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HETEROGENEITY IN THE FLUIDITY OF INTACT ERYTHROCYTE MEMBRANE AND ITS HOMOGENIZATION UPON HEMOLYSIS

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SUMMARY

Intact erythrocytes were spin-labeled with various classes of phospholipid label. The ESR spectrum for phosphatidylcholine spin label was distinctly different from those for phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid spin labels. The overall splitting for the former (52.5 G) was markedly larger than those for the others (approx. 47 G), suggesting a more rigid phosphatidylcholine bilayer phase and more fluid phosphatidylethanolamine and phosphatidylserine phases in the erythrocyte membrane. Evidence for asymmetric distribution of phospholipids in the membrane was obtained. Spin-labeled phosphatidylcholine incorporated into erythrocytes was reduced immediately by cystein and Fe^{3+}, while the reduction of spin-labeled phosphatidylserine was very slow. The present results therefore suggest asymmetric fluidity in erythrocyte membrane; a more rigid outer layer and a more fluid inner layer. The heterogeneity in the lipid structure was also manifested in the temperature dependence of the fluidity. The overall splitting for phosphatidylcholine spin label showed two inflection points at 18 and 33 °C, while that for phosphatidylserine spin label had only one transition at 30 °C.

When the spin-labeled erythrocytes were hemolyzed, the marked difference in the ESR spectra disappeared, indicating homogenization of the heterogeneous fluidity. Mg^{2+} or Mg^{2+}+ATP prevented the hemolysis-induced spectral changes. Ca^{2+} did not prevent the homogenization and acted antagonistically to Mg^{2+}. The heterogeneity preservation by Mg^{2+} was nullified by trypsin, pronase or *N*-ethylmaleimide added inside the cell. Some inner proteins may therefore be involved in maintaining the heterogeneous structure. The protecting action of Mg^{2+} was dependent on hemolysis temperature, starting to decrease at 18 °C and vanishing at 40 °C. The present study suggests that the heterogeneity in the fluidity of intact erythrocyte membranes arises from interactions between lipids and proteins in the membrane and also from interactions between the membrane constituents and the inner proteins. Concentration of cholesterol in the outer layer may also partly contribute to the heterogeneity.

INTRODUCTION

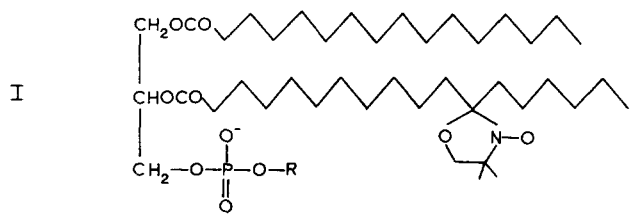
The organization and physical characteristics of lipids are intimately related to a variety of physiological functions of biological membranes [1–7]. These membranes contain various classes of lipids, each of which appears to play its own characteristic role. For example, it has been demonstrated that phosphatidylserine is a Ca^{2+}-receptive lipid and causes ionotropic lateral phase separations [8–11]. The distribution of lipids can be heterogeneous as a result of interactions with proteins and with lipids. The thermotropic [1, 12, 13] and ionotropic [8–11, 14] lateral phase separations into crystalline and liquid-crystalline phases are such examples. Distribution in fluid liquid-crystalline phase can also be heterogeneous due to preferred interactions. In the case of intact erythrocyte membrane, an asymmetric phospholipid distribution has been suggested by the studies using 'non-permeable' reagents and lipolytic enzymes [15, 16].

In order to study the dynamic behavior of individual lipids, we synthesized spin-label analogues for various classes of phospholipids and studied some phospholipid mixture membranes [8–11, 14]. In the present investigation, we have spin-labeled intact erythrocyte and studied ESR spectra under various conditions. The results clearly indicate heterogeneity in the fluidity of phospholipids in intact erythrocyte membrane and its homogenization upon hemolysis.

