 *M* KCl–0.01 *M* 2-mercaptoethanol–0.01 *M* Tris-HCl (pH 7.4).

[*cholesterol*-4-^{14}C]cholesteryl oleate (56 mCi/mmol), and [9,10-^{3}H]oleic acid (4.89 Ci/mmol) were from New England Nuclear. Radioactivity was counted with a fluor consisting of 10 ml of a toluene–Triton scintillator (Patterson and Greene, 1965) and 1 ml of aqueous buffer.

Phospholipids. Egg PC was purified from egg yolks according to Litman (1973). Synthetic DOPC[1] was prepared as described by Robles and van den Bergh (1969). [^{3}H]DOPC was synthesized by using radioactive oleic acid anhydride, prepared from [9,10-^{3}H]oleic acid, in place of the unlabeled anhydride. All lecithins gave a single spot after thin-layer chromatography on silica gel with chloroform–methanol–water (65:25:4, v/v) as the solvent.

Erythrocyte Ghosts. Ghosts were prepared from human red blood cells after lysis in dilute phosphate buffer or Tris buffer according to standard procedures (Hanahan and Ekholm, 1974; Steck and Kant, 1974). Equivalent results were obtained with both lysis procedures.

Microsomes. Unlabeled rat liver microsomes were prepared in large quantities by a modification of the procedure of Wirtz and Zilversmit (1969). Livers (170 g) were rinsed with ice-cold 0.25 *M* sucrose–1 m*M* Na_2EDTA (pH 7.4), minced, and homogenized with 10 vol of the same buffer. This was followed by three centrifugations with the Sorvall GSA rotor (20 min at 1000*g*, 10 min at 15,000*g*, 20 min at 15,000*g*) in which the supernatants were saved and the pellets discarded. The resulting post-mitochondrial supernatant was centrifuged for 6.5 hr at 19,000 rpm in the Beckman 19 rotor. The microsomal pellets were homogenized with 400 ml of 10 m*M* Tris-HCl (pH 8.6), and centrifuged for 100 min in the Beckman 21 rotor at 21,000 rpm. These pellets were homogenized with 400 ml of 1 m*M* Tris-HCl (pH 8.6) and centrifuged as before. The final pellets were homogenized with 30 ml of 0.25 *M* sucrose–1 m*M* Na_2EDTA (pH 7.4).

Microsomes labeled with [^{3}H]PC were prepared according to Wirtz and Zilversmit (1969) from the liver of a rat that had been injected intraperitoneally with 5 mCi of [*methyl*-^{3}H]choline chloride 1 hr before it was sacrificed. A radiochromatogram of a lipid extract of one such preparation showed that greater than 97% of the ^{3}H cochromatographed with a PC standard. These microsomes (2–4 μCi/mg of protein) were used directly for the experiments in which [^{3}H]PC was introduced into vesicles from microsomes by the exchange protein. For the routine assay used for exchange protein purification, labeled microsomes were diluted with unlabeled microsomes to a specific activity of 1.2×10^{5} dpm/mg of protein. Microsomes were stored at −20°.

Phosphatidylcholine Vesicles. Appropriate lipid mixtures were lyophilized from benzene solution, dispersed in buffer using a vortex mixer, and sonicated for at least 15 min with a Branson or MSE probe sonicator under an N_2 atmosphere in a water-jacketed cell at 4°. The sonicate was centrifuged at 15,000*g* for 15 min to remove titanium particles shed from the probe. These dispersions are referred to as "unsized" vesicles, although chromatography on Sepharose 4B columns showed that at least 90% of the PC in these dispersions was present as fraction II homogeneous vesicles (Huang, 1969). For some experiments, unsized vesicles were chromatographed on Sepharose 4B columns (Huang, 1969) before use to obtain completely homogeneous preparations, which are referred to as "sized" vesicles. Thin-layer chromatography of chloroform–methanol extracts of vesicle dispersions revealed that no chemical degradation had taken place during the preparative procedure. Sephadex G-75 and Sepharose 4B columns were saturated with about 50 mg of egg PC before use (Huang, 1969) in order to minimize the binding of vesicles to the column. No measurable exchange of gel-bound PC with vesicle PC or elution of gel-bound PC takes place during vesicle chromatography (unpublished experiments).

Phospholipid Exchange Protein. A PC-specific exchange protein was purified from a calf liver pH 5.1 supernatant by pH 3 precipitation followed by chromatography on DEAE-cellulose, CM-cellulose, and Sephadex G-50 essentially as described by Wirtz et al. (1972). PC exchange activity was assayed by a modification of their original procedure: 0.6 mg of microsomes, labeled with 70,000 dpm of [^{3}H]PC, and 0.2 μmol of unsized egg PC vesicles, containing a trace of [^{14}C]cholesteryl oleate as a nonexchangeable marker, were incubated in polyethylene centrifuge tubes with protein fractions in a final volume of 0.6 ml of 0.25 *M* sucrose–1 m*M* Na_2EDTA–10 m*M* Tris-HCl (pH 7.4). After 15 min at 20°, the reaction was stopped by adding 0.2 ml of 0.2 *M* pH 5.0 acetate buffer. The precipitated microsomes were pelleted in an Eppendorf 3200 centrifuge within 3 min. Supernatant lipids were extracted (Bligh and Dyer, 1959) and transferred in chloroform–methanol to a glass counting vial. Triton-X-100 (10 mg) was added and the solvents were evaporated by heating at 90° for at least 15 min. The residue was dissolved with scintillation fluid and counted. Exchange was determined from the $^{3}H/^{14}C$ ratio, and units of activity were calculated from the known specific radioactivities. One unit is defined as a rate of exchange of 1 nmol of PC/min in this assay (Kamp et al., 1973).

