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Effect of Drugs on Human Erythrocytes. III.[1] Protecting Effect of Chondroitin Sulfate on Drug-induced Hemolysis[2]

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An attempt has been made to prevent drug-induced hemolysis and to clarify the mechanism of the protective effect of chondroitin sulfate. As a result of these studies, it was found that chondroitin sulfate (10^{-4} and 5×10^{-4} M) had a protective effect on chlorpromazine-induced hemolysis and K^+ efflux. Scanning electron microscopic observations indicated that chondroitin sulfate reduced the cell swelling at relatively lower concentrations of chlorpromazine and significantly prevented the shrinking and sinking of the cells at higher concentrations. The erythrocytes pretreated with chondroitin sulfate, however, had little protective effect against osmotic and heat-induced hemolysis. The experiment on 1-anilinonaphthalene-8-sulfonate binding to the ghosts pretreated with chondroitin showed that the hydrophobic environment of the membrane was not significantly affected by the exposure. The cells aggregated in the medium with chondroitin sulfate added, probably due to electrostatic repulsion. The aggregation appears partially to protect the cells from contact with the drug, as indicated by the data that chondroitin sulfate at 10^{-4} and 5×10^{-4} M slightly blocked the binding of the drug to the cell membrane, and to decrease drug-induced hemolysis. Another protecting effect is ascribed to the inhibitory effect of the compound on hemoglobin diffusion.

Keywords——human erythrocytes; protecting of hemolysis; drug-induced hemolysis; chondroitin; chlorpromazine; aggregative effect of chondroitin

At present many kinds of drugs are known to cause hemolysis to varying degrees at higher concentrations,[4] while in very low concentrations these compounds stabilize erythrocytes against hypotonic hemolysis.[4a,b,5] The mechanism of this stabilizing effect is explained as the membrane expansion[4a] or the increase in critical volume with drugs.[5k] The mechanism of hemolysis of the cell, however, at higher drug concentrations has been only partially clarified: Hexachlorophen induces the efflux of Na^+ and K^+ from red cells by directly altering the permeability of the cellular membrane, and secondarily induces osmotic swelling and subsequent hemolysis[6]; ellipticine-induced hemolysis appears to be due to disruption of mem-

1) Part II: T. Ogiso, M. Kurobe, H. Masuda, and Y. Kato, *Chem. Pharm. Bull.* (Tokyo), **25**, 1078 (1977).
2) This work was presented at the 97 th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April, 1977.
3) Location: *Mitahora, Higashi-5-6-1, Gifu.*
4) *a*) P. Seeman and J. Weinstein, *Biochem. Pharmacol.*, **15**, 1737 (1966); *b*) P. Seeman, *Biochem. Pharmacol.*, **15**, 1753 (1966); *c*) J. Dausset and L. Contu, *Ann. Rev. Med.*, **18**, 55 (1967); *d*) I.P. Lee, *J. Pharm. Exptl. Therap.*, **196**, 525 (1976); *e*) T. Ogiso, M. Watanabe, K. Yamauchi, T. Sato, and Y. Kato, *Nippon Yakurigaku Zasshi*, **72**, 145 (1976).
5) *a*) H. Chaplin Jr., H. Crawfold, M. Cutbush, and P.L. Mollison, *J. Clin. Pathol.*, **5**, 91 (1952); *b*) O. Schales, *Proc. Soc. Exptl. Biol. Med.*, **83**, 593 (1953); *c*) P.M. Seeman and H.S. Bialy, *Biochem. Pharmacol.*, **12**, 1181 (1963); *d*) G. Zografi, D.E. Auslander, and P.L. Lytell, *J. Pharm. Sci.*, **53**, 573 (1964); *e*) L.L.M. van Deenen and R.A. Demel, *Biochim. Biophys. Acta*, **94**, 314 (1965); *f*) A.R. Freeman and M.A. Spirtes, *Biochem. Pharmacol.*, **11**, 161 (1962); *g*) A.R. Freeman and M.A. Spirtes, *Biochem. Pharmacol.*, **12**, 47 (1963); *h*) A.R. Freeman and M.A. Spirtes, *Biochem. Pharmacol.*, **12**, 1235 (1963); *i*) J.D. Judah, "Drugs and Enzymes, CIBA Symposium," ed. by J.L. Mongar and A.V.S. de Reuck, Little Brown, Boston, 1962, p. 359; *j*) S. Roth and P. Seeman, *Biochim. Biophys. Acta*, **255**, 190 (1972); *k*) J. van Steveninck, W.K. Gjösûnt, and H.L. Booij, *Biochem. Pharmacol.*, **16**, 837 (1967); *l*) S. Roth and P. Seeman, *Nature New Biol.*, **231**, 284 (1971); *m*) F. Okumura, J. Koh, and I. Ueda, *Masui*, **17**, 186 (1968).
6) T.L. Miller and D.R. Buhler, *Biochim. Biophys. Acta*, **352**, 86 (1974).

