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Effect of Bile Salts on the Rectal Absorption of Sodium Ampicillin in Rats[1)]

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The rectal absorption of sodium ampicillin in the presence of various bile salts, such as dehydrocholate, dihydroxycholate, trihydroxycholate, and their glycine and taurine conjugates, was investigated using the *in situ* rat rectal loop method. A marked but variable absorption-promoting effect was observed with dihydroxycholate, whereas trihydroxy and triketo bile salts did not enhance the rectal absorption of sodium ampicillin. These results suggest that the promoting efficacy of bile salts for rectal absorption of sodium ampicillin depends upon their chemical structures and/or their physicochemical properties.

Solubilizing activity, hemolytic activity, lipophilicity and calcium ion sequestration capacity of the bile salts were measured *in vitro*, and the relations between promoting efficacy and physicochemical properties were investigated. The bile salts having higher hemolytic activity, higher lipophilicity, and higher values of lipophilicity (*Rm* value) × calcium ion sequestration capacity showed higher promoting effects on rectal absorption of sodium ampicillin.

Keywords——rectal absorption; rat rectal loop method; ampicillin; bile salt; rectal absorption-promoting effect; hemolytic activity; lipophilicity; calcium ion sequestration capacity

It is well known that bile salts have many important roles as physiologic surfactants in the intestinal absorption of lipid and lipid-soluble vitamins.[2-4)] Bile salts have also been reported to produce changes in the permeability to many drugs of the small intestine,[5-7)] rectum,[8)] vagina,[9)] and naris.[10)] However, these reports have dealt with relatively few bile salts.

In the present study, the effects of nine bile salts on the rectal absorption of sodium ampicillin (ABPC Na) were investigated and the relations between the promoting efficacy for rectal absorption of ABPC Na and the physicochemical properties were also studied. ABPC Na was used as a model compound which is poorly absorbable through rat rectal membrane. The physicochemical properties of bile salts investigated were solubilizing activity, hemolytic activity, lipophilicity, and calcium ion sequestration capacity.

Experimental

Materials——Sodium ampicillin (ABPC Na, 912 μg/mg) was obtained from commercial sources and used without further purification. The nine bile salts used were sodium cholate (C), sodium taurocholate (TC), sodium glycocholate (GC), sodium deoxycholate (DC), sodium taurodeoxycholate (TDC), sodium chenodeoxycholate (CDC), sodium glycochenodeoxycholate (GCDC), sodium ursodeoxycholate (UDC), and sodium dehydrocholate (DHC). Cholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, and dehydrocholic acid were purchased from Sigma Chemical Company, and were recrystallized according to the literature.[11)] These bile acids were transformed to the sodium salts by reaction with an equimolar amount of sodium hydroxide in a small volume of deionized water with subsequent dilution to a desired final concentration in the buffer solution used in animal or physicochemical studies. GC and TC were purchased from Nakarai Chemicals, Ltd. and TDC and GCDC were purchased from Sigma Chemical Company. Conjugated bile salts were used without further purification. All bile salts used were confirmed to be chromatographically pure.[12)] All other reagents and solvents were of reagent grade and

were used without further purification.

Animal Study——Male Wistar rats weighing 180—200 g were used. Following an overnight fast, each rat was anesthetized with pentobarbital by intraperitoneal injection and fixed on a water bed kept at 37 °C to maintain normal body temperature during experiments. The rectum was exposed by a midline abdominal incision and was cannulated with polyethylene tubing (PE 50, Clay Adams) at the distal end of the colon. The anus was closed with surgical adhesive paste (Aronalpha, Sankyo Co., Ltd.). The length of rectal loop was about 3 cm. Drug solution was introduced into the rectal lumen *via* polyethylene tubing at the volume of 1 ml/kg. Blood samples (0.15 ml) were collected from the jugular vein at 2.5, 5, 10, 20, 40, 60, 90, and 120 min after rectal administration of ABPC Na. Drug solutions (60 mg ABPC Na/ml) containing various concentrations of bile salts were prepared with pH 8.0, 1/20 M Tris-HCl buffer. Their osmolarities were adjusted to 560 mOsm/kg·H_2O with NaCl. Osmolarity was determined with a digital micro osmometer (Osmotron-10, Orion-Riken Co., Ltd., Tokyo). The dose of ABPC Na was 60 mg/kg.

