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Effect of Drugs on Human Erythrocytes. II.[1] A Possible Mechanism of Drug-induced Hemolysis[2]

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Many kinds of drugs cause lysis of erythrocytes at higher concentrations. To clarify the mechanism of hemolysis, the effect of chlorpromazine and clemastine on the structure and arrangement of the membrane proteins and phospholipids was studied using circular dichroism (CD) and some hydrophobic probes, in addition to studies on the alterations in the components of the cells and using "pink ghosts" containing α-amylase. Initial experiments demonstrated that human erythrocytes were hemolyzed within several minutes at a higher drug concentration (1×10^{-3} or 8×10^{-4}M), while a somewhat lower concentration (6 or 5×10^{-4}M) there was a lag phase for about 30 min and then resulted in severe hemolysis. Dramatic hemolysis occurred at a chlorpromazine concentration which approximately corresponds with the critical micellar concentration. The content of sulfhydryl groups in the cells and calcium in the membrane was not affected by the drug treatment. At above 2×10^{-4}M of both drugs, components I and II (spectrin) were released from the membrane. These drugs, however, had little or no effect on the CD spectra of the ghosts, suggesting that the drug-induced hemolysis is not due to the denaturation of the proteins. 1-Anilinonaphthalene 8-sulfonate (ANS)-ghost fluorescence and 2,4,6-trinitrobenzenesulfonate (TNBS) binding to aminophospholipids were extremely enhanced by the drug exposure. The enhancement of the ANS-ghost fluorescence and TNBS binding with drugs was almost paralleled with that of the hemolytic effect. These drugs thus disturb the arrangement of phospholipids and the hydrophobic interactions between lipids and proteins, and alter the membrane permeability, thereby appear to induce hemolysis.

Keywords——erythrocytes; mechanism of hemolysis; drug-induced hemolysis; phospholipids of membrane; hydrophobic probes; CD spectra

Many kinds of drugs, such as tranquilizers, antihistaminics and anesthetics,[4] in lower concentrations stabilize erythrocytes against hypotonic hemolysis, however, these drugs cause lysis at higher concentrations.[1,4j,o,5] In the case of anesthetics, the bulk concentrations inducing osmotic stabilization correspond rather well to pharmacological anesthetic

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concentrations.[6] The stabilization phase has been ascribed to a reversible fluidization of membrane lipids[7] and there is evidence that such fluidization can be induced in lipid model membranes by anesthetics such as halothane,[8] tetracaine and butacaine,[9] probably due to the increased disordering of the lipid structure.[9] On the other hand, it is suggested that the labilization of the cell membrane at high drug concentrations appears to due to denaturation of membrane proteins.[10] It is also indicated that certain drugs and chemicals alter the permeability or integrity of the red cell membrane resulting in the destruction of the cells[11] and drugs seem to be able to induce erythrocyte hemolysis either through a direct attack on the red cell membrane[11b,12] or indirectly, by inhibition of energy metabolism or oxidation of cellular components.[11,12] Hexachlorophene-induced hemolysis is reported to be probably a secondary event resulting from osmotic swelling subsequent to increased membrane permeability, according to inducing the efflux of Na^+ and K^+ from red cells.[13]

Previous work indicated that chlorpromazine and clemastine induced morphological changes of human erythrocytes and increased the fragility.[1] In order to further clarify the mechanism of the drug-induced hemolysis, a study on the structure of the proteins and the arrangement of the phospholipids, using circular dichroism (CD), a hydrophobic fluorescent prode, 1-anilinonaphthalene 8-sulfonate (ANS),[14] and a non-penetrating probe, 2,4,6-trinitrobenzenesulfonate (TNBS),[15] has been initiated. The measurement of the components of the erythrocyte membrane exposed with these drugs and the preparation of "pink ghosts" containing α-amylase were also carried out for this purpose.

Experimental

Materials——Chlorpromazine hydrochloride (Nihon Shinyaku) and clemastine fumarate (Sankyo Co.) were used throughout this experiment. ANS and TNBS were purchased from Tokyo Kasei Kogyo Co., Ltd. Crystalline bacterial α-amylase (95100 units/g by Blue value method[16]) was obtained from Daiwa Kasei Kogyo Co., Ltd.

Preparation of Erythrocyte Suspension——Human erythrocyte suspensions were prepared by the same method as described in a previous paper.[1] Erythrocytes were suspended in isotonic NaCl solution,[17] pH 7.4, to make finally $40\pm1\%$ hematocrit value.

