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Intestinal Absorption of a New Anticholinergic Agent, Timepidium Bromide. I. Various Factors Influencing Absorption

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Various factors influencing intestinal absorption of a quaternary anticholinergic agent, timepidium bromide (TB), were studied in rats using *in situ* intestinal loop technique. Bile, mucin and intestinal mucous material significantly decreased the amount of TB absorbed from intestinal loops. This decrease was assumed to be due to bonding of TB cation to anionic groups of bile constituents, mucin and intestinal mucous membranes. Gastric juice and hydrochloric acid significantly increased the absorption of TB. Organic acids, such as citric, malic and acetic acid, also increased the absorption of TB. The greatest increase in absorption of TB was observed in the presence of citric acid. Citric acid had this facilitatory effect only when added to the medium in relatively high concentrations (0.05 M) and at relatively low pH. However, this increased absorption was shown not to be simply the result of an acidic intestinal environment or irreversible changes in the gut wall.

Keywords——intestinal absorption; quaternary anticholinergic drug; timepidium bromide; bile and bile constituent; gastric mucine and mucous material; gastric acid; organic acid; *in situ* intestinal loop technique; citric acid; EDTA

It is well known that, as a generality, anticholinergic quaternary ammonium compounds are not well absorbed following oral administration.[2] In a study of absorption, distribution and excretion of an anticholinergic quaternary ammonium compound, 1,1-dimethyl-5-methoxy-3-(dithien-2-ylmethylene)piperidinium bromide, timepidium bromide (TB),[3] this drug was shown to be absorbed approximately 12% from the gastrointestinal tract of rats within 4 hr.[4] In order to modify the rate of the gastrointestinal absorption of the drug, the intestinal absorption studies were extended, using the method of *in situ* intestinal loops. It is generally assumed that poor absorption of quaternary ammonium compounds is due to their ionized character. Presumably, anionic groups present in intestinal mucous membranes retain the cations of quaternary ammonium compounds by electrostatic bonding and displacement is slow by an ion exchange mechanism. Levine *et al.*[5] have shown that mucin and mucous material of the gastrointestinal tract act as barriers to quaternary absorption by formation of stable complexes. Cavallito and O'Dell[6] reported that with increasing hydrogen ion concentrations, appreciably less fixation of quaternary at polyanionic receptors of mucin was

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evident and that oral effectiveness of a quaternary hypotensive agent in dogs was significantly increased by increasing the acidity of contents of loops of intestine. This paper deals with various factors influencing absorption of TB.

Experimental

^{14}C-TB and Reagents——^{14}C-TB was prepared by the quaternization of the tertiary amine of TB with ^{14}C-methyl bromide. The specific activity of ^{14}C-TB was 3.0 μCi/mg, and the radiochemical purity was more than 98% as determined by thin-layer chromatography (TLC).[7] All of the chemicals employed in this study were reagent grade unless otherwise specified.

Experimental Procedures——Male rats of the Sprague-Dawley strain, weighing between 180—250 g were fasted for 16 hr before the experiments. Water was giving *ad libitum* and coprophagy was prevented by using cages with wide-mesh floors. The degree of intestinal absorption was determined using *in situ* intestinal loop method of Levine *et al.*[5b] The rats were anesthetized approximately 1 hr prior to surgery with urethane using an *i.p.* injection of 1 g/kg. The small intestine was exposed by a midline abdominal incision, and the proximal ligature placed about 8 cm from the pylorus. The length of the loop (about 10 cm) was determined *in vivo* by counting off 4 mesenteric blood vessels between the proximal and distal ligatures. After the drug solution was injected quantitatively into the loop (without puncturing the gut wall between ligatures), the proximal ligature was secured and the incision closed. At the end of the relevant time interval, the incision was reopened, and the loop was removed.

In some loop preparations, the intestinal loop was perfused before the ligatures were secured. Distilled water, in two equal portions of 0.5 ml each, was injected into the loop. After each injection the fluid was removed along the loop and past the distal ligature by means of a milking technique. The distal ligature was tied only after the second portion of water had been through the loop. The drug solution was then quantitatively injected into the loop and the proximal ligature secured. Manipulation controls for the perfusion procedure were carried out in a similar manner only omitting the injection of the water.

Mucous material was gently scraped from the small intestines of several rats. This material was pooled and water homogenates containing 200 mg (wet) per ml were made. Gastric mucin powder (from porcine stomach) was used in the form of a suspension in water. Bile sample was collected from the common bile ducts of several rats anesthetized with urethane. Gastric juice was collected from several pylorus-ligated rats.

