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A SPIN-LABEL STUDY OF THE CORRELATION BETWEEN STOMATOCYTE FORMATION AND MEMBRANE FLUIDIZATION OF ERYTHROCYTES

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Abstract—Change in the membrane fluidity of human erythrocytes on transformation to stomatocytes was observed by ESR spectroscopy using 12-doxyl stearic acid or its methyl ester as a probe. When the transformation to stomatocytes was induced by four qualitatively different methods, i.e. (a) addition of cationic amphiphilic agents such as chloropromazine, tetracaine, chloroquine or primaquine, (b) addition of Triton X-100, a non-ionic detergent, (c) lowering the pH, and (d) depleting membrane cholesterol, membrane fluidization was always observed. This indicates the existence of a close correlation between stomatocyte formation and increase in the membrane fluidity. Furthermore, since the stomatocytes fixed by diamide treatment exhibited membrane fluidization only in the presence of the reagent, the enhanced membrane fluidity was a direct consequence of the reagent interacting with, and changing the state of, the lipid bilayer itself, and not through the influence of some structural alteration of spectrin. These results provide experimental support for the theoretical prediction made by Brailsford *et al.* [*J. theoret. Biol.* **86**, 531 (1980)]. Plausible mechanisms for the discocyte–stomatocyte transformation are discussed.

A normal resting erythrocyte has a unique biconcave discoid shape (discocyte), which is easily transformed to a crenated form (echinocyte) or to a cup form (stomatocyte) depending upon the pH or the type of amphiphilic agent added to the cell suspension. Deuticke [1] first demonstrated that echinocytes are induced by anionic amphiphilic agents, e.g. fatty acids, whereas stomatocytes are induced by cationic agents such as phenothiazines. Later, Sheetz and Singer [2] proposed the bilayer couple hypothesis as an explanation of the mechanism by which the morphological change is induced by amphiphilic agents. According to this hypothesis, the echinocytogenic substances bind preferentially into, and expand the outer leaflet of, the membrane bilayer, while the stomatocytogenics bind preferentially into, and expand, the inner leaflet. This produces a positive or a negative precurvature of the cell membrane, inducing transformation to echinocytes and stomatocytes respectively. With their hypothesis, a number of experimental results on morphological changes of erythrocytes have been interpreted [3–10].

However, in their studies of theoretical models for such transformations, Brailsford *et al.* [11] have found that the echinocyte is theoretically reproducible only as the result of a strong negative precurvature of the membrane, combines with a high ratio of bending to shear stress. This is in contrast to the premise of a positive precurvature in the bilayer couple hypothesis. Furthermore, they pointed out in their analysis that the stomatocyte formation could not be explained as a change in the intrinsic precurvature of the membrane or the change in the bending-to-shear modulus ratio, but instead the stomatocyte would result from a loss of bending resistance of the membrane, probably caused by a sliding of the inner and outer leaflets of the lipid bilayer with respect to one another [12]. On the basis of this theoretical analysis, they predicted that the action of stomatocytogenic substances such as chlorpromazine should be to increase the fluidity of the hydrophobic interior of the lipid bilayer so that sliding is facilitated.

Earlier, Glaser and a co-worker postulated, using a different reasoning, that high fluidity in the membrane is a necessary condition for stomatocytic transformation [13, 14].

In the present study, we attempt to see if their prediction of a fluidity increase as a step in discocyte–stomatocyte transformation in erythrocytes has experimental support.

A number of stomatocytogenic substances and the several ways known to produce stomatocytes [1, 15] may be classified into the following five categories according to the presumed mode of action in changing the membrane state: (a) addition of cationic agents; (b) addition of non-ionic agents; (c) lowering the pH; (d) depleting membrane cholesterol; and (e) treatment with the enzyme phospholipase C.

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We have measured the change in membrane fluidity by using the ESR spin-labeling method with 12-doxyl stearic acid (or its methyl ester) as a probe. This is a well-established method for detecting a small change in the dynamic state of the red cell membrane [16].

We have found that membrane fluidity increases in all cases when discocytes are transformed into stomatocytes by the addition of chlorpromazine, an ethanol–chloroform mixture (1:1), several local anesthetics, anti-malarials, and Triton X-100, by the reduction of the cholesterol level in the membrane, or by lowering the pH of the cell suspension. These results appear to support the prediction proposed by Brailsford *et al.* [12]. In the case of the phospholipase C induced stomatocytes, however, the change of membrane fluidity could not be detected for reasons which we shall discuss later.

To elucidate the mechanism of membrane fluidization, we have also measured the change of membrane fluidity in the stomatocyte, in which cell shape is fixed by a spectrin cross-linking reagent, diamide [9, 17].