Physicochemical Properties of Bile Salts——Solubilizing Activity: Solubilizing activity of bile salts was measured by determining the solubility of Sudan III in a solution containing a bile salt. Excess of powdered Sudan III (about 100 mg) was added to 2 ml of isotonic phosphate buffer (pH 7.4) containing a bile salt at the concentration of 12.5 mM. The solution was shaken mechanically overnight in a room kept at 25 °C. After solubility equilibrium was established, an aliquot was filtered through 0.45 μm filteres (HA, Millipore). The concentration of Sudan III in the filtrate was determined spectrophotometrically at 550 nm after appropriate dilution with 50% aqueous ethanol solution.

Hemolytic Activity: Hemolytic activity was measured according to the method of Ogiso *et al.*[13] with a small modification. Sheep blood was centrifuged for 20 min at 3000 rpm. The plasma was removed together with the buffy coat of leucocytes, and erythrocytes were washed 3 times with pH 7.4 isotonic phosphate buffer. Erythrocytes were resuspended in pH 7.4 isotonic phosphate buffer at the concentration of 10 v/v%. To 0.5 ml of erythrocytes suspension, 0.5 ml of isotonic phosphate buffer containing a bile salt at various concentrations was added. The mixture was incubated for 30 min at 37 °C and then centrifuged for 20 min at 3000 rpm. The supernatant was diluted with 0.9% NaCl solution, if necessary, and the concentration of hemoglobin in the supernatant was determined spectrophotometrically at 410 nm.

Determination of Lipophilicity: As a measure of lipophilicity, the theoretical *Rm* value was selected. The *Rm* value of bile salts was measured according to the method of Vandamme and Vaets.[14] Aliquots (10 μl) of ethanol solutions of bile salts were spotted on KC_{18} reversed phase plates (Whatman), and the plates were developed with a mixture of pH 7.4 phosphate buffer and acetone. From the *Rf* value obtained with each developing mixture, the *Rm* value was calculated from the following equation: $Rm = \log(1/Rf - 1)$. A plot of the *Rm* values against the concentration of acetone in the mobile phase was linear in the range of 50 to 85% acetone. The theoretical *Rm* values corresponding to 0% acetone were obtained by extrapolation.

Calcium Ion and Magnesium Ion Sequestration Capacity: The calcium ion and magnesium ion sequestration capacities of bile salt were measured by the dye indicator method at pH 10.0 (1/200 M NH_4Cl–NH_4OH) at 20 °C.[15] A 10 μl aliquot of bile salt solution (2%, pH 10.0) was added in a stepwise manner to a mixture of 0.1 ml of 1/400 M $CaCl_2$ or $MgCl_2$ (pH 10.0) and 0.1 ml of 0.01% eriochrome black T solution (pH 10.0). A blue color indicates the end point of the reaction.

Analytical Method——Whole blood was diluted 6 times with deionized water, and the concentration of ABPC Na was microbiologically determined by the paper disc method with *Bacillus subtilis* ATCC 6633 as the test organism.

Results and Discussion

Effect of Bile Salts on the Rectal Absorption of ABPC Na

The effect of bile salts at various concentrations on the rectal absorption of ABPC Na was investigated at the dose of 60 mg ABPC Na/kg. In the preliminary experiments, rectal absorption of ABPC Na from aqueous solution without any adjuvant was studied in rats. ABPC Na was not detected microbiologically in the whole blood after doses of less than 50 mg/kg. As it was a dose at which ABPC Na certainly appeared in the blood, 60 mg/kg was used in the present study. Considering the dose of ABPC Na and the volume introduced into the rectal loop, the osmolarity of the solution applied to the rectal loop was adjusted to 560 mOsm/kg·H_2O (twice the isotonic value). As a typical example, the blood concentrations of ABPC Na after rectal administration with various concentrations of CDC are shown in Fig. 1(a). In the presence of CDC, the blood level of ABPC Na was increased markedly. The maximum level was obtained 10—20 min after rectal administration. Figure 1(b) also indicates that a higher dose of CDC resulted in a higher blood level of ABPC Na with a maximum level of about 30 μg/ml. Upon addition of CDC at doses of more than 12.5 mM, the