The volume of ^{14}C-TB solution with or without additives injected into a loop was 0.5 ml in all experiments. Unless otherwise stated, all compounds studied, were dissolved in distilled water. The pH determinations were carried out with a Radiometer pH meter (Copenhagen Denmark).

Determination of ^{14}C-TB——To determine the residual ^{14}C-TB in the lumen of the loop and the bound drug in the intestinal wall, an excised loop was cut open and the lumen contents were carefully drained, collected, and measured. The mucosal surface was washed with 20 ml of physiological saline, and the washings were combined with the drained lumen contents. The washed intestinal wall was then homogenized in 20 ml of methanol and centrifuged. An aliquot of the lumen contents and the supernatant of the homogenates was determined for ^{14}C with an Aloka liquid scintillation spectrometer LSC-502 equipped with an automatic quenching monitor system. The extent of circulatory uptake, which refers to material taken into blood or lymphatic fluid, was calculated from the difference between the amount of drug placed in the intestine and that recovered from the intestinal tissue and contents. To determine whether there was metabolic destruction of ^{14}C-TB in the loop during the time period of the experiments, the lumen contents and the tissue homogenates were extracted with $CHCl_3$ and then subjected to TLC with a solvent system of chloroform–methanol–acetic acid (70: 20: 5).[7] TLC showed that none of ^{14}C-TB was metabolized in the intestinal loop.

Binding of ^{14}C-TB to Mucin, Mucous Material and Bile——The degree of binding was measured by equilibrium dialysis and Sephadex gel filtration. The equilibrium dialysis was carried out according to the procedure of Cavallito and O'Dell.[6] The gel filtration experiments were made with columns (1.6 × 20 cm) packed with Sephadex G-25.[8] After equilibration of the column with 0.05 M NaCl, 1 mM of ^{14}C-TB in 0.05 M NaCl with or without additives was put on a column in a volume of 10 ml. Elution was then started with 0.05 M NaCl and the effluent was collected in fractions of 3.0 ml.

Results

The time course of distribution of ^{14}C–TB in *in situ* intestinal loop is shown in Fig. 1. The circulatory uptake of the drug continued until 90 min and thereafter ceased. The tissue

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binding also achieved its maximum level at 90 min, and then fell gradually. The lumenal content fell for the first 90 min and rose concomitantly with the decrease in bound material. It seems likely to consider that ^{14}C–TB which remained bound was unavailable for circulatory uptake and could only be released back into the gut lumen.

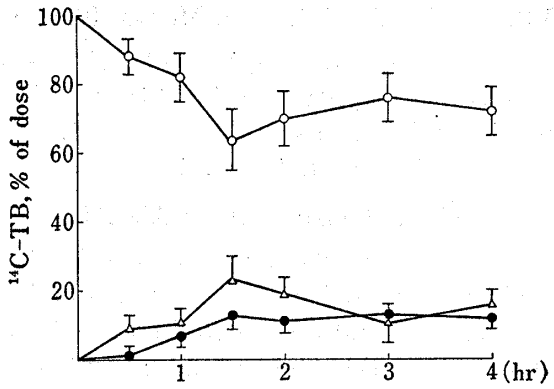

Fig. 1. Time Course of ^{14}C–TB in *in Situ* Intestinal Loops

—○—, residue in intestinal lumen; —△—, tissue binding; and —●—, circulatory uptake. Each point represents the mean of 3 to 4 fasted rats with S.D.

Effect of Fasting and Perfusion of Intestinal Lumen (Table I)

Washing out the intestinal loop significantly increased both the circulatory uptake and tissue binding only in the fasted animals. Manipulation of the loop had no influence. When the intestinal loop was washed with water, the circulatory uptake was significantly greater in non-fasted than in fasted rats while the tissue binding was greater in fasted rats.

TABLE I. Effect of Perfusion upon Absorption of TB from *in Situ* Intestinal Loops in Non-Fasted and Fasted Rats

	Non-fasted rats		Fasted rats	
	Circulatory uptake % of dose/loop	Tissue binding % of dose/loop	Circulatory uptake % of dose/loop	Tissue binding % of dose/loop
Untreated	20.9±14.0	14.5±7.4	10.7±3.3	19.1±4.9
Perfused[a)]	26.8± 1.5*	21.0±5.1*	17.7±2.6**	33.7±6.1**
Manipulated[b)]			12.8±4.8	13.4±5.0

All studies are for 2-hour periods. A dose of 0.2 mg of ^{14}C–TB in 0.5 ml of water is placed in loop. Results were expressed as the mean percentage of the dose in 4 to 8 rats with S.D.

a) Loop was perfused twice with 0.5 ml of distilled water.

b) Manipulation controls for the perfusion procedure were carried out in a similar manner only omitting the injection of the water.