The preparation used here was 2000-fold purified over the pH 5.1 supernatant and had a specific activity of 1400 units/mg. It was stored at −20° in a 1:1 mixture of glycerol and 50 m*M* citrate–0.1 *M* Na_2HPO_4–10 m*M* 2-mercaptoethanol (pH 5.0). No activity was lost over a 5-month period. Exchange protein was either used directly from the 50% glycerol buffer (after the pH was adjusted to 7.4 with 1 *N* NaOH) or after dialysis into KCl-Tris buffer (0.1 *M* KCl–10 m*M* 2-mercaptoethanol–10 m*M* Tris-HCl (pH 7.4)). Equivalent results were obtained. Before dialysis, bovine serum albumin was added (0.2 mg/ml) to the exchange protein solution to prevent loss of activity which otherwise occurs. In agreement with previous work (Wirtz and Zilversmit, 1969), we have found that albumin has no PC-exchange activity (unpublished results).

Recently, a new procedure suitable for a routine exchange protein assay has been developed. Ghosts and unsized vesicles of [^{3}H]DOPC, containing [^{14}C]cholesteryl oleate as an unexchangeable marker, are incubated with exchange protein fractions under conditions similar to Figure 3 and the amount of exchange is determined (see below). With this technique, 50- to 100-fold stimulation of exchange by exchange protein over blank values is reproducibly observed.

Determination of Exchange between Vesicles and Ghosts. Appropriate mixtures of exchange protein, bovine serum albumin, ghosts, and labeled vesicles were sealed under N_2 (to minimize autoxidation of lipids) and incubated at 37°. At appropriate times, 0.4-ml aliquots were removed and the ghosts were pelleted within 3 min with an Eppendorf 3200 centrifuge. Three-tenths milliliter of the supernatant was counted, and the pellet was resuspended with 1 ml of KCl-Tris buffer and then recentrifuged. The washed ghost pellet was suspended with 0.1 ml of water and

was transferred to a scintillation counting vial. The centrifuge tube was rinsed twice with 0.1 ml of water each, and the rinses were pooled in the vial and counted.

All vesicle preparations contained [^{14}C]cholesteryl oleate as a nonexchangeable marker (Kamp et al., 1973) and [^{3}H]PC as a substrate for the exchange protein. To show that [^{14}C]cholesteryl oleate is nonexchangeable, sized vesicles formed from synthetic bis-9,10-dibromostearoylphosphatidylcholine, containing [^{14}C]cholesteryl oleate, were incubated with sized [^{3}H]DOPC vesicles with or without exchange protein, and the two vesicle types (differing in density but of similar size) were then separated on 5–20% sucrose velocity gradients. No redistribution of the cholesterol ester was observed (to be published).

The amount of exchange was calculated as follows. For supernatants, the fraction of [^{3}H]PC remaining with the vesicles after the reaction was taken to be the supernatant $^{3}H/^{14}C$ ratio divided by the $^{3}H/^{14}C$ ratio for the starting vesicles. For pellets, all ^{14}C is due to contaminating vesicles. It was assumed that the vesicles which contaminate the pellet have exchanged to the same extent as those which remain in the supernatant after centrifugation. From these premises it can be shown that the fraction of vesicle [^{3}H]PC which had been transferred to ghosts is $(H_p - C_pR_v)/(H_{tot} - C_pR_v)$, where H_p is the total ^{3}H in the pellet, C_p is the total ^{14}C in the pellet, R_v is the $^{3}H/^{14}C$ ratio in the starting vesicles, and H_{tot} is the total ^{3}H in the aliquot that was centrifuged. Exchange values determined from assay of the supernatants and of the pellets agreed well in most cases.

Analytical Determinations. Protein was measured by the method of Lowry et al. (1951). Vesicle PC concentrations were determined directly by measuring organic phosphate, from radioactivity of [^{3}H]DOPC of known specific activity, or from radioactivity of cholesteryl oleate in vesicles with known ratios of ^{14}C to PC. For erythrocyte ghosts, total phospholipid was assayed by P determination (Gomori, 1942) of a quantitative lipid extract (Burger et al., 1968). Alternatively, the acetylcholinesterase activity (Ellman et al., 1961) of a ghost suspension was compared with an equivalent volume of the packed red cells from which the ghosts were made. Ghost phospholipid concentration was then calculated using the known packed cell phospholipid concentration (Hanahan, 1969). Both techniques gave equivalent results. The ghost PC concentration was calculated from the total phospholipid concentration and the known (Rouser et al., 1968) mole fraction of phospholipid which is PC, 0.29.

Results

Incubation of Vesicles with Excess Ghosts. When labeled PC vesicles are mixed with unlabeled ghosts and exchange protein, the specific radioactivity of all exchangeable PC molecules equalizes given sufficient time. Thus, if the exchangeable pool of ghost PC greatly exceeds that of the vesicle, it follows that the fraction of radioactive PC removed from the vesicle and introduced into the ghost by exchange protein is, after equilibration, the same as the fraction of PC in the vesicle which is available for exchange.

