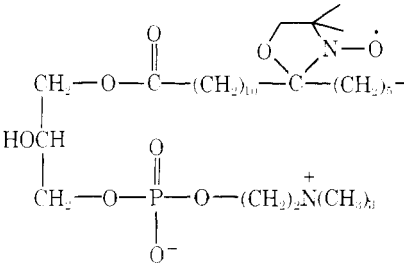
Interaction of Spin-Labeled Lysophosphatidylcholine with Rabbit Erythrocytes†

Hideo Utsumi,‡ Keizo Inoue, Shoshichi Nojima, and Takao Kwan*,‡

ABSTRACT: Synthetic spin-labeled lysophosphatidylcholine had hemolytic activity in rabbit erythrocytes. The hemolysis was slower than that caused by egg yolk lysophosphatidylcholine and depended on temperature. Electron spin resonance spectra indicated that both monomers and micelles of spin-labeled lysophosphatidylcholine could interact with erythrocyte membranes. Monomers of spin-labeled lysophosphatidylcholine were incorporated into the lipid bilayer of cell membranes, without causing any appreciable hemolysis. The resonance line arising from micelles of spin-labeled lysophosphatidylcholine gradually broadened when the lysophosphatidylcholine was incubated with erythrocytes at 37 °C. Similar line broadening was observed when spin-labeled lysophosphatidylcholine micelles were incubated with phosphatidylcholine liposomes. The line was broadest when the lysophosphatidylcholine was incubated with liposomal membranes that were in a state of phase separation. The time course and temperature dependence of the line broadening observed with erythrocytes corresponded closely to those of hemolysis. The line broadening seems to be due to dilution of micelles of spin-labeled lysophosphatidylcholine with membrane lipids, since similar line broadening has been observed previously in mixed micelles formed from spin-labeled lysophosphatidylcholine and dimyristoylphosphatidylcholine. A possible mechanism of hemolysis by spin-labeled lysophosphatidylcholine is proposed: lysophosphatidylcholine micelles may take away some lipid components from the cell membranes and this may induce a change in molecular organization of the membranes, causing hemolysis.

Lysophosphatidylcholine is known to react strongly with membranes. Exogenous lysophosphatidylcholine induces morphological changes in cells (Bergenhem & Fåhraeus, 1936; Singer, 1940; Klibansky & de Vries, 1963; Sato & Fujii, 1974) and facilitates fusion of various cell types (Ahkong et al., 1972); moreover at a sufficiently high concentration, it causes hemolysis (Bergenhem & Fåhraeus, 1936; Singer, 1940; Gottfield & Rapport, 1963). From studies on the hemolytic activities of several synthetic lysophosphatidylcholines, Reman et al. suggested that the hemolysis may be correlated with the critical micellar concentration (cmc) (Reman et al., 1969). Using lysophosphatidylcholine analogues with a benzylated hydroxyl group, Weltzien postulated the need for adsorption of the compound to the cell surface for a hemolytic reaction (Weltzien, 1973; Weltzien et al., 1976). Inoue and Kitagawa showed that lysophosphatidylcholine causes severe damage to artificial lipid membranes (liposomes) and suggested that the permeability changes of membranes are related to the interaction of lysophosphatidylcholine with the lipid bilayer (Inoue & Kitagawa, 1974). The mechanism of this interaction, however, is still poorly understood.

A spin label is a stable organic free radical, and its electron spin resonance (ESR)[1] spectrum can provide useful information on its interaction with cell membranes (Keith et al., 1973; Berliner, 1976). We have previously shown that 1-[12′-(*N*-oxyl-4″,4″-dimethyloxazolidine)stearoyl]-*sn*-glycero-3-phosphocholine (spin-labeled lysophosphatidylcholine), in which the spin-label moiety is covalently attached to the fatty acyl chain, forms micelles at above a certain concentration (Utsumi et al., 1977). Spin-labeled lysophosphatidylcholine should react with membranes, because its structure is similar



to that of egg lysophosphatidylcholine. In the present work, we studied the interaction of spin-labeled lysophosphatidylcholine with rabbit erythrocytes. The ESR spectra of spin-labeled lysophosphatidylcholine incubated with liposomal membranes were also measured and compared with the spectral changes during hemolysis. The possible mechanism of hemolysis induced by lysophosphatidylcholine is discussed.

Materials and Methods

Buffer. Veronal-buffered saline consisting of 145 mM sodium chloride, 1.82 mM sodium 5,5-diethylbarbiturate, and 3.12 mM 5,5-diethylbarbituric acid, pH 7.5, was used throughout.

Erythrocytes. Erythrocytes were prepared by centrifugation (350*g*, 5 min) of heparinized rabbit blood and were used within 3 days.[2] The buffy coat was removed and the remaining precipitate was washed three times with Veronal-buffered saline. For micromeasurement of hemolysis, cells were labeled with radioactive chromate by the method of Weinrach et al. (1958)

† From the Division of Physico-Chemical Technology (H.U. and T.K.) and the Department of Health Chemistry (K.I. and S.N.), Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113, Japan. *Received June 22, 1977; revised manuscript received November 10, 1977.*

‡ Present address: Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Tsukui, Kanagawa, 199-01, Japan.

