Two Nonspecific Phospholipid Exchange Proteins from Beef Liver. 2. Use in Studying the Asymmetry and Transbilayer Movement of Phosphatidylcholine, Phosphatidylethanolamine, and Sphingomyelin in Intact Rat Erythrocytes†

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ABSTRACT: The nonspecific exchange protein from beef liver catalyzes the exchange of phospholipids between intact rat erythrocytes and unilamellar vesicles. The transfer of phospholipid from red cells (labeled in vivo with $^{32}P_i$ and/or in vitro with [^{3}H]arachidonic acid) to unilamellar vesicles is followed as a function of time. Of the [^{32}P]phosphatidylcholine, 63% is transferred rapidly (k = 1.34 h^{-1}) and 37% is transferred slowly (k = 0.061 h^{-1}), whereas all of the [^{3}H]arachidonate-labeled phosphatidylcholine is transferred with a rate constant of 0.155 h^{-1}. Likewise, all of the [^{32}P]sphingomyelin is transferred with a rate constant of 0.23 h^{-1}. The entirety of both ^{32}P-labeled and [^{3}H]arachidonate-labeled phosphatidylethanolamine is transferred very slowly (k < 0.025 h^{-1}). Increased concentrations of exchange protein have no effect on the transfer of [^{3}H]arachidonate pulse labeled phosphatidylcholine and [^{3}H]arachidonate-labeled [^{32}P]phosphatidylethanolamine but increase the initial rate of transfer of [^{32}P]phosphatidylcholine and the rate of transfer of [^{32}P]-sphingomyelin. Preincubation of the labeled red cells for 3 h in the absence of exchange protein produces no change in the transfer of [^{32}P]phosphatidylcholine and [^{32}P]sphingomyelin, a minimal increase in the rate of transfer of [^{32}P]- and [^{3}H]arachidonate-labeled phosphatidylethanolamine, and a large increase in the initial rate of transfer of [^{3}H]arachidonate-labeled phosphatidylcholine. The results are consistent with an asymmetric distribution of sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine in the rat erythrocyte membrane. All of the sphingomyelin, 63% of the phosphatidylcholine, and very little of the phosphatidylethanolamine appear to be localized in the outer monolayer.

Since it was first suggested that the human erythrocyte membrane had an asymmetric distribution of phospholipids, on the basis of chemical labeling (Bretscher, 1972; Gordesky & Marinetti, 1973) and phospholipase hydrolysis (Verkleij et al., 1973), the distribution and transbilayer movement of phospholipids (Rothman & Lenard, 1977; Bergelson & Barsukov, 1977) in both natural and artificial membranes have been the subject of extensive investigation. There has been an interest in whether asymmetry is a general property of biological membranes and, if so, how it originates, how it is maintained, and what function it serves.

Phospholipid exchange proteins (Wirtz, 1974; Zilversmit & Hughes, 1976) have been used increasingly as probes of membrane phospholipid distribution and transbilayer movement. Johnson et al. (1975), using an exchange protein purified from beef heart, studied the exchange of [^{32}P]phosphatidylcholine between unilamellar vesicles and mitochondria. They found that 60% of the label was transferred rapidly, while the remaining 40% was transferred much more slowly with a half-time of several days. They concluded that the rapidly removed label represented phosphatidylcholine localized in the outer exposed monolayer, while the slowly removed pool of label represented phosphatidylcholine masked from the exchange protein because of its localization in the inner monolayer. The rate of removal of the label localized in the inner monolayer was then equal to the rate of translocation. This result was also found by Rothman & Dawidowicz (1975).

More recently, the use of exchange proteins for the measurement of phospholipid distribution and translocation has been extended to biological membranes. Rothman et al. (1976) looked at the asymmetry and transbilayer movement of lipids in the influenza virus membrane using two types of phospholipid exchange protein in conjunction with phospholipases. Using an exchange protein isolated from beef heart, Bloj & Zilversmit (1976) studied phosphatidylcholine distribution and translocation in resealed rat erythrocyte ghosts and inside-out vesicles prepared from rat erythrocytes. On the basis of kinetic analyses of exchangeable pools, they concluded that 65–75% of the phosphatidylcholine was localized in the external monolayer, while the remaining 25–35% was localized in the cytoplasmic monolayer and had an apparent half-time of translocation of 3 and 8 h in resealed ghosts and inside-out vesicles, respectively. Recently, a nonspecific exchange protein from rat liver has been used to show rapid translocation of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol in the rat liver microsomal membrane (Zilversmit & Hughes, 1977). This has been confirmed for phosphatidylcholine with the specific beef liver exchange protein (Van den Besselaar et al., 1978) and for phosphatidylinositol with the phosphatidylinositol exchange protein from beef brain (Brophy et al., 1978).

In the present study we have determined the kinetics of transfer of lipids from labeled red cells to unilamellar vesicles with the nonspecific exchange protein from beef liver whose purification and characterization were reported in the preceding paper (Crain & Zilversmit, 1980). This protein, in contrast to previously studied exchange proteins (Rothman & Dawidowicz, 1975; Bloj & Zilversmit, 1976; Hellings et al., 1974), is capable of exchanging lipid from intact red cells.

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It is also capable of transferring a variety of lipids and is therefore useful in studying the asymmetry and translocation of all of the major membrane phospholipids. The results are compared with previous findings concerning phosphatidylcholine asymmetry and transbilayer movement in resealed rat erythrocyte ghosts studied with phospholipid exchange protein (Bloj & Zilversmit, 1976) and in intact rat erythrocytes studied with phospholipases (Renooij et al., 1976).

