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FLUIDITY OF HUMAN ERYTHROCYTE MEMBRANE AND EFFECT OF CHLORPROMAZINE ON FLUIDITY AND PHASE SEPARATION OF MEMBRANE

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The fluidity of human erythrocyte membrane, and the effect of chlorpromazine at prelytic and lytic concentrations on the fluidity have been studied by using three kinds of fatty acid spin labels and measuring the temperature dependence of Mg^{2+}-ATPase activity. The Arrhenius plot of the apparent rotational correlation time, τ_c, for probes I(12,3) and I(5,10) showed an abrupt discontinuity at about 30°C, and the plot for I(1,14) at 25°C, indicating that a large difference in the fluidity exists between the interior and the outer surface of the lipid bilayer. The portions of the fatty acid chain near the ten carbon bond lengths removed from the bilayer surface became more fluid by chlorpromazine treatment; there was a decrease in the break point to around 26°C following treatment with 0.6 or 1 mM of the drug. Two breaks at 21 and 30°C in the Arrhenius plot of the Mg^{2+}-ATPase activity were observed in normal erythrocyte membrane. The activation energy of the Mg^{2+}-ATPase reaction has the values of 3.0 and 22.1 kcal/mol above the upper break and below the lower break, respectively. The drug exposure induced only a slight shift in the break temperatures, while the treatment significantly enhanced the associated activation energies of the reaction. These results suggest that the boundary phospholipids of the Mg^{2+}-ATPase in the membrane are probably more rigid than the bulk lipids.

Introduction

The cell membrane is thought to be fluid under physiological conditions, and so integral membrane proteins are mobile in the plane of the plasma membrane [1]. Such movements are of importance in determining the functional activities of the cell, and the organization and physical characteristics of lipids of biological cell membranes are intimately related to a variety of physiological functions of the membranes, such as specific transport processes [2], immunochemistry [3], temperature adaptation [4,5] and egg fertilization [6,7]. In addition, the activity of membrane-bound enzymes may be affected in various ways by the lipid environment, one of which is a change in the physical state of the lipid phase. Phospholipid fluidity therefore seems to markedly affect the activity of a number of membrane-bound enzymes [8–10], including ATPase (EC 3.6.1.3) [8, 11].

The spin label technique has been widely used to investigate molecular phenomena in the biological membrane [12]. Various studies have been made on the erythrocyte membrane; the effect of hormones and drugs on the fluidity of the membrane [13–15], the application to the diagnosis of the myotonic disease [16,17] and the virus-induced fusion of the erythrocytes [18]. Since temperature-induced phase changes are known to affect the kinetics of many membrane-associated enzyme systems [19], Arrhenius plots of the membrane-bound enzyme activities have yielded various break temperatures depending on the enzyme assayed [8,11,20–25]. The fact that such discontinuities appear to reflect the phase transi-

Abbreviations. Spin labels are defined in Materials.

tions of membrane lipids has been demonstrated by the studies which have shown a correlation between the discontinuity temperatures and independently measured phase transition temperatures of the intact membranes and the extracted lipids [8,21,26–30].

We have been studying the mechanism of drug-induced hemolysis of human erythrocytes [31–33]. To further clarify the mechanism, attention has been given to the studies designed to elucidate the changes in the membrane fluidity induced by chlorpromazine. In this paper, we report the fluidity in the different parts (depth) of the membrane and the effect of the drug at prelytic and lytic concentrations, using three kinds of fatty acid label, and the temperature dependence of Mg^{2+}-ATPase activity. The activation energies in an Arrhenius plot of the ATPase activity were also measured after drug treatment of the membrane.

Materials and Methods

Materials

Chlorpromazine hydrochloride (Nihon Shinyaku, Kyoto) was used throughout this experiment. The spin labels, 2-(14-carboxytetradecyl)-2-ethyl- and 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as I(1,14) and I(12,3), respectively) were purchased from Syva Co. and used without further purification. *N*-Oxyl-4,4-dimethyloxazolidine derivative of 12-ketostearic acid (I(5,10)) was synthesized according to the procedure of Waggoner et al. [34]. Human serum albumin was obtained from Miles Laboratories Inc. (fraction V, fatty acid-free). ATP disodium salt and ouabain were obtained from Sigma Chemical Co. and E. Merck, respectively.

Methods

Preparation of erythrocyte suspension and hemoglobin-free erythrocyte ghosts. Human erythrocyte suspensions and erythrocyte ghosts were prepared by the same method as described in the previous paper [31] and the method of Dodge et al. [35], respectively. The ghosts obtained were immediately resealed by the procedure of Mueller and Morrison [36].