[1] Abbreviations used: ESR, electron spin resonance; cmc, critical micellar concentration.

[2] Erythrocytes are well known to lose intracellular ATP upon aging and consequently to change in membrane fragility. However, in the present experiments, we could not observe any difference between fresh and 3-day-old erythrocytes.

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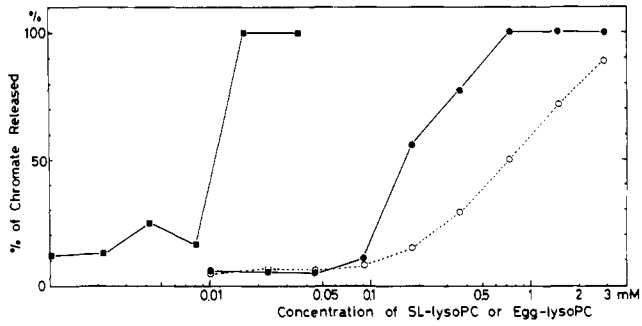

FIGURE 1: Effect of the concentration of lysophosphatidylcholine on lysis of rabbit erythrocytes. Rabbit erythrocytes labeled with radioactive sodium chromate (5×10^7 cells/mL) were incubated with egg lysophosphatidylcholine (■—■) or spin-labeled lysophosphatidylcholine (●—●) for 2 h at 37 °C. Rabbit erythrocytes (10^9 cells/mL) were also incubated with spin-labeled lysophosphatidylcholine for 2 h at 37 °C (O- - -O). Then the mixtures were centrifuged (350*g*, 5 min) and the radioactivities of the supernatants were measured.

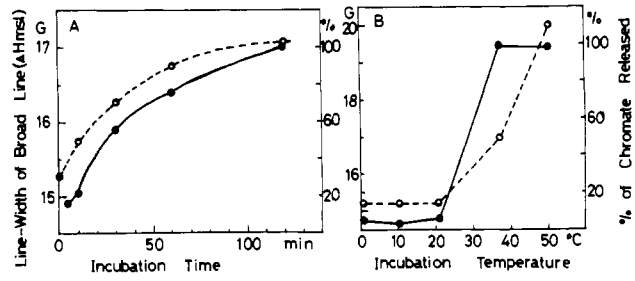

FIGURE 2: Time-course (A) and termperature dependence (B) of hemolysis (●—●) and line broadening (O- - -O). (A) Rabbit erythrocytes (10^9 cells/mL) were incubated with 6.7 μmol/mL of spin-labeled lysophosphatidylcholine at 37 °C for various times. (B) Rabbit erythrocytes (10^9 cells/mL) were incubated with 6.7 μmol/mL of spin-labeled lysophosphatidylcholine for 90 min at different temperatures. Hemolysis was measured as described in the legend of Figure 1. Line widths were measured on the spectra in Figures 3A and 3B (shown by arrows in Figures 3A and 3B).

and Sanderson (1965) as modified by Inoue et al. (1972). To 2 mL of packed cells was added 2 mL of Fisher's medium (Nissui Seiyaku Co., Tokyo), and the suspension was incubated with about 100 μCi of radioactive chromate (Daiichi Radioisotope Labs Co. Ltd.) for 2 h at 37 °C. The cells were collected by centrifugation and washed with Veronal-buffered saline three times.

Erythrocyte Ghosts and Membrane Lipids. Membranes from rabbit erythrocytes were isolated by the method of Dodge et al. (1963). Total lipids of erythrocyte membranes were extracted by the procedure of Bligh & Dyer (1959).

Spin-Labeled Lysophosphatidylcholine. Spin-labeled lysophosphatidylcholine was synthesized as described previously (Utsumi et al., 1977) and was stocked as a chloroform solution at −20 °C. The solution of the lysophosphatidylcholine in Veronal-buffered saline was prepared as follows. An appropriate quantity of the stock solution of spin-labeled lysophosphatidylcholine was put into a test tube; the chloroform was removed in a rotary evaporator, and the tube was kept under reduced pressure for more than 1 h; the dried lipid film obtained was then dissolved in Veronal-buffered saline with shaking in a Vortex mixer.

Liposomes. Egg yolk phosphatidylcholine and egg yolk lysophosphatidylcholine were prepared in our laboratory as described previously (Inoue, 1974; Inoue & Kitagawa, 1974). D,L-α-Dipalmitoylphosphatidylcholine was purchased from Sigma Chemical Co. All phospholipids used in the present study showed a single spot on thin-layer chromatograms. Liposomes were prepared as described previously (Utsumi et al., 1976).

