Accelerated Transbilayer Movement of Phosphatidylcholine in Sickled Erythrocytes

A reversible process*

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The transbilayer mobility of phosphatidylcholine (PC) molecules in the membrane of homozygous reversible sickle cells (RSCs) was studied using a PC-specific exchange protein from beef liver.

In deoxygenated RSCs, all of the PC present in the membrane of the intact cell is rapidly available for exchange, mediated by this protein. Since a substantial amount of the PC is present in the inner membrane leaflet of these cells, this observation implies that the PC molecules in their membranes do experience rapid transbilayer movements.

To determine the actual rate of transbilayer movement of the PC, radioactive PC was introduced into the outer monolayer of oxygenated RSCs using the PC-specific exchange protein. Subsequently, the cells were incubated at 37°C under oxygen- and deoxygenating conditions to enable the PC to equilibrate within the bilayer. At various time intervals, samples were taken and treated with phospholipase A₂, which selectively degrades the PC in the outer monolayer. Analysis of the specific radioactivities of the lyso-PC thus produced, as well as of the residual PC, enabled us to follow the rate of the radioactive PC previously introduced into the outer membrane layer. The half-time value for transbilayer equilibration of the PC in deoxygenated RSCs was determined to be 3.5 h, which is about four times lower than that for oxygenated RSCs. This increased transbilayer mobility of PC, observed in deoxygenated RSCs, is immediately restored to the normal low rate upon reoxygenation of the cells, indicating a complete reversibility of this phenomenon.

The phospholipids in the normal human erythrocyte membrane are distributed over both halves of the membrane bilayer in an asymmetric fashion. More than 75% of each of the two choline-containing phospholipids, PC and sphingomyelin are found in the outer monolayer, whereas the inner layer comprises 80% of the PE and all of the PS. Earlier studies indicated an almost complete absence of transbilayer movement of PC molecules in the normal human red cell membrane (3-5), but more recent studies reported half-time values for transbilayer equilibration of PC and lyso-PC at 37°C of approximately 13 and 27 h, respectively (6, 7). Although such half-time values represent slow translocation rates, the process may be significant when compared to the average life time (120 days) of the human erythrocyte.

The maintenance of the asymmetric phospholipid distribution during the life span of the cell may be found in direct interactions of (part of) the phospholipids with the membrane-bound proteins, which are known to possess an absolute asymmetry in their orientation with respect to the plane of the membrane (37). Oxidative cross-linking of the proteins, and in particular of spectrin by treatment of intact human erythrocytes with diamide or tetrathionate, appears to result in an increased accessibility of the aminophospholipids (PE and PS) towards the action of exogenous phospholipase A₂ (8, 9). Moreover, recent experiments involving a purified PC-specific exchange protein have shown that such a cross-linking of the membrane-bound proteins results in a complete exchangeability of all of the PC, whereas in control cells only the PC pool located in the outer monolayer is exchangeable (10). Hence, these observations demonstrate that cross-linking of membrane proteins enhances the rate of transbilayer movement of the PC molecules considerably.

Abnormalities in the availability of the phospholipids to exogenous probes have also been shown to occur in sickled erythrocytes. The accessibility of both aminophospholipids for chemical (11, 12) as well as enzymatic (13) probes appears to be increased, not only in irreversibly sickled cells, but also in deoxygenated (sickled) RSCs, when compared to both normal cells or oxygenated (discoid) RSCs. As is also the case in diamide- or tetrathionate-treated erythrocytes (9), these changes appeared to be confined to the glycerophospholipids, since the availability of sphingomyelin to sphingomyelinase C is always identical with that in normal cells (13). Furthermore, it is of interest that RSCs not only possess the ability to readopt their discoid shape upon reoxygenation, but that this process also for the greater part restores the degree of accessibility of the glycerophospholipids to exogenous probes to the levels found in normal erythrocytes (12, 13). These changes in accessibility of phospholipids in the outer membrane layer observed in sickled erythrocytes and the earlier observation that in irreversibly sickled cells the structure of the cytoskeletal network is permanently altered (14) may support the view that this cytoskeletal protein network is...
involved in maintaining the asymmetric phospholipid distribution and transversal mobility in the normal erythrocyte membrane.

