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## Studies of Absorption Promoters for Rectal Delivery Preparations. II. A Possible Mechanism of Promoting Efficacy of Enamine Derivatives in Rectal Absorption<sup>1)</sup>

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The effect of calcium ions on the promoting efficacies of enamine derivatives for rectal absorption of water-soluble  $\beta$ -lactam antibiotics was investigated *in vivo* and *in vitro*.

Ethyl acetoacetate enamine derivatives of phenylglycine and ampicillin, which are membrane-permeable and have calcium binding ability, enhanced the rectal absorption of water-soluble  $\beta$ -lactam antibiotics. The promoting efficacy of the enamine derivative for ampicillin was larger than that of water-soluble chelating agents such as EDTA 2Na and trisodium citrate.

The blood level-time profile of ampicillin after rectal administration of the enamine derivative of ampicillin was not influenced by the presence of calcium chloride in the suppository. However, the promoting potency of the enamine derivative for rectal absorption of co-existing ampicillin decreased in the presence of calcium chloride in the suppository.

The enamine derivative of phenylglycine-Ca<sup>2+</sup> complex was proved to be permeable through the rat rectal sac *in vitro*, although the complex had no promoting potency for the permeation of ampicillin through the rectal sac. The loss of absorption promoting potency upon calcium complex formation by the enamine was also found in *in vivo* experiments.

This finding suggests that the role of enamine derivatives as rectal absorption promoters for water-soluble antibiotics is based upon their calcium binding ability.

**Keywords**—rectal absorption of ampicillin; rectal absorption of cephalothin; absorption promoter for rectal absorption; enamine promoter of phenylglycine; enamine prodrug of ampicillin; effect of calcium ion on enamine promoter; chelating agents

In order to enhance the intestinal absorption of poorly absorbable or non-absorbable drugs, many studies have been carried out using chelating agents<sup>2,3)</sup> and surfactants.<sup>4,5)</sup>

We previously reported that, in rabbits,  $\beta$ -dicarbonyl enamine derivatives of amino acids and ampicillin enhanced the rectal absorption of water-soluble  $\beta$ -lactam antibiotics which are poorly permeable through the rectal mucous membrane, and that their promoting efficacies for rectal absorption of water-soluble  $\beta$ -lactam antibiotics were greater for antibiotics having small partition coefficients.<sup>6)</sup>

The previous results suggest that chelation with calcium ions in the epithelial surface may be a possible mechanism for the absorption promoting action of the enamine derivatives of amino acids and ampicillin, because calcium ions play an important role as cross linkage agents in mucous structure<sup>7,8)</sup> and in the cellular junction between epithelial cells.<sup>9,10)</sup>

In the present work, the effect of calcium ion on the promoting efficacy of enamine derivatives for the rectal absorption of water-soluble  $\beta$ -lactam antibiotics was investigated.

### Experimental

**Materials**—Commercial sodium ampicillin (ABPC Na) and sodium cephalothin (CET Na) were used as supplied. All other reagents and solvents were commercial products of reagent grade and were used without further purification.

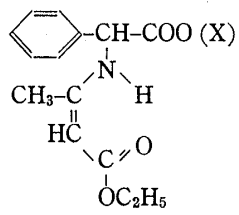
**Synthesis of Enamine Derivatives**—Sodium *N*-(1-methyl-2-ethoxycarbonylvinyl)ampicillin (ABPC EtAA Na) and sodium *N*-(1-methyl-2-ethoxycarbonylvinyl)-*D*-phenylglycine (PG EtAA Na) were synthesized following the method described previously.<sup>6,11)</sup>

PG EtAA-Ca<sup>2+</sup> Complex: Calcium *N*-(1-methyl-2-ethoxycarbonylvinyl)-*D*-phenylglycine was similarly prepared from the calcium salt of *D*-phenylglycine.<sup>6)</sup> One gram of the compound was dissolved in 50 ml of methanol. Then, the solution was saturated with CaCl<sub>2</sub>. After standing overnight at room temperature, the solution with excess undissolved CaCl<sub>2</sub> was filtered through a sintered glass filter (3G). The filtrate was poured into an excess of ethyl ether to obtain a precipitate. The precipitate was collected and recrystallized from ethanol to give PG EtAA-Ca<sup>2+</sup> complex. Recrystallization was repeated twice. The complex was dissolved in water to determine calcium ion. The same amount of the complex was dissolved in acetone to determine PG EtAA. Calcium ion was determined with a clinical test kit (Calcium C-Test WAKO, Wako Pure Chemical Industries, Ltd., Osaka). PG EtAA was spectrophotometrically determined at 289 nm. The analytical results showed that 0.75 mol ion of calcium bound per 1 mol of PG EtAA.

