Differential Effects of Sulfate and Sulfobutyl Ether of β-Cyclodextrin on Erythrocyte Membranes *in Vitro*

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The hemolytic activity of β-cyclodextrin (β-CyD) on rabbit erythrocytes was reduced by the introduction of negatively-charged groups onto the hydroxyls of β-CyD; the membrane disrupting abilities decreased in the order of β-CyD > 2-hydroxypropyl-β-CyD (HP-β-CyD) > sulfobutyl-β-CyD (SB-β-CyD) >> β-CyD sulfate (S-β-CyD). Under pre-hemolytic concentrations, both β-CyD and SB-β-CyD induced shape changes of membrane invagination on the erythrocytes. In sharp contrast, S-β-CyD showed biphasic effect on the shape of the erythrocytes; *i.e.* the crenation at relatively low concentrations and the invagination at higher concentrations. The S-β-CyD-induced membrane crenation arose from a direct action on the membranes rather than cell metabolism-mediated effects. Unlike β-CyD, S-β-CyD was found to bind to the erythrocytes and may be confined to the outer surface of the membrane bilayer, which may expand the exterior layer relative to the cytoplasmic half, thereby inducing the cells to crenate. On the other hand, the membrane invagination mediated by the three β-CyDs was initiated by extracting specific membrane lipids from the cells, depending upon their inclusion abilities, subsequently leading to the lysis of the cells. These results indicate that SB-β-CyD and S-β-CyD interact with the erythrocyte membranes in a differential manner and possess lower membrane disrupting abilities than the parent β-CyD and HP-β-CyD.

KEY WORDS: β-cyclodextrin sulfate; sulfobutyl-β-cyclodextrin; rabbit erythrocytes; hemolysis; morphological changes; lipid solubilization.

INTRODUCTION

The sulfation of a majority of hydroxyl groups of CyDs gives such derivatives unique biological activities similar and sometimes superior to those of heparin (1,2). Sodium salts of CyD sulfates (S-CyDs) are highly hydrophilic amorphous mixtures with distributions of the degree of substitution by sulfate groups (3), and less hemolytic than the parent CyDs (4). Our previous studies have shown that S-CyDs were less toxic than the parent CyDs and dextran sulfate when administered parenterally in the rat (5), and protected the rat against the aminoglycoside-induced acute renal failure (6). Since the highly hydrated and negatively-charged sulfate groups of S-CyDs are located near the entrance of the cavity, the access of guest molecules to the cavity is considerably restricted (7). To overcome such drawbacks, a series of sulfoalkyl ether derivatives of CyDs have been prepared, in which the sulfonate groups are appropriately spaced from the CyD cavity with an alkyl side chain as the substituents do not interfere with the inclusion process (8).

Recent studies have described that the cytotoxicity of CyD derivatives toward fibroblasts (9) and P388 murine leukaemic cells (10) increase in proportion to their hemolytic activity. The effects of chemically modified CyDs on the biological membranes must be different from those of the parent CyDs. Following on from these studies, we examined the effects of S-β-CyD and sulfobutyl ether of β-CyD (SB-β-CyD) on rabbit erythrocytes *in vitro* and compared their effects with those of the parent β-CyD and 2-hydroxypropyl-β-CyD (HP-β-CyD) to gather additional data in support of parenteral use of such derivatives.

MATERIALS AND METHODS

Materials

α-CyD, β-CyD, γ-CyD, and HP-β-CyD were donated by Nihon Shokuhin Kako Co. Ltd. (Tokyo, Japan). S-CyDs were prepared by a non-regional selective method as described previously (3). SB-β-CyD was prepared by the procedure reported previously (8). The structures and abbreviations of CyDs used were listed in Table 1. Dextran sulfate with a sulfur content of 17.5 % and flufenamic acid were purchased from Sigma Chemical Co. (MO, U.S.A.).

Hemolysis Assays

Hemolytic activities of CyDs were assessed as described previously (11). From freshly drawn citrated rabbit blood, erythrocytes were separated by centrifugation at 1000 × g for 5 min, washed three times with phosphate buffered saline (154 mM sodium chloride, 10 mM 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 CyDs. After 30 min-incubation at 37 °C, the release of hemoglobin from the cells was measured spectrophotometrically at 543 nm.

