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Relationship between Hemolytic Concentrations and Physicochemical Properties of Basic Drugs, and Major Factors Inducing Hemolysis

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To clarify the differences in hemolytic activities between basic drugs and to identify the major factors inducing hemolysis, some tranquilizers and antihistaminics were compared with each other as regards hemolytic activities, van der Waals volume (V), pK_a, partition coefficient (octanol/water, P), lipid spin labeling and membrane/buffer partition ratio ($P_{m/b}$), and correlation analyses were carried out among these parameters. The best correlation was between logarithmic $P_{m/b}$ for drugs and $1/C_1$ (C_1; the initial concentration inducing hemolysis, $r=0.877$, $p<0.01$) or $1/C_{50}$ (C_{50}; the concentration inducing 50% hemolysis, $r=0.924$, $p<0.01$). Less good correlations were observed between the logarithmic V ($r=0.708$, $p<0.05$; $r=0.780$, $p<0.05$) or the fluidity of lipids ($r=0.711$, $p<0.05$; $r=0.619$) and $1/C_1$ or $1/C_{50}$, respectively. No correlation, however, was found between P and hemolytic concentrations. These results indicate that the amount of drugs penetrated into the membrane is probably a major factor in the induction of hemolysis, and that large molecular volume of a drug, increased fluidity of the membrane lipids and un-ionized concentration of the drug significantly affect the drug-induced hemolytic process.

Keywords——correlation between hemolysis and physicochemical property of basic drug; drug-induced hemolysis; basic drug; membrane/buffer partition coefficient; van der Waals volume; pK_a; fluidization

Many kinds of drugs, such as tranquilizers, antihistaminics and anesthetics, stabilize erythrocytes against hypotonic hemolysis at low concentrations,[1-13] but these drugs cause lysis at higher concentrations.[9,13-16] All of these drugs therefore exert a biphasic drug concentration-dependent effect on the erythrocyte membrane. The mechanism of hemolysis of erythrocytes at higher drug concentrations has been studied.[15,17] We have proposed that the hemolysis induced by chlorpromazine and clemastine is probably attributable to the increased membrane permeability and to disruption of the membrane structure as a result of drastic changes in the arrangement of phospholipids and a perturbation of lipid–protein interactions in the membrane.[18] We also find that the fluidity of interior portions in the membrane is greatly increased by chlorpromazine and that the increased fluidity may be closely related to the drug-induced hemolysis.[19]

Previously, we have found differences in hemolytic activity between drugs, such as tranquilizers and antihistaminics;[16,20] all of these drugs have different effects on the rate and extent of hemolysis. In order to clarify the reason why characteristic differences arise in hemolytic effect between drugs and to identify the major factors involved in hemolysis, a series of phenothiazines and some tranquilizers were compared with each other as regards hemolytic activities and physicochemical properties, *i.e.* van der Waals volume of the drug, pK_a, partition coefficient in the octanol/water system, membrane/buffer partition ratio and lipid spin labeling, and correlation analyses were carried out among the factors.

Experimental

Drugs——Promazine hydrochloride (Hokuriku Pharmaceutical Co., Fukui), promethazine hydrochloride, prochlorperazine methanesulfonate, levomepromazine hydrochloride (Shionogi Pharmaceutical Co., Osaka), trimeprazine tartrate (Dai-ichi Pharmaceutical Co., Tokyo), perazine dimalonate (Morishita Pharmaceutical Co., Osaka), chlorpromazine hydrochloride, prothipendyl hydrochloride (Nippon Shinyaku, Kyoto), homochlorcyclizine hydrochloride (Eizai Co., Tokyo) and carpipramine hydrochloride (Yoshitomi Pharmaceutical Co., Osaka) were used throughout this experiment. The spin label, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as I (1, 14)) was supplied by Syva Co. (Palo Alto, U.S.A.) and was used without further purification. The other reagents were of special or analytical grade.

