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EFFECT OF DRUGS ON HUMAN ERYTHROCYTES—4.
PROTECTING EFFECT OF DEXTRAN ON DRUG-INDUCED HEMOLYSIS

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Abstract—The protection of drug-induced hemolysis was designed in an attempt to prevent adverse reactions of drugs. The dextrans (mol. wt of 7,600, 18,500 and 23,200) were used for this purpose. These dextrans produced a protecting effect on drug-induced hemolysis *in vitro*. Electron microscopic observations indicated that addition of the dextrans at higher concentrations to the cells partially prevented the cell shrinking, most of the cells retained smooth spheres at 0.8 mM chlorpromazine, at which concentration 100% hemolysis was produced in the absence of the dextrans. Although the dextrans had no stabilizing effect on the cell membrane, they strongly inhibited the diffusion of hemoglobin and K^+ and decreased quantities of the drug molecules adsorbed to the cells. There was a good correlation between the viscosity of the dextrans and the protecting effect on drug-induced hemolysis. The protecting effect of the dextrans thus is due to inhibition of diffusion of hemoglobin and K^+ and the decrease in quantities of drug molecules adsorbed to the cells, probably ascribed to removing solvent volume accessible to the diffusing hemoglobin, the increased viscosity and the sheath of dextran molecules.

Many kinds of drugs, such as tranquilizers, antihistaminics and anesthetics, at low concentrations protect erythrocytes against hypotonic hemolysis [1–14], but at high levels cause complete cell disruption [12, 13, 15–17]. The mechanism of this protective effect was explained as the membrane expansion or the increase in critical volume by drugs [12, 11]. Additionally, the stabilization phase is ascribed to a reversible fluidization of membrane lipids and there is evidence that such fluidization can be induced in lipid model membranes by anesthetics such as halothane, tetracaine and butacaine, probably due to the increased disordering of the lipid structure [18–20]. The mechanism of hemolysis of the cells at higher drug concentrations has been partially clarified: Hexachlorophen induces the efflux of Na^+ and K^+ from red blood cells by directly altering the permeability of the cellular membrane, and secondarily induces osmotic swelling and subsequent hemolysis; ellipticine-induced hemolysis appears to be due to disruption of membrane structure as a result of the drug-phospholipid and protein interactions [21, 16]. We also proposed that hemolysis induced with chlorpromazine and clemastine is probably due to changes of the arrangement of the phospholipids and to an increase in the permeability of the membrane concomitant to a perturbation of lipid–protein interactions [22]. In most cases, drug-induced hemolysis has not been thought severe enough to cause any significant clinical problems. However, drug-induced hemolysis may be representative of the membrane injury in various tissues in the region where the drugs were dosed. It is shown that some drugs at higher concentrations have a lytic action on lysosomes [23, 24]. The protection of drug-induced hemolysis thus is of great importance in preventing such adverse reactions of drugs. However, the studies on protecting effect against drug-induced hemolysis have been scarcely carried out. We found that some dextrans protected hemolysis induced with chlorpromazine and clemastine. This report deals with protection of drug-induced hemolysis with dextrans and the mechanism of the protective effect.

MATERIALS AND METHODS

Drugs and dextrans. Chlorpromazine hydrochloride (Nihon Shinyaku, Kyoto) and clemastine fumarate (Sankyo Co., Tokyo) were used throughout this experiment. Dextran KL, XM and X of the properties shown in Table 1 were obtained from Meito Sangyo Co., Tokyo.

Preparation of erythrocyte suspension. Human erythrocytes were prepared by the same method as described in a previous paper [26]. Hematocrit value was ordinarily 40 ± 1 per cent and in some experiments 48 ± 1 per cent.

Drug-induced hemolysis. To a mixture consisting of 1 ml drug solution (0.1–1 mM in the incubation medium) and 2 ml dextran (6–20 mM for dextran KL and 0.5–5 mM for dextran XM and X in the medium) 0.3 ml erythrocyte suspension (hematocrit value, 40 ± 1 per cent) was added and mixed immediately.

