Effect of Oral Pretreatment with Antibiotics on the Hydrolysis of Salicylic Acid–Tyrosine and Salicylic Acid–Methionine Prodrugs in Rabbit Intestinal Microorganisms

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We examined the hydrolysis mechanism of salicylic acid–tyrosine (salicyl-tyrosine) and salicylic acid–methionine conjugate (salicyl-methionine) in rabbits by exploring their behavior following intraduodenal and intracecal administration (72 and 36 μmol/kg, respectively: salicylic acid equivalent). A large amount of salicyl-methionine was absorbed following intraduodenal administration of salicyl-methionine, without being metabolized to salicylic acid in the small intestinal mucosa. On the contrary, salicylic acid was detected in the blood following intraduodenal administration of salicyl-tyrosine, suggesting that salicyl-tyrosine was metabolized in the small intestinal mucosa. After oral pretreatment of rabbits with kanamycin sulfate (6 × 400 mg) or tinidazole (6 × 160 mg), the hydrolysis of salicyl-tyrosine and salicyl-methionine following intracecal administration was inhibited significantly, indicating that the intestinal microorganisms were responsible for the biotransformation of these prodrugs. Furthermore, in rabbits orally pretreated with both kanamycin sulfate and tinidazole, a significant inhibition of salicylic acid formation from salicyl-tyrosine and salicyl-methionine following intracecal administration was observed.

Keywords salicylic acid; salicylic acid–tyrosine conjugate; salicylic acid–methionine conjugate; antibiotic; hydrolysis; prodrug; rabbit; intestinal microorganism

In our previous investigation,[1] we examined the behavior of salicylic acid–tyrosine (salicyl-tyrosine) and salicylic acid–methionine conjugate (salicyl-methionine) in rabbits following oral, intravenous, intracecal and rectal administration, with the aim of developing a potent prodrug of salicylic acid to prolong its blood concentration, and suggested that these prodrugs were metabolized to salicylic acid by intestinal microorganisms. However, the precise mechanism by which salicyl-tyrosine and salicyl-methionine were metabolized to salicylic acid remains to be elucidated. In our series of investigations,[1–5] it is unknown what class of bacteria was responsible for the hydrolysis of salicylic acid–amino acid conjugate.

In the present study, we investigated the effect of oral pretreatment with antibiotics on the salicylic acid formation from salicyl-tyrosine and salicyl-methionine following intracecal administration in rabbits. Moreover, we examined the behavior of salicyl-tyrosine and salicyl-methionine following intraduodenal administration to determine the involvement of metabolism by small intestinal mucosa in the presystemic de-conjugation of these prodrugs.

Experimental

Chemicals Acetylsalicylic acid, L-tyrosine, L-methionine, acetonitrile, acetic acid, methanol and *o*-anisic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Kanamycin sulfate and tinidazole were obtained from Meiji Seika Kaisha, Ltd. (Tokyo, Japan) and Sigma Chemical Co. (St. Louis, MO, U.S.A.), respectively. All other chemicals were of reagent grade.

Syntheses of Salicyl-tyrosine and Salicyl-methionine Salicyl-tyrosine and salicyl-methionine were synthesized by coupling of tyrosine or methionine methyl ester, and acetylsalicylic acid, respectively, using a carbodiimide as described previously.[2] Finally, 17.5% of salicyl-tyrosine or 38.5% of salicyl-methionine was obtained as white crystals: mp 172 and 95 °C; $[\alpha]_D^{20}$ −50.0 and −19.3° (c=1% (w/v) in acetone), respectively. Chemical structures of the products were ascertained by nuclear magnetic resonance, mass spectrum and elemental analyses. Analysis of salicyl-tyrosine: Calcd for $C_{16}H_{15}NO_5$: C, 63.78; H, 5.02; N, 4.65. Found: C, 63.43; H, 5.08; N, 4.63. Electron impact-mass spectra (EI-MS) m/z: 301. Analysis of salicyl-methionine: Calcd for $C_{12}H_{15}NO_4S$: C, 53.52; H, 5.61; N, 5.20. Found: C, 53.31; H, 5.48; N, 5.21. EI-MS m/z: 269. Nuclear magnetic resonance and mass spectra were taken on a JEOL FX90Q Fourier transform spectrometer (JEOL, Ltd., Tokyo, Japan) and a JEOL JMS-DX303 mass spectrometer (JEOL, Ltd.), respectively. Elemental analyses were performed by the Center for Organic Elemental Micro-analysis, Nagasaki University.