The results indicate that the increase in membrane fluidity is due change in the state of the lipid bilayer itself and not to the influence of some structural alteration of spectrin. The fact that the induced stomatocyte shape can be maintained by diamide fixation after removal of the drug implies that the effect of the drug reaches spectrin and alters the discocytic state of spectrin into the stomatocytic. Based on the present experimental results as well as the theoretical model by Brailsford *et al.* [12], we discuss plausible mechanisms for the discocyte–stomatocyte transformation.

MATERIALS AND METHODS

Chemicals. Chlorpromazine hydrochloride (CPZ), procaine hydrochloride, tetracaine hydrochloride, chloroquine diphosphate, primaquine phosphate, dipyridamole, diamide [diazine dicarboxylic acid bis-(dimethylamide)], L-α-phosphatidylcholine from egg yolk, and chromatographically purified phospholipase C from *Clostridia perfringens* were all purchased from the Sigma Chemical Co. (St. Louis, MO). Triton X-100 was obtained from the Research Products International Corp. (Elk Grove Village, IL), and the spin label 12-doxyl stearic acid (5,10) [2-(10-carboxypropyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl], its methyl ester, and 3-doxylandrostanol (17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3-yloxyl) were from the Syva Corp. (Palo Alto, CA). The reagents were used without further purification. All other chemicals and solvents used were of analytical grade purity.

An appropriate amount of each agent was dissolved in medium A, containing 90 mM KCl, 45 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, and 44 mM sucrose (pH 7.4). For CPZ stock solutions, the pH value was adjusted to 6.9, since CPZ precipitates out at higher pH values [18].

Preparation of erythrocyte suspensions. Freshly drawn heparinized human blood was washed three times with 5 mM phosphate buffer (pH 7.4) containing 154 mM NaCl (medium B) following removal of the buffy coat and plasma by suction [1, 3, 5].

Spin-labeling of erythrocytes for ESR measurements. Spin-labeled packed erythrocytes (hematocrit 90–92%) were obtained by incubating the washed packed cells for 10 min at 37° in a flask which was coated inside with a thin film of the spin label. The spin-labeled cell suspension at 35% hematocrit for ESR measurements was prepared by adding an appropriate amount of the stock solution of a drug to the spin-labeled pack cells. The final concentration of the spin label in a cell suspension was adjusted to 6.5×10^{-5} M, at which concentration no morphological change was observed, as shown in Fig. 1A (also, see Ref. 19).

Preparation of cholesterol-depleted erythrocytes. Intact erythrocytes were depleted of cholesterol, using the liposome of L-α-phosphatidylcholine from egg yolk, according to the method of Chailley *et al.* [20]. Lipid dispersions (liposomes) were prepared by sonicating 100 mg of egg lecithin in 10 ml of medium A at 42° under a stream of nitrogen gas for 40 min at a power level of 65 W with a Branson Sonifier model W185 equipped with a standard micro-tip. Fatty acid free bovine albumin (5 mg/ml) was then added to the dispersion in order to remove lysolecithin produced during sonication. The resultant dispersion was centrifuged at 35,000 *g* for 1 hr to remove titanium particles and non-dispersed lipids. Erythrocytes (hematocrit 20%) were incubated with an appropriate amount of liposomes in a buffer solution containing 90 mM KCl, 45 mM NaCl, 10 mM glucose, 35 mM sucrose, 50,000 I.U./ml penicillin, 10 mM phosphate and 1 mM $MgCl_2$ at 37° for 7 hr in a shaking water bath. The incubated cells were washed three times with medium A and were spin-labeled at room temperature for 10 min with a final spin-label concentration of 6.5×10^{-5} M. To determine the remaining cholesterol-to-phospholipid molar ratio (C/P), the treated cells were extracted with a mixture of 2-propanol and chloroform (11:7, v/v) [21]. The extract (5 ml) was dried under a nitrogen gas flow and dissolved in a 30-μl 10% sodium taurodeoxycholate solution. The amount of cholesterol was determined according to the method of Allain *et al.* [22], using the Sigma Kit (No. 350) for enzymatic determination of cholesterol. The amount of phosphate in the extract was determined according to the method of Bartlett [23] with the use of the Sigma Kit (No. 670) for the colorimetric determination of inorganic phosphorus.

