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DECREASED VISCOSITY OF HUMAN ERYTHROCYTE SUSPENSION INDUCED BY CHLORPROMAZINE AND ISOXSUPRINE

TAKEO SUDA, DAIZABURO SHIMIZU,* NOBUJI MAEDA and TAKESHI SHIGA†
Department of Physiology and *Central Research Laboratory, Medical School, Ehime University, Shigenobu, Onsen-gun, Ehime 791–02, Japan

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Abstract—The effects of two cationic drugs, chlorpromazine and isoxsuprine, on the suspension viscosity of human erythrocytes were studied. Chlorpromazine and isoxsuprine reduced the suspension viscosity, the former being more effective than the latter (i.e. a concentration of isoxsuprine about fifteen times higher than of chlorpromazine was needed to obtain similar effects). The cells transformed to stomatocytes and then to spherostomatocytes, depending on the drug concentration. The decrease in suspension viscosity paralleled the appearance of spherostomatocytes. Membrane fluidity, monitored by the motion of fatty acid spin labels, was increased by chlorpromazine at high concentrations, but was unaffected by isoxsuprine. Quantitative data of drug uptake by erythrocytes and ghosts revealed that the drugs were incorporated into both membrane and cytoplasm (partly bound to hemoglobin, as shown by equilibrium dialysis). The above drug effects appeared to depend primarily on the drug concentration in the membrane. In short, the transformation to spherostomatocytes, induced by drug incorporation into the erythrocyte membrane, was essential for the decrease in suspension viscosity.

The viscosity of blood may be influenced by many factors [1, 2], such as cell count, erythrocyte shape, rigidity of the membrane, and state of the cell contents, even in the absence of cell aggregation. For example, the viscosity of an erythrocyte suspension changes when the osmolarity of the medium is modified [3], probably because of the shape alteration; or when the membrane cholesterol is artificially augmented the suspension viscosity increases slightly [4, 5], due to the decreased membrane fluidity; also, the viscosity of the blood of Hb S patients is increased [6]. On the other hand, certain drugs, e.g. isoxsuprine, have been shown to decrease blood viscosity [7–9]. During the course of our *in vitro* study on the action of isoxsuprine, however, an alteration of the cell shape was found that was quite similar to that induced by chlorpromazine, a drug known to induce "stomatocytosis" [10, 11]. In contrast, Meiselman [12] has shown that nitrophenols increase the suspension viscosity because of echinocytic transformation.

To clarify the rheological effects of the stomatocytic transformation, the relations between suspension viscosity, membrane fluidity, and cell shape were analyzed as a function of the drug concentration. Further, to explain the differences in effective doses, drug incorporation into membrane was measured quantitatively. This paper describes the decrease in suspension viscosity induced by chlorpromazine and isoxsuprine, the alteration of cell shapes by them, and the increase in membrane fluidity produced by chlorpromazine. In addition, the incorporation of these drugs into the erythrocyte membrane was estimated and the mechanism is discussed.

MATERIALS AND METHODS

Erythrocytes. Fresh, heparinized human blood was drawn by venipuncture; after centrifugation, the plasma and buffy coat were removed, and the cells were washed twice. An isotonic phosphate-buffered saline solution (90 mM NaCl, 50 mM Na-phosphate buffer, 5 mM KCl, 1 mM $MgSO_4$, and 10 mM glucose; pH 7.4) was used throughout (this solution was similar to that of Allen and Rasmussen [13], but the buffering capacity was increased and Ca^{2+} was omitted to avoid precipitation).

Ghosts. ACD-blood, obtained from a local Blood Bank, was washed with isotonic Tris–HCl buffer (172 mM; pH 7.6) three times, then hemolyzed with 15 vol. of hypotonic Tris–HCl buffer (11 mM; pH 7.6), and centrifuged for 40 min at 20,000 *g* [14]. The ghosts were washed with the hypotonic buffer three times and then, resealed by incubation with the isotonic-buffered saline for 30 min at 37°.

Hemoglobin. The hemolysate was passed through a Sephadex G-25 column equilibrated with 0.1 M NaCl, to remove organic phosphate. The concentration of hemoglobin was determined by a CN-methemoglobin method [15].

Reagents. Isoxsuprine·HCl was supplied by the Dai-ichi Seiyaku Co. (Tokyo), chlorpromazine·HCl by the Yoshitomi Pharmaceutical Co. (Osaka), and isoproterenol·HCl by the Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Viscosity measurement. A cone-plate viscometer (Tokyo Keiki Co., Tokyo, model E, mounted 0.8° cone) was used at 37°. The erythrocyte concentrations of the different samples were adjusted on the basis of hematocrit. Hematocrit was determined by

centrifugation for 5 min at 9000 *g* (Kubota Manuf. Co., Tokyo model KH-120M), without correction for plasma trapping.