* Significantly different ($p<0.05$) from comparable value for fasted rat.

** Significantly different ($p<0.05$) from comparable value for untreated.

TABLE II. Influence of Bile, Mucin, Mucous Material and Gastric Juice on Absorption of TB from *in Situ* Intestinal Loops

Additive	Amount of additive	No. of animals	Circulatory uptake % of dose/loop	Tissue binding % of dose/loop
Control		8	10.7±3.3	19.1±4.9
Bile	0.1 ml	4	2.3±2.0*	6.5±2.6*
Bile	0.4 ml	4	0.7±0.6*	6.0±1.2*
Mucin	10 mg	4	6.8±2.4	6.9±2.3*
Mucin	40 mg	4	2.1±0.8*	7.6±0.7*
Mucous material	20 mg	4	4.3±2.5*	8.5±3.0*
Mucous material	80 mg	4	0.7±0.6*	6.3±1.1*
Gastric juice	0.2 ml	4	18.4±4.1*	30.5±6.3

All studies are for 2-hour periods. A dose of 0.2 mg of ^{14}C–TB was injected in a total volume of 0.5 ml. Results were expressed as the mean percentage of the dose with S.D.

* Significantly different ($p<0.05$) from comparable value for control.

Effect of Mucin, Intestinal Mucus, Bile and Gastric Juice (Table II)

The circulatory uptake of TB was significantly decreased by bile, mucin and mucous material as compared with controls. The tissue binding of TB was also impeded by the bile, mucin and mucous material. On the contrary, the presence of gastric juice significantly increased the circulatory uptake and tissue binding.

Effect of Bile Constituents (Table III)

When taurocholic acid was added to the medium as sodium salt, the circulatory uptake slightly decreased, but when it was added as free form, that significantly increased. Sodium glycocholate and taurine had no effect on the circulatory uptake. Sodium deoxycholate and cholate increased the circulatory uptake and decreased the tissue binding. All of the intestinal loops containing cholate or deoxycholate were found to be distended with fluid at the time of excision.

Table III. Effect of Bile Constituents on Absorption of TB from *in Situ* Intestinal Loops

Bile constituent	Molarity of additive	pH of solution injected	Circulatory uptake % of dose/loop	Tissue binding % of dose/loop
Control		5.8	10.7±3.3	19.1±4.9
Sodium glycocholate	0.1 M	6.8	11.3±3.6	19.0±9.5
Sodium taurocholate	0.1 M	7.1	7.3±5.4	19.4±3.4
Taurocholic acid	0.03M	1.6	20.1±5.7*	9.4±3.1*
Sodium cholate	0.03M	6.6	25.0±2.3*	4.9±0.9*
Sodium deoxycholate	0.03M	7.5	49.9±8.7*	3.1±1.0*
Taurine	0.03M	5.5	13.7±5.4	28.9±2.4*

All studies are for 2-hour periods. A dose of 0.2 mg of ^{14}C–TB was injected in a total volume of 0.5 ml. Results were expressed as the mean percentage of the dose in 4 to 8 rats with S.D.
* Significantly different ($p<0.05$) from comparable value for control.

Effect of Various Acids (Table IV)

All of the acids studied markedly increased the circulatory uptake of TB. When the drug was administered alone, it was absorbed scarcely during 30 min (about 2%), but, when administered with acids, the degree of circulatory uptake rose to values ranging from 10 to 39%. The greatest increase in the circulatory uptake was observed in the presence of citric acid.

Table IV. Effect of Various Acids on Absorption of TB from *in Situ* Intestinal Loops

Acid	Molarity of acid	pH of solution injected	Circulatory uptake % of dose/loop	Tissue binding % of dose/loop
Control		5.8	1.9±1.2	9.3±3.0
Acetic acid	0.1M	2.9	10.1±3.2*	3.9±0.4*
Glucuronic acid	0.1M	2.2	16.5±1.0*	6.9±2.1
Lactic acid	0.1M	2.4	19.2±4.3*	5.4±1.3
HCl	0.1M	1.0	25.5±6.3*	4.8±1.1*
Malic acid	0.1M	2.2	30.6±8.4*	7.2±3.3
Citric acid	0.1M	2.0	38.7±5.2*	10.6±3.4

All studies are for 30 min periods. A dose of 0.2 mg of ^{14}C–TB was injected in a total volume of 0.5 ml. Results were expressed as the mean percentage of the dose in 4 to 8 animals with S.D.
* Significantly different ($p<0.01$) from the comparable value for control.

