Asymmetry and Transposition Rates of Phosphatidylcholine in Rat Erythrocyte Ghosts†

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ABSTRACT: Purified phospholipid exchange protein from beef heart cytosol is used to accelerate the exchange of phospholipids between labeled sealed ghosts and phosphatidylcholine/cholesterol liposomes. The purified protein accelerates the transfer of phosphatidylcholine and, to a lesser degree, that of sphingomyelin, phosphatidylinositol, and lysophosphatidylcholine. The presence of exchange protein does not accelerate the exchange of phospholipids between intact red blood cells and liposomes, but 75% of the phosphatidylcholine of sealed ghosts is readily available for exchange. The remaining 25% is also exchangeable but at a slower rate. When the exchange is assayed between inside-out vesicles and liposomes, 37% of the phosphatidylcholine is readily available, and 63% is exchanged at a slower rate. These results are consistent with an asymmetric distribution of phosphatidylcholine in isolated erythrocyte membrane fractions. The sum of the forward and backward transposition of phosphatidylcholine between the inside and outside layers of sealed ghost membranes amounts to 11% per hour, and the half-time for equilibration is 2.3 h. Significantly lower values are obtained for the inside-out vesicles (half-time for equilibration: 5.3 h). These results suggest that, during the formation of the vesicles, the asymmetry of phosphatidylcholine is partially preserved, but structural changes occur in the membrane that affect the rate of membrane transposition of phosphatidylcholine.

The asymmetric distribution of phospholipid in both natural and artificial membranes has attracted increasing interest in recent years. Bretscher (1972) proposed an asymmetric distribution of amino phospholipids in the membrane of human erythrocytes. His proposal was corroborated by the work of Gordesky and Marinetti (1973). Both groups based their conclusions on chemical labeling with relatively nonpermeant reagents. Verkleij et al. (1973) carried out a study on intact cells and washed ghosts with the use of phospholipases, and their conclusions were in agreement with the earlier investigations: the outside of the membrane is composed mainly of PC[1] and sphingomyelin, whereas phosphatidylethanolamine and phosphatidylserine are confined to the inner side. Kahlenberg et al. (1974) confirmed these results by using inside-out vesicles generated from leaky ghosts and subjecting them to digestion with phospholipase A_2 and C.

The rate of phospholipid transposition across natural and artificial membranes is a question intimately related to the problem of lipid asymmetry. Kornberg and McConnell (1971) explored this problem with a spin-labeled analogue of PC. Recently, the possibility of using radioactively labeled PC to measure the rate of phospholipid transposition was demonstrated by Johnson et al. (1975). These investigators used a purified protein isolated from beef heart cytosol, which accelerates the exchange of phospholipids between natural and artificial membranes (Wirtz and Zilversmit, 1968; Wirtz and Zilversmit, 1969; Zilversmit, 1971; Ehnholm and Zilversmit, 1973). They incubated liposomes made from ^{32}P-labeled PC with an excess of mitochondria in the presence of the purified exchange protein and found that only about 60% of the label was rapidly transferred to the mitochondria, the other 40% being transferred with a half-life of several days. They concluded that the rapidly exchanging pool was the PC of the outer layer of the liposome bilayer and that the very slow exchange of the remaining 40% represented the transposition of PC from the inner to the outer monolayer of the liposome. Essentially the same results have been reported by Rothman and Dawidowicz (1975).

In this paper, we report the use of the phospholipid exchange protein as a tool to explore the structure and dynamics of phospholipids in sealed ghosts and inside-out vesicles derived from rat erythrocytes.

Materials and Methods

Lipids. [^{14}C]Triolein was obtained from Applied Science Laboratories (State College, Pa.) and purified by silica gel H thin-layer chromatography with hexane–diethyl ether–acetic acid (60:40:1, v/v/v). The triolein band was eluted with chloroform and stored at −20 °C. [*Methyl*-^{14}C]Phosphatidylcholine was obtained from rat liver 1 h after injection with [*methyl*-^{14}C]choline chloride (New England Nuclear, Boston, Mass.; 150 μCi/100 g of body weight). PC was purified by neutral alumina chromatography (Johnson and Zilversmit, 1975) and stored in chloroform–methanol (2:1, v/v) under N_2 at −20 °C. Nonradioactive soy PC (Nattermann, Köln, Germany) was purified and stored under similar conditions. Cholesterol (Sigma Chemical Co., St. Louis, Mo.) was purified as the dibromide derivative and crystallized in methanol. It was stored at 4 °C in ethanol. Butylated hydroxytoluene (Nutritional Biochemical Corp., Cleveland, Ohio) was dissolved in chloroform and used without further purification.

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[1] Abbreviations used are: PBS, 150 mM NaCl–5 mM sodium phosphate (pH 8.0); 5P8, 5 mM sodium phosphate (pH 8.0); 0.5P8, 0.5 mM sodium phosphate (pH 8.0); 5P8-Mg, 5 mM sodium phosphate–1 mM $MgSO_4$ (pH 8.0); AcChase, acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7); PC, phosphatidylcholine.