Preparation of Hemoglobin-free Erythrocyte Ghosts——Hemoglobin-free erythrocyte ghosts were prepared according to the method of Dodge, *et al.*[18] The ghosts were resealed by the procedure of Mueller and Morrison.[19]

Drug-induced Hemolysis and Exposure of Ghosts with Drugs——Drug-induced hemolysis was measured as described in a previous paper.[1] In the experiment using ghosts, to 1 ml of ghost suspension (2.5 mg protein/ml for ANS fluorescence and 4.0 mg protein/ml for TNBS binding) 5.0 ml of the drug solution was added in their final concentrations ranging from 10^{-5} to 10^{-3}M in isotonic NaCl solution and incubated at 37° for 1 hr, followed by centrifugation at $20000\times g$ for 30 min. The ghost bottom was washed with 10 ml of isotonic NaCl solution and centrifuged at the same conditions.

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Determination of Sulfhydryl (SH) Groups and Calcium——The SH groups in the erythrocytes were assayed according to the method of Ellman.[20] After incubation at 37° for 1 hr, to the incubation medium containing erythrocytes and drugs 1 ml of 21.5% trichloroacetic acid was added, mixed for 30 sec and centrifuged. A portion (0.5 ml) of the supernatant was subjected to the assay. For the determination of calcium, the erythrocytes treated with drugs were centrifuged at $1500 \times g$ for 5 min or $20000 \times g$ for 30 min. To the pellet, following washing once with isotonic NaCl solution, 5 ml of HNO_3 and 1.5 ml of 60% $HClO_4$ were added and digested. The final solution was brought to 10 ml by adding 2 ml of 5N HNO_3, 1 ml of 75 mM $SrCl_2$ and the appropriate volume of water. The amount of calcium was determined with a Hitachi atomic absorption spectrophotometer at 422.7 nm.

Preparation from Intact and Drug-induced Erythrocytes of "Pink Ghosts" Containing α-Amylase——Pink ghosts containing α-amylase were prepared by lysing 2.0 ml aliquots of intact and drug-treated (at 7×10^{-4}M and 37° for 1 hr) erythrocytes (hematocrit 40 ± 1%) in 10 ml of ice-cold 31 mosM phosphate buffer, pH 7.4, containing 10 mg of bacterial α-amylase and 2 mM $CaCl_2$. After lysis was complete, 1 ml of 9% NaCl in 0.04M tris–HCl buffer, pH 7.4, was added to restore isotonicity. The suspensions were incubated at 37° for 2 hr, which resulted in resealing of the membranes. After centrifugation at $14000 \times g$ for 20 min, the supernatant layers were carefully removed by aspiration and the ghost pellets were resuspended in fresh 310 mosM phosphate buffer, pH 7.4. The pellets were washed 4 times by resuspension in 310 mosM buffer and sedimentation. Finally the pellets were resuspended in the same buffer to make 10 ml.

Analysis of Protein Components Released from Drug-treated Erythrocytes——After treatment of erythrocytes with drugs, the suspension was centrifuged at $1500 \times g$ for 10 min and the supernatant obtained was dialyzed and lyophilized. The residues were analysed by sodium dodecyl sulfate (SDS) disc electrophoresis and stained according to the procedure of Fairbanks, *et al.*[21]

Surface Tension Measurements of Drugs——Surface activities of varying concentrations of drugs in isotonic NaCl solution were determined at 25° by means of the ring method. The duNouy surface tensiometer was used for the surface tension measurements.

Measurements of CD Spectra——CD spectra of the erythrocyte membranes were measured in a JASCO J-20 automatic recording spectropolarimeter with a cuvette of 0.1 mm-optical path. The erythrocyte ghost suspensions were diluted with isotonic NaCl solution, pH 7.4, to give a concentration of about 2.5—2.8 mg of protein per ml. A mixture consisted of an equal volume of the ghost suspension and the drug solution (at final concentrations of 10^{-5} to 8×10^{-4}M) was incubated at 37° for 1 hr and then sonicated for 40 sec under chilling with ice-NaCl. The amount of α-structure was calculated by following approximate relation[22]: $f_H = [\theta]_{222}/40000$. The mean residue ellipticity, $[\theta]$, was calculated using a mean residue molecular weight of 114.[23]

Measurements of ANS-ghost Fluorescence——After drug-exposure and centrifugation, the ghost bottom, washed once with isotonic NaCl solution, was resuspended in 5.0 ml of isotonic sodium phosphate buffer, pH 7.0, by swirling and 2 ml of the suspension was mixed with an equal volume of 50 μM ANS dissolved in the isotonic sodium phosphate buffer in a quartz cell of 1.0 cm-optical path. Fluorescence of ANS associated with the ghost membrane was measured in a Shimadzu RF-501 recording spectrofluorophotometer. The excitation wavelength was held constant at 380 nm and the emission wavelength was 480 nm.

