Characterization of the Inclusion Mode of β-Cyclodextrin Sulfate and Its Effect on the Chlorpromazine-Induced Hemolysis of Rabbit Erythrocytes

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The inclusion mode of β-cyclodextrin sulfate (β-CyD-sul) with a cationic drug, chlorpromazine, was investigated, and the effect of β-CyD-sul on the hemolytic activity of chlorpromazine was compared with that of parent β-CyD. The interaction of β-CyD-sul with chlorpromazine was weaker than that of parent β-CyD, probably because of the steric or electrostatic repulsion between anionic sulfate groups and hydrophobic phenothiazine moiety. Spectroscopic studies, including pH- and salt-effects, as well as thermodynamic parameters, suggested that both electrostatic and hydrophobic interactions are operative in the inclusion complexation of β-CyD-sul with chlorpromazine. The inhibiting effect of parent β-CyD on the chlorpromazine-induced hemolysis of rabbit erythrocytes was accounted for by the decreased fraction of free drug through the complexation. In the case of β-CyD-sul, the hemolysis and binding of the drug to the erythrocyte membrane was higher than those estimated from the fraction of free drug, probably due to the increased hydrophobicity of the drug through the complexation. However, the chlorpromazine-induced shape change of the erythrocytes was significantly suppressed by β-CyD-sul, and its inhibiting effect was greater than that of β-CyD, because of the counterbalance of the opposite effects, *i.e.*, internalization and externalization induced by chlorpromazine and β-CyD-sul, respectively.

Keywords β-cyclodextrin sulfate; inclusion mode; chlorpromazine; hemolysis; morphological change

Recently, extensive efforts have been directed toward the development of more functional and safer cyclodextrin (CyD) derivatives as parenteral drug carriers.[1,2] Of the CyD derivatives examined so far, 2-hydroxypropylated CyDs[3,4] and branched CyDs[5,6] have gained some acceptance for parenteral use. More recently, polysulfated CyDs have been evaluated as a new class of parenteral drug carrier,[7,8] because CyD sulfates (CyD-suls) are highly hydrophilic,[9] and less hemolytic than the parent CyDs.[10] Our previous studies have shown that CyD-suls were less toxic than the parent CyDs and dextran sulfate when administered parenterally into rats,[8] and they protected against aminoglycoside-induced acute renal failure.[7] Since the highly hydrated and negatively-charged sulfate groups of CyD-suls are located near the entrance of the cavity, the inclusion ability of CyD-suls may be different from that of non-ionizable CyDs such as parent, alkylated, hydroxyalkylated and branched CyDs. In this study, therefore, the interaction of β-CyD-sul with chlorpromazine, a typical cationic drug, was investigated spectrophotometrically in order to compare it with the interaction mode of parent β-CyD reported previously.[11] Furthermore, we examined the effect of β-CyD-sul on the hemolytic activity of chlorpromazine, which interacted with β-CyD-sul most significantly among the drugs employed.[12]

Materials and Methods

Materials Chlorpromazine hydrochloride was obtained from Nacalai Tesque Co., Ltd. (Kyoto, Japan). β-CyD was donated by Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). β-CyD-sul was a gift from Kaken Pharmaceutical Co. (Shizuoka, Japan). The average degree of substitution of sulfate groups in β-CyD-sul was 10.7, as calculated from a ratio of existent values obtained by fast-atom bombardment mass spectrometry recorded in a negative ion mode using triethanolamine as a matrix.[9] This value was in good agreement with the sulfur content (about 16%) of β-CyD-sul determined by the method of oxygen flask combustion.

The Interaction between Chlorpromazine and β-CyDs Ultraviolet (UV) and circular dichroism (CD) spectra were recorded with a Hitachi U-3200 spectrophotometer (Tokyo, Japan) and a Jasco J-600 recording polarimeter (Tokyo, Japan), respectively. The stability constants (K_c) of complexes of chlorpromazine with β-CyDs at 15—50 °C were determined spectrophotometrically. UV absorption changes of chlorpromazine in the presence of β-CyDs ($1.0—7.0 \times 10^{-3}$ M) in 0.01 M phosphate buffer (ionic strength 0.05—0.5, pH 3.0—7.4) were measured at appropriate wavelengths of UV absorption. The K_c and ε_c values were determined according to the following Scott equation[13]:

$$a \cdot b/d = 1/K_c \cdot \varepsilon_c + b/\varepsilon_c$$

where a is the total concentration of chlorpromazine, b is the total concentration of β-CyDs, ε_c is the difference in molar absorptivities for free and complexed chlorpromazine, and d is the change in absorbance of chlorpromazine by the addition of β-CyDs. The stoichiometry of the complexes in an isotonic phosphate buffer (pH 7.4) at 25 °C was determined by a continuous variation method, analyzing the intensity of the induced CD.[14]