Table I: Transfer of Phospholipid Radioactivity from Erythrocytes, Sealed Ghosts, and Leaky Ghosts to PC/Cholesterol Liposomes.[a]

Membrane	Lipid ^{32}P Transferred to Liposomes (%)		
	Without Exchange Protein	With Exchange Protein	Net Acceleration due to Protein
Erythrocytes	12.0	15.1	3.1
Sealed ghosts	6.6	29.2	22.6
Leaky ghosts	8.5	28.7	20.2

[a] Labeled erythrocytes, sealed ghosts, and leaky ghosts (10 μg of phospholipid phosphorus) were incubated with PC/cholesterol liposomes (50 μg of phospholipid phosphorus) at 37 °C in 2 ml of PBS, with or without phospholipid exchange protein (4.9 units). After 60 min, the supernatants were isolated by centrifugation, and partitioned with 10 ml of chloroform–methanol (2:1, v/v). The lower phase was dried down, and radioactivity was determined in a liquid scintillation counter in the medium of Gordon and Wolfe (1960). Recovery of liposomes was monitored by the [^{14}C] triolein incorporated during their preparation, and the phospholipid transfers were corrected accordingly.

Liposome Preparation. Appropriate volumes of the stock solutions were mixed and dried at 30 °C under a stream of N_2. The final lipid mixture contained 12.5 mg of PC, 5 mg of cholesterol, 0.625 mg of butylated hydroxytoluene, and a trace of [^{14}C]triolein. The dried lipid was resuspended in 3.5 ml of diethyl ether and redried under N_2 to form a thin film in a test tube. Four milliliters of buffer (5P8 or PBS) was added, and a lipid suspension was formed by agitation for 10 min with a Vortex mixer. The sealed test tube containing the lipid suspension under N_2 was sonicated in a sonicating bath (Branson HD-50 or Laboratory Supply Co., Hicksville, N.Y.) for 60 min held between 4 and 20 °C. After sonication, 1 ml of bovine serum albumin (2.5 g % in the corresponding buffer) was added. The liposomes were prepared the day before the experiment and were centrifuged for 20 min at 27 700*g* before use.

Sealed Ghosts, Leaky Ghosts, and Inside-out Vesicles. Male rats were injected intraperitoneally with ^{32}Pi (0.5 mCi/100 g of body weight). Seventy-two hours after the injections, blood was drawn by heart puncture and collected into centrifuge tubes containing 1 ml of 100 mM Na_2EDTA as anticoagulant. All subsequent operations were performed at 0–5 °C unless specified otherwise. Plasma and the "buffy" coat were separated from cells by centrifugation at 1000*g* for 15 min. The red blood cells were washed three times with 3–5 volumes of 0.9% NaCl, and the top portion of the cells was discarded after each wash. Sealed ghosts and inside-out vesicles were prepared by the method of Steck (1974a) as follows: sealed ghosts were obtained by hemolysis of 1 volume of packed cells in 100 volumes of 5P8-Mg, followed by centrifugation at 12 000*g* for 10 min. In some experiments, the sealed ghosts were prepared by hemolysis of the erythrocytes in 40 volumes of 5P8, centrifugation, and resuspension of the membranes in 40 volumes of PBS. Sealing was achieved by incubation at 37 °C for 60 min. The sealed ghosts were collected by centrifugation and washed once with PBS. Ghosts prepared by either method were purified by subjecting the preparation to a Dextran gradient in the appropriate buffer as described below for inside-out vesicles.

Leaky ghosts were obtained by hemolysis in 5P8 (1 volume of packed cells in 40 volumes of buffer) and three additional washes with the same buffer. The membranes were pelleted after hemolysis and washed by centrifugation at 300 000 mol wt, Nutritional Biochemical Corp., Cleveland, Ohio) was used. Ten milliliter linear gradients (5 ml of 0.5P8 + 5 ml of Dextran, 8% w/v, in 0.5P8) were prepared were then centrifuged and stored at 0 °C overnight. They were resuspended in a minimal volume of 0.5P8 and passed five times through a 27-gauge needle. To separate the sealed vesicles from the unsealed ones and from residual leaky ghosts, a continuous gradient of Dextran (200 000–300 000 mol wt, Nutritional Biochemical Corp., Cleveland, Ohio) was used. Ten milliliters linear gradients (5 ml of 0.5P8 + 5 ml of Dextran, 8% w/v, in 0.5P8) were prepared at room temperature in Spinco SW41 rotor tubes with a SG 201 Multiple Sucrose Gradient Maker (Hoefer Scientific Instruments, San Francisco, Calif.).