Preparation of Erythrocyte Suspension and Hemoglobin-Free Erythrocyte Ghosts——Acid citrate dextrose (ACD)-blood, obtained from healthy adult donors, was washed three times with isotonic NaCl–phosphate buffer (NaCl 90.0 g, $NaH_2PO_4 \cdot 2H_2O$ 3.43 g and $Na_2HPO_4 \cdot 12H_2O$ 34.425 g/l, pH 7.4), and erythrocytes were resuspended in the washing solution to make $40 \pm 1\%$ hematocrit. The erythrocyte ghosts were prepared by the method of Dodge *et al.*[21]

Drug-Induced Hemolysis——A 0.3 ml aliquot of the erythrocyte suspension (hematocrit value, $40 \pm 1\%$) was added to 3 ml of drug solution at various concentrations and mixed immediately. The test solution was ordinarily prepared in isotonic NaCl–phosphate buffer, pH 7.4. The mixture was incubated for 60 min at 37 °C and after centrifugation the percentage hemolysis was determined by measuring the absorbance of hemoglobin in the supernatant at 543 nm.

Calculation of van der Waals Volume——The van der Waals volume of each drug used was calculated by the method of Bondi.[22]

Measurement of Partition Coefficient in the *n*-Octanol/Water System——A drug was dissolved at 0.2 mM in 10 ml of *n*-octanol (saturated with 1 N NaOH) and the solution was added to 10 ml of 1 N NaOH (saturated with *n*-octanol). After vigorous shaking for 60 min at 20—25 °C the phases were separated by centrifugation. The substance in both phases was measured by means of spectrophotometry at the extinction maximum for each drug. The partition coefficient (P) was calculated according to the formula:

$$P = \frac{\text{concentration of drug in } n\text{-octanol}}{\text{concentration of drug in water}}$$

Measurement of pK_a Value——The pK_a value of each drug was measured by using the spectroscopic method of Albert and Serjeant[23] at 20—25 °C. Some of the pK_a value were taken from reference data.[24]

Measurement of Membrane/Buffer Partition Ratio——The partition of each drug between the ghost membrane and isotonic solution, pH 7.4, was carried out as follows. A ghost suspension (2 ml; 5 mg protein/ml) was added to 10 ml of 0.24 mM drug in isotonic buffer, and the mixture was incubated for 60 min at 37 °C. The ghost membrane was quantitatively washed twice with 10 volumes of isotonic buffer. Following centrifugation, 4 ml of 0.01 N NaOH was added to the ghost pellet and the whole was mixed vigorously for 30 s. The suspension was extracted with 15 ml of water-saturated ethyl acetate in a 40 ml centrifuge tube by shaking for 15 min, and the two layers were separated by centrifugation. Ten ml of the ethyl acetate layer was pipetted off, 5 ml of 0.1 N HCl was added to the solvent layer, and the drug was extracted into 0.1 N HCl by shaking followed by centrifugation. The aqueous layer was carefully and as completely as possible transferred to another tube. The remaining ethyl acetate layer was again extracted with 5 ml of 0.1 N HCl. Subsequently, the drug concentration in both aqueous layers was spectrophotometrically determined at the extinction maximum of the drug. The membrane partition ratio ($P_{m/b}$) was calculated by means of the following equation:

$$P_{m/b} = \frac{\text{amount of drug in membrane}}{\text{amount of drug added}} \times 100$$

Since the drug extraction ratio by this method was different for each drug, the ratio was determined by the same method after addition of a definite amount of drug to the ghost membrane, and the partition ratio for each drug was corrected by using the extraction ratio thus obtained.

Electron Spin Resonance (ESR) Measurement——The method of preparing spin-labeled I (1, 14) ghosts was described previously.[19] The ESR spectra of the packed sample were recorded by using a JEOL JES-PE-3X ESR spectrometer (at 8.0 GHz, 100 kHz field modulation) with a variable temperature accessory. The rotational correlation time, τ_c, was calculated according to the previous method.[19]

Protein Determination——Protein concentration was determined by the procedure described by Lowry *et al.*[25] with bovine albumin, fraction 5, as a standard.

Data Analysis——The calculation and statistical analyses were carried out with the aid of a Sharp MZ-80B personal computer. Correlation analyses were done by the least-squares linear regression method. Correlation coefficients were examined for significance ($p < 0.05$) by means of the t-test.