Electron spin resonance (e.s.r.) measurements. Three fatty acid spin labels, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl, 2-(10-carboxydecyl)-2-hexyl- and 2-(14-carboxydecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as I (12, 3), I (5, 10) and I (1, 14), respectively), were purchased from the Syva Co. (Palo Alto, CA). The methods of preparing spin-labeled erythrocytes were described previously [16, 17]. A Varian E-3 spectrometer (Palo Alto, CA) with a variable temperature accessory was used. The spectral parameters were calculated from the e.s.r. spectra by the conventional manner [18, 19].

Scanning electron microscopy. The cells that were treated with drugs were immersed in 15 vol. of the isotonic solution containing 1% glutaraldehyde. Further, the cells were fixed with 1% OsO_4 for 30 min and dehydyrated by ethanol. The samples were coated with Pt by a Hitachi critical point dryer (model HCP-1, Hitachi-city). A Hitachi S-500A scanning electron microscope (Hitachi-city) was used.

Measurements of drug incorporation (or binding). (i) The partition of drugs between the isotonic solution and *n*-hexane was carried out as follows. Drug was dissolved in the isotonic solution (2 ml) and mixed with the same volume of *n*-hexane; the mixture was shaken vigorously for 30 min at 20°. The drug concentration in the isotonic solution was determined by u.v. spectroscopy (for chlorpromazine ε_{253nm} = 25,300 and for isoxsuprine ε_{267nm} = 2,690 respectively). (ii) The incorporation of a drug into the erythrocytes and ghosts was quantified as follows. The cells (0.5 ml, hematocrit of 47 and 23%) were incubated with the isotonic solution (4.5 ml) containing a drug for 30 min at 37° with gentle stirring and then centrifuged; the drug concentration in the supernatant fraction was measured by u.v. spectroscopy. The erythrocytes were hemolyzed slightly with chlorpromazine (less than 3%) and isoxsuprine (less than 1%), and the contribution of hemoglobin to the u.v. spectra was corrected. Further details of the calculation of partition parameters are described in the Appendix. (iii) The binding constant between hemoglobin and a particular drug was determined by equilibrium dialysis using cellulose tubing (Union Carbide Co., Chicago, IL) for 16 and 22 hr at 4° in a dark room; hemoglobin (5 ml, 0.68 mM solution of the tetramer) was dialyzed against 15 ml of isotonic solution containing various amounts of drugs. About 10 per cent of the chlorpromazine was lost during dialysis (probably bound to tubing), and the calculation of the binding constant was corrected for the loss; no loss of isoxsuprine was observed. Equimolar binding, i.e. drug:hemoglobin (tetramer) = 1:1, was tentatively assumed. Two sets of data (16- and 22-hr dialysis) agreed; thus, equilibrium was established under these experimental conditions.

RESULTS

Viscosity of the erythrocyte suspension. Washed erythrocytes were incubated with various drug concentrations in isotonic solution for 30 min at 37° (the hematocrits were *ca.* 10%); at higher drug concentrations (e.g. above 0.5 mM chlorpromazine), hemolysis was evident. The concentration of isoxsuprine was limited by poor solubility. The apparent viscosity (η_{app}) of the erythrocyte suspension (the hematocrits were adjusted to 28.87 ± 0.03%) decreased as the concentration of chlorpromazine or isoxsuprine was increased (Fig. 1), but it was unchanged by isoproterenol. Chlorpromazine seemed to be more effective than isoxsuprine, with respect to the effective concentration. The representative data are summarized in Table 1.

At high shear rates ($38 < \dot{\gamma} < 752$ sec^{-1}), the Casson plots ($\sqrt{\tau}$ vs $\sqrt{\dot{\gamma}}$; $\tau = \dot{\gamma} \cdot \eta_{app}$) gave straight lines [1, 20, 21]. As the drug concentrations were increased, the Casson viscosity (η_c), which was shown to be independent of $\dot{\gamma}$ [4], decreased. With increasing hematocrit (from *ca.* 10 to 50%), the apparent viscosity increased; the plot of log (η_c) vs hematocrit was almost linear, and the slope of the plot became smaller for the drug-treated cells.