Treatment of cells with phospholipase C. Phospholipase C was dissolved in a 1% albumin solution in medium B (5 units/ml) at 0° to form a stock solution. This enzyme stock solution was diluted, prior to incubation of the cells, in a 10 mM Tris buffer (pH 7.4) containing 90 mM KCl, 45 mM NaCl, 5 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 44 mM sucrose to make up a concentration series of 0.01 to 0.1 units/ml. Nineteen volumes of diluted enzyme solution were added to one volume of the washed packed cells. The progress of the morphological changes in cells in the suspension was followed under the microscope at room temperature. When many cells were transformed into stomatocytes, the cells were washed with a 10 mM EDTA isotonic solution

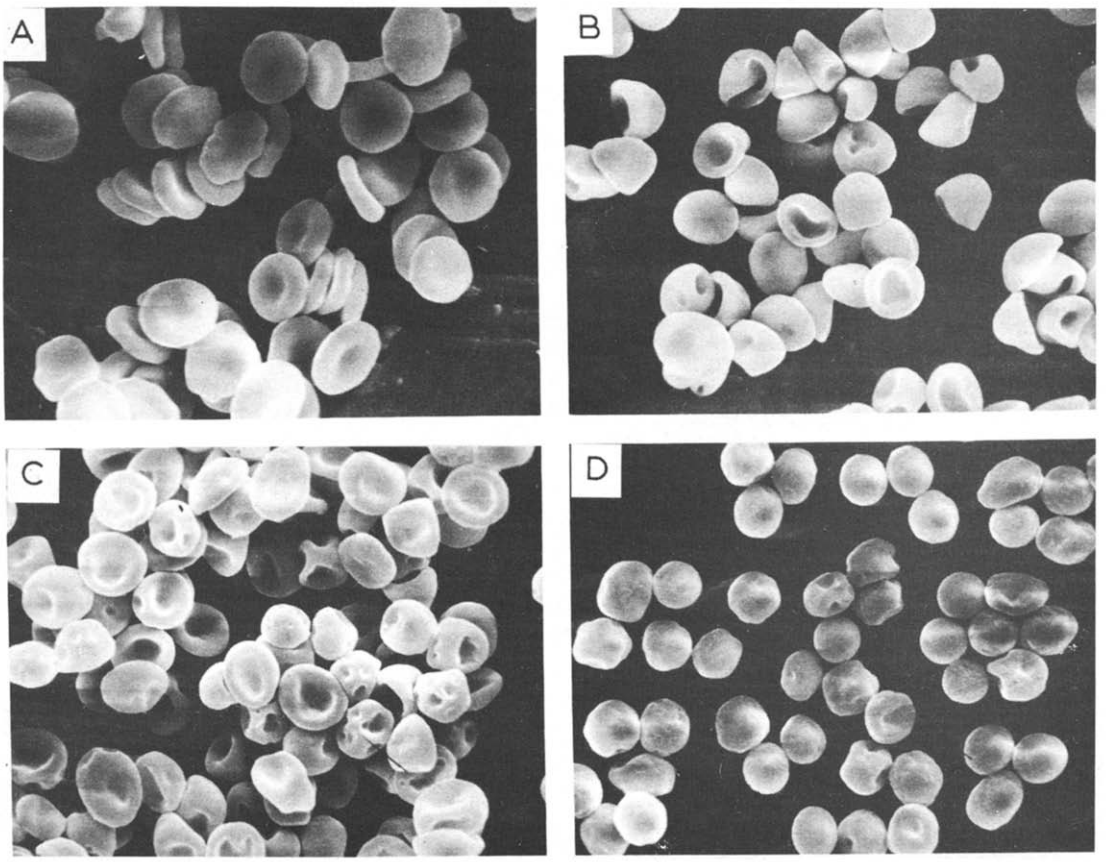

Fig. 1. Scanning electron micrographs of human erythrocytes in the presence of (A) 0 mM, (B) 0.32 mM, (C) 0.48 mM, and (D) 1.20 mM chlorpromazine.

(the pH varied from 6 to 7.4) in order to quench the enzyme reaction. The packed cells thus obtained were spin-labeled at room temperature for 10 min for ESR measurements.

Preparation of diamide-treated cells. A plasma and buffy coat-free cell suspension (25% hematocrit) in medium A (pH = 8.0) was incubated with 10 mM iodoacetate for 15 min at 37° to block the intracellular glutathione [17] and was subsequently washed three times with medium A. The iodoacetate-treated cells (i.e. either discocytes, or stomatocytes induced by CPZ) were incubated with 10 mM diamide in medium A (pH = 8.0) for 15 min at 37° [17], followed by three washings with medium A (pH 7.4) to remove excess diamide (and CPZ in the induced stomatocyte suspension). The diamide-treated cells were spin-labeled as described above, and ESR observations were made in the presence (0.48 mM) or absence of CPZ.

Scanning electron microscopy. The cells were fixed with 0.5% glutaraldehyde in an isotonic buffer solution (pH = 7.4) at room temperature and were cooled to 4°. The cells were washed twice with Millonig's phosphate buffer (pH = 7.4) and were fixed in 1% osmium tetroxide in the buffer for 1 hr at room temperature. After washing three times with distilled water, the cells were suspended in two changes of 50% propylene oxide and transferred to 100% propylene oxide. The samples were air-dried, coated with gold by Mini-coater (Commonwealth Scientific Co., Alexandria, VA), and observed with a Mini-SEM scanning electron microscope (International Scientific Instruments, Avon, CT).