Preparation of the spin-labeled ghost membrane and ESR measurement. The spin label (1.4 mg) was dissolved in diethyl ether in a test tube and the solvent was evaporated in a nitrogen stream. 1 ml human albumin solution (50 mg/ml) was added to the test tube and stood for 6–24 h at 4°C. 0.5 vol. each of the spin label albumin and the isotonic Tris-saline buffer (37 mM Tris-HCl/112 mM NaCl/44 mM glucose, adjusted to pH 7.4) was added to 1 vol. ghost suspension (5 mg protein/ml) and the mixture was incubated for 1.5 h at 0°C. The spin-labeled ghost membranes were quantitatively washed three times with 20 vol. isotonic buffer. 10 ml isotonic solution of chlorpromazine was added to 1 ml spin-labeled ghost suspension (5 mg protein/ml) in the final drug concentrations ranging from 0.2 to 1 mM and incubated for 30 min at 37°C, followed by centrifugation. The ghost pellet was briefly washed once with 20 vol ice-cold isotonic buffer in order to remove the drug adsorbed on the membrane and packed in a hematocrit capillary tube (Corning Co., 1.1 mm inner diameter, nonheparinized) by centrifugation at 12000 rev./min for 5 min at 4°C. The ESR spectra of the packed samples were recorded by the JEOL JES-PE-3X ESR spectrometer (at 6.3 GHz, 100 KHz field modulation) equipped with a variable temperature accessory. The temperature was controlled by the nitrogen gas stream. The portion of the packed membrane was placed in the variable temperature accessory. The parallel ($T_{\parallel}$) and perpendicular ($T_{\perp}$) principal values of the hyperfine tensor of an axially symmetrical spin Hamiltonian were estimated from the ESR spectra and the order parameter, S, was calculated using the relation, $S = a(T_{\parallel} - T_{\perp})/(T_{zz} - T_{xx})$, $a = (T_{zz} + 2T_{xx})/(T_{\parallel} + 2T_{\perp})$, where T_{zz} (32.9 G) and T_{xx} (5.9 G) are the hyperfine principal values of the nitroxide radical [37]. For these spectra, the rotational correlation time, τ_c, was calculated according to the following formula [38]: $\tau_c = 6.5 \cdot 10^{-10} W_0[(h_0/h_{-1})^{1/2} - 1]$, where W_0 and h_0 are the width and height, respectively, of the medium line of the first derivative spectrum, and h_{-1} is the height of the high field line.

Assay of Mg^{2+}-ATPase activity. For the measurement of Mg^{2+}-ATPase activity, the erythrocyte ghosts (prepared by using 40 mM Tris-saline buffer, pH 7.4, instead of phosphate buffer) were treated with the drug solutions as described above and washed once with 40 mM Tris-saline buffer and the ghost pellet was frozen and thawed twice. Mg^{2+}-ATPase activity of the membrane was assayed by measuring the

inorganic phosphate released from ATP during the incubation at various temperatures in the presence of 1 mM ouabain according to the method of Kramer et al. [39], except that the activity was assayed at pH 7.7. The activity was expressed as μg P_i/mg protein per h.

Protein determination. Protein concentration was determined by the procedure described by Lowry et al. [40] with bovine albumin, fraction V, as a standard.

Results

Fluidity of erythrocyte membrane and effect of chlorpromazine treatment

Figs. 1–3 show the representative ESR spectra obtained with the erythrocyte membrane, labeled respectively with spin labels I(12,3), I(5,10) and I(1,14), after the drug treatment. The spectra of the untreated membrane are similar to those of erythrocytes obtained by other workers [41,42]. These spectra indicate the anisotropic motion as observed

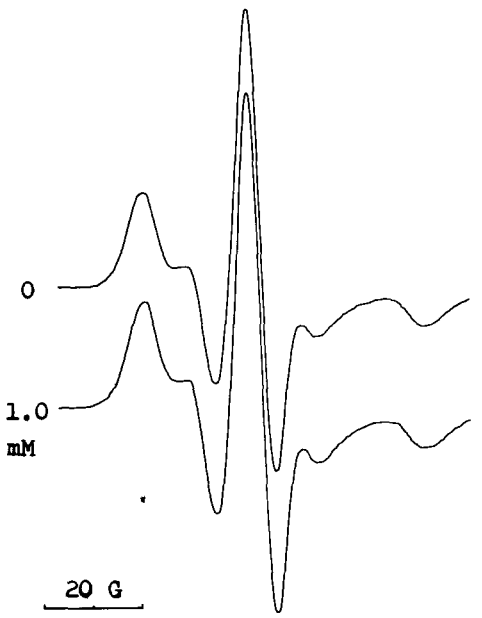

Fig. 1. ESR spectra of I(12,3)-labeled erythrocyte membrane at 24.5°C following treatment with chlorpromazine. The spin-labeled ghosts (5 mg protein/ml) were treated with 1 mM chlorpromazine for 30 min at 37°C. 0, untreated ghosts.

with spin probes inserted in the lipid bilayers. The intensity of the spectrum increased steadily with the temperature, indicating a smooth increase of the fluidity of the bulk phospholipid matrix. The treatment of the membrane with 0.2–1 mM chlorpromazine produced significant changes in the spectra of both probes I(5,10) and I(1,14), the high field extreme of the spectra was markedly enhanced with increasing drug concentrations, while the treatment induced no detectable change in the spectra of probe I(12,3). These results suggest that the drug has little or no effect on the fluid state of the bilayer near the phospholipid head groups, but extremely increases the molecular motion of the hydrocarbon core. Each experiment was repeated at least four times and a very good reproducible result was obtained.

