Effect of Cyclodextrins on Biological Membrane. II. Mechanism of Enhancement on the Intestinal Absorption of Non-absorbable Drug by Cyclodextrins

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The effects of two kinds of cyclodextrins (CyDs), α- and β-CyD, on biological membranes were investigated by measuring changes in the absorption of a non-absorbable drug, sulfanilic acid (SA), from the rat small intestine, using *in situ* and *in vitro* experiments. After pretreatment with a mucolytic agent, *N*-acetyl-L-cysteine (N-Ac), only β-CyD increased the absorption of SA significantly compared to the absorption without pretreatment.

The mechanism of the enhancing effect of CyDs on the absorption of SA was discussed. Almost no morphological change in the small intestine was observed by pretreatment with N-Ac alone, N-Ac or α- or β-CyD combinations. The liberation of membrane components differed among the CyDs, *e.g.*, α-CyD selectively released phospholipid while β-CyD released mainly cholesterol from the intestinal membrane. It is suggested that the interaction of membrane components with CyDs may be at least partly responsible for the enhanced absorption of SA. Moreover it was found from *in vitro* electrophysiological experiment, that the alteration in enhanced permeability caused by β-CyD occurred primarily in the transcellular pathways, rather than in the paracellular pathways of the small intestine.

These results suggest that the enhancement of intestinal absorption by β-CyD, after removal of the mucin layer from the intestinal surface, is due to the interaction between the membrane components and CyD. This interaction would induce disorder in cell membrane lipid, resulting in the increased permeability of the transcellular route.

Keywords cyclodextrin; intestinal absorption; non-absorbable drug; sulfanilic acid; transcellular route; paracellular route; inclusion complex; membrane component

Introduction

Cyclodextrins (CyDs), which include many kinds of hydrophobic molecules, are now widely used in pharmaceutical formulations. It is well recognized that the bioavailability of slightly soluble drugs administered in oral or suppository form is improved when the drugs are given in the form of CyD complexes.[1,2] Transdermal transport can be improved by reducing the barrier function of skin with the aid of penetration enhancers or accelerants.[3] Di-*O*-methyl-β-CyD, a reducer of skin barrier function, significantly enhanced the absorption of drugs from the skin.[4] Similarly, Hirai *et al.* reported that administration of insulin containing α-CyD as a nasal spray achieved a much greater absorption of insulin than when insulin was used alone.[5]

On the other hand, it has been reported that CyDs induce morphological changes and haemolytic activity in human erythrocytes.[6] Also, CyDs have been reported to enhance the membrane permeability of phosphatidylcholine or phosphatidylcholine–cholesterol liposomes.[7] We have reported that the intestinal absorption of a non-absorbable drug, sulfanilic acid (SA), is enhanced in the presence of β-CyD, but not α-CyD. This phenomenon occurred on the mucus layer free intestinal membrane obtained from the treatment with a mucolytic agent, *N*-acetyl-L-cysteine (N-Ac), or adjuvants.[8] However, the direct effect of CyDs on the biological membrane at the absorption site of a drug is not fully understood.

From this point of view, we investigated the interaction of CyDs with biological membranes in order to clarify the mechanism of the enhancement of intestinal absorption of drugs. Membrane function and the pathways (transcellular and paracellular routes) of enhanced drug absorption were also evaluated from the electrophysiological parameters of rat intestinal mucosa in the presence and absence of CyDs.

Experimental

Materials SA, β-CyD and N-Ac were purchased from Wako Pure Chemical Co. and α-CyD was purchased from Nacalai Tesque, Inc. Other chemicals were of reagent grade.

Absorption Experiment In the pretreatment experiment, 1% N-Ac in pH 6.5 phosphate buffer solution was perfused for 10 min, or 1% N-Ac was perfused for 10 min and β-CyD solution was perfused for 50 min through the small intestine. Then, SA solution (2 mg/ml) was perfused immediately; blood samples were taken just before the start of perfusion and at intervals of 10 min thereafter. The SA concentration in the blood was determined spectrophotometrically.

Measurement of Phospholipid and Cholesterol in Perfusate The whole intestine pretreated with N-Ac was washed out with 50 ml physiological saline at 37 °C, and then phosphate buffer solution, with or without CyDs, was perfused for 60 min. Liberated phospholipid and cholesterol in the perfusate were then determined.

Morphological Change by Optical Microscope At the end of the perfusion, a small piece of the intestine was surgically excised and washed with cold physiological saline solution. The excised intestine was then fixed with 10% formaldehyde in physiological saline solution, and cut into slices. The slices were stained with hematoxylin–eosin solution and were observed under an optical microscope.