TNBS Binding to Aminophospholipids——TNBS binding to aminophospholipids was carried out according to the method of Bonsall and Hunt[24] with a slight modification. One ml of drug-treated ghosts suspended in isotonic NaCl solution was mixed with an equal volume of 0.3% TNBS in isotonic phosphate buffer, pH 7.4, at 20°. After 15 min, the reaction was stopped by the addition of 7 volumes of a 1:1 mixture of $CHCl_3$: MeOH to the suspension and vigorously mixed for 2 min. The solution was filtrated and the residues on the filter paper were washed with 3 volumes of the $CHCl_3$: MeOH mixture. The extract was washed with 2 ml of 0.9% NaCl and standed overnight. The lower phase was evaporated to dryness and the remained lipid was dissolved in small amount of the solvent. Individual phospholipid class was separated by thin-layer chromatography (TLC) on Kieselgel 60 HR (Merck) as described in a previous paper.[25]

Results

Time Course of Hemolysis

The time course of *in vitro* hemolysis in the presence of varying concentrations of the drugs is shown in Fig. 1. At a higher concentration of drugs, 10^{-3} M chlorpromazine and 8

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$\times 10^{-4}$ M clemastine, the hemolysis was rapid and 100% hemolysis completed within several minutes, while at a slightly lower concentration, 6×10^{-4} M of the former and 5×10^{-4} M of the latter, the hemolysis within the first 30 min period was extremely slight and beyond the period there was a rapid increase. These results suggest that drug-induced hemolysis of erythrocytes results from direct association of the drugs with the membrane at their higher concentrations and at the relatively lower concentrations it requires for some time to produce damage of the membrane.

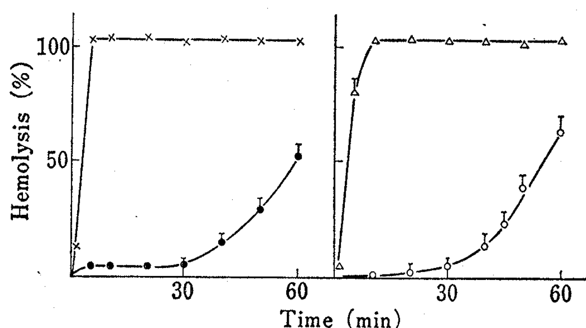

Fig. 1. Time Course of Hemolytic Pattern of Erythrocytes incubated with Drugs

Experimental conditions are described in text. Points represent the mean ±S.D. of 3 experiments. ●: 6×10^{-4}M chlorpromazine, ×: 10^{-3}M chlorpromazine, ○: 5×10^{-4}M clemastine· △: 8×10^{-4}M clemastine

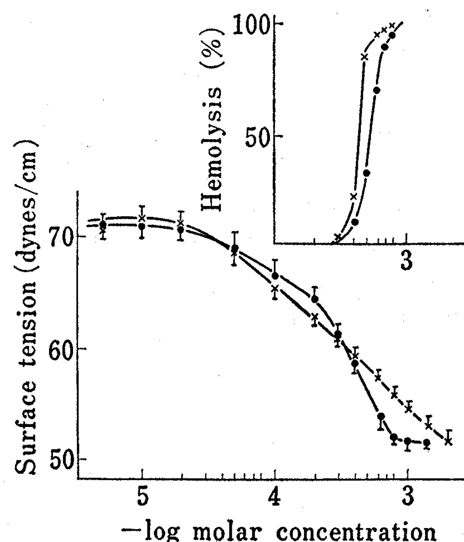

Fig. 2. Relationship between Surface Tension of Drugs and Degree of Hemolysis as a Function of Drug Concentrations

top: percent hemolysis, bottom: surface tension (dynes cm^{-1}) changes as a function of log drug concentration. Points represent the mean ±S.D. of 3 experiments. ●: chlorpromazine; ×: clemastine

Relationship between Surface Tension and Degree of Hemolysis

It has been observed that the therapeutic effects of many phenothiazines and local anesthetics are related to the biphasic properties of these drugs on the red cell membrane.[26] Therefore, it is of interest to study the relationship between the surface tension and degree of hemolysis of the drugs. The results are shown in Fig. 2 with drug-induced hemolysis. These drugs lowered the surface tension with rising drug concentrations. The hemolysis was initiated at 3—4×10^{-4} M of the drugs, at which concentrations the drugs showed much lower surface tension, and dramatic hemolysis occurred at chlorpromazine concentration which approximately corresponded to the critical micellar concentration (CMC). In the case of clemastine, CMC was not observed in concentrations ranging from 10^{-5} to 2×10^{-3} M, however, 100% hemolysis occurred at concentrations above 6×10^{-4} M. These suggest that the hemolytic actions of the drugs are displayed at much lower surface tension and the lowered surface activity is clearly related to the increased hemolysis.