Fluidity of the erythrocyte membrane. The spin-labeled erythrocytes were incubated with drugs for 30 min at 37° (final hematocrit ≃ 11%) and then were packed in the sample tube (i.d. = 1.1 mm) for e.s.r. measurement [16, 17]. Isoxsuprine and isoproterenol did not modify the e.s.r. spectra of all spin labels in the membrane. The addition of higher concentrations of chlorpromazine, however, increased the spin-label motion, as judged from the e.s.r. spectra of all spin labels. The representative spectra are shown in Figs. 2 and 3. The order par-

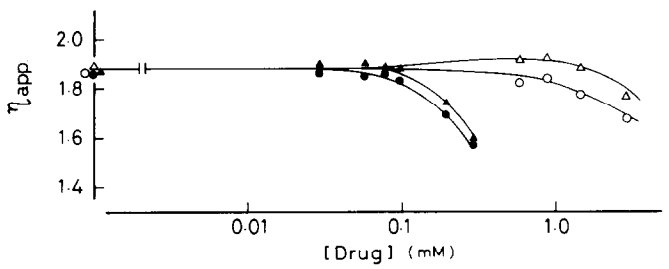

Fig. 1. Changes of the apparent viscosity (η_{app}) of erythrocyte suspensions as a result of incubation with chlorpromazine or isoxsuprine. Viscosity was measured at 37°, an hematocrit of 29.87 ± 0.03%, and two shear rates ($\dot{\gamma}$), with chlorpromazine at $\dot{\gamma}$ = 752.2 (●) and at $\dot{\gamma}$ = 150.5 sec^{-1} (▲), and with isoxsuprine at $\dot{\gamma}$ = 752.2 (○) and at $\dot{\gamma}$ = 150.5 sec^{-1} (△).

Table 1. Effects of chlorpromazine and isoxsuprine on the viscosity of erythrocyte suspension*

Shear rate (sec^{-1})	Apparent viscosity (η_{app}) Chlorpromazine†			Isoxsuprine‡		
	Control	0.1 mM	0.3 mM	Control	1.5 mM	3.6 mM
752.2	1.874	1.839	1.576	1.995	1.870	1.771
376.1	1.945	1.900	1.656	2.071	1.963	1.838
150.5	1.865	1.887	1.591	2.138	1.951	1.817
75.2	1.827	1.857	1.553	2.123	1.922	1.832
37.6	1.918	1.948	1.644	2.368	2.086	1.817
18.8	2.131	2.070	1.766	2.562	2.086	2.026
Casson viscosity	1.873	1.822	1.578	1.916	1.849	1.783

* The values of apparent viscosity (η_{app}) are in cP (centipoise), at 37°. Hematocrit was adjusted to 28.87 ± 0.03. Values are averages of duplicate experiments.

† The mean cell volume of this series was 101.1 ± 3.3 μ^3, which was unchanged by drug.

‡ The mean cell volume of the control sample was 89.2 μ^3. Upon incubation with drug (>1 mM), it decreased to *ca.* 84 μ^3.

ameter (S) of I (12, 3) and I (5, 10) decreased and the correlation time (τ_c) of I(1, 14) decreased, as the chlorpromazine concentration increased.

The temperature dependencies of the order parameters (S) are shown in Fig. 4. The slopes of the plots, S vs $1/T$, were independent of the chlorpromazine concentration.

Erythrocyte shapes. The scanning electron micrographs of the same erythrocytes as those used for the viscosity measurements are shown in Fig. 5. As the drug concentration was increased, the shape of the cell transformed from "stomatocytosis" to "spherostomatocytosis", according to Bessis's expression [10, 11, 22]. The change to stomatocytes did not alter the suspension viscosity, but the change to spherostomatocytes decreased the suspension viscosity as the transformation proceeded. According to the semiquantitative morphological index of Fujii *et al.* [11], the viscosity decreased at "invagination degree −3 and −4".

Concerning the drug-induced transformation, isoxsuprine again was less effective than chlorpromazine (apparently a fifteen times higher concentration of isoxsuprine was needed to obtain a shape change similar to that of chloropromazine). In addition, the cell volume (calculated from the packed volume and the cell count) was unchanged by chlorpromazine, but it was slightly decreased by isoxsuprine as the transformation proceeded (at maximum, 5 per cent).