***In Vitro* Experiment for Electrophysiological Investigation** A: The transmural flux rate of SA was measured according to the method of Yamashita *et al.*[9] A portion of jejunum, isolated from the rat, was perfused either with 1% N-Ac for 10 min and isotonic phosphate buffer (30 min) or with N-Ac (10 min) and 10 mM CyDs (30 min) by the *in situ* single perfusion method; this portion was then mounted between two Lucite half chambers. Ringer solution containing SA (10 mM) was introduced to the mucosal side. A sample solution was taken from the serosal side every 10 min for 1 h. The mucosal to serosal flux rate of SA was calculated from the rate of increase in the serosal concentration of SA.

B. Voltage–Clamp Experiments: After introducing SA-containing Ringer solution, potential difference between the mucosal and serosal sides was clamped immediately to an arbital values (−20—+30 mV) by applying electric fields externally; the flux rate of SA was then measured.

Analytical Method SA was determined spectrophotometrically, as described previously.[10] Total phospholipid and cholesterol in the perfusate were extracted according to the method of Folch *et al.*[11] The lipids extracted with mixed solvent (chloroform–methanol (2:1)) were washed with 0.1 M KCl, and concentrated by evaporating under N_2 stream. Cholesterol and total phospholipid in the perfusate were determined according to the methods of Zlatkis and Zak[12] and Bartlett,[13] re-

spectively.

Data Analysis All mean values of the data were presented with their standard errors (S.E.). Student's *t*-test was used to determine significant differences.

Results and Discussion

To obtain information on the mechanism of the enhancing effects of β-CyD, intestinal absorption of SA after pretreatment with N-Ac alone or with N-Ac and β-CyD solution, was investigated by *in situ* single perfusion method as shown in Fig. 1. As the blood level of SA after pretreatment with N-Ac was almost the same as that of the control, N-Ac itself had no influence on the SA absorption. However, after pretreatment with both N-Ac and β-CyD, the SA absorption increased significantly with time, while in the initial stage of the perfusion of β-CyD and SA after pretreatment with N-Ac, the blood level of SA increased gradually, and thereafter more rapidly.[8] α-CyD and β-CyD without N-Ac, on the other hand, were unable to enhance the absorption of SA through the small intestine.[8]

These results indicate that enhanced SA absorption was not due to the formation of an inclusion complex between β-CyD and SA.[4,8] Therefore, β-CyD may act directly on the mucus-free intestinal membrane.

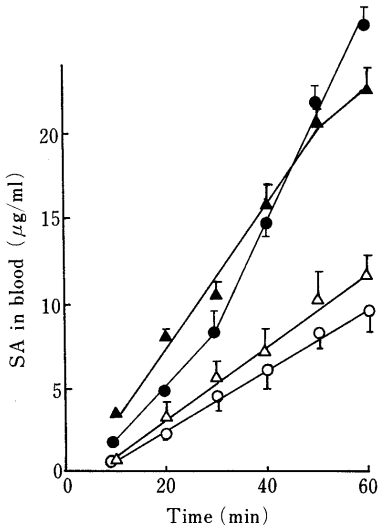

Fig. 1 Effect of Pretreatment with β-CyD on the Absorption of SA

—○—, control; —△—, SA perfusion after pretreatment with N-Ac; —▲—, SA perfusion after pretreatment with N-Ac and β-CyD; —●—, SA and β-CyD perfusion after pretreatment with N-Ac. Each bar represents the mean±S.E.

Liberation of Lipid Components It has been reported that β-CyD interacted with endogeneous compounds, bile acids,[14] cholesterol, and phospholipids.[6] Figure 2 shows the effect of CyDs on the release of phospholipid (A) and cholesterol (B) from intestinal tissue. Isotonic phosphate buffer liberated small amounts of phospholipid and cholesterol as N-Ac did alone. After pretreatment with N-Ac a large amount of phospholipid was liberated by α-CyD, but not by β-CyD. The release of cholesterol was observed under β-CyD perfusion, and it was found that the release of intestinal membrane components depended on the cavity size of the CyDs. Furthermore, the liberated protein after pretreatment with N-Ac was also determined according to the method of Lowry *et al.*,[15] but protein in perfusate was not measured by this method.