Results

Drug-Induced Hemolysis

Plots of percentage hemolysis against drug concentration are shown in Fig. 1. The drugs were found to show characteristic differences in hemolytic activities; prochlorperazine and carpipramine produced the severest lysis, while prothipendyl had less effect. The initial concentration (C_1) at which hemolysis was initiated was determined from the intercept obtained by extrapolating the plot to zero per cent hemolysis. The C_1 and C_{50} (the concentration inducing 50% hemolysis) values are shown in Table I.

Relationship between van der Waals Volume (V) and C_1 or C_{50} Value

The calculated van der Waals volumes of these drugs were compared with the C_1 or C_{50} values. The plots of the logarithmic V *vs.* reciprocal of C_1 or C_{50} value are shown in Fig. 2. The relationship was approximately linear and there was a rough correlation between the parameters ($r=0.708$ for $1/C_1$ and $r=0.780$ for $1/C_{50}$, $p<0.05$ in each case). This suggests that a large molecular volume drug in the membrane may encourage hemolysis.

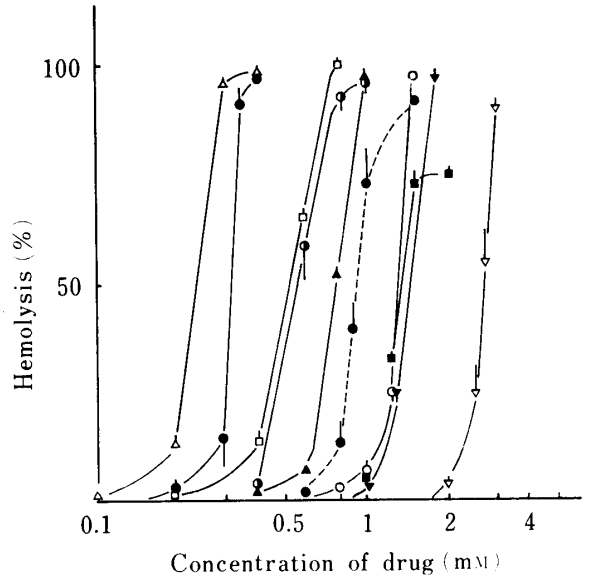

Fig. 1. Lytic Effect of Various Basic Drugs on Human Erythrocytes

Experimental conditions are described in the text. Each point represents the mean ± S.D. of four experiments. Promazine (○), promethazine (■), trimeprazine (▼), perazine (◑), chlorpromazine (□), prochlorperazine (△), levomepromazine (---●), prothipendyl (▽), homochlorcyclizine (▲), carpipramine (●).

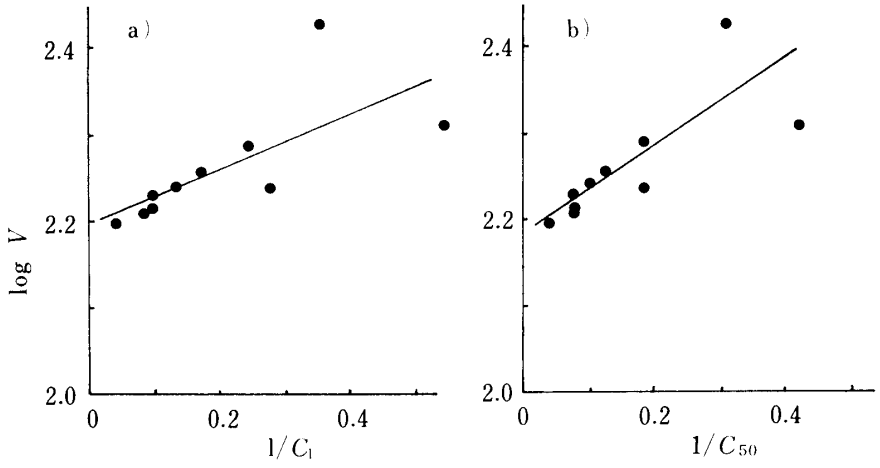

Fig. 2. Relationship between van der Waals Volume and C_1 or C_{50} Value

a) $r=0.708$ ($p<0.05$), $\log y=0.308(1/C_1)+2.201$; b) $r=0.780$ ($p<0.05$), $\log y=0.432(1/C_{50})+2.195$; y, van der Waals volume.

