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Permeation and Hydrolysis of Trichloroethyl Phosphate in the Rat Intestine

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The hydrolysis and permeation of trichloroethyl phosphate were examined *in vitro* by using everted sacs of rat intestine. The concentration of trichloroethanol was higher in serosal solutions than in mucosal solutions at a high concentration of the phosphate ester whereas the opposite was the case at a low concentration of the ester at pH 7.4. The concentration of trichloroethanol in serosal solutions differed in different regions of the small intestine. The phosphate ester permeated into serosal solutions as such at pH 3.5. Therefore the ester is expected to permeate into serosal solutions as such and then to be hydrolyzed at a high concentration of the phosphate ester at pH 7.4. Percentage losses of the phosphate ester from the intestinal loop *in situ* differed slightly in different regions of intestinal tracts. The ester is expected to be hydrolyzed in the intestinal lumen before absorption under normal conditions, or be absorbed as such when present at a high concentration.

Keywords——trichloroethyl phosphate; phosphate ester; trichloroethanol; phosphatase; permeation; hydrolysis; everted sac; rat intestine; intestinal loop; absorption

Drugs are often administered in the form of prodrugs and the majority of the prodrugs are ester-type derivatives. The prodrugs of phosphate ester type have a much higher water solubility than their parent drugs because of the presence of an ionized group. Further, most phosphate esters are sufficiently stable to hydrolytic cleavage to allow the formulation of solutions with practical shelf-lives,[2] and some of the phosphate esters can be used in formulations for injection.[3] Phosphate esters of clindamycin[3] and corticosteroids[2] have been employed clinically. A further advantage of the phosphate esters is the absence of tissue irritation,[3,4] which is often caused by the parent drugs.

2,2,2-Trichloroethyl phosphate is the phosphate ester of 2,2,2-trichloroethanol, a sedative-hypnotic. The phosphate is a solid and is easier to handle than trichloroethanol, which is a liquid.

The hydrolytic behavior of trichloroethyl phosphate in the gastrointestinal tract of rats has been reported previously.[5] In this study, permeation as well as hydrolysis of the phosphate ester was examined.

Experimental

Materials——Monosodium trichloroethyl phosphate (a product of Glaxo Laboratories, Middlesex, England) was generously supplied through Torii Yakuhin Co., Tokyo, and *p*-nitrophenyl phosphate dipotassium salt hexahydrate, alkaline phosphatase from calf intestine, and *β*-glucuronidase from *E. coli* were purchased from Sigma Chemical Co., St. Louis. All other chemicals were of analytical grade. These materials were employed as received.

Animals——Male Wistar rats (200—300 g) were used after fasting for 16—20 hr.

Everted Sac Experiments——Under pentobarbital anesthesia, the small intestines, from the pyloric region to the ileo-cecal junction, were quickly removed from the rats. A ten-cm segment from the pyloric region, a ten-cm segment twenty cm away from the jejunal segment were used as doudenal, jejunal, and ileal segments, respectively. Each segment was cut off and washed with cold 0.9% NaCl solution. All segments were everted by means of a wire inserted through the lumen. Each everted sac was ligated at one end, then 1 ml of 0.27 M Tris-HCl buffer solution, pH 7.4, was introduced, and the other end was ligated. Four everted sacs of each segment (four rats were used at a time) were placed in 150 ml of isotonic Tris-HCl buffer solution, pH 7.4, containing 1 mg/ml monosodium trichloroethyl phosphate (equivalent to 596 μg/ml trichloroethanol)