The order parameter, S, of probe I(12,3) and apparent rotational correlation time, τ_c, of the three labels were calculated from ESR spectrum. In the present case, τ_c values do not represent true correlation time because the molecular orientation and motion are anisotropic in the membrane. Fig. 4 shows the plots of order parameter vs. temperature and apparent rotational correlation time vs. temperature for I(12,3) spectra of drug-treated membranes. Both curves represent the temperature dependence of S and τ_c values, indicating the increased fluidity of lipids at higher temperatures. Significant changes in both parameters after the drug treatment were not observed at temperatures over the range 3–45°C. Arrhenius plots of τ_c values showed an abrupt inflection at 29.7 ± 0.7 ($n = 8$) for the untreated and drug-treated membranes. The S and τ_c values of the incorporated spin labels following drug treatment are compared with one another at the three temperatures in Table I. S and τ_c values of probe I(12,3) were essentially similar for the drug-treated and untreated membranes. In addition, the width of the outer extrema, $2T_{\|}$, of I(12,3) was not altered by the drug treatment. These results support an assumption that the fluidity of the bilayer near the phospholipid head groups hardly changes during the drug treatment.

In Fig. 5, the effect of temperature on the motion parameter of probes I(5,10) and I(1,14) for the drug-treated and untreated membranes is shown. It is recognized that (1) the spin labels in the drug-treated membrane are always more mobilized than those in the untreated membrane, (2) the spin label mobility

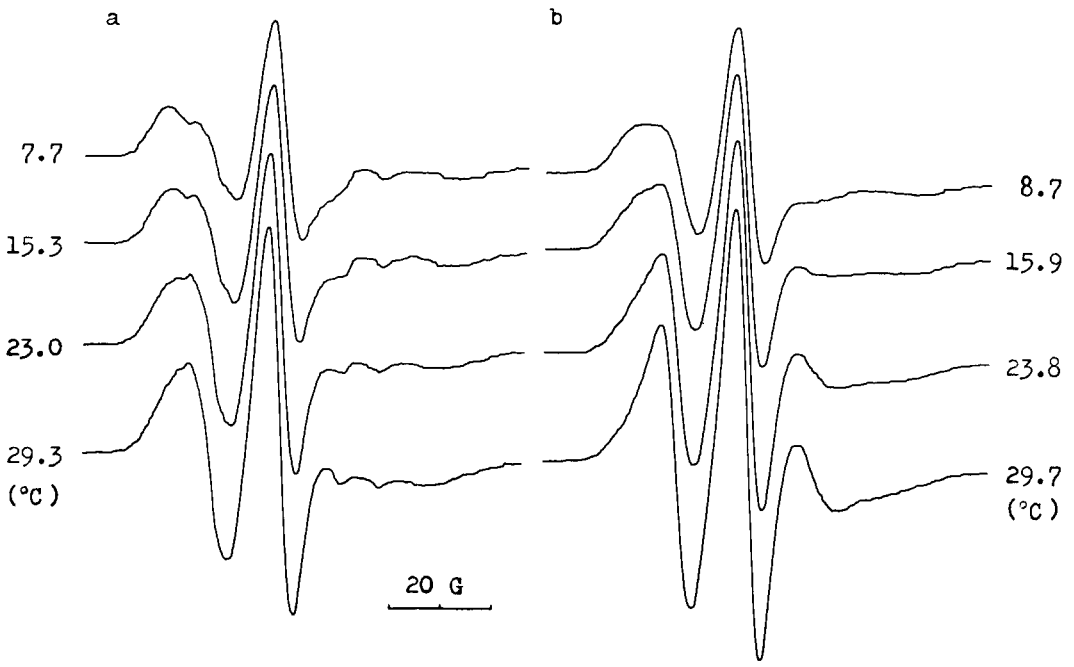

Fig. 2 ESR spectra of I(5,10)-labeled erythrocyte membrane at various temperatures following treatment with chlorpromazine The spin-labeled ghosts (5 mg protein/ml) were treated with 0.6 mM chlorpromazine for 30 min at 37°C (a) untreated ghosts, (b) drug-treated ghosts.