brane structure as a result of the drug-phospholipid and protein interactions.[4d] We also proposed that hemolysis induced with chlorpromazine (CP) 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.[1] 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. Therefore, the drugs which give rise to hemolysis may cause many kinds of adverse reactions. It is shown that some drugs at high concentrations have a lytic action on lysosomes.[7] The protection of drug-induced hemolysis thus is of great importance in preventing such adverse reactions of drugs. In an attempt to obtain information bearing on protection of hemolysis, a study of the effects of some compounds on the stability of human erythrocytes has been initiated. This report deals with protection of drug-induced hemolysis with chondroitin sulfate and some mechanisms of the protective effect.

Experimental

Materials——Chlorpromazine hydrochloride (Nihon Shinyaku) was used in this experiment. Chondroitin sulfate sodium salt (special grade) and 1-anilinonaphthalene-8-sulfonate (ANS) were obtained from Seikagaku Kogyo Co., Ltd. and Tokyo Kasei Kogyo Co., Ltd., respectively.

Preparation of Erythrocyte Suspension——Human blood collected from hematologically normal adult donors, utilizing sodium citrate as an anticoagulant, was washed 3 times with isotonic NaCl solution, pH 7.4, by the method described in a previous paper.[8] Hematocrit value was ordinarily $40\pm1\%$ and in some experiments $48\pm1\%$.

Preparation of Hemoglobin-free Erythrocyte Ghosts——Hemoglobin-free erythrocyte ghosts were prepared according to the method of Dodge, *et al.*[9] The ghosts were resealed by the procedure of Mueller and Morrison.[10]

Drug-induced Hemolysis——To a mixture consisted of 2 ml of the drug solution (10^{-4}—10^{-3} M in the incubation medium) and 1 ml of chondroitin sulfate (calculated as a molecular weight of 50000) solution 0.3 ml of the erythrocyte suspension (hematocrit value, $40\pm1\%$) was added and mixed immediately. The test and chondroitin solutions were usually prepared in isotonic NaCl solution. 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.[8]

Electron Microscopy——Erythrocytes, treated with the drug and centrifuged, were fixed with 1.5% glutaraldehyde in isotonic phosphate buffer, pH 7.2. The cells were washed 3 times with isotonic phosphate buffer, pH 7.2, and dried with increasing concentrations of acetone (60 to 100%, v/v). The specimens were coated at continuously varying angles with gold and viewed with a Nihon Denshi scanning electron microscope, Model JEM-100B.

Potassium Measurements——To 2 ml of the supernatant obtained in the experiment on drug-induced hemolysis an equal volume of 10% trichloroacetic acid was added. The mixture was stood overnight at 4° and centrifuged at $1500\times g$ for 10 min. Potassium released was measured with a Hitachi atomic flame absorption spectrophotometer at 7665 Å.