Changes in phospholipid distributions, as observed in the RSC membrane, may involve relatively fast transbilayer movements of these molecules. The following two possibilities may occur: (i) a fast momentary transbilayer movement takes place only during the process of sickling, after which the new situation is stabilized, or (ii) increased transbilayer movements continue to exist as long as the cell is in its sickled configuration. In this paper, we present experimental evidence for the second possibility. First, it will be shown that, in contrast to the normal discoid cell, all of the membranous PC in the deoxygenated (sickled) RSC is available for rapid exchange, mediated by the PC-specific exchange protein from beef liver. In a second type of experiment, the actual rate of transbilayer movement of the PC molecules is determined in oxy-, deoxy-, and reoxygenated RSCs by using a technique which has been developed recently (6). Radioactive PC is introduced into the outer membrane half of oxygenated (discoid) RSCs during a relatively short incubation of cells with 14C-labeled PC/cholesterol vesicles in the presence of PC-specific exchange protein. After several washes, the cells are subsequently incubated at 37°C, under either oxygenating (deoxygenating (sickling)) conditions. After various time intervals, the distribution of the newly inserted (radioactive) PC over the outer and inner layer is determined by a nonlytic treatment of the cells with pure phospholipase A2.

MATERIALS AND METHODS

Erythrocytes—After obtaining informed consent, fresh venous blood from normal (AA) and homozygous sickle cell (SS) patients were collected in heparinized tubes. The cells were pelleted by centrifugation for 5 min at 2500 g and the buffy coat was carefully removed by aspiration. They were resuspended in a 4-fold volume of an isotonic buffer containing 90 mM KCl, 45 mM NaCl, 44 mM sucrose, 10 mM glucose, and 10 mM Tris/HCl, pH 7.4 (referred to as buffer throughout), and washed twice. All experiments were carried out with unfractionated populations of erythrocytes. In all cases, sickle cell populations contained less than 5% of cells in the irreversible sickled form.

Phosphatidylcholine Donor Systems—Two types of PC donor systems have been used. Rat liver microsomes were used in some experiments to study the exchangeability of the PC in the erythrocytes because under these conditions the exchange process proceeds at considerably higher rates than when PC/cholesterol vesicles are used as a donor system (35). PC/cholesterol vesicles, however, have been more thoroughly characterized, and sufficiently high amounts of radioactive PC can be incorporated into the outer monolayer of the erythrocyte membrane within reasonably short times of incubation, despite the slower exchange rate (35).

Rat liver microsomal membranes containing [methyl-3H]PCphosphatidylcholine were obtained from Wistar rats and prepared following the procedure of Kamp and Wirtz (16). Before use, the microsomes were sonicated for 2 min with a Branson Sonifier (70 watts) under nitrogen and cooled in ice. Previous studies (17, 18) have shown that in the microsomal membranes thus prepared, all of the PC is available for exchange. The [methyl-3H]PC label of 9°C was found in the PC fraction (25.5 ± 0.3 pmol/mg). The sonicated microsomal suspension was centrifuged for 10 min at 10,000 g to remove the heavier fractions that may co-sediment with erythrocytes at low speeds of centrifugation. The supernatant was used as the donor system.

When unilamellar vesicles were used as the donor system, they were prepared as follows. Equimolar amounts of cholesterol and phospholipid (97% egg PC and 3% egg phosphatidic acid, mole ratios) were dissolved in chloroform/methanol (2:1, v/v) and egg [methyl-3H]PC (91 ± 1 pmol/mg) and glycerol [3H]trioleate (0.91 ± 0.01 pmol/mg of vesicle PC), the latter as a nonexchangeable marker, were added and the mixture was dried at 37°C under 4 ml of nitrogen. The dried lipid was dispersed in buffer by vortexing. Vesicles were subsequently prepared by ultrasonication with a Branson Sonifier (70 watts) for 15 min under nitrogen, while the temperature was kept between 4 and 20°C. Larger lipid aggregates and metal particles released from the sonicator probe were removed by centrifugation for 60 min at 100,000 × g. The final unilamellar vesicle suspension contained approximately 10 pmol of PC/ml.