Table 1. Properties of dextrans

Lot No.	KL	XM	X
Loss on drying (%)	9.5	3.9	4.0
Inherent viscosity (dl/g 25°)	0.096	0.175	0.219
Reducing sugar (%)	2.82	1.15	0.92
$\overline{Mn}$*	7600	18,500	23,200

* Determined by the method of Isbell [25].

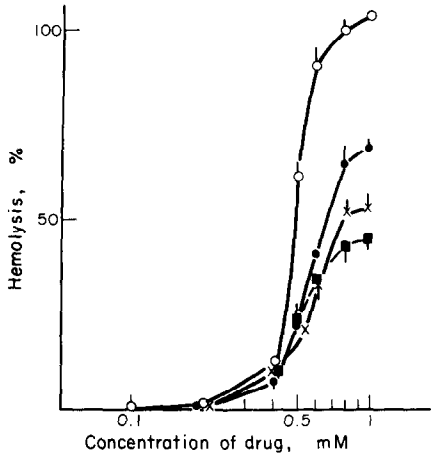


Fig. 1. Effect of dextrans on chlorpromazine-induced hemolysis. Experimental conditions are described in text. Points represent the mean ± S.D. of three experiments. Key: control (○); 20 mM dextran KL (■); 5 mM dextran XM (●); and 5 mM dextran X (×).

The test and dextran solutions were usually prepared in isotonic NaCl solution (0.9% in phosphate buffer, pH 7.4) [27]. The mixture was incubated for 60 min at 37° and after centrifugation the percentage hemolysis was determined by the method described in a previous paper [28].

Electron microscopy. Scanning electron microscopy of the cells was observed according to the method described in a previous paper [26].

Potassium measurement. The determination of potassium was done by the method described previously [26]. Potassium in dextrans and trichloroacetic acid was assayed by the same method.

Measurement of drug adsorption to erythrocytes. To a mixture consisting of 1 ml drug solution (at final concentrations of 0.05–3 mM) and 2 ml dextran solution at indicated concentrations 0.3 ml erythrocyte suspension (hematocrit value, 40 ± 1 per cent) was added and incubated for 30 min at 37°. Following centrifugation the concentration of chlorpromazine in the supernatant was determined spectrophotometrically by measuring the absorbance at 255 nm. The absorbance at 255 nm was corrected for the absorption of dextrans and proteins released, which was calculated from the absorbance at 280 nm.

Measurement of diffusion rate of hemoglobin. The diffusibility of hemoglobin was tested according to the method described in a previous paper [26].

Measurement of viscosity of dextrans. The viscosity of dextrans was measured using an Ostwald-type viscometer, with a flow time of 28 sec with deionized water.

Protein determination. Protein concentration was determined by the procedure described by Lowry *et al.* [29] with bovine albumin, fraction 5, as a standard.

RESULTS

Protective effect of dextrans on hemolysis and K^+ efflux induced with chlorpromazine and clemastine. As shown in Fig. 1, dextran KL at 20 mM and