at 37° and the mucosal solution was bubbled through with O_2–CO_2 (95 : 5 v/v) for one hr. At the end of the incubation period, the sacs were opened at one end and the serosal solutions were collected from each segment. Each sac was rinsed twice with 1 ml of Tris-HCl buffer solution, pH 7.4, and the washing solution was combined with the serosal solution. Each mucosal solution was also sampled. Two milliliters of a 30% trichloroacetic acid solution was added to the collected serosal solutions (12 ml), and one milliliter of the acid solution was added to each mucosal solution (1 ml). Proteins were removed by centrifugation. Each supernatant was diluted appropriately. Experiments with 100 μg/ml trichloroethyl phosphate and 300 μg/ml trichloroethanol were carried out in the same way as in the experiment with 1 mg/ml trichloroethyl phosphate. The Michaelis barbital–acetate buffer solution[6] was used for the experiments at pH 3.5. Each diluted sample was divided into three parts; the first part was hydrolyzed with alkaline phosphatase, the second part was hydrolyzed with 250 Sigma units[7] of β-glucuronidase at 37° overnight, and the third part was not subjected to hydrolysis. Trichloroethanol in each part was assayed. In the enzymatic hydrolysis, the pH of each solution was adjusted to neutral with 2 N NaOH.

Hydrolysis Experiments——The everted sac was incubated in the drug-free solution at pH 7.4 in the same way as in the everted sac experiments described above. At the end of the incubation period, 4 ml of the mucosal solution was sampled and diluted to 20 ml with Tris-HCl buffer solution, pH 7.4. The serosal solution (4 ml) from the four sacs was also diluted to 20 ml for each segment. Five milliliters of mucosal or serosal solution for the duodenal and jejunal segments was pipeted into a 10 ml volumetric flask, 1 ml of 20 mg/ml *p*-nitrophenyl phosphate was added to it, and the total volume was adjusted to 10 ml with Tris-HCl buffer solution, pH 7.4. In the case of the ileal segment, 1 ml of 20 mg/ml *p*-nitrophenyl phosphate was diluted to only 10 ml with either the mucosal or serosal solution. Each solution was incubated at 37° and sampled at appropriate times. *p*-Nitrophenol produced in each solution was assayed spectrophotometrically.

Absorption Experiments *in Situ*——Each rat was anesthetized with pentobarbital, and the intestine was exteriorized through a central mid-line incision. Loops of ten-cm length were prepared from the duodenum, jejunum, and ileum (defined in the same way as the everted sac segments). Each loop was ligated at one end, and 1 ml of 1 mg/ml trichloroethyl phosphate solution was introduced with a syringe, then the other end was ligated. The loops were then returned to the abdomen. After 30 min, the solution in each loop was recovered after removing the loops from the rat, and each loop was rinsed twice with 1 ml of Tris-HCl buffer solution, pH 7.4. The washing solution was combined with the solution from the loop. The combined solution was subjected to hydrolysis with 6 mg of alkaline phosphatase by incubation at 37° and pH 7.4 for 12 hr. Total trichloroethanol in each solution was assayed, and the percentage of the drug remaining unabsorbed was calculated.

Analytical Method——Trichloroethanol was determined as reported previously.[5] To measure total trichloroethanol, trichloroethyl phosphate was hydrolyzed with alkaline phosphatase to form trichloroethanol, which was assayed by gas chromatography.

p-Nitrophenyl phosphate was hydrolyzed to *p*-nitrophenol by incubation with alkaline phosphatase. After hydrolysis, 1 ml of 12 N HCl was added to the solution prior to extraction of *p*-nitrophenol with methylene chloride, and then the phenol was reextracted from the organic layer to the aqueous layer with 2 N NaOH. The absorbance of the aqueous layer was measured at 420 nm.

The concentration of the phosphate ester was calculated in terms of the parent compound before and after enzyme hydrolysis.

Statistical Analysis——Analysis of variance (the two-way design) and Student's *t*-test[8] were employed for the data obtained in the permeation experiments and the absorption experiments *in situ*, respectively.

Results and Discussion

Permeation of Trichloroethyl Phosphate *in Vitro*

It has been reported that the rate of hydrolysis of trichloroethyl phosphate in rat intestine is dependent on the region of the tract.[5] As regards the permeative behavior in the small intestine, the serosal concentrations of free trichloethanol were dependent on the region of the intestine ($p/2>0.01$ in the F test of significance), and the concentrations of free trichloroethanol in the serosal solutions were higher than those in the mucosal solutions ($p/2>0.01$ in the F test of significance, Fig. 1). If trichloroethyl phosphate is hydrolyzed in the mucosal solution and then permeates into the serosal solution as trichloroethanol, the concentration of free trichloroethanol in the mucosal solution must be equal to or higher than that in the serosal solution, provided that only a passive transport mechanism operates.