Materials and Methods

Lipids. Glycerol [9,10-^{3}H]trioleate and glycerol [^{14}C]-trioleate (Applied Science Laboratories, State College, PA) were purified by thin-layer chromatography on silica gel H with hexane–diethyl ether–acetic acid (60:40:1 v/v). The glycerol trioleate was eluted with hexane and stored at −20 °C. Butylated hydroxytoluene (Nutritional Biochemical Corp., Cleveland, OH) was dissolved in chloroform and used without further purification. Phosphatidylcholine and phosphatidylethanolamine (Lipid Products, South Nutfield, England) were diluted with chloroform and stored at −20 °C. Sphingomyelin (Avanti Biochemicals, Birmingham, AL) was dissolved in chloroform and stored at −20 °C. Cholesterol (Sigma Chemical Co., St. Louis, MO) was purified as the dibromide derivative, crystallized from methanol, and stored at −20 °C in ethanol. The purity of all lipids was greater than 99% as determined by thin-layer chromatography.

Unilamellar Vesicle Preparation. Appropriate volumes of stock solutions of lipids were mixed and dried as a thin film at 30 °C under a stream of nitrogen. The phospholipid composition was generally phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine (62:28:10 mol %). Cholesterol was included at a phospholipid to cholesterol molar ratio of 1:0.75. A trace of radioactively labeled glycerol trioleate was included as a nonexchangeable marker, and 0.1 wt % butylated hydroxytoluene was included as an antioxidant. A clear suspension of lipid (10 μmol of phospholipid per mL) was formed by sonication for 15 min at 20 °C in a solution of 2% cholic acid (Sigma Chemical Co., St. Louis, MO) in SHNG buffer[1] (220 mM sucrose, 10 mM Hepes, 10 mM NaCl, 10 mM glucose, 2 mM EGTA, and 3 mM NaN_3) at pH 7.4. Unilamellar vesicles were formed by extensive dialysis against SHNG buffer, pH 7.4, to remove cholate (Kagawa et al., 1973).

Red Cells. Male rats were injected intraperitoneally with 2–3 mCi of $^{32}P_i$ per 100 g of body weight. After 72 h, blood was drawn by cardiac puncture and collected in 0.5 mL of 200 mM Na_2EGTA, pH 7.4, per 10 mL of whole blood as the anticoagulant. Cells were sedimented by centrifugation at 1000*g* for 10 min. Plasma and buffy coat were removed, and the cells were washed 3 times with 3–5 volumes of saline. The same process was performed on red cells from unlabeled rats. At this point ^{32}P-labeled cells, which were to be used for exchange experiments, were washed once with SHNG buffer.

Red cells were labeled with [^{3}H]arachidonic acid (63 Ci/mmol; New England Nuclear, Boston, MA) in vitro by a modification of the method of Renooij & Van Golde (1977). [^{3}H]Arachidonic acid (60 μCi) was dried under nitrogen and suspended in 6 mL of saline containing 10 mM glucose and 1% by weight crystalline Pentex bovine albumin (Miles Laboratories, Inc., Elkhart, IN). To this was immediately added 6 mL of washed, packed red cells (either ^{32}P labeled or unlabeled). After gentle shaking for 1 h at 37 °C, 20 mL of cold saline was added. Cells were sedimented by centrifugation at 1000*g* for 10 min and washed 3–4 times at 4 °C with 5–10 volumes of 1% BSA in saline. Cells were washed once with SHNG buffer and used immediately for exchange experiments. In one experiment [^{3}H]arachidonate- and ^{32}P-labeled packed red cells were preincubated for 3 h at 37 °C in 1 volume of saline containing 1% BSA and 10 mM glucose before the final wash in SHNG buffer.

Purified Phospholipid Exchange Protein. Nonspecific phospholipid exchange protein was purified from beef liver cytosol as described in the preceding paper (Crain & Zilversmit, 1980). After column chromatography on octyl-agarose, the exchange protein was dialyzed against SHNG buffer. One unit of phosphatidylethanolamine exchange activity is defined as the transfer of 0.1 μmol of phosphatidylethanolamine per h at 37 °C (Zilversmit & Hughes, 1976). Details of the procedures involved in measuring the exchange of labeled phospholipid from rat erythrocytes to unilamellar vesicles are described in the legends to the figures.

Calculations. Parameters for the exponential curves were calculated by a nonlinear regression of the Levenberg–Marquardt type as described by Dell et al. (1973). An exponential term was declared nonsignificant if the least-squares error about the fitted line was not significantly reduced as judged by an *F* test. Pool sizes and rate constants were calculated from these as described by Bloj & Zilversmit (1976).

Results

Transfer of ^{32}P-Labeled Phospholipids from the Red Cell Membrane to Unilamellar Vesicles. Red cells, labeled in vivo for 72 h with $^{32}P_i$, were incubated at 37 °C with exchange protein and unilamellar vesicles containing phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and cholesterol. The phospholipid composition of the unilamellar vesicles was adjusted to reflect that of the outer monolayer of the red cell membrane (Renooij et al., 1976). Cholesterol was included to maintain the phospholipid to cholesterol molar ratio of the red cell membrane which was determined to be 1:0.75. Incubations were done in SHNG buffer because of the inhibition of the nonspecific exchange protein from beef liver by high ionic strength buffers (Crain & Zilversmit, 1980). In incubations containing unilamellar vesicles and multilamellar vesicles, red cell ghosts and unilamellar vesicles, or red cells and unilamellar vesicles, the beef liver nonspecific exchange protein catalyzed 4–8 times as much transfer of phospholipid per unit of time in low ionic strength buffer (less than 0.025 M) as compared to saline. As shown in Table I, very little phospholipid transfer occurred in the absence of exchange protein. Addition of 4.0 units of the phosphatidylcholine-specific exchange protein from beef liver resulted in a small increase in the transfer of phosphatidylcholine but resulted in no change in the transfer of either phosphatidylethanolamine or sphingomyelin. Addition of 2.5 units of the nonspecific exchange protein from beef liver (4 units if based on phosphatidylcholine exchange activity) to the incubations resulted in very little change in phosphatidylethanolamine transfer but increased dramatically the transfer of both sphingomyelin and phosphatidylcholine (Table I).