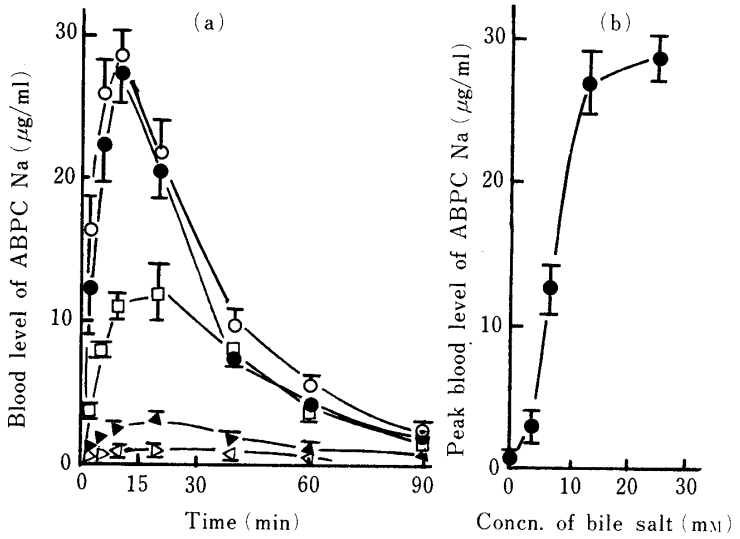

Fig. 1. Enhanced Rectal Absorption of ABPC Na in the Presence of Sodium Chenodeoxycholate in Rats

(a): Blood levels of ABPC Na–time.
(b): Enhanced peak blood levels of ABPC Na–concentration of bile salts.
Dose: 60 mg ABPC Na/ml/kg. Solution: pH 8.0, 1/20 M Tris-HCl buffer, 560 mOsm/kg·H_2O.
Concentration of sodium chenodeoxycholate: —○—, 25 mM; —●—, 12.5 mM; —□—, 6.25 mM; —▲—, 3.125 mM; —△—, 0 mM.
Each point represents the mean ± S.E. for 3—4 rats.

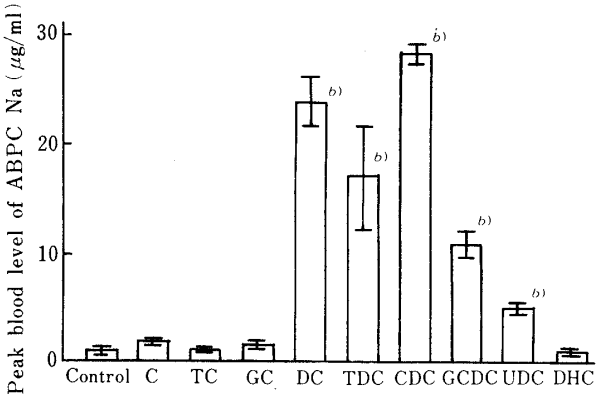

Fig. 2. Peak Blood Levels of ABPC Na after Rectal Administration in the Presence of Various Bile Salts (12.5 mM) in Rats

Dose: 60 mg ABPC Na/ml/kg. Solution: pH 8.0, 1/20 M Tris-HCl buffer, 560 mOsm/kg·H_2O.
C, sodium cholate; TC, sodium taurocholate; GC, sodium glycocholate; DC, sodium deoxycholate; TDC, sodium taurodeoxycholate; CDC, sodium chenodeoxycholate; GCDC, sodium glycochenodeoxycholate; UDC, sodium ursodeoxycholate; DHC, sodium dehydrocholate.
Each point represents the mean ± S.E. for 3—4 rats.
Significant at *a*) $p<0.05$ and *b*) $p<0.01$ as compared to the control.

absorption-promoting efficacy of CDC remained almost constant.