MATERIALS AND METHODS

Phosphatidylcholine spin label was prepared by the reaction of egg lysophosphatidylcholine with anhydride of 12-nitroxide stearic acid according to Hubbell and McConnell [17]. 12-Nitroxide stearic acid was synthesized by the method of Waggoner et al. [18] with slight modification [10]. Phosphatidylserine spin label, a gift of Dr. Tadanao Ito, was synthesized by the reaction of spin-labeled CDP-diglyceride and L-serine in the presence of phosphatidylserine synthetase [10]. Phosphatidylethanolamine and phosphatidylglycerol spin labels were prepared by substitution of choline group in the phosphatidylcholine spin label with ethanolamine and glycerol, respectively, under catalytic action of phospholipase D. These labels were a gift of Dr. Toyozo Maeda. Phosphatidic acid spin label was prepared by the action of phospholipase D on the phosphatidylcholine spin label [10, 14]. Spin-labeled phosphatidylcholine was synthesized by condensation of phosphatidic acid, derived from egg phosphatidylcholine, with tempocholine according to Kornberg and McConnell [19]. Spin-labeled phosphatidylserine was prepared as amide of ox brain phosphatidylserine and *N*-oxyl-2,2,5,5-tetramethyl-3-carboxypyrrolidine and donated by Dr. Toyozo Maeda. The chemical formula of these phospholipid spin labels are given in Fig. 1.

Human red blood cells were supplied from Kyoto Red Cross Blood Center through the courtesy of H. Saji and used within several days after drawing. The cells were washed three times with 0.9 % NaCl and the buffy coat was removed by aspiration. Hemoglobin-free erythrocyte ghosts were prepared according to Dodge et al. [20]. Total lipid was extracted from the ghost with chloroform/methanol (1 : 2) by the method of Bligh and Dyer [21]. Amphotericin B was a gift of Dr. Yoshinori Nozawa.



a) R= $-CH_2CH_2\overset{+}{N}(CH_3)_3$ b) R= $-CH_2CH(\overset{+}{N}H_3)COO^-$

c) R= $-CH_2CH_2\overset{+}{N}H_3$ d) R= $-CH_2CH(OH)CH_2OH$

e) R= $-H$

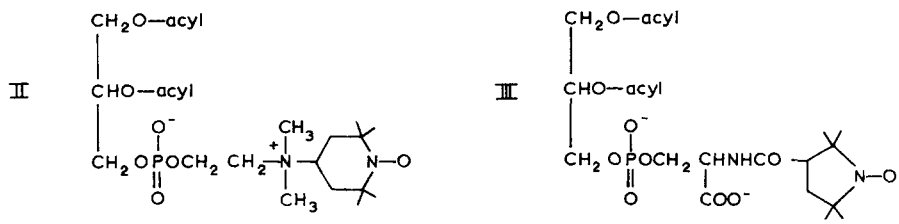


Fig. 1. Chemical formulae for phospholipid spin labels. I (a) Phosphatidylcholine; (b) phosphatidylserine; (c) phosphatidylethanolamine; (d) phosphatidylglycerol; (e) phosphatidic acid. II. Spin-labeled phosphatidylcholine. III. Spin-labeled phosphatidylserine. I and II were derived from egg phosphatidylcholine and III from ox brain phosphatidylserine.

Spin-labeling of red blood cells was carried out by incubation with sonicated dispersions of phospholipid spin labels. A suspension of the label, 0.5 mg/ml of 150 mM NaCl and 40 mM Tris or *N*-tris(hydroxymethyl)methylglycine (pH 7.4), was sonicated at 4 °C for 2 min under N_2, with a tip-type sonicator (Kaijo Denki T-A-4201), and centrifuged at $1500 \times g$ at 4 °C for 5 min. The supernatant (0.1 ml) was mixed with 0.1 ml of packed erythrocyte and incubated at 37 °C for 2.5 h. The spin-labeled cells were washed three times with 150 mM NaCl and 40 mM Tris by centrifugation at $1500 \times g$ at 4 °C for 5 min. In the spin-labeling with phosphatidylethanolamine label, a mixture of the label and egg phosphatidylcholine (1 : 4) or the label and ox brain phosphatidylserine (1 : 4) was used, since the label alone was not well dispersed. Phosphatidylethanolamine spin label was not easily incorporated by the incubation, in contrast to the other phospholipid labels. The ESR signal for the phosphatidylethanolamine label was therefore rather weak so that a detailed examination of the spectral shape was difficult.

For a detailed study of the effect of hemolysis conditions, 0.1 ml of spin-labeled packed erythrocyte was mixed at various temperatures with 2.0 ml of hypotonic salt solution containing 5 mM Tris (pH 6.9) and divalent cations, ATP,

brought back to isotonic condition by dilution, hemolysis occurred and the spin-label signal changed.