Vesicle homogenates derived from 1–2 ml of ghosts were layered on the gradient and centrifuged for 2 h at 40 000 rpm at 22 °C. After centrifugation, the sealed vesicles were collected from the upper portion of the gradient, diluted with 5P8, and washed twice with the same buffer. The vesicles were stored in this buffer at 0 °C and used within 4 days of preparation. AcChase activity with and without Triton X-100 was used as a marker for sidedness and sealing of the vesicles. The method of Ellman et al. (1961), as modified by Steck (1974a), was used with the following changes: 5P8 was used instead of 100 mM sodium phosphate buffer, and the final concentration of 5-5′-dithiobis(2-nitrobenzoic acid) was 0.25 mM. The latency of the AcChase activity, defined as 100[1 − (activity without Triton/activity with Triton)], is taken as an index of the purity of the preparation. For seven different preparations, it was 77.7 ± 4.5 (average ± SD). The contamination was considered to be right-side-out vesicles because the unsealed species pelleted at the bottom of the gradient.

Purified Phospholipid Exchange Protein. Phospholipid exchange protein was purified from beef heart cytosol and assayed as described by Johnson and Zilversmit (1975). It was dialyzed against 50 volumes of 5P8 or PBS overnight before use. One unit of phospholipid exchange protein is defined as the transfer of 1 nmol of PC/min at 37 °C (Zilversmit and Hughes, 1976). The incubation conditions and analytical procedures are described in the respective tables and figures.

Calculations. The calculations were performed on the basis of a two-pool model. The mathematical treatment is given in Appendix.

Results

Transfer of Phospholipids from Red Blood Cell Membranes to PC/Cholesterol Liposomes. Red blood cells, sealed ghosts, and leaky ghosts were incubated at 37 °C with PC/cholesterol liposomes (Table I). Cholesterol was incorporated into the liposomes in order to avoid loss of sterol from the natural membranes (Bruckdorfer et al., 1968). In two experiments with red blood cells, 12% of ^{32}P-labeled phospholipid was transferred to the liposomes, whereas with sealed ghosts and leaky ghosts, a somewhat smaller amount of labeled phospholipid was transferred to the liposomes after 60 min of incubation. The presence of 4.9 units of phospholipid exchange protein notably enhanced the transfer between sealed ghosts or leaky ghosts and liposomes but did not affect the transfer between intact red blood cells and liposomes. This was not due to inactivation of the exchange protein by incubation with intact cells: after the protein had been incubated for 40 min with intact

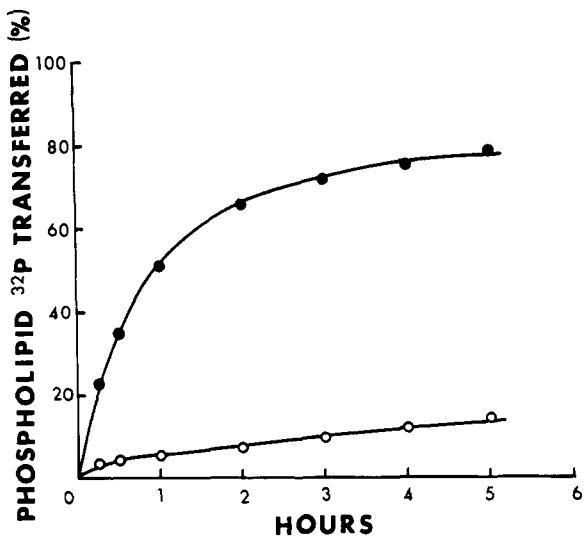

FIGURE 1: Transfer of phospholipid from sealed ghosts to PC/cholesterol liposomes. ^{32}P-Labeled ghosts (5 μg of phospholipid phosphorus) were incubated with PC/cholesterol liposomes (50 μg of PC phosphorus) in 2 ml of PBS at 37 °C without (O—O) and with (●—●) purified phospholipid exchange protein (10.5 units). At each time point, the membranes were pelleted (5 min at 15 000 g) and resuspended in fresh supernatant to continue the incubations. The supernatants were partitioned with 10 ml of chloroform-methanol (2:1, v/v). The lower phase was dried down, and radioactivity was determined as described in Table I. Recovery of liposomes (higher than 90%) was monitored by the [^{14}C]triolein incorporated in the liposomes during their preparation, and the values were corrected accordingly.