The enamine derivatives synthesized are listed in Table I together with some physicochemical properties.

In an nuclear magnetic resonance (NMR) study, the proton signals of PG EtAA-Ca<sup>2+</sup> complex in dimethyl sulfoxide-*d*<sub>6</sub> solution were identical with those of PG EtAA Na.

TABLE I. Properties of PG EtAA Na and PG EtAA-Ca<sup>2+</sup> Complex



(X); Na PG EtAA Na  
(X); (Ca) PG EtAA-Ca<sup>2+</sup> complex

| Property                | PG EtAA Na | PG EtAA-Ca <sup>2+</sup> complex |
|-------------------------|------------|----------------------------------|
| $\lambda_{\max}^a$ (nm) | 289.0      | 289.0                            |
| $t_{1/2}^b$ (min)       | 26.1       | Stable                           |
| <i>Rm</i> <sup>c)</sup> | 1.7        | 1.8                              |
| P.C. <sup>d)</sup>      | 7.4        | 3.5                              |

a)  $\lambda_{\max}$  was measured in ethyl alcohol.

b)  $t_{1/2}$  was measured in pH 7.4 phosphate buffer ( $\mu=0.15$ ) at 25°C.

c) The *Rm* value was determined in pH 7.4 veronal buffer with silica gel G as the solid support, and liquid paraffin as the stationary support.

d) Apparent partition coefficient was measured in Menzel's buffer-butyl alcohol phase at pH 9.5, 25°C.

**Surface Tension of ABPC EtAA Na and PG EtAA Na Aqueous Solutions**—To avoid hydrolysis of the enamine moiety, ABPC EtAA Na or PG EtAA Na was dissolved in pH 9.5 phosphate buffer. The surface tension was measured with a Shimadzu ST-1 surface tensiometer at room temperature.

**Calcium Binding Activity**—To avoid hydrolysis of the enamine moiety, the calcium binding activity was measured using eriochrome black T solution (Universal BT, Wako Pure Chemical Industries, Ltd.) at pH 10.0 at room temperature (Table II). To obtain the correct endpoint from blue to red, spectrophotometric measurement was done at 570 nm.

TABLE II. Apparent Calcium Ion Binding Activity at pH 10.0

| Agent             | Consumption of calcium ion,<br>mg/100 g of agent |
|-------------------|--|
| EDTA 2Na          | 11100  |
| Trisodium citrate | 44.1   |
| Sodium tartrate   | 9.0  |
| Sodium oxalate    | 7.2  |
| EtAA              | 18.0   |
| ABPC EtAA Na      | 12.1   |
| PG EtAA Na        | 10.0   |

Each value was obtained using eriochrome black T. To avoid hydrolysis of the enamine moiety, the binding activity was measured at pH 10.0, at room temperature. The binding activity of lithium acetoacetate was not measured by this method, because the lithium ion of lithium acetoacetate binds to eriochrome black T.

**Animal Study**—Four to five overnight-fasted male albino rabbits weighing 2.5–3.0 kg were used for each *in vivo* experiment. Three to five overnight-fasted male rats of the Wistar albino strain weighing 180–200 g were used for rectal membrane permeation experiments *in vitro*. The doses of antibiotics for rabbits were fixed at 15 mg/kg and 50 mg/kg equivalent to ABPC Na and CET Na, respectively.

**Rectal Administration:** Rectal suspensions of drugs were prepared at 40°C immediately prior to the experiments by dispersing a drug into fused rectal base with a mortar and pestle. The concentrations of drugs in the rectal suspension were 10% for ABPC Na and ABPC EtAA Na, and 20% for CET Na. Three bases, Witpsol H-15 (Dynamit Nobel Chemicals, Troisdorf-Oberlat, West Germany), a mixture of equal amounts of liquid paraffin and white petrolatum, and aqueous 1% methyl cellulose solution were used. Administration of rectal suspensions was performed as reported previously.<sup>6)</sup>