Relationship between Partition Coefficient (P) in *n*-Octanol/Water System and C_1 or C_{50} Value

Seeman[26] found an excellent correlation between the membrane/buffer partition coefficient of various anesthetics and the octanol/water partition coefficient. We also estimated the correlation between the partition coefficient and C_1 or C_{50} value. The correlation coefficient was -0.133 or -0.130 between P and C_1 or C_{50}, -0.086 or -0.082 between $1/P$ and C_1 or C_{50} and -0.076 or -0.120 between P and $1/C_1$ or $1/C_{50}$, respectively. These results suggest that there is not a close correlation between the hemolytic activity and partition coefficient of drugs.

Relationship between pK_a and C_1 or C_{50} Value

Most drugs used are weak bases and exist in solution as an equilibrium between the un-ionized and ionized forms, and only un-ionized nonpolar drug is assumed to penetrate the membrane. The fraction un-ionized in solution depends on both the pH and pK_a of drugs, according to the Henderson–Hasselbach equation. Therefore, the correlation between pK_a and hemolytic concentration was examined. The correlation coefficients between $1/\text{p}K_a$ and $1/C_1$ or $1/C_{50}$ were 0.633 and 0.651, respectively, so that there was no significant correlation between these parameters. It seems likely, however, that the hemolytic activity of drugs is influenced by the concentration of un-ionized drug, which is controlled by the pK_a, at pH 7.4. Thus, the concentrations of un-ionized drugs at pH 7.4 were calculated using the pK_a values (for 7 drugs) by use of the Henderson–Hasselbach equation. A good correlation was obtained between the concentration of un-ionized drug and $1/C_1$ ($r=0.851$, $p<0.01$, $y=30.689\ (1/C_1)-2.157$) or $1/C_{50}$ ($r=0.882$, $p<0.01$, $y=42.295\ (1/C_{50})-2.357$).

Relationship between $P_{m/b}$ and C_1 or C_{50} Value

It is known that drugs penetrate into the cell membrane and disorder or change the membrane structure, resulting in membrane damage and hemolysis. The $P_{m/b}$ values for basic drugs were therefore compared with the hemolytic concentrations. There was a significant correlation between logarithmic $P_{m/b}$ and $1/C_1$ ($r=0.877$, $p<0.01$) or $1/C_{50}$ ($r=0.924$, $p<0.01$), as shown in Fig. 3, and between $P_{m/b}$ and $1/C_1$ ($r=0.857$, $p<0.01$) or $1/C_{50}$ ($r=0.922$, $p<0.01$), which suggests that there is a close relation between the amount of drug penetrated into the membrane and hemolytic activity, and that drugs which are highly partitioned into the cell membrane can produce strong hemolytic action (*e.g.* prochlorperazine, carpipramine and perazine).

To estimate the synergetic contribution of the partition ratio and molecular volume of