Measurements of ANS-ghost Fluorescence——A mixture consisted of 4 ml of erythrocyte ghost suspension (3.9—4.0 mg protein/ml) and 2 ml of 1.5×10^{-3} M chondroitin solution was incubated for 10 min at 37°. To 1 ml of the mixture 5 ml of the drug solution was added and incubated for 60 min at 37°. After centrifugation for 30 min at $20000\times g$, the ghost bottom was washed once with isotonic NaCl solution and resuspended in 5 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 solution. The fluorescence of ANS-ghost was measured by the same method as described previously.[1]

Measurements of Drug Adsorption to Erythrocytes——To a mixture consisted of 2 ml of the drug solution (5×10^{-5}—3×10^{-4} M in the incubation medium) and 1 ml of chondroitin solution 0.3 ml of erythrocyte suspension (hematocrit value, $40\pm1\%$) was added and incubated for 30 min at 37°. After centrifugation the concentration of chlorpromazine in the supernatant was determined spectrophotometrically by measuring

7) D.A. Lewis, *J. Pharm. Pharmacol.*, **22**, 902 (1970); D.A. Lewis, A.M. Symons, and R.J. Ancill, *J. Pharm. Pharmacol.*, **22**, 909 (1970).
8) T. Ogiso, S. Imai, R. Hozumi, M. Kurobe, and Y. Kato, *Chem. Pharm. Bull.* (Tokyo), **24**, 479 (1976).
9) J.T. Dodge, C. Mitchell, and D.J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).
10) T.J. Mueller and M. Morrison, *Biochemistry*, **14**, 5512 (1975).

the absorbance at 255 nm. The absorbance at 255 nm was corrected for the absorption of chondroitin and proteins in the supernatant. The contribution of proteins, mainly hemoglobin, to the absorbance at 255 nm was calculated from the absorbance ratio (1.23) of 280 nm to 255 nm.

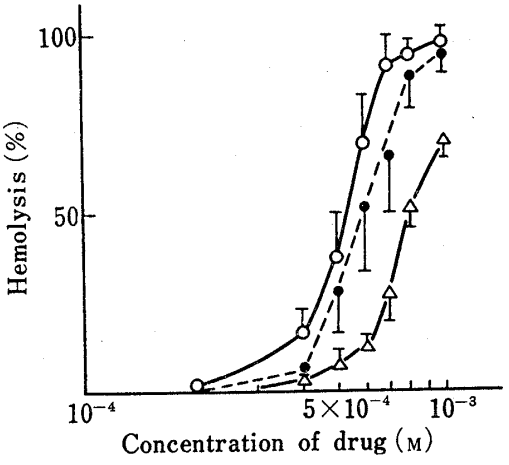
Measurements of Diffusion Rate of Hemoglobin——The diffusibility of hemoglobin was tested according to the method described by Seeman[11] with a slight modification. Erythrocyte suspension (hematocrit value, 40±1%) was frozen and thawed, and the membranes were discarded after centrifugation at 20000×*g* for 30 min. The final concentration of hemoglobin, assayed by CN-methemoglobin method,[12] in the hemolysate was 14% (13.4—14.4%). An aliquot of 0.2 ml was placed at the bottom of a test tube (0.7×10 cm). A 1.5 ml of chondroitin solution (10^{-6}—10^{-3} M) 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.

Protein Determination——Protein concentration was determined by the procedure described by Lowry, *et al.*[13] with bovine albumin, fraction V, as a standard.

Results

Protective Effect of Chondroitin Sulfate on Hemolysis and K^+ Efflux Induced with CP

As shown in Fig. 1, chondroitin sulfate at 10^{-4} and 5×10^{-4} M reduced the hemolysis induced with CP. A higher concentration of chondroitin sulfate, 5×10^{-4} M, increased the protective effect significantly. Due to the high viscosity of the solution, concentrations higher than 5×10^{-4} M were not tested. The effect of adding chondroitin sulfate on the loss of hemoglobin and K^+ induced with CP is shown in Fig. 2. With higher concentrations of chondroitin sulfate, *i.e.* 10^{-4} and 5×10^{-4} M, there was a significant decrease in the release of hemoglobin and K^+, but in all cases was K^+ loss more significant than hemoglobin.