A stability experiment was carried out in 0.1 M phosphate buffer solutions of pH 2.5, 6.0 and 7.5 at 37 °C at a drug concentration of 100 μg/ml as salicylic acid. The pH of buffer solution was adjusted to the desired value by using a buffer system consisting of 0.1 M H_3PO_4 and 0.1 M $Na_2HPO_4 \cdot 12H_2O$. Salicyl-tyrosine and salicyl-methionine were completely stable (100% remained) at each pH after a 24-h incubation.

Animals Male albino rabbits weighing 1.7—3 kg of 4—5 months of age were used throughout the study. The animals were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co., Ltd., Tokyo, Japan).

***In Vivo* Experiments** The rabbits were starved for about 24 h prior to use for experiments but had free access to water. Salicyl-tyrosine and salicyl-methionine were dissolved in aqueous NaOH solution (equivalent to salicyl-tyrosine or salicyl-methionine, pH 12). Following intraduodenal and intracecal administration of drug, blood was collected with a heparinized syringe at appropriate time intervals from an ear vein and centrifuged at 8000 × *g* for 5 min. Blood concentration of drug was calculated as salicylic acid from the calibration curve.

Intraduodenal Administration of Drug: Animals were anesthetized with sodium pentobarbital (25 mg/kg), given intravenously, *via* an ear vein. After complete anesthesia, the lower abdomen was cut open. The intestine was ligated about 5 cm above the ileo-cecal valve. The drug solution (72 μmol/kg: salicylic acid equivalent) was then administered by direct injection into the duodenum about 7 cm below the pylorus. No leakage of drug solution at the injection site was observed. The abdomen was closed with operative stitching.

Intracecal Administration of Drug: Animals were anesthetized with sodium pentobarbital (25 mg/kg), given intravenously, *via* an ear vein. After complete anesthesia, a midline incision (2—3 cm) was made, and the drug solution (36 μmol/kg: salicylic acid equivalent) was administered by direct injection into the cecum by syringe. No leakage of drug solution at the injection site was observed. The abdomen was closed with operative stitching.

Oral pretreatment of rabbits with kanamycin sulfate or tinidazole before intracecal administration of prodrugs was carried out as follows. Rabbits were divided into four groups (each n=4): untreated (control), pretreated with kanamycin sulfate, tinidazole and both kanamycin sulfate and tinidazole. The administration time of kanamycin sulfate was based on the method established by Gingell *et al.*[6] The animals received 6 × 400 mg/animal of kanamycin sulfate which was dissolved in distilled water. Kanamycin sulfate was given orally twice daily for 2 d before salicyl-tyrosine or salicyl-methionine administration and then 4 h before and 4 h after salicyl-tyrosine and salicyl-methionine administration. The administration time of tinidazole was the same as that of kanamycin

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sulfate. The animals received 6 × 160 mg/animal of tinidazole which was suspended in 1% carboxymethyl cellulose sodium salt.

Analytical Method Salicyl-tyrosine, salicyl-methionine and salicylic acid in blood were analyzed by high performance liquid chromatography according to the method of Cham *et al.*[7] with slight modifications. Details of the analytical method were described in our previous paper.[2] The chromatographic mobile phase consisted of a mixture of acetic acid–methanol–water (4:35:65 and 4:45:55, v/v/v, for detection of salicyl-tyrosine and salicyl-methionine, respectively). In the detection of salicyl-tyrosine, the retention times of salicyl-tyrosine, salicylic acid and the internal standard *o*-anisic acid were 14.7, 12 and 6.8 min, respectively. For salicyl-methionine, the retention times of salicyl-methionine, salicylic acid and the internal standard *o*-anisic acid were 9.7, 7.4 and 4.3 min, respectively.

Results and Discussion

The main purpose of this research is to clarify the mechanism by which salicyl-tyrosine and salicyl-methionine are metabolized to salicylic acid following oral, intracecal and rectal administration. The possible participation of intestinal microorganisms in the presystemic de-conjugation of these prodrugs was suggested previously,[1] however, further investigation was required to elucidate this point in detail.