Hemolysis Assays The hemolytic activity of chlorpromazine was assessed as described previously.[15] From freshly drawn citrated rabbit blood, erythrocytes were separated by centrifugation at $1000 \times g$ for 5 min, washed three times with phosphate buffered saline (0.154 M sodium chloride, 0.01 M phosphate, pH 7.4) and resuspended in the buffer solution to give a hematocrit of 5%. The cell suspension (0.1 ml) was added to the buffer solution (2 ml) containing chlorpromazine at various concentrations. Each mixture was incubated for 30 min at 37 °C and then centrifuged at $1000 \times g$ for 5 min. The release of hemoglobin from the cells was measured spectrophotometrically at 543 nm. Results were expressed as the percentage of total efflux of hemoglobin, which was obtained when water was used instead of the buffer solution.

Binding Test of Chlorpromazine to Erythrocytes Under the pre-hemolytic condition, the binding test of chlorpromazine to rabbit erythrocytes in the absence and presence of β-CyDs was performed according to the method described previously.[16] After the addition of 1 ml of the drug (3.0×10^{-4} M) and 1 ml of β-CyDs ($0.25—1.0 \times 10^{-3}$ M) to the erythrocyte suspension (20%, 1 ml), the mixture was incubated for 5 min at 37 °C, and then centrifuged at $1000 \times g$ for 5 min. The amount of drug in the supernatant was determined by the HPLC method under the following conditions: pump, Hitachi 635 A (Hitachi, Ltd., Tokyo, Japan); UV detector, Hitachi 655 A-21 (Hitachi Ltd., Tokyo, Japan); column, YMC packed column (AM-312, S-5, 120 Å, ODS, Kyoto,

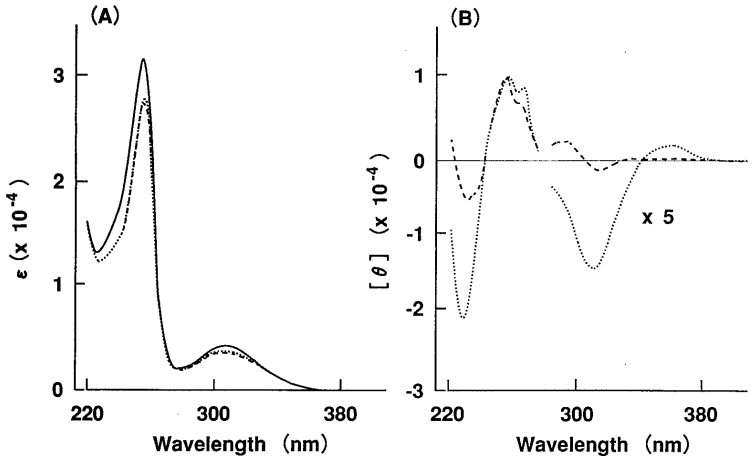

Fig. 1. UV (A)[a] and Induced CD (B)[b] Spectra of Chlorpromazine in the Absence and Presence of β-CyDs (5.0×10^{-4} M) in Isotonic Phosphate Buffer (pH 7.4) at 25 °C

——, without CyDs; ------, with β-CyD; ·····, with β-CyD-sul. *a*) The concentration of chlorpromazine was 2.5×10^{-5} M. *b*) The concentration of chlorpromazine was 1.0×10^{-4} M.

Japan); wavelength, 255 nm; mobile phase, acetonitrile: 0.05 M ammonium acetate (65 : 35 V/V); flow rate, 1.4 ml/min.

Morphological Observation of Erythrocytes The cell suspension (5%, 0.1 ml) was incubated with the buffer solution (2 ml) containing CyDs or chlorpromazine at 37 °C up to 60 min, and then fixed with 2% glutaraldehyde solution (5 ml). After standing for 1 h at room temperature, the fixed cells were washed three times with water, dried under reduced pressure for 16 h and coated with gold. The preparation was then observed by a scanning electron microscope (Akashi, MSM4C, Tokyo, Japan). The degree of shape changes of the cells was expressed by morphological indices as described by Fujii *et al.*,[17] where discocytes were assigned a score of 0, echinocytes were assigned a score of 1 to 4, and stomatocytes were assigned a score of −1 to −4. The average score for a field of about 25 cells was called its morphological index.