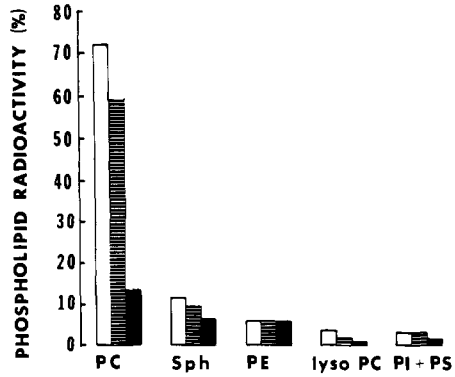

FIGURE 2: ^{32}P distribution in membrane phospholipid after incubation with liposomes in the presence or absence of phospholipid exchange protein. Labeled sealed ghosts (10 μg of phospholipid phosphorus) were incubated with PC/cholesterol liposomes (100 μg of PC phosphorus) in 4 ml of PBS at 37 °C with or without phospholipid exchange protein (36 units). After 4 h, the membranes were sedimented by centrifugation and washed once with PBS. Phospholipids were extracted with 20 volumes of chloroform-methanol (2:1, v/v). A mixture of pure phospholipids (50 μg of total lipid phosphorus) with a composition similar to that of red blood cells was added as carrier. The phospholipids were separated by thin-layer chromatography on silica gel H with chloroform-methanol-acetic acid-water (75:45:12:6, v/v). The spots were visualized by brief exposure to I_2 vapors. After evaporation of the I_2, the silicic acid was transferred to counting vials. (□) sealed ghosts incubated in buffer; (▤) sealed ghosts incubated with liposomes; (■) sealed ghosts incubated with liposomes and phospholipid exchange protein. PC: phosphatidylcholine; Sph: sphingomyelin; PE: phosphatidylethanolamine; lyso PC: lysophosphatidylcholine; PI: phosphatidylinositol; PS: phosphatidylserine.

erythrocytes and separated by centrifugation, it was still fully active when assayed in the leaky ghost-liposome system.

The transfer of labeled phospholipid from sealed ghosts to PC/cholesterol liposomes is shown in Figure 1. This transfer was greatly stimulated when purified phospholipid exchange protein was added to the incubation medium. More than 50% of the phospholipid radioactivity was transferred in the first hour of incubation. After 5 h, the total radioactivity transferred was about 80%.

In an additional experiment, sealed ghosts were incubated with liposomes (Figure 2); after 4 h, the phospholipids of the sealed ghosts were extracted and separated by thin-layer chromatography. In the original sealed ghost fraction, PC accounted for about 75% of the phospholipid radioactivity. The labeled PC was lost from the membrane fraction to the greatest extent, whereas sphingomyelin and phosphatidylinositol[2] were transferred to a lesser degree. Lysophosphatidylcholine was extensively transferred to the liposomes in the absence of exchange protein. This was not due to the presence of albumin since the same results were found when this protein was omitted from the incubation medium. The transfer of lyso PC was also stimulated by the presence of the exchange protein. No transfer of phosphatidylethanolamine was detected under these conditions.

Exclusion of Phospholipid Exchange Protein by Sealed Ghosts. The distribution of PC between the two sides of the erythrocyte membrane was investigated by exchanging the labeled PC from the outside monolayer of sealed ghosts and inside-out vesicles. This could only be done if these membranes were impermeable to the phospholipid exchange protein. The following experiments tested this requirement.

The sealed ghosts did not release significant amounts of entrapped hemoglobin during the incubations, indicating that they remained impermeable to a protein of 64 000 mol wt. The phospholipid exchange protein, however, has a mol wt of 21 000 (Ehnholm and Zilversmit, 1973) and might be accessible to the phospholipids of the internal portion of the phospholipid bilayers. The degree of exclusion of the exchange protein from the sealed ghosts was measured by incubating nonradioactive sealed ghosts with the exchange protein at 37 °C. After the incubation, the suspension was centrifuged, and the dilution of the exchange activity in the supernatant was measured as described in Table II. In the same experiment, the exclusion of phospholipid exchange protein by freshly prepared leaky ghosts was measured for comparison. No corrections were made for the small volume of interstitial space between the packed membranes that were added to the solutions of exchange protein. The results (Table II) show that the sealed ghosts excluded 93% of the exchange protein, whereas the freshly prepared leaky ghosts excluded none. The exclusion of exchange protein by inside-out vesicles was ascertained on the basis of AcChase activities (see below).

Exchange of PC between Sealed Ghosts and PC/Cholesterol Liposomes. The exchange of PC between sealed ghosts and PC/cholesterol liposomes was studied in detail. For this purpose, ^{32}P-labeled sealed ghosts were incubated with an excess of liposomes in the presence of a large amount of exchange protein (84 units).

In order to avoid any reverse flow of label from the liposomes back to the ghosts, the suspension was centrifuged at

[2] Phosphatidylinositol and phosphatidylserine were not separated by this system. In one experiment, this mixture was rechromatographed with chloroform-methanol-7 N NH_4OH (65:35:5, v/v); phosphatidylinositol accounted for 70% of the total radioactivity. Thus, some transfer of this phospholipid must occur to account for the 50% diminution of radioactivity in the phosphatidylinositol + phosphatidylserine fraction after incubation. From these results, it cannot be ascertained whether the protein accelerates the exchange of phosphatidylserine.