The transfer of [^{32}P]phospholipid from the red cell membrane to unilamellar vesicles was examined as a function of time (Figure 1). A fivefold excess of phospholipids in unilamellar vesicles was included to prevent return of the label from unilamellar vesicles to the erythrocyte. At each time point, the cells were sedimented by centrifugation, and the incubation was continued at 37 °C with fresh exchange protein and unilamellar vesicles after removal of the supernatant. At

[1] Abbreviation used: SHNG buffer, 220 mM sucrose, 10 mM Hepes, 10 mM NaCl, 10 mM glucose, 2 mM EGTA, and 3 mM NaN_3, pH 7.4.

Table I: Transfer of [^{32}P] Phospholipid from Erythrocytes to Unilamellar Vesicles[a]

phospholipid	% [^{32}P] phospholipid transferred: without exchange protein	beef liver "specific" exchange protein[b]	beef liver "nonspecific" exchange protein
phosphatidylcholine	1.0	5.5	30.2
phosphatidylethanolamine	0.6	0.6	1.1
sphingomyelin	1.0	1.1	11.1

[a] Red cells were labeled in vivo with $^{32}P_i$ as described under Materials and Methods. 0.25 mL of packed red cells was incubated with unilamellar vesicles as described in Figure 1 in the presence of 2.5 units of the beef liver nonspecific exchange protein (4 units if expressed as phosphatidylcholine exchange activity), 4 units of the beef liver phosphatidylcholine specific exchange protein, or no exchange protein. After 30 min the cells were sedimented by centrifugation, the supernatant was extracted by the method of Dligh & Dyer (1959), and the transfer of labeled phospholipid was measured by liquid scintillation counting after separation by thin-layer chromatography. [b] Purified as described by Kamp et al. (1973).

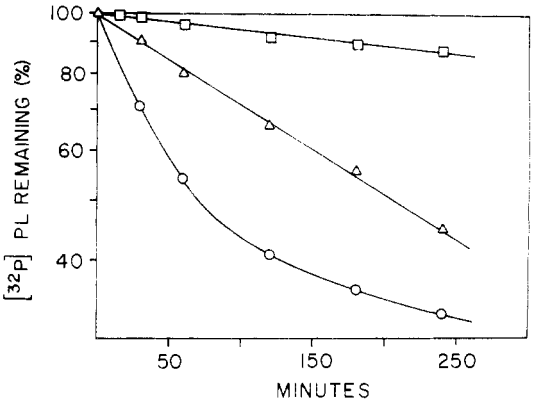

FIGURE 1: Semilogarithmic plot of the extensive transfer of red cell [^{32}P]phospholipids to unilamellar vesicles catalyzed by beef liver nonspecific exchange protein (2.5 units). Red cells were labeled in vivo with $^{32}P_i$ as described under Materials and Methods. 0.25 mL of packed red cells (about 1 μmol of total phospholipid) was incubated with unilamellar vesicles (4 μmol of total phospholipid) composed of 35.4 mol % phosphatidylcholine, 16.0 mol % sphingomyelin, 5.7 mol % phosphatidylethanolamine, and 42.8 mol % cholesterol (glycerol [^{3}H]trioleate was included as a nonexchangeable marker) in the presence of exchange protein (2.5 units) in a total volume of 2 mL of SHNG buffer at 37 °C. At each time point, cells were sedimented by centrifugation. After removal of the supernatant (containing the unilamellar vesicles), the cells were incubated at 37 °C in the presence of fresh exchange protein and fresh unilamellar vesicles. From the supernatant, an aliquot was counted to determine the unilamellar vesicle recovery. The optical density of another sample was determined at 540 nm as a measure of cell lysis. Finally, an aliquot was extracted by the method of Bligh & Dyer (1959), and the phospholipid was separated by thin-layer chromatography in chloroform–methanol–glacial acetic acid–water (25:15:4:2 v/v) and quantitated by liquid scintillation counting after elution (Crain et al., 1978). Recoveries were generally 90% or better. The amount remaining was determined by subtraction of the amount exchanged from the amount present in red cells at time 0. This figure is representative of four experiments. Each point is the average of duplicate determinations. (□) Phosphatidylethanolamine; (△) sphingomyelin; (○) phosphatidylcholine.

all points, recovery of the unilamellar vesicles was calculated to be greater than 95%, based on the recovery of glycerol [^{3}H]trioleate. Spectrophotometric measurement of released hemoglobin was used to analyze for cell hemolysis, which was less than 10% after 4 h. The amounts of labeled phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin transferred were measured in unilamellar vesicles by scintillation counting after separation of the phospholipid classes by thin-layer chromatography. The amount of label remaining in the red cell was determined based on the total at time 0.

The time courses of transfer of [^{32}P]phosphatidylethanolamine and [^{32}P]sphingomyelin could be approximated by a single exponential term. The rate constant for the transfer of [^{32}P]sphingomyelin was 0.23 h^{-1} while the rate constant for the disappearance of [^{32}P]phosphatidylethanolamine was 0.024 h^{-1} (Table II).

The time course for transfer of [^{32}P]phosphatidylcholine, however, was best approximated by the sum of two exponential terms. The parameters for these were calculated by a nonlinear regression (Dell et al., 1973). Pool sizes and kinetic constants were calculated as described by Bloj & Zilversmit (1976) based on a two-pool model (see Appendix). It was found that when less than 2 units of exchange protein was included in the incubations, the rapidly exchangeable pool of label was not transferred to the unilamellar vesicles fast enough to allow accurate calculation of the pool size and kinetic constants for the slowly exchangeable pool. When 2 or more units of exchange protein was included, reproducible values for these were obtained. It was found that 63% of the [^{32}P]phosphatidylcholine was transferred rapidly with a rate constant of 1.34 h^{-1} (Table II). The remaining 37% was transferred more slowly. The rate constant for transfer of this pool was calculated to be 0.061 h^{-1} (Table II).