Effect of Drugs on Erythrocyte Membrane

1) Changes in Amount of SH Groups and Calcium in Erythrocytes and Ghost membrane——Since a number of investigators by chemical modification studies of erythrocyte membranes have shown that SH groups are important to maintenance of different membrane

26) P. Seeman, *Pharmacol. Rev.*, **24**, 583 (1972).

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functions,[27] SH content in the erythrocytes or the hemolysates after drug treatment was assayed. The amount of SH groups in the suspension was found to be little or no change in all samples (controls; 0.61—0.79 μmole, the drug-treated erythrocytes; 0.65—0.81 μmole/ml of the suspension), suggesting that the drugs have no effect on the erythrocyte SH groups. The SH groups of the drug-treated (at 10^{-5}—3×10^{-4} M) erythrocytes, separated and washed with isotonic NaCl solution, was also assayed and the result similarly showed that the drugs had no effect on the content.

Calcium ions were found to exert protective effect on hypotonic hemolysis of human erythrocytes,[28] therefore, the amount of calcium in erythrocytes and ghost membrane was assayed. As shown in Table I, the drug treatment decreased the calcium content in the erythrocytes with increasing the drug concentrations and both drugs at 7×10^{-4} M decreased the content by about one half as compared with the controls, probably due to efflux of the inner calcium by destruction of the membrane functions. On the other hand, the calcium content of the ghosts treated with drugs was shown not to change (controls and chlorpromazine-treated ghosts; 0.065—0.076, clemastine-treated ghosts; 0.068—0.079 μg/ml of ghost suspension), indicating that the drugs were not capable of diminishing the calcium tightly bound to the membrane.

Table I. Calcium Content of Erythrocytes treated with Drugs

Drug concentration (M)	Chlorpromazine		Clemastine	
	μg/ml cells[a]	%	μg/ml cells[a]	%
None	0.23 ± 0.03	100	0.22 ± 0.03	100
2×10^{-4}	0.22 ± 0.03	97 ± 4	0.22 ± 0.03	102 ± 2
3×10^{-4}	0.23 ± 0.03	100 ± 3	0.22 ± 0.02	100 ± 2
4×10^{-4}	0.20 ± 0.02	88 ± 8	0.18 ± 0.03	80 ± 6
5×10^{-4}	0.16 ± 0.02	68 ± 10	0.12 ± 0.02	54 ± 7
6×10^{-4}	0.13 ± 0.02	55 ± 5	0.11 ± 0.02	49 ± 5
7×10^{-4}	0.13 ± 0.02	56 ± 5	0.11 ± 0.02	48 ± 2
H_2O	0.11 ± 0.02	49 ± 2	0.11 ± 0.01	48 ± 5

After the drug treatment at 37° for 1 hr, the suspension was centrifuged at $1500\times g$ for 5 min or $20000\times g$ for 30 min and the content in the pellet was assayed. Values are means ±S.D. of 3 experiments. *a*) Ca μg/ml of erythrocyte suspension

2) **Packing of α-Amylase into "Pink Ghosts" treated with Drugs**——To clarify the extent of the membrane damage with drugs and the reversibility, a technique packing α-amylase into the erythrocytes treated with and without drugs was used. The α-amylase activity of the pink ghosts and the supernatant after incubation at 37° for 30 min was assayed. As shown in Table II, pink ghosts from drug-treated erythrocytes contained much smaller enzyme activity, about one-eighth to one-tenth of untreated ghosts and the low activity is due to unsealing of the cell membrane, while untreated ghosts had a high activity and the enzyme activity released from the pink ghosts under the condition was slight as compared with the drug-treated ghosts. The results indicate that the damage of the cell membrane with these drugs was by far drastic and the resealing of the drug-treated membrane was not complete, meaning irreversibility of the alteration of the membrane on the drug treatment.

27) *a*) R.I. Weed and C.F. Reed, *Am. J. Med.*, **41**, 681 (1966); *b*) A.F. Rega, A. Rothstein, and R.I. Weed, *J. Cell. Physiol.*, **70**, 45 (1967); *c*) R.M. Sutherland, A. Rothstein, and R.I. Weed, *J. Cell. Physiol.*, **69**, 185 (1967); *d*) J. van Steveninck, R.I. Weed, and A. Rothstein, *J. Gen. Physiol.*, **48**, 617 (1965).
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Table II. Release of α-Amylase from Pink Ghosts Containing the Enzyme and Activity in Pink Ghosts

Sample		Activity (units/ml)[a)		
		Control	Chlorpromazine[b)]	Clemastine[b)]
Released during incubation[c)]	A	0.31	0.63	0.74
	B	0.29	0.65	0.81
Pink ghosts[d)]	A	22.19	2.68	2.25
	B	22.73	2.51	2.29

a) units/ml of pink ghost suspension
b) The drug concentration was 7×10^{-4}M.
c) incubated at 37° for 30 min in isotonic phosphate buffer, pH 7.4
d) After pink ghosts were lysed in 8 ml of 31 mosM phosphate buffer, pH 7.4, the activity was assayed.