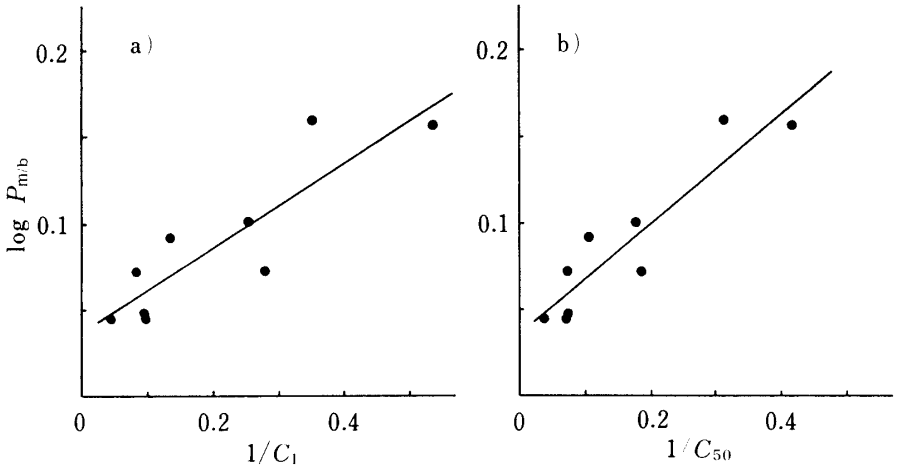

Fig. 3. Relationship between $P_{m/b}$ and C_1 or C_{50} Value

a) $r=0.877$ ($p<0.01$), $\log y=2.384(1/C_1)+0.379$; b) $r=0.924$ ($p<0.01$), $\log y=3.207(1/C_{50})+0.356$; y, $P_{m/b}$.

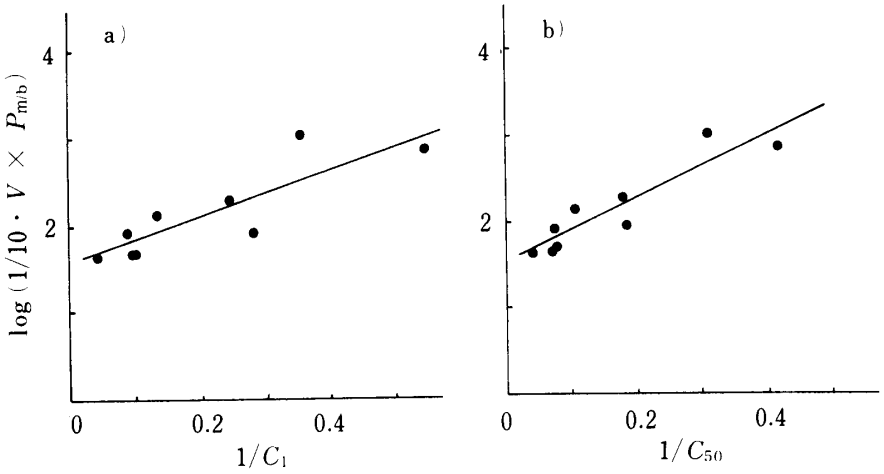

Fig. 4. Relationship between $1/10 \cdot V \times P_{m/b}$ and C_1 or C_{50} Value

a) $r=0.866$ ($p<0.01$), $\log y=2.693(1/C_1)+1.579$; b) $r=0.917$ ($p<0.01$), $\log y=3.641(1/C_{50})+1.550$; y, $1/10 \cdot V \times P_{m/b}$.

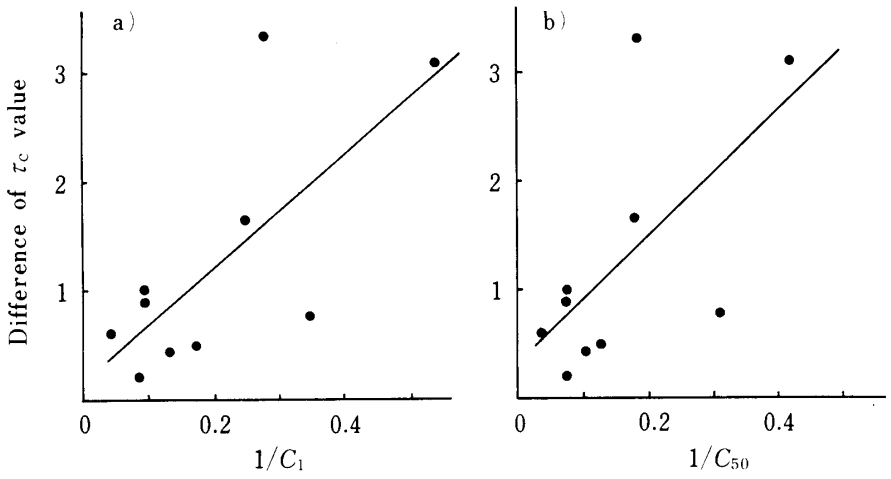

Fig. 5. Relationship between Difference of τ_c and C_1 or C_{50} Value

a) $r=0.711$ ($p<0.05$), $y=5.125(1/C_1)+0.201$; b) $r=0.619$, $y=5.686(1/C_{50})+0.350$; y, difference of τ_c.

drugs penetrated into the cell membrane to drug-induced hemolysis, the relationship between $P_{m/b}$ multiplied by V and C_1 or C_{50} value was estimated. The results obtained are shown in Fig. 4. Based on this result, it seems reasonable to conclude that the molecular volume does make some contribution to the hemolytic process.