[^{3}H]DOPC vesicles containing [^{14}C]cholesteryl oleate as a nonexchangeable marker were made by sonication followed by chromatography on a Sepharose 4B column. Figure 1 is typical of the elution profiles obtained. The high ratio of $^{14}C/^{3}H$ in the void volume (fraction 11) indicates either the presence of cholesterol ester particles or the preference of the cholesterol ester for multilayers. The latter possibility is similar to results found for thiocholesterol (Huang et al., 1970). For experiments with ghosts, vesicle fractions 19 and 20 were chosen. It is known from previous work on egg PC vesicles (Huang, 1969) that preparations from this region of the elution profile consist of structurally homogeneous bilayer vesicles.

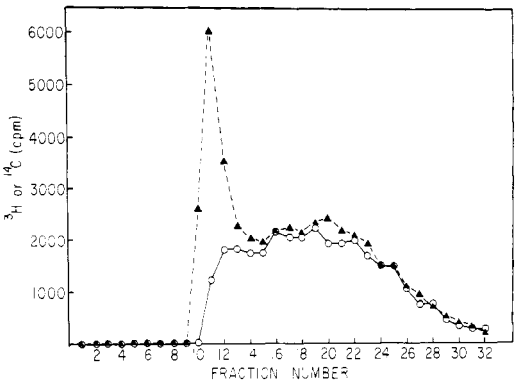

FIGURE 1: Elution profile of a sonicated dispersion of ^{3}H-labeled 1,2-dioleoylphosphatidylcholine from Sepharose 4B. Unsized vesicles were made from 1.5 μCi of [^{14}C]cholesteryl oleate (80 μCi/mg) and 1.1 μmol of [^{3}H]DOPC (25 μCi/μmol) in 4.0 ml of KCl-Tris buffer. The unsized vesicles were applied to a Sepharose 4B column (2.5 cm × 23 cm) at 4° and eluted with KCl-Tris buffer. Fractions (5 ml) were collected and 100-μl aliquots of each fraction were counted. Fractions 19 and 20 were used in subsequent experiments: (▲) [^{14}C]cholesteryl oleate; (○) [^{3}H]PC.

Figure 2 shows the time course of exchange between Sepharose 4B sized vesicles and excess ghosts (ghost to vesicle PC ratio about 80) catalyzed by exchange protein. A saturating value of about 70% of the vesicle [^{3}H]PC is transferred to ghosts. This result has been obtained three times, using both sized and unsized PC vesicles. We believe that the poor kinetics of the early points is due to the difficulty of terminating the reaction by centrifugation.[2] When exchange protein was omitted from a reaction otherwise identical with Figure 2, no exchange was observed. The amount of ghost PC used did not limit the amount of vesicle [^{3}H]PC that could be exchanged. Thus, duplicate aliquots of the reaction of Figure 2 were taken at 60 min, fresh ghosts were added, and the reaction was continued for 60 min more. It was found that doubling the ghost concentration caused no additional [^{3}H]PC to be exchanged. That the shut-off of exchange at 70% is not due to a loss of activity of exchange protein or to a change in the reactivity of the ghosts during the incubation is demonstrated by the following experiment. When ghosts and exchange protein are preincubated without vesicles at 37° for 130 min (i.e., after the point where exchange has shut off) under conditions similar to Figure 2, and then vesicles are added for 10 min at 37°, 54% of the vesicle [^{3}H]PC is transferred to ghosts; if the preincubation is omitted, 55% transfer occurs. Indeed,

[2] The difficulty in obtaining accurate kinetics in the initial phase of the exchange reaction has been experienced by other workers (Hellings et al., 1974), who have also cited the time lag involved in stopping the reaction as the likely source of error (Hellings et al., 1974). The principal conclusions of the present study rest upon the time-independent, saturating values obtained from the time courses of exchange, and not at all on the kinetics or initial velocities. Our attempts to stop the exchange reaction by procedures other than centrifugation have failed or have impaired the ultimate separation of the donor and the acceptor membranes. For example, water-soluble inhibitors of exchange protein structurally related to lecithin were not found, protease digestion of exchange protein is too slow, and acid or ammonium sulfate treatment coprecipitates vesicles with ghosts and exchange protein.

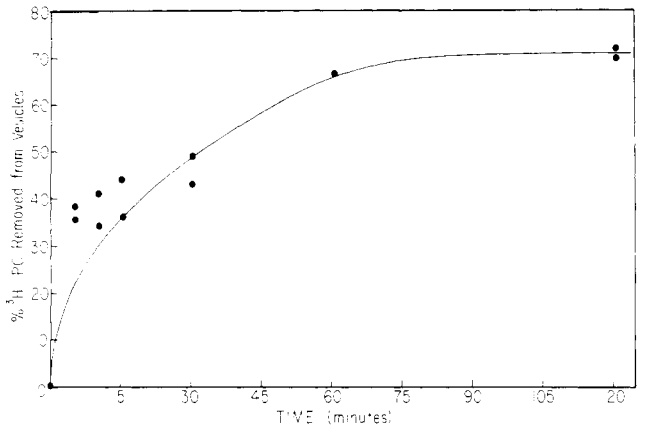

FIGURE 2: Time course of exchange between sized vesicles and excess ghosts. Sized vesicles from fraction 20 of Figure 1 (8.5 nmol of PC) were incubated with ghosts (680 nmol of PC), dialyzed exchange protein (63 units), and bovine serum albumin (0.36 mg) in a total volume of 8.0 ml of KCl-Tris buffer at 37°. At various times, duplicate samples were taken and the amount of exchange was determined as described under Materials and Methods. Here, the percent of [^{3}H]PC that was transferred to ghosts from vesicles (determined from the ghost pellet) is plotted vs. time of incubation. When exchange protein was omitted from the reaction, no exchange took place over a 2-hr period.