Concentration of drug (M)

Fig. 1. Effect of Chondroitin Sulfate on Chlorpromazine-induced Hemolysis

Experimental conditions are described in text. Points represent the mean ± SD of 3 experiments. ○, no chondroitin; ●, 5×10^{-5}M chondroitin; △, 5×10^{-4}M chondroitin.

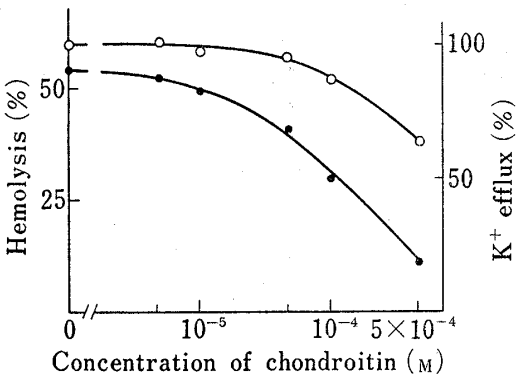


Fig. 2. Effect of Chondroitin Sulfate on Hemolysis and K^+ Efflux Induced with Chlorpromazine

This is a representative datum. The values of hemolysis and K^+ released in several experiments were not averaged, since the values varied to some extent. The drug concentration was 6.0×10^{-4}M. ●, hemolysis; ○, K^+ efflux.

Scanning Electron Microscopic Observations

Results of scanning electron microscopy are shown in photomicrographs reproduced in Fig. 3. Examination of these photomicrographs reveals that CP induces visual shape changes in the red cells which lead to eventual hemolysis, and that this result agrees well the data reported previously.[8] The addition of chondroitin sulfate to the cell suspension resulted in

11) P. Seeman, *Can. J. Physiol. Pharmacol.*, **51**, 226 (1973).
12) T. Fujii, T. Watanabe, and M. Okuda, "Rinsho Kagaku Soron," Hirokawa, Tokyo, 1975, p. 118.
13) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

the prevention of shape changes to some extent as shown in Fig. 3 e), f) and g). The sequence of shape changes induced with CP in the presence or absence of 5×10^{-4} M chondroitin sulfate can be summarized as follows; 1) at 10^{-4} M of CP the cells with chondroitin sulfate added show a less swelling as compared with that treated with CP alone, 2) at 2×10^{-4} M both cells become almost smooth spheres, 3) at 4×10^{-4} M the shrinking and sinking of the cells in the presence of chondroitin sulfate are relatively less, 5) at 6×10^{-4} and 8×10^{-4} M there are relatively more smooth spheres which are not hemolyzed by addition of chondroitin sulfate, whereas all cells are changed to highly contracted globules and ghosts in its absence (Fig. 3d mainly shows the contracted cells).