When the tonicity of the suspending medium was gradually reduced, the spectral change and hemolysis started to occur concurrently at about 150 ideal mosM. The overall splitting for phosphatidylcholine label decreased gradually from 52.5 G and reached 49.5 G at about 50 ideal mosM. In the intermediate osmolarities, the spectra can be interpreted as sums of the spectra for intact erythrocytes and erythrocyte ghosts at various ratios.

Amphotericin B (25 μg/ml) and saponin (250 μg/ml) gave no appreciable effects on the spectrum of phosphatidylcholine spin label in erythrocyte ghost.

The hemolysis-induced homogenization was also observed for the other mammalian erythrocytes. The overall splitting for phosphatidylcholine and phosphatidylglycerol spin labels became 48.5 and 49.0 G for pig and 51.5 and 52.0 G for ox, respectively.

ESR spectra of various phospholipid spin labels incorporated in total lipid bilayer membrane were similar to each other. The overall splitting values were 48.5 G with an exception for phosphatidylethanolamine label (47 G) (see Table I). The spectra were somewhat sharper than those for the labels in erythrocyte membranes. The choline-containing phospholipids, phosphatidylcholine and sphingomyelin, were isolated from the total lipid extract. Phosphatidylcholine spin label incorporated in a mixture membrane of cholesterol and the phospholipids (1.5 : 1) gave considerably large overall splitting (49.5 G).

Preservation by Mg^{2+} or Mg^{2+}+ATP of the heterogeneity in membrane fluidity

The spin-labeled erythrocyte was hemolyzed at various temperatures in the presence of various additives and its ESR spectrum was measured (see Table III). It was found that Mg^{2+} prevented the hemolysis-induced spectral change. When the

TABLE III

EFFECT OF ADDITIVES IN HEMOLYSIS BUFFER ON THE SPECTRAL CHANGE IN SPIN-LABELED INTACT ERYTHROCYTES

The spin-labeled cells were hemolyzed at 0 °C in 5 mM Tris (pH 6.9) containing the indicated additives and, after restoration of isotonicity by NaCl, incubated at 37 °C for 30 min for resealing. The hemolysis was complete. ESR spectra were measured at 23 °C and the overall splitting value for phosphatidylcholine spin label was given in G.

	Overall splitting
None	50.0
Mg^{2+} (2 mM)*	52.5
Mg^{2+} (2 mM)+ATP (2 mM)	52.5
Ca^{2+} (2 mM)**	49.0
Mg^{2+} (2 mM)+Ca^{2+} (2 mM)	49.0
Mg · ATP (2 mM)+*N*-ethylmaleimide (5 mM)	49.5
Mg · ATP (2 mM)+trypsin (10 μg/ml)	49.0
Mg · ATP (2 mM)+pronase (10 μg/ml)	49.0

* The overall splitting for phosphatidylserine spin label was 47.5 G.
** The overall splitting for phosphatidylserine spin label was 49.0 G.

erythrocyte was subjected to a hypotonic condition at 0 °C in the presence of Mg^{2+}, the overall splitting values for phosphatidylcholine and phosphatidylserine spin labels remained unchanged, 52.5 and 47.5 G, respectively, in spite of complete hemolysis. Mg^{2+}+ATP also protected the spectral change. Although the splitting values were the same as those in Mg^{2+} alone, the spectral shape was slightly but definitely different. A control experiment, hemolysis at 0 °C in the absence of Mg^{2+}, showed a decrease in the overall splitting for phosphatidylcholine spin label. Ca^{2+} showed no protecting action. Hemolysis at 0 °C in the presence of Ca^{2+} caused homogenization of the membrane fluidity; a decrease in the overall splitting for phosphatidylcholine label to 49.0 G and an increase in the splitting for phosphatidylserine spin label to 49.0 G. Ca^{2+} acted antagonistically to Mg^{2+}. When both divalent cations were present in the hemolysis buffer, the homogenization of the membrane fluidity was observed.

Some protein-modifying reagents added in the hemolysis solution nullified the protecting action of Mg^{2+}. The overall splitting for phosphatidylcholine spin label decreased to 49 G when hemolysis was carried out at 0 °C in the presence of *N*-ethylmaleimide, trypsin or pronase in addition to Mg^{2+}. The same reagents failed to give any effects when added in isotonic erythrocyte suspension without causing hemolysis, as mentioned in a previous section. Addition of trypsin to the resealed ghosts that had been hemolyzed at 0 °C in the presence of Mg^{2+} and incubated at 37 °C in isotonic buffer did not affect the spectrum, the splitting value for phosphatidylcholine label remaining 52.5 G. The reagents therefore prevent the heterogeneity-preserving action of Mg^{2+} only when they are present inside the cells.