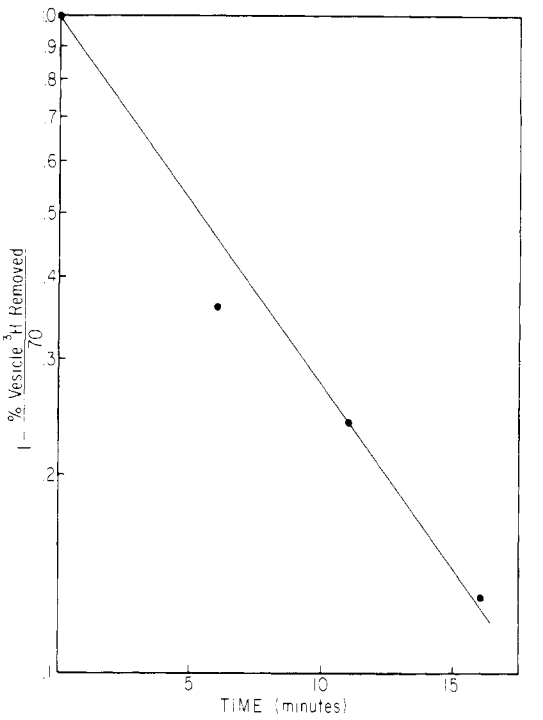

FIGURE 3: Kinetics of exchange between vesicles and excess ghosts. Eighty nanomoles of [^{3}H]DOPC (25 μCi/μmol) and 0.1 μCi of [^{14}C]cholesteryl oleate (80 μCi/mg) were colyophilized and unsized vesicles were prepared in 4 ml of KCl-Tris buffer. Unsized vesicles (0.5 ml) (10 nmol of PC) were incubated at 37° with 0.2 ml of ghosts (260 nmol of PC), 0.5 ml of undialyzed exchange protein (17 units), 0.2 ml of bovine serum albumin (0.4 mg in KCl-Tris buffer), and 2.6 ml of KCl-Tris buffer. At stated times, aliquots were removed and the amount of exchange determined. Values from a control reaction in which the exchange protein was replaced with an equivalent volume of 50% glycerol storage buffer were subtracted from the corresponding values of the complete reaction to give the data presented here. The graph plots $[1 - (E(t)/70)]$ on a logarithmic scale vs. time of incubation. $E(t)$ is the percent of vesicle [^{3}H]PC which has been transferred to ghosts by time t, as determined from the supernatant. For isotopic equilibration, this plot should, theoretically, yield a straight line (see text).

in the presence of 2-mercaptoethanol and bovine serum albumin, exchange protein retains full activity after 24 hr at 37° (unpublished results). It is concluded that the shut-off of exchange at 70% is due to an inherent property of the vesicles, namely that only 70% of the PC is accessible to the exchange protein. Because 70% of the PC molecules are on the outer monolayer, the conclusion that exchange protein can exchange the outer but not the inner monolayer is strongly suggested (see Discussion).

For isotopic equilibration between two pools, namely radioactive donor vesicles in which only 70% of the isotope can exchange and excess acceptor membranes (ghosts), one can calculate that $E(t) = 70(1 - \exp(-t/T))$ where E is the percent of vesicle [^{3}H]PC which has been transferred to ghosts at time t, and T is the reciprocal rate constant. Thus, a semilog plot of $1 - E/70$ vs. time should, theoretically, yield a straight line. Figure 3 shows such a plot for an experiment, similar to that of Figure 2, which was chosen because it fits the theoretical kinetic expression well.

Removal of PC Introduced into Vesicles by Exchange Protein. The above experiments show that only 70% of the radioactivity of a uniformly labeled PC vesicle is exchangeable, corresponding to the outer monolayer. A strong prediction is that any PC introduced into vesicles via exchange protein should be accessible to exchange during a second incubation, provided that equilibration with the nonexchangeable inner monolayer (flip-flop) takes place very slowly.

^{3}H-Labeled PC was incorporated into unlabeled DOPC vesicles by the exchange protein using biosynthetically labeled microsomes as a donor membrane. The microsomes were removed by centrifugation, and the supernatant was passed through a Sephadex G-75 column to separate the vesicles from exchange protein. The vesicles were located in the void volume and were then incubated with exchange protein and ghosts, and the time course of exchange was determined.

Rapid redistribution of [^{3}H]PC between the two membranes took place until an equilibrium was reached whereupon no further transfer of radioactivity occurred with prolonged incubation. The fraction of [^{3}H]PC transferred to the ghosts at this point depended on the ratio of ghost PC to vesicle PC in a predictable fashion. Let p_g be the percent of exchangeable PC in the reaction which is in ghost membranes, and let p_v be the corresponding percent for vesicles ($p_v + p_g = 100$). We assume that all the [^{3}H]PC introduced by exchange protein into the vesicle is still exchangeable. Then, for isotopic equilibration between the vesicle and ghost exchangeable PC pools, $R(t) = p_v + p_g \exp(-t/T)$, where $R(t)$ is the percent of vesicle [^{3}H]PC which remains in the vesicle at time t, and T is the reciprocal rate constant. Rearrangement of this equation shows that $[R(t) - p_v]/p_g$ plotted on a log scale vs. time should yield a straight line. Figure 4 presents this plot for one such experiment. p_v and p_g were calculated by assuming (1) that all PC in the ghosts was exchangeable and (2) that all [^{3}H]PC but only 70% of the total PC molecules in the vesicles were exchangeable.