Phosphatidylcholine-specific Exchange Protein—PC-specific exchange protein was purified from beef liver according to Kamp and Wirtz (16). Glycerol, routinely used at a 50% (v/v) concentration during storage of the exchange protein at −20°C, was removed by overnight dialysis at 4°C against 300 volumes of buffer. To avoid nonspecific adherence, the dialysis bag was filled with a 0.15 M NaCl solution of bovine serum albumin. After dialysis, the volume of the protein solution was reduced by covering the dialysis bag with solid polyethylene glycol (Aquacide III, Cali-schem-Behring) until the desired protein concentration (150 µg/ml) was reached.

Introduction of PC in Normal (AA) and Deoxygenated Sickled (SS) Erythrocytes—Suspensions of normal (AA) and sickled (SS) erythrocytes were simultaneously incubated under gentle shaking at 37°C in the presence of exchange protein (2 nmo1/100 µl of packed cells). While the normal erythrocytes were incubated under room air, a nitrogen atmosphere was applied to the sample containing the sickle cells. After 15 min, in the latter sample more than 95% of the cells showed the characteristic sickled morphology, the exchange reaction was started by the addition of the [3H]PC-labeled microsomal membranes (100 nmol of PC/100 µl of packed cells). The final cell concentration was 33%. Incubations were continued under the above conditions. At various time intervals, aliquots were taken from the incubation mixtures and the exchange reaction terminated by centrifugation for 5 min at 2500 × g. Residual microsomal membranes and exchange protein were removed by two additional washes of the cells with buffer.

Introduction of [methyl-3H]PC into Intact RSCs, Transbilayer Equilibration under Oxy-, Deoxy-, and Reoxygenating Conditions, and Subsequent Treatment of Intact Cells with Phospholipases A2—For the introduction of [3H]PC into the outer membrane layer, a 33% suspension of oxygenated RSCs was incubated for 1 h at 37°C in the presence of [3H]PC/cholesterol (100 nCi of [3H]PC/100 µl of packed cells) and 2.5 mg of PC-specific exchange protein (1 nmol/100 µl of packed cells). The exchange reaction was stopped by centrifugation (5 min, 2500 × g), followed by two additional washes of the cells with buffer to remove residual exchange protein and lipid vesicles. After resuspending the cells in buffer (33% suspension), part of this suspension was exposed to a nitrogen atmosphere to induce the cells to Sickles.

The [3H]PC was enabled to equilibrate between both halves of the membrane bilayer by subsequent incubation of the two cell suspensions at 37°C, under either room air or humidified nitrogen. In some experiments, after 2 h of incubation under nitrogen, the deoxygenated cells were reoxygenated by exposing the suspension to pure oxygen. The RSCs redated their discoid shape within a couple of minutes, after which the incubation was continued under room air. At various time intervals, samples (comprising 310 µl of packed cells) were taken from the above incubation mixtures. After collecting the erythrocytes by centrifugation, they were resuspended in 3 µl of the following buffer: 90 mM KCl, 45 mM NaCl, 10 mM CaCl2, 0.25 mM MgCl2, 2 mM sucrose, 10 mM Tris/HCl, pH 7.4. A mixture of highly purified phospholipase A2 from N. naja and bee venom was added, each at a concentration of 6 IU/100 µl of packed cells. Selective hydrolysis of the PC in the outer membrane layer was achieved by incubating these suspensions for 1 h at 37°C. The cells were subsequently collected by centrifugation (5 min, 2500 × g), and the action of the phospholipases was terminated by resuspending the cells in 0.5 ml of 100 mM EDTA in 0.9% NaCl. Finally, the cells were lysed by the addition of a solution of EDTA saturated with sodium dodecyl sulfate. The intact membranes were spun down at 2500 × g for 20 min and stored at
Transbilayer Movement of Phosphatidylethanoline in Sickled Cells

—20 °C until extraction and analysis of their lipids.