Effect of Molarity and pH of Citric Acid (Table V)

The circulatory uptake of TB was found to increase significantly as the concentration of citric acid was increased until 0.1 M. When the concentration of citric acid was more than 0.2 M, the intestinal loops were found to be distended with fluid at the time of excision, and the increase in the circulatory uptake was slightly smaller than that when the concentration of citric acid was 0.1 M. When the pH of the citric acid solution to be used in the loop was raised to 3.7 and 8.6, by addition of NaOH, or when the concentration of citric acid was lowered to 0.02 M, there was no effect on the control level of the circulatory uptake of TB. When NaCl or $CaCl_2$ was added to citric acid, or when the buffer solution (pH 2.0) which consisted of 0.1 M disodium citrate and 0.1 N HCl was used, the increase in the circulatory uptake was smaller than that when citric acid was used alone.

EDTA significantly increased the circulatory uptake of TB. Addition of an equimolar amount of $CaCl_2$ to EDTA had no effect on the circulatory uptake observed in the presence of EDTA.

TABLE V. Effect of Molarity and pH of Citric Acid on Absorption of TB from *in Situ* Intestinal Loops

Medium	Molarity of citric acid	pH of solution injected	Circulatory uptake % of dose/loop	Tissue binding % of dose/loop
Control		5.8	1.9±1.2	9.3±3.0
Citric acid	0.02M	2.4	2.5±1.1	12.5±1.1
Citric acid	0.05M	2.2	17.9±6.2*	11.1±3.7
Citric acid	0.1 M	2.0	38.7±5.2*	10.6±3.4
Citric acid	0.2 M	1.8	34.8±4.7*	6.3±3.4
Citrate buffer[a)]	0.1 M	2.0	18.0±4.0*	9.1±3.1
Citric acid and NaCl	0.1 M	2.1	23.3±4.9*	11.5±3.5
Citric acid and $CaCl_2$	0.1 M	1.9	19.9±6.6*	9.9±2.5
Monosodium citrate	0.1 M	3.7	1.2±0.2	6.9±1.1
Trisodium citrate	0.1 M	8.6	0.9±0.4	7.4±2.7
EDTA[b)]	0.1 M	4.4	10.1±1.8*	9.7±1.3
EDTA[b)] and $CaCl_2$	0.1 M	4.1	10.0±3.0*	4.7±0.8*

All studies are for 30 min periods. A dose of 0.2 mg of ^{14}C-TB was injected in a total volume of 0.5 ml. Results were expressed as the mean percentage of the dose in 4 to 8 animals with S.D.
a) Citrate buffer consists of 0.1 M disodium citrate and 0.1 N HCl.
b) Disodium salt.
* Significantly different ($p<0.01$) from the comparable value for control.

In another series of experiments, the pH in the loops was measured during the course of the experiments. Within 30 min after injection of any of low pH (2.0—2.9) acid solutions, the pH within the lumen of all experimental loops was back to the pH of the adjacent control loops (6.0 to 6.6). Therefore, the time course of the circulatory uptake was investigated to determine what relationship there might be between absorption and pH change within the gut. The results are shown in Fig. 2. The rate of absorption of the quaternary was greatest during the first 15 min in the case of citric acid. In the case of sodium citrate, additional TB was absorbed up to about 90 min. Throughout the 2-hr period the amount of TB uptaken in the case of citric acid was significantly greater than that in the case of sodium citrate, despite the fact that with citric acid there was no increase in degree of the circulatory uptake beyond 30 min.