ESR measurements. ESR spectra were obtained in a silicone-coated 50 μl capillary tube on a Varian model E-109 X-band ESR spectrometer combined with a JEOL cylindrical resonant cavity (JES UCX2). The amplitude of 100 KHz field modulation with this cavity was 2 G. The microwave power was kept at 40 mW. The sample-containing capillary tube was surrounded by a single-jacketed quartz dewar flask, through which dry nitrogen gas of a constant temperature (22 ± 0.05°) was passed at a rate of 6 l/min. Under this condition, the sample temperature was maintained at 24° with fluctuations of less than ± 0.05° over the period of experiments. The temperature was measured by a Bailey digital thermometer (BAT-9). The measured parallel peak separation was the same as that obtained at a lower microwave power level (e.g. 10 mW) within the reading error (± 0.2 G).

In order to complete a series of measurements with varied drug concentrations in a reasonable length of time (*ca.* 2 hr), only the necessary regions of the spectrum were scanned around the lower and the higher field parallel peaks. Each peak was scanned three times with the scan-time setting at 4 min (for 100 G full span), and the amplifier time

constant at 0.25 sec. Three curves were completely superimposable.

RESULTS

Stomatocyte formation induced by amphiphilic agents. Among the many substances that induce transformation to stomatocytes (see Refs. 1 and 15), we chose the following as typical agents: chlorpromazine (CPZ) [1, 2, 7, 9, 18]; the local anesthetics, procaine and tetracaine [1]; the antimalarials, primaquine [1, 24] and chloroquine [1]; the non-ionic detergent Triton X-100 [15]; and an organic solvent mixture methanol–chloroform (1:1, v/v).

Figure 1 shows the dependence of the cell shape of the spin-labeled erythrocytes on CPZ concentration. With increase in the CPZ concentration, the cells were transformed from discocytes (Fig. 1A) to spherocytes (Fig. 1D) through stomatocytes (panels B and C), and eventually hemolysis took place. Qualitatively similar shape changes to those shown in Fig. 1 were observed for other stomatocytogenic agents, although the concentration at which the transformation occurred varied depending upon the agents.

ESR spectral change of spin-labeled cells in the presence of chlorpromazine. Figure 2 shows the typical ESR spectra of an erythrocyte suspension labeled with 12-doxyl stearic acid at 35% hematocrit in the absence and presence of 0.48 mM CPZ. In the latter condition, the cells were transformed to stomatocytes (Fig. 1C). Both anisotropic spectra indicate that the spin labels were incorporated into the membrane and were undergoing anisotropic rotation along the fatty acid chain [25, 26]. The separation between the outermost lines, which corresponds to twice the parallel hyperfine separation $2A_{\parallel}$, was smaller in the presence, than in the absence, of CPZ. In general, the decrease in $2A_{\parallel}$ occurred due to the following: (1) a greater distribution of molecular orientations relative to the membrane surface, and/or (2) the faster random motion of the nitroxide moiety [25, 26]. The spectral change observed was so small that it was difficult to determine which factor was dominant in this case, but the decrease in $2A_{\parallel}$ was, in any case, indicative of an increase in membrane "fluidity" [16, 26]. Similar spectral changes were observed when the stomatocytes were induced by other agents.

When the methyl ester of 12-doxyl stearic acid was used instead of the fatty acid spin label, a greater change in the spectrum was observed, as shown in Fig. 3. The spectrum of the ester spin lables in the membrane appears pseudo-isotropic [27], indicating that the labels were intercalated into more disordered parts of the lipid bilayer than those in which the acid labels reside. Since there exists a flexibility gradient in the membrane [25], in such a way that the degree of order in the lipid array decreases with increasing distance from the polar head group, the ester labels seemed to be dissolved deep in the hydrocarbon parts of the bilayer. The main changes in the spectrum of ester-labeled cells under the action of CPZ were a decreased line-width of the peak at the lowest field and a reduced intensity of the peak at the highest field. These changes also indicate that the molecular ordering of the lipid chains was perturbed by the presence of CPZ and/or that the spin labels underwent more rapid rotation in the presence of CPZ than in its absence. Both of these effects are expressions of an enhanced membrane "fluidity".