Morphological Observation of Erythrocytes

The cell suspension (5 %, 0.1 ml) was incubated with the buffer solution (2 ml) containing CyDs or flufenamic acid at 37 °C up to 60 min and then fixed with 2 % glutaraldehyde solution (5 ml). After being stood 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 under the 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. (12), 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.

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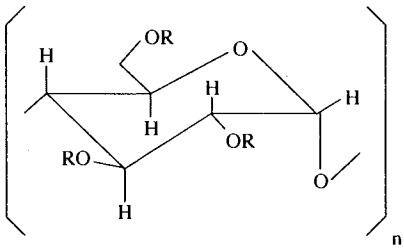
Table 1. Cyclodextrin derivatives used in this study



Compound	Abbreviation	n	R	DS[a]
β-cyclodextrin	β-CyD	7	H	
2-hydroxypropyl-β-cyclodextrin	HP-β-CyD	7	H or $[CH_2CH(CH_3)O]_mH$ (m = 1, 2, 3, ---)	4.8
α-cyclodextrin sulfate	S-α-CyD	6	H or SO_3Na	11.2
β-cyclodextrin sulfate	S-β-CyD	7	H or SO_3Na	10.7
γ-cyclodextrin sulfate	S-γ-CyD	8	H or SO_3Na	13.3
sulfobutyl-β-cyclodextrin	SB-β-CyD	7	H or $(CH_2)_4SO_3Na$	3.5

[a] The average degree of substitution.

Cellular ATP Determination

The cell suspension (14 %, 7 ml) was incubated with 3 mM CyDs at 37 °C for 2 h. After centrifugation at 1000 × g for 5 min, the cells were completely lysed by the addition of the buffer solution (0.1 ml) containing 10 % titron X-100. The cellular ATP content of the lysate was measured by the luciferin-luciferase procedure using an assay kit (ATP bioluminescence CLS, Boehringer Mannheim Co., Germany).

Binding Studies of CyDs to Erythrocyte Membranes

The buffer solution (1 ml) containing 15 μM CyDs was added to 10 % or 20 % cell suspension (1 ml). This condition was chosen to detect the binding of CyDs to the cells with high accuracy and to minimize their membrane disrupting effects. The mixture was incubated at 37 °C for 30 min and was centrifuged at 1000 × g for 5 min. The concentrations of CyDs in the supernatant were determined by the anthrone method (13) with slight modification.

Phase Solubility Diagrams of Cholesterol-CyD Systems

Solubility measurements were carried out according to the method reported by Higuchi and Connors (14). Excess amounts of cholesterol were added to the buffer solution containing various concentrations of CyDs, and the mixtures were shaken at 25 °C for 10 days. After equilibrium was attained, an aliquot was centrifuged and the supernatant (3 ml) was filtrated through a membrane filter (DISMIC-25CS, Toyo Roshi Kaisha Ltd., Tokyo, Japan). The concentration of cholesterol in the filtrate was determined by the cholesterol oxidase method (Cholesterol C-Test Wako, Wako Pure Chemical Ind., Ltd, Osaka, Japan).

Release of Cholesterol from Erythrocytes

The cell suspension (5 %, 0.2 ml) was added to the buffer solution (3.8 ml) containing 3 mM CyDs. The mixture was incubated at 37 °C for 30 min and centrifuged at 2000 × g at 4 °C for 40 min. The amount of cholesterol in the supernatant was determined by the cholesterol oxidase method.