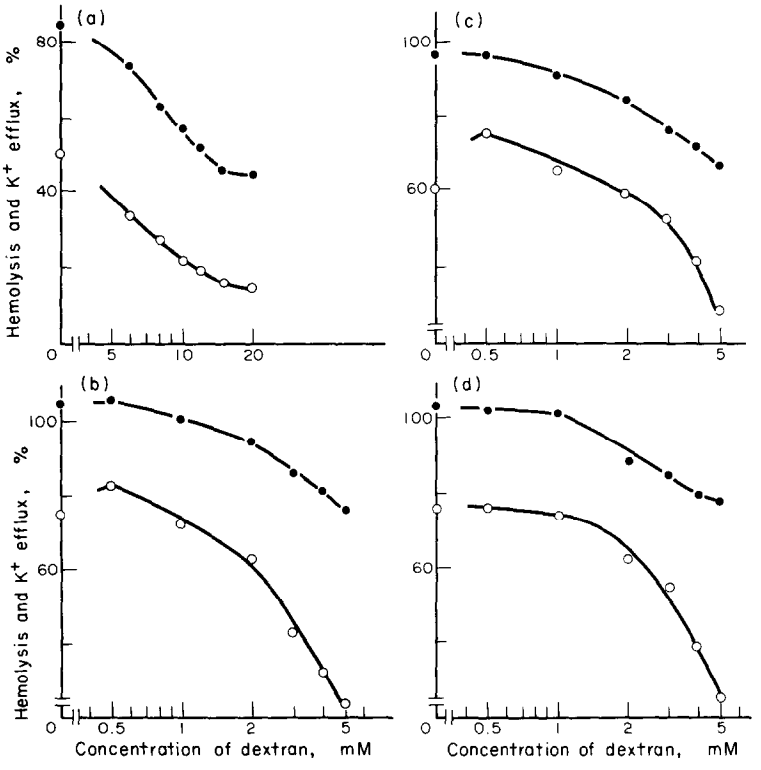
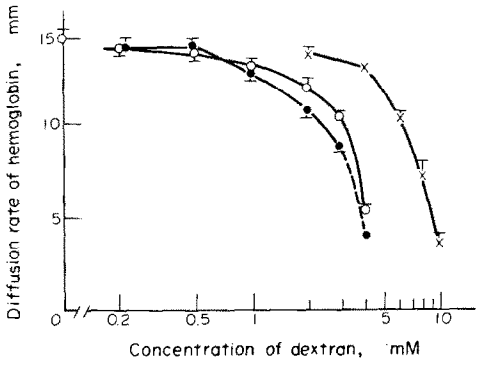


Fig. 2. Effect of dextrans on hemolysis and K^+ efflux induced with drugs. These are representative data. The values of hemolysis and K^+ released in several experiments were not averaged, since the values varied to some extent. The drug concentration was 0.6 mM for chlorpromazine and 0.47 mM for clemastine. Key: hemolysis (○) and K^+ efflux (●). Panel A, dextran KL; panel B, dextran XM; panels C and D, dextran X.

dextran XM and X at 5 mM each reduced chlorpromazine-induced hemolysis. These dextrans also showed a similar effect on clemastine-induced hemolysis. Due to the high viscosity and the solubility of dextrans, concentrations higher than 20 mM dextran KL and 5 mM dextran XM and X were not tested. The effect of adding dextrans on the loss of hemoglobin and K^+ induced with the drugs is shown in Fig. 2. Dextran KL at concentrations higher than 8 mM and dextran XM and X at above 1 mM each produced a significant decrease in the release of hemoglobin and K^+, but in all cases there was K^+ loss more significant than hemoglobin. The



experiments were carried out three times and all results were in good agreement.

Scanning electron microscopic observations. Results of scanning electron microscopy are shown in photomicrographs reproduced in Fig. 3. Examination of these photomicrographs reveals that chlorpromazine induces visual shape changes in the cells which lead to eventual hemolysis. The addition of dextrans to the cell suspension resulted in prevention of shape changes as shown in Fig. 3 D–L. The sequence of shape changes induced with chlorpromazine in the presence or absence of 20 mM dextran KL and 5 mM each dextran XM and X can be summarized as follows: (1) at 0.4 chlorpromazine, at which concentration the hemolysis is initiated, the cells were partially shrunk, induced by the release of K^+ and H_2O from the cells. At 0.8 mM all cells are changed to highly contracted and rigid spheres or ghosts, (2) the cells with 20 mM dextran KL added become almost smooth spheres and even at 0.8 mM drug, at which concentration 100 per cent hemolysis was produced, many cells retained the smooth spheres, (3) the cells with 5 mM each dextran XM and X partially swelled in the presence of 0.4 mM drug and partially shrunk at 0.8 mM drug, although the extent of shrinking was much more in the cells with dextran XM added than those with dextran X.