Determination of Hemolytic Activity. In a small centrifuge tube, 100 μL of chromate-labeled erythrocyte suspension (5×10^7 to ~1×10^9 cells/mL) was mixed with 50 μL of a solution of spin-labeled lysophosphatidylcholine in Veronal-buffered saline. The mixture was incubated at 37 °C for 2 h and then centrifuged at 350*g* for 5 min. Fifty microliters of supernatant was removed for counting the radioactivity of chromate in an Auto-Gamma Counter (Aloka, JDC-751). The percentage of chromate released was calculated as an index of the hemolytic activity (Weinrach et al., 1958; Sanderson, 1965; Inoue et al., 1972).

ESR Measurement. Erythrocytes were incubated with various amounts of spin-labeled lysophosphatidylcholine and samples were transferred to sealed capillary tubes for ESR measurements. The ESR spectra were measured at room temperature (20 °C) with a JEOL, JES-PE-1X (X-band, 100 kHz field modulation) instrument. For interaction of monomeric spin-labeled lysophosphatidylcholine with erythrocytes, a heavy suspension of erythrocytes (5×10^9 cells/mL) was incubated with the lysophosphatidylcholine at 37 °C for various times. The precipitates obtained by centrifugation (350*g*, 5 min) were washed once with Veronal-buffered saline and transferred to capillary tubes for ESR measurements.

Electron Micrography. Liposomes (10 mM phospholipid) were incubated with excess amounts of egg yolk lysophosphatidylcholine at 25 °C for 10 min. A drop of the reaction mixture was placed on the 180 mesh copper grid previously coated with carbon film. After excess samples had been removed with a filter paper, 2% sodium phosphotungstate (pH 6.5) was applied to the grid. An excess of staining solution was removed with a filter paper after 10–15 s and the grid was examined under an electron microscope (Hitachi HU 11-B).

Results

Hemolytic Activity of Spin-Labeled Lysophosphatidylcholine. The hemolytic activity of spin-labeled lysophosphatidylcholine was compared with that of egg yolk lysophosphatidylcholine by incubating various amounts of lysophosphatidylcholine with rabbit erythrocytes (5×10^7 cells/mL) at 37 °C for 2 h (Figure 1). Spin-labeled lysophosphatidylcholine was also incubated with a higher concentration of erythrocytes (1×10^9 cells/mL). Spin-labeled lysophosphatidylcholine showed some lytic activity, but less than that of egg yolk lysophosphatidylcholine. The minimum concentration of spin-labeled lysophosphatidylcholine required for hemolysis was about 1×10^{-4} M at both concentrations of erythrocytes, but this value is one order of magnitude higher than the value for egg yolk lysophosphatidylcholine (about 1×10^{-5} M). The cmc of spin-labeled lysophosphatidylcholine and egg yolk lysophosphatidylcholine were shown to be around 0.1 and 0.01 mM, respectively (Utsumi et al., 1977), and these values are similar to the minimum concentrations for hemolysis. Thus the hemolytic activity of lysophosphatidylcholine may depend on micellar formation.

The time course of hemolysis by spin-labeled lysophosphatidylcholine is shown in Figure 2A. Spin-labeled lysophosphatidylcholine reacted very slowly and the time needed for 50% hemolysis was 30 min, while hemolysis by egg yolk lysophosphatidylcholine was very rapid and reached a maximum within 5 min. This difference may be due to its spin-label moiety.