It has been reported that ^{14}C-labelled β-CyD was not absorbed in its intact form from either the stomach or the small intestine of the rat.[16] The interaction of CyDs with lipids takes place at the surface of the mucosal membrane and would include those lipid molecules on the sublayer of membrane. Similarly, Irie *et al.* reported that the stability constant for the inclusion complex of cholesterol with α-CyD is significantly small when compared with those for β- and γ-CyD.[6] Miyajima *et al.* showed that membrane components are responsible for the leakage of marker compound from liposome; the effect of α-CyD decreased concomitantly with the increase of cholesterol content in the liposome.[17]

Morphological Change in the Small Intestine This study was undertaken to clarify whether N-Ac or CyDs and their combination cause morphological changes in the intestine. When the mucosa of intestine comes in direct contact with N-Ac and/or CyDs, structural change of the membrane takes place. The results of microscopic observation are presented in Fig. 3. A section of mucosa after perfusion with isotonic phosphate buffer alone is shown in Fig. 3A. Normal clear images of epithelial cells and the crypt of goblet cells are observed. As in each group treated with N-Ac and/or CyDs, there no changes are observable in the epithelial cells or the goblet cells under optical microscope.

***In Vitro* Experiments** Figure 4 shows the cumulative

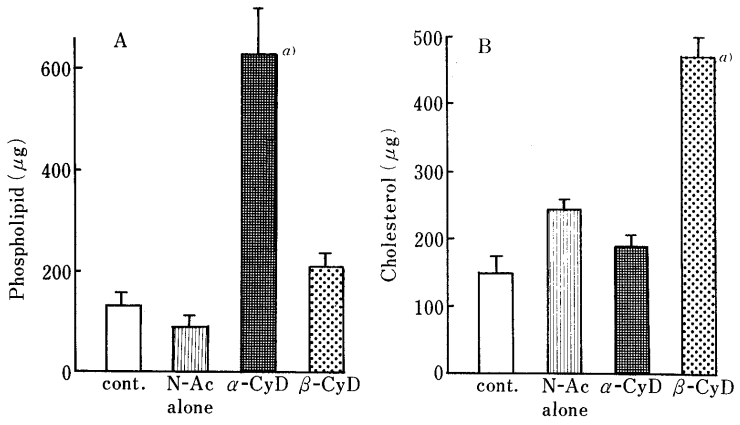

Fig. 2. Effect of CyD on the Release of Phospholipid (A) and Cholesterol (B) from Intestinal Membrane

Control, isotonic phosphate buffer; N-Ac, pretreatment with N-Ac alone; α-CyD, 10 mM α-CyD after pretreatment with N-Ac; β-CyD, 10 mM β-CyD after pretreatment with N-Ac. Each bar represents the mean±S.E. Significantly different from control, $p<0.05$.

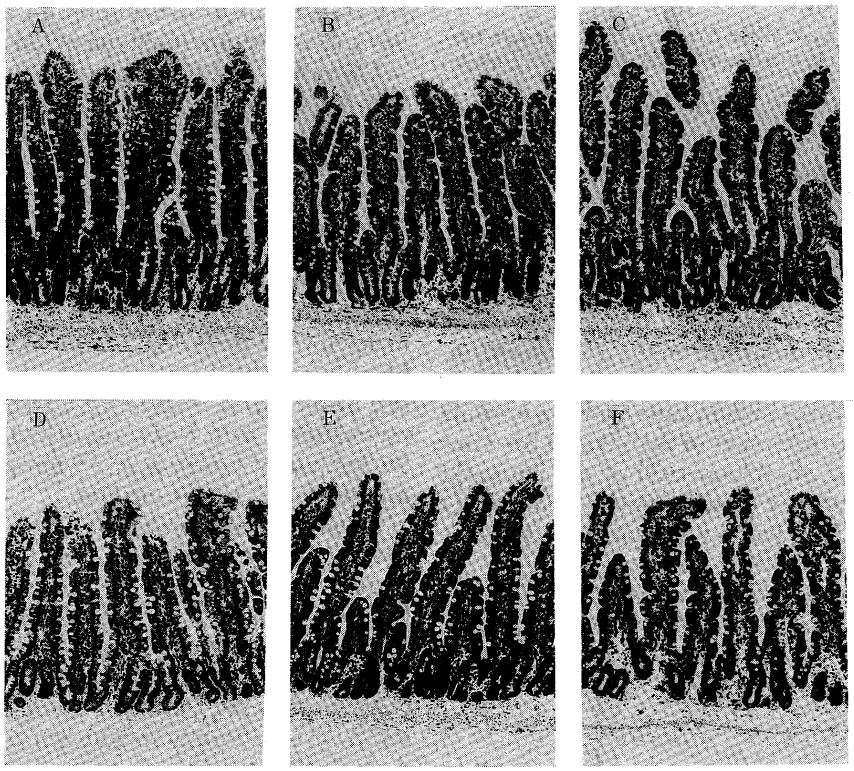

Fig. 3. Microphotographs of Intestinal Mucosa after Pretreatment with CyDs and/or N-Ac

A, control (isotonic phosphate buffer); B, 10 mM α-CyD; C, 10 mM β-CyD; D, pretreatment with N-Ac alone; E, 10 mM α-CyD after pretreatment with N-Ac; F, 10 mM β-CyD after pretreatment with N-Ac.