Promoting efficacy of other bile salts for rectal absorption of ABPC Na was studied at the dose of 12.5 mM. Peak blood levels of ABPC Na after rectal administration were used as a measure of the absorption-promoting efficacy of bile salts. As shown in Fig. 2, the results were diverse. Dihydroxy bile salts such as CDC, GCDC, DC, TDC, and UDC enhanced the rectal absorption of ABPC Na markedly, while trihydroxy and triketo bile salts did not.

It has been reported that bile salts influence the permeability of physiological membranes to many drugs. Kakemi *et al.* reported that enhancement of the intestinal absorption of sulfaguanidine was caused by direct action of bile salts on the mucosal membranes, and they found that the effect was reversible.[16] Feldman *et al.* reported that DC enhanced the absorption of phenol red markedly by altering the permeability of the intestinal membranes.[17] As to the mechanism of action of the bile salts in altering the permeability of the intestinal tract to various drugs, interaction of bile salt micelles with membrane lipid, particularly phospholipid, has been suggested,[18] but the mechanism of action of bile salts

TABLE I. Properties of Bile Salts

Bile salts	Solubilizing[a] activity (mg/l)	$ED_{50\%}$[b] hemolysis (mM)	$ED_{100\%}$[b] hemolysis (mM)	*Rm*[c]	Affinity for[d] calcium ion (gram ion/M)
Cholate	6.78	17.0	18.0	2.04	0.060
Taurocholate	22.79	25.5	36.0	0.98	0.075
Glycocholate	29.76	—	—	1.05	0.090
Deoxycholate	13.75	1.9	2.1	2.66	0.083
Taurodeoxycholate	15.43	2.0	3.5	1.50	0.163
Chenodeoxycholate	10.92	2.0	2.3	2.48	0.091
Glycochenodeoxycholate	25.80	2.0	2.3	1.41	0.152
Ursodeoxycholate	2.83	4.3	6.8	2.46	0.040
Dehydrocholate	0.94	>100	>100	1.27	ND

a) The solubility of Sudan III in the presence of 12.5 mM bile salt in pH 7.4 isotonic phosphate buffer, 25 °C.
b) Hemolytic activity of bile salt was determined using 10% sheep erythrocyte suspensions in pH 7.4 isotonic phosphate buffer, 37 °C.
c) The extrapolated *Rm* value was determined in pH 7.4 phosphate buffer on a reversed phase silica gel plate (KC_{18}, Whatman).
d) The calcium ion sequestration capacity of bile salt at pH 10.0, 20 °C.
ND: not detectable.

remains to be fully elucidated.

In the present study, we examined the relations between the absorption-promoting efficacy of bile salts and their physicochemical properties in order to clarify the mechanism of action of bile salts. The physicochemical properties studied in the present report are summarized in Table I.

Relations between the Absorption-Promoting Efficacy of Bile Salts and Their Physicochemical Properties

Solubilizing Activity of Bile Salts——Bile salts form micelles with various aggregation numbers and help to solubilize and to disperse poorly water-soluble compounds at concentrations above their critical micelle concentrations (cmc).[19] The values of cmc in water were reported to be about 1—1.5 mM for dihydroxy bile salts and 5—10 mM for trihydroxy bile salts.[20] It was also reported that DHC does not form micelles.[19,20] The concentration of bile salts of 12.5 mM used in the animal study and in the solubilizing activity experiment is more than the cmc of the various bile salts in water, except for DHC. The solubilizing activity of conjugated bile salts at the concentration of 12.5 mM was higher than that of unconjugated bile salts. There was no relation between the solubilizing activity and enhanced peak blood level of ABPC Na.

Hemolytic Activity of Bile Salts——Gullikson *et al.* studied the effect of anionic surfactants including bile salts on hamster small intestinal membrane structure and function.[20] They observed that dihydroxy bile salts showed a dose-response type inhibition of water transport in everted hamster jejunal segments. They observed no activity with trihydroxy and triketo bile salts and found that the relative effects on water transport were paralleled by the abilities to lyse erythrocytes.

In the present study, we determined the potency of bile salts for erythrocyte lysis using sheep erythrocyte aqueous suspension, and we compared these potencies with those for enhancement of the rectal absorption of ABPC Na in the rat.

As shown in Fig. 3, rather good linear relations were observed between peak blood concentration of ABPC Na and reciprocal value of the concentration of bile salts causing 100% hemolysis. The following two regression equations were obtained.