Relationship between Difference of τ_c Value and C_1 or C_{50} Value

We have previously proposed that the increased fluidity of erythrocyte membrane caused by chlorpromazine is closely related to the drug-induced hemolysis.[19] Araki and Rifkind[27] suggested that the fluidity of the lipid bilayer correlates with the rate of hemolysis induced by chlorpromazine, Triton X-100 and ethanol. In order to clarify the relationship between the membrane fluidity and hemolytic activity, the difference of τ_c value (the τ_c for untreated membrane minus the τ_c for treated membrane) was compared with the hemolytic concentration. As shown in Fig. 5, a rough correlation was observed between the difference of τ_c and $1/C_1$ ($r=0.711, p<0.05$). This result provides some support to the view that the increased fluidity caused by drugs might be related to the drug-induced hemolysis.

All parameters obtained in this study are summarized in Table I.

TABLE I. Hemolytic Concentrations and Physicochemical Parameters of Drugs

Drug	C_1 (0.1 mM)	C_{50} (0.1 mM)	V (cm^3/ml)	P	pK_a	$P_{m/b}$	$1/10 \cdot V \times P_{m/b}$	Difference of τ_c value[a]
Promazine	11.7	13.3	164.13	94.85	9.4[b]	5.28	86.66	0.216
Promethazine	10.6	13.5	164.12	123.60	9.1	3.00	49.24	0.898
Trimeprazine	10.5	13.8	170.02	26.00	8.6	2.78	47.27	1.031
Perazine	4.0	5.6	195.71	102.50	8.8	10.10	197.67	1.652
Chlorpromazine	3.6	5.4	173.61	124.76	9.3	5.26	91.32	3.355
Prochlorperazine	1.8	2.4	205.19	21.82	8.1[b]	36.55	749.97	3.094
Levomepromazine	7.5	9.5	175.03	17.46	9.5	8.17	143.00	0.427
Prothipendyl	23.3	27.0	160.40	32.60	—[c]	2.81	45.07	0.596
Homochlorcyclizine	5.8	8.0	181.42	16.65	8.5	—	—	0.486
Carpipramine	2.8	3.2	268.42	—	—[c]	38.96	1045.76	0.761

V is the van der Waals volume of a drug. C_1 and C_{50} are the drug concentrations initiating hemolysis and producing 50% hemolysis, respectively. P is the octanol/water partition coefficient of a drug. $P_{m/b}$ is the membrane partition ratio of a drug at pH 7.4. Each value is the mean of four experiments.

a) The τ_c value of drug-untreated membrane minus that of drug-treated membrane.

b) The pK_a value was taken from "The Extra Pharmacopoeia, Martindale," 27th ed. by the Pharmaceutical Press, London.

c) The pK_a value could not be measured by the method described in the text.

Discussion

It is well documented that a number of drugs exert a hemolytic effect on erythrocytes at high concentrations[9,13-16] and that different drugs have different effects on the rate and extent of hemolysis.[13,16,20] In the present study, the drugs used all showed clear-cut differences in their hemolytic action. Thus, in order to elucidate the differences in the hemolytic activities between drugs and also the major factors inducing hemolysis, some experiments were carried out on 10 basic drugs with human erythrocytes.

In the present paper, we found the best correlation between $P_{m/b}$ of drugs and $1/C_1$ or $1/C_{50}$ (Fig. 3). This indicated that a drug with a high $P_{m/b}$ value had a greater affinity for the membrane and penetrated much more into the membrane, even at low concentrations. Therefore, the amount of drugs penetrated into the membrane was probably a main factor inducing hemolysis. The increase of drug molecules in the membrane appears to induce disturbance or rupture of the bilayer structure due to hydrophobic interactions with phospholipids and proteins, as demonstrated previously,[18] and ultimately to result in hemolysis. This also suggests that the rate-limiting process which determines the rate and extent of hemolysis involves rupturing of the bilayer structure.