**Rectal Membrane Permeability *in Vitro*:** Permeation of drugs with or without adjuvants was studied *in vitro* according to Wilson *et al.*<sup>12)</sup> with some modifications. A roughly 2 cm long section of rat rectum was isolated and washed with a sufficient volume of cold Krebs–Ringer's solution (pH 7.4). One end was ligated, and 0.1 g of rectal suspension containing antibiotics and/or a promoter was forced into the mucosal side of the rectum with a 1 ml syringe, then the other end was also ligated tightly. The rectal sac was bathed in 50 ml of Krebs–Ringer's solution maintained at 37°C and the serosal fluid was continuously gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Aliquots (1 ml) of serosal fluid were pipetted out at suitable intervals for 45 min, and the concentration of antibiotic and/or promoter which had permeated through the rectal membrane was determined.

**Analytical Method**—Antibiotics: The concentrations of antibiotics in blood samples or in aqueous solution samples were determined by microbioassay with *Sarcina lutea* ATCC 9341 as the test organism.

**EtAA:** EtAA was determined as equivalent fraction of enamine derivative. An aliquot of sample solution or serosal fluid containing enamine derivative was acidified with 1 N HCl to hydrolyze the enamine derivative. The hydrolysis was completed in 1 min. EtAA, the hydrolyzed product, was determined by a spectrophotometric method according to Frommer.<sup>13)</sup>

## Results and Discussion

Surfactants and some compounds having metal chelating potency have been used to enhance the gastrointestinal absorption of poorly absorbable drugs.<sup>2,4)</sup> As possible mechanisms, decrease of the surface tension between the gastrointestinal fluid and the epithelial cells, which are supposed to be a physical barrier to drug absorption, and an increase of fluidity of mucous compounds on the epithelial tissues have been reported.<sup>14,15)</sup>

Enamine derivatives of ABPC and amino acids have remarkable promoting potency for the rectal absorption of water-soluble drugs.<sup>6,11)</sup> They have surface tension lowering ability and metal chelating potency. Thus, in order to clarify the mechanism of their absorption promoting potency, the surface activity of enamine promoter was studied. As is known generally, enamine derivatives are susceptible to acid hydrolysis.<sup>16)</sup> The enamine promoters under study are also easily hydrolyzed in acid conditions. Thus the surface tension was determined at pH 9.5 to avoid possible hydrolysis.

The surface tensions of ABPC EtAA Na and PG EtAA Na at the concentration of 1% were 55 and 50 dyn/cm, respectively. This finding suggests that the promoting efficacy of enamine derivatives may not be based upon their surface activity alone.

On the other hand,  $\beta$ -dicarbonyl compounds are known to have metal binding ability,<sup>17)</sup> and have been classified as lipophilic chelating agents. Amino acids and penicillins are also known to be water-soluble chelating agents. Thus, the calcium binding ability of PG EtAA Na was measured in alcohol and aqueous solution. Its calcium binding ability was similar to that of sodium oxalate.<sup>17,19)</sup>

This finding suggests that calcium binding ability may be related to rectal absorption promoting potency for water-soluble drugs.

To study whether compounds having metal chelating potency enhance the rectal absorption of water-soluble drugs, the effects of EDTA 2Na and sodium citrate tribasic on the rectal absorption of ABPC Na were studied in rabbits (Fig. 1).

Without these chelating agents in the suppository, as reported previously,<sup>6)</sup> ABPC was not detectable in blood after rectal administration of ABPC Na alone. The rectal absorption of ABPC Na was slightly enhanced by the coadministration of water-soluble chelating agents

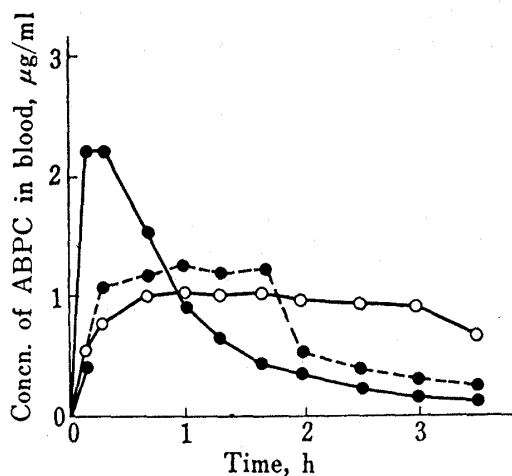


Fig. 1. Blood Level of ABPC in Rabbits following Rectal Administration of ABPC Na at a Dose of 15 mg/kg in the Presence of EDTA 2Na (1%) (—○—), Trisodium Citrate (5%) (—●—), or Lithium Acetoacetate (5%) (—●—) suspended in Liquid Paraffin-white Petrolatum

Each dose was 0.15 g/kg of suppository. Each point represents the mean for four rabbits. The coefficient of variation of each point is less than 11%.