The preservation of heterogeneity by Mg^{2+} was dependent on hemolysis temperature. Fig. 5 shows the overall splitting for phosphatidylcholine spin label when the spin-labeled erythrocyte was hemolyzed at various temperatures in the presence of Mg^{2+}. The protecting action began to diminish at about 18 °C and disappeared at about 40 °C. The hemolysis temperature, but not the temperature of the following incubation, governed the effect. Thus, when the labeled erythrocyte was hemolyzed at 0 °C for 3 min in Mg^{2+} and then incubated at 37 °C for 30 min in the same solution, the overall splitting remained 52.5 G. On the other hand, hemolysis at

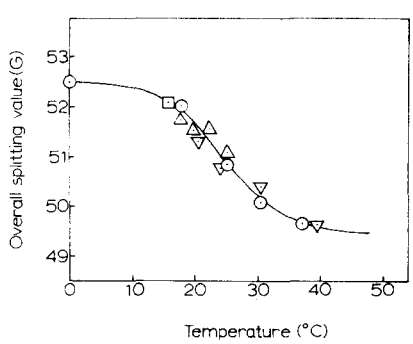


Fig. 5. The overall splitting for phosphatidylcholine spin label in erythrocytes as a function of hemolysis temperature in the presence of Mg^{2+}. The spin-labeled erythrocyte was hemolyzed at the indicated temperature in 5 mM Tris (pH 6.9) containing 2 mM $MgCl_2$ and, after restoration of isotonicity, was incubated at 37 °C for 30 min. ESR spectra were measured at 23 °C. Various marks represent data from different series of experiments.

N-ethylmaleimide or proteolytic enzyme. After standing for 30 min at the indicated temperature, the tonicity was restored by adding 0.2 ml of 9 % NaCl and incubated at 37 °C for 30 min for resealing of the ghost [22–24]. The hemolysate was centrifuged at 4 °C for 5 min at $1500 \times g$ or for 15 min at $12\,000 \times g$. The hemolysis was almost complete at 0 °C and about 80 % at 37 °C.

ESR spectra of the spin-labeled erythrocytes were measured at 23 °C routinely or at various temperatures with a commercial X-band spectrometer (JEOLCO Model ME). The filed scan was calibrated with Mn^{2+} in MgO. The overall splitting given in the text represents the value at 23 °C unless otherwise noted.

RESULTS

Heterogeneity in the fluidity of phospholipids in intact erythrocyte membrane

ESR spectra of intact erythrocyte spin-labeled with various phospholipid spin labels are reproduced in Fig. 2. The major features of the spectra indicate that the labels are undergoing anisotropic motion and suggest that these labels are incorporated in the lipid bilayer portion of the membrane. The overall splitting equals twice the parallel principal value of the ^{14}N hyperfine tensor and is related to the flexibility of the alkyl chains. We used the splitting value as a simple measure of the fluidity of membrane. Larger or smaller splitting corresponds to more rigid or fluid membranes.

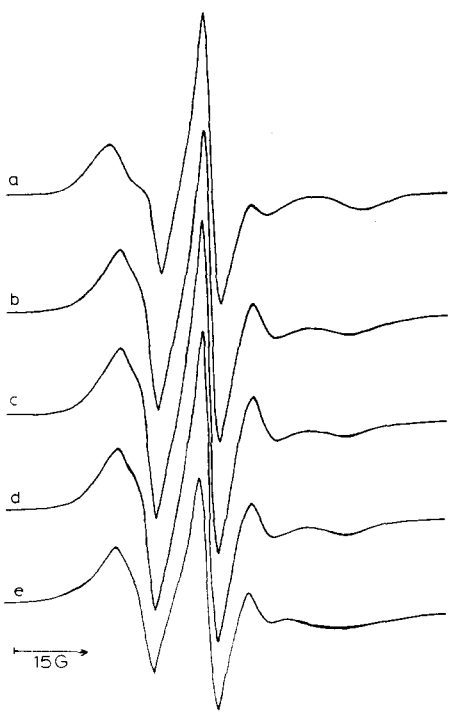


Fig. 2. ESR spectra of intact erythrocytes spin-labeled with (a) phosphatidylcholine, (b) phosphatidylserine, (c) phosphatidylglycerol, (d) phosphatidic acid and (e) phosphatidylethanolamine spin labels. The spectra were measured at 23 °C.