TABLE I. Effects of CyD on the Mucosal-to-Serosal Flux Rate of SA in Intestinal Membranes

	SA flux rate (nmol/cm^2·min)				*AUC* of SA[a] (μg·min·ml^{-1})	
	None	Ratio	N-Ac pretreat.	Ratio	N-Ac pretreat.	Ratio
Control	2.32±0.17	1	3.05±0.16	1	272.5±20.0	1
α-CyD	2.73±0.36	1.2	3.57±0.32	1.2	344.6±26.0	1.3
β-CyD	2.82±0.18	1.2	5.70±0.23[b]	1.9	665.8±10.9[c]	2.5

Each value represents the mean±S.E. *a*) *AUC* of SA up to 60 min was obtained by *in situ* single perfusion method. Significantly different from control *b*) $p<0.001$, *c*) $p<0.05$.

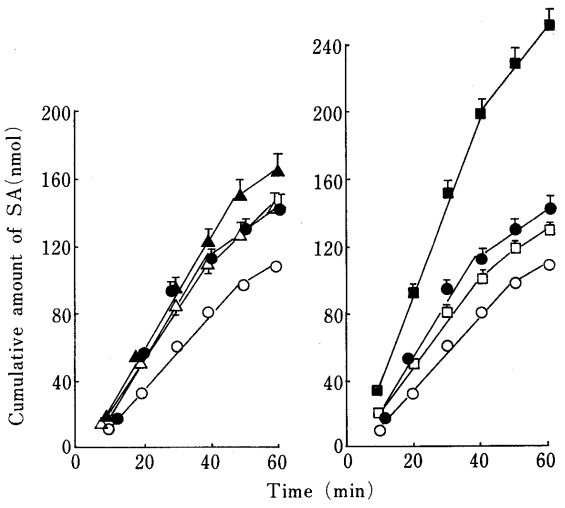

Fig. 4. Effect of CyD on the Mucosal-to-Serosal Transfer of SA

—○—, control; —●—, pretreatment with N-Ac alone; —△—, 10 mM α-CyD solution; —▲—, 10 mM α-CyD after pretreatment with N-Ac; —□—, 10 mM β-CyD solution; —■—, 10 mM β-CyD after pretreatment with N-Ac. Each point represents the mean of 4—7 experiments, with the S.E.

amount of SA transferred across the jejunal membrane from the mucosal side to the serosal side as a function of time. Since in all cases the regression lines become linear after a lag time of 10 min, SA flux rates were calculated from the linear part of each plot; the mean values are listed in Table I. The transferred amount of SA is increased slightly compared with control, but not significantly, by pretreatment with each of α-CyD and β-CyD. The intestinal membrane treated with N-Ac or N-Ac and α-CyD also showed a slight increase in SA transfer. But after pretreatment with N-Ac and β-CyD the flux rate of SA increased significantly; about 2 fold compared to the other groups. These result were consistent with those of *in situ* experiments.[8]

Changes in the electrical paramerers such as potential difference (PD) and membrane resistance (R_m) of the intestinal membrane were measured after treatment with N-Ac and/or CyDs. Similar patterns in PD were observed in every case. In the control R_m was maintained nearly constant (6—8 Ω cm^2) during the experimental period. When the intestine was treated with both N-Ac and CyDs

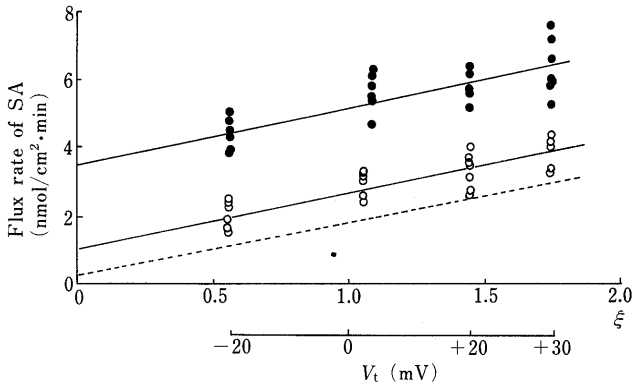

Fig. 5. Effect of Externally Applied PD on the Flux Rate of SA across Jejunal Membrane

---, control; —○—, pretreatment with N-Ac alone; —●—, 10 mM β-CyD after pretreatment with N-Ac. The intercept (transcellular flux) and slope (paracellular flux) of each line are summarized in Table II.