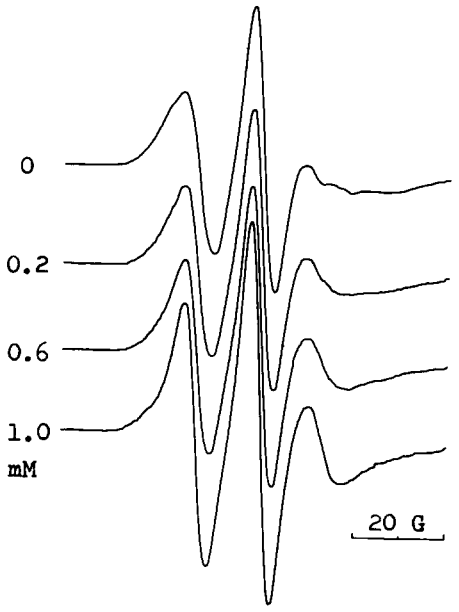

Fig. 3. ESR spectra of I(1,14)-labeled erythrocyte membrane at 37°C following treatment with chlorpormazine. The spin-labeled ghosts (5 mg protein/ml) were treated with 0.2, 0.6 or 1.0 mM chlorpormazine for 30 min at 37°C. 0, untreated ghosts.

is increased with the increasing drug concentrations, and (3) the probe I(5,10) displays relatively more molecular motion than the deeper probe I(1,14) in the drug-treated membrane. The result in Table II also indicates that the molecular motion of the probe I(5,10) is extremely increased by the drug treatment and that the motion is enhanced with increasing drug concentrations. There was more fluidity near the terminal methyl group of the fatty acid side chains than there was closer to the phospholipid head group in the untreated membrane, as indicated by the decrease in the τ_c values for the spectra. Additionally, an apparent temperature break for spin label I(5,10) can be seen at 29.9 ± 0.7°C (n = 5) for the untreated membrane and at 27.1 ± 1.2 (n = 4), 25.7 ± 1.2 (n = 5) and 25.6 ± 0.8°C (n = 4) for the 0.2, 0.6 and 1 mM drug-treated membranes, respectively. The temperature break for the probe I(1,14) was at 25.1 ± 0.8°C (n = 4) and the value was little affected by the drug. The temperature range of the break obtained from the plots was reproducible for several experiments. The τ_c value of probe I(5,10) after 1 mM drug

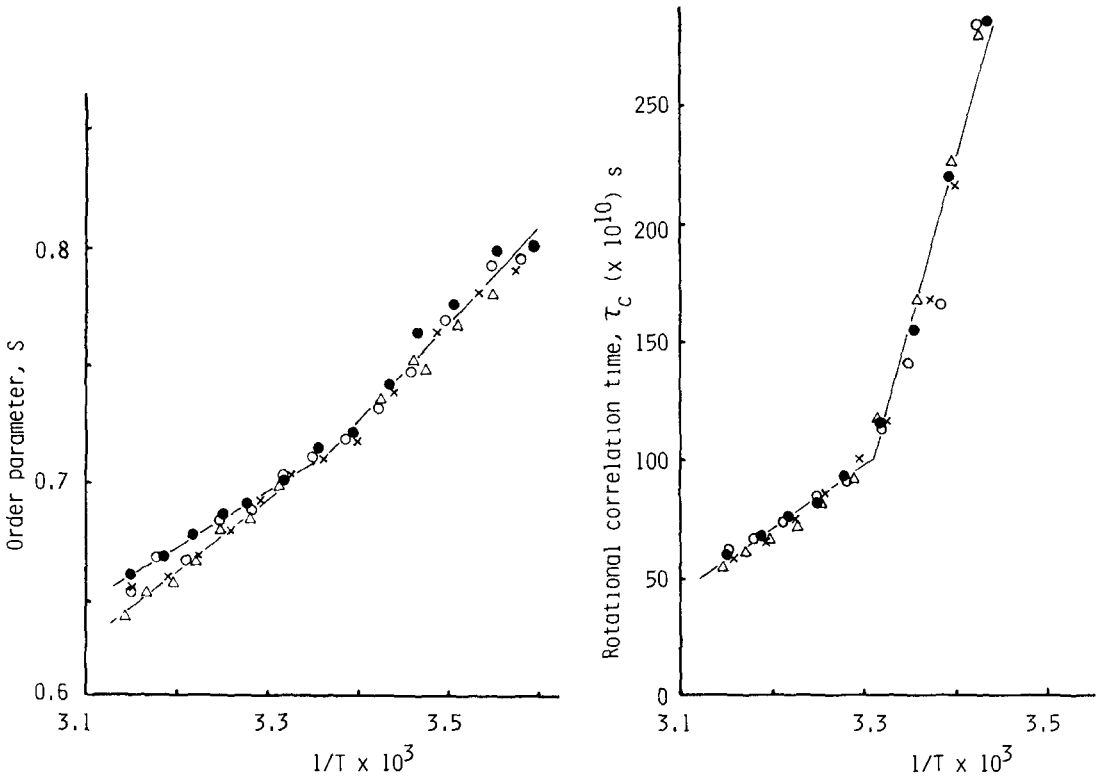

Fig. 4 Temperature dependence of the motion parameters of I(12,3) and the effect of chlorpromazine treatment. Left, the order parameter (S) vs. $1/T$; Right, the apparent rotational correlation time (τ_c) vs. $1/T$. Spin-labeled erythrocyte ghosts (5 mg protein/ml) were treated with chlorpromazine (•, untreated; ○, 0.2 mM; X, 0.6 mM and △, 1 mM) for 30 min at 37°C.