In the transport experiment with trichloroethanol, there was little difference between the concentration of trichloroethanol in the mucosal solution and that in the serosal solution (Fig. 2). This observation indicates that trichloroethanol is transported by a passive diffusion mechanism through the membrane to the serosal solution.

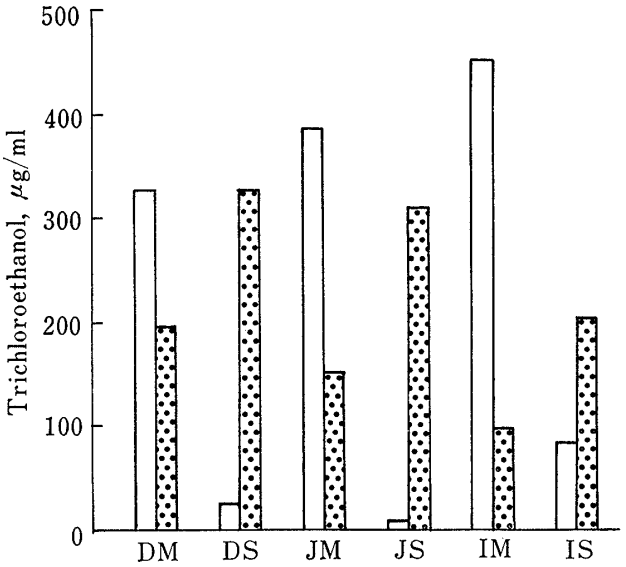

Fig. 1. Mucosal and Serosal Concentrations of Trichloroethyl Phosphate and Trichloroethanol at pH 7.4 after Incubation of 1 mg/ml Trichloroethyl Phosphate for 1 hr

Results (mean values of duplicate experiments) are expressed in terms of the concentration of trichloroethanol ▭, phosphate ester; ▨, free trichloroethanol; D, duodenal segment; J, jejunal segment; I, ileal segment; M, mucosal solution; S, serosal solution.

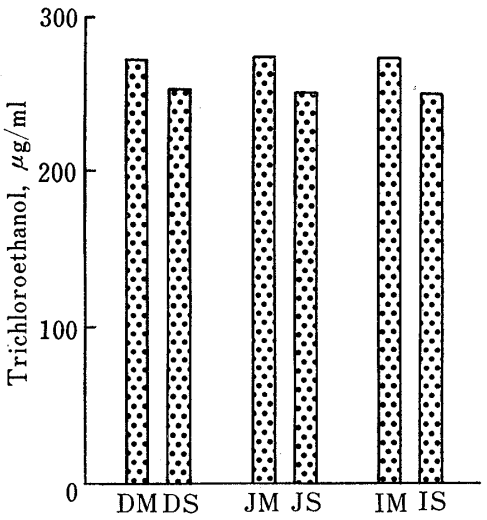

Fig. 2. Mucosal and Serosal Concentrations of Trichloroethanol at pH 7.4 after Incubation of 300 μg/ml Trichloroethanol for 1 hr

Symbols are the same as in Fig. 1.

In order to examine the permeation of the ester, a permeation experiment with trichloroethyl phosphate was carried out at pH 3.5 instead of pH 7.4, where trichloroethyl phosphate in the mucosal solution is expected to be easily hydrolyzed by alkaline phosphatase released from the epithelial cells.[5,9] In the experiment, the phosphate ester was clearly found as unhydrolyzed ester in the serosal solution (Fig. 3).