Transfer of [^{3}H]Arachidonate-Labeled Phospholipids from the Red Cell Membrane to Unilamellar Vesicles. In vivo labeling of red cells with $^{32}P_i$ results in labeling of both monolayers of the membrane. After 72 h, the labeled phospholipids in the two monolayers have similar specific activities (Bloj and Zilversmit, unpublished experiments). In order to study the transbilayer movement of phospholipid, rat erythrocytes were labeled in vitro with [^{3}H]arachidonic acid. Fatty

Table II: Kinetic Parameters for the Transfer of Labeled Phospholipid from Red Cells to Unilamellar Vesicles[a]

phospholipid	pool size (% of total): pool a	pool b	rate constants (h^{-1}): k_0	k_{ab}	$t_{1/2}$[d] (h)
[^{32}P] phosphatidylcholine[b]	63 ± 2	37 ± 2	1.34 ± 0.14	0.061 ± 0.026	7.2
[^{3}H] arachidonate-labeled phosphatidylcholine[c]	0	100		0.155 ± 0.029[e]	2.8
[^{32}P] spingomyelin[b]	100	0	0.23 ± 0.04		
[^{32}P] phosphatidylethanolamine[b]	0	100		0.024 ± 0.007	
[^{3}H] phosphatidylethanolamine[c]	0	100		0.023 ± 0.005	

[a] The experimental procedures are described in Figures 1 and 2. Calculations were done as described under Materials and Methods. [b] Mean ± standard deviation; $N = 4$. [c] Mean ± standard deviation; $N = 6$. [d] $t_{1/2}$ equilibration = $0.693/(k_{ab} + k_{ba})$. [e] Significantly different ($p < 0.01$) from [^{32}P]phosphatidylcholine by the Student's t test.

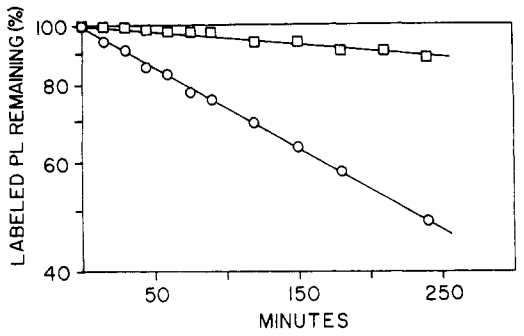

FIGURE 2: Semilogarithmic plot of the transfer of red cell [^{3}H]arachidonate-labeled phospholipid to unilamellar vesicles catalyzed by beef liver nonspecific exchange protein (2.5 units). Red cells were labeled in vitro with [^{3}H]arachidonic acid as described under Materials and Methods. The transfer of labeled phospholipids from red cells to unilamellar vesicles was performed and measured as described in Figure 1 except that glycerol [^{14}C]trioleate was included as the nonexchangeable marker of unilamellar vesicles in these experiments. This figure is representative of six experiments. Each point is the average of duplicate determinations. (□) Phosphatidylethanolamine; (○) phosphatidylcholine.

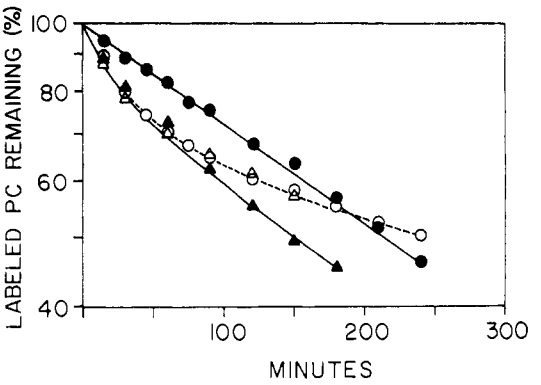

FIGURE 3: Semilogarithmic plot of the effect of preincubation on the transfer of red cell [^{3}H]arachidonate-labeled [^{32}P]phosphatidylcholine to unilamellar vesicles catalyzed by beef liver nonspecific exchange protein (2.0 units). ^{32}P-Labeled red cells were labeled with [^{3}H]-arachidonic acid as described under Materials and Methods. The transfer of label was measured as a function of time (as described in Figure 1) before (●, ○) and after (▲, △) preincubation for 3 h at 37 °C in 1 volume of saline. Each point is the average of duplicate determinations. (●, ▲) [^{3}H]Arachidonate-labeled phosphatidylcholine; (○, △) [^{32}P]phosphatidylcholine.

acids have been shown (Renooij et al., 1976, 1974; Renooij & Van Golde, 1977) to be incorporated in the inner surface of the erythrocyte membrane.

The time courses for the transfer of [^{3}H]arachidonate-labeled phosphatidylethanolamine and phosphatidylcholine are shown in Figure 2. Data for sphingomyelin could not be obtained because insufficient arachidonic acid was incorporated into this phospholipid class. Kinetic analysis revealed a single exponential term for both phosphatidylcholine transfer and phosphatidylethanolamine transfer. The rate constant for the transfer of [^{3}H]phosphatidylcholine was 0.155 h^{-1} (Table II). The transfer of [^{3}H]arachidonate-labeled phosphatidylethanolamine was very slow (k = 0.023 h^{-1}) and appeared almost identical with that for [^{32}P]phosphatidylethanolamine.

The kinetics of the transfer of [^{3}H]arachidonate-labeled phosphatidylcholine (Figure 2) can be explained by three models. A single exponential would be expected if (1) all of the [^{3}H]arachidonate-labeled phosphatidylcholine is present in the outer, available monolayer, (2) the labeled phosphatidylcholine is present in both monolayers, but the rate constant for transfer of polyenoic phosphatidylcholine is so slow (10-fold compared to [^{32}P]phosphatidylcholine) that it becomes rate limiting, and (3) most of the labeled phosphatidylcholine is present in the inner monolayer and translocation is rate limiting. Two additional experiments were done in order to differentiate between these models.