TABLE I

OVERALL SPLITTING OF ESR SPECTRA OF PHOSPHOLIPID SPIN LABELS INCORPORATED IN INTACT ERYTHROCYTES, ERYTHROCYTE GHOSTS AND TOTAL LIPID BILAYER MEMBRANE

Approximate range of error was ±0.5 G. Human red blood cells (0.1 ml packed volume) were incubated at 37 °C for 2.5 h with 0.1 ml of sonicated dispersion of phospholipid spin labels (0.5 mg/ml) in isotonic buffer. In the case of phosphatidylethanolamine label, a mixture of the label and egg phosphatidylcholine (1 : 4) was used. The spin-labeled cells were washed and ESR spectrum was measured at 23 °C. The spin-labeled cells were hemolyzed and washed according to Dodge et al. [20] and the ESR spectrum of the resulting ghosts was measured at 23 °C. Total lipid extract and a small amount of phospholipid label were dissolved in benzene and put on Millipore filter [9]. The lipid-impregnated filter was soaked in isotonic buffer and the ESR spectrum measured at 23 °C.

Spin label	Intact erythrocyte (G)	Erythrocyte ghost (G)	Total lipid (G)
Phosphatidylcholine	52.5	49.0	48.5
Phosphatidylserine	47.5	49.0	48.5
Phosphatidylglycerol	47.0	49.0	48.5
Phosphatidylethanolamine	46–47	–	47

We did not employ the order parameter, since the inner splitting was not accurately determined for some spectra.

Comparing the spectra in Fig. 2, it is noted that the spectrum for phosphatidylcholine spin label is remarkably different from those for the other phospholipid spin labels. The overall splitting for phosphatidylcholine label (52.5 G) was markedly larger than those for the other labels (approx. 47 G) (see Table I). The spectra for phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid labels were not the same, but were similar to each other. The splitting values were not markedly different. The isotropic hyperfine coupling constant was nearly the same for the phospholipid labels (14.7 G for phosphatidylcholine and 14.2 G for phosphatidylserine spin labels), indicating that the nitroxide moiety experiences nearly the same non-polar environment. The results therefore strongly suggest heterogeneity in fluidity of intact erythrocyte membrane; a more rigid phosphatidylcholine phase and more fluid phosphatidylserine and phosphatidylethanolamine phases. Phosphatidylglycerol and phosphatidic acid, which are not contained in appreciable amounts in the erythrocyte membrane, behaved quite similarly to phosphatidylserine and phosphatidylethanolamine. In some of the following experiments, phosphatidylglycerol spin label was used instead of phosphatidylserine spin label, just to save the latter.

The heterogeneity in the membrane fluidity existed in a wider range of temperature. ESR spectra of the spin-labeled erythrocyte was measured at various temperatures from 4 to 50 °C and the overall splitting value was plotted against temperature (see Fig. 3). The splitting value for phosphatidylcholine spin label was always larger than that for phosphatidylserine (or phosphatidylglycerol) spin label. The temperature dependence of the fluidity discloses another feature related to the membrane heterogeneity. There are two inflection points for phosphatidylcholine label (18 and 33 °C), while only one point is observed for phosphatidylserine label (30 °C). The inflection point can be an indication of some change in the physical state of the lipids.

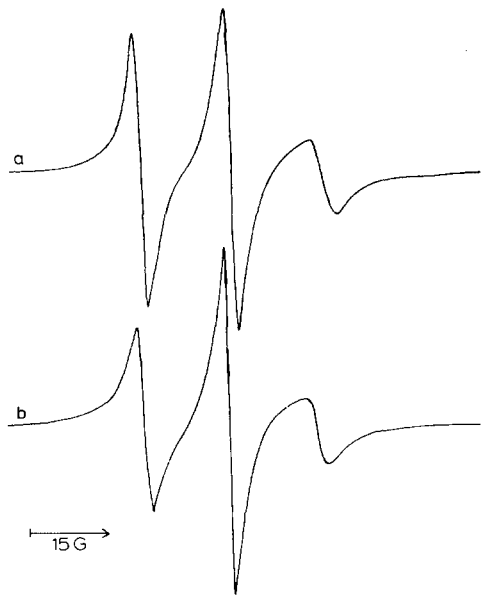


Fig. 6. ESR spectra of (a) spin-labeled phosphatidylcholine (II) and (b) spin-labeled phosphatidylserine (III) incorporated in intact erythrocytes measured at 23 °C.