RESULTS AND DISCUSSION

Figure 1 shows the hemolytic effects of the four types of β-CyD derivatives toward rabbit erythrocytes in phosphate buffered saline (pH 7.4) for 30 min of incubation at 37 °C. The efflux of hemoglobin from the cells treated with each of β-CyDs started on the first contact and left to proceed for the period of incubation. The hemolytic activity of β-CyD was reduced by the introduction of negatively-charged polar groups onto the hydroxyl groups of β-CyD in the order of β- > SB-β- >> S-β-CyD. The hemolytic activity of SB-β-CyD or S-β-CyD was significantly lower than that of HP-β-CyD, a derivative which showed less local tissue irritancy than the parent β-CyD and was well tolerated by animals and human even when given parenterally (15,16). In particular, S-β-CyD showed no measurable hemolysis even at higher concentrations of more than 100 mM. The lack of hemolytic activity of S-β-CyD is in agreement with a recent finding described by

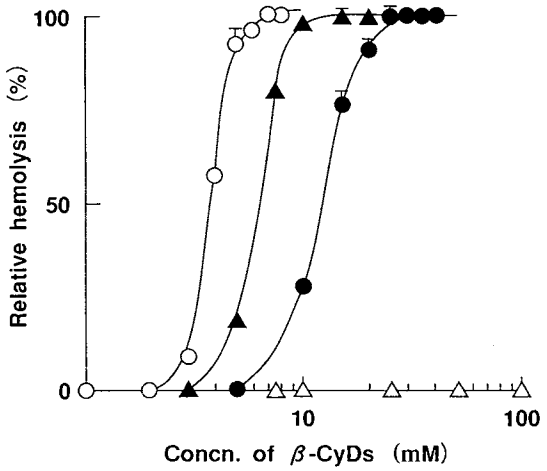


Fig. 1. Hemolytic effects of β-CyDs on rabbit erythrocytes in phosphate buffered saline (pH 7.4) at 37 °C. ○: β-CyD, ●: SB-β-CyD, △: S-β-CyD, ▲: HP-β-CyD. Each point represents the mean ± S.E. of 3–6 experiments.