3) **Release of Proteins from Drug-treated Erythrocytes**——The data on release of proteins from drug-treated erythrocytes are shown in Fig. 3. At lower drug concentrations, a slight amount of component V (band nomenclature adapted from Fairbanks, *et al.*[21)]) was released from the cells with hemoglobin and at above 2×10^{-4} M components I and II (spectrin) released. The initial concentration of the drugs inducing the dissolution of spectrin was close to the drug concentrations (3—4×10^{-4} M) initiating hemolysis.

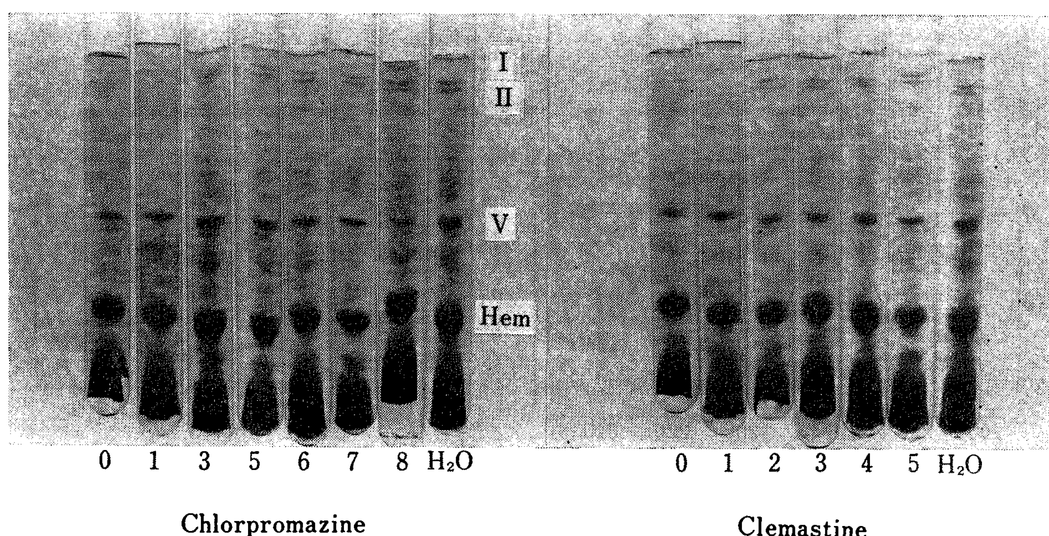

Fig. 3. Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoretic Patterns of Released Proteins from Human Erythrocyte Membrane treated with Drugs

0: no drug, 1: 1×10^{-4}M, 2: 2×10^{-4}M, 3: 3×10^{-4}M, 4: 4×10^{-4}M, 5: 5×10^{-4}M, 6: 6×10^{-4}M, 7: 7×10^{-4}M, 8: 8×10^{-4}M, H_2O:in H_2O. I, II, V: components, Hem: hemoglobin

4) **CD Spectra of Ghost Membrane treated with Drugs**——A presumption, drawn from the studies on stabilization of the erythrocyte membrane by anti-inflammatory drugs,[29)] that the denaturation of the membrane protein may cause the hemolysis made us measure the CD spectra of drug-treated ghosts. The result is shown in Fig. 4 and Table III. The CD spectrum of the untreated ghosts exhibited two negative extrema at 208 and 222 nm, which are indicative of helical polypeptide conformation. The magnitude of $[\theta]$ at 222 nm was found to be -15973, and the helical content of the intact ghost proteins was estimated

29) *a*) Y. Mizushima and S. Sakai, *J. Pharm. Pharmacol.*, **21**, 327 (1969); *b*) Y. Mizushima, S. Sakai, and M. Yamamura, *Biochem. Pharmacol.*, **19**, 227 (1970); *c*) A.D. Inglot and E. Wolna, *Biochem. Pharmacol.*, **17**, 269 (1968).

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to be 39.9%, if a value of $[\theta]_{222}$ of −40000 is assumed for completely helical polypeptide.[22] The value is consistent with the data obtained by many investigators.[30] The drugs, under the conditions used, had little or no effect on the CD spectra and the value of $[\theta]_{222}$ for the proteins, even at a higher concentration of 8×10^{-4} M, at which concentration erythrocytes were completely hemolyzed. This suggests that these drugs have no denaturating effect on the membrane proteins under the conditions tested and the drug-induced hemolysis is not due to the denaturation of the membrane proteins.