Results and Discussion

Interaction of Chlorpromazine with β-CyD-sul Figure 1 shows the UV and CD spectra of chlorpromazine in the absence and presence of β-CyD or β-CyD-sul. The UV absorption of chlorpromazine at 255 nm was shifted to a slightly longer wavelength (about 2 nm), with a concomitant decrease in the molar absorption coefficient, by the addition of β-CyDs. The UV intensity at 307 nm of the drug decreased in the presence of β-CyDs. These spectral changes were similar to those when the drug was dissolved in less polar solvents such as ethanol.[18] In the CD spectra, the optical activity of chlorpromazine was induced at about 230, 257 (with shoulder), 315 and 365 nm, with an alternative sign by the addition of β-CyDs. Since chlorpromazine is optically inactive and both β-CyDs have no CD band at wavelengths longer than 220 nm under these experimental conditions, the observed optical activities were attributable to the induced Cotton effect of chlorpromazine by binding to β-CyDs. The CD pattern of the β-CyD-sul system resembled that of the parent β-CyD system. The larger Cotton effect of the former, compared with the latter, may be ascribed to the high polarizability of the O–S bond or to the different conformation of β-CyD-sul.[19,20] Figure 2 shows the continuous variation plots[14] of changes in the ellipticity of chlorpromazine at 250 nm (β-CyD) and 265 nm (β-CyD-sul). Both β-CyD systems gave a peak at a 1 : 1 molar ratio of the guest and host, indicating a 1 : 1

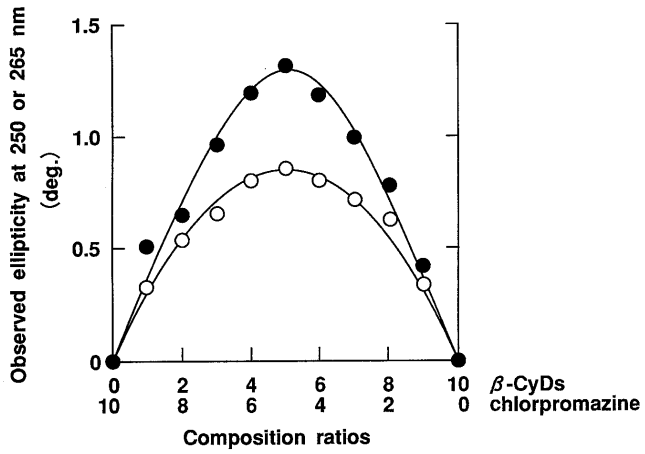

Fig. 2. Continuous Variation Plots for Chlorpromazine–β-CyDs Systems in Isotonic Phosphate Buffer (pH 7.4) at 25 °C

○, β-CyD system; ●, β-CyD-sul system.

stoichiometry of the complexes. These results suggest that the inclusion mode of the chlorpromazine-β-CyD-sul complex is generally similar to that of the parent β-CyD complex.[11] To elucidate the inclusion characteristic of β-CyD-sul in detail, the effects of pH, ionic strength and temperature on the complex formation of chlorpromazine with β-CyD-sul were investigated. As shown in Fig. 3A, the K_c value of the β-CyD-sul complex decreased with an increase in the pH of the solution, whereas that of the β-CyD complex was little changed. Since the pK_a of the dimethylamino moiety of chlorpromazine is 9.30,[21] the decrease in K_c value with the pH change suggests that the cationic site of chlorpromazine takes part in the interaction with β-CyD-sul, *i.e.*, the electrostatic interaction between the dimethylammonium cation of the guest and the sulfate anion of the host. This electrostatic interaction was further supported by the salt effect on the complexation of chlorpromazine with β-CyDs. As shown in Fig. 3B, the K_c values decreased with the increasing ionic strength of the solutions, where the effect was much more significant in the β-CyD-sul complex than in the β-CyD complex,