In the experiment of Figure 4, after equilibration, 69% of vesicle radioactivity was removed. Higher ratios of ghost to vesicle PC allow for more transfer of radioactivity. For isotopic exchange with no chemical transfer, the percent of ^{3}H transferred from vesicle to ghost at equilibrium (E_∞) will be identical with p_g. Table I presents the experimental values E_∞ and theoretical values p_g calculated above.

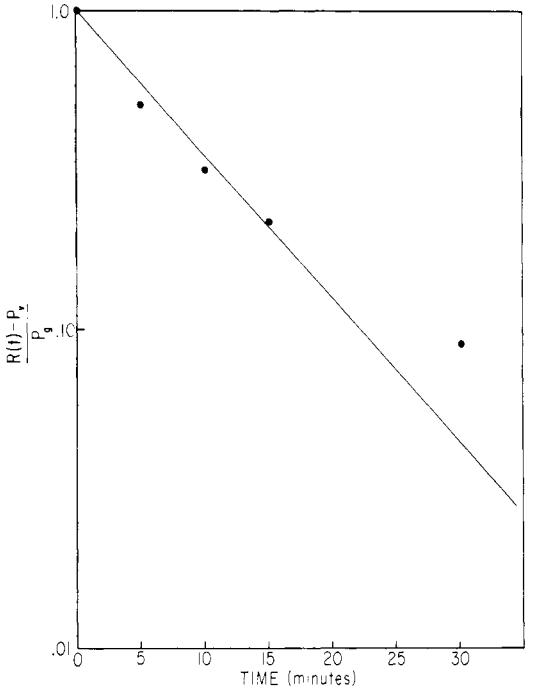

FIGURE 4: Kinetics of removal of [^{3}H]PC introduced into vesicles by the exchange protein. Unsized vesicles were prepared from 16 μmol of unlabeled DOPC and 1 μCi of [^{14}C]cholesteryl oleate (80 μCi/mg) in 4.0 ml of KCl-Tris buffer. Labeling of vesicles with [^{3}H]PC was accomplished as follows: 0.8 ml of unsized vesicles, 0.8 ml of microsomes labeled with [^{3}H]PC (4.8 mg of protein/ml, 2 μCi/mg, in 0.25 *M* sucrose–1 m*M* Na_2EDTA (pH 7.4)) and 0.5 ml of undialyzed exchange protein (17 units) were incubated at 37°. After 2 hr, the mixture was layered above 4.0 ml of 10% (w/v) sucrose in KCl-Tris buffer, and centrifuged at 4° for 90 min at 32,000 rpm in a Beckman SW 39 rotor. The upper layer was passed through a Sephadex G-75 column (1 cm × 22 cm) at 4° to separate the vesicles from the exchange protein. The column was eluted with KCl-Tris buffer, and 1.5-ml fractions were collected. The void volume (containing vesicles) was located by counting aliquots of fractions. For the exchange reaction with ghosts, G-75 purified, exchange protein-labeled [^{3}H]PC vesicles (0.48 μmol of PC) were incubated with ghosts (0.51 μmol of PC), dialyzed exchange protein (38 units), and bovine serum albumin (0.23 mg) in a final volume of 6.0 ml of KCl-Tris buffer at 37°. At the stated times, samples were taken and the amount of exchange was determined. When exchange protein was omitted, no exchange took place. In this experiment, a limiting value of 69% of the vesicle [^{3}H]PC was transferred from vesicle to ghost. Time points after isotopic equilibration was established are not shown here. p_g was calculated to be 60% and p_v to be 40%. Plotted here is $[R(t) - p_v]/p_g$ on a logarithmic scale vs. time (see text for definitions of symbols). Theoretically, this should yield a straight line.

The close agreement between the predictions and experimental results implies that all of the [^{3}H]PC introduced by the exchange protein is available for exchange. This is most strikingly demonstrated by experiment 3 of Table I in which 96% of the vesicle [^{3}H]PC has been transferred to the ghosts.

Movement of PC between the Surfaces of a Vesicle. The results presented thus far show that only PC molecules in the outer monolayer of a vesicle are accessible to the exchange protein. A corollary of this is that the movement of PC across the bilayer (flip-flop) must be a slow process. The following experiments were undertaken in order to quantitate the rate of this process.

Asymmetric PC vesicles, labeled with [^{3}H]PC exclusively in the outer monolayer, were prepared by exchange with radioactive microsomes as described above. Exchange protein was separated from these vesicles by gel filtration. The asymmetric vesicles were preincubated at 37° for various

Table I: Comparison of Predicted with Experimental Values for Isotopic Equilibration of Vesicle [^{3}H]PC, Introduced by Exchange Protein, with Ghosts.[a]

Expt	Ghost PC Concn (nmol/ml)	Vesicle PC Concn (nmol/ml)	Calcd p_g (%)	Measured E_∞ (%)
1	85	29	81	83
2	85	80	60	69
3	134	8.9	96	96

[a] All three experiments were carried out in a fashion similar to Figure 4. Different ghost and vesicle preparations were used in each experiment. The stated concentrations are those during the incubation of Sephadex G-75 purified [^{3}H]PC vesicles with ghosts and exchange protein. E_∞ is the percent of vesicle [^{3}H]PC which is transferred to ghosts when isotopic equilibration has been reached. E_∞ was determined from the average of at least two data points widely separated in time (about an hour) in the region of the time course in which no significant net transfer of ^{3}H took place. p_g is the percent of exchangeable PC in the reaction which is in the ghost membranes, calculated from the ghost and vesicle PC concentrations as described in the text.