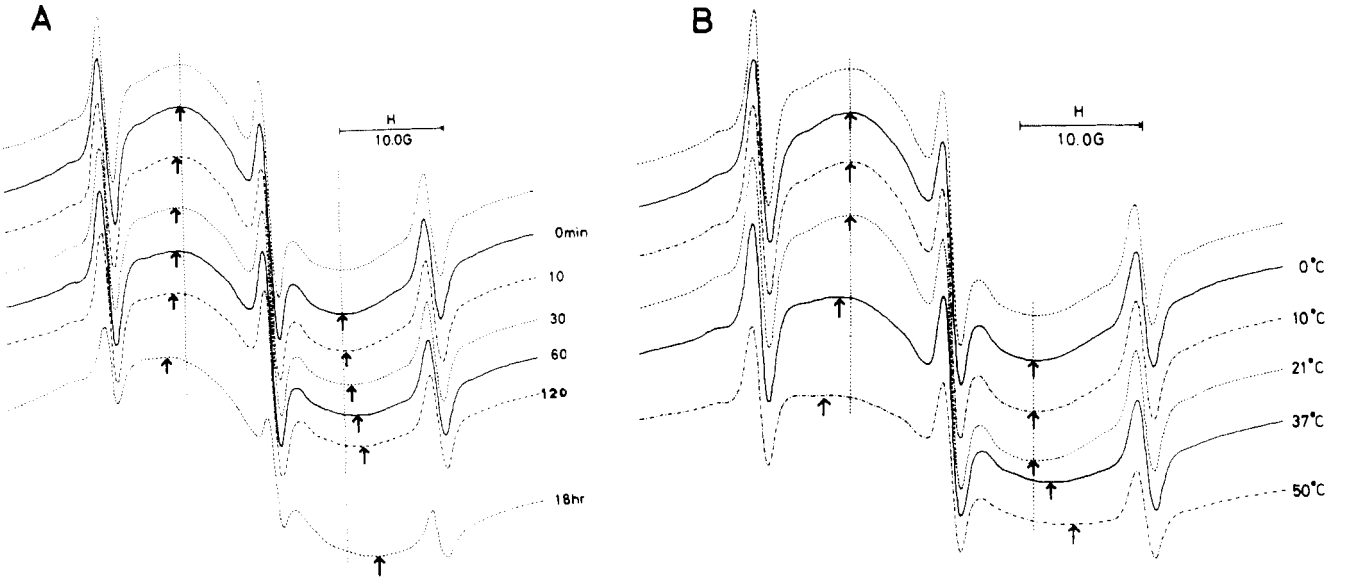

FIGURE 3: Typical ESR spectra of spin-labeled lysophosphatidylcholine incubated with rabbit erythrocytes. (A) Rabbit erythrocytes (10^9 cells/mL) were incubated with 6.7 μmol/mL of spin-labeled lysophosphatidylcholine at 37 °C for various times. Then the mixtures were transferred to capillary tubes and their ESR spectra were measured at room temperature. As a control, the spectrum of spin-labeled lysophosphatidylcholine solution without erythrocytes was measured. (B) Rabbit erythrocytes (10^9 cells/mL) were incubated with 6.7 μmol/mL of spin-labeled lysophosphatidylcholine for 90 min at different temperatures. ESR spectra were measured as described above. The arrows indicate the line widths of the broad resonance line, and the dotted vertical lines indicate the line width of the control spectrum.

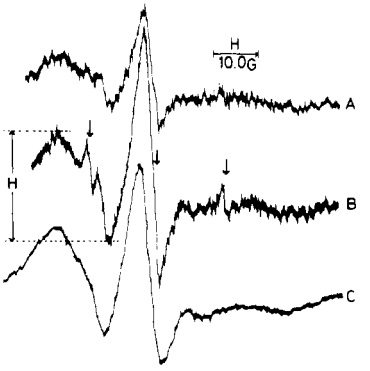

FIGURE 4: Typical ESR spectra of spin-labeled lysophosphatidylcholine incorporated into erythrocyte membranes (A and B), and that of spin-labeled phosphatidylcholine (C). Rabbit erythrocytes (5×10^9 cells/mL) were incubated with 26 nmol/mL of spin-labeled lysophosphatidylcholine (A) or 51 nmol/mL of spin-labeled lysophosphatidylcholine (B) at 37 °C for 30 min. Then the mixtures were centrifuged (350*g*, 5 min) and the ESR spectra of the precipitates were measured at room temperature. (C) Rabbit erythrocytes were labeled with spin-labeled phosphatidylcholine as described by Maeda et al. (1975), and their ESR spectra were measured at room temperature. The arrows indicate the "three sharp lines", which is due to monomeric spin-labeled lysophosphatidylcholine remaining in the sample.

The effect of temperature on the hemolysis by spin-labeled lysophosphatidylcholine is shown in Figure 2B. The rate of hemolysis was very high at 37 and 50 °C, but negligible at 0–20 °C.

ESR Spectra of Spin-Labeled Lysophosphatidylcholine Incubated with Rabbit Erythrocytes. (1) Interaction of Micellar Lysophosphatidylcholine with Erythrocytes. Figure 3A shows the ESR spectra of the lysophosphatidylcholine after incubation with an erythrocyte suspension at 37 °C for various times. The same ESR spectra as shown in Figure 3A were observed in the supernatant obtained by centrifugation, and under the same conditions of ESR measurement any signal could be hardly observed in the pellet which was washed with Veronal-buffered saline. In these spectra, the broad single resonance line seems to be superimposed on three sharp resonance lines. As reported previously (Utsumi et al., 1977), we considered that the former resonance line is due to micelles of spin-labeled lysophosphatidylcholine, while the latter resonance lines are due to the lysophosphatidylcholine dissolved in water as monomers. The line width (peak to peak) of the broad resonance line (shown by arrows in Figure 3A) gradually broadened, while the three sharp lines did not change appreciably.

The change in the line width of the broad resonance line due to micellar spin-labeled lysophosphatidylcholine is plotted against the incubation time in Figure 2A. It closely coincides with the time course of the hemolysis.

The line broadening was also observed when spin-labeled phosphatidylcholine was incubated with erythrocytes which did not contain chromate ion, and the same relation as shown in Figure 2A was obtained between the line broadening and hemolysis estimated from the absorbance of hemoglobin.

Figure 3B shows the ESR spectra of spin-labeled lysophosphatidylcholine incubated with erythrocyte suspensions for 90 min at various temperatures. The ESR spectra of the lysophosphatidylcholine incubated with erythrocytes at 0, 10, and 20 °C were the same as the spectrum of the lysophosphatidylcholine without erythrocytes. However, the ESR spectra obtained after incubation at 37 or 50 °C were different. The line widths of the broad resonance lines were 17.0 and 20.0 G after incubation at 37 and 50 °C, respectively. Figure 2B shows the relationship between the temperature dependence of line broadening and of hemolysis. The temperature dependence of the hemolysis again corresponded well with that of line broadening of the ESR signal.