To determine whether the effect of citric acid on absorption of TB were the result of irreversible changes in the gut wall, a series of experiments was run in which citric acid was placed in the loop prior to the addition of TB. Thirty min after 0.5 ml of water, 0.1 M citric

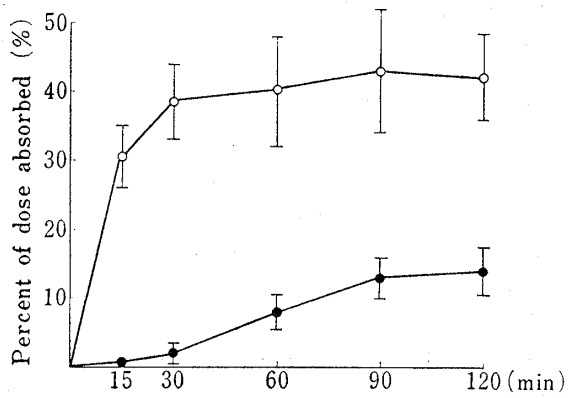

Fig. 2 Time Course of Absorption of ^{14}C–TB from *in Situ* Intestinal Loops

—○—, 0.1 M citric acid pH 2.0 and —●—, 0.1 M sodium citrate pH 3.7. Each point represents the mean of 3 to 4 rats with S.D.

Table VI. Absorption of TB from *in Situ* Intestinal Loops Following Treatment with Citric Acid or EDTA

	Circulatory uptake % of dose/loop	Tissue binding % of dose/loop
Pretreatment with water	10.0±2.0	32.5±7.1
Pretreatment with citric acid	9.7±2.6	29.6±4.2
Pretreatment with disodium EDTA*	29.2±5.0	4.5±0.8

A volume of 0.5 ml of water, 0.1 M citric acid or 0.1 M disodium EDTA was injected into the loops. After 30 min, 0.5 ml of ^{14}C–TB (0.2 mg) was quantitatively added to the loop. At the end of 2 hr, the circulatory uptake and tissue binding were determined. Results were expressed as the mean percentage of the dose in 4 animals with S.D.

* The intestinal loops were distended with fluid at the time of excision.

acid or 0.1 M disodium EDTA was injected into the loop, 0.5 ml of ^{14}C–TB was quantitatively added to each loop. There was no significant difference at 2 hr between the circulatory uptake from the loops treated with water and that from those treated with citric acid, whereas the circulatory uptake of TB from the loops treated with EDTA markedly increased, as shown in Table VI.

Binding of ^{14}C-TB to Mucin, Mucous Material and Bile (Table VII)

It was shown by both gel filtration and equilibrium dialysis that TB was bound to the mucin, mucous material and bile. The addition of citric acid to the mucin, mucous material and bile mediums markedly reduced the apparent binding of TB to the mucin, mucous material and bile.

Table VII. Degree Binding of ^{14}C–TB to Mucin, Mucous Material and Bile

Medium	pH of medium	Gel filtration %	Equilibrium dialysis %
Mucin[a)]	6.1	22.7±1.8	29.5±3.1
Mucin and citric acid	1.3	3.0±0.7	1.4±0.7
Mucous material[b)]	6.8	25.4±1.5	33.3±3.4
Mucous material and citric acid	1.8	3.5±0.8	1.0±0.6
Bile[c)]	8.7	18.3±2.4	23.5±0.6
Bile and citric acid	2.3	2.0±0.3	2.2±0.3

a) One % suspension of gastric mucin in 0.05 M NaCl.
b) One g of intestinal mucus was homogenized with 9 volumes of 0.05 M NaCl.
c) Fifty % solution of bile in 0.05 M NaCl.
The final concentrations of ^{14}C–TB and citric acid are 1 mM and 0.1 M respectively. Data represent the mean of 3 experiments with S.D.

Discussion

Some of the materials added to the intestinal loops along with TB significantly decreased the circulatory uptake of TB as compared with controls. That mucin and mucous material had this effect was not surprising, since Levine, *et al.*[5a,b)] had already presented evidence that this decrease could occur through formation of stable quaternary salt complexes with mucin

and mucous material. The formation, *in vitro*, of stable complexes of acidic polysaccharides with quaternary ammonium compounds has also been demonstrated by Scott.[9] Levine[10] reported that bile and bile salts had no effect on the *in vivo* absorption of benzomethamine, a quaternary ammonium drug. Pfeffer and Schor[11] reported that bile did not affect the circulatory uptake but decreased the tissue binding. In the present study, however, the addition of rat bile significantly decreased both the circulatory uptake and tissue binding of TB although major bile salts, glycocholate and taurocholate, had no effect. It was also shown *in vitro* that bile, like mucin and mucous material, formed stable complexes with TB. Therefore, the decrease in tissue binding seen with bile, which, in turn, probably causes the decrease in the circulatory uptake, may be explained by taking account of that TB cation could combine with anionic bile constituents with a greater affinity than it has for binding sites of the intestinal wall. This was supported by the fact that washing out the intestinal loop significantly increased tissue binding of TB.