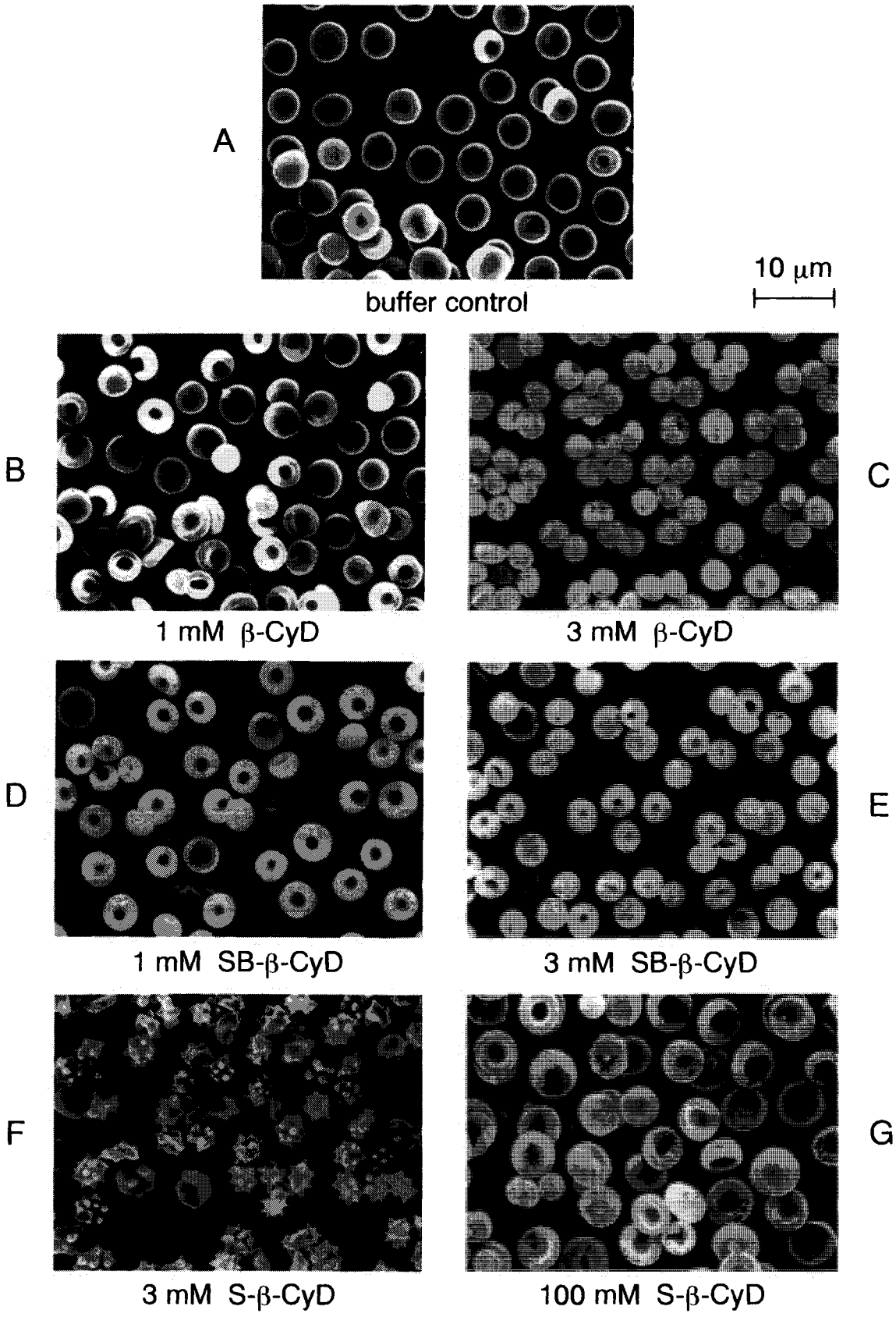


Fig. 2. Scanning electron micrographs of rabbit erythrocytes treated with β-CyDs in phosphate buffered saline (pH 7.4) at 37 °C.

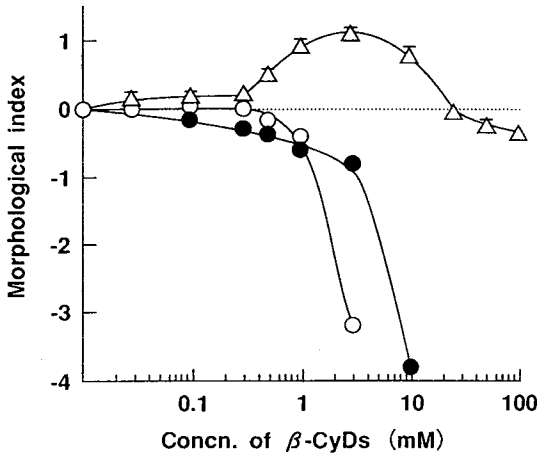


Fig. 3. Shape changes in rabbit erythrocytes as a function of initial concentrations of β-CyDs in phosphate buffered saline (pH 7.4) at 37 °C. ○: β-CyD, ●: SB-β-CyD, △: S-β-CyD. Each morphological index represents the mean ± S.E. of 4 experiments.

Macarak et al. using β-CyD tetradecasulfate and human erythrocytes (4).

Under pre-hemolytic conditions, CyDs and their hydrophilic derivatives with electrically-neutral substituents are found to induce shape changes of membrane invagination on the erythrocytes in a concentration-dependent manner (11,17). Figure 2 shows some scanning electron micrographs of the erythrocytes treated with β-CyD and its anionic derivatives, and the extent of shape changes of the cells as a function of initial concentrations of β-CyDs added to the medium was depicted in Fig. 3. When β-CyDs were added to the cell suspension, the shape changes of the cells were induced instantly, and no further progress of the shape changes was observed during 60 min of incubation at 37 °C. Both β-CyD and SB-β-CyD transformed biconcave discocytes into monoconcave stomatocytes. The extent of the shape changes progressed with increasing concentrations of β-CyD and SB-β-CyD, the former being more pronounced, especially at more than 1 mM. At the critical concentration of each of the two β-CyDs to cause partial lysis of the cells, the majority of the cells was transformed into spherocytes (Fig. 2-C and 2-E). In sharp contrast, the shape change of the cells