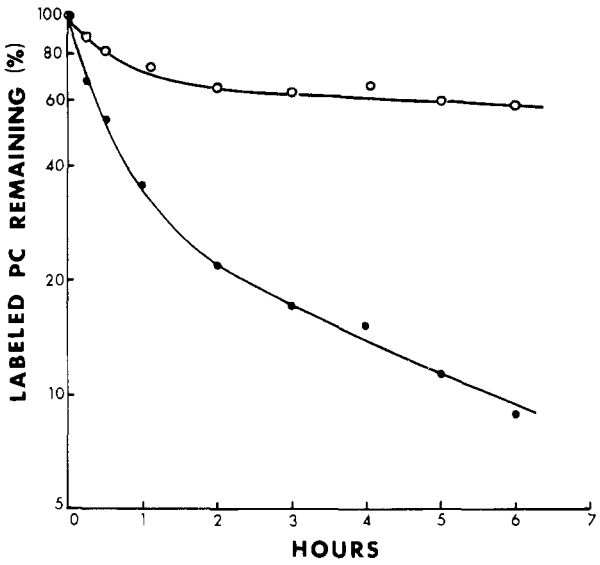

FIGURE 3: Exchange of PC between sealed ghosts and liposomes (●—●) and between inside-out vesicles and liposomes (○—○). Labeled sealed ghosts (12.5 μg of phospholipid phosphorus) were incubated with PC/cholesterol liposomes (500 μg of PC phosphorus) and 84 units of phospholipid exchange protein in 10 ml of 5P8-Mg. The [^{14}C]triolein marker for the liposomes was omitted because it was established in separate experiments that no liposomes sedimented under the conditions described. After 1 h, the sealed ghosts were separated by centrifugation for 5 min at 15 000*g*, and fresh supernatant was added. At different time points, aliquots of the mixture (1.2 ml) were centrifuged for 5 min at 27 700*g*, and 1 ml of the supernatant was partitioned with 5 ml of chloroform–methanol (2:1, v/v). The chloroform phase was dried under N_2, and PC was separated by thin-layer chromatography and counted as described in the legend to Figure 2. Recovery of PC was checked by adding a known amount of [^{14}C]PC before extraction, and the values were corrected accordingly. An identical procedure was used for the inside-out vesicles, except that the medium was 5P8, and the supernatant was not replaced after the first hour of incubation.

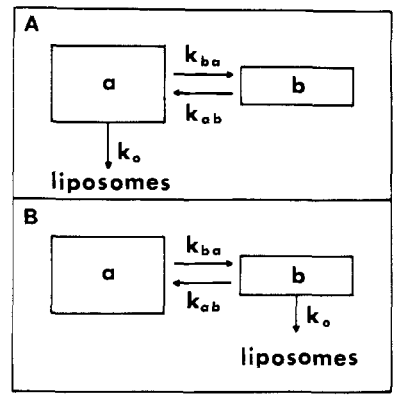

FIGURE 4: Two-pool model for the exchange of PC between sealed ghosts (A) or inside-out vesicles (B) and liposomes. Only the flow of labeled PC is indicated.

Table II: Degree of Phospholipid Exchange Protein Exclusion by Sealed and Leaky Ghosts.[a]

	Sealed Ghosts		Leaky Ghosts	
Incubation Mixture	^{32}P Transferred (%)	Exclusion[b] (%)	^{32}P Transferred (%)	Exclusion[b] (%)
(A) Protein	14.2		12.3	
(B) Protein + buffer	7.2		6.5	
(C) Protein + ghosts	13.8	93	6.1	0

[a] A solution of phospholipid exchange protein was assayed (A), (B) diluted with an equal volume of PBS, or (C) added to a pellet of sealed or of freshly prepared leaky ghosts to final hematocrits of 50%. Samples were incubated for 2 h at 37 °C and centrifuged, and 0.2 ml of the supernatants was assayed for exchange activity in 2 ml of PBS containing ^{32}P-labeled ghosts (10 μg of phospholipid phosphorus) and PC/cholesterol liposomes (50 μg of PC phosphorus). After 60 min at 37 °C, the samples were centrifuged, and the amount of ^{32}P transferred to the liposomes was measured. Under these conditions, up to 17% of the radioactivity transferred is in the linear portion of the assay curve. [b] No decrease of the exchange activity after the incubation indicates 100% exclusion of the exchange protein by the membrane fraction.

4 °C after 60 min of incubation. The incubation was continued for 4–5 h with fresh liposomes and exchange protein at 37 °C. At different times, aliquots of the suspension were chilled, centrifuged, and the supernatants, containing the liposomes, were partitioned with 5 volumes of chloroform–methanol 2:1. The PC was isolated by thin-layer chromatography, and the radioactivity was counted. The disappearance of labeled PC from the sealed ghosts is shown in Figure 3.

The kinetic analysis of the curve shows that the experimental points can be fitted by the sum of two exponentials. This indicates that the PC of the sealed ghosts is distributed in two pools. The pool sizes and rate constants, derived from the two-pool model analysis (Figure 4), are shown in Table IV. These results are consistent with an asymmetric distribution of PC in the membrane, the larger pool (75% of total PC) facing the outside and being easily exchangeable. If this were the case, the transposition of PC molecules from the inside to the outside monolayer would be the rate-limiting step for the process.

From the values of the rate constants and pool sizes, it follows that the sum of the forward and backward transposition of PC between the inside and outside layers of the membrane amounts to 11% of the total membrane PC per hour. The half-time for equilibration of PC in the two monolayers is 2.3 h (see Appendix).