Lipid Extraction and Analysis—Lipids were extracted from erythrocytes or ghost membranes following the method of Rose and Ockander (19). After evaporation of the solvent under reduced pressure, the lipids were taken up in a small volume of chloroform/methanol (2:1, v/v) and separated by two-dimensional thin layer chromatography according to Broekhuysen (20). The amounts of PC and lyso-PC were determined by phosphate analysis according to Romers et al. (21), after ashing the corresponding TLC spots with 70% perchloric acid. When the radioactivity in PC and lyso-PC had to be determined, the extracted lipids were separated by one-dimensional TLC on silica gel plates, using chloroform, methanol, 25% ammonia, water (90:5:4:5:5.5, v/v) as solvent system. Spots, corresponding with PC and lyso-PC, were quantitatively transferred into counting vials. After addition of toluene, containing 2,5-diphenyloxazole (5 g/liter), methyl-1,4-bis(5-phenyloxazolyl)benzene (0.25 g/liter), and Bio-Solv (2%, v/v), radioactivity was measured by the °C single channel rate method, using a Packard liquid scintillation spectrometer. Radioactivity in total lipid extracts was determined by °H/C dual label procedure.

Calculations—Calculations of the exchangeable pool(s) of PC in the erythrocyte membranes(s) as well as the necessary corrections of the analytical data for contamination of the erythrocytes with microsomes were carried out as described by Van Meer et al. (5). Contamination never exceeded 2% of the total erythrocyte phospholipid.

During the incubation of oxygenated (discoid) RSCs with [14C]PC-containing vesicles in the presence of the exchange protein, the radioactive PC is exclusively introduced into the outer monolayer of the red cell membrane. In case the PC molecules do experience transbilayer movements in this membrane, a time-dependent fall in the specific radioactivity of these molecules in the outer monolayer, coupled with an increase in that in the inner layer, will be observed during subsequent incubation of the cells in the absence of vesicles. This process, known as a two-pool closed system (22), will finally result in a stable permanent equilibrium in which the specific radioactivity of the PC at either side of the membrane is the same. Incubation of the cells with phospholipase A2 under nonlytic conditions enabled us to discriminate between PC molecules present in the outer and inner monolayers. Since these probes do not cross the bilayer, the hydrolytic conversion of PC into its lyso-derivative thus achieved remains confined to those molecules which are present in the outer membrane leaflet. Full details of the mathematical background of the calculations are published elsewhere (6). The half-time value for the equilibration of PC molecules over both halves of the membrane, the so-called "flip-flop" rate, can be easily determined from a semilogarithmic plot of (SAo - SA)/((SAo - SA) - SA) versus time of incubation, in which SAo is the specific radioactivity of the lyso-PC produced by treatment of the intact cells with phospholipase A2, which treatment started at time t; and SA is the specific radioactivity of the lyso-PC at indefinite time of incubation at °C, when complete equilibration of the entire PC pool within the erythrocyte membrane(s) as well as the necessary corrections of the data of the exchange process depicted in Fig. 1. Therefore, the experiments described below were undertaken to simplify this problem to a two-pool closed system.

Transbilayer Movement of 14C]PC in the Membranes of Oxy- and Deoxygenated RSCs—[14C]PC was introduced into the outer monolayer of oxygenated (discoid) RSCs by incubation of these cells with egg [14C]PC-containing egg PC/cholesterol vesicles in the presence of the PC-specific exchange protein (see "Materials and Methods"). The amount of radioactivity thus introduced ranged from 20 to 25 dpm/nmol of erythrocyte PC. The trace amount of glycerol [3H] trioleate in the PC donor vesicles was used as a nonexchangeable marker to determine the ultimate extent of contamination of the erythrocytes with vesicles. The amount of contaminating vesicle PC never exceeded 2% of the total erythrocyte phospholipids. In all subsequent experiments, the analytical data were corrected for this contamination, prior to their use in the calculations.

In order to enable the newly introduced PC to redistribute over both halves of the membrane bilayer, the RSCs, washed free of vesicles and exchange protein, were reincubated at 37 °C in fresh buffer. One fraction of the cell suspension was kept under room air (discocytes) and a second fraction under humidified nitrogen (sickled cells). At various time intervals, samples were taken and the localization of the newly introduced PC was determined by °H/C dual label procedure.
duced $[^{14}C]PC$ within the bilayer was detected by nonlytic treatment of the cells with a mixture of N. naja and bee venom phospholipase $A_2$. This mixture was used to eliminate possible differences in molecular species substrate specificities (23). The lys-PC thus produced is representative of the PC fraction located in the outer monolayer. However, complete hydrolysis of all of the PC molecules in the outer membrane layer can only be achieved when sphingomyelin present in that layer is also degraded by sphingomyelinase (1, 2, 24).