TABLE III. The Values of $[\theta]_{222}$ and α-Helical Composition of Ghosts treated with Drugs

Chlorpromazine (M)	$[\theta]_{222}$	α-Helix (%)	Clemastine (M)	$[\theta]_{222}$	α-Helix (%)
None	15973	39.9	None	15973	39.9
1×10^{-5}	16487	41.1	1×10^{-5}	16943	42.4
5×10^{-5}	16440	41.1	5×10^{-5}	16335	40.8
1×10^{-4}	16511	41.3	1×10^{-4}	15638	39.1
2×10^{-4}	16182	40.5	2×10^{-4}	16183	40.5
3×10^{-4}	16314	40.8	3×10^{-4}	15662	39.1
4×10^{-4}	16277	40.7	4×10^{-4}	16031	40.1
6×10^{-4}	15524	38.8	6×10^{-4}	16616	41.5
8×10^{-4}	16356	40·9	8×10^{-4}	15355	38.4

Figures are means of values in 3 experiments.

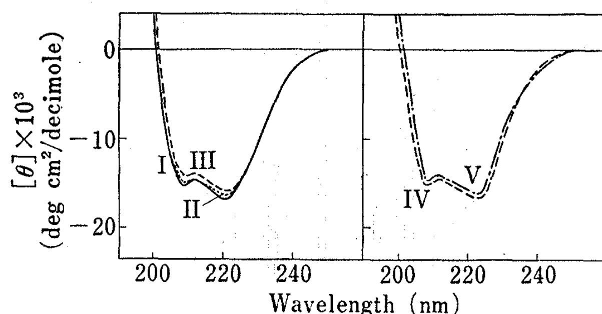

Fig. 4. Far-ultraviolet CD Spectra of Ghosts treated with Drugs

I: control, II: 2×10^{-4}M chlorpromazine, III: 8×10^{-4}M chlorpromazine, IV: 2×10^{-4}M clemastine, V: 6×10^{-4}M clemastine

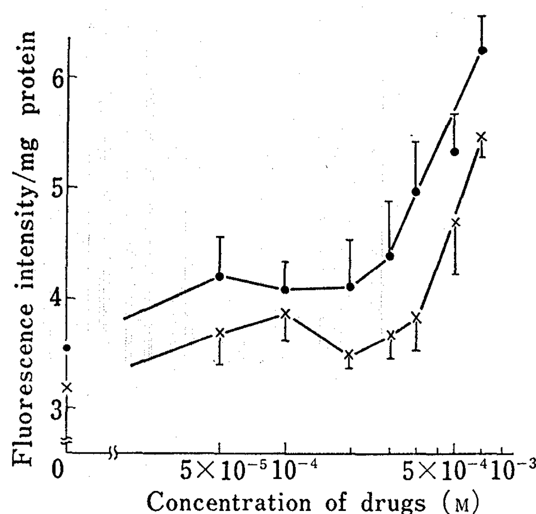

Fig. 5. ANS Binding to Ghosts Treated with Drugs

Points represent the mean ±S.D. of 4 experiments.
●: chlorpromazine ×: clemastine

5) **ANS-ghost Fluorescence**——ANS, a hydrophobic fluorescent probe,[14] was used to study the conformational changes of drug-treated ghost membrane. As shown in Fig. 5, ANS-ghost fluorescence was significantly enhanced in the ghosts treated with drugs at above 4×10^{-4} M, probaly due to an increase in the membrane fluidity. This suggests that these drugs caused a significant alteration of the arrangement of the hydrophobic region in the membrane. It is noteworthy that the enhancement of the fluorescence intensity with the drugs was almost paralleled with the increase in hemolysis.

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6) TNBS Binding to Aminophospholipids——TNBS has been used to study the outside of the erythrocyte membrane,[27d,31] and it cannot penetrate the permeability barrier of the membrane and easily binds to amine groups of phospholipids and proteins on the outer membrrane.[24] To clarify the effect of the drugs on arrangement of the membrane aminophospholipids of resealed ghosts, phospholipids of the ghosts treated with chlorpromazine were separated by TLC and the extent of reaction of TNBS with the aminophospholipids was estimated by ninhydrin reaction. The result shown in Table IV indicates that a minor part (10%) of phosphatidylserine (PS) and a greater part (68%) of phosphatidylethanolamine (PE) in untreated ghosts reacted with the reagent, in accord with the result of Bonsall and Hunt[24] that PE are preferentially located at the outer surface and PS at the inside of the ghost membrane. The treatment of the ghosts with the drug induced an increase in the amount of the PS and PE reacted with the reagent; the drug at above 2×10^{-4} M produced an extreme increase in trinitrophenylated phospholipids and at above 4×10^{-4} M essentially all the PE could react with the reagent. An increase in the accessibility of PS and PE to TNBS may be due to extreme alterations of the membrane permeability. The data also approximately agreed with those of the ANS-fluorescence and the hemolysis.