Incorporation or binding of drugs to erythrocytes, ghosts and hemoglobin. To understand the mech-

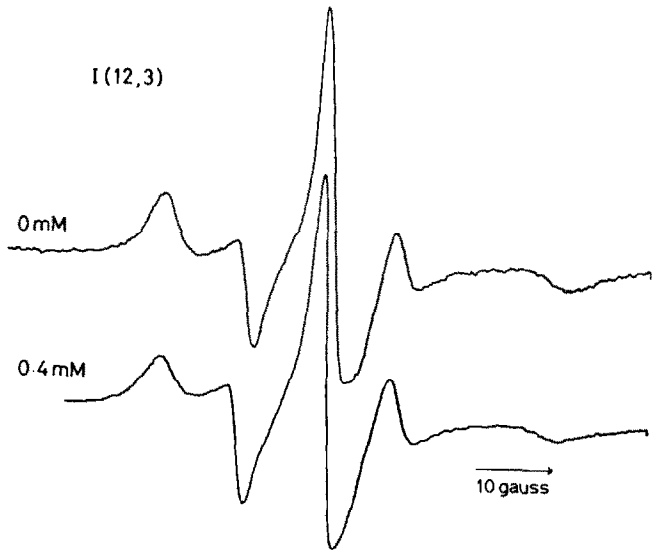

Fig. 2. Electron spin resonance spectra of I (12, 3)-labeled erythrocytes. (Top) control, (bottom) chlorpromazine 0.4 mM. Conditions: incident microwave power, 5 mW; modulation, 100 kHz, 1 G; at 38°.

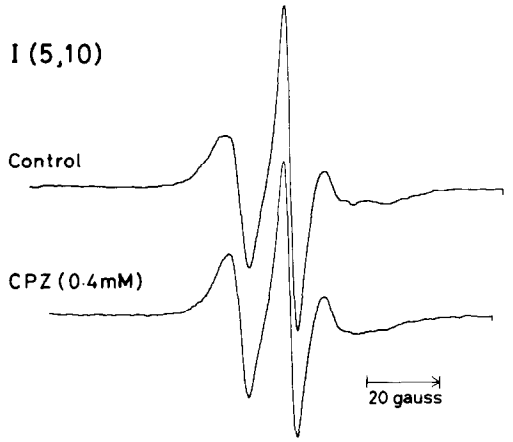

Fig. 3. Electron spin resonance spectra of I (5, 10)-labeled erythrocytes. (Top) control, (bottom) chlorpromazine 0.4 mM. Conditions: same as in Fig. 2.

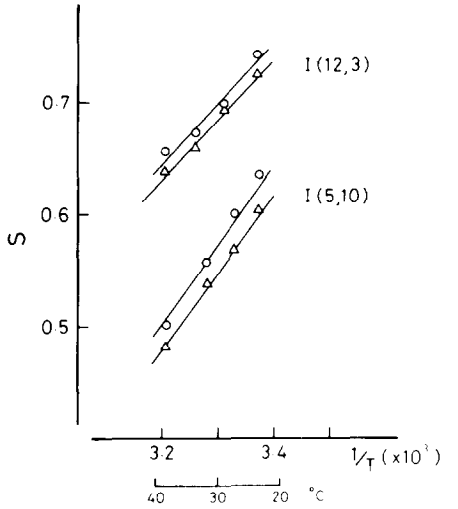

Fig. 4. Temperature dependence of order parameters (S). (Top) I (12, 3): control (○), and chlorpromazine 0.4 mM (△). (Bottom) I (5, 10): control (○), and chlorpromazine 0.4 mM (△).

anism of the above phenomenon and the difference between the effective concentrations of the drugs, we measured the apparent partition of each drug between the erythrocytes (or ghosts) and the isotonic solution, the binding of each drug to purified hemoglobin, and the *n*-hexane/buffered saline partition. The results are summarized in Table 2. The details of the calculations are described in the Appendix.

Many studies have already been done of chlorpromazine uptake by ghost membranes [23, 24], e.g. Kwant and Seeman [23] distinguished two sets of adsorption sites; the first one was bound strongly, in a hyperbolic fashion with respect to drug concentration, and the second was a "lytic" site that was bound weakly and was probably incorporated into the membrane by a partition mechanism. In our measurements, however, the sensitivity of the technique (u.v. spectroscopy) restricted the lower limit of drug concentration; thus, determination of the first site of Kwant and Seeman was abandoned. In addition, at low concentrations of chlorpromazine, at which the first binding was operative, no change in suspension viscosity or in membrane fluidity was observed.

Comparing chlorpromazine and isoxsuprine, the results shown in Table 2 can be summarized as follows: (i) the partition constants of chlorpromazine to erythrocytes, ghosts and *n*-hexane were greater than those of isoxsuprine, though to different extents, and (ii) the binding constant of chlorpromazine to purified hemoglobin was twelve times greater than that of isoxsuprine. Therefore, the apparent incorporation of drug into whole erythrocytes was a complex measure and was not suitable for discussing the mechanism.