Exchange of PC between Liposomes and Inside-out Vesicles Derived from Erythrocyte Membranes. In a parallel set of experiments, the exchange of phospholipids between inside-out vesicles and PC/cholesterol liposomes was investigated. The experiment shown in Table III indicates that an appreciable portion of the total phospholipid radioactivity (52%) can be transferred from the vesicles to PC/cholesterol liposomes in the presence of the phospholipid exchange protein. In order to obtain reliable results, it is essential that the vesicles do not disintegrate or become leaky during the incubation for several hours at 37 °C. According to the data of column 2, the recovery of total AcChase in the vesicles sedimented after 5 h of incubation was close to 100%. This indicates that, in the presence of liposomes and exchange protein, no disintegration of vesicles took place. The latency of the AcChase activity of the original vesicles preparation was 83% and was unchanged by incubation. This implies that the orientation of the vesicles was preserved and that the vesicles remained impermeable to acetylthiocholine and therefore would also exclude the phsopholipid exchange protein.

The exchange of PC between labeled inside-out vesicles and liposomes was studied under the same conditions as those used for sealed ghosts. The percentage of right-side-out vesicles was obtained from the measurements of AcChase activity, and the exchange values were corrected by assuming that the right-side-out vesicles exchange PC at

Table III: Stability of Inside-out Vesicles during Incubation at 37 °C.[a]

Incubation Conditions	^{32}P Remaining (%)	Acetylcholinesterase Activity[c] In Presence of Triton X-100	In Absence of Triton X-100	Latency[b]
Vesicles in buffer (0 °C)	100	0.190	0.032	83
Vesicles in buffer (37 °C)	95	0.176	0.042	76
Vesicles + liposomes (37 °C)	86	0.192	0.032	83
Vesicles + liposomes + exchange protein (37 °C)	43	0.198	0.038	81

[a] Labeled inside-out vesicles (5 μg of phospholipid phosphorus) were incubated at 0 or at 37 °C in 4 ml of 5P8 buffer. Where indicated, the medium also contained liposomes (200 μg of phosphorus) and 16.2 units of phospholipid exchange protein. After 5 h, the vesicles were pelleted for 5 min at 27 700*g*. The vesicles were resuspended and adjusted to a final volume of 1 ml with 5P8 buffer. Aliquots (0.2 ml) were taken for acetylcholinesterase activity measurement in the presence or absence of Triton; 0.4-ml aliquots were taken for the determination of radioactivity. [b] Defined as: 100[1 − (activity without Triton X-100/activity with Triton X-100)]. [c] Micromoles of substrate hydrolyzed/h per μg of lipid phosphorus.

Table IV: Kinetic Parameters for the Exchange of PC between Sealed Ghosts and Inside-out Vesicles with PC/Cholesterol Liposomes.[a]

	Rate Constants (h^{-1})			Pool Sizes (% of total PC)	
	k_0	k_{ab}	k_{ba}	a	b
Sealed ghosts	1.82 ± 0.87	0.225 ± 0.017	0.077 ± 0.013	74.7 ± 1.9	25.3 ± 1.9
Inside-out vesicles	2.29 ± 1.29	0.078 ± 0.019	0.047 ± 0.018	63.1 ± 3.0	36.9 ± 3.9

[a] The kinetic parameters were calculated as described in the Appendix. The values are average ± SD of three separate experiments.

the same rate as sealed ghosts.[3] As with sealed ghosts, the curve obtained can be fitted by the sum of two exponential terms (Figure 3). The faster exchanging pool, or the outside monolayer of the inside-out vesicles, comprises 37% of the PC.

The values of k_{ab} and the pool size of a are significantly lower ($p < 0.01$) for inside-out vesicles than for sealed ghosts (Table IV). The half-time of PC equilibration in the two monolayers of the inside-out vesicles is 5.3 h.

Discussion

In the present paper, the phospholipid exchange protein has been used to study the degree of asymmetry and of inside–outside transposition of PC in membranes derived from rat erythrocytes. The results indicate that, in sealed ghosts, 75% of the PC is readily available for exchange by the purified exchange protein. This amount of the membrane PC is probably exposed to the outer surface of the membrane since the protein is unable to penetrate the sealed ghosts. The remaining 25% is also exchangeable but at a much slower rate. The possibility that this pool is also exposed to the outer surface but in a different structural arrangement (i.e., more closely associated with membrane proteins, which would make it less available for exchange) seems unlikely in view of the results obtained with the inside-out vesicles: when the membrane is turned inside-out, the faster exchangeable pool is the smaller one, about 37% of the total PC (Table IV). These results suggest that this phospholipid is asymmetrically distributed in the cell membrane of rat erythrocytes and that the asymmetry is largely maintained during the formation of inside-out vesicles.

It would have been of interest to perform exchange experiments with leaky ghosts, in which both sides of membrane phospholipids are available to the exchange protein. However, we have consistently found that leaky ghosts reseal when exposed at 37 °C for 2 h or more in PBS or 5P8. The degree of resealing was judged by the position of the membranes in the Dextran gradient after incubation. Extensive washing of the leaky ghosts with 5P8, 11 mM Tris-HCl buffer, or inclusion of Na_2EDTA during the preparation did not prevent resealing, although the washing with Na_2EDTA seemed to slow down the process.