Table IV. TNBS Reaction with Aminophospholipids on Ghosts treated with Chlorpromazine

Drug concentration[a] (M)	PS reacted with TNBS (%)	PE reacted with TNBS (%)
None	9.8	68.4
5×10^{-5}	7.4	76.9
1×10^{-4}	8.1	78.2
2×10^{-4}	18.7	81.6
4×10^{-4}	54.5	96.1
6×10^{-4}	76.3	97.4
8×10^{-4}	80.2	97.7

Each value represents the mean of 3 experiments.
a) concentration of drug in the incubation medium

Discussion

Studies on the effect of drugs on erythrocytes have been promoted by a number of investigators. Many kinds of drugs cause lysis at higher concentrations,[1,4j,o,5] however, the mechanism for drug-induced hemolysis at higher concentrations has scarcely been clarified. Thus, attention has turned to studies designed to elucidate the mechanism of hemolysis in an attempt to prevent an adverse reaction of drugs, such as a lytic action on cells. Morphological changes and the increase in fragility of erythrocytes treated with some drugs, including chlorpromazine and clemastine, have already been reported by the present authors.[1]

In the present study, chlorpromazine and clemastine at higher concentrations, 10^{-3} and 8×10^{-4} M respectively, took an immediate hemolytic action on the drug exposure, while at somewhat lower concentrations, 6×10^{-4} and 5×10^{-4} M respectively, there was a lag phase for about 30 min and then resulted in severe hemolysis (Fig. 1). A possible interpretation is that the drugs at higher concentrations result in the destruction of the cell membrane through direct association, while at somewhat lower concentrations (6 or 5×10^{-4} M) they appear to induce the membrane damage indirectly or directly after drug penetration into the membrane. Their hemolytic action at higher concentrations is probably, at least partially, due to their surface activities, because dramatic hemolysis occurred at drug con-

31) A.H. Maddy, *Biochim. Biophys. Acta*, **88**, 390 (1964); H.C. Berg, *Biochim. Biophys. Acta*, **183**, 65 (1969).

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centrations which showed much lower surface tensions. A significant correlation between the degree of hemolysis and surface activities of these drugs suggests that the nature of drug-phospholipid interaction appears to be due to their hydrophobic interactions.

It is suggested that SH groups play a role in hydrophobic bonding of proteins to erythrocyte membrane and many of the effects attributed to SH binding agents may be related to nonspecific structural alterations in the membrane.[32] Haest and Deuticke postulated that disulfide bond fromation between membrane protein SH groups leads to an alteration in protein-phospholipid interactions.[33] However, the present study showed that SH groups in the membrane was not affected by the drug treatment, suggesting that the drug-induced hemolysis is not involved in the alteration of SH groups, such as disulfide formation.

The effect of the drugs on calcium in erythrocyte membrane is of considerable interest because of a possible involvement of Ca^{2+} in erythrocyte deformability and destruction[34]; introduction of Ca^{2+} into resealed ghosts from fresh erythrocytes causes significant volume loss[35] and transforms them into rigid spheres.[34a] Palek, *et al.* have suggested that a Ca^{2+}-activated adenosine triphosphatase (ATPase) might be involved in the contraction of the cell.[36] Our experiments on the effect of the drugs on the calcium content of erythrocytes and the membrane indicated that the drugs had no effect on the content of calcium tightly bound to the membrane, although Ca^{2+} in the cells increasingly released with rising the drug concentrations (Table I). Therefore, a possibility that the hemolysis of the erythrocytes may be related to the loss of calcium in the membrane is ruled out.

The experiment using "pink ghosts" containing α-amylase showed that the erythrocyte membrane exposed with 7×10^{-4} M drugs were subjected to severe damage and the pore size produced was larger than the molecular size of bacterial α-amylase and hemoglobin. The view is strongly supported by electron microscopic observations as reported previously.[1] This result thus provides an evidence of the irreversibility of the membrane alteration induced with the drugs.

We found that the components I and II (spectrin) which localized at the inner surface of red cell membrane[37] released from the cells during an incubation with the drugs at concentrations higher than 2×10^{-4} M. It is not clear on the basis of the result obtained whether or not the release of spectrin on drug treatment can lead that of hemoglobin. The reports, however, that spectrin may form filamentous networks adherent to the cytoplasmic surface and such networks would offer structural support to the deformable lipid bilayer structure,[38] and profound alterations in the membrane structure were induced following spectrin removal[39] should be noted in consideration of the present result. This result that components I, II and V released during drug treatment is of interest in comparison with the data presented by Fairbanks, *et al.* that incubation of ghosts at low ionic strenght could produce the selective release of components I, II and V.[21] These components may be tenuously related to the membrane, therefore, they might be easily released from the membrane on the drug treatment.