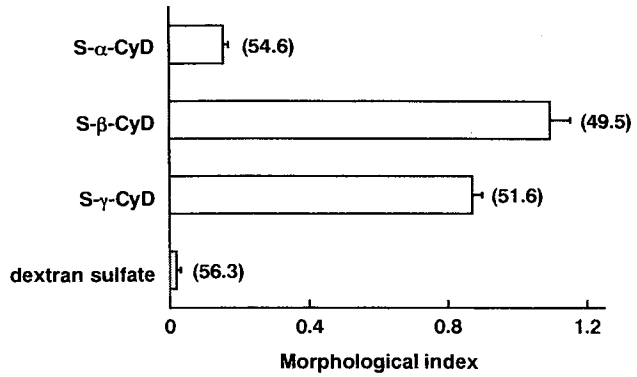


Fig. 4. Shape changes in rabbit erythrocytes treated with S-CyDs and dextran sulfate (3 mM) in phosphate buffered saline (pH 7.4) at 37 °C. Each value in parenthesis is the content of sulfate groups by weight (W/W%).

Table 2. Equilibrium bindings of β-CyDs[a] to rabbit erythrocytes in phosphate buffered saline (pH 7.4) at 37 °C

Compound	Hematocrit (%)	Amount of β-CyDs bound to the cells[b] (nmol/ml cell volume)
β-CyD	10	<0.5
	20	<0.5
SB-β-CyD	10	37.1 ± 3.5
	20	18.1 ± 2.7
S-β-CyD	10	109.9 ± 0.2
	20	54.2 ± 0.1

[a] The initial concentration of β-CyDs was 15 μM.
[b] Each value represents the mean ± S.E. of 3 experiments.

exposed to S-β-CyD was biphasic. When the cells were incubated with S-β-CyD at relatively low concentrations ranging from 1 to 10 mM, the cells became spiculate echinocytes (Fig. 2-F), while S-β-CyD induced slight membrane invagination of the cells at the higher concentrations (Fig. 2-G).

Figure 4 compares the echinocytogenic effects of three S-CyDs and dextran sulfate with similar extent of sulfation. S-α-CyD with a smaller size of cavity was less effective than S-β- and S-γ-CyDs, whereas dextran sulfate, a linear sulfated polysaccharide was unable to induce crenation under the same concentration. This probably arises from the spatial constraints imposed on the sulfates by the macrocyclic rings of the CyDs (18). The observed cavity size dependence indicates that the S-CyD-mediated membrane crenation may be required for an appropriate charge density of anionic groups of the CyDs.

The membrane crenation of the erythrocytes is known to be induced by a variety of conditions including calcium loading, metabolic depletion, and asymmetric distribution of amphiphatic compounds across the plasma membrane (19). When the cells were incubated with 3 mM β-CyDs for 2 h at 37 °C, the remaining percentages of cellular ATP were 99.7 ± 13.5 %, 99.1 ± 9.1 %, and 96.1 ± 5.1 % with β-CyD, SB-β-CyD, and S-β-CyD respectively. This result, taken together

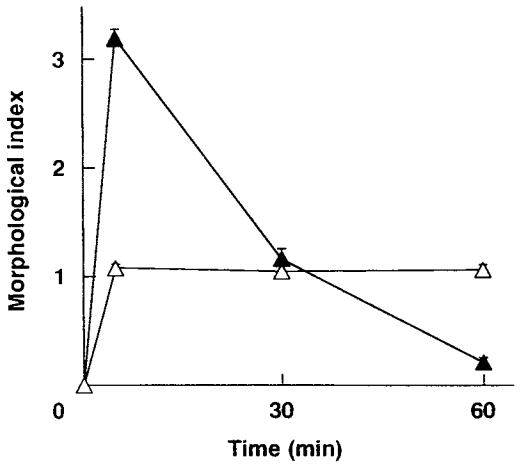


Fig. 5. Time courses of shape changes of rabbit erythrocytes treated with S-β-CyD (3 mM) and flufenamic acid (0.5 mM) in phosphate buffered saline (pH 7.4) at 37 °C. △: S-β-CyD, ▲: flufenamic acid. Each morphological index represents the mean ± S.E. of 4 experiments.