DISCUSSION

Blood viscosity may be affected by changes in plasma components, erythrocyte count, aggregation of cellular components, and alteration of erythrocyte properties. Among these factors, the rheological properties of erythrocytes depend primarily on cell shape, membrane fluidity (or flexibility), and viscosity of the cell contents. Chlorpromazine and isoxsuprine certainly decreased the viscosity of the erythrocyte suspension (Fig. 1 and Table 1) at high shear rates. Braasch [25] demonstrated that the viscosity augmentation induced by cholic acid or fatty

Table 2. Partition constants and binding constants*

	Partitioning to:			
	Erythrocytes (P_{cell})	Membrane $(Q_m)^+$	*n*-Hexane	Binding to hemoglobin (K_{ass})‡
Chlorpromazine	34 ± 5 (12)	30 (2)	8.3 ± 1.9 (8)	(2.0 ± 0.4) × 10^3 M^{-1} (4)
Isoxsuprine	3.3 ± 0.5 (6)	1 (2)	0.033 ± 0.010 (7)	(1.7 ± 0.6) × 10^2 M^{-1} (4)
Factor§	10	30	250	12

* Values are means ± S. D.; the number of experiments is given in parentheses after the values.
† Relative values (see Appendix).
‡ Assuming hemoglobin (tetramer): drug = 1:1.
§ The ratio of chlorpromazine/isoxsuprine.