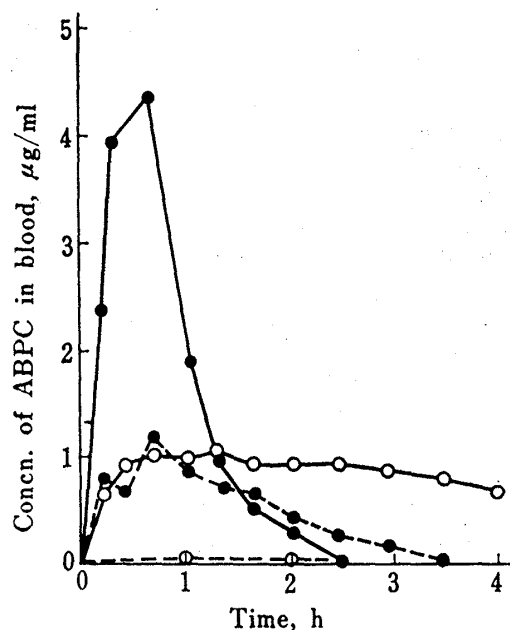


Fig. 2. Blood Level of ABPC in Rabbits following Rectal Administration of ABPC Na at a Dose of 15 mg/kg in the Presence of PG EtAA Na (10%) (—●—), PG EtAA-Ca<sup>2+</sup> Complex (10%) (—●—), EDTA 2Na (1%) (—○—), or Ca-EDTA 2Na (5%) (—○—) suspended in Liquid Paraffin-white Petrolatum

Each dose was 0.15 g/kg of suppository. Each point represents the mean for four rabbits. The coefficient of variation of each point is less than 16%.

such as EDTA 2Na and sodium citrate. The enhancing effect of lithium acetoacetate on rectal absorption of ABPC Na was larger in the early stage than that of EDTA 2Na and sodium citrate. Chelating agents used in the above experiments, like ABPC Na, are very water-soluble and are considered to be released readily from the base in the rectum.

From these results, compounds which have calcium binding ability may be considered to have promoting efficacy for the rectal absorption of water-soluble chelating agents does not appear to correlate directly with their calcium ion chelating ability. To explain the difference of the promoting efficacy of these chelating agents, other characteristics such as affinity for the rectal membrane must be taken into consideration. To study the importance of calcium ion chelating ability to the absorption promoting potency in more detail, the effect of PG EtAA-Ca<sup>2+</sup> complex or Ca-EDTA-2Na on the rectal absorption of ABPC Na was studied using a mixture of liquid paraffin-white petrolatum (1:1 by weight) as a suppository base.

As shown in Fig. 2, in contrast to the case of Ca-EDTA-2Na, PG EtAA-Ca<sup>2+</sup> complex did not completely lose its absorption-enhancing ability. This phenomenon may be attributable to possible formation of ABPC EtAA Na, which will be readily absorbed, resulting from the EtAA exchange reaction between PG EtAA-Ca<sup>2+</sup> complex and ABPC Na.

To examine this possibility, a study was made with CET Na, which has no active amino group which can form an enamine with  $\beta$ -lactam antibiotics (Fig. 3).

A 1% methyl cellulose enema suspension was used in the experiment to ensure the release of ingredients from the suppository. Almost complete loss of absorption promoting potency was observed. Thus, incomplete loss of the promoting ability of PG EtAA-Ca<sup>2+</sup> complex for the absorption of ABPC Na can be partly explained on the basis of the EtAA exchange reaction.