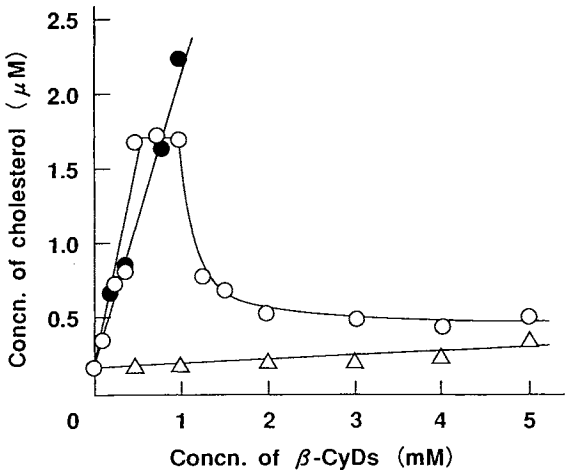


Fig. 6. Phase solubility diagrams of cholesterol-β-CyDs systems in phosphate buffered saline (pH 7.4) at 25 °C. ○: β-CyD, ●: SB-β-CyD, △: S-β-CyD.

with the rapidity of the shape changes of the cells exposed to each of β-CyDs suggests the direct actions of these β-CyDs on the membrane surfaces rather than cell metabolism-mediated effects.

CyDs have the ability to solubilize specific lipids from the erythrocyte membranes (11) or liposome (20) through the rapid and reversible formation of inclusion complexes. This process occurred without entry of the CyDs into the membranes, a mechanism of solubilization/lysis different from that of detergents, which first enter the membranes (21). Other research has shown that S-β-CyD protected human erythrocytes against lysis induced with a wide variety of substances in a non-specific manner, indicating probably through protective interaction between S-β-CyD and the membranes (22). Thus, we examined the binding ability of the anionic β-CyDs to the erythrocyte membranes. In agreement with our previous finding using ^{14}C-labeled compound (11), β-CyD did not bind to and/or adsorb onto the membrane surface. On the other hand, considerable amounts of SB-β-CyD and S-β-CyD were bound to the cell membranes, the latter being more effective (Table 2).

Generally, anionic amphiphiles are known to concentrate primarily in the outer half of the membrane bilayer due to their repulsion by anionic phosphatidylserine located in the inner half, and expand the outer half relative to the inner half of the bilayer, thus inducing crenation (23). S-β-CyD bound to the cells was displaced by a typical anionic amphiphile, flufenamic acid. When 28 μM S-β-CyD was incubated with the cells in the presence of 28 μM flufenamic acid, the amount of S-β-CyD bound to the cells was reduced by 71.4 ± 9.3 %. Because of the limited interaction of S-β-CyD with flufenamic acid, the displacement could be explained by the competitive binding of the two anionic compounds. Above results indicate that S-β-CyD may bind preferentially to the outer half of the membrane bilayer, and thus induce crenation in a similar manner as anionic amphiphiles. The possibility of transbilayer distribution of S-β-CyD was examined by monitoring the time-dependence of the cell morphology. As shown in Fig. 5, the cells incubated with flufenamic acid crenated initially and then reverted to discoid shape within 60 min, indicating the translocation of the drug into inner half or cytoplasm upon incubation (12,24). In contrast, cells exposed to S-β-CyD retained stable echinocytic shape during 60 min of incubation, suggesting an inability of S-β-CyD to permeate the membrane bilayer, probably due to its highly hydrophilic nature and macrocyclic structure. These results suggest that S-β-CyD may be confined to the outer surface of the membranes, a situation which may expand the exterior layer relative to the cytoplasmic half, and thereby inducing the cells to crenate.