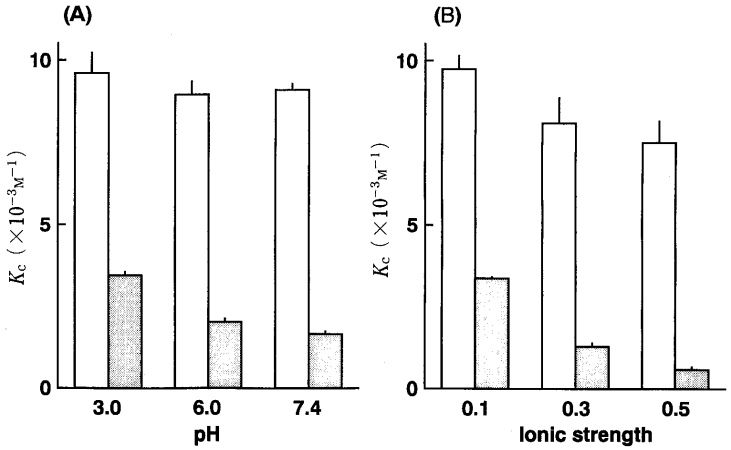

Fig. 3. Effects of pH (A; ionic strength=0.16) and Ionic Strength (B; pH=3) on Stability Constants of Complexes of Chlorpromazine with β-CyDs in Phosphate Buffer at 25 °C

□, with β-CyD; ⬚, with β-CyD-sul. Each value represents the mean±S.E. of 3—4 experiments.

TABLE I. Stability Constants and Thermodynamic Parameters of Complexes of Chlorpromazine with β-CyDs in Isotonic Phosphate Buffer (pH 7.4)

System	K_c (M^{-1})[a]				$\Delta G^{b)}$	$\Delta H^{b)}$	$\Delta S^{b)}$
	15 °C	25 °C	37 °C	50 °C	($kJ \cdot mol^{-1}$)	($kJ \cdot mol^{-1}$)	($J \cdot K^{-1} \cdot mol^{-1}$)
β-CyD	12700	9050	5800	3530	−22.6	−28.4	−19.5
β-CyD-sul	1780	1640	1320	1020	−18.4	−13.7	15.8

a) Average of at least 3 experiments. Accuracy: $K_c < \pm 7.7\%$. b) Thermodynamic parameters (ΔG, ΔH, and ΔS, at 25 °C) were calculated from the mean K_c values.

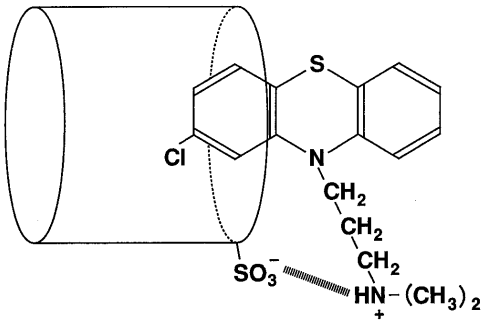

Fig. 4. Proposed Interaction Mode of Chlorpromazine–β-CyD-sul Complex

indicating an ionic character in the guest/host interaction.

Table I summarizes the stability constants, K_c=[complex]/[guest][host], of the 1:1 complexes at various temperatures, as well as the thermodynamic parameters for the complexation. The K_c values were determined by analyzing the UV changes as a function of β-CyDs concentration by means of the Scott method.[13] The plots of the Scott equation were linear (correlation coefficient (r)>0.99), supporting the 1:1 complex formation. The thermodynamic parameters were determined from van't Hoff plots of the K_c values (r=0.995). Interestingly, the K_c values of the β-CyD-sul complex were much smaller than those of the β-CyD complex, and the difference was pronounced at a lower temperature. The changes in enthalpy ($\Delta H = -28.4\,kJ \cdot mol^{-1}$) and entropy ($\Delta S = -19.5\,J \cdot K^{-1} \cdot mol^{-1}$) for the complexation with parent β-CyD were within the range observed for other CyD complexations, *i.e.*, a non-classical hydrophobic effect may be predominantly operative in the β-CyD system.[22] On the other hand, the enthalpy change ($-13.7\,kJ \cdot mol^{-1}$) of the β-CyD-sul complex was smaller than that of the β-CyD complex, and the entropy change reversed to a positive value ($15.8\,J \cdot K^{-1} \cdot mol^{-1}$), suggesting the destruction of a water structure around the guest and host molecules. Figure 4 shows the proposed interaction mode of the β-CyD-sul complex. As reported previously,[11] the chlorobenzene moiety of chlorpromazine is snugly fitted to the cavity of parent β-CyD, resulting in a large contact area between the guest and host molecules, which gives negative ΔH and ΔS values, *i.e.*, a non-classical hydrophobic effect. In the case of the β-CyD-sul complex, the dimethylammonium cation of the guest may be at first anchored at the sulfate anion sites of the CyD rim, and then the chlorobenzene moiety may be included in a mode similar to that reported for the intramolecular complexes of guest-appended CyD derivatives.[23] In this structure, the inclusion of the chlorobenzene may be shallow because of the repulsion between the charged sulfate anion and hydrophobic phenothiazine moiety and/or the steric hindrance of sulfate groups. Furthermore, some water molecules may be released from the ionic binding site by the complexation, giving small negative ΔH and positive ΔS values. The importance of electrostatic interaction in the complexation with β-CyD-sul was supported by the fact that β-CyD-sul barely interacted with acidic and neutral drugs such as flufenamic acid, flurbiprofen, diazepam and steroids.[12,24] It was difficult to determine which side of the cavity, the primary or secondary hydroxyl side, interacts with chlorpromazine, because β-CyD-sul is a multi-component mixture with different degrees of substitution of sulfate groups. Studies on regioselectively prepared CyD-sul isomers or CyD-suls with different degrees of substitution will provide more detailed information on the interaction mode with chlorpromazine.