Effect of Preincubation on the Transfer of [^{3}H]Arachidonate-Labeled [^{32}P]Phospholipid from the Red Cell Membrane to Unilamellar Vesicles. If the ^{32}P label is symmetrically distributed, whereas the [^{3}H]arachidonate label is preferentially localized in the cytoplasmic monolayer, one would expect that preincubation would have different effects on the transfer of these two labels from red cells labeled with both. Preincubation would not be expected to cause any change in the transfer of [^{32}P]phospholipid while it should cause an increase in the initial transfer rate of [^{3}H]arachidonate-labeled phospholipid. As shown in Figure 3 for the transfer of phosphatidylcholine from red cells to unilamellar vesicles, this was observed. After incubation for 3 h, [^{32}P]phosphatidylcholine transfer was not changed while [^{3}H]arachidonate-labeled phosphatidylcholine transfer was increased and the shape of the curve was changed. After preincubation, the data for the transfer of [^{3}H]arachidonate-labeled phosphatidylcholine were best approximated by the sum of two exponentials, indicating that a portion of the label was present in the external monolayer. Mass ratios of the phosphatidylcholine present in monolayers a and b and kinetic constants were calculated based on a two-pool model as described in the Appendix. Sixty-seven percent of the phosphatidylcholine was calculated to be present in the outer monolayer, based on the ratio of the rate constants k_{ab} and k_{ba}. This is in good agreement with the value of 63% calculated from exchange experiments using ^{32}P-labeled red blood cells (Table II). The value of k_{ab} (0.33 h^{-1}) calculated from preincubated red cells was greater than the value (0.19 h^{-1}) obtained from the same labeled erythrocytes which had not been preincubated. The statistical significance of this difference cannot be determined since only one such set of experiments was done, but it is possible that the 3-h preincubation facilitated subsequent translocations of phosphatidylcholine across the membrane. In data not shown, the transfer of [^{32}P]sphingomyelin was not changed by preincubation, whereas the rates of transfer of [^{32}P]phosphatidylethanolamine and [^{3}H]arachidonate-labeled phosphatidylethanolamine were increased very slightly to about the same extent.

Effect of the Concentration of Exchange Protein on the Transfer of [^{3}H]Arachidonate-Labeled [^{32}P]Phospholipid from the Red Cell Membrane to Unilamellar Vesicles. In order to confirm that [^{3}H]arachidonate-labeled phospholipid is asymmetrically distributed and that the rate of transfer from red cells to unilamellar vesicles is determined by the rate of transbilayer movement, the exchange of [^{3}H]arachidonate-labeled [^{32}P]phospholipid was examined at two concentrations of phospholipid exchange protein. If the [^{3}H]arachidonate-labeled phospholipid is localized predominantly in the cytoplasmic monolayer and if the exchange protein does not influence the rate of transbilayer movement of phospholipid, little difference would be expected in the rate of transfer caused by increased levels of exchange protein. On the other hand, [^{32}P]phosphatidylcholine and sphingomyelin which are predominantly present in the outer monolayer should show an increased rate of transfer at the higher protein concentration. As shown in Figure 4, almost no difference was seen in the transfer of [^{3}H]arachidonate-labeled phosphatidylcholine at twice the exchange protein concentration, whereas an increased rate was observed for the transfer of [^{32}P]phosphatidylcholine and sphingomyelin. The transfer of both [^{3}H]arachidonate-

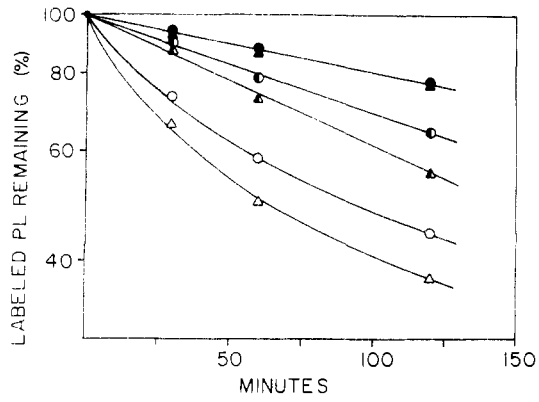

FIGURE 4: Semilogarithmic plot of the transfer of red cell [^{3}H]arachidonate-labeled [^{32}P]phospholipids to unilamellar vesicles catalyzed by two concentrations of beef liver nonspecific exchange protein (2.5 and 5 units). ^{32}P-Labeled red cells were labeled with [^{3}H]arachidonic acid. The transfer of label from red cells to unilamellar vesicles composed of 37.5 mol % phosphatidylcholine, 17.0 mol % sphingomyelin, and 45.4 mol % cholesterol was measured as a function of time by using 2.5 (○, ◐, ●) and 5.0 (△, ▲, ▲) units of exchange protein. The transfer of labeled phospholipid was measured as described in Figure 1. Each point is the average of duplicate determinations. (○, △) [^{32}P]Phosphatidylcholine; (◐, ▲) [^{32}P]sphingomyelin; (●, ▲) [^{3}H]arachidonate-labeled phosphatidylcholine.

labeled phosphatidylethanolamine and [^{32}P]phosphatidylethanolamine was low (less than 5%) and showed no significant change at the increased level of exchange protein (data not shown).