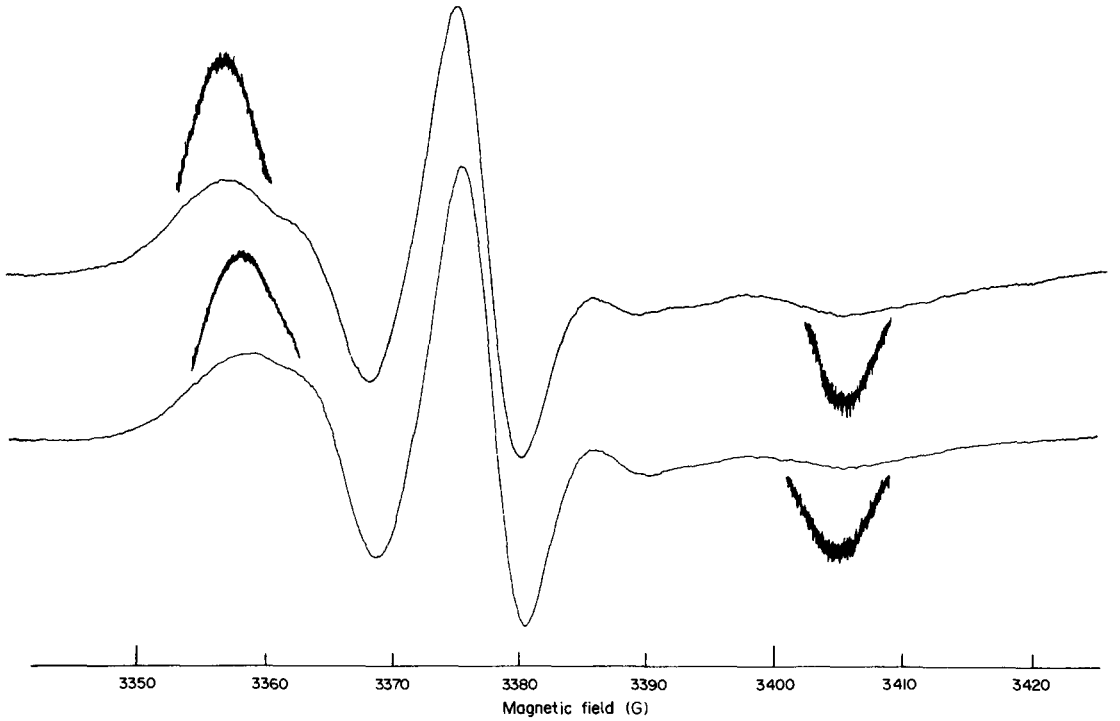

Fig. 2. ESR spectra of erythrocytes spin labeled with 12-doxyl stearic acid in the presence of 0 mM (upper) and 0.48 mM (lower) chlorpromazine.

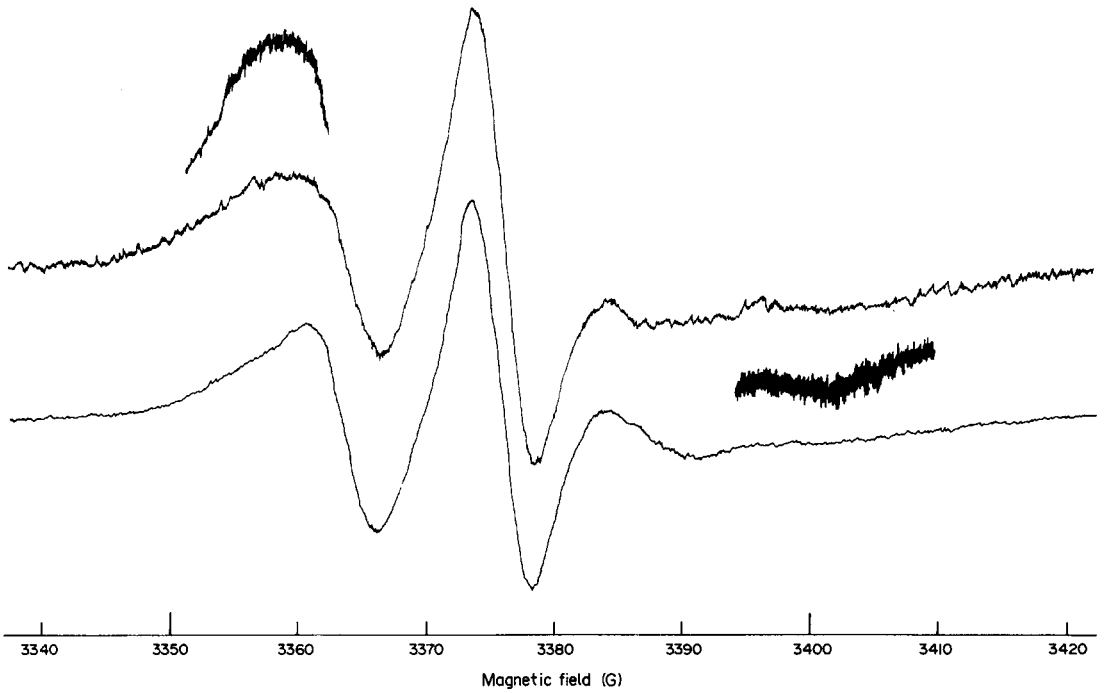

Fig. 3. ESR specta of erythrocytes spin labeled with the methyl ester of 12-doxyl stearic acid in the presence of 0 mM (upper) and 0.48 mM (lower) chlorpromazine.