To gain insight into the differential effects of the three types of β-CyDs at higher concentrations on the shape and stability of the erythrocytes, we investigated the interaction of β-CyDs with cholesterol, a lipid which is known to act as the main rigidifier in lipid bilayers and to modify the enzyme activity and the permeability properties (25). The phase solubility diagrams for cholesterol with the three types of β-CyDs are shown in Fig. 6. The solubility curve for cholesterol with β-CyD was of the Bs-type (14), with precipitation of microcrystalline complex at higher β-CyD concentrations. This is consistent with the data reported by Frijlink et al. (26). The stoichiometry of the cholesterol-β-CyD complex was estimated from the plateau region to be 3:1, which was confirmed by isolation and analysis of the crystalline complex. The higher order complexation may be due to self-association of cholesterol (27) and/or partial inclusion of the side chain of cholesterol into the β-CyD cavity (28). In sharp contrast, the solubility of cholesterol increased linearly as a function of SB-β-CyD concentrations used, while S-β-CyD, which has the electric charges located near the cavity, was the least effective. The apparent stability constants (Kc) of complexes of cholesterol with the three β-CyDs, assuming that a 1:1 complex will be initially formed, were calculated from the initial straight-line portion of the solubility diagrams; the Kc values were 16100 M^{-1}, 11100 M^{-1}, and 190 M^{-1} for β-CyD, SB-β-CyD, and S-β-CyD, respectively. As shown in Fig. 7, the three β-CyDs extracted cholesterol from the erythrocyte membranes in the order of β- > SB-β- >> S-β-CyD, which clearly fit with the sequence of their inclusion abilities for cholesterol. This process could be maintained by a thermodynamic equilibrium, where the lipid extraction may be triggered by the enhanced desorption of the lipid from the cells into aqueous phase, rather than the transfer during transient contact between CyDs and the cells in a

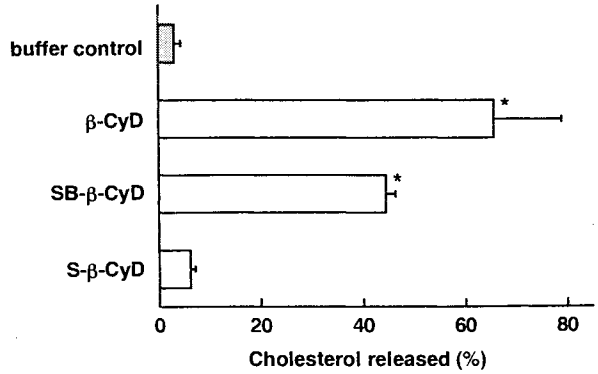


Fig. 7. Release of cholesterol from rabbit erythrocytes treated with β-CyDs (3 mM) in phosphate buffered saline (pH 7.4) at 37 °C. Each value represents the mean ± S.E. of 3–6 experiments.

similar manner as reported in the lipid transfer between lipid-containing vesicles (29). CyDs possibly increase the poor size of the lipid molecules present in the aqueous phase through inclusion complexation. The removal of cholesterol from the cells may result in an increase in membrane fluidity (30), which would induce the membrane invagination through a loss of the bending resistance, and consequently lead to the lysis of the cells. In addition, our previous studies have shown that CyDs removed phospholipids especially phosphatidylcholine and sphingomyelin from the outer half of the membrane bilayer, leading to imbalance of the bilayer and which may contribute in part to the formation of stomatocytes through an inward bending of the membranes (20).

On the other hand, the close proximity of the charge to the CyD cavity appears to decrease the solubilizing capacity of S-β-CyD. In addition, S-β-CyD protected the erythrocytes against osmotic and heat-induced hemolysis. In the presence of 10 mM S-β-CyD, the effluxes of hemoglobin from the cells treated with hypotonicity of 121 ± 3 mOsm and heat at 55 ± 0.5 °C were decreased by 20.3 ± 1.5 % and 45.3 ± 5.8 %, respectively. Thus, the lack of hemolytic activity of S-β-CyD may be due to the minimal capacity to solubilize the membrane lipids, together with the protective interaction with the membranes.

The limited data obtained here suggest that SB-β- and S-β-CyDs interact with the erythrocyte membranes in a differential feature and possess lower membrane disrupting abilities than the parent β- and HP-β-CyDs. An additional advantage of SB-β-CyD is to retain the favorable binding and solubilizing characteristics as a drug carrier and to establish electrostatic interaction of anionic sulfobutyl groups with cationic guest molecule (31).

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