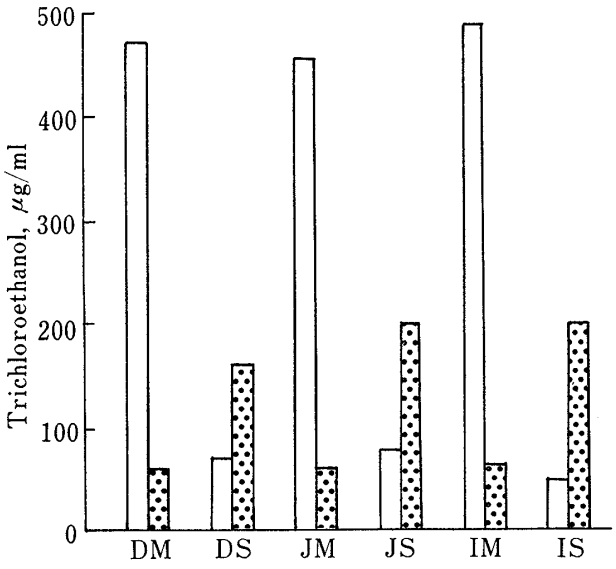

Fig. 3. Mucosal and Serosal Concentrations of Trichloroethyl Phosphate and Trichloroethanol at pH 3.5 after Incubation of 1 mg/ml Trichloroethyl Phosphate for 1 hr

The data are mean values of duplicate experiments. Symbols are the same as in Fig. 1.

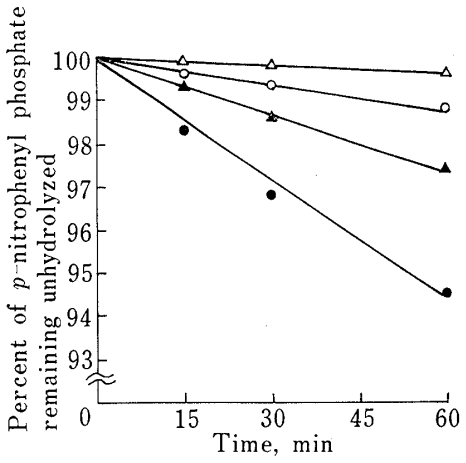

Fig. 4. Rates of Hydrolysis of 2 mg/ml *p*-Nitrophenyl Phosphate Dipotassium Salt Hexahydrate in Mucosal and Serosal Solutions in the Duodenal and Jejunal Regions at 37° and pH 7.4

○, duodenal mucosal solution;
●, duodenal serosal solution;
△, jejunal mucosal solution;
▲, jejunal serosal solution.

This suggests that some of the phosphate ester in the buffer at pH 3.5 passes through the epithelial cell without being hydrolyzed. Therefore it is also likely that some of the phosphate ester in the buffer at pH 7.4 is taken up in the epithelial cell and transferred into the serosal solutions without being hydrolyzed.

Hydrolytic Behavior

The phosphatase activity was examined in experiments with *p*-nitrophenyl phosphate. Phosphatase activity was also observed in the serosal solutions (Figs. 4 and 5), so a part of the free trichloroethanol in the serosal solutions must be produced by hydrolysis of permeated trichloroethyl phosphate by the phosphatase activity in the serosal solutions.

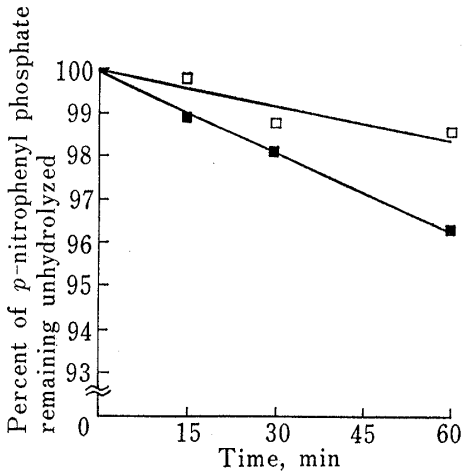

Fig. 5. Rates of Hydrolysis of 2 mg/ml *p*-Nitrophenyl Phosphate Dipotassium Salt Hexahydrate in Mucosal and Serosal Solutions in the Ileal Regions at 37° and pH 7.4

□, mucosal solution;
■, serosal solution.