(2) Incorporation of Monomeric Lysophosphatidylcholine into Erythrocyte Membranes. We have not yet obtained any evidence for the incorporation of spin-labeled lysophosphatidylcholine into membranes or its adsorption to the membranes; the signal intensity of the lysophosphatidylcholine incorporated into erythrocyte membranes may be so weak that it is concealed by the broad resonance line of micellar lysophosphatidylcholine. When a low concentration of spin-labeled lyso-

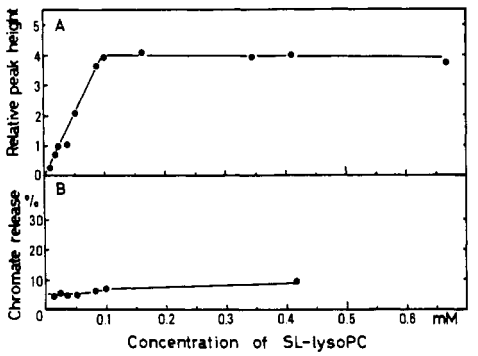

FIGURE 5: Effect of the concentration of spin-labeled lysophosphatidylcholine on the relative signal intensity of spin-labeled lysophosphatidylcholine which was incorporated into rabbit erythrocyte membranes (A) and on hemolysis after incubation at 37 °C for 2 h (B). The final erythrocyte concentration was 5×10^9 cells/mL. The relative signal intensity was estimated from the peak height as described in text (shown as H in Figure 4B).

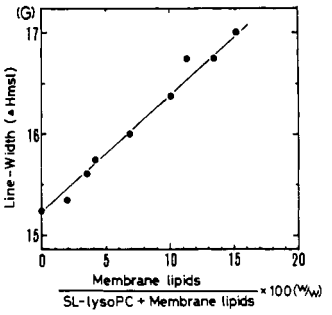

FIGURE 6: Change in line width of mixed micelles consisted of spin-labeled lysophosphatidylcholine and total lipids of erythrocyte membrane. For the line width of ESR signal the widths from peak to peak were measured as described in the legend of Figure 3.

phosphatidylcholine was incubated with a heavy suspension of erythrocytes (5×10^9 cells/mL) at 37 °C and the ESR signal of packed cells was measured at various times, weak ESR spectra could be observed immediately after incubation, though the spectra did not change on incubation for up to 2 h (Figures 4A and 4B). These spectra were characterized by the presence of the anisotropic signal superimposed on the three sharp lines. This anisotropic signal was in fair agreement with the spectrum of spin-labeled phosphatidylcholine incorporated into rabbit erythrocyte membranes (Figure 4C), which is established to be due to a rapid anisotropic rotational motion in the lipid bilayer (Hubbell & McConnell, 1971; Maeda et al., 1975). The overall splitting, estimated from the spectra shown in Figures 4A, 4B, and 4C, decreased from 52 to 49 G, equally on hemolysis under hypotonic conditions (data not shown). Therefore, spin-labeled lysophosphatidylcholine may be present in a similar region of rabbit erythrocyte membranes to spin-labeled phosphatidylcholine.

It is rational that the relative intensities of these signals may be roughly estimated from their peak heights, since the pattern and widths of each resonance lines are quite the same among these spectra (Berliner, 1976). We measured the relative peak height of the anisotropic resonance line in the lower magnetic field (see Figure 4B) as an index of the amount of the lysophosphatidylcholine incorporated into erythrocyte membranes, and plotted it against the concentration of lysophosphatidylcholine added (Figure 5A). The signal intensity due to the incorporated lysophosphatidylcholine increased linearly with increase in the concentration of lysophosphatidylcholine up to 0.1 mM, but further addition of lysophosphatidylcholine

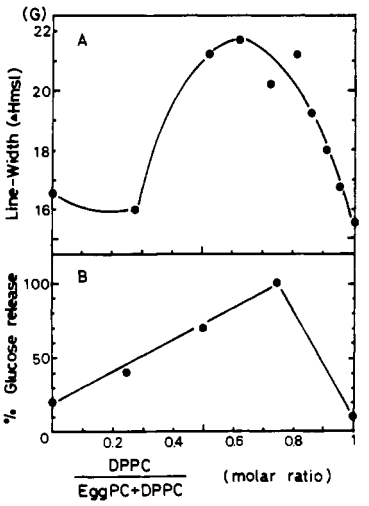

FIGURE 7: Broadening of the ESR signal due to spin-labeled lysophosphatidylcholine micelles (A), and glucose permeability of liposomes induced by egg yolk lysophosphatidylcholine (B) (Inoue & Kitagawa, 1974). (A) Liposomes (20 μmol/mL phospholipids) were incubated with spin-labeled lysophosphatidylcholine (4 μmol/mL) at 20 ° for 30 min, and the ESR spectra of the mixtures were observed. Line widths were estimated as described in the legend of Figure 3.

could no longer increase the intensity of anisotropic signal.