Intraduodenal Administration of Salicyl-tyrosine and Salicyl-methionine Salicyl-tyrosine and salicyl-methionine were administered intraduodenally in rabbits to determine the possibility of de-conjugation of these prodrugs in the small intestinal mucosa. In this experimental system, the metabolism by intestinal microorganisms in the cecum and colon was omitted because the small intestinal contents including drug solution cannot reach the area where microorganisms exist in large amounts. Therefore, this experimental system is useful since it enables us to evaluate small intestinal absorption and subsequent elimination processes of drug independent of the influence of intestinal microorganisms in the cecum and colon. Both unchanged salicyl-tyrosine and salicyl-methionine were detected in the blood following absorption by small intestine (Figs. 1a and 1b). A trace amount of salicylic acid (<0.03 μg/ml) was detected with salicyl-methionine. In the previous report,[1] the hydrolysis of salicyl-methionine and salicyl-tyrosine was not observed in the *in vitro* experiment of incubation with small intestinal (jejunum, upper ileum and lower ileum) contents. Thus, it was suggested that presystemic de-conjugation in the small intestinal mucosa was not involved in the hydrolysis of salicyl-methionine. On the contrary, a considerable amount of salicylic acid was observed in the blood following intraduodenal administration of salicyl-tyrosine, indicating that a part of the salicyl-tyrosine was metabolized to salicylic acid presystemically in the small intestinal mucosa. The metabolism ratio of salicyl-tyrosine in the small intestinal mucosa was calculated to be 0.7%, by dividing the area under the blood concentration–time curve (*AUC*)/dose of salicylic acid that appeared following intraduodenal administration of salicyl-tyrosine by that following intravenous administration of salicylic acid. In the case of oral administration of salicyl-tyrosine,[1] no lag time for appearance of salicylic acid in the blood was observed. This phenomenon might be the result of rapid de-conjugation of salicyl-tyrosine in the small intestinal mucosa.

In our series of investigations,[1-5] we examined the behavior of several amino acids or dipeptide conjugates

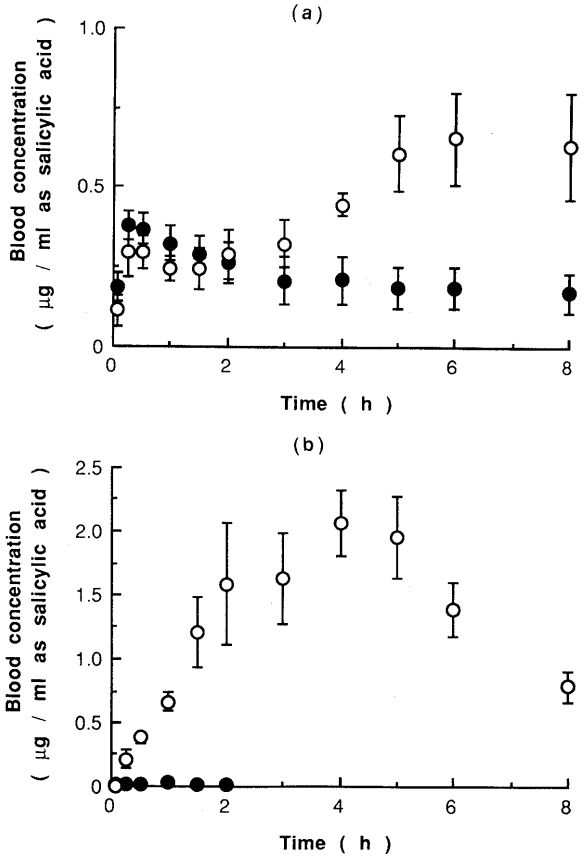

Fig. 1. Blood Concentration of Salicyl-tyrosine or Salicyl-methionine, and Salicylic Acid Following Intraduodenal Administration of Salicyl-tyrosine (a) and Salicyl-methionine (b) (72 μmol/kg: Salicylic Acid Equivalent) to Rabbits

(a) ○, salicyl-tyrosine; ●, salicylic acid. (b) ○, salicyl-methionine; ●, salicylic acid. Each point represents the mean ± S.E. of 4 experiments.

of salicylic acid in rabbits. Accordingly, we calculated the absorption and metabolism ratio of these prodrugs following oral administration in rabbits to evaluate their usefulness. The absorption ratios of salicyl-tyrosine, salicyl-methionine, salicylic acid–L-alanine (salicyl-L-alanine), salicylic acid–glycylglycine (salicyl-glycylglycine) and salicylic acid–glutamic acid conjugate (salicyl-glutamic acid) were calculated to be 62, 75, 66, 26 and 29%, respectively, by dividing the *AUC*/dose of prodrugs following oral administration by those following intravenous administration. On the other hand, the metabolism ratios of salicyl-tyrosine, salicyl-methionine, salicyl-L-alanine, salicyl-glycylglycine and salicyl-glutamic acid were calculated to be 38, 10, 26, 74 and 71%, respectively, by dividing the *AUC*/dose of salicylic acid that appeared following oral administration of prodrugs by those following intravenous administration of salicylic acid. Since the metabolism of these prodrugs in systemic circulation and small intestinal mucosa was extremely small,[1,2,4,5] the metabolism ratio of these prodrugs can be regarded as the hydrolysis ratio in the intestinal microorganisms. Furthermore, these results confirm that low absorbability of prodrug from the gastrointestinal tract led to the enhancement of hydrolysis to salicylic acid in the intestinal microorganisms.