Effect of β-CyD-sul on the Interaction of Chlorpromazine with Erythrocyte Membrane Figure 5 shows effects of

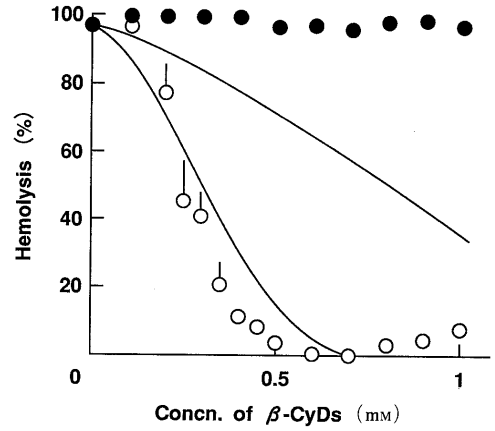

Fig. 5. Effects of β-CyDs on Chlorpromazine (3.0×10^{-4} M)-induced Hemolysis of Rabbit Erythrocytes in Isotonic Phosphate Buffer (pH 7.4) at 37 °C

○, with β-CyD; ●, with β-CyD-sul. The solid lines represent the theoretical curves assuming that the hemolytic activities of the chlorpromazine–β-CyDs systems arise from the free drug.

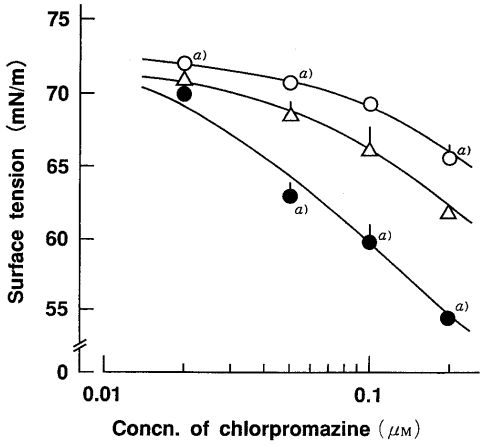

Fig. 7. Effects of β-CyDs (5.0×10^{-4} M) on Surface Activity of Chlorpromazine in Isotonic Phosphate Buffer (pH 7.4) at 25 °C

△, chlorpromazine alone; ○, with β-CyD; ●, with β-CyD-sul. Each point represents the mean ± S.E. of 3 experiments. *a)* $p < 0.05$ *versus* chlorpromazine alone.

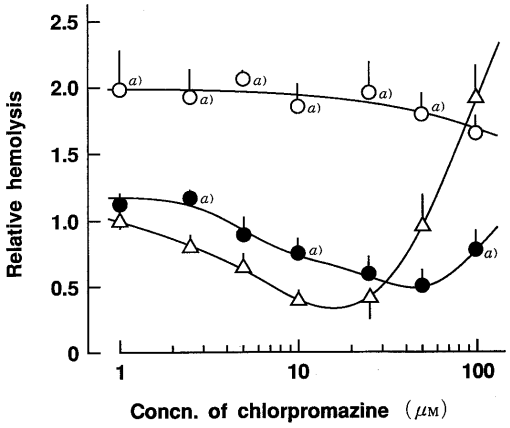

Fig. 6. Stabilizing Effects of Chlorpromazine on Hypotonic (121 ± 3 mOsm/kg) Hemolysis of Rabbit Erythrocytes in the Absence and Presence of β-CyDs (1.0×10^{-3} M) in Phosphate Buffer (pH 7.4) at 37 °C

△, chlorpromazine alone; ○, with β-CyD; ●, with β-CyD-sul. Each point represents the mean ± S.E. of 3—6 experiments. *a)* $p < 0.05$ *versus* chlorpromazine alone.