During incubation for 30 min, no appreciable hemolysis was observed, although some hemolysis occurred after 2-h incubation (Figure 5B).

Interaction of Spin-Labeled Lysophosphatidylcholine with Erythrocyte Ghosts and with Membrane Lipids. ESR signal of micellar lysophosphatidylcholine showed line broadening on incubating with erythrocyte ghosts. The line width of the ESR signal increased from 15.3 to 16.2 G when the lysophosphatidylcholine solution (8 μmol/mL) was incubated at 37 °C for 2 h with erythrocyte ghosts, the concentration of which corresponded to 1×10^9 cells/mL.

To estimate the contribution of membrane lipids to line broadening, mixed micelles of spin-labeled lysophosphatidylcholine with total membrane lipids were prepared and observed with ESR spectroscopy as described previously (Utsumi et al., 1977). The line width of the broad resonance line was plotted against membrane lipids content in Figure 6.

Interaction of Lysophosphatidylcholine with Liposomal Membranes. Broadening of the ESR signal due to micellar spin-labeled lysophosphatidylcholine and the incorporation of monomeric spin-labeled lysophosphatidylcholine into membranes were also observed when the lysophosphatidylcholine was incubated with liposomes prepared from mixtures of dipalmitoylphosphatidylcholine and egg yolk phosphatidylcholine. Liposomes thus prepared were incubated with spin-labeled lysophosphatidylcholine at 20 °C, and the mixtures were measured with ESR spectroscopy. The broadening of the resonance line was maximal when the lysophosphatidylcholine was incubated with liposomes containing both dipalmitoylphosphatidylcholine and egg yolk phosphatidylcholine in the molar ratio of 1:1 to 3:1 (Figure 7A). The results obtained here were compatible with the observation that liposomal damage was induced by exogenous egg yolk lysophosphatidylcholine (Figure 7B) (Inoue & Kitagawa, 1974). On the other hand, the signal intensity of monomeric spin-labeled lysophosphatidylcholine incorporated into membranes was strongest in liposomes consisting of egg yolk phosphatidylcholine (data not shown).

The interaction of egg yolk lysophosphatidylcholine with

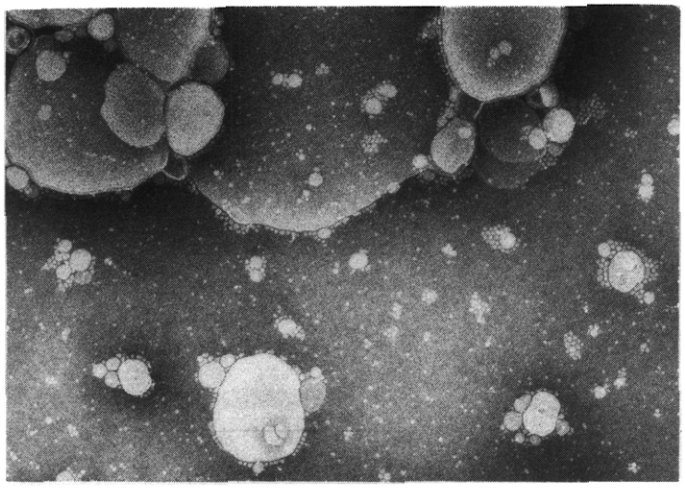

FIGURE 8: Electron microgram of egg phosphatidylcholine liposomes treated with exogenous egg yolk lysophosphatidylcholine in a Veronal-buffered saline, negatively stained with sodium phosphotungstate. Scale: 100 nm.

liposomes was also studied by electron microscopy using negative staining. Liposomes composed of egg yolk phosphatidylcholine and cholesterol in the molar ratio of 1:1 were mixed with lysophosphatidylcholine whose final concentration was above cmc. The mixtures were incubated for 10 min at 25 °C before being placed on the grid. Under the electron micrograms, globular micelles of lysophosphatidylcholine having about 80 Å radius, which were already observed previously (Inoue et al., 1977), were found to attach onto the surface of multi-lamellar liposomes (Figure 8). The globular micelles were observed only on the surface, but not in the bilayers. It was also observed that most of the liposomes remained rather intact. Liposomes composed of egg yolk phosphatidylcholine and dipalmitoylphosphatidylcholine were completely broken and gave quite heterogeneous structures such as various sized globular or cylindrical structures when treated with lysophosphatidylcholine. It is probable that we could observe the "intermediate" state of the interaction of lysophosphatidylcholine with liposomes, because the liposomes were rather insensitive to lysophosphatidylcholine as described above.