Effect of Oral Pretreatment with Kanamycin Sulfate and Tinidazole In the previous study,[1] salicyl-tyrosine and salicyl-methionine were administered directly into cecum,

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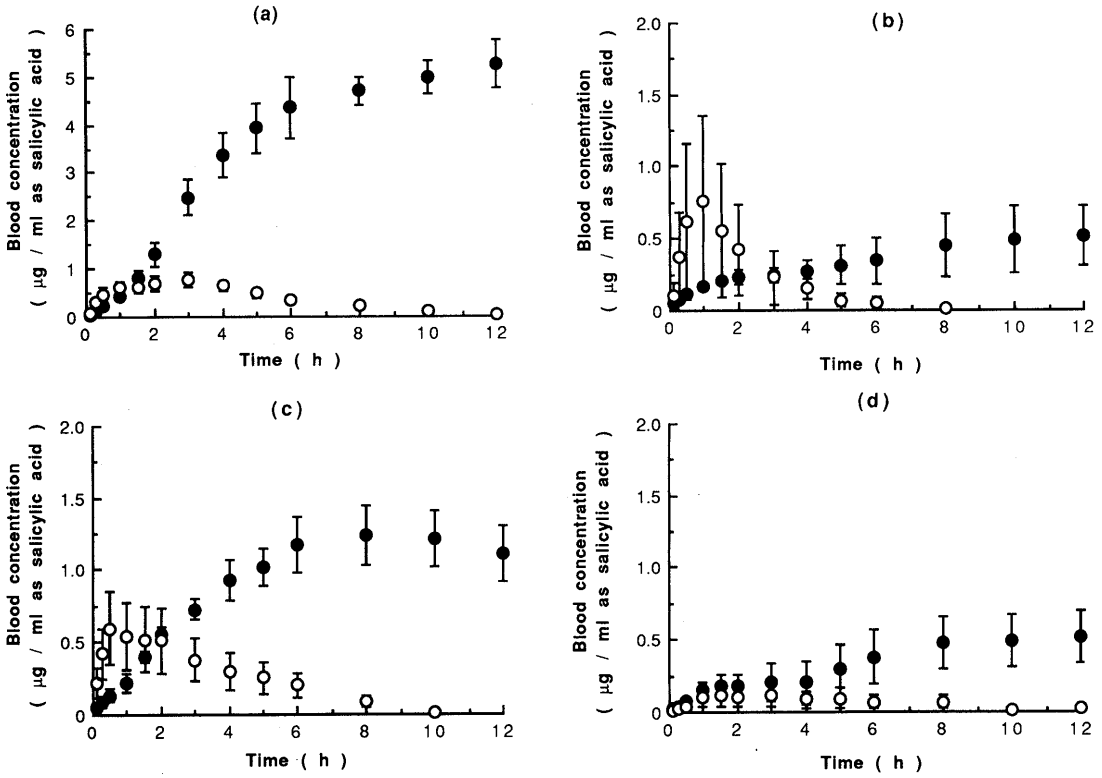

Fig. 2. Blood Concentration of Salicyl-tyrosine and Salicylic Acid Following Intracecal Administration of Salicyl-tyrosine (36 μmol/kg: Salicylic Acid Equivalent) to Rabbits Untreated (a) or after Oral Pretreatment of Rabbits with Kanamycin Sulfate (b), Tinidazole (c) and Both Kanamycin Sulfate and Tinidazole (d)

○, salicyl-tyrosine; ●, salicylic acid. Each point represents the mean ± S.E. of 4 experiments.