Although the ESR spectral change of the ester spin label was larger than that of the acid, we used the latter label in most of the experiments, since the spectral change in the acid label is easily quantitated by simply measuring the change of the parallel hyperfine separation $2A_{\parallel}$.

Another spin label, 3-doxylandrostanol, incorporated similarly gave a spectrum showing considerable mobility of the probe. Addition of 0.5 to 0.7 mM chlorpromazine caused a small spectral change, which is consistent with an enhanced fluidity, in the region of the parallel hyperfine structure [27], but the overall change was much less than with the other two labels and was difficult to quantitate.

Dependence of the parallel hyperfine splitting upon the agent concentration. To show the dependence of the parallel hyperfine splitting $2A_{\parallel}$ on the drug concentration, the relative change $R = 2A_{\parallel}/2A_{\parallel,0}$ was plotted in Figs. 4 and 5, against the total concentration of the agent used, $2A_{\parallel,0}$ being the value measured in the control. Although the details of the profile were different for an individual agent, they showed a common characteristic of a decrease in R with increasing amounts of the agent, and each curve exhibited a stepwise decrease at a concentration of 0.7 mM for CPZ, 0.9 mM for Triton X-100, 1.2 mM for primaquine, and 4.1 mM for tetracaine and chloroquine. The first decrease of R down to 0.95, e.g. for CPZ, corresponds to stomatocyte formation (stomatocytes I–III [18]), as was microscopically observed. The following gradual decrease corresponds to the transformation to spherocytes. When the R value reaches *ca.* 0.93, hemolysis is observed.

On the other hand, when echinocytes were formed by salicylate, a typical echinocytogenic agent [1, 9], virtually no change in R was observed, as shown in Fig. 5. Similarly, with procaine, which does not induce stomatocytes in the concentration range up to 11 mM, the R value remained unchanged at R = 1 (Fig. 5). It is interesting to note that stomatocyte formation was observed in the presence of a 1% methanol–chloroform (1:1) mixture, in which condition the R value was measured to be 0.94, indicating a similar increase in the membrane fluidity. Such an effect of a methanol–chloroform mixture has not been reported previously.

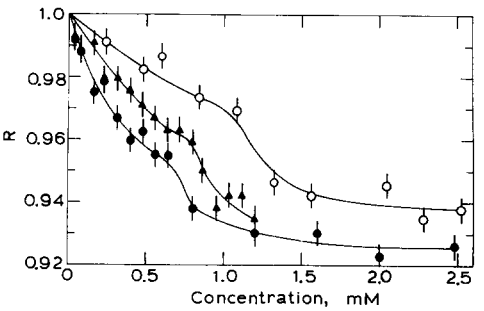

Fig. 4. Relative change (R) of the parallel hyperfine splitting constant versus the total concentration of chlorpromazine (●), Triton X-100 (▲), and primaquine (○). Hematocrit 35%. $2A_{\parallel,0} = 51.4$ G with reading error ± 0.2 G. Titration with Triton X-100 was stopped at 1.25 mM, since further increments caused an increasing degree of hemolysis. Each curve represents a typical result of repeated experiments (chlorpromazine, four times; Triton X-100, three times; and primaquine, two times). Repeated runs showed qualitatively the same profiles. The error bar indicates the uncertainty in measuring the spectral peak separations, which depends upon the signal-to-noise ratio and was constant throughout the experiments.

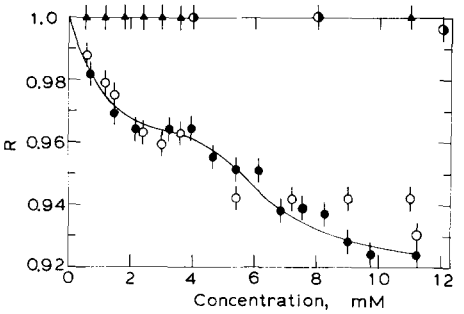

Fig. 5. Relative change (R) of the parallel hyperfine splitting constant versus the total concentration of tetracaine (○), chloroquine (●), procaine (▲), and salicylate (◑). Hematocrit 35%. The $2A_{1,0}$ value is the same as in Fig. 4. Curves are the typical results of duplicate experiments.