TABLE II. Transcellular (Intercept) and Paracellular (Slope) Flux Rate of SA

Group	Intercept	Slope	Correlation coefficient
Control	0.23	1.58	0.97
1% N-Ac alone	1.06	1.61	0.81
1% N-Ac and 10 mM β-CyD	3.50[b)]	1.68	0.74
Control[a)]	0.75	0.91	0.88
10 mM diclofenac sodium[a)]	2.80[c)]	1.67[c)]	0.87
EDTA[a)]	0.95	4.06[c)]	0.97

a) Ca free Ringer's solution was used. Significantly different from control: *b*) $p<0.05$, *c*) $p<0.01$.

a small decrease of R_m was observed, but there were no significant differences between α-CyD and β-CyD (data not shown).

Voltage-Clamp Experiments We previously reported that the voltage-clamp experiment was useful for separation of the permeation route of a drug, because this method can evaluate the amount of drug transferred from the transcellular and paracellular route, respectively.[18] The mucosal-to-serosal flux rates of SA were measured under various externally applied potential differences and are shown in Fig. 5. The intercept and the slope of each line which means the flux rate through the transcellular route and the paracellular route, respectively,[10] are summarized in Table II. Straight lines have high regression coefficients: $r=0.97$, 0.81 and 0.74 for control, N-Ac, and N-Ac and β-CyD, respectively. As seen in Table II, SA penetrates the intestinal membrane by both pathways (control). Treatment with N-Ac alone produces a 4-fold increase in the transcellular flux rate, while the paracellular flux rate is less affected. After pretreatment with N-Ac, β-CyD induces a 15-fold selective increase in the transcellular flux rate.

In our previous report, ethylenediaminetetraacetic acid (EDTA) enhanced the permeation of drug through the paracellular route only, while diclofenac sodium enhanced both routes.[18] The mechanism of the enhancing effects of β-CyD may be different from those of EDTA and diclofenac sodium.

To elucidate the enhancement mechanism of β-CyD morphological changes in the intestine and interaction with membrane lipids were measured. SA absorption from the intestine was little influenced in the presence of α-, β- or γ-CyDs alone.[8] However, enhanced absorption of SA was observed in the absence of the mucin layer. Mucin adheres to the luminal surface of the gastro–intestinal tract as a gel or high viscosity solution; this may disturb the direct interaction of CyDs with the membrane lipids.

Many studies concerning the permeability change of drugs have been performed with either model or animal membranes; these have demonstrated that filipin interacts specifically with sterols forming stoichiometric compounds.[19,20] As a consequence of such interaction, an increase in cell membrane permeability is often evidenced. Cholesterol influences the membrane permeability of phospholipid bilayer accompanied by a change in the fluidity of this bilayer. The greater of cholesterol content with phospholipid increases the fluidity of membrane in a gel state, or decreases it in a liquid–crystalline state.[21,22] When the mucin layer is removed by adjuvants, CyD molecules recognize the lipid molecules in the membrane by virtue of their cavity size. They withdraw the lipid molecules by forming inclusion complexes, thereby enhancing membrane permeability. The release of cholesterol rather than phospholipid causes enhancement in the absorption of SA. From the results shown in Fig. 1 and the results of liposome experiments,[17] the enhanced absorption of SA may be based on direct action of CyDs on model or animal membranes in the absence of mucus. However, under the optical microscope morphological changes of the small intestine were not observed in any cases.

Two transport pathways, the transcellular and paracellular routes, are possible ways of inorganic and organic ion absorption in the small intestine.[23] It is reasonable that 10 mM β-CyD, after pretreatment with N-Ac, enhanced mainly the permeability of the transcellular pathway (Table II). There is considerable evidence that many functions of biological membranes are influenced by the composition and physical state of the membrane lipids.[24] Shinitzky and Inbar demonstrated the direct correlation between changes in membrane fluidity and changes in the cholesterol content of lymphocytes.[25] The lowering of the cholesterol content of the membrane by β-CyD may induce perturbation of the lipid layer in the membrane, leading to an increased permeability in the transcellular route. Although the detailed mechanism of CyDs action in enhancing the transcellular route has not yet been clarified, it seems likely that the regular arrangement of lipid molecules, which constitute the cell membrane, is perturbed by the interaction of membrane lipids and CyDs.

Additional studies using the brush border membrane of the small intestine are in progress to clarify the relationship between the change of membrane permeability and the physicochemical properties of membrane.

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