treatment was a half of the value of the untreated membrane at 25.5°C as shown in Table II. Since values of τ_c at a given temperature serve as an index of membrane lipid viscosity [43], assuming that the break temperature represents the phase transition or phase separation of the membrane, it is suggested that this drug treatment decreases a phase separation temperature from the gel to the liquid-crystalline phase

TABLE I

THE FREEDOM OF MOTION OF LIPID SPIN LABEL I(12,3) IN ERYTHROCYTE MEMBRANE TREATED WITH CHLORPROMAZINE

The order parameter, S, and apparent rotational correlation time, τ_c, were calculated from the respective equations described in the text. Values are expressed as mean ± S D. of four experiments.

Drug treatment (mM)	S			τ_c (ns)	
	16°C	25.5°C	37°C	25.5°C	37°C
None	0.76 ± 0.01	0.71 ± 0.01	0 66 ± 0.00	14.87 ± 0.74	8.25 ± 0.31
0.2	0.76 ± 0 01	0.71 ± 0.01	0.67 ± 0.00	14.65 ± 0.60	8.13 ± 0.20
0.6	0.76 ± 0.01	0.71 ± 0 02	0.67 ± 0.00	14.67 ± 0.21	8.04 ± 0.25
1 0	0.76 ± 0.01	0.71 ± 0.01	0.67 ± 0.01	14.73 ± 0.48	8.14 ± 0.31

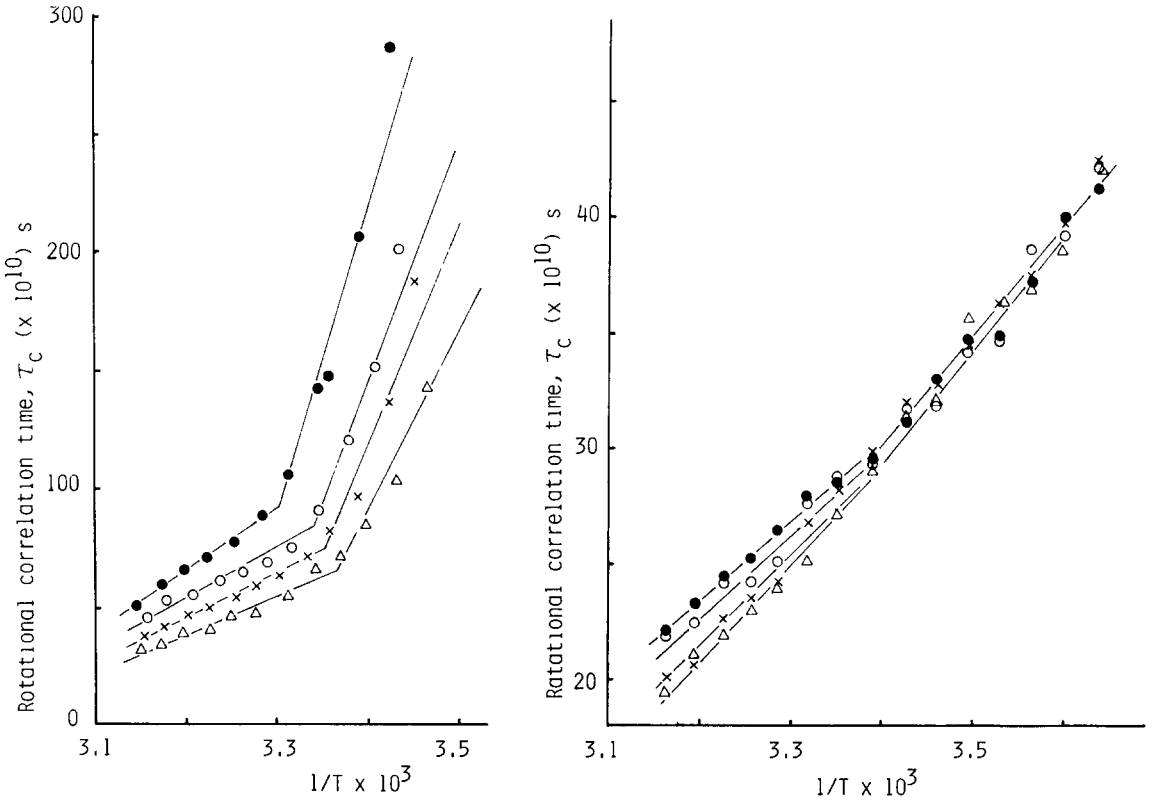

Fig 5. Temperature dependence of the motion parameter of I(5,10) and I(1,14) and the effect of chlorpromazine treatment. Left, I(5,10)-labeled ghosts; Right, I(1,14)-labeled membrane, τ_c vs. $1/T$. The drug treatment is described in Fig. 4.

and that the incorporation of the drug into the membrane makes the lipid environment more fluid than in the normal membrane, this effect on the fluidity of the lipid phase is significant over the lower temperature range.