Discussion

The results presented here demonstrate that the [^{32}P]-phosphatidylcholine in the rat erythrocyte membrane exists in two pools, whereas [^{32}P]sphingomyelin, [^{3}H]arachidonate-labeled [^{32}P]phosphatidylethanolamine, and [^{3}H]arachidonate pulse labeled phosphatidylcholine each exist in one pool. It has previously been shown in unilamellar vesicles that phospholipid exchange proteins utilize only phospholipid that is in the outer monolayer (Johnson et al., 1975; Rothman & Dawidowicz, 1975). Since it is unlikely that the red blood cell is permeable to a phospholipid exchange protein of M_r 14 000, the readily available pool of [^{32}P]phosphatidylcholine (63%) is considered to be in the outer monolayer, whereas the slowly exchangeable pool of [^{32}P]phosphatidylcholine (37%) is considered to be in the inner monolayer. Bloj & Zilversmit (1976) have previously found that 75% of the labeled phosphatidylcholine in ^{32}P rat erythrocyte ghosts was rapidly transferred to phosphatidylcholine–cholesterol unilamellar vesicles in the presence of exchange protein. The remaining 25% was exchanged slowly. In inside-out vesicles prepared from ^{32}P rat erythrocytes, 37% was transferred rapidly and 63% was transferred slowly. These values are in good agreement with the results of the present investigation. The relatively small discrepancies in pool sizes may be due, in part, to changes in the membrane caused by ghost formation and/or membrane inversion. Studies with phospholipases have also shown that a large fraction of the phosphatidylcholine is present in the outer monolayer of the rat erythrocyte. Treatment with phospholipase A_2 and sphingomyelinase was found to hydrolyze 62% of the phosphatidylcholine before cell lysis (Renooij et al., 1976). Similarly, in human erythrocytes, 63% of the phosphatidylcholine has been found to be exchangeable with plasma lipoproteins (Reed, 1968).

For the remaining phospholipids, present in only one pool, it is most likely that the majority of each is present in a single membrane monolayer. We conclude that [^{32}P]sphingomyelin is present exclusively in the outer monolayer. This is consistent with the lack of effect of preincubation on sphingomyelin exchange and the increase in exchange rates with increasing exchange protein concentrations. Renooij et al. (1976) have also concluded that 100% of the sphingomyelin exists in the outer monolayer, based on its accessibility to hydrolysis by sphingomyelinase in intact rat erythrocytes. The possibility cannot be ruled out that a portion of the sphingomyelin is localized in the cytoplasmic monolayer but undergoes rapid translocation ($k > 0.23$ h^{-1}).

The extremely low rate constant for transfer of the entirety of both the [^{3}H]arachidonate-labeled phosphatidylethanolamine and the ^{32}P-labeled phosphatidylethanolamine, which is not a consequence of the specificity of the exchange protein (Crain & Zilversmit, 1980), suggests that both are predominantly localized in the cytoplasmic monolayer. Renooij et al. (1976) have reported that when red cells were treated with phospholipase A_2 and sphingomyelinase, 20% of the phosphatidylethanolamine was hydrolyzed before cell lysis. The present results do not demonstrate any phosphatidylethanolamine which is present in a readily accessible pool. One possible explanation for this discrepancy is that the orientation of the phosphatidylethanolamine in the membrane is changed by the hydrolysis of sphingomyelin. In the absence of sphingomyelinase, very little phosphatidylethanolamine (8%) is hydrolyzed by phospholipase A_2 (Renooij et al., 1976). Likewise, in human red cells, 68% of the phosphatidylcholine and no phosphatidylethanolamine are hydrolyzed by phospholipase A_2 action on intact red cells (Verkleij et al., 1973). Treatment with sphingomyelinase, which hydrolyzes 85% of the sphingomyelin, exposes 20% of the phosphatidylethanolamine to hydrolysis by phospholipase A_2. By the action of nonpenetrating chemical probes on human erythrocytes, it has also been suggested that little phosphatidylethanolamine is present in the outer monolayer (Gordesky et al., 1975; Whiteley & Berg, 1974). On the other hand, procedures which disturb the phospholipid–protein interactions in the membrane increase the availability of phosphatidylethanolamine to phospholipase hydrolysis or chemical labeling (Haest & Deuticke, 1975, 1976; Marinetti & Crain, 1978).

From the kinetics of transfer, the effect of exchange protein concentration, and the effect of preincubation, it is concluded that [^{3}H]arachidonate-labeled phosphatidylcholine is formed exclusively in the cytoplasmic monolayer. This has previously been shown in rat erythrocytes (Renooij et al., 1976; Renooij & Van Golde, 1977) and human erythrocytes (Renooij et al., 1974) by means of phospholipases. Labeled phosphatidylcholine will be introduced into the outer monolayer by translocation. From the rate constants ($k_{ab} = 0.155$ h^{-1} and $k_{ba} = 0.091$ h^{-1}) for transbilayer movement of arachidonate-labeled phosphatidylcholine, half-times of 4.5 and 7.6 h, respectively, are obtained. These are in agreement with the value of 4–5 h ($t_{1/2}$ calculated from k_{ba}) obtained from phospholipase studies on rat red cells labeled in vitro with [^{32}P]phospholipid (Renooij et al., 1976). On the other hand, the rate constant (k_{ab}) for transbilayer movement of phosphatidylcholine calculated for red blood cells labeled in vivo with ^{32}P$_i$ is 0.061 h^{-1}. Similarly, Bloj & Zilversmit (1976) calculated values for k_{ab} of 0.225 h^{-1} in resealed rat erythrocyte ghosts and 0.078 h^{-1} in inside-out vesicles formed from rat erythrocytes.

The difference between rates of transbilayer movement calculated from exchange of [^{3}H]arachidonate-labeled and ^{32}P-labeled phosphatidylcholine may be a reflection of the difference in transbilayer movement for different molecular classes of phosphatidylcholine. Renooij & Van Golde (1977)

calculated that in phosphatidylcholine molecules containing labeled palmitic acid, the rates of translocation of the monoenoic, dienoic, and polyunsaturated molecular classes were 2, 2.7, and 3.2, respectively, times the rate of translocation found for disaturated phosphatidylcholine. A second possible cause for the difference in the rate of transbilayer movement is that newly synthesized phospholipid exists in a distinct pool which undergoes rapid translocation. This is unlikely since the rate of translocation of newly synthesized polyunsaturated phosphatidylcholine found here is similar to that for the polyunsaturated phosphatidylcholine exchanged from lipoprotein (Renooij et al., 1976).