$Y=49.463X-0.623$ for all points ($r=0.869$)

$Y=60.293X-1.518$ for points except GCDC ($r=0.977$)

where Y is peak blood level of ABPC Na (μg/ml) and X is 100% hemolytic activity of bile salts (reciprocal value of the concentration of bile salt, 1/mM).

These results suggest that the mechanism causing hemolysis may be related to that of the rectal absorption-promoting effect of bile salts.

Lipophilicity of Bile Salts——In the present study, the *Rm* values of bile salts were used as a measure of lipophilicity, and were compared with the potencies for enhancing rectal absorption of ABPC Na (Fig. 4). The relation between *Rm* value and peak blood level of ABPC Na obtained was not good.

Calcium Ion Sequestration Capacity——In previous reports, we suggested that compounds that can permeate through membranes and that have calcium ion sequestration

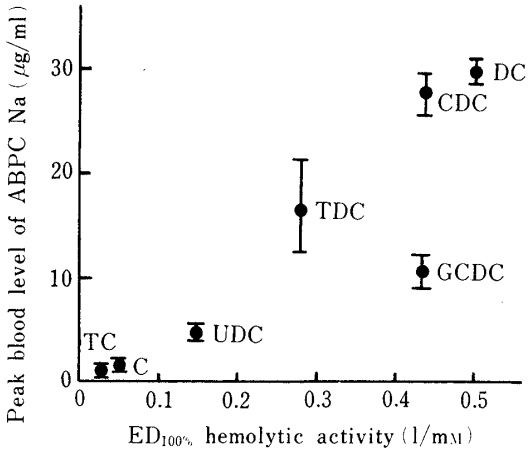

Fig. 3. Relations between Enhanced Peak Blood Levels of ABPC Na in the Presence of Bile Salts (12.5 mM) and Hemolytic Activities of Various Bile Salts

The concentration of a bile salt required to cause 100% hemolysis in 30 min at 37 °C was determined using 10 v/v% sheep erythrocytes suspension in pH 7.4 phosphate buffer ($\mu=0.15$). The reciprocal of the concentration was used as 100% hemolytic activity.

Regression line: $Y=49.463X-0.623$ for all points ($r=0.869$). $Y=60.293X-1.518$ for points except GCDC ($r=0.977$), where Y is enhanced peak blood level of ABPC Na (μg/ml) and X is 100% hemolytic activity of bile salts (reciprocal concentration of bile salt, 1/mM). See Fig. 2 for enhanced peak blood levels of ABPC Na.

Abbreviations are the same as in Fig. 2.

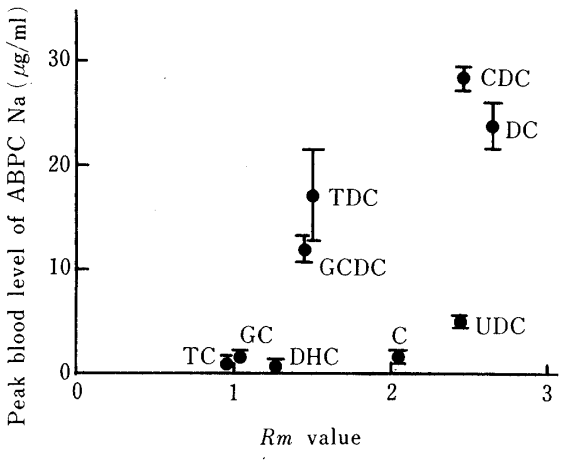

Fig. 4. Relations between Enhanced Peak Blood Levels of ABPC Na in the Presence of Bile Salts (12.5 mM) and *Rm* Values of Bile Salts

Rm value was determined by reversed phase thin layer chromatography (KC_{18}, Whatman).

See Fig. 2 for enhanced peak blood levels of ABPC Na.

Abbreviations are the same as in Fig. 2.