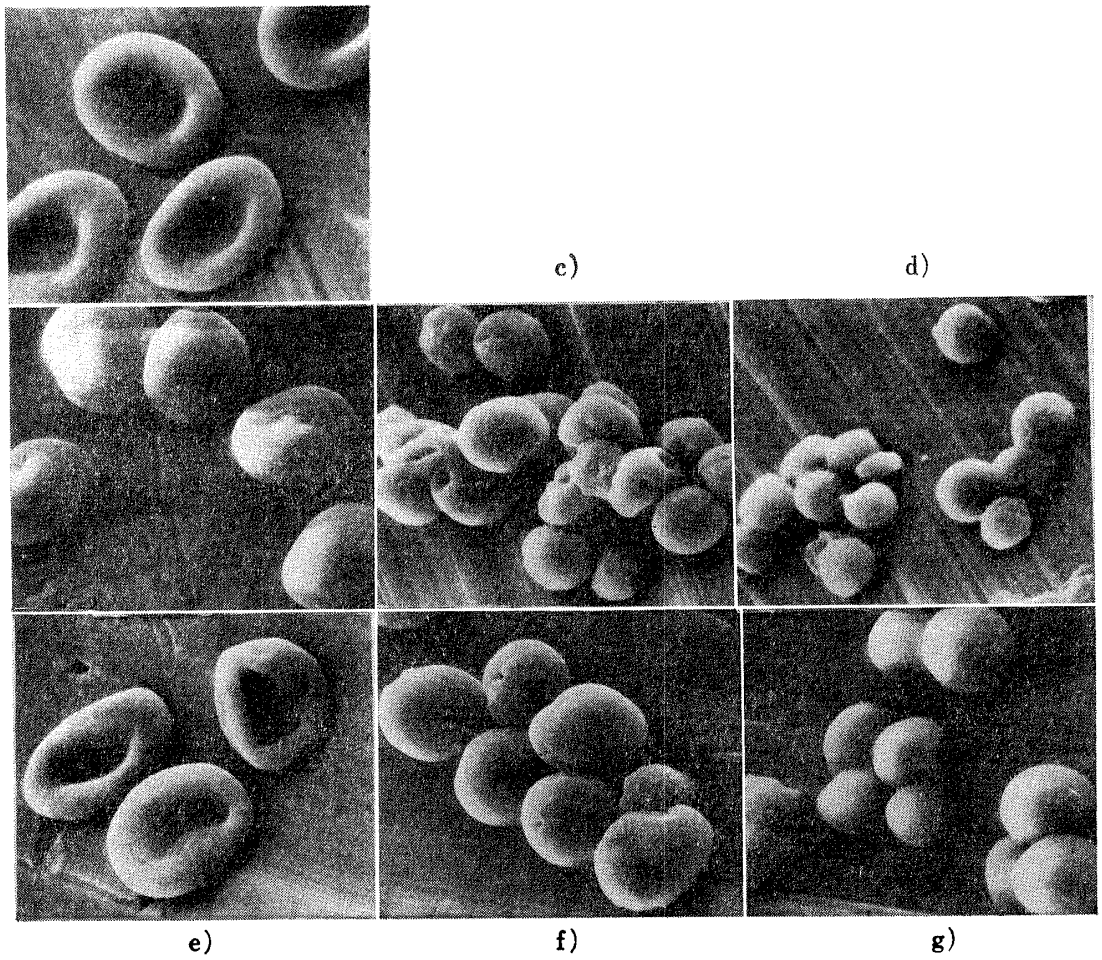


Fig. 3. Scanning Electron Micrographs of Erythrocytes Treated with Chlorpromazine and with the Drug and Chondroitin Sulfate

a) no drug treatment; b) 10^{-4}M CP; c) 6×10^{-4}M CP; d) 8×10^{-4}M CP; e) 10^{-4}M CP plus chondroitin; f) 6×10^{-4}M CP plus chondroitin; g) 8×10^{-4}M CP plus chondroitin.
The concentration of chondroitin sulfate was 5×10^{-4}M for all. Magnification $\times7148$.

In order to clarify the mechanism of the anti-hemolytic effect of chondroitin sulfate, some experiments were carried out.

Stability of Erythrocytes Pretreated with Chondroitin Sulfate

Erythrocytes were pretreated with 2×10^{-5}, 10^{-4} and 5×10^{-4} M chondroitin sulfate for 10 min at 37° and osmotic and heat fragility of the cells was tested. Consequently, osmotic fragility of the cells pretreated with chondroitin sulfate was not significantly decreased, even at its high concentration, 5×10^{-4} M. Similarly, heat stability of the cells was not increased

with chondroitin sulfate and inversely the cells were found to become unstable to a lesser extent.