Phospholipase treatment and chemical labeling have been used to study phospholipid asymmetry in human erythrocyte membranes. Verkleij et al. (1973) reported that only 68% of the PC can be degraded by phospholipase A_2 (*N. naja*) in intact cells without hemolysis. No degradation of phosphatidylethanolamine or phosphatidylserine was observed. Sphingomyelinase (*S. aureus*) caused 85% degradation of sphingomyelin. Phospholipase C (*B. cereus*) produced no degradation of phospholipid in the intact cell. When washed ghosts were subjected to digestion by phospholipase A_2 or C, total degradation of PC, phosphatidylethanolamine, and phosphatidylserine was observed.

These results led to the conclusion that the phospholipids are asymmetrically distributed in the red blood cell membrane, with the total sphingomyelin fraction, 68% of PC, and some phosphatidylethanolamine forming the outer monolayer of the membrane. However, the possibility that alterations due to hemolysis were responsible for the increased degradation of phospholipid cannot be ruled out in those experiments. In addition, extensive alterations of the membrane, as revealed by electron microscopy, occur during some of the phospholipase treatments. Kahlenberg et al. (1974) exposed inside-out vesicles derived from human red cell ghosts to phospholipases and found that 30–40% of the PC was degraded. No data as to whether the inside-out vesicles remained sealed during the treatment are given.

Chemical labeling with trinitrobenzenesulfonate has been used to study the distribution of amino-containing phospholipids in the red blood cell membrane (Gordesky and Marinetti, 1973). However, there is some controversy regarding

[3] Three attempts to prepare right-side-out vesicles from leaky ghosts of rat erythrocytes yielded preparations with more than 50% contamination with inside-out vesicles.

the extent to which this chemical penetrates into the intact cell (Gordesky et al., 1975). Labeled imidoesters of different penetrabilities seem to be a more promising tool to study the phospholipid distribution in intact cells (Whiteley and Berg, 1974).

Johnson et al. (1975) and Rothman and Dawidowicz (1975) used the phospholipid exchange protein to measure the rate of inside-outside transposition of PC in liposomes and obtained a half-time of several days for this process. With the same technique, we obtained a much faster rate for the transposition of PC through the membrane of sealed rat ghosts. We could not perform the experiments on intact red cells since the phospholipid exchange protein does not significantly accelerate the exchange of phospholipids between intact cells and liposomes. The lack of PC exchange by intact red blood cells was also reported by Hellings et al. (1974) and by Rothman and Dawidowicz (1975) using human erythrocytes and PC exchange protein purified from beef liver. This indicates that, in the preparation of sealed ghosts, some structural changes have occurred in the membrane, even though the selective permeability has been restored (Steck, 1974a). The magnitude of these changes and its influence on the interpretation of chemical labeling of membrane components is a subject of controversy (Bretscher, 1973; Wallach et al., 1974; Cabantchik et al., 1975; Staros et al., 1974). Woodward and Zwaal (1972) have reported differences in the susceptibility to degradation by phospholipases between phospholipids in the intact cell and in sealed ghosts. Thus, one should be cautious in extrapolating the results obtained with a derivative of the red blood cell to the intact cell.

Our results suggest that the transposition of PC through natural membranes, which contain a complex mixture of lipids and protein, is much faster than through a bilayer of pure PC liposomes. Differences for the process between liposomes and natural membranes and within different natural membranes is also suggested by the results of Kornberg and McConnell (1971) and McNamee and McConnell (1973), obtained with spin-label analogues of PC.

The experiments with inside-out vesicles suggest that the asymmetry of PC, although still present, has been significantly altered during the generation of the inside-out vesicles. Also, the membrane transposition of PC is 2.4 times slower. Factors related to the membrane structure are probably involved: the membrane proteins are asymmetrically distributed preferentially located on the inner side of the erythrocyte membrane (Steck, 1974b; Marchesi, 1975). Loss of these proteins, primarily spectrin, occurs during the inversion and vesiculization to which the leaky ghosts are subjected in order to obtain the inside-out vesicles. Profound alterations in the membrane structure following spectrin removal have been described by Elgsaeter and Branton (1974) and by Steck (1974b). It is not implausible that the rate of phospholipid transposition is altered under these circumstances.

Acknowledgments

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APPENDIX

Definitions. g_1 and g_2 are the fast and slow exponential constants, respectively.

k_{ab}, k_{ba}, and k_0 are rate constants for models A and B in Figure 4.

q_a = labeled PC in pool a at time t

q_{a0} = labeled PC in pool a at time $t = 0$

q_b = labeled PC in pool b at time t

q_{b0} = labeled PC in pool b at time $t = 0$

r_a = fraction of PC mass present in pool a. This is also equal to the fraction of PC label at $t = 0$ present in a, $q_{a0}/(q_{a0} + q_{b0})$, if (as is assumed to be the case) the specific activities of PC in the inner and outer layers of the red cell membrane are equal.