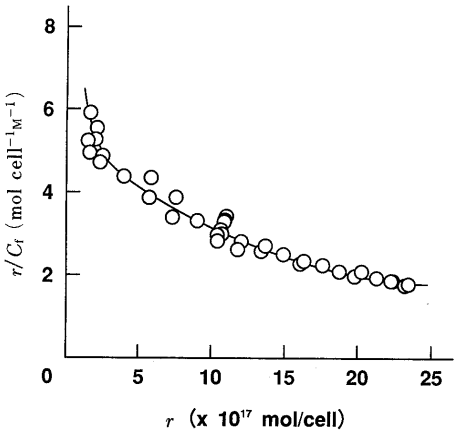

Fig. 8. Scatchard Plots for Binding of Chlorpromazine to Rabbit Erythrocytes in Isotonic Phosphate Buffer (pH 7.4) at 37 °C

β-CyD-sul and parent β-CyD on the chlorpromazine (3.0×10^{-4} M)-induced hemolysis of rabbit erythrocytes. The hemolytic activity of chlorpromazine decreased with increasing concentrations of parent β-CyD, and was completely suppressed in the presence of about 6.0—7.0 × 10^{-4} M β-CyD. The hemolysis profile for the β-CyD system was in agreement with the theoretical profile (solid line in Fig. 5) which was depicted, assuming that free chlorpromazine participates in the hemolysis (the concentration of the free drug was calculated using the stability constant ($5800\ M^{-1}$) described above).[16] The slightly increased activity, above 7.0×10^{-4} M β-CyD, may be ascribed to the hemolytic action of β-CyD itself. In contrast to the case of β-CyD, β-CyD-sul showed no inhibiting effect on the chlorpromazine-induced hemolysis under these experimental conditions, and the activity was rather high compared with that estimated by the free concentration of the drug. Since β-CyD-sul has no hemolytic activity below at least 0.1 M,[12] this may be ascribed to the dissociation of the complex due to competitive inhibition with the membrane components. Figure 6 shows the effects of chlorpromazine on the hypotonic (121 mOsm/kg) hemolysis of rabbit erythrocytes in the absence and presence of β-CyDs (1.0×10^{-3} M). Chlorpromazine showed typical biphasic hemolysis, *i.e.*, the stabilizing and destabilizing effects on membranes at lower and higher concentrations, respectively, with a minimum hemolytic activity at about 1.5×10^{-5} M chlorpromazine. The minimum hemolytic activity of the β-CyD-sul system shifted to about 5.0×10^{-5} M, suggesting the decreased stabilizing effect of chlorpromazine. On the other hand, no stabilizing effect was observed in the β-CyD system under these concentration ranges.

The surface activity of chlorpromazine and its binding behavior to erythrocyte membranes were both investigated in order to gain insight into the drug-induced hemolysis mechanism in the presence of β-CyDs. Figure 7 shows the effects of β-CyDs on the surface tension of chlorpromazine. The surface activity of chlorpromazine solution decreased as its concentration increased due to the amphiphilic character of the drug. The surface activity decreased by the addition of β-CyD, which may be ascribed to the increased hydrophilicity of the drug due to the inclusion