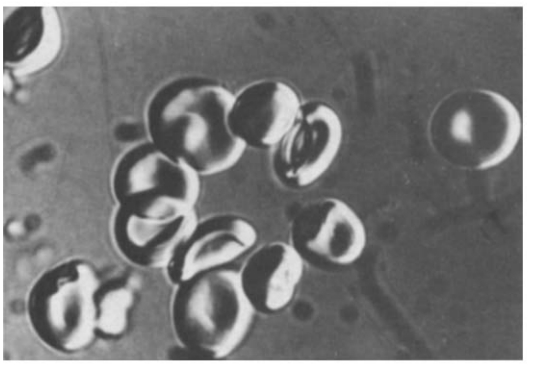

Fig. 6. Red cells treated with 0.1 units of phospholipase C per ml of red cell suspension for 15 min at 37°. The photomicrograph was taken by using a Nikon microscope (Biophot) equipped with differential interference contrast optics.

It must be pointed out that such a comparison of the effects of drugs as shown in Figs. 4 and 5 has only a relative significance, since the binding constants of most of these drugs are unknown and since the fatty acid spin label used is known to promote echinocyte formation [19], thereby providing an antagonistic effect to some extent which may depend on the individual drug.

Fluidity change in stomatocytes induced by lowering the pH, or by cholesterol depletion. Erythrocytes are known to become stomatocytes when the pH value of the cell suspension is in the range between 6.0 and 4.5 [1, 15]. The R values in stomatocytes obtained at pH 6.0 and 5.5 were estimated to be 0.95 and 0.94 respectively. On the other hand, the R value measured at a high pH value between 7.4 and 10.0 remained unchanged at 1.0, while the cells were transformed to echinocytes at pH 10. Furthermore, when the methyl ester of 12-doxyl stearic acid was used, a similar spectral change to the one described above for the effect of CPZ (Fig. 3) was observed by lowering the pH from 7.4 to 6.0. The ESR spectral change of fatty acid spin labels dissolved in lecithin dispersions, when the pH was changed, was interpreted to be due to the shift in location of the labels on ionization of their carboxylic acid groups [28, 29]. The absence of such spectral change in lecithin dispersions, when the methyl ester label was used, seems to support the explanation. However, the fact that we have observed a spectral change with a methyl ester label in red cell membranes when the pH was lowered indicates that the decrease in R in the latter system was due to an increased membrane fluidity, possibly with some contribution from a shift in the label location. Thus, we may conclude that the membrane fluidity was enhanced also in stomatocytes produced by lowering the pH of the suspension.

Recently, Chailley *et al.* [20] reported that removal of 30–40% of cholesterol also induces stomatocytes. We have confirmed their result as follows. When cells were incubated with ~1 and ~3 mg per ml of liposomes, the cholesterol–phospholipid molar ratio (C/P) was reduced to 0.65 and 0.55, respectively, from 1.1 in the control. In both conditions, the cells were transformed to stomatocytes. The R values were estimated to be 0.96 and 0.94, respectively, for the cells having C/P molar ratios of 0.65 and 0.55. These results also indicate an increase in the membrane fluidity in stomatocytes produced by chlolesterol depletion.

Another known method to induce stomatocyte formation is treatment with phospholipase C [8, 30]. The enzyme attacks the membrane phospholipids and produces 1,2-diacylglycerol, which diffuses rapidly to the inner leaflet of the membrane. Formation of stomatocytes in this case was explained by Allan *et al.* [6], on the basis of the consequent expansion of the inner leaflet. However, we could not detect the enhancement of membrane fluidity in suspensions that contained stomatocytes produced by the phospholipase C treatment. It should be pointed out, however, that the cell shapes induced by the treatment with phospholipase C were considerably inhomogeneous, as shown in Fig. 6, and included substantial populations of discocytes and spherocytes as well as stomatocytes. The R value measured in such an inhomogeneous system was apparently unchanged. Furthermore, the R value observed for the suspension containing a homogeneous population of spherocytes, as the result of prolonged treatment with phospholipase C, was also unchanged, in contrast to the observations with the spherocytes produced by addition of agents (Figs. 3 and 4).

It is of interest to note that, when phospholipase C treated cells were washed, to stop the reaction, with an EDTA buffer solution at pH 6.6, the R values showed some decrease compared with the control. Although slightly acidic, the pH value was not sufficiently low to induce transformation to stomatocytes, by itself. This fact may indicate that the effect of the pH on morphological change is related to the number of head groups of the phospholipids located on the outer surface of the membrane.

Membrane fluidity of the diamide-fixed stomatocytes and discocytes. Haest *et al.* [9] recently found that the shape of an agent-induced stomatocyte can be retained by reaction with diamide, which cross-links preferentially the cytoskeletal protein spectrin, even after the inducer is removed by washing. If the membrane of diamide-fixed stomatocytes remains

Table 1. R values of diamide-fixed discocytes and stomatocytes in the presence and absence of CPZ*

Treatment	Intact cells	Iodoacetate-treated cells	Diamide-fixed discocytes	Diamide-fixed stomatocytes
Without CPZ	1.00	1.03	1.02	0.98 (1.00)†
With 0.48 mM CPZ	0.96	0.95	0.94	0.93 (0.93)†

* A typical result from three experiments repeated under the same conditions. The experimental error of ± 0.01 is due to uncertainty in measuring the spectral peak separations. $2A_{\|,0} = 51.4 \pm 0.2$ G.