Unfortunately, and in contrast to normal erythrocytes, the combined action of phospholipase $A_2$ and sphingomyelinase C causes extensive lysis of sickled erythrocytes (13). Nevertheless, it should be noted that even an incomplete hydrolysis of the PC in the outer monolayer will have no consequences for the determination of the specific radioactivity of the lys-PC produced. Table I shows that under the experimental conditions, the extent of PC hydrolysis caused by treatment of the cells with phospholipase $A_2$ is fairly constant regardless of the morphology of the cells (i.e. discoid under room air or sickled under nitrogen) or the time of preincubation. Table I also shows that the total amount of $[^{14}C]PC$ in either of the two cell suspensions does not change during the 8-h incubation.

Fig. 2 shows the time-dependent changes in specific radioactivity of the lys-PC produced in the outer monolayer by subsequent treatment of the cells with phospholipase $A_2$, as well as that of the residual PC not degraded by these enzymes. As discussed above, the fraction of residual PC does not completely represent the inner pool of this phospholipid, but also contains a small part of the PC in the outer layer which is not degraded by phospholipase $A_2$. This accounts for the radioactivity detected in the residual PC at zero time (Fig. 2).

Moreover, it should be realized that some of the $[^{14}C]PC$ introduced into the outer layer will, although at a low rate, start to equilibrate with the PC in the inner layer during the exchange incubation and subsequent washing of the oxygenated RSCs.

In the oxygenated (discoid) RSCs, a relatively slow decrease in the specific radioactivity of the lys-PC is observed concomitant with an increase in the specific radioactivity of the residual PC (Fig. 2). It should be noted that at complete equilibrium, the specific radioactivity of the lys-PC in the outer monolayer should be equal to that of the residual PC in the inner layer. At that time, the specific radioactivity should have reached a value which is 0.76 times the initial specific radioactivity of the PC in the outer monolayer, since in discoid erythrocytes this layer contains 76% of the total membranous PC.

In deoxygenated RSCs, which have been induced to sickle under nitrogen at zero time (Fig. 2), the changes in specific radioactivity of both the lys-PC and residual PC proceed at much faster rates when compared to the oxygenated RSCs. This obviously indicates a higher equilibration rate and therefore an increased transbilayer mobility of the PC molecules in the sickled RSCs, which confirms the conclusion derived from the PC exchange experiments described above. It is of particular interest to note that at zero time (Fig. 2) the specific radioactivity of the residual PC in the deoxygenated (sickled) RSCs does not significantly deviate from that of the residual PC in the oxygenated (discoid) RSCs. If the process of sickling would have been accompanied by a momentary redistribution of a considerable fraction of the PC from the outer to the inner monolayer, a significantly higher specific radioactivity of the residual PC in the deoxygenated RSCs should have been observed at zero time.

In order to determine the actual rate of transbilayer movement of the PC molecules, the time-dependent changes in specific radioactivity of the (lys-)PC in the outer monolayer are recorded in a semilogarithmic plot (Fig. 3). Fig. 3A shows the average results of three independent experiments. Half-time values for the transbilayer equilibration of the PC molecules can be easily determined from the slope of the lines depicted in this figure. These are approximately 14 and 3.5 h for discoid and sickled RSCs, respectively. It is of considerable interest to note that the immediate increase in transbilayer mobility of the PC molecules, observed when the RSCs are transformed from discocytes to sickled cells by deoxygenation (Fig. 3A), appears to be completely reversible. When RSCs that have been incubated under nitrogen for 2 h were rapidly reoxygynated by a brief exposure to pure oxygen, they recovered their discoid morphology almost immediately. During

### Table I

<table>
<thead>
<tr>
<th>Preincubation of RSCs</th>
<th>Total radioactivity of $[^{14}C]PC$</th>
<th>PC hydrolyses by phospholipase $A_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$dpm/nmol$</td>
<td>%</td>
</tr>
<tr>
<td>Room air</td>
<td>22.2</td>
<td>62.4</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>22.2</td>
<td>59.0</td>
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<td>21.9</td>
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<td></td>
<td>22.7</td>
<td>53.0</td>
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<td></td>
<td>21.8</td>
<td>57.9</td>
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The lyso-PC thus produced is representative of the PC fraction located in the outer monolayer under nitrogen at zero (Fig. 2), specific radioactivity of the (lyso-)PC in the outer monolayer, since in discoid erythrocytes this layer contains 76% of the total membranous PC.