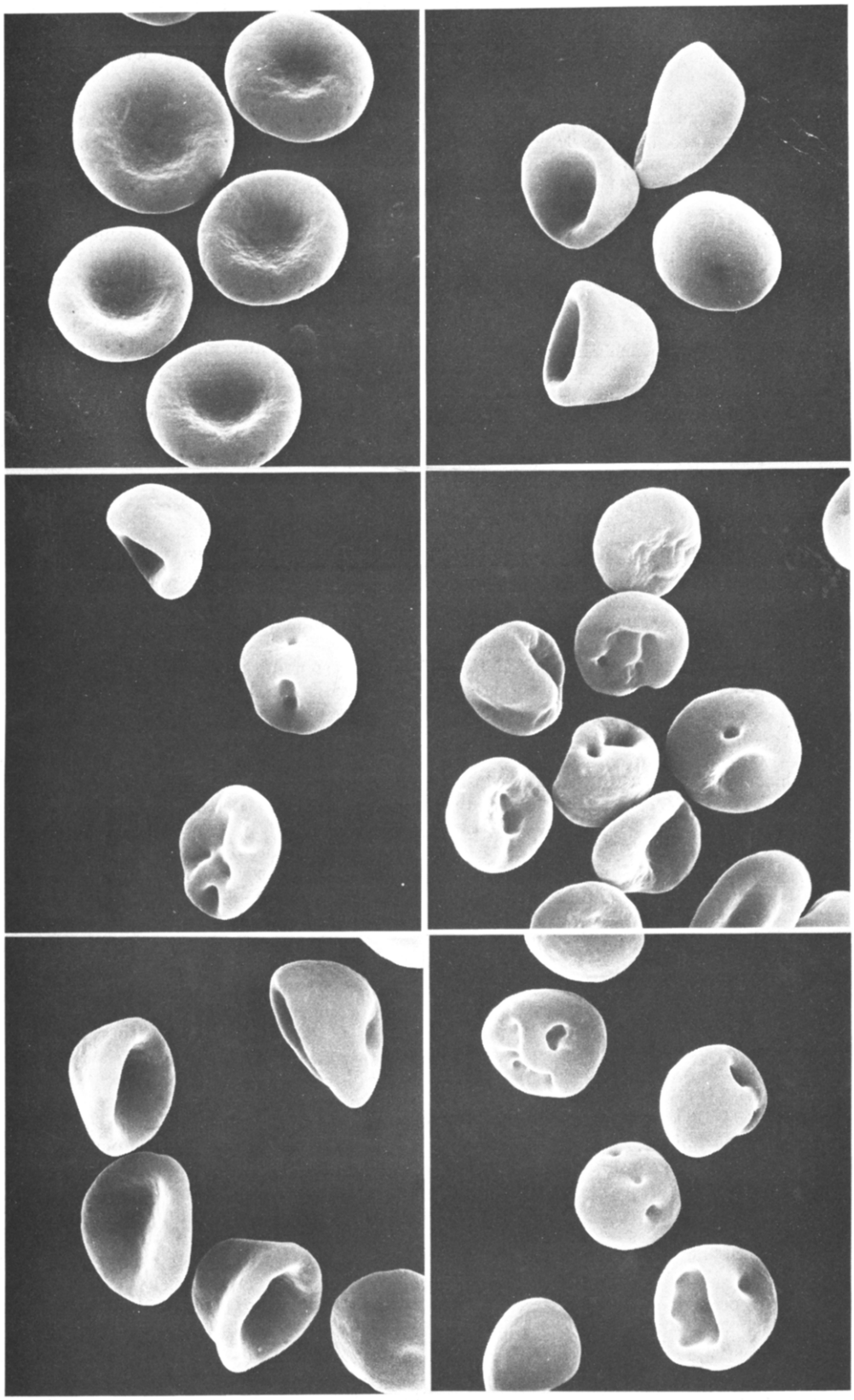

Fig. 5. Scanning electron micrographs of drug-treated erythrocytes. (Left top) control; (right top) chlorpromazine, 0.1 mM; (left middle) chlorpromazine, 0.2 mM; (right middle) chlorpromazine, 0.3 mM; (left bottom) isoxsuprine, 0.6 mM; and (right bottom) isoxsuprine, 3.6 mM.

acid was eliminated by chlorpromazine. In general, erythrocyte aggregation did not occur at high shear stress. Therefore, the reduced viscosity of an erythrocyte suspension in a drug-containing medium resulted primarily from changes in the properties of individual erythrocytes.

Main cause of the decrease in suspension viscosity. The change in membrane fluidity, as detected by the motion of fatty acid spin labels, certainly affected the suspension viscosity, e.g. when the membrane cholesterol was artificially augmented without shape change, the membrane fluidity decreased [26, 27] and the suspension viscosity increased [4, 5]. In the present study, however, both chlorpromazine and isoxsuprine modified the cell shape in a similar manner (Fig. 5), but the membrane fluidity was not influenced by isoxsuprine. Isoxsuprine decreased cell volume slightly, but chlorpromazine did not. The decreased viscosity, therefore, could be related mainly to the transformation to "spherostomatocytosis" or "invagination degree −3 and −4", although the increased membrane fluidity (by chlorpromazine) and the decreased cell volume (by isoxsuprine) might contribute to some extent.

Meiselman [12] showed that the suspension viscosity of echinocytic erythrocytes increased in relation to the degree of echinocyte formation at low shear rates, but that it was nearly identical to normal, biconcave cells at high shear rates. 2,4-Dinitrophenol, used by Meiselman, as well as trinitro-benzene sulfonate, immobilized the spin-label motion, but lysolecithin fluidized the membrane, though these compounds similarly induced the echinocytic transformation and increased the suspension viscosity (unpublished observation). These facts strongly suggest that the main cause of the increased viscosity was echinocyte formation, and, in contrast, that the reason for decreased viscosity was the transformation to spherostomatocytes.

Sheetz and Singer [28, 29] have postulated a "bilayer couple" mechanism, for drug-induced erythrocyte transformation whereby, when a drug is preferentially incorporated into the inner half of the bilayer membrane, the area of the inner layer increases above that of the outer layer, causing stomatocytosis (invagination or internalization) to occur. Chlorpromazine and isoxsuprine are cationic molecules, and they may be incorporated preferentially into the inner layer and result in investigation, as suggested by Elferink [30] for chlorpromazine.

On the other hand, interaction between the drug and the membrane proteins must be taken into consideration. Gazitt *et al.* [31] reported that chlorpromazine induced ATP depletion, intramembrane particle aggregation, and exposure (i.e. of phospholipids extracted easily by dry ether), of chicken and rat erythrocytes. The modification of surface proteins was detected earlier by Sandberg and Piette [32] using SH-spin-labeled bovine ghost membrane. Later, Manian *et al.* [33] and also Leterrier *et al.* [34] suggested that changes of the protein–lipid interface were induced by chlorpromazine. Leterrier *et al.* [34], however, detected increased membrane fluidity at low concentrations of chlorpromazine using I (12, 3). When much more drug was applied, the fluidity increased, as shown in the present study and by Araki and Rifkind [35].

At the present stage, it is impossible to clarify the reason for transformation, but it appears that "spherostomatocytosis" leads to decreased suspension viscosity.

Incorporation of drugs into erythrocytes. Both drugs, chlorpromazine and isoxsuprine, were incorporated by whole erythrocytes. If chlorpromazine had been present only in the membrane, however, the drug/membrane-lipid molar ratio would have been higher than 1 at the maximum; this was not the case. Banaschak and Bluth [36] showed that noradrenaline, incorporated by erythrocytes, was probably distributed both in membrane and in cytoplasm (partly bound to hemoglobin), but they could not estimate the amount in the membrane.