Discussion

Spin-labeled lysophosphatidylcholine at concentrations above cmc (0.1 mM) showed hemolytic activity against rabbit erythrocytes at 37 °C. However, hemolysis by spin-labeled lysophosphatidylcholine was much slower than that by egg yolk lysophosphatidylcholine. The slow reaction of spin-labeled lysophosphatidylcholine is very convenient for analysis of the mechanism of hemolysis, as shown in the cases of benzyllysophosphatidylcholine (Weltzien, 1973) and L-α-dilauroylphosphatidylcholine (Kitagawa et al., 1977). As reported previously (Utsumi et al., 1977), spin-labeled lysophosphatidylcholine can form micelles and exists both as micelles and as monomers above cmc. Monomers and micelles of the lysophosphatidylcholine were shown to interact with erythrocyte membranes in different ways.

When a heavy suspension of erythrocytes was treated with a low concentration of the lysophosphatidylcholine, a new ESR signal appeared immediately, the pattern of which suggests that the lysophosphatidylcholine exists in the lipid bilayer of erythrocyte membranes. The intensity of this signal increased as increase of the lysophosphatidylcholine added up to cmc. Further addition, however, did not change the signal intensity significantly, indicating that the amount of lysophosphatidylcholine incorporated into membranes remains constant above cmc. This result might be explained as follows. The amount of lysophosphatidylcholine in the membranes may be a function of the partitioning of monomers between the aqueous and membrane phases. As the concentration of monomers in the aqueous phase increases so will the concentration of lysophosphatidylcholine in the membrane increase. When the cmc is reached, however, an increase in total lysophosphatidylcholine added to the system does not significantly increase the concentration of monomers in the aqueous phase and therefore does not increase the concentration of lysophosphatidylcholine in the membranes.

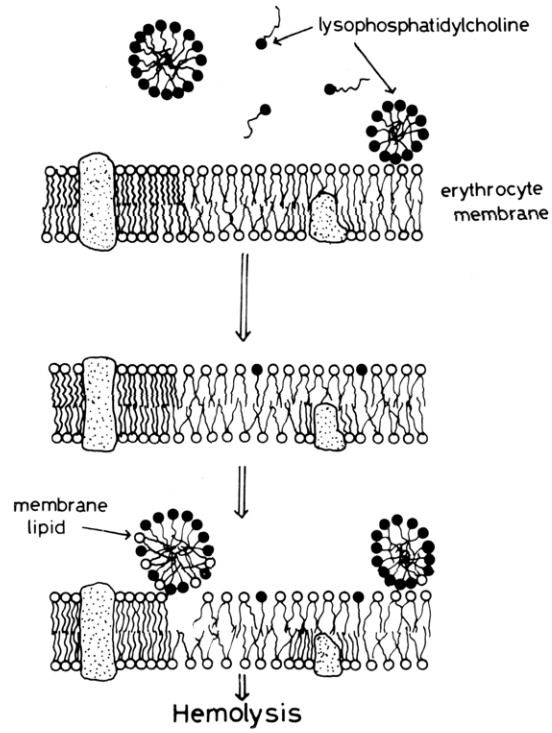

FIGURE 9: Hypothetical mechanism of hemolysis induced by spin-labeled lysophosphatidylcholine.

The incorporation of lysophosphatidylcholine into erythrocyte membranes has already been measured from the experiments using thin-layer chromatography (Sato, 1973), radioactive lysophosphatidylcholine (Weltzien, 1973; Weltzien et al., 1976), or NMR spectroscopy (Chapman et al., 1968). However, the net amount of lysophosphatidylcholine incorporated into membranes could not be estimated from these experiments since, from the results obtained by the former two methods, it is uncertain whether or not lysophosphatidylcholine is incorporated into membranes, and it is also impossible from NMR spectrum to estimate the amount of lysophosphatidylcholine incorporated. To our knowledge, this is the first report to show the "net amount" of lysophosphatidylcholine incorporated into erythrocyte membranes. No direct relation was found between hemolysis and the incorporation of lysophosphatidylcholine. Thus incorporation of monomeric lysophosphatidylcholine is probably not responsible for hemolysis.

When a high concentration of the lysophosphatidylcholine was incubated with erythrocytes at above 37 °C, the resonance line due to micellar lysophosphatidylcholine gradually broadened. Based on the appearance of the new ESR signal, Scandella et al. postulated that spin-labeled phosphatidylcholine liposomes might diffuse into sarcoplasmic reticulum membranes (Scandella et al., 1972). The spectral change observed here was quite different from their observation. Thus

when spin-labeled lysophosphatidylcholine micelles interact with erythrocytes, they may exist as micelles without diffusing into the membranes.

As reported previously (Utsumi et al., 1977), the resonance line due to micellar lysophosphatidylcholine broadened linearly with increase in the contents of D,L-α-dimyristoylphosphatidylcholine in lysophosphatidylcholine micelles. Similar line broadening was also observed by addition of total membrane lipids to the micelles. This line broadening can be explained as a result of a decrease in the spin-exchange interaction between the lysophosphatidylcholines (Bolton, 1972; Carrington & McLachlan, 1967).

The line broadening was also observed when spin-labeled lysophosphatidylcholine micelles were incubated with liposomes. Therefore, the line broadening observed during hemolysis may be interpreted as due to the interaction of the lysophosphatidylcholine with membrane lipids, which causes a decrease in the collision rate of the lysophosphatidylcholine in micelles.