37 °C in the presence of Mg^{2+} and the following incubation at 0 °C caused a decrease in the splitting value to 49.5 G. It is interesting to note that the temperature for the onset of decrease in the protecting action coincides well with the lower characteristic temperature for the fluidity change of the phosphatidylcholine phase.

Reduction of spin-labeled phosphatidylcholine and phosphatidylserine incorporated into erythrocyte membranes

Spin-labeled phosphatidylcholine and phosphatidylserine, incorporated in intact erythrocyte, gave ESR spectra consisting of three rather sharp lines with the splitting of 16.8 and 15.5 G, respectively (see Fig. 6). The spectra indicate rapid

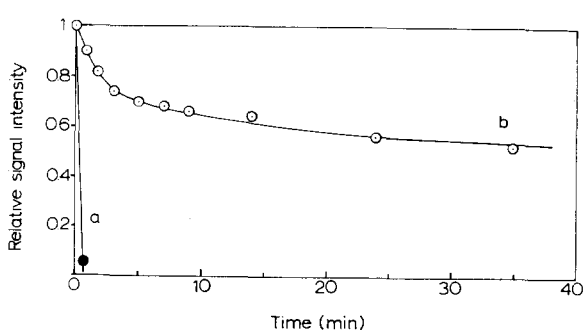


Fig. 7. ESR signal intensity of (a) spin-labeled phosphatidylcholine and (b) spin-labeled phosphatidylserine incorporated in intact erythrocyte membrane against incubation time at 23 °C with 0.3 mM cystein and 0.01 mM $FeCl_3$ in isotonic buffer (pH 7.6).

rotational motions of the labels, which are consistent with an expected geometry of the nitroxide moiety facing an aqueous medium. When a reducing reagent, cystein and Fe^{3+}, was added to the spin-labeled erythrocyte suspension, the ESR signal for spin-labeled phosphatidylcholine disappeared immediately, while the signal for spin-labeled phosphatidylserine decayed very slowly (see Fig. 7). Both labels were immediately reduced by the reagent when incorporated in erythrocyte ghost membrane and also in egg phosphatidylcholine bilayer membrane. Ascorbate, which is usually used to reduce nitroxide radicals [19], was not able to reduce spin-labeled phosphatidylserine.

DISCUSSION

The spin labels for various classes of phospholipids have sensitively revealed differences in the individual environments in intact erythrocyte membrane. The lipid bilayer portion of the membrane is not homogeneous; it is more rigid when examined by phosphatidylcholine spin label and more fluid by phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid spin labels. The heterogeneity in phospholipid structure was also manifested in the temperature dependence of the fluidity.

Several chemical and enzymatic studies have indicated asymmetric distribution of phospholipids in erythrocyte membranes: predominant localization of phosphatidylcholine and sphingomyelin on the exterior and phosphatidylethanolamine and phosphatidylserine on the interior [15, 16]. The observed marked difference in the reduction rates of take off spin-labeled phosphatidylcholine and phosphatidylserine also supports the asymmetric distribution. The present study therefore suggests asymmetric fluidity in the intact erythrocyte membrane: a more rigid outer layer and a more fluid inner layer. This heterogeneity was homogenized upon hemolysis.

The heterogeneity arises from interactions between the lipids and the proteins in the membrane and also probably from interactions between the membrane constituents and the inner proteins. The bilayer membrane consisting only of the erythrocyte lipids did not show the heterogeneous fluidity. Mg^{2+} or Mg^{2+}+ATP prevented the hemolysis-induced homogenization. Proteolytic enzymes and *N*-ethylmaleimide nullified the protecting action of Mg^{2+} when added inside the cell, but gave no effect on the membrane fluidity when added outside the cell. Our preliminary observation indicated that heavy meromyosin taken from skeletal muscle also nullified the protecting action when added inside the cell.

Some polymerizable polypeptides have been isolated from the erythrocyte cells. Spectrin, [26–28], myosin-like and actin-like polypeptides [29] may form filamentous networks adherent to the cytoplasmic surface, and such networks would offer structural support to the deformable lipid bilayer structure [30]. Interaction of spectrin with a membrane protein has been suggested by an electron microscopic study on the translational mobility of the protein [31]. It is highly probable that the inner proteins that modify the membrane fluidity are some of these polymerizable proteins. Decrease in the intracellular level of Mg^{2+} upon hemolysis may cause structural changes in the inner proteins and modify interactions with the membrane constituents. Or some inner proteins may be released from the cell. It should be biologically important that erythrocyte membrane fluidity is controlled by modification of the inner proteins.