In a two-pool system (see Figure 4A), in which all the isotope is present in pool a at time $t = 0$, the distribution of isotope in a and b as a function of time is:

$$\frac{q_a}{q_{a0}} = H_1 e^{-g_1 t} + H_2 e^{-g_2 t} \quad (1)$$

$$\frac{q_b}{q_{a0}} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} \quad (2)$$

If pool b contained all the isotope at $t = 0$, the distribution of isotope would be:

$$\frac{q_b}{q_{b0}} = H_1' e^{-g_1 t} + H_2' e^{-g_2 t} \quad (3)$$

$$\frac{q_a}{q_{b0}} = K_1' e^{-g_1 t} + K_2' e^{-g_2 t} \quad (4)$$

If, as is the case in the red blood cell, the distribution of isotope in a and b at $t = 0$ is proportional to the amounts of PC in the two layers, one can add eq 1–4 after weighting each one by factors (r_a and $1 - r_a$) proportional to the label initially present in compartments a and b and obtain:

$$\frac{q_a + q_b}{q_{a0} + q_{b0}} = (r_a H_1 + K_1' - r_a K_1' + r_a K_1 + H_1' - r_a H_1') e^{-g_1 t} + (r_a H_2 + K_2' - r_a K_2' - r_a K_2 - H_2 - r_a H_2) e^{-g_2 t} \quad (5)$$

The values of H's and K's in terms of rate constants are obtained by standard solution of the differential equations (Shipley and Clark, 1972, p 215):

$$H_1 = \frac{k_{ab} - g_1}{g_2 - g_1} \quad H_1' = \frac{k_{ba} + k_0 - g_1}{g_2 - g_1}$$

$$K_1 = \frac{k_{ba}}{g_2 - g_1} \quad K_1' = \frac{k_{ab}}{g_2 - g_1}$$

$$H_2 = \frac{k_{ab} - g_2}{g_1 - g_2} \quad H_2' = \frac{k_0 + k_{ba} - g_2}{g_1 - g_2}$$

$$K_2 = \frac{k_{ba}}{g_1 - g_2} \quad K_2' = \frac{k_{ab}}{g_1 - g_2}$$

which, when substituted in eq 5, gives:

$$\frac{q_a + q_b}{q_{a0} + q_{b0}} = \frac{(r_a k_0 - g_2)}{g_1 - g_2} e^{-g_1 t} + \frac{(g_1 - r_a k_0)}{g_1 - g_2} e^{-g_2 t} \quad (6)$$

and the following relationships:

$$g_1 + g_2 = k_{ab} + k_{ba} + k_0 \quad (7)$$

$$g_1 g_2 = k_{ab} k_0 \quad (8)$$

$$\frac{g_1 - r_a k_0}{g_1 - g_2} = C = \frac{\text{intercept with the } Y \text{ axis of the final linear portion}}{\text{of the radioactivity curve}} \tag{9}$$

and

$$r_a = \frac{k_{ab}}{k_{ab} + k_{ba}} \quad \text{based on the equality of the PC exchange in both directions} \tag{10}$$

Substitution of eq 10 into eq 9 gives:

$$k_{ab} + k_{ba} = \frac{g_1 g_2}{g_1 - (g_1 - g_2)C_2}$$

From this equation and eq 7, we obtain k_0, after which k_{ab}, k_{ba}, and r_a are readily obtained. Note: The solution of the equations for the inside-out vesicles is the same except that k_{ab} is substituted for k_{ba}, k_{ba} for k_{ab}, and r_b for r_a.

Half-Time of Transposition. If one considers the two-pool system in Figure 4A during a time when, after compartment A is depleted of label, the two pools are allowed to equilibrate without exchange of liposomes, the system behaves as a two-pool closed model (cf. Shipley and Clark, 1972, p 133). In this instance

$$\frac{q_b}{q_{b0}} = H_1' e^{-g_1 t} + H_2' e^{-g_2 t}$$

taking into account that $g_2 = 0$ and replacing H_1' and H_2'

$$\frac{q_b}{q_{b0}} = \frac{-k_{ba} + g_1}{g_1} e^{-g_1 t} + \frac{k_{ba}}{g_1}$$

Since in this case $g_1 = k_{ab} + k_{ba}$

$$\frac{q_b}{q_{b0}} = \frac{k_{ab}}{k_{ab} + k_{ba}} e^{-(k_{ab}+k_{ba})t} + \frac{k_{ba}}{k_{ab} + k_{ba}} \tag{11}$$

When equilibrium is reached ($t = \infty$)

$$\frac{q_{be}}{q_{b0}} = \frac{k_{ba}}{k_{ab} + k_{ba}}$$

and

$$\frac{q_b - q_{be}}{q_{b0} - q_{be}} = e^{-(k_{ab}+k_{ba})t}$$

That is, the rate constant of PC transposition is the sum of the two rate constants. From this value, the half-time of equilibration of PC can be calculated as

$$t_{1/2} = 0.693/(k_{ab} + k_{ba})$$

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