Loss of absorption promoting potency was observed in a study of the effect of CaCl<sub>2</sub> on

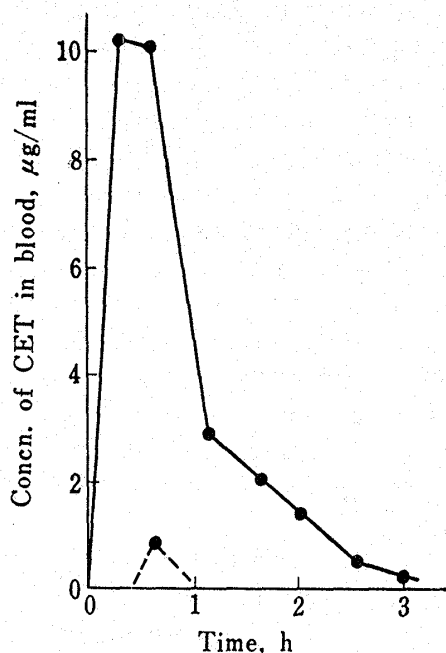


Fig. 3. Blood Level of CET in Rabbits following Rectal Administration of CET Na at a Dose of 50 mg/kg in the Presence of PG EtAA Na(10%) (—●—) or PG EtAA-Ca<sup>2+</sup> Complex (10%) (—○—) suspended in 1% Methyl Cellulose Aqueous Solution

Each dose was 0.25 g/kg of solution. Each point represents the mean for four rabbits. The coefficient of variation of each point is less than 5%.

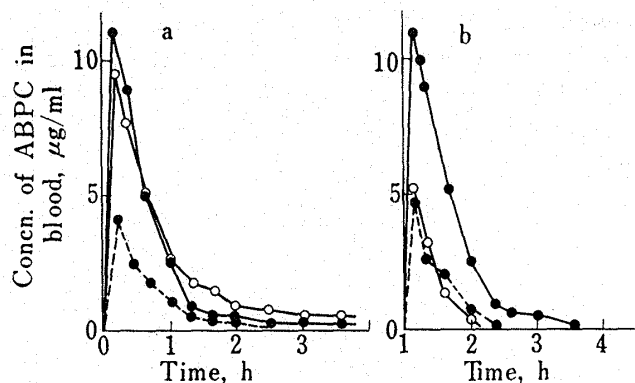


Fig. 4. (a) Blood Level of ABPC in Rabbits following Rectal Administration of ABPC EtAA Na (—●—), a Mixture of 1/2 ABPC EtAA Na and 1/2 ABPC Na (—○—), or 1/2 ABPC EtAA Na (—●—) at a Dose Equivalent to 15 mg/kg of ABPC Na, (b) Blood Level of ABPC in Rabbits following Rectal Administration of ABPC EtAA Na (—●—), a Mixture of 1/2 ABPC EtAA Na and 1/2 ABPC Na (—○—), or 1/2 ABPC EtAA Na (—●—) in the Presence of 10% CaCl<sub>2</sub> suspended in Liquid Paraffin-white Petrolatum at a Dose Equivalent to 15 mg/kg of ABPC Na

Each dose was 0.15 g/kg of suppository. Each point represents the mean for four rabbits. The coefficient of variation of each point is less than 24%.

the absorption promoting efficacy of ABPC EtAA Na.

As shown in Fig. 4(a), the blood level of ABPC obtained from suppositories containing 7.5 mg/kg of ABPC Na and 9.8 mg/kg of ABPC EtAA Na (equivalent to 7.5 mg/kg of ABPC Na) was almost the same as that obtained from suppositories containing 19.6 mg/kg ABPC EtAA Na (equivalent to 15.0 mg/kg of ABPC Na). The blood level of ABPC obtained from suppositories containing 9.8 mg/kg ABPC EtAA Na alone was about half that obtained with the double dose.

As reported previously,<sup>11)</sup> when ABPC EtAA Na was rectally administered, the enamine derivative was rapidly hydrolyzed during the absorption processes and only ABPC was detected in the blood. So, these findings suggested that the absorption of ABPC EtAA Na simultaneously enhanced the permeation of ABPC Na.

As shown in Fig. 4(b), the existence of CaCl<sub>2</sub> in the suppository did not influence the absorption of ABPC EtAA Na at a dose of 19.6 mg/kg or 9.8 mg/kg. However, the blood level of ABPC obtained from a suppository containing 7.5 mg/kg of ABPC Na and 9.8 mg/kg of ABPC EtAA Na was decreased by the addition of CaCl<sub>2</sub> to the level obtained from 9.8 mg/kg ABPC EtAA Na alone.

These findings show that absorption of ABPC EtAA Na did not change after the loss of its Ca<sup>2+</sup> chelating ability, but its absorption promoting efficacy was lost.