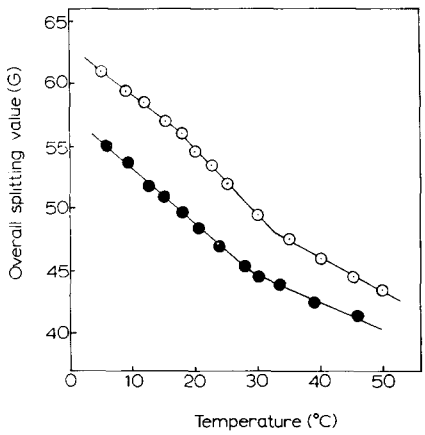


Fig. 3. Temperature dependence of the overall splitting of ESR spectrum for phosphatidylcholine spin label (○) and for phosphatidylserine spin label (●) incorporated in intact erythrocytes.

TABLE II

EFFECT OF VARIOUS TREATMENTS ON ESR SPECTRA OF SPIN-LABELED INTACT ERYTHROCYTES

The overall splitting values at 23 °C are given in G.

	Phosphatidylcholine spin label	Phosphatidylglycerol spin label
Intact erythrocyte*	52.0	46.8
Removal of cholesterol (20 %)**	50.0	47.0
Amphotericin B (25 μg/ml)***	50.0	47.5
Saponin (100 μg/ml)	50.0	47.2

* In 300 mM sucrose.

** Cholesterol was partially depleted by incubation at 37 °C for 2 h of 1 ml of packed erythrocyte with 1 ml of sonicated dispersion of egg phosphatidylcholine (15 mg/ml) in 150 mM NaCl and 40 mM Tris (pH 7.4). No hemolysis occurred. The cholesterol content was 80 % of the initial value according to assay by the method of Zlatkis et al. [25]. The cells were then spin-labeled with phospholipid labels.

*** Amphotericin B, dissolved in dimethylsulfoxide at a concentration of 10 mg/ml, was added to 1 ml of spin-labeled erythrocytes (0.15 ml packed volume) suspended in 300 mM sucrose. Dimethylsulfoxide alone at this concentration did not affect the spectrum.

Spin-labeled erythrocytes in isotonic media were subjected to a variety of treatments without causing hemolysis and its ESR spectrum was examined (Table II). Partial depletion of cholesterol caused a decrease in the overall splitting for phosphatidylcholine spin label (from 52 to 50 G). However, the depletion did not appreciably affect the splitting for phosphatidylglycerol spin label. Amphotericin B and saponin caused similar decreases in the overall splitting for phosphatidylcholine label (from 52 to 50 G). Treatment of the spin-labeled erythrocyte with *N*-ethylmaleimide (5 mM), trypsin (10 μg/ml) or pronase (10 μg/ml) had no effect on the spectra.

The heterogeneity in membrane fluidity was also observed for other mammalian erythrocytes. The overall splitting value for phosphatidylcholine spin label was always larger than that for phosphatidylglycerol spin label; 52.0 and 47.0 G for pig and 56.0 and 50.5 G for ox, respectively.

Homogenization of the membrane fluidity induced by hemolysis

ESR spectra of the spin-labeled erythrocytes were remarkably changed upon hemolysis. Fig. 4 shows an example of the spectral changes for phosphatidylcholine and phosphatidylserine spin labels incorporated in human erythrocytes. The overall splitting for phosphatidylcholine spin label was decreased from 52.5 to 49.0 G and that for phosphatidylserine label was increased from 47.0 to 49.0 (see Table I). The spectra for various phospholipid spin labels thus became similar to each other. The spectral changes were not due to the polarity change of the nitroxide environment, since the isotropic hyperfine constant was unchanged (14.3 G for phosphatidylcholine and phosphatidylserine labels).