To discuss the differences between the dose-response relations of two drugs and to elucidate the mechanism of action, estimation of the drug concentrations in the membrane and in the cell interior is important. As shown in the Appendix, the overall, apparent drug incorporation (solid lines of Fig. 7 in the Appendix) can be separated into two compartments, i.e. membrane and cytoplasm. For the calculation, the following assumptions were made: (i) the properties of the cell membrane are the same for intact erythrocytes and ghosts concerning drug partition, in spite of possible differences in membrane constituents, and (ii) the contribution of specific drug binding to the membrane (if any, *vide supra*) is ignored, because it seems to be far less than the non-specific partitioning.

Based on the above assumptions, the present results can be summarized as follows: (i) chlorpromazine was easily incorporated into the whole cell compared with isoxsuprine (*ca.* ten times), (ii) when the quantities of overall drug uptake were similar (e.g. *ca.* 3.4 mmoles/l of packed erythrocytes), about 60 per cent of incorporated chlorpromazine was present in membrane whereas about 80 per cent of incorporated isoxsuprine was in the cell interior (as shown by the column in Fig. 7), because the partition of chlorpromazine in the membrane was greater than that of isoxsuprine (*ca.* thirty times), and (iii) the binding constant of chlorpromazine to hemoglobin was greater than that of isoxsuprine (*ca.* twelve times). In short, chlorpromazine was more soluble in membrane and more strongly bound to hemoglobin. For example, the addition of either *ca.* 0.2 mM chlorpromazine or *ca.* 3 mM isoxsuprine (to an erythrocyte suspension with a hematocrit of 29%, see Results) produced similar shape changes and reductions of suspension viscosity (Fig. 1), but the drug concentrations in membrane were estimated to be similar (2 to 2.5 mmoles/l of packed volume), which corresponded roughly to one drug molecule/three lipid molecules in membrane.

In summary, it is suggested that (i) spherostomatocytosis, rather than changes in membrane fluidity, was the main cause of the decreased suspension viscosity, and (ii) the degree of transformation was dependent upon the drug concentration in membrane, rather than upon the overall drug uptake.

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APPENDIX

Measurement of drug partition between cellular compartments. The overall drug incorporation by erythrocytes could be separated into two compartments, i.e. membrane and cytoplasm; the latter consisted of hemoglobin-bound and free drug. To separate these, the following experiments were carried out as described in Materials and Methods (i) determination of drug incorporation by whole erythrocytes and (ii) by ghosts, and (iii) measurement of hemoglobin–drug binding.

As shown in Fig. 6, an amount of erythrocyte suspension (packed volume of V_x) was mixed and equilibrated with isotonic solution (volume V_0) containing various concentrations of drugs ($[D]_0$). After centrifugation, the supernatant fraction (volume V_y, $[D]_{free}$) was obtained. The mean drug concentration in whole erythrocytes ($\overline{[D]}_{cell}$, regardless of compartmentation) could be calculated as follows,

$$[D]_0 V_0 = \overline{[D]}_{cell} V_x + [D]_{free} V_y \quad (1)$$

thus

$$\overline{[D]}_{cell} = [D]_0 V_0/V_x - [D]_{free} V_y/V_x \quad (2)$$

The apparent partition constant (P_{cell}) between erythrocytes and solution may be defined as,

$$P_{cell} \equiv \overline{[D]}_{cell}/[D]_{free} \quad (3)$$

The experimental results with chlorpromazine and isoxsuprine are shown in Fig. 7 (solid lines), where

$$P_{cell} = 34 \pm 5 \quad \text{for chlorpromazine}$$

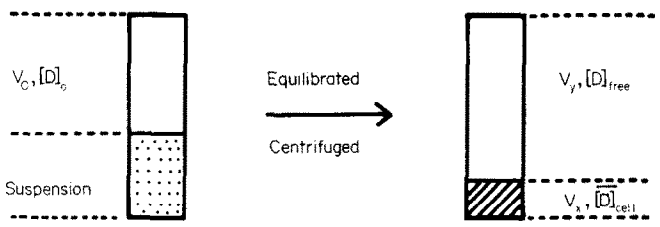

Fig. 6. Experimental procedure for measuring the partition constant. See Appendix.