ANS-ghost Fluorescence

To clarify whether or not chondroitin sulfate induces alterations of the hydrophobic environment of the ghost membrane and the alterations may be involved in the protective effect, ANS, hydrophobic fluorescent probe,[14] was adapted to the ghosts pretreated with chondroitin sulfate. As a result, ANS binding to the ghosts pretreated with chondroitin sulfate was greatly enhanced with rising concentration of the drug, however, the untreated ghosts were also increased the binding in the same manner, as reported previously,[1] although a slight difference was shown in the fluorescence intensity; the ghosts pretreated with chondroitin sulfate had a slightly higher intensity over a range of the drug concentrations tested in comparison with untreated ghosts. This indicates that the hydrophobic environment of the membrane was little affected by chondroitin treatment.

Aggregation of Erythrocytes Treated with Chondroitin Sulfate

It was found during these experiments that the erythrocytes were precipitated quickly in the presence of chondroitin sulfate. To resolve this phenomenon the photomicrographs of the cells in the presence or absence of 10^{-4} and 5×10^{-4} M chondroitin sulfate and 2×10^{-4} M CP were taken and the result is shown in Fig. 4. There is a general trend for the cells to aggregate in the medium with chondroitin sulfate added. At the concentration of 5×10^{-4} M chondroitin sulfate the cells formed large aggregates. The aggregation probably leads to protection of the cells from contact with the drug molecules and to the decreased hemolysis.

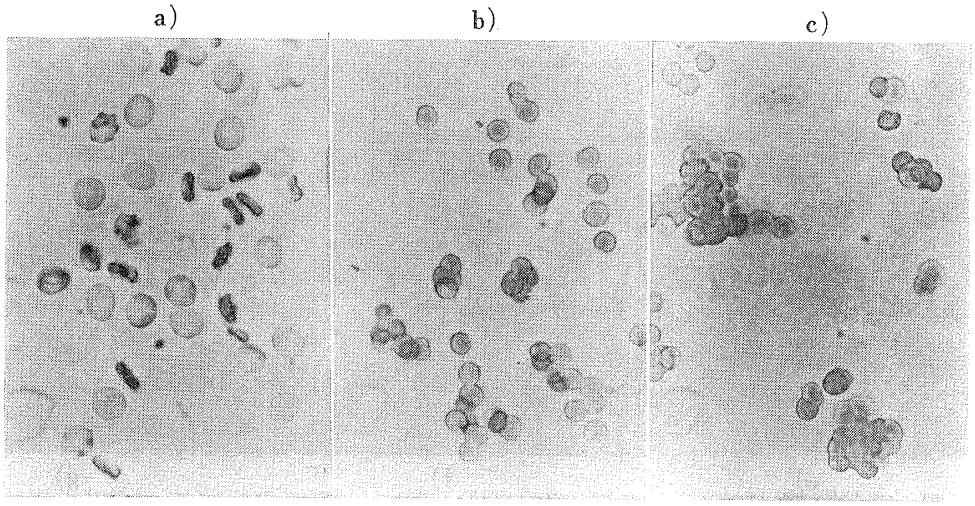


Fig. 4. Photomicrographs of Erythrocytes Treated with Chlorpromazine and Chondroitin Sulfate

a) normal erythrocytes; b) 2×10^{-4}M CP plus 10^{-4}M chondroitin sulfate. c) 2×10^{-4}M CP plus 5×10^{-4}M chondroitin sulfate. Magnification ×650.

Adsorption of CP to Erythrocytes in the Presence of Chondroitin Sulfate

A presumption to explain the protective effect is that chondroitin sulfate diminishes quantities of CP adsorbed at the surface of the cell membrane as a result of the aggregation. This is tested and the result is shown in Table I. The quantities of CP adsorbed to the cells were little changed in the presence of 2×10^{-5} M chondroitin sulfate. However, the increased concentration (10^{-4} and 5×10^{-4} M) of chondroitin sulfate induced a slightly decreased drug

14) T.E. Elling and R.P. DiAugustine, *Biochem. J.*, **123**, 539 (1971).

adsorption, a decrease of approximately 13% in the presence of 5×10^{-4} M chondroitin sulfate as compared with that of the control with 3×10^{-4} M drug added.