The spectral change was induced not only by hypotonic hemolysis but also by hemolysis in isotonic media. Thus, hemolysis by sonication at 4 °C for 30 s, by freeze-thawing, by incubation in isotonic Tris buffer for several hours at 37 °C,and in isotonic histidine-imidazole buffer caused the same decrease in the overall splitting for phosphatidylcholine spin label. ESR spectrum of the spin-labeled erythrocyte was not affected in a hypertonic phosphate buffer (3100 ideal mosM). When the medium was

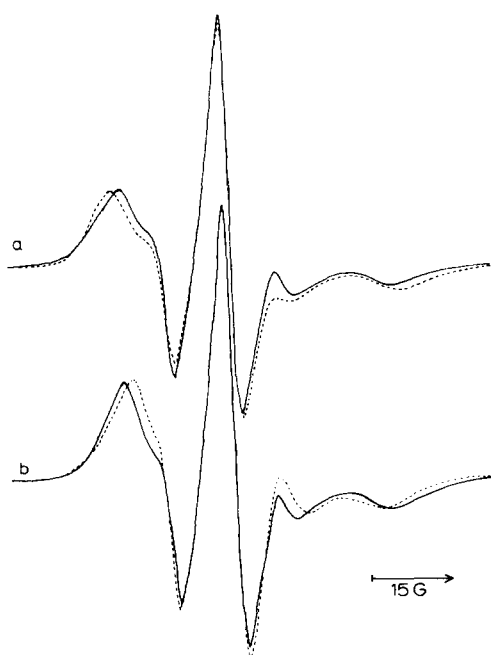


Fig. 4. Changes in ESR spectra of spin-labeled intact erythrocyte induced by hemolysis. The dotted spectrum changed into the full-line spectrum. (a) Phosphatidylcholine spin label; (b) phosphatidylserine spin label. Hemolysis and washing of the spin-labeled erythrocyte were carried out according to Dodge et al. [20]. ESR spectra were measured at 23 °C.

The rigidness of the outer phosphatidylcholine phase may be partly due to concentration of cholesterol in that phase. Partial depletion of cholesterol from erythrocytes caused a decrease in the fluidity of the phosphatidylcholine phase. Similar fluidizing effect of amphotericin B also supports the role of cholesterol, since this antibiotic has been shown to interact specifically with cholesterol [32]. However, the rigidity was not completely reproduced by a mixture membrane consisting of cholesterol and the choline-containing phospholipids from erythrocytes. Phosphatidylcholine in the mixture membrane had greater fluidity than that in the intact erythrocyte membrane. The concentration of cholesterol in the phosphatidylcholine phase would make the fluidity of the inner layer even greater than that of the total lipid bilayer membrane. It is interesting to note that a similar asymmetric disposition of cholesterol has been put forward for myelin by an X-ray diffraction study [33]. The asymmetric disposition is compatible with an observation that cholesterol was rapidly exchanged between erythrocytes and plasma [34].

The fatty acid composition of human erythrocyte lipids varies with the phospholipid class. The choline-containing phospholipids had markedly larger fractions of saturated acyl chains than those for the other lipids: 0.78 for sphingomyelin, 0.51 for phosphatidylcholine, 0.37 for phosphatidylethanolamine and 0.20 for phosphatidylserine [35]. The heterogeneous composition may also partly contribute to the rigidity of the outer layer and the fluidity of the inner layer.

The fluidity of the phosphatidylcholine phase of erythrocyte membranes changed characteristically at 18 and 33 °C. The heterogeneity preservation by Mg^{2+} appears to be correlated with the structural change, since the preservation decreased in the same temperature range. It has been reported that transfer of phospholipid from hemagglutinating virus of Japan to erythrocyte membranes began to occur at 19 °C and almost saturated at 37 °C [36]. The rate of glucose transport in erythrocytes showed a break at 19 °C [37]. The fluidity change in the outer erythrocyte membrane may therefore govern some membrane physiological phenomena.

The erythrocyte ghost membrane can be drastically different from the intact erythrocyte membrane, in view of the marked change in the fluidity upon hemolysis. Diffusion, carrier-mediated transport of molecules and activity of membrane-bound enzymes can be greatly modified. The trigger for the homogenization is probably modification of interactions between the membrane constituents and the inner proteins and might result in delocalization of cholesterol. There is a possibility for homogenization of phospholipid distribution since the flip-flop motion could be facilitated.

Stearic acid spin labels failed to detect the marked changes [38]. We have confirmed that the spectra of erythrocytes spin-labeled with stearic acid labels, their methyl esters and a steroid label were superposable on those of erythrocyte ghosts. These labels may have given some average spectra. Or perturbations by these classes of spin labels may have altered the membrane fluidities. Decreased osmotic fragility and altered surface topology of erythrocyte by these labels have recently been observed [39].

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