The structures of the basic drugs used are shown in Fig. 6. Some of the drugs have bulky substituents and long side chains. Although a correlation was observed between logarithmic V and $1/C_1$ or $1/C_{50}$ value, the correlation coefficients were not good ($r=0.708$ and 0.780, respectively) (Fig. 2). The van der Waals volume of the drug molecule may have some effect on the hemolytic activity; a bulky molecule, such as carpipramine or prochlorperazine, in the membrane would presumably accelerate the disordering of the lipid bilayer, since most of the drug molecules are probably present in the lipid bilayer. Cerbon[28] has shown that tetracaine interacts with phospholipids with a positively charged amine group lying close to the lipid head groups and the remainder of the molecule being buried within the membrane, parallel to the fatty acid chains, and Lee[29] suggested that the interaction of chlorpromazine with phospholipids should be similar to that of tetracaine. The contribution of the molecular volume to the drug-induced hemolysis, however, was less significant than that of amount of drug penetrated into the membrane, as judged from the relationship between the V and $1/C_1$

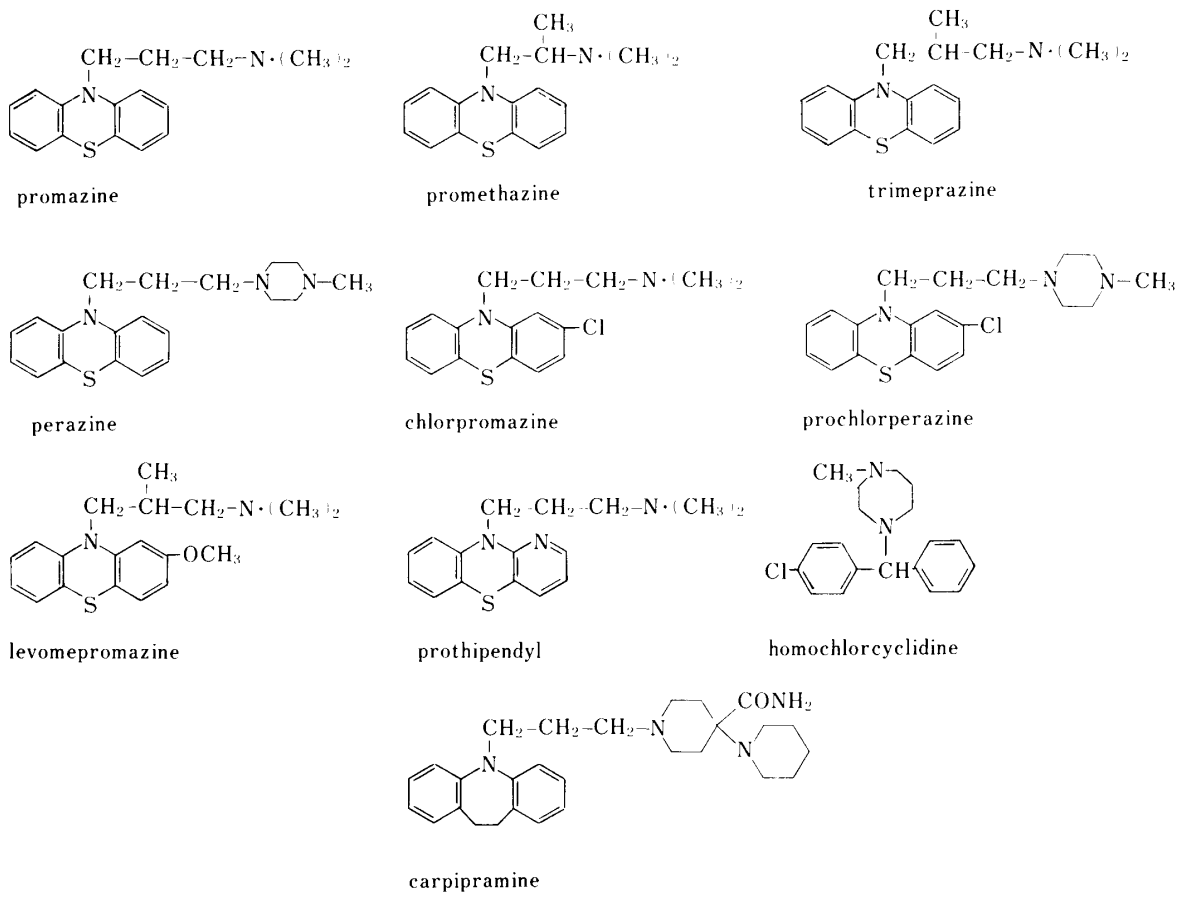

Fig. 6. Structures of Drugs Used in the Experiments

or $1/C_{50}$ value, but there is a possibility that bulky molecular volume and amount of drug may synergetically contribute to the hemolysis. This possibility is supported by the finding of a good correlation between logarithmic $(1/10 \cdot V \times P_{m/b})$ and 1/hemolytic concentration.

ESR spin-label studies have previously demonstrated that the fluidity of the lipid bilayer[27] and of the erythrocyte membrane[19] correlates with the hemolytic activity of drugs. In the present study, it was also shown that increased fluidity of membrane lipids caused by drugs was associated with the hemolytic activity (Fig. 5). Although this supported the view expressed previously,[19,27] the correlation coefficients of 0.714 and 0.621 suggest that increased fluidity of the membrane lipids is partially involved in the hemolysis by drugs. The slight correlation may be ascribed to differences in amount of drugs in the membrane, since although the drug treatment was done at the same concentration (0.2 mM) of drugs, the drugs have different rates of membrane penetration, as shown in Fig. 3.