TABLE II

THE FREEDOM OF MOTION OF LIPID SPIN LABELS I(5,10) AND I(1,14) IN ERYTHROCYTE MEMBRANE TREATED WITH CHLORPROMAZINE

The apparent rotational correlation time, τ_c, was calculated from the equation described in the text. Values are expressed as mean ± S.D. of 4–5 experiments.

Drug treatment (mM)	τ_c (ns)				
	I(5,10)		I(1,14)		
	25 5°C	37°C	16°C	25.5°C	37°C
None	13.66 ± 0 62	6.74 ± 0.23	3.18 ± 0.11	2.90 ± 0.04	2.47 ± 0 03
0.2	8.56 ± 0.49	5.25 ± 0.51	3.20 ± 0.03	2.88 ± 0.02	2.44 ± 0.08
0.6	7.21 ± 0.45	4.66 ± 0.30	3.18 ± 0.06	2.87 ± 0.04	2.30 ± 0.05
1.0	6.93 ± 0.23	4.17 ± 2 27	3.13 ± 0.07	2.73 ± 0.06	2 25 ± 0.08

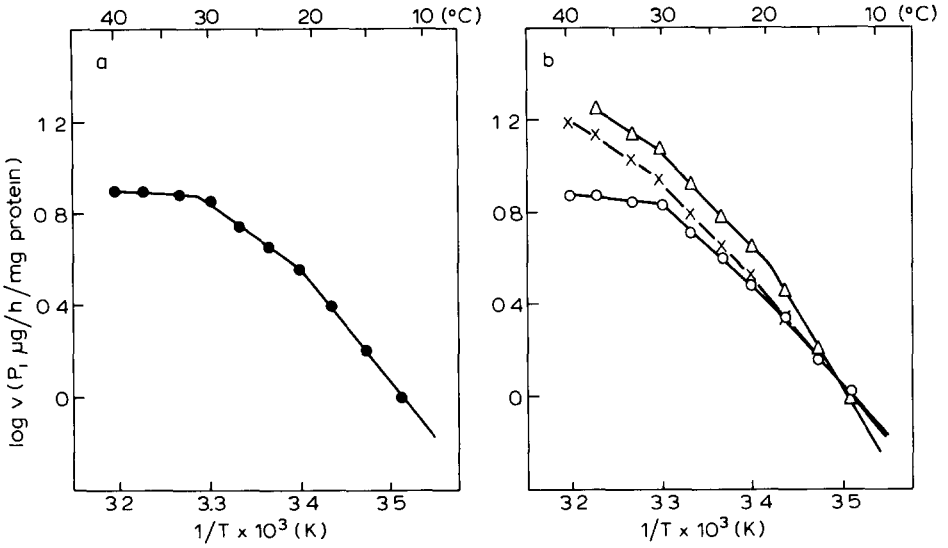

Fig. 6. Temperature dependence of erythrocyte membrane Mg^{2+}-ATPase activity. The ghost membrane was treated or untreated with chlorpromazine, followed by washing once with isotonic buffer, pH 7.4, and the activity was assayed. (a) normal ghosts, (b) drug-treated ghosts (○, 0.2 mM; X, 0.4 mM, △, 0.6 mM).

Effect of chlorpromazine on Arrhenius plots of Mg^{2+}-ATPase activity

The activity of Mg^{2+}-ATPase, which is a membrane-bound enzyme and not inhibited by chlorpromazine [33], was measured following the treatment of membrane with the drug at prelytic (0.2 mM) and lytic (0.6 and 1 mM) concentrations at various temperatures from 12 to 43°C. When the results were plotted according to Arrhenius, two breaks in the slope were observed in all cases as shown in Fig. 6. The two characteristic breaks in the normal membrane were at 21.0 ± 0.5 and 30.1 ± 0.2°C, respectively. The slope of the Arrhenius plots became almost flat above the upper break point in the normal membrane. The drug treatment has little affected the upper break temperature, while it affected slightly

TABLE III

BREAK TEMPERATURES IN ARRHENIUS PLOTS AND ACTIVATION ENERGIES OF Mg^{2+}-ATPase REACTIONS AFTER DRUG TREATMENT

E_1, E_2 and E_3 are the activation energies below the lower break, between the two breaks and above the upper break, respectively, of the slope in Fig. 6. Values are mean ± S.D., the numbers of experiments are in parentheses.