**Fig. 2.** Time-dependent changes in specific radioactivity in the inner and outer PC pools in oxygenated and deoxygenated RSCs. $[^{14}C]PC$ was introduced into the outer monolayer of oxygenated RSCs by incubating these cells for 1 h in the presence of the PC-specific exchange protein and egg $[^{14}C]PC$/cholesterol vesicles. After washing, the cells were incubated at $37^\circ C$ under either room air or nitrogen. At the times indicated, samples were taken and the PC in the outer monolayer converted into lys-PC by treatment of the intact cells with phospholipase $A_2$. After inhibition of the phospholipase $A_2$, the lipids were extracted, and separated by TLC, and the specific radioactivities of the lys-PC and residual PC were determined. For full details see "Materials and Methods." Specific radioactivities of lys-PC (LPC) ($\bullet$, $\triangle$) and residual PC (C, $\Delta$) from oxygenated (O) and deoxygenated (A, A) RSCs are presented as a function of time.
Indeed, studies involving lipid model systems have shown that by oxidative cross-linking of the membrane proteins, it supports the hypothesis that the structural integrity of the bilayer significantly increased flip-flop rates. Since this is comparable to the situation that can be induced in normal erythrocytes, it can be concluded that in the inner monolayer are weakened, these phospholipids may experience an increased freedom of transbilayer mobility. Therefore, it is possible that during prolonged incubations of sickled cells with phospholipases increasing amounts of these substrates may become available for degradation.

Increased transbilayer mobility of PC in deoxygenated (sickled) RSCs relative to that in oxygenated (discoid) RSCs was also demonstrated by following the fate of radioactive PC that had been previously introduced into the outer membrane layer of these cells under oxygenated conditions. Transbilayer movements will cause a time-dependent decrease in the specific radioactivity of the PC in the outer layer, concomitant with an increase of that in the inner one. This time-dependent change was greatly enhanced when the RSCs were incubated under hypoxic (sickling) conditions (Fig. 2). This enhanced decrease is attenuated by an enhanced, time-dependent increase in the specific radioactivity of the residual PC, of which the greater part is representative of the PC fraction in the inner layer (Fig. 2). The actual flip-flop rate of the PC in the membrane of oxygenated RSCs is approximately 14 h. In deoxygenated RSCs, the half-time value is only 3.5 h. This indicates that the transbilayer movement of PC in sickled cells is approximately four times faster than that in discoid RSCs. The reversibility of this process is most striking. The enhanced transbilayer mobility of the PC in sickled RSCs is restored to the original low level observed in discoid RSCs immediately after reoxygenation of these cells (Fig. 3B). This reversibility indicates that these alterations are indeed associated with the sickling process.

Fig. 3. Transbilayer movement of PC in oxygenated, deoxygenated, and reoxygenated RSCs. Introduction of [3H]PC, incubation of the RSCs at 37°C, and subsequent treatment of the cells with phospholipase A2 were similar as described in the legend to Fig. 2. Relative specific radioactivity of the PC in the outer monolayer, calculated as (SAo - SA1)/(SA1 - SA0) (see "Calculations"), is plotted semilogarithmically versus time. A, oxygenated (○) and deoxygenated (△) RSCs; B, deoxygenated (○) and reoxygenated (□) RSCs. Reoxygenation (△) was achieved by brief exposure of RSCs to oxygen, after incubation of these cells for 2 h under nitrogen. Data represent the means ± S.D. of three independent experiments.

DISCUSSION

A relatively low rate of transbilayer mobility is considered to be responsible for the fact that in normal human erythrocytes only that fraction of the PC located in the outer monolayer is available for rapid, protein-mediated exchange (Refs. 5 and 10 and Fig. 1). The present study demonstrates that in deoxygenated (sickled) RSCs the entire PC complement is available for such an exchange process, thus indicating that in contrast to normal erythrocytes, the transbilayer movement of PC no longer hampers the exchange of the PC from the inner monolayer. Hence, it can be concluded that in the membrane of sickled RSCs the PC molecules experience significantly increased flip-flop rates. Since this is comparable to the situation that can be induced in normal erythrocytes by oxidative cross-linking of the membrane proteins (10), it supports the hypothesis that the structural integrity of the cytoskeleton which appears to be disturbed in sickled cells (14) is essential for stabilization of the lipid bilayer (9, 25). Indeed, studies involving lipid model systems have shown that spectrin is able to interact directly with phospholipids, and in particular with those carrying a negative charge, like PS (26-30).