One might, however, consider that the line broadening might arise from the interaction of spin-labeled lysophosphatidylcholine with proteins or paramagnetic ions such as chromate ion. These possibilities, however, can be easily eliminated, since line broadening was also observed when the lysophosphatidylcholine was incubated with liposomes or erythrocytes without chromate. We could not yet observe line broadening when the lysophosphatidylcholine micelles were incubated with such proteins as bovine serum albumin, cytochrome *c*, polylysine, lysozyme, or hemoglobin.[3]

From Figures 2A and 6, it can be roughly estimated that the lysophosphatidylcholine micelles might contain about 10% (w/w) of erythrocytes lipids when almost all erythrocytes hemolyzed. In the present experiments, 400 μg of lipids was approximately calculated to be incorporated into the micelles, since 4 mg of the lysophosphatidylcholine was incubated with 10^9 cells. According to Nelson (1967), 10^9 cells may contain 400 μg of membrane lipids, suggesting that almost 100% of lipids were taken away from erthrocyte membranes by the micelles. It is worthwhile to note that this calculation was based on the supposition that the micelles take away all kinds of membrane lipids homogeneously. There is a possibility that the micelles may interact with some particular lipid components. If the interaction proceeds in the latter way, this calculation seems not to be reliable, since the degree of line broadening depends on lipid species incorporated. In our preliminary experiments, the line broadening due to the interaction with cholesterol was much less than that observed in the interaction with dimyristoylphosphatidylcholine. In addition to the interaction mentioned above, the alternative interaction of the lysophosphatidylcholine with "unknown materials" might partially contribute to the line broadening, since further increase of the line width was observed when the reaction mixture was incubated at 37 or 50 °C after the complete hemolysis.

The rates of hemolysis and line broadening showed similar temperature dependences. Thus the interaction of the lysophosphatidylcholine with membranes which causes line broadening may also induce hemolysis.

The signal of micellar lysophosphatidylcholine was broadest when lysophosphatidylcholine was incubated with liposomes containing both dipalmitoylphosphatidylcholine and egg yolk phosphatidylcholine. It is well known that liposomes consisting of both dipalmitoylphosphatidylcholine and egg yolk phosphatidylcholine should be in a state of phase separation at 20 °C (Chapman & Urbina, 1974; Shimshick & McConnell, 1973; Phillips et al., 1972). It was reported that there are some kinds of phase separation in biological membranes including erthrocyte membrane at physiological temperature (Tanaka & Ohnishi, 1976). It is, therefore, possible that lysophosphatidylcholine micelles might interact preferentially with regions of membranes that show a kind of phase separation.

The presumed interactions of lysophosphatidylcholine with erythrocytes can be summarized as shown in Figure 9.

(1) Lysophosphatidylcholine can form micelles and exist both as monomers and as micelles above cmc.

(2) The monomers and micelles interact with erythrocyte membranes in different ways.

(3) The monomers can be incorporated into more fluid regions of lipid bilayers without causing hemolysis.

(4) The micelles may interact with the lipid bilayer of membranes and be diluted with some lipid components of erythrocytes. In other words, the micelles may take away some lipid components from erythrocyte membranes. Regions showing phase separation may be the most sensitive to "attack" by the micelles. This interaction may induce some changes in the molecular organization of the cell membranes, resulting in hemolysis.

This mode of action of spin-labeled lysophosphatidylcholine toward erthrocytes was quite different from that of such compounds as sodium dodecyl sulfate (Kondo, 1976), fatty acid (Utsumi, 1976), or ether-deoxylysophospholipid (Weltzien et al., 1977). In the case of hemolysis by these compounds, their minimum concentrations required for hemolysis depend on erythrocyte concentration. There are some relationships between the amount of compounds incorporated and the hemolytic activity. The discrepancy between the results obtained by using the compounds and present results might be due to the difference of the dissociation constants of the compounds, that is, the amount of monomers which have the ability of incorporating into membranes.

In the present experiments, it remains to be clarified whether or not an interaction of lysophosphatidylcholine with membranes causing line broadening triggers the hemolysis directly.

Acknowledgments

We wish to thank Mr. K. Suzuki, National Institute of Health (Japan), for taking electron micrograms, and Professor M. Sato, Teikyo University, for valuable comments.

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[3] Bovine serum albumin is well known to combine with egg yolk lysophosphatidylcholine (Switzer & Eder, 1965; Klopfestein, 1969). In fact, spin-labeled lysophosphatidylcholine interacted with serum albumin; however, this interaction immobilized the spin-labeled lysophosphatidylcholine and resulted in a completely different ESR spectrum from that shown in Figure 3. Some line broadening was observed when spin-labeled lysophosphatidylcholine micelles were incubated with methemoglobin, which is well known as a strong paramagnetic protein. In the present experiment, the effect of methemoglobin on line broadening may be neglected, since methemoglobin is hardly present in fresh erythroyctes.

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