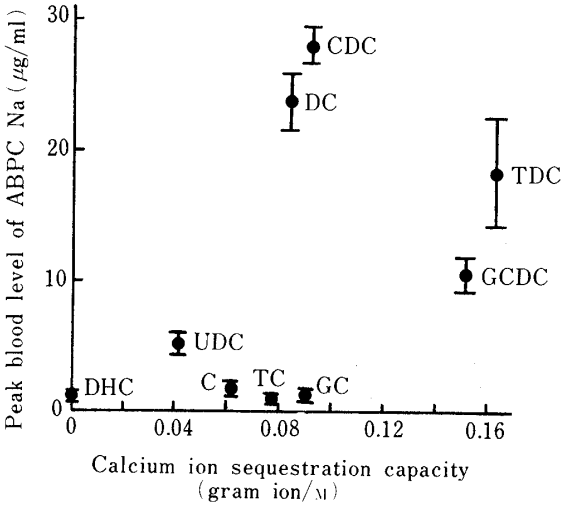

Fig. 5. Relations between Enhanced Peak Blood Levels of ABPC Na in the Presence of Bile Salts (12.5 mM) and Calcium Ion Sequestration Capacities of Bile Salts

Calcium ion sequestration capacity of bile salts was measured by the dye indicator method at pH 10.0 at 20 °C using eriochrome black T.

See Fig. 2 for enhanced peak blood levels of ABPC Na.

Abbreviations are the same as in Fig. 2.

capacity might promote the paracellular transfer of water-soluble drugs of low molecular weight.[21,22] It has been reported that disodium ethylenediamine tetraacetate (EDTA), which may produce some alterations in the cell membrane structures of mammalian cells by removing calcium ions from them, causes an increase of membrane permeability to some water-soluble compounds such as phenol red in the rat intestine.[23-25] Cassidy and Tidball reported that EDTA placed in the rat lumen evoked a five-fold increase in membrane permeability to phenol red and at the same time the mucosal contents of magnesium and calcium were decreased significantly.[26] They also observed rounded swellings on the microvilli in the area of the junctional complexes between adjacent epithelial cells, and widening of intercellular channels, particularly in the region of the intermediate junctions, in the presence of EDTA by electron microscopy.

An increase in membrane permeability of rat rectum to water-soluble drugs such as *p*-aminobenzoic acid, phenol red, and ABPC Na was found to be produced by EDTA and other absorption promoters, and the increase was studied from the viewpoint of calcium ion sequestration capacity.[27] Therefore, a similar investigation was carried out on bile salts. As shown in Fig. 5, the contribution of calcium ion sequestration capacity of bile salts to their promoting effect on rectal absorption of ABPC Na is not clear. Calcium ion sequestration capacity of bile salts is lower than that of EDTA, but the promoting effect of EDTA on rectal absorption of ABPC Na is less than that of dihydroxy bile salts.[27] It is possible that the combination of lipophilicity and calcium ion sequestration capacity is necessary for effectiveness. The peak blood level of ABPC Na is plotted against *Rm* value $\times$ calcium ion sequestration capacity (CH) in Fig. 6. Bile salts having a higher value of (*Rm*) $\times$ (CH) indeed showed a higher promoting effect on rectal absorption of ABPC Na. The value of (*Rm*) $\times$ (CH) is considered to reflect the potency of bile salts to sequestrate calcium ions in the membranes.

It has been proposed that EDTA and anions of long chain fatty acids bind calcium in the membrane and open the tight junction between the duodenal enterocytes.[28] Further ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), which binds calcium, is known to increase paracellular conductance in the ileum.[29] Munck and Rasmussen also suggested that materials with high affinity for binding calcium ions make the tight junctions between luminal ends of the enterocytes more permeable to water than they ordinarily are.[30]