The rate of transbilayer equilibration $0.693/(k_{ab} + k_{ba})$ calculated for [^{3}H]arachidonate-labeled phosphatidylcholine ($t_{1/2}$ = 2.8 h) and for [^{32}P]phosphatidylcholine ($t_{1/2}$ = 7.2 h) may be compared to a number of studies on natural and artificial membranes.[2] Translocation of phosphatidylcholine measured in unilamellar vesicles by a number of techniques has been found to be very slow (Johnson et al., 1975; Rothman & Dawidowicz, 1975; DiCorleto & Zilversmit, 1979; Kornberg & McConnell, 1971; De Kruijff & Wirtz, 1977; Shaw et al., 1977). The rate of transmembrane movement may be increased by inducing phospholipid asymmetry (De Kruijff & Wirtz, 1977; De Kruijff & Baken, 1978). Incorporation of glycophorin was also found to enhance the rate of transbilayer movement (Van Zoelen et al., 1978; De Kruijff et al., 1978), though cytochrome oxidase had no effect (DiCorleto & Zilversmit, 1979). As the number of biological membranes studied has increased, it has become apparent that no generalizations regarding the transmembrane movement of phospholipid can be made. In the influenza virus, the half-time for the equilibration of phosphatidylcholine between the outer and inner monolayers was found to be greater than 10 days (Rothman et al., 1976), while in spikeless virions of the vesicular stomatitis virus, it was measured at 7–11 h (Shaw et al., 1979). In the inner mitochondrial membrane (Rousselet et al., 1976a) and the LM plasma membrane (Sandra & Pagano, 1978), the rate of transmembrane movement was found to be quite slow. In membranes from electroplax (McNamee & McConnell, 1973), *Acholeplasma laidlawii* (Grant & McConnell, 1973), and rat liver microsomes (Zilversmit & Hughes, 1977; Van den Besselaar et al., 1978), however, the rate of transbilayer movement of phosphatidylcholine has been found to be quite rapid. In red cells from humans (Renooij et al., 1974; Rousselet et al., 1976b; Steck et al., 1976) and rabbits (Renooij, 1977), the translocation of phosphatidylcholine at 37 °C has been found to be quite slow relative to that found for rat red cells (Renooij et al., 1976).

The transbilayer movement of phospholipid has previously been described as flip-flop (Kornberg & McConnell, 1971), in which the polar head group of the phospholipid passes through the hydrophobic core of the bilayer. Such a mechanism would be expected to be thermodynamically unfavorable, and this is undoubtably responsible for the slow rate of transbilayer movement found in unilamellar vesicles. However, it cannot explain the large variation found in the rate of transbilayer movement of phospholipids in biological membranes. Specific proteins have been hypothesized which might catalyze the transfer of phospholipids between membrane monolayers (Bretscher, 1973; Rothman & Lenard, 1977). A second possible mechanism involves lateral diffusion of phospholipid along membrane pores formed by transmembrane proteins (Zilversmit & Hughes, 1977). Such a mechanism could explain the variation found in the rate of transbilayer phospholipid movement by differences in membrane composition. It is supported by the effect found for glycophorin on translocation of phospholipid in unilamellar vesicles (Van Zoelen et al., 1978; De Kruijff et al., 1978).

In the many membranes in which the transbilayer movement of phospholipids has been found, asymmetry cannot be maintained as with proteins by an absolute lack of transmembrane movement. The asymmetric distribution of phospholipids found in many membranes may then result from asymmetric synthesis and degradation (Rothman & Lenard, 1977; Marinetti & Crain, 1978) or a thermodynamic equilibrium in which the equilibrium constant for phospholipid distribution is shifted toward one monolayer by factors (protein–lipid interactions and the pH or ionic composition of the environment) which lower the free energy of a particular phospholipid class on that membrane surface (Zilversmit & Hughes, 1977; Rothman & Kennedy, 1977). In the rat red blood cell, the former mechanism is not likely to be of major importance.

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Appendix

The following model was used:

$$\text{b(inside)} \underset{k_{ba}}{\overset{k_{ab}}{\rightleftharpoons}} \text{a(outside)} \xrightarrow{k_0} \text{liposomes}$$

Definitions. g_1 and g_2 are the fast and slow exponential constants, respectively, for the transfer of [^{3}H]arachidonate-labeled phosphatidylcholine from red blood cells after 3-h preincubation (Figure 3). k_{ab}, k_{ba}, and k_0 refer to kinetic constants for the model above. q_a = labeled phosphatidylcholine in pool a at time t. $q_{a(0)}$ = labeled phosphatidylcholine in pool a at time $t = 0$. q_b = labeled phosphatidylcholine in pool b at time t. $q_{b(0)}$ = labeled phosphatidylcholine in pool b at time $t = 0$. X = fraction of labeled phosphatidylcholine in pool b after 3-h preincubation.

If one considers a two-pool closed system in which all of the isotope is present in pool b at $t = 0$, as is the case in red blood cells pulse labeled with [^{3}H]arachidonate and incubated without exchange protein or unilamellar vesicles, the distribution of labeled phosphatidylcholine in pool b as a function of time is (Shipley & Clark, 1972)

$$\frac{q_b}{q_{b(0)}} = H_1 e^{-g_1 t} + H_2 \tag{1}$$

where

$$g_1 = k_{ab} + k_{ba}$$

$$H_1 = \frac{k_{ab}}{k_{ab} + k_{ba}} \qquad H_2 = 1 - H_1$$

At 3 h

$$X = \frac{q_b}{q_{b(0)}} = \frac{k_{ab}}{k_{ab} + k_{ba}} e^{-3(k_{ab}+k_{ba})} + 1 - \frac{k_{ab}}{k_{ab} + k_{ba}} \tag{2}$$

After the 3-h preincubation, label is present in both pools a and b. If the red blood cells are then incubated with uni-

[2] It is frequently difficult to ascertain which physical parameters are referred to by literature values for half-times.