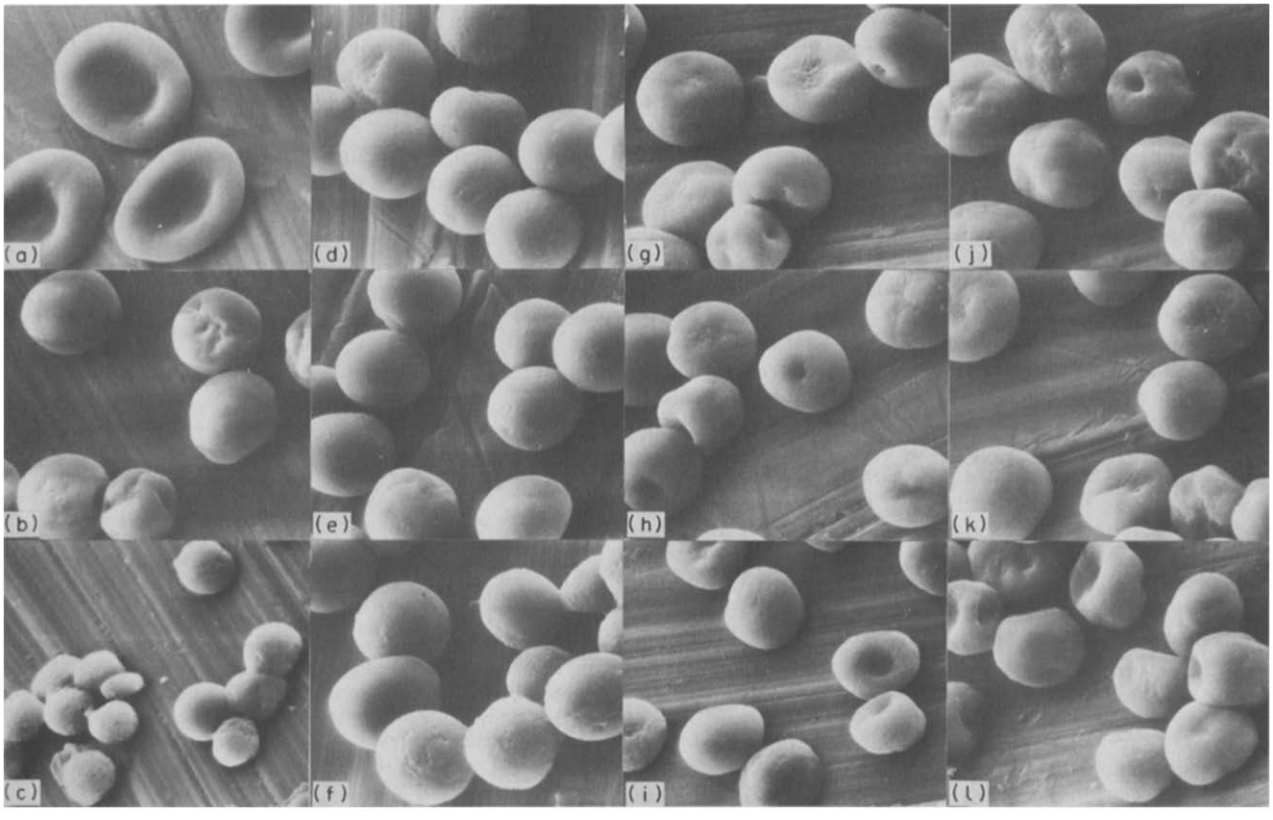


Fig. 3. Scanning electron micrographs of erythrocytes treated with chlorpromazine and with the drug and dextrans. The concentration of dextran was 20 mM for dextran KL and 5 mM each for dextran XM and X. The magnification of the photographs is 7148. (A) control; (B) 0.4 mM drug; (C) 0.6 mM drug; (D) 0.4 mM drug plus dextran KL; (E) 0.6 mM drug plus dextran KL; (F) 0.8 mM drug plus dextran KL; (G) 0.4 mM drug plus dextran XM, (H) 0.6 mM drug plus dextran XM; (I) 0.8 mM drug plus dextran XM; (J) 0.4 mM drug plus dextran X; (K) 0.6 mM drug plus dextran X; and (L) 0.8 mM drug plus dextran X.