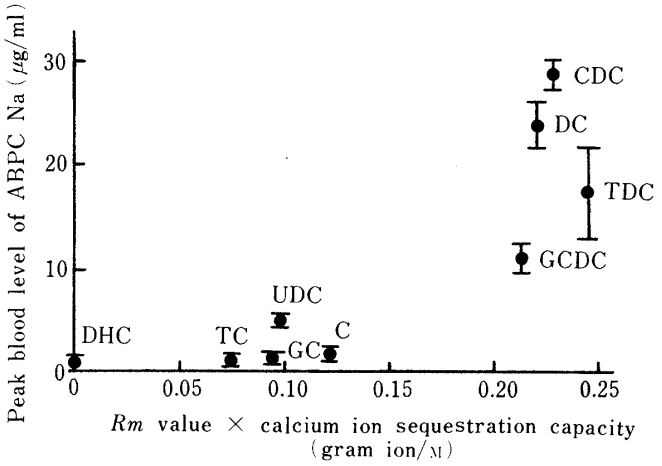

Fig. 6. Relations between Enhanced Peak Blood Levels of ABPC Na in the Presence of Bile Salts (12.5 mM) and *Rm* Value $\times$ Calcium Ion Sequestration Capacity of Bile Salts

See Fig. 2 for enhanced peak blood levels of ABPC Na.
See Table I for *Rm* values and calcium ion sequestration capacities of bile salts.
Abbreviations are the same as in Fig. 2.

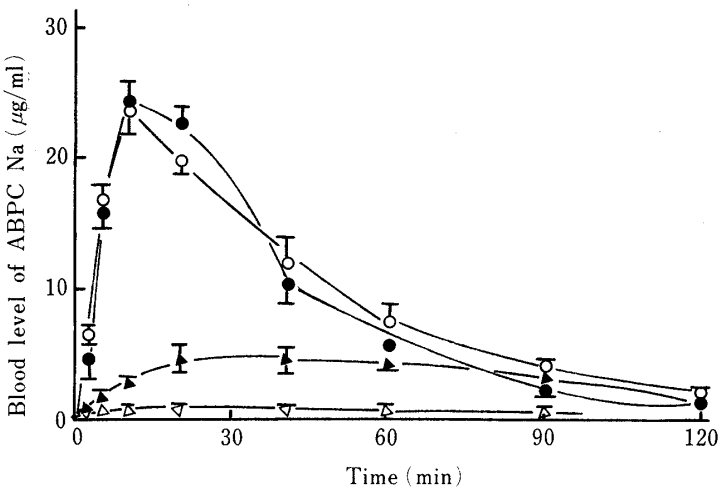

Fig. 7. Effect of Magnesium or Calcium Ion on the Enhanced Blood Levels of ABPC Na in the Presence of Sodium Deoxycholate (12.5 mM) in Rats

Dose: 60 mg ABPC Na/ml/kg. Solution: pH 8.0, 1/20 M Tris-HCl buffer, 560 mOsm/kg·H_2O. Concentration: $MgCl_2$, 6.25 mM; $CaCl_2$, 6.25 mM.
—○—, DC; —●—, DC + $MgCl_2$; —▲—, DC + $CaCl_2$; —△—, no additive.
Each point represents the mean ± S.E. for 3—4 rats.

To further investigate whether the calcium sequestration capacity of bile salts correlates with the promoting efficacy for rectal absorption of ABPC Na, the effect of $CaCl_2$ on the promoting effect of CDC on rectal absorption of ABPC Na was studied. As shown in Fig. 7, the promoting effect of CDC on rectal absorption of ABPC Na was markedly decreased in the presence of $CaCl_2$ with bile salts in the solution applied to the rectal loop. In contrast, addition of $MgCl_2$ did not decrease the effect of CDC. The magnesium ion sequestration capacity of CDC was examined by the same method, but no complex formation of CDC with magnesium ion was observed.

In conclusion, two findings emerged from this study. First, it was demonstrated that bile salts such as dihydroxy bile salts markedly promote the rectal absorption of water-soluble materials such as ABPC Na. Second, the role of bile salts as rectal absorption promoters for water-soluble materials such as ABPC Na is due at least partly to their effect upon the binding of calcium ions to the rectal membranes.

Bile salts have many other effects on mucosal membranes, such as producing release of desoxyribonucleic acid (DNA), phospholipid and cholesterol, and increasing the forward diffusion of sodium ions.[31-33] However, it was reported that DC caused no morphological changes in the concentration range of 5.4—10.8 mM as determined by light microscopy, though some damage to the microvilli of the cells of rat jejunal mucosa was detected electron-microscopically at the concentration of 27 mM.[34] Further investigation is necessary to clarify the mechanism of action of bile salts as rectal absorption promoters.

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