In the case of PG EtAA-Ca<sup>2+</sup> complex, it would also fail to show absorption promoting efficacy if it was not released from the suppository. However, it was difficult to examine this possibility *in vivo*. Thus, a study was made *in vitro* using rectal sac. The promoting efficacy of PG EtAA Na for rectal absorption of CET Na was also observed in rats,<sup>1)</sup> so experiments on the permeation of CET Na and/or PG EtAA through the rectal membrane were carried out with rectal sac of rats.

PG EtAA Na or PG EtAA-Ca<sup>2+</sup> complex was suspended with CET Na in melted Witepsol H-15. The rectal suspension was forced into a freshly isolated rat rectal sac. The sac was suspended in Krebs-Ringer's solution. CET or EtAA hydrolyzed from the PG EtAA-Ca<sup>2+</sup> complex or PG EtAA Na in the serosal solution was determined at appropriate intervals.

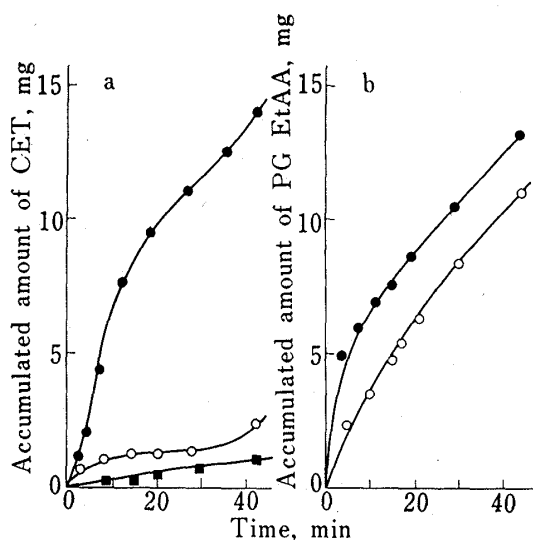


Fig. 5. (a) Effect of PG EtAA Na or PG EtAA-Ca<sup>2+</sup> Complex on the Permeation of CET through Rat Rectal Sac at 37°C

Rp; CET Na (20%) (—■—), CET Na (20%) and PG EtAA Na (10%) (—●—), or CET Na (20%) and PG EtAA-Ca<sup>2+</sup> complex (10%) (—○—) suspended in witepsol H-15.

Dose: 0.1 g of suppository/rectal sac.

Each point represents the mean for three experiments. The coefficient of variation of each point is less than 14%.

(b) Permeation of EtAA through Rat Rectal Sac at 37°C

—●—: the accumulated amount-time profile of PG EtAA Na.  
—○—: the accumulated amount-time profile of PG EtAA-Ca<sup>2+</sup> complex.

Each enamine derivative was suspended in witepsol H-15 at a concentration of 10%. Each point represents the mean for three experiments. The coefficient of variation of each point is less than 17%.

soluble  $\beta$ -lactam antibiotics is based at least partly upon the binding of calcium ions during permeation through the rectal mucosa. The coexisting antibiotics in the suppository permeate through the resultant leaky barrier. Other membrane-permeable materials having calcium ion binding ability might similarly promote the paracellular transfer of water-soluble drugs.

As shown in Fig. 5(a), CET permeated through the rectal sac to some extent. However, permeation of CET was enhanced in the presence of PG EtAA Na. The enhancement was not observed in the presence of PG EtAA-Ca<sup>2+</sup> complex. These results correspond to those of the *in vivo* study.

On the other hand, as shown in Fig. 5(b), PG EtAA-Ca<sup>2+</sup> complex permeated through the rectal sac to about the same extent as PG EtAA Na.

This finding suggests that the loss of absorption promoting efficacy of PG EtAA-Ca<sup>2+</sup> complex is mainly attributable to the inactivation of calcium ion binding in the rectal fluid and mucosa.

It was reported that calcium ions play an important role as a cross-linking agent in intercellular structures,<sup>9</sup> and that free calcium ions in the cell contents seem to regulate cell-to-cell communication through the gap junction.<sup>19,20</sup>

It appears that the enamine derivatives used in the present experiments can bind calcium ions and permeate through the rectal membrane freely even if they form a Ca<sup>2+</sup> complex, but the complex does not show promoting efficacy for the rectal absorption of water-soluble drugs such as ampicillin and cephalothin.

In conclusion, the role of enamine derivatives as rectal absorption promoters for water-

## References and Notes

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