Drug treatment (mM)	Break temperature	Activation energy (kcal/mol)		
		E_1	E_2	E_3
None	21.0 ± 0.5 (7) 30.1 ± 0 2 (6) 21.0 ± 0.4 (5)	22.1 ± 2.3	8.7 ± 1.9	3.0 ± 0.3
0.2	30 3 ± 0 3 (5) 18.9 ± 0.8 (6)	20 8 ± 2.5	15.0 ± 1.9	3.9 ± 0 5
0.6	30.8 ± 0.7 (5) 18.5 ± 1.3 (5)	30.5 ± 3 4	20.4 ± 2.1	12.0 ± 1 4
1.0	30.8 ± 1.4 (4)	35.8 ± 2.0	22.9 ± 0.4	12.0 ± 0.5

the lower break temperature with a minor shift to lower temperatures. The slopes of these plots were reproducible within one membrane preparation, but slightly varied from one batch to the other. Because of the slight variations in different membrane preparations, we shall show the results of a typical experiment in the figure. In Table III, the calculated activation energies above and below the break temperatures are shown together with the break temperatures in the Arrhenius plots. In all cases the activation energy above the upper break temperature is less than that below the temperature. In the normal membrane, the average values for the activation energies of 22.1 ± 2.3, 8.7 ± 1.9 and 3.0 ± 0.3 kcal/mol were obtained below the lower break, between the two breaks and above the upper break, respectively. The activation energies for Mg^{2+}-ATPase activity were significantly increased by the drug exposure and the enhancement was drastic in the activation energies above the upper break. The break temperatures and activation energies, respectively, were approximately similar for the membranes treated with 0.6 and 1 mM drug, which are the concentrations to lyse human erythrocytes, and clearly different from those for the membrane treated at the prelytic (0.2 mM) drug concentration and the normal membrane.

Discussion

In the previous studies [31–33], we have clarified that tranquilizers and antihistaminics caused lysis of erythrocytes at relatively higher concentrations, above 0.3 mM, and that the mechanism was attributed to the increased membrane permeability and disruption of the membrane structure as a result of the drastic changes in the arrangement of the phospholipids and a perturbation of lipid-protein interactions in the membrane. To further clarify the mechanism of the drug-induced hemolysis, the effect of the drug on the membrane fluidity and phase separation was investigated in this study by using the spin label method and estimating the temperature dependence of Mg^{2+}-ATPase activity.

Assuming that the probe molecules align themselves parallel to the phospholipid hydrocarbon chains with their polar group at the membrane-water interface, the three spin labels will monitor at different planes within the erythrocyte lipid bilayer. In this study, spin labeling technique shows that the deeper probe, I(1,14) displays more molecular motion than the superficial one, I(12,3), and that the lipid chains become much more mobile and 'fluid' with increasing temperatures. This is consistent with the data which were studied by spin labeling and deuterium NMR techniques [44–46]. The fluidity of the membrane changes dramatically in the temperature range where a crystalline-liquid crystalline phase transition or phase separation occurs [21,29,47,48]. Our experiments showed that the Arrhenius plots of the apparent rotational correlation time, τ_c, for probes I(12,3) and I(5,10) featured an abrupt inflection at about 30°C and that the plots for I(1,14), the inflection of which is not sharp, at 25°C in the untreated membrane. There is an important possibility that the apparent temperature break may be an artifact because the parametrization may not be valid for the anisotropic molecular orientation and motion. These break points, however, are in close agreement with the data obtained by other workers [42] and the present result obtained from the Arrhenius plot of Mg^{2+}-ATPase activity. Therefore, the break points may reflect the phase separations of the membrane lipids at different planes within the bilayer, the bulk lipids near the lipid-water interface may be in a liquid-crystalline phase above 30°C and the lipids within the hydrocarbon core may be in the same phase above 25°C. This result shows that a large difference in fluidity exists between the interior and the outer surface of the lipid bilayer of the membrane.

The drug treatment of the membrane decreased the break temperature in the Arrhenius plots of τ_c values for the probe I(5,10) spectra, while the treatment had no significant effect on the break temperature for the probe I(1,14) spectra, although the τ_c values were lowered with increasing temperatures. This indicates that the portions of the fatty acid chain near the ten carbon bond lengths removed from the bilayer surface become more fluid when the drug is incorporated into the membrane, probably due to the localization of the drug molecules in the portions, and the tranquilizer induces a further 'melting' of the bilayer over the range of relatively lower temperatures. It is shown from fluorescence measurements that chlorpromazine and propranolol reduced the temperature of the gel to liquid-crystalline phase transition in dipalmitoylphosphatidylcholine and