of a hydrophobic phenothiazine moiety in the cavity.[16] On the other hand, β-CyD-sul increased the surface activity of chlorpromazine. As described above, the cationic site of the drug is neutralized by the sulfate anions of the host through ionic interaction, which may increase the hydrophobicity of the drug, enhancing its affinity to erythrocyte membranes. Figure 8 shows the Scatchard plot for the binding of chlorpromazine to rabbit erythrocytes in the absence of CyDs in an isotonic phosphate buffer (pH 7.4) at 37 °C. The binding profile was analyzed assuming the presence of two binding sites as reported previously[25,26] and the following binding parameters were obtained: $K_1 = 1.13 \times 10^5$ M^{-1} and $n_1 = 4.93 \times 10^{-17}$ mol/cell for the primary binding site and $K_2 = 3.50 \times 10^3$ M^{-1} and $n_2 = 6.11 \times 10^{-16}$ mol/cell for the secondary binding site. Figure 9 shows the effects of β-CyDs on the binding of chlorpromazine (1.0×10^{-4} M) to erythrocytes, where the binding amount (r) of the drug decreased with increasing CyD concentration. The observed Scatchard plot for the parent β-CyD system was in good agreement with the theoretical profile depicted, assuming only free drug binds to the membrane (the concentration of free drug was calculated using the stability constant of the chlorpromazine–β-CyD complex and the binding parameter of the drug to the membrane described above). On the other hand, the inhibiting effect of β-CyD-sul on the binding was much smaller than that of β-CyD, and the binding amount of chlorpromazine was larger than that estimated from the concentration of free drug. The

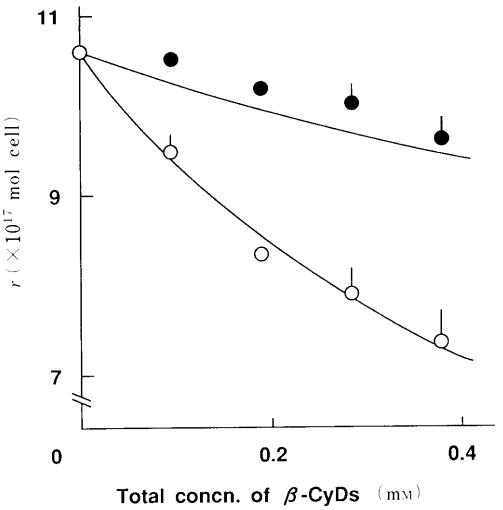

Fig. 9. Effects of β-CyDs on Binding of Chlorpromazine (1.0×10^{-4} M) to Rabbit Erythrocytes in Isotonic Phosphate Buffer (pH 7.4) at 37 °C

○, chlorpromazine with β-CyD; ●, chlorpromazine with β-CyD-sul. The solid lines represent the theoretical curves assuming that β-CyDs and their complexes do not bind to erythrocytes. Each point represents the mean ± S.E. of 3–6 experiments.