In a previous communication (13), we interpreted the increased accessibility of the aminophospholipids in intact sickled erythrocytes to phospholipases in terms of a permanent alteration in the asymmetric phospholipid distribution within the membrane of these cells. In view of the present observations, the highly increased transbilayer movement of phospholipid molecules in sickled erythrocytes should also be considered to be responsible for this phenomenon. When the interactions which normally retain the aminophospholipids in the inner monolayer are weakened, these phospholipids may experience an increased freedom of transbilayer mobility. Therefore, it is possible that during prolonged incubations of sickled cells with probes like phospholipases increasing amounts of these substrates may become available for degradation.

Relatively long incubation times were found to be required to hydrolyze greater amounts of PE and a significant fraction of the PS during treatment of sickled cells with phospholipase

\[ (S_{Ao} - S_{A1})/(S_{A1} - S_{A0}) = \text{semilogarithmic plot} \]
A$_2$ (13). In addition, the time curve for the hydrolysis of PE and PS in sickled cells appeared not to end up on a clear plateau, not even after 4 h of incubation (13). In contrast, the hydrolysis of substrates during incubation of normal human erythrocytes reaches a plateau after 2 h (1). These results suggest that in normal cells the products of hydrolysis in the outer leaflet of the bilayer do not exchange with parent molecules on the inner leaflet, whereas in sickled cells such transbilayer movements may be possible.

If the quantity of phospholipids on both sides of the bilayer were to remain equal, a considerable increase in the amount of aminophospholipids in the outer monolayer should have to be compensated for by the reversed migration of part of the PC from the outer to the inner membrane layer. Using bee venom phospholipase A$_2$, we have previously reported data consistent with that possibility. However, a momentary redistribution of a considerable fraction of the PC from the outer to the inner layer, taking place during the process of sickling, would have resulted in an immediate and significant increase in the specific radioactivity of the residual PC in deoxygenated RSCs at zero time in the experiment depicted in Fig. 2. Since this is not the case, a more gradual redistribution of the PC in the sickled cell membrane should be considered. However, a considerable decrease in the pool size of the PC in the outer layer would ultimately result in a smaller fraction of the PC that is available for hydrolysis by the combination of phospholipases A$_2$ used. Table I clearly shows that the phospholipase A$_2$-degradable fraction of the PC does not change, not even after 8 h of incubation of the RSCs under nitrogen. This inconsistency suggests that the decreased PC degradation noted with bee venom phospholipase A$_2$ represents a decrease in PC availability to the enzyme and not necessarily a change in PC organization within the bilayer.

Our studies indicate the presence of rapid transbilayer movement of PS in sickled cells. Similarly, it is possible that transbilayer movements of PS and PE exist. Such movements of (glycerophospholipids may be due to weakened or lost interactions with the cytoskeletal network. The abnormalities in the organization of aminophospholipids we have previously reported to exist in sickled cells (12, 13) may represent enhanced accessibility to nonpenetrating probes rather than a reorganization of these phospholipids within the lipid bilayer.

Nevertheless, under certain conditions the outer membrane leaflet of the sickled cell may contain PE and/or increased quantities of PE. For instance, it may be possible that the extracorpulcular environment may favorably retain these phospholipid classes in the outer layer, under conditions where their stabilization in the inner monolayer is lost. In this context, it is worth mentioning the enhanced adherence of sickled cells to endothelial cells (32–34). This process might be accomplished when a certain fraction of the red cell aminophospholipids has been stabilized in the outer membrane layer by an interaction with the endothelial cell. Obviously, such processes require extensive transbilayer movements of the (glycerophospholipids. These conditions seem to be fulfilled in the sickled erythrocyte.

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Transbilayer Movement of Phosphatidylcholine in Sickle Cells

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