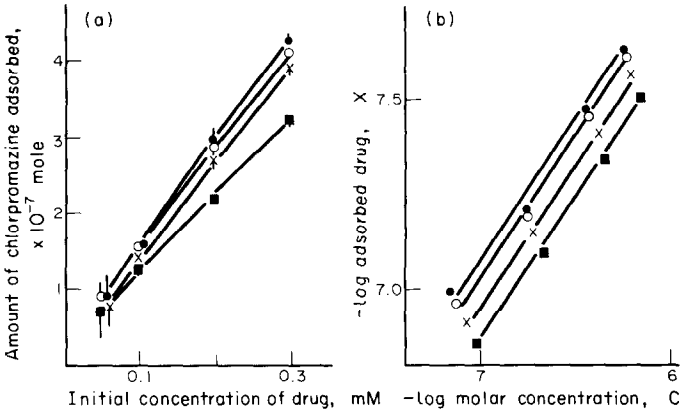


Fig. 4. Adsorption of chlorpromazine to erythrocytes in the presence or absence of dextran X. Experimental conditions are described in text. Points represent the mean ± S.D. of three experiments. Panel A, plots of initial drug concentrations against adsorbed drug (x) and panel B, logarithmic plots of unbound drug concentrations (C) and x. Key: no dextran (●); 0.5 mM dextran (○); 2 mM dextran (×); and 5 mM dextran (■).

In order to clarify the mechanism by which the dextrans inhibit the drug-induced hemolysis, some experiments were done.

Effect of dextrans on fragility of erythrocytes. Erythrocytes were pretreated with 20 mM dextran KL or 5 mM each dextran XM and X for 10 min at 37° and osmotic and heat fragility of the cells was tested. As a result, osmotic fragility of the cells pretreated with these dextrans was not changed; both the control and pretreated cells showed 62–65 per cent hemolysis in 0.4% NaCl solution and completely lyzed in 0.3% NaCl solution. Heat fragility of the cells pretreated was not increased; all cells were subjected to a 13.6–15.6 per cent hemolysis by the exposure for 10 min at 50° and complete hemolysis for the same period at 55°. These results suggest that the dextrans had no stabilizing effect on the cell membrane.

Adsorption of chlorpromazine to erythrocytes in the presence of dextrans. A presumption that dextrans diminish quantities of the drug adsorbed at the surface of the cell membrane was tested and the result is shown in Fig. 4. The quantities of chlorpromazine adsorbed to the cells were decreased with rising concentration of dextran X, approximately 25 per cent decrease in the presence of 5 mM dextran X in comparison with the control, probably due to the decrease in the contact of the drug molecules with the membrane by a sheath of dextran molecules and the enhanced viscosity. Logarithmic plots of unbound drug concentrations (C) against absorbed drug (x), which were linear, show that the adsorption of the drug to the cells follows the adsorption equation of Freundlich, $\log x = \log k + n\log C$ where n and k are constant.

Effect of dextrans on diffusion rate of hemoglobin. Seeman [30] interpreted that the protecting effect of macromolecules such as albumin, dextran and ferritin against hypotonic hemolysis is due to inhibition of hemoglobin release from hemolyzing erythrocytes rather than reduction of the membrane slits [30]. To clarify whether dextran exerts such a protecting effect in the isotonic medium, the effect of the dextrans on diffusion rate of hemoglobin was tested. The result in Fig. 5 indicates that concentrations higher than 4 mM of dextran KL and 1 mM each of dextran XM and X respectively decreased the diffusion rate of hemoglobin, 20 mM dextran KL showed the most protecting effect. In equimolar concentrations, however, dextran X which has the highest mol. wt showed the most inhibitory effect on hemoglobin diffusion. The inhibitory effect accordingly seems to be proportional to the mol. wt of dextrans tested in equimolar concentrations. The inhibition of hemoglobin diffusion agreed well with the inhibitory effect of the dextrans on the drug-induced hemolysis shown in Fig. 2.