lamellar vesicles and exchange protein (Figure 3), the fraction of label remaining in the erythrocyte as a function of time is (Bloj & Zilversmit, 1976)

$$\frac{q_a + q_b}{q_{a(0)} + q_{b(0)}} = \frac{k_0(1 - X) - g_2}{g_1 - g_2} e^{-g_1 t} + \frac{g_1 - k_0(1 - X)}{g_1 - g_2} e^{-g_2 t} \quad (3)$$

and

$$g_1 + g_2 = k_{ab} + k_{ba} + k_0 \quad (4)$$

$$g_1 g_2 = k_{ab} k_0 \quad (5)$$

$C_2 = [g_1 - (1 - X)k_0]/(g_1 - g_2) =$
Y intercept of the final log linear portion of the curve

$$X = \frac{C_2(g_1 - g_2) - g_1 + k_0}{k_0} \quad (6)$$

Substituting X into eq 3 gives

$$\frac{C_2(g_1 - g_2) - g_1}{g_1 g_2} = \frac{e^{-3(k_{ab}+k_{ba})} - 1}{k_{ab} + k_{ba}} \quad (7)$$

g_1, g_2, and C_2 were determined by a nonlinear least-squares fit of the data in Figure 3 (Dell et al., 1973):

$$g_1 = 2.34\ h^{-1} \qquad g_2 = 0.22\ h^{-1} \qquad C_2 = 0.872$$

k_{ab} and k_{ba} were determined iteratively from eq 7 by Newton's method. k_0 was obtained by substituting these values of k_{ab} and k_{ba} into eq 4.

The ratio of mass (M_a and M_b) of phosphatidylcholine in pools a and b was determined from the equality of transposition in both directions:

$$k_{ab}[M_b] = k_{ba}[M_a] \quad (8)$$

$$k_{ab} = 0.33\ h^{-1} \qquad k_{ba} = 0.67\ h^{-1} \qquad k_0 = 1.57\ h^{-1}$$

$$M_a = 0.67 \qquad M_b = 0.33$$

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Light-Scattering Measurements of Hemoglobin Binding to the Erythrocyte Membrane. Evidence for Transmembrane Effects Related to a Disulfonic Stilbene Binding to Band 3†

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ABSTRACT: Hemoglobin binding to isolated human erythrocyte membranes was studied by using light scattering. The light-scattering change induced upon binding gave saturation curves which were quantitatively comparable to those obtained by centrifugation measurements under stoichiometric conditions. Binding isotherms were constructed as a function of both hemoglobin and membrane concentrations. Two classes of binding sites were identified. A high-affinity class, consisting of $\sim 1 \times 10^6$ hemoglobin tetramer sites per cell, was shown to be coincident with the glyceraldehyde-3-phosphate dehydrogenase binding site on the cytoplasmic portion of band 3, the membrane protein involved in anion transport. A second class of $\sim 4 \times 10^6$ sites/cell had a very much lower affinity and are presumably located elsewhere on the inner surface of the membrane. Following these characterizations, DIDS [4,4′-bis(isothiocyano)-2,2′-stilbenedisulfonate] binding to externally exposed portions of band 3 is shown to alter hemoglobin binding by changing the isotherms from noncooperative to apparent negative cooperative at low membrane concentrations. These results suggest that DIDS binding to band 3, presumably at the anion transport site, results in transmembrane conformational changes which affect the affinity of protein binding sites on the cytoplasmic side of the membrane.

Recent studies have suggested that hemoglobin may bind to band 3 (Shaklai et al., 1977a,b; Salhany & Shaklai, 1979), the transmembrane protein of the erythrocyte involved in anion transport (Cabantchik et al., 1978). Techniques are required which can be used to study hemoglobin binding at concentrations approaching those present within the erythrocyte in order to test the possibility that this interaction may occur under physiological conditions. A relatively unexplored technique which has this potential is light scattering. Since light scattering from particles the size of erythrocytes and their ghosts arises from size and particularly shape changes, binding could be studied if hemoglobin binds to a particular site on the membrane and produces a change in the "shape" of the ghost. Then, the only requirement would be that the resultant hemoglobin-induced light-scattering change be a linear function of the total hemoglobin added under stoichiometric conditions.

After fluorescence measurements were published indicating the conditions for hemoglobin binding (Shaklai et al., 1977a), one of us (J.M.S.) discovered a very large hemoglobin-induced light-scattering effect under those conditions. Myoglobin, on the other hand, caused no light-scattering change under the same conditions. Furthermore, there was no effect of hemoglobin on light scattering at alkaline pH. Following this discovery, Salhany & Shaklai (1979) used the effect preliminarily as an adjunct to measurements of the functional properties of membrane-bound hemoglobin. In the present study, we provide a more detailed characterization of the hemoglobin-induced light-scattering effect. We then use this effect to show that hemoglobin binding to the cytoplasmic surface of the membrane can be influenced by DIDS[1] binding to the site involved in the inhibition of anion transport. This site is located on the outer aspect of band 3 and is thought to be coincident with the anion transport site (Cabantchik et al., 1978).

Materials and Methods

Freshly outdated whole blood or packed cells for this and other projects were obtained from the Omaha chapter of the American Red Cross. Most of the other reagents used have been described in previous publications from this laboratory (Salhany & Swanson, 1978; Salhany et al., 1978; Salhany & Shaklai, 1979). The proteolytic enzymes and G3PD were from Sigma, as was the inhibitor PMSF. DIDS was from Pierce Chemical Co.

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[1] Abbreviations used: G3PD, glyceraldehyde-3-phosphate dehydrogenase; DIDS, 4,4′-bis(isothiocyano)-2,2′-stilbenedisulfonate; PMSF, phenylmethanesulfonyl fluoride; 5P(pH), 5 mM orthophosphate, with the pH given in parentheses; PBS, 5 mM orthophosphate + 150 mM NaCl, pH 8; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

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