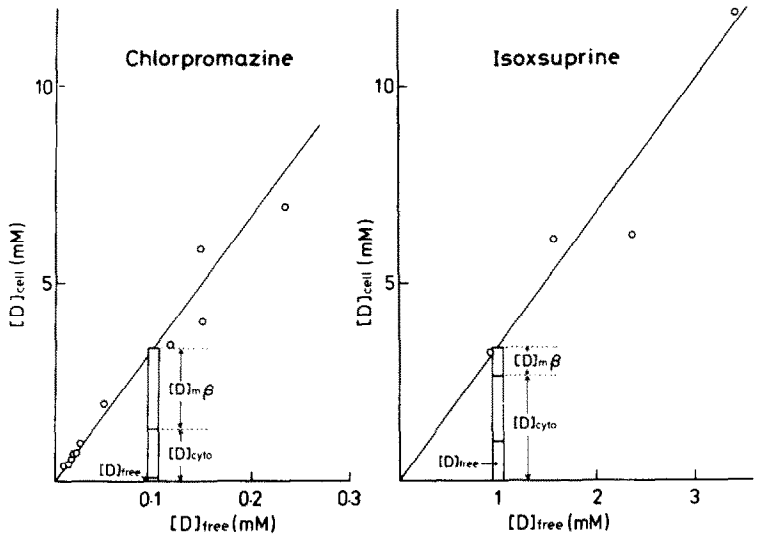

Fig. 7. Drug incorporation into whole erythrocytes. Solid lines are experimental results, $[D]_{free}$ vs $\overline{[D]}_{cell}$; the columns are calculated estimates of $[D]_m$ and $\overline{[D]}_{cyto}$.

and

$$P_{cell} = 3.3 \pm 0.5 \quad \text{for isoxsuprine.}$$

Now, the incorporated drug should be distributed in membrane (volume fraction in packed cells is β; concentration is $[D]_m$) and in cytoplasm (volume fraction $1 - \beta$; mean concentration $\overline{[D]}_{cyto}$), thus

$$\overline{[D]}_{cell} V_x = [D]_m \beta V_x + \overline{[D]}_{cyto}(1 - \beta)V_x. \qquad (4)$$

In the last term β may be neglected since $\beta \ll 1$.

Next, similar experiments were performed with ghosts. In this case, the space of cytoplasma was replaced by the incubation medium, and the volumes and the concentrations were expressed with a prime.

$$[D]'_0 V'_0 = [D]'_m \beta' V'_x + [D]'_{free}(V'_{total} - \beta' V'_x) \qquad (5)$$

where $V'_{total} = V'_x + V'_y$. $\beta' V'_x$ in the last term may be neglected, thus

$$[D]'_m \beta' = [D]'_0 V'_0 / V'_x - [D]'_{free} V'_{total} / V'_x \qquad (6)$$

Here, $[D]'_m \beta'$ is the amount of drugs in the membrane/ghost volume. With our experimental conditions (centrifuged 5 min for erythrocytes and 20 min for ghost, at 9,000 g), the lipid contents (both phospholipids and cholesterol) per packed volume of erythrocytes and of ghosts were similar, *ca.* 6×10^{-3} moles/l of packed volume, thus $\beta = \beta'$. The ratio Q_m was defined as

$$Q_m \equiv [D]'_m \beta' / [D]'_{free}$$

$Q_m = 21$ for chlorpromazine

and $Q_m = 0.7$ for isoxsuprine were obtained.

Then substituting Q_m into equation 4 for erythrocytes,

$$\overline{[D]}_{cell} V_x = Q_m [D]_{free} V_x + \overline{[D]}_{cyto} V_x. \qquad (7)$$

Therefore, we obtain

$$\overline{[D]}_{cyto} = \overline{[D]}_{cell} - Q_m [D]_{free} \qquad (8)$$

and

$$\overline{[D]}_m \beta = \overline{[D]}_{cell} - \overline{[D]}_{cyto}. \qquad (9)$$

The calculated values of $[D]_m \beta$ and $\overline{[D]}_{cyto}$ are shown in Fig. 7. When the mean drug concentrations in whole cells were the same ($\overline{[D]}_{cell}$=3.3 mM), about 60 per cent of the chlorpromazime was present in membrane, whereas about 20 per cent of the isoxsuprine was present (as shown in Fig. 7 by columns).

Finally, from $\overline{[D]}_{cyto}$ and $[D]_{free}$, the binding constant between intracellular hemoglobin ($\simeq$5 mM) and a drug may be calculated as follows,

$$K_{ass} = \frac{\overline{[D]}_{cyto} - [D]_{free}}{(0.005 - \overline{[D]}_{cyto} + [D]_{free}) \times [D]_{free}} \qquad (10)$$

where the volume occupied by hemoglobin was neglected for simplicity. The calculated values are

$K_{ass} = 2 \times 10^3\ M^{-1}$ for chlorpromazine

$K_{ass} = 5 \times 10^2\ M^{-1}$ for isoxsuprine.

These estimates are close to the experimental values obtained by the equilibrium dialysis (at 4°, with diluted, purified hemoglobin) shown in Table 2.