A correlation between viscosity of dextrans, protecting effect of them and diffusion rate of hemoglobin. It is of interest to investigate whether or not a correlation exists between the viscosity and pro-

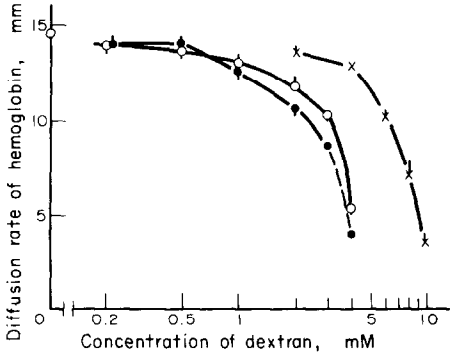


Fig. 5. Effect of concentrations of dextrans on diffusion rate of hemoglobin. Erythrocyte suspension (hematocrit value, 40 ± 1%) was frozen, thawed and centrifuged at 20,000 g for 30 min. An aliquot of 0.2 ml of the supernatant obtained (hemoglobin was 13.4–14.4%) was placed at the bottom of a test tube (0.7 × 10 cm). A 1.5 ml of dextran solution was added over the hemoglobin solution. The height in mm of the sharp frontier of hemoglobin diffusing in the vertical direction after 120 hr at 4° was measured. Points represent the mean ± S.D. of three experiments. Dotted line was drawn approximately, because distinct interface was not obtained. The rate for 5 mM each of dextran XM and X could not be measured because of the inversion of the phase. Key: dextran KL (×); dextran XM (○); and dextran X (●).

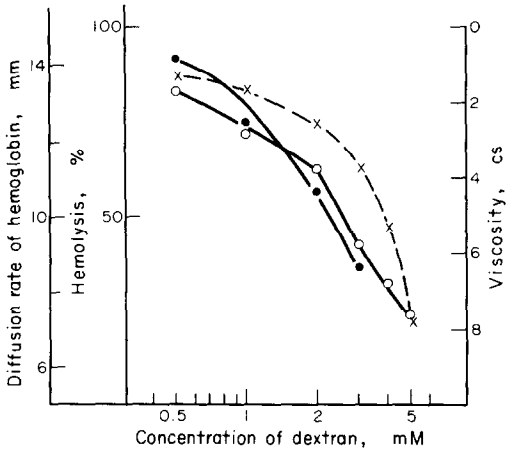


Fig. 6. A correlation between viscosity of dextran X, protecting effect and diffusion rate of hemoglobin. Key: viscosity (×); hemolysis (○); and diffusion rate (●).

tecting effect of the dextrans on drug-induced hemolysis. The viscosity of these dextrans at 5 mM in the isotonic buffer is shown in Table 2 with the percentage hemolysis. In equimolar concentrations, dextran X having the highest viscosity showed the most significant protecting effect on drug-induced hemolysis and dextran KL being the lowest viscosity was the least effect. Figure 6 shows a relationship between the viscosity, the protecting effect and the decrease in diffusion rate of hemoglobin with dextran X. There was a good correlation between them, indicating that consequently an increase in the viscosity of dextran results in a decrease in diffusion rate of hemoglobin and hemolysis.

DISCUSSION

The protection of drug-induced hemolysis was designed in an attempt to prevent adverse reactions of drugs. An extremely wide variety of drugs stabilizes erythrocytes against hypotonic hemolysis [1–14]. Hemolysis induced by H_2O_2, heat and u.v. irradiation was inhibited with anti-inflammatory drugs, and ellipticine-induced hemolysis was protected with citrate, EDTA and oxytetracycline [31–34, 16]. The hemolysis induced with hexachlorophen was markedly delayed by addition of the non-penetrating solute sucrose [21]. Cholesterol sulfate and certain analogs can protect the red blood cells against hypotonic hemolysis [35]. High mol. wt dextrans are shown to inhibit acid hemolysis of paroxysmal nocturnal hemoglobinuria erythrocytes, of which hemolysis appears to be the production of an initial "hole" in the membrane sufficiently large to permit the direct egress of hemoglobin and the inhibition with the dextran is associated with inhibition of K^+ loss [36, 37]. The mechanism of the protective effect is explained as: (1) membrane expansion as shown in the protective effect of drugs against hypotonic hemolysis [12], (2) inhibition of hemoglobin release with macromolecules, albumin, dextran and ferritin [30], (3) providing an external osmotic force based on inability to cross the cell membrane, as in the case with sucrose [21] and phosphorylated compounds [38], (4) providing energy in the form of adenosine triphosphate, as in the case with glucose and adenosine [21, 38]. However, little is known of the compounds which inhibit hemolysis induced with drugs, including tranquilizers and antihistaminics, at higher concentrations.