Table I. Adsorption of Chlorpromazine to Erythrocytes in the Presence or Absence of Chondroitin Sulfate

Drug concentration (M)	Amount of adsorbed drug ($\times10^{-8}$ mol/0.3 ml cell suspension) Chondroitin (M)			
	None	2×10^{-5}	10^{-4}	5×10^{-4}
5×10^{-5}	9.5	9.6	9.1	8.2
10^{-4}	18.0	18.2	17.0	15.5
2×10^{-4}	30.7	30.7	29.2	27.0
3×10^{-4}	43.7	43.8	41.2	38.0

The values were corrected for the absorption of proteins released and chondroitin. Values are means of 2 experiments.

Table II. Effect of Shaking on Protective Effect of Chondroitin Sulfate

Drug concentration ($\times10^{-4}$M)	Hemolysis (%)		
	Chondroitin (5×10^{-4}M) Shaking times		No chondroitin Shaking times
	12	3	3
2	2.7±0.1	0.7±0.3	2.2± 1.4
4	12.8±0.7	3.8±0.7	15.0± 4.1
6	56.4±2.5	12.9±0.6	70.1±17.1
8	83.2±1.1	50.9±5.4	93.7± 6.9
10	87.7±1.2	70.5±2.2	96.8± 6.3

The tube was inversed 3 times every 5 or 20 min during incubation for 1 hr at 37°. Values are means ±SD of 3 experiments.

Effect of Shaking on Protective Effect of Chondroitin Sulfate

To clarify whether the reduction in hemoglobin release is mainly due to the aggregative effect of chondroitin sulfate based on the electrostatic repulsion, the mixture consisted of erythrocytes, the drug and chondroitin sulfate was repeatedly shaken by inversion at intervals of 5 min, which prevented the precipitation of the cells, during incubation for 1 hr and compared with normal shaking at intervals of 20 min. The result is shown in Table II. The increased shaking resulted in a significant decrease in the protective effect of chondroitin sulfate, although some protective effects were still retained.

Effect of Chondroitin Sulfate on Diffusion Rate of Hemoglobin

The result in Fig. 5 indicates that higher concentrations (above 5×10^{-4} M) of chondroitin sulfate decreased the diffusion rate of hemoglobin, but not to a significant degree in the concentrations below 2×10^{-4} M. Chondroitin sulfate at 10^{-3} M showed the highly inhibitory effect on the diffusion of hemoglobin. The higher concentrations of chondroitin sulfate thus appear to

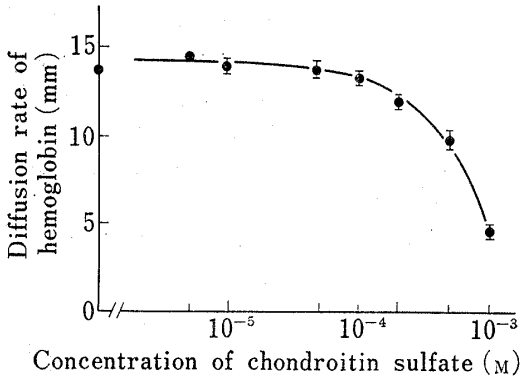


Fig. 5. Effect of Chondroitin Sulfate on Diffusion Rate of Hemoglobin

Points represent the mean ±SD of 3 experiments.

prevent the drug-induced hemolysis by the inhibition of hemoglobin diffusion. The inhibitory effect on hemoglobin diffusion from hemolyzing erythrocytes agreed well with the inhibition of K^+ efflux with the compound.