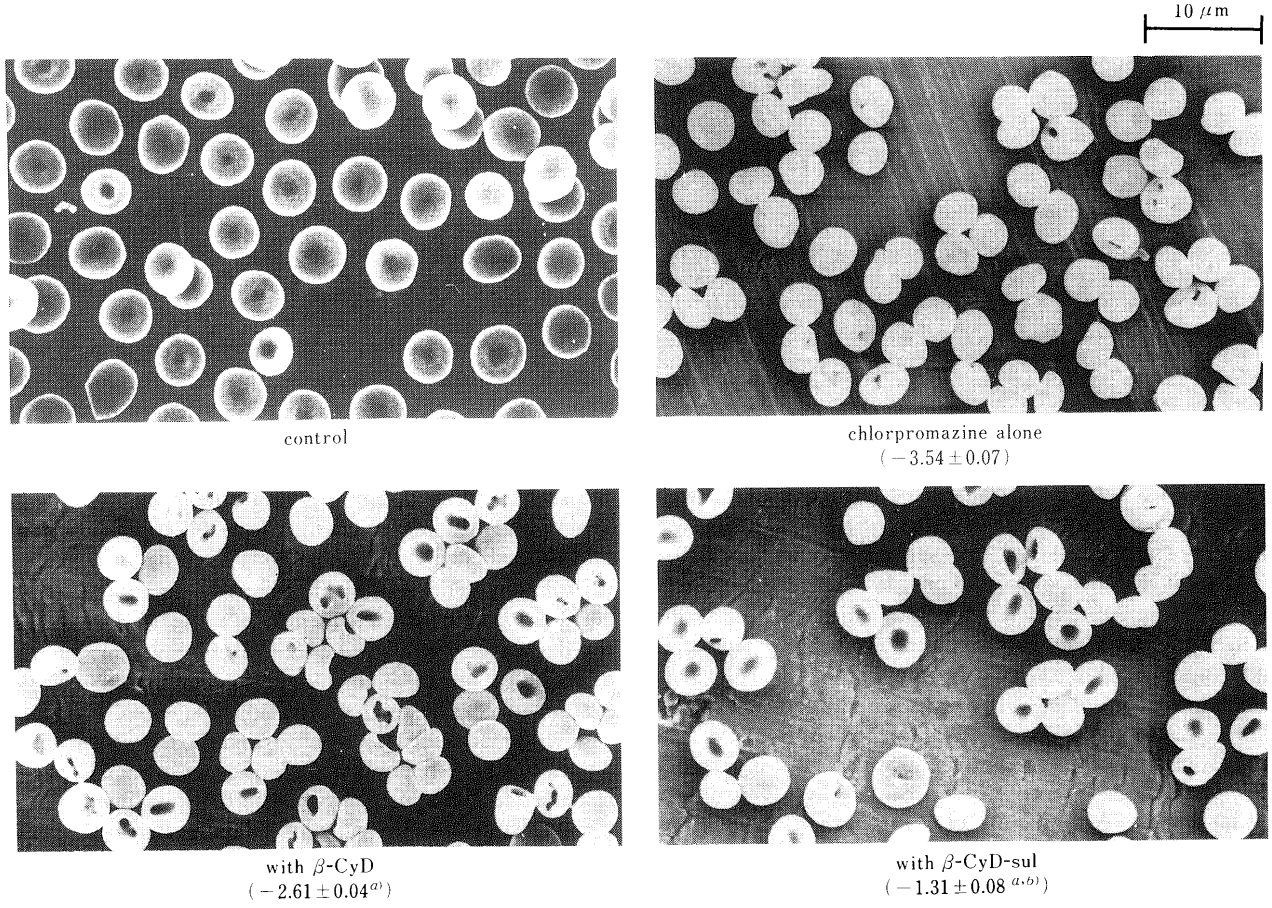

Fig. 10. Effects of β-CyDs (1.0×10^{-3} M) on Chlorpromazine (7.5×10^{-5} M)-induced Shape Changes of Rabbit Erythrocytes in Isotonic Phosphate Buffer (pH 7.4) at 37 °C

The value in parenthesis is the morphological index (the mean ± S.E. of 4 experiments). *a*) $p < 0.05$ *versus* chlorpromazine alone. *b*) $p < 0.05$ *versus* chlorpromazine with β-CyD.

increased binding of chlorpromazine in the presence of β-CyD-sul may be due to a number of causes, which include a possible binding of the complex to the membrane because of its increased hydrophobicity and a displacement of the drug from the complex. Furthermore, the binding of β-CyD-sul to erythrocytes may influence the drug binding, since β-CyD-sul had a relatively higher affinity to erythrocytes, whereas parent β-CyD had no affinity.[12,27] These factors may be responsible for the apparently negligible effect on drug-induced hemolysis. The detailed binding mechanism of β-CyD-sul will be reported elsewhere.

Figure 10 shows the scanning electron micrographs of rabbit erythrocytes treated with chlorpromazine in the absence and presence of β-CyDs. Chlorpromazine at a concentration of 7.5×10^{-5} M caused the erythrocytes to become stomatocytes, scored as a morphological index (MI) $= -3.54$ (± 0.07). The drug-induced deformation of the erythrocytes was suppressed by the addition of both β-CyDs (1.0×10^{-3} M), where the inhibiting effect of β-CyD-sul (MI $= -1.31$ (± 0.08)) was greater than that of β-CyD (MI $= -2.61$ (± 0.04)), in spite of the weak complexation ability of the former. Since parent β-CyD had a negligible effect (MI $= -0.43$ (± 0.05)) on the shape-change at this concentration,[12] the observed inhibition may be attributable to the decreased concentration of free chlorpromazine through the complexation. On the other hand, β-CyD-sul at 1.0×10^{-3} M caused an opposite shape-change of the erythrocytes, *i.e.*, echinocytes scored as MI $= +0.94$ (± 0.08).[12] Therefore, the drug-induced internalization of the erythrocytes seems to be counterbalanced by the β-CyD-sul-induced externalization.

The present results suggest that β-CyD-sul retains the inclusion ability, particularly to cationic drugs, by means of electrostatic and hydrophobic interactions, although its binding ability was inferior to that of parent β-CyD. Furthermore, the membrane-binding behavior of β-CyD-sul and the inhibiting effect on drug-induced hemolysis seem to be different from those of parent β-CyD. Thus, β-CyD-sul may be useful as a parenteral drug carrier because of its higher water-solubility and lower membrane disrupting ability than parent β-CyD.

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