dipalmitoylphosphatidylethanolamine liposomes [49]. Boggs et al. [50] pointed out that the concentration of anesthetics at which significant increases in fluidity occur corresponds more closely to those causing lysis of erythrocyte ghosts than to those for anesthesia. In our study, the fluidity of the membrane was significantly increased by chlorpromazine treatment, when compared with that of untreated membranes at the same temperature, as indicated by the decrease in τ_c values of probe I(5,10). The extent of the decrease in τ_c values of I(5,10) was approximately proportional to the concentration of the drug, whereas there was no alteration of τ_c values of I(12,3) and I(1,14) at the same temperature except for the change in the values of the latter above 25°C. This suggests that a significant disordering of the local lipid structure is produced by the drug at relatively higher concentrations, consequently the drug treatment probably causing the disruption of the membrane. The greater effect of chlorpromazine on the membrane fluidity is presumably the result of a greater hydrophobicity and molecular size of the drug. Therefore, the increased fluidity of the membrane induced by chlorpromazine appears to be closely related to the lysis of erythrocytes. This consideration is noteworthy in comparison with the result obtained by Lowe and Coleman [51] that the bile canaliculus plasma membrane of low fluidity was less readily lysed by bile salts than the membrane of higher fluidity. The fact that τ_c values of I(5,10) were significantly decreased at lower temperatures after drug treatment is of particular interest out of consideration for the result obtained by Lee [49] that addition of chlorpromazine to lipid bilayers in the liquid-crystalline state produced no significant change in fluorescence intensity. Our results thus suggest that the fluidity of the erythrocyte bilayer in liquid-crystalline state may not be significantly changed by the drug.

It is tempting to investigate the effect of chlorpromazine on the temperature dependence of the Mg^{2+}-ATPase activity in order to clarify dynamic changes in the membrane physical state. As a result, two well-defined breaks, at 21 and 30°C, in the Arrhenius plot of Mg^{2+}-ATPase activity were observed in the normal erythrocyte membrane. The two discontinuities in the Arrhenius plot may be attributed to the temperature-induced alterations in tightly associated lipids with the enzyme protein, and the discontinuities are considered as the transition of the physical state of the associated lipids. The lipids probably exist as the fluid, liquid-crystalline state at the temperature higher than 30°C, the gel, crystalline state below 21°C and the mesomorphic state between 21 and 30°C. These two inflection temperatures approximately agreed with the break points at 18 and 33°C for phosphatidylcholine spin label and at 30°C for phosphatidylserine label in erythrocytes, obtained by Tanaka and Ohnishi [42]. Zimmer and Schirmer [52] described that a nonlinearity in the changes of viscosity with temperature was at 18–19°C in sonicated human erythrocyte membranes and the extracted membrane lipids. Temperature studies of the acetylcholine hydrolysis by erythrocyte enzyme showed a discontinuity in the Arrhenius plot at approx. 20°C [53]. In biological membranes, such as a transition from the gel to the liquid-crystalline state may lower the activation energies of enzymatic reactions of membrane-bound enzymes at above the critical transition temperature [54]. The activation energy of the Mg^{2+}-ATPase reaction has values of 3.0 and 22.1 kcal/mol at temperature ranges above the upper break and below the lower break, respectively, and the energy is 8.7 kcal/mol between the two break temperatures, indicating that the activation energy of the activity is considerably low in the liquid state of the lipids. The drug treatment of the erythrocyte membrane did not dramatically change the break temperatures in comparison with the alterations observed in ESR studies, although there was a minor shift of the lower break point to the low temperature. The reason why the two break temperatures were only slightly shifted by chlorpromazine may be explained by the concept that the enzyme molecule in the membrane is enclosed by a halo of the phospholipid matrix which differs from the bulk lipid matrix of the membrane as it is in a quasi-crystalline state below 30°C and undergoes a transition into a fluid state at 30°C, and that the boundary phospholipids of the Mg^{2+}-ATPase in the membrane are probably more rigid than the bulk lipids.

The increased activation energies after the drug treatment may be accounted for because the drug abnormally changes the conformational flexibility of the enzyme protein, presumably a certain minimum rigidity of the bilayer being essential for maintaining

the active form of the protein, or it locally increases the fluidity of the membrane lipids, thereby somehow interfering with the passage of substrate. Lee [55] suggested that addition of local anesthetics might trigger a change in the surrounding lipids to the liquid-crystalline phase, the new fluid environment then allowing the sodium channel in the nerve to relax into a more stable state, in which the sodium channel is effectively closed.

In conclusion, therefore, the present results lead us to postulate that the bulk lipids near the lipid-water interface of human erythrocyte membrane appear to be in a liquid-crystalline phase above about 30°C, the lipids within the hydrocarbon core appearing to be in the same phase above 25°C as shown by ESR studies. The fluidity of the interior portions in the membrane was extremely increased and the apparent phase separation temperature was decreased by chlorpromazine treatment. The increased fluidity will be closely related to the drug-induced hemolysis. The membrane lipids surrounding the Mg^{2+}-ATPase probably exist in the gel state below 21°C, the mesomorphic state between 21 and 30°C and the fluid, liquid-crystalline state above 30°C. Treatment of the membrane with chlorpromazine only slightly changed the two break temperatures, while the activation energies of the Arrhenius plots were markedly enhanced.

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