† Controls were treated identically, but without diamide.

fluidized even in the absence of a stomatocytogenic agent, it is reasonable to assume that some stomatocytic change in the physical state of spectrin may be the cause of the increase in the membrane fluidity. On the other hand, if the diamide treatment does not arrest the fluidized state when the agent is removed, it should be the agent itself that affects directly the structure of the bilayer and brings about an enhanced membrane fluidity. As shown in Table 1, when CPZ was removed from the stomatocyte membrane fixed by diamide, the R value increased back to 0.98 of that in the control, which is the iodoacetate-treated cells washed with a buffer solution following the CPZ treatment. Thus, the fluidity was almost completely recovered when the drug was removed, demonstrating that the presence of CPZ was indispensable to keeping the membrane fluidized, and that the spectrin was perhaps passively modified from the discocyte to the stomatocyte state by the CPZ treatment. This conclusion is confirmed by a further observation that, when the discocytes fixed with diamide were treated with 0.48 mM CPZ, the R value decreased to 0.94 without any morphological change.

DISCUSSION

The present work was motivated by the results of the theoretical analysis made by Brailsford *et al.* [12]. Using experimentally obtained shapes of stomatocytes, they calculated the mechanical energy of the membrane system and compared it with the corresponding energy in discocytes. They showed that the calculated energy of the stomatocyte becomes lower than that of the discocyte only when the bending resistance is made vanishingly small and suggested that one way to achieve the condition may be for the membrane to be fluidized so that the two adjacent layers, in which the bending resistance arises, can slide over each other. If their prediction is correct, it is expected that the membrane fluidity would increase independently of the method by which stomatocytes are produced.

We have measured the change in the membrane fluidity of stomatocytes, induced according to the five qualitatively different methods reported in the past. Our results in four out of the five such cases unambiguously demonstrated such correlation, the only exception being the reaction with phospholipase C, in which a homogeneous stomatocyte suspension could not be obtained. Hence, the theoretical prediction by Brailsford *et al.* [12] seems to have reasonable experimental support. Furthermore, since we have shown by diamide fixation that the observed fluidity state of the membrane is not determined by cell shape, we may assume that the enhanced fluidity is not the result of cup formation but, instead, is likely to be the cause of the transformation.

Such increase in membrane fluidity has been observed in various model membranes, red cell ghosts, and in intact cells in the presence of anesthetics, etc., using ESR, NMR, or a fluorescence depolarization method [31–35]. Also a similar increase in membrane fluidity as a result of cholesterol depletion has been reported by Cooper *et al.* [36] and Borochov *et al.* [37]. Thus, the phenomenon itself is not uncommon, but the correlation with stomatocyte formation has not been pointed out previously.

The detailed mechanism as to how the membrane fluidization actually induces cup formation is not clearly understood at present. There seem to be at least two possibilities, depending upon the source of the bending resistance. If the bending resistance arises in the phospholipid bilayer, as is usually postulated, the straightforward mechanical consideration such as the one presented by Brailsford *et al.* [12] may apply, since the fluidity change we observe pertains to the bilayer.

On the other hand, if the interaction of the cytoskeletal network (spectrin-actin-band 4.1) [38] with the bilayer is the dominant source of the bending resistance [12], the possible link from the bilayer fluidization to stomatocyte formation may involve several biochemically mediated steps. For example, Chailley *et al.* [20] recently reported that, during discocyte–stomatocyte transformation induced by cholesterol depletion, there is a specific decrease in spectrin phosphorylation and an increased phosphorylation in the lipid. They attributed the effect to the change in the activity of the membrane bound protease and kinase [20]. Also, we have just shown that cholesterol depletion leads to a rise in membrane fluidity. It is generally known that change in mem-

brane fluidity modifies the activity of the membrane bound enzymes [39–42]. These observations together suggest as a possibility that the enhanced membrane fluidity may alter the phosphorylation state of the spectrin complex. There are several lines of evidence which imply that the spectrin-actin-band 4.1 complex interaction is modulated by phosphorylation [43, 44]. Thus, if the enhanced fluidity in the membrane causes a decrease in the spectrin complex phosphorylation, it may modify the interaction of the network with the whole bilayer. A loss of bending resistance thus occurs which will cause the transformation.

Much more work is needed to anser the question as to which of the two, or some other, possibilities really is the case.

Finally, existence of a positive correlation between membrane fluidization and stomatocyte formation is support for, but not a positive proof of, the theoretical model by Brailsford *et al.* [12] and, therefore, the increase in membrane fluidity by itself is not necessarily incompatible with the bilayer couple hypothesis.

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