Many investigators suggested that stabilization of erythrocyte membrane by anti-inflammatory drugs may arise from a stabilizing effect of the drugs on some proteins in the cell membrane.[29a,c,40] To clarify an involvement of denaturation of the membrane protein

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37) *a*) T.J. Mueller and M. Morrison, *J. Biol. Chem.*, **249**, 7568 (1974); *b*) R.L. Juliano, *Biochim. Biophys. Acta*, **300**, 341 (1973).
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to drug-induced hemolysis, the CD spectra of the membrane were measured in the presence of chlorpromazine and clemastine. The result indicated that no denaturation of the membrane proteins was found during the drug treatment, even at which concentration 100% hemolysis occurred (Fig. 4 and Table III). Thus, the drug-induced hemolysis is not related to denaturation of the membrane proteins.

The enhancement of ANS-ghost fluorescence and TNBS binding on the drug treatment suggests that the erythrocyte membrane was subjected to drastic alterations with drugs (Fig. 5 and Table IV). There are several findings to suggest that ANS-microsome fluorescence is largely attributable to ANS binding to membrane phospholipids[14,41]; phospholipase C or D markedly decreased ANS-microsome fluorescence and quantum yield of ANS-microsome fluorescence appears to be related directly to phospholipid content of microsomes.[14] ANS is also considered to be located near the membrane interface and to probe the glycerol region of the lipid bilayer.[42] Therefore, the enhancement of ANS-ghost fluorescence by the drug treatment is probably due to the increase in phospholipids in the membrane, which are able to react with ANS. The view is strongly supported by the present data that TNBS binding to aminophospholipids was significantly increased by chlorpromazine treatment and all the PE was modified with the reagent in the presence of above 4×10^{-4} M chlorpromazine, in accordance with the result that chlorpromazine-membrane interaction is hydrophobic.[43] It is suggested that the rate of the reaction of PE with TNBS is used as an indicator for such an alteration of lipid-protein interactions, and the increase in accessibility of PE resulted from changes of its arrangement of lipid-protein interactions normally stabilized by glycolytic metabolism.[44] Thus, these drugs dramatically alterate the arrangement of phospholipids, including the asymmetric distribution, in the erythrocyte membrane and consequently affect the specific lipid-protein interactions, which play a direct role in a variety of membrane functions.[45] Lenard and Singer[46] treated erythrocyte membrane with phospholipase C and found that in spite of the profound perturbation of the physical state of the residual fatty acid chains, the average protein conformation in the membrane, as determined by CD measurements, is unaffected. This is of interesting in comparison with our data that despite the drastic alteration of phospholipid arrangement the conformation of the membrane proteins remained unaffected. The data shown by us and Lenard and Singer[46] indicate that the phospholipids and proteins of the membrane do not interact strongly.

The functional properties of many proteins in many different biological membranes seem to be intimately related to the microenviroment provided by membrane lipids[47] and the proteins of the erythrocyte membrane are no exception. Although the involvement of the glycolytic and active transport enzymes to the drug-induced hemolysis is unable to neglect, their involvement seems to be a little on the basis of the data on the time course of hemolysis, CD spectra, ANS-ghost fluorescence and TNBS binding.

One important fact that the intact cell and resealed ghost may differ with respect to certain biochemical parameter[37b] and the accessibility of lipids[48] should be noted in this study. However, it is clear that certain ghost preparations can closely mimic the permeability of intact red cells, including asymmetric activation of $(Na^+—K^+)$ATPase,[49] active ion

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uptake,[50] facillitated diffusion of glucose[51] and relatively slow passive cation fluxes,[52] arrangement of proteins[37b] and orientation of the membrane.[37a] Despite the qualified nature of such a study, it seems possible, nevertheless, to draw many limited conclusions from the results of this study.

In conclusion, therefore, the present results lead us to posturate that the drugs bind to the hydrophobic region of the membrane and immediately or following penetration disturb the arrangement of phospholipids, including the asymmetric distribution, in the membrane, thereby weaken or cleave the hydrophobic interactions between lipids and proteins, and induce the disruption of the membrane structure and the formation of pores, through which hemoglobin is released out. The consequently increased membrane permeability, shown by the accelerated efflux of K^+ from red cells as reported previously,[1] appears to be, at least partially, related to the destruction of the membrane functions.

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