Discussion

The protection of drug-induced hemolysis seems to be important in terms of a reduction of adverse reactions of drugs. It is known that an extremely wide variety of drugs stabilizes erythrocytes against hypotonic hemolysis.[4a,b,5] Hemolysis induced by H_2O_2[15] and heat[16] was inhibited with anti-inflammatory drugs, and ellipticine (an antineoplastic agent)-induced hemolysis was protected with citrate, EDTA and oxytetracycline.[4d] The hemolysis induced with hexachlorophen was markedly delayed by addition of the nonpenetrating solute sucrose to the incubation mixture.[6] The mechanism of the protective effect is explained as 1) membrane expansion as shown in the protective effect of drugs against hypotonic hemolysis,[4a] 2) providing an external osmotic force based on inability to cross the cell membrane, as in the case with sucrose[6] and phosphorylated compounds,[17] 3) providing energy in the form of adenosine triphosphate, as in the case with glucose and adenosine.[6,17] However, little is known of the compounds which inhibit hemolysis induced with drugs such as anesthetics, tranquilizers and antihistaminics at higher concentrations.

In the present study chondroitin sulfate was found to have a protecting effect on drug-induced hemolysis. In order to clarify the mechanism of this protective effect, some experiments were carried out. The erythrocytes pretreated with chondroitin sulfate had little protective effect against osmotic and heat-induced hemolysis, indicating that chondroitin sulfate had no stabilizing effect on the cell membrane and that this protective effect is due to an indirect action on the membrane rather than to direct one. The experiment on ANS binding to ghosts shows that the hydrophobic environment of the membrane is not significantly affected by the exposure with chondroitin sulfate, although a slight enhancement of ANS-ghost fluorescence intensity is observed on the pretreatment with chondroitin sulfate. The slight alterations of the hydrophobic region observed may be not involved in the protective effect. The aggregation of erythrocytes in the presence of chondroitin sulfate is probably attributed to electrostatic repulsion based on the negative charge of the compound, since erythrocytes have weak negative charges.[18] Therefore, the aggregated cells may be avoided from continuous contact with the drug molecules and retain the hydrophobic arrangement of the membrane, thereby at least partially decrease the lysis. One possibility that chondroitin sulfate inhibits the binding of the drug to the membrane and accordingly protects the drug-induced hemolysis as a result of the aggregation was partially demonstrated by the data that chondroitin sulfate at concentrations of 10^{-4} and 5×10^{-4} M slightly blocked the binding of the drug (Table I). However, this action may be unable to explain all of the protecting effect observed herein. Probably there may be another mechanism of the effect with chondroitin sulfate, since the protecting action with the compound was significantly decreased but not lost by the repeated shaking (Table II). Seeman 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 (100—1000 Å) accompanying changes in conformation of the membranes.[11] High concentrations of the macromolecules, which are extensively hydrated, were proposed to remove solvent volume accessible to the diffusing hemo-

15) S. Otomo and E. Fujihira, *Yakugaku Zasshi*, **90**, 1347 (1970).
16) D.A. Kalbhen and R. Loyen, *Arzneim.-Forsh.*, **23**, 945 (1973); Y. Mizushima, S. Sakai, and M. Yamamura, *Biochem. Pharmacol.*, **19**, 227 (1970).
17) D.N. Mohler, *Blood*, **30**, 449 (1967).
18) H. Yoshikawa and Y. Nakao (ed), "Ketsueki No Seikagaku," Asakura, Tokyo, 1969, pp. 79—83.

globin.[11] Chondroitin sulfate used in this experiment was found to exert a similar action in the isotonic medium (Fig. 5). This compound thus may inhibit diffusion of hemoglobin and K^+ from lysing cells by exclusion of solvent.

Based on these results, it seems likely that the protective effect of chondroitin sulfate is due to the aggregation of the cells, the inhibition of diffusion of hemoglobin and the decrease in K^+ efflux.