times. Before the preincubation all [^{3}H]PC is available for exchange, as has been demonstrated in the previous section. If flip-flop occurs during the preincubation, this will no longer be the case. The specific radioactivity of PC in the outer monolayer will decrease from its initial value as the [^{3}H]PC moves to the inner monolayer and is replaced by unlabeled PC. The specific radioactivity of PC in the outer monolayer, and therefore flip-flop, was assayed as a function of time of preincubation by incubating the preincubated vesicles with ghosts and exchange protein. The percent of [^{3}H]PC transferred to the ghosts during this incubation is proportional to the specific radioactivity of the outer monolayer. The results of this experiment are presented in Table II. No significant change of the amount of transfer to ghosts, 22.0 ± 1.3%, was observed over the 5-hr preincubation period. If complete equilibration of [^{3}H]PC between the two surfaces had taken place, the specific radioactivity of the outer monolayer would have been 0.70 of its initial value (see Discussion), so that 15.4% transfer would have occurred in the incubation. Isotopic equilibration by flip-flop follows simple exponential kinetics, so one may write $E(t) = 15.4 + 6.6 \exp(-t/T_f)$, where E is the amount of vesicle [^{3}H]PC transferred to ghosts after preincubation time t and T_f is the time constant for the flip-flop process. If the amount of transfer in the incubation had decreased to as little as 19.4% (the mean less two standard deviations) after the 5-hr preincubation, it would almost certainly have been detected, but was not. Using the above equation, a lower limit for T_f of 10 hr may be calculated. This corresponds to a half-time in excess of 7 hr at 37° for the flip-flop process.

It was not possible to extend the preincubation period in the above experiment significantly, since anomalies due to hydrolysis of the cholesterol ester occurred in the incubations. When preincubations of a day or longer were attempted, it was found that the $^3H/^{14}C$ ratio of the vesicle supernatant after the control incubations without exchange protein was paradoxically greater than that of the preincubated vesicles. Thin-layer chromatography indicated that [^{14}C]cholesterol had been formed by hydrolysis of the [^{14}C]cholesteryl oleate. Presumably, the [^{14}C]cholesterol produced by hydrolysis exchanges with unlabeled cholesterol in the ghost membrane, but is not accompanied by

Table II: Measurement of Outside–Inside Transition Rate (Flip-Flop) in Phosphatidylcholine Vesicles at 37°.[b]

Time of Preincubation (hr)	% of Vesicle [^{3}H]PC Transferred to Ghosts during Incubation	
	Without Exchange Protein in Incubation	With Exchange Protein in Incubation
0.5	3.9, 3.9	23.3, 21.6
1.0	4.1, 4.1	20.3, 18.8
2.0	3.1, 4.3	23.2, 21.9
3.0	3.9, 4.4	22.3, 21.5
4.0	3.4, 3.9	23.9, 21.8
5.0	4.0, 4.5	22.7, 22.5
	4.0 ± 0.4[a]	22.0 ± 1.3[a]

[a] Mean values ± standard deviation. [b] Phosphatidylcholine vesicles, containing [^{3}H]PC in the outer monolayer only, were prepared as described in Figure 4, except that 0.2 ml of exchange protein and 0.1 ml of serum albumin (2 mg/ml of KCl-Tris buffer) were used in the 1-hr microsome labeling reaction. As before, the labeled vesicles were freed from exchange protein on Sephadex G-75. The vesicles were then preincubated at 37° (0.4 μmol of PC/ml) under nitrogen for the stated time, at which point aliquots were removed for a short incubation to measure the specific radioactivity of the outer monolayer of the vesicles. Thus, 0.2 ml of preincubated vesicles (80 nmol of PC), 20 μl of ghosts (25 nmol of PC), 20 μl of serum albumin (2 mg/ml in KCl-Tris buffer), 80 μl of KCl-Tris buffer, and 100 μl of undialyzed exchange protein (3.4 units, in the 50% glycerol storage buffer) were incubated at 37°. After 15 min, the ghosts were sedimented and the percent of vesicle [^{3}H]PC transferred to the ghosts was determined from the pellet as described under Materials and Methods. Control incubations in which the exchange protein was replaced by an equivalent volume of the 50% glycerol storage buffer were carried out in parallel. All incubations were done in duplicate. The table shows that the specific radioactivity of the outer surface of the vesicles did not decrease detectably during the entire preincubation period, so no flip-flop was observed. If the half-time for transbilayer isotopic equilibration were less than 7 hr, a decrease in the percent transfer of vesicle [^{3}H]PC to ghosts would have been observed (see text).

[^{3}H]PC, since the exchange protein is absent.

In a complementary series of experiments, asymmetric DOPC vesicles whose outer monolayer was depleted of [^{3}H]DOPC relative to the inner monolayer were prepared by reacting uniformly labeled vesicles with ghosts and exchange protein. The ghosts were removed by centrifugation, and the exchange protein was separated from the vesicles by gel filtration. The asymmetric vesicles were preincubated for various times at 37° in the presence or absence of freshly added exchange protein. If flip-flop were to take place during the preincubation, the specific radioactivity of the outer monolayer would increase from its initial value as [^{3}H]DOPC moves from the inner to outer monolayer in exchange for unlabeled DOPC. The specific radioactivity of the outer monolayer, and thus flip-flop, was measured by a short incubation of the preincubated vesicles with ghosts and exchange protein, as in the previous experiment. The amount of exchange protein present in the incubation was the same whether the vesicles were preincubated in the presence or absence of exchange protein. Control reactions in which the exchange protein was omitted in both the preincubation and the incubation were performed throughout.