In the present study these dextrans were found to have a profound protecting effect on drug-induced hemolysis (Figs. 1–3). To clarify the mechanism of this protective effect, some experiments were done. The erythrocytes pretreated with the dextrans had little protective effect against osmotic and heat-induced hemolysis, indicating that the dextrans had no stabilizing effect on the cell membrane and that this protective effect was due to an indirect action on the membrane rather than to a direct one. Electron microscopic observations indicate that the cells were shrunk at concentrations above 0.4 mM chlorpromazine however addition of these dextrans at higher concentrations partially prevented the shrinking and thereby hemolysis. At 20 mM dextran KL most of the cells retained smooth spheres which were not hemolyzed (Fig. 3), probably due to inhibition of the release of K^+, H_2O and hemoblobin. A possible explanation for the inhibitory effect on hemoblobin diffusion is that the dextrans which are extensively hydrated remove solvent volume accessible to the diffusing hemoblobin, as proposed by Seeman [30], and the increased viscosity and the produced sheath of dextran prevent its diffusion. This inhibitory effect of the dextrans seems to be similarly exerted against the cation release from the cells. This is demonstrated by the data that the dextrans protected K^+ efflux from the cells (Fig. 2). Once distributive and diffusive processes have brought a drug molecule to the surface of the cells, random thermal agitation produces multiple collisions with that surface. The drug molecules in the presence of dextran are surrounded wholly or partly by the macromolecules. The surface of the cells, likewise, can be thought of as covered by a sheath of dextran molecules. Therefore, the random thermal agitation of the drug molecules must be prevented by the high viscosity and the sheath of dextran, thereby the binding of the drug to the cells is decreased. Although an important protecting effect of dextran against drug-induced hemolysis is

Table 2. Comparison between viscosity* and protecting effect of dextrans in equimolar concentrations

	Control	Dextran KL	Dextran XM	Dextran X
Hemolysis (%)	60.0 ± 5.0	47.9 ± 5.3	26.8 ± 3.8	22.7 ± 4.3
Viscosity (cs)	1.041 ± 0†	1.423 ± 0.028	4.207 ± 0.073	7.706 ± 0.039

* Viscosity was presented by kinematic viscosity (cs).

† Viscosity of isotonic NaCl solution. Dextran and chlorpromazine concentrations were 5 and 0.59 mM, respectively. Each value represents the mean ± S.D. of three experiments.

due to inhibition of release of K^+ and hemoglobin, as shown in Figs 2 and 6, a decrease in the thermal agitation of the drug molecules with macromolecules such as dextran and consequent decrease in the drug molecules adsorbed to the cells are also an unquestionable and significant effect against drug-induced hemolysis. This is strongly suggested by the data that a decrease in the adsorbed drug was parallel with the increased viscosity of the dextran. The decreased adsorption of the drug therefore probably results in the reduced concentration of the drug in the membrane and thereby the declined disturbance of the arrangement of phospholipids and the hydrophobic interactions between lipids and proteins of the membrane, the disturbance is a main cause of drug-induced hemolysis [22].

On the basis of the results presented in the paper, we conclude that the protective effect of dextrans is due to inhibition of diffusion of hemoglobin and K^+ and the decrease in quantities of drug molecules adsorbed to the cell membrane, probably based on removing solvent volume accessible to the diffusing hemoglobin, the increased viscosity and the sheath of dextran molecules.

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