Addition of deoxycholate or cholate increased the circulatory uptake of TB and decreased the tissue binding. This increase in the circulatory uptake and the decrease in the tissue binding seem to be due to disruption of the intestinal epithelium, since it is known that deoxycholate disrupts the cell membrane. Gibaldi and Grundhofer[12] investigated effect of epithelium disruption by sodium deoxycholate on the *in situ* absorption of various compounds from the small intestine of rats, and demonstrated that the epithelial border of the small intestine was a critical anatomical barrier to the *in situ* intestinal absorption of both charged and uncharged poorly lipid-soluble compounds including a quaternary of N,N-bis(phenylcarbamoyl)dimethyl ammonium chloride.

When the intestinal loop was washed with water, it was found that the circulatory uptake of TB was greater in non-fasted than in fasted rats while the tissue binding was greater in fasted rats. This led us to study the influence of gastric juice on intestinal absorption of TB. Addition of gastric juice into the intestinal lumen significantly increased not only binding of TB but also the circulatory uptake. This fact suggested that HCl in the gastric juice might have facilitating effect on absorption of TB. In fact, administration of a solution of the quaternary in 0.1 N HCl significantly increased the amount of TB absorbed in 30 min. Furthermore, all of the organic acids studied had facilitating effect on absorption of TB. The facilitating effect of organic acids was the highest in citric acid, middle in malic acid and the lowest in acetic acid. This fact indicates that the more the number of the carboxylic group is, the greater the facilitating effect is. In other words, the lower pH the acids have, the greater the facilitating effect is. However, the facilitating effect of HCl was lower than those of citric and malic acid although the pH of the former was lower than those of the latters. When the pH of the citric acid solution was raised to 3.7, or when the concentration was lowered to 0.02 M, which was 20 times greater than the concentration of TB, there was no effect on the control level of absorption of TB. This fact indicates that citric acid must be used in relatively high concentrations and at relatively low pH. This was in good agreement with the results of Levine[10] that the saturated water–soluble fatty acids, acetic, propionic and butyric acid, increased the absorption of benzomethamine and these acids had this facilitatory effect only when added to the medium in relatively high concentrations (0.2 M) and at relatively low pH. Reuning, *et al.*[13] observed that the intestinal absorption of the quaternary ammonium compound, N-acetyl-procaine amide ethobromide, from isotonic buffered solution of pH 2 occurred more greatly than from those of pH 3.75, 7 and 9, and suggested that the acidic medium has a direct effect on the permeability of the intestinal membrane to the quaternary.

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13) R.H. Reuning, B.L. Ross, B.J. Shoemaker, and S.S. Watson, *Pharmacologist*, **13**, 195 (1971).

An acid medium, while not appreciably affecting the ionization of the quaternary, might influence the ionization of anionic groups of intestinal mucous membranes, mucin and bile constituents, making them less negative and thereby leaving more quaternary free for absorption, since the results of the present study showed *in vitro* that there was less bonding of TB cation to mucous material, mucin and bile in a low pH solution than in a high pH solution. However, the increased absorption of TB in the presence of citric acid cannot be explained only on the basis of a low pH persisting within the lumen for a longer period of time in the case of citric acid than in the case of the buffer. The fact that the facilitatory effect of citric acid on absorption of TB was decreased by the addition of either NaCl or $CaCl_2$, suggests that citric acid might alter the permeability of the intestinal mucous membrane through the removal of inorganic cations. On the other hand, EDTA,[14] which has been shown to increase the intestinal absorption of phenol red by depleting calcium and magnesium of the mucosa, also increased the circulatory uptake of TB. Moreover, prior treatment of intestinal loops with citric acid did not produce any significant difference in the degree of absorption of TB as compared with appropriate control, whereas pretreatment with EDTA markedly increased the absorption of TB. These facts indicate that the mechanism by which citric acid enhances the intestinal absorption is different from that by which EDTA does. The present study also showed that citric acid facilitated quaternary absorption without producing irreversible changes in the gut wall.

From a consideration of the facts described above, it seems reasonable to consider that citric acid facilitates quaternary absorption by decreasing binding of quaternary with anionic groups of bile constituents, mucin and intestinal mucous membrane and by altering the permeability of intestinal mucous membrane through the removal of inorganic cations.

In any event, from these facts described above, it is clear that intestinal absorption of TB is facilitated by the addition of acidic compounds such as citric acid. This has led us to study the effect of citric acid on gastrointestinal absorption of TB when administered orally, and the results of that study are to be reported separately.

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