The results of this experiment are shown in Table III. The amount of [^{3}H]PC transferred from vesicles to ghosts in the incubation did not vary detectably during the 5-day preincubation period, so no flip-flop was observed. Furthermore, the exchange protein did not measurably enhance the

Table III: Measurement of Inside to Outside Transition Rate (Flip-Flop) in Phosphatidylcholine Vesicles at 37° in the Presence and Absence of Exchange Protein.[b]

Time of Preincubation (hr)	% of Vesicle [^{3}H]PC Transferred to Ghosts during Incubation	
	Exchange Protein in Preincubation and Incubation	Exchange Protein in Incubation Only
0	33	25
2	28	28
6.5	23	21
15	18	19
24	21	23
38	20	23
66	24	24
121	26	29
	24.1 ± 4.5[a]	24.0 ± 3.1[a]

[a] Mean value ± standard deviation. [b] Unsized vesicles were prepared as described in Figure 3. One milliliter of vesicles, 1.0 ml of ghosts (1.3 μmol of PC), 0.2 ml of serum albumin (0.4 mg in KCl-Tris buffer), and 34 units of undialyzed exchange protein (in 1.0 ml of 50% glycerol storage buffer) were incubated at 37° for 2 hr. The mixture was then centrifuged at 15,000*g* for 30 min at 4° to pellet the ghosts. The supernatant was applied to a Sephadex G-75 column (1 × 22 cm) to separate the vesicles from exchange protein. The column was eluted with KCl-Tris buffer at 4°, and vesicles were located in the void volume and diluted with the KCl-Tris buffer. The vesicles were then preincubated at 37° in the presence or absence of the exchange protein for the stated time. For preincubation with exchange protein (preincubation A), 3.0 ml of vesicles (1.5 nmol of PC) was mixed with 0.5 ml of serum albumin (1 mg in KCl-Tris buffer), 0.70 ml of undialyzed exchange protein (24 units), and 0.8 ml of KCl-Tris buffer. An identical mixture was used for preincubation without exchange protein (preincubation B) except that the exchange protein was replaced with 50% glycerol storage buffer, and the total volume was doubled. After preincubation, 0.5-ml aliquots of the vesicles were incubated with the ghosts, with or without additional exchange protein, for 10 min at 37°. When the exchange protein was present both in the preincubation and incubation, 0.5 ml of vesicles from preincubation A (containing 2.4 units of exchange protein) was incubated with 20 μl of ghosts (26 nmol of PC) and 70 μl of 50% glycerol storage buffer. When the exchange protein was present only during the incubation, 0.5 ml of vesicles from preincubation B was incubated with 20 μl of ghosts and 70 μl of undialyzed exchange protein (2.4 units). Control incubations were performed in this case by replacing the exchange protein with 70 μl of the 50% glycerol storage buffer. The amount of exchange during the incubation was determined from the washed ghost pellet as described under Materials and Methods. The results presented are for the percent of vesicle [^{3}H]PC transferred to ghosts during the incubation as a function of preincubation time. These values are independent of time to within the error expected from the scintillation counting; statistical fluctuations in counting rates in this experiment (of one standard deviation) cause the values of exchange to vary by about ±3–4% exchange, and the standard deviations of the observed values for exchange over the 5-day preincubation period are in this range. The control incubation gave values for exchange which did not vary significantly with time. The mean and standard deviation were 7.3 ± 3%. The exchange protein evidently has not lost activity during the 5-day preincubation, since the amount of exchange in the incubation with preincubated exchange protein (24.1 ± 4.5) is identical with that using fresh exchange protein (24.0 ± 3.1).

flip-flop rate, since the amount of transfer for vesicles preincubated with exchange protein, 24.1 ± 4.5%, was identical with that for vesicles preincubated without exchange protein, 24.0 ± 3.1%. The anomalous effects on the control incubations (in which the exchange protein was omitted) which are attributable to cholesterol ester hydrolysis were not observed in these experiments. This observation, coupled with the invariance of the amount of transfer during

the incubation, argues that the structure of the vesicles has not changed throughout the course of the experiment.

An upper limit for the flip-flop rate can be estimated from these data provided that the amount of [^{3}H]PC transferred during the incubation from vesicles whose outer and inner surfaces are completely equilibrated is known. This quantity was determined by performing incubations identical with those in the flip-flop experiment, except that the preincubated vesicles were replaced by an equal amount of the original vesicles from which the asymmetric vesicles had been prepared, whose inner and outer monolayers have the same specific radioactivities: 46 ± 1% of the [^{3}H]PC of these vesicles was transferred to ghosts. Twenty-four percent of the preincubated vesicle [^{3}H]PC was transferred to ghosts, independent of preincubation time, as shown in Table III. Therefore, the following equation may be written: $E(t) = 46 - 22 \exp(-t/T_f)$, where E, t, and T_f are defined as above. If the amount of transfer had increased to 30% (the mean plus two standard deviations) during the 5-day preincubation, it would almost certainly have been detected, but was not. Using the equation with $t = 5$ days and $E = 30\%$ provides an estimate of a lower limit for T_f of 16 days. This corresponds to a half-time of 11 days.

In summary, flip-flop proceeds with a half-time in excess of 11 days at 37°, and is not detectably enhanced by the exchange protein.