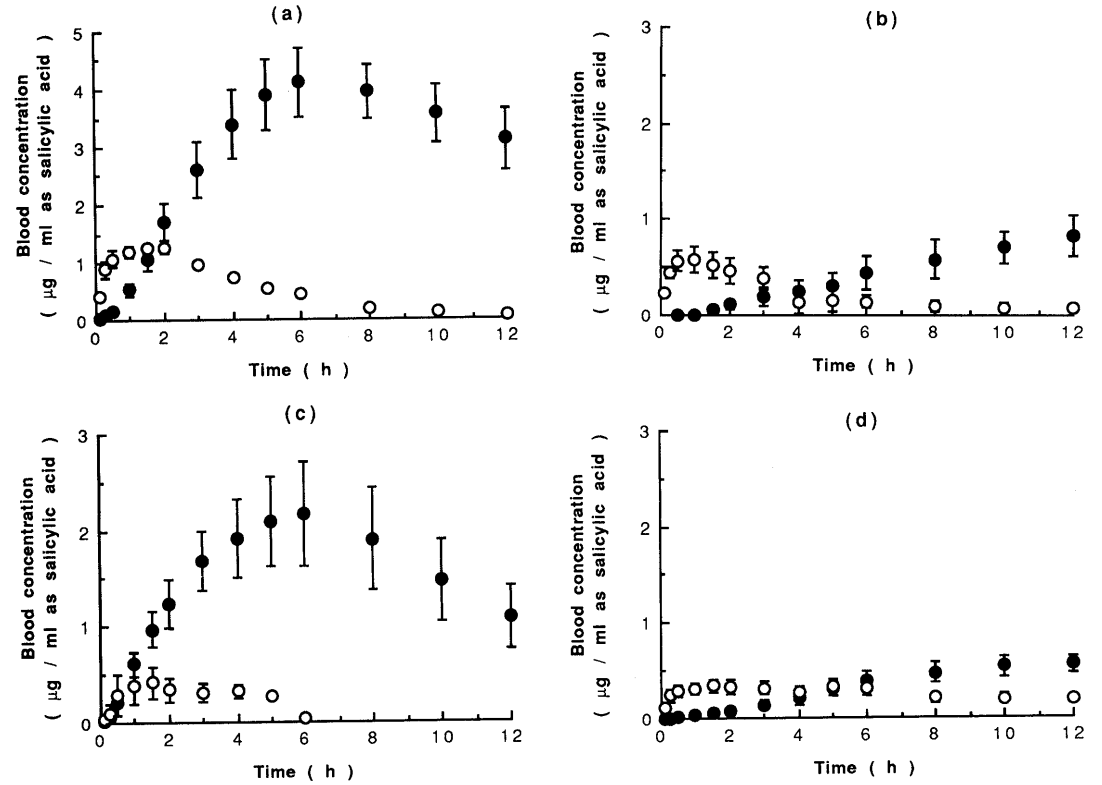

Fig. 3. Blood Concentration of Salicyl-methionine and Salicylic Acid Following Intracecal Administration of Salicyl-methionine (36 μmol/kg: Salicylic Acid Equivalent) to Rabbits Untreated (a) or after Oral Pretreatment of Rabbits with Kanamycin Sulfate (b), Tinidazole (c) and Both Kanamycin Sulfate and Tinidazole (d)

○, salicyl-methionine; ●, salicylic acid. Each point represents the mean ± S.E. of 4 experiments.

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in which a large amount of microorganisms exist. The extensive salicylic acid formation from salicyl-tyrosine and salicyl-methionine in the cecum suggests that they were hydrolyzed by the intestinal microorganisms.

In the present study, effect of oral pretreatment of rabbits with kanamycin sulfate and tinidazole on the blood concentration of salicyl-tyrosine or salicyl-methionine, and salicylic acid following intracecal administration was investigated to determine the hydrolysis mechanism of these prodrugs. Kanamycin sulfate and tinidazole are known to inhibit the growth of aerobic and anaerobic bacteria selectively, respectively.[8] Blood concentration profiles of salicyl-tyrosine or salicyl-methionine, and salicylic acid in rabbits orally pretreated with kanamycin sulfate (b), tinidazole (c) and both kanamycin sulfate and tinidazole (d) are shown in Figs. 2 and 3, respectively, together with those of control (a) for comparison.[1] The oral pretreatment of rabbits with kanamycin sulfate resulted in significant reduction of salicylic acid formation from salicyl-tyrosine and salicyl-methionine, judging from the decreased blood concentration of salicylic acid up to 12 h. This result indicates that aerobic bacteria was involved in the hydrolysis of these prodrugs. Although tinidazole pretreatment inhibited the salicylic acid formation from salicyl-tyrosine and salicyl-methionine to some extent, its inhibitory effect on this formation was smaller than that of kanamycin sulfate in both prodrugs, suggesting that anaerobic bacteria did not play an important role in the hydrolysis of these prodrugs. In addition, simultaneous oral pretreatment of rabbits with kanamycin sulfate and tinidazole reduced the salicylic acid formation from salicyl-tyrosine and salicyl-methionine to a degree similar to that obtained with kanamycin sulfate. These results suggest that aerobic bacteria was mainly responsible for the presystemic de-conjugation of salicyl-tyrosine and salicyl-methionine.

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