The $1/pK_a$ of drugs showed little correlation with $1/C_1$ or $1/C_{50}$ value. However, there was a good correlation between the concentration of un-ionized drug at pH 7.4 (used in this study) and $1/C_1$ or $1/C_{50}$ value. The un-ionized drug molecules may be rather easily incorporated into the membrane, although there are differences in penetrating rate between the drugs. The correlation between the un-ionized drug concentration and hemolytic concentration indirectly supports our conclusion that drug-induced hemolysis is strongly affected by the membrane/buffer partition ratio of the drugs.

The fact that no correlation was observed between the partition coefficients in the octanol/water system and hemolytic concentrations is very surprising. This may be because the drug penetration into the erythrocyte membrane at high drug concentrations depends strongly on the membrane/buffer partitioning and the concentration of un-ionized drug rather

than on the partition coefficient in the octanol/water system; the membrane–basic drug interaction in the hemolytic process is not always hydrophobic in nature and there is an involvement of other factors. This is also suggested by the finding that no good correlation existed between the P and $P_{m/b}$ ($r = -0.358$).

The reason why prochlorperazine, perazine and carpipramine penetrated more easily into the membrane is not clear on the basis of the results obtained. However, it is of particular interest that the former two drugs have a piperazinyl ring and the latter has a bipiperizine residue in the molecule, and prochlorperazine has a bulky chlorine residue, as in chlorpromazine, while the less penetrating drug, prothipendyl, has a pyridyl ring which ionizes at pH 7.4.

In addition to the above considerations, our results are also of some relevance to the mechanism of hemolysis by basic drugs. Other factors than those looked at in this study, *e.g.* the polarity and oxidizing capacity of drugs, may be important; however, it seems possible, nevertheless, to draw limited conclusions on the hemolytic activity of basic drugs from the results obtained.

In conclusion, therefore, the present results lead us to consider that the most important factor inducing hemolysis was the amount of drug present in the erythrocyte membrane, and that drug molecules concentrated in the membrane probably increased the fluidity, disordering and rupturing the membrane lipid and bilayer structure. Bulky molecules of drug penetrated into the membrane probably also promote the hemolytic process. The differences in hemolytic activity between drugs appear to be ascribable to the different affinities of drugs for the membrane, perhaps for lipids; the amount and rate penetrating into the membrane, the disordering effect and the molecular volume of the drug may also be important, in addition to the concentration of un-ionized drug in the medium.

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