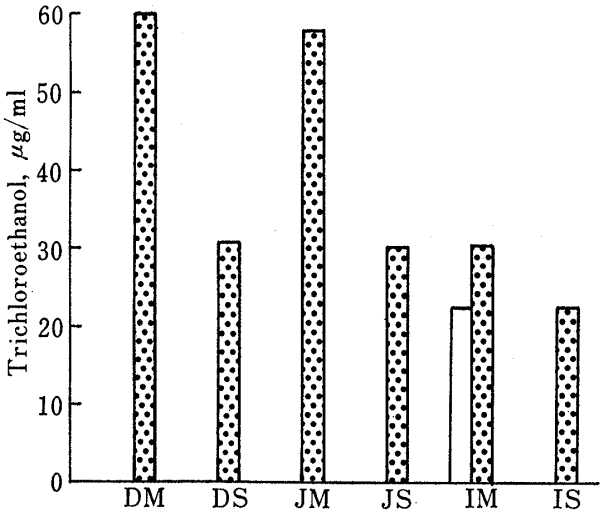

Fig. 6. Mucosal and Serosal Concentrations of Trichloroethyl Phosphate and Trichloroethanol at pH 7.4 after Incubation of 100 μg/ml Trichloroethyl Phosphate

The data are means of duplicate experiments. Symbols are the same as in Fig. 1. The phosphate ester was not detected in mucosal solutions of the duodenal and jejunal segments or in the serosal solutions of all segments.

In addition, the effect of the concentration of trichloroethyl phosphate on its hydrolytic behavior was examined at pH 7.4. When the concentration of the phosphate ester was reduced to one-tenth of that used in Fig. 1, no phosphate ester was found in the mucosal solutions in the duodenal and jejunal segments. Free trichloroethanol concentrations were higher in the mucosal solutions than in the serosal solutions ($p/2<0.01$ in the F test, Fig. 6). It is suggested that the phosphate ester did not permeate throught the membrane because its concentration was low, and it was hydrolyzed rapidly to trichloroethanol in the mucosal solution.

In the permeation experiments with trichloroethyl phosphate, there was only a small difference between the concentrations of trichloroethanol before and after hydrolysis with β-glucuronidase in the mucosal and serosal solutions. This indicates that there is little glucuronide in the solution.

Absorption of Trichloroethyl Phosphate *in Situ*

The percentages of the drug remaining unabsorbed from loops in the duodenal, jejunal, and ileal regions in 30 min were 1.9±1.9, 13±8, and 12±5% (mean ±S.E.M., $n=5$), respectively. There was a slight difference between the duodenal and the other two regions ($0.05>$

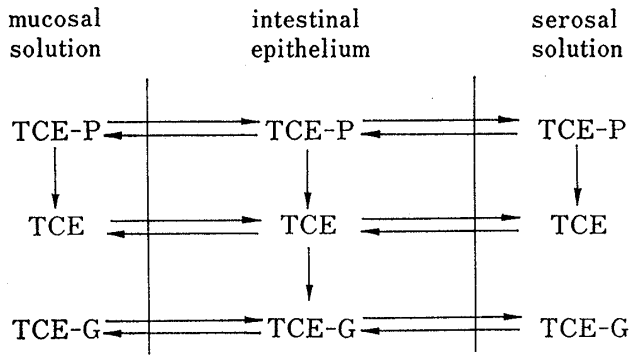

Fig. 7. Scheme Describing the Permeation and Hydrolysis of Trichloroethyl Phosphate in Everted Sac Experiments

TCE-P, trichloroethyl phosphate; TCE, trichloroethanol; TCE-G, trichloroethyl glucuronide.

$p>0.1$), but there was no significant difference between the jejunal and ileal regions. Greater absorption in the duodenal region than in the jejunal and ileal regions may be attributed to higher phosphatase activity[10] and resulting greater availability of trichloroethanol in the duodenal region than in the jejunal and ileal regions.

The above discussion may be summarized as shown in Fig. 7. In the intestinal lumen a part of the phosphate ester exists as the ester and the rest is hydrolyzed to trichloroethanol. Both species are taken up by the epithelial cells. Some of the taken-up ester is hydrolyzed to trichloroethanol in the cells, while the rest is absorbed without hydrolysis. A small portion of trichloroethanol is expected to be conjugated with glucuronic acid in the cells and absorbed or released into the lumen.

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References and Notes

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