Discussion

Exchange Protein Can Exchange Only PC on Its Side of a Vesicle. The experiments just described demonstrate that only 70% of the phospholipid in DOPC vesicles is available for exchange. Careful work by Huang and collaborators has shown that egg PC vesicles are homogeneous single bilayer spheres containing 2700 PC molecules (Huang, 1969) with an outer radius of 105 Å (Huang and Thompson, 1974). The inner monolayer radius can be calculated from the bilayer thickness of 36 Å (Wilkins et al., 1971). Assuming the area per PC is the same in both monolayers, it follows that 70% of the PC must be on the outer surface of the vesicle. This value is in agreement with direct measurements made with magnetic resonance techniques (Bystrov et al., 1971; Huang et al., 1974). From the internal aqueous volume of DOPC vesicles (as measured by Dr. R. E. Pagano) in comparison with egg PC vesicles, one can calculate that 67–68% of the phospholipid is in the outer monolayer for values of bilayer thickness ranging from 35 to 41 Å.

Kornberg and McConnell (1971b) discovered that rapid lateral diffusion of PC takes place in vesicles. They found that the translational step for this process occurs much more frequently than 3000 times/sec at 0°. Therefore, on the time scale at which exchange protein-mediated PC exchange takes place (minutes), all PC molecules in each monolayer are structurally equivalent. It follows that either the whole outer monolayer or the entire vesicle must be available for exchange, and that no intermediate cases can exist. Our results therefore prove that the exchange protein can only exchange the outer monolayer of a PC vesicle.

While this work was in its final stages, we became aware of experiments conducted independently by Hellings et al. (1974). These investigators have analyzed the early kinetics of exchange between vesicles by making the assumption that only the outer monolayer can exchange, and have found that their data can fit this model. The fit is not unique, since a model in which both outer and inner monolayers can exchange fits the data equally well. Their results can therefore not be considered to prove that only the outer surface exchanges, although they are consistent with this.

Flip-Flop Is a Very Slow Process. The first attempt to measure the rate of transbilayer movement of phospholipids was reported 4 years ago in an influential paper by Kornberg and McConnell (1971a). These authors observed that flip-flop of a spin-labeled nitroxide derivative of PC proceeded with a half-time of 6.5 hr at 30°, a very slow rate compared with that for lateral diffusion (Kornberg and McConnell, 1971b). Here we report a half-time for flip-flop in excess of 11 days at 37°. We believe that the most likely cause of this difference is that the spin-labeled PC molecule, though similar to authentic PC, is not in fact isomorphous with it. However, other factors may be involved. Recent spin-label experiments, similar to those of Kornberg and McConnell, with the exception that the reducing agent, ascorbate, is covalently linked to a high polymer, have shown that the half-time for the flip-flop of the spin-labeled phosphatidylcholine is as long as 20 hr at 25° (Dr. W. Hubbell, personal communication). The difference between the flip-flop rates of PC and its spin-labeled analog may mean that the extremely short flip-flop times reported for spin-labeled analog PC in natural membranes (on the order of minutes for *Acholeplasma laidlawii*, erythrocytes, and vesicles derived from the electroplax of *Electrophorus electricus*) by McNamee and McConnell (1973) and by Grant and McConnell (1973) are gross underestimates of the flip-flop times for the native PC molecules. Indeed, experiments which argue that fatty acid is incorporated into phospholipid exclusively at the inner surface of the erythrocyte membrane suggest that the PC flip-flop in erythrocytes has a half-time of hours or longer (Renooij et al., 1974).

After this manuscript had been completed, we became aware of measurements of flip-flop related to those described here which have subsequently been published (Johnson et al., 1975). The results obtained by these authors are in agreement with those reported in the present study.

Several different lines of evidence have led Bretscher (1972b) to suggest that mammalian cell surface membranes are asymmetrical bilayers, in that the cytoplasmic monolayer is comprised mainly of aminophosphatides, whereas cholinephosphatides are chiefly in the external monolayer. In our opinion, this asymmetry would most likely be a reflection of an asymmetric deposition of the lipids during membrane biosynthesis. From this point of view, it is significant that flip-flop in a model system proceeds with a half-time of several days or longer, since this could account for the maintenance of asymmetry over biologically relevant time periods.

Erythrocytes vs. Ghosts as Substrates for the Exchange Protein. In agreement with Hellings and co-workers (1974), we have found that intact human erythrocytes do not act as substrates for the exchange protein. Nonlytic trypsinization was without effect, so that charge repulsion by sialic acid (Jackson et al., 1973) or protection of PC by protein is most likely not involved. Nevertheless, washed ghosts of erythrocytes are excellent substrates for the exchange protein. This may indicate that there are lysis-induced alterations of membrane structure that may be relevant to the interpretation of labeling (Bretscher, 1972a; Gordesky and Marinetti, 1973) and enzymatic digestion experiments (Verkleij et al., 1973) which compare whole with lysed red cells, a possibility considered by some authors (Bretscher, 1972a). It is noteworthy that the availability of

PC for exchange in ghosts as compared to intact cells parallels properties possessed by phospholipase C from *Bacillus cereus* (Woodward and Zwaal, 1972), so a structural relationship between these two enzymes may exist. Hellings et al. (1974) noted that sphingomyelinase treatment of whole red cells made PC available for exchange. However, factors other than the presence of sphingomyelin must be involved in the inhibition of PC exchange, since ghosts and whole cells have the same lipid composition.

A principal result of this paper, that the exchange protein can utilize only PC on its own side of a membrane, can be exploited to measure PC flip-flop rates and possible PC asymmetry in natural membranes, without altering the structure of the membrane being studied. This represents a major advantage over other techniques which are being applied to these problems, such as chemical labeling (Bretscher, 1972a; Gordesky and Marinetti, 1973), phospholipase digestion (Verkleij et al., 1973), and spin labeling (McNamee and McConnell, 1973).

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