# Basic Studies on 5-(7-Hydroxy-3-*O*-phosphonocholyl)aminosalicylic Acid for the Evaluation of Microbial Overgrowth

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**A newly synthesized conjugate of 7-hydroxy-3-*O*-phosphonocholic acid (ursodeoxycholic acid monophosphate) with 5-aminosalicylic acid (5-ASA–UDCA monophosphate) was investigated to determine its suitability for the evaluation of enteric bacteria. This compound, 5-ASA–UDCA monophosphate, was efficiently deconjugated by cholylglycine hydrolase to release 5-ASA, whereas it was completely resistant to deconjugation by pancreatic and intestinal mucosal enzymes. In everted gut sac experiments, 5-ASA–UDCA monophosphate was not actively absorbed from any part of the small intestine. In animal experiments, urinary excretions of *N*-acetyl-5-ASA (Ac-5ASA) were measured for 24 h following the oral administration of 20 mg of 5-ASA–UDCA monophosphate. Control rats excreted 276.3 ± 89.0 μg (mean ± S.E.) of Ac-5ASA, whereas the rats with intestinal bacterial overgrowth excreted more (1224.1 ± 231.5 μg; $p < 0.01$). These basic studies indicate that this compound is likely to offer a simple method for the evaluation of microbial overgrowth without the use of radioisotopes or expensive, special apparatus.**

**Key words** 5-aminosalicylic acid; ursodeoxycholic acid; intestinal microorganism; bacterial overgrowth; ulcerative colitis

Intestinal microflora is considered to have a great effect on the health, life-span, and carcinogenesis of colonic cancer[1,2] and systemic immunity[3,4] in humans. For example, intestinal bacterial overgrowth in the blind loop syndrome causes the malabsorption of vitamin $B_{12}$, fat and other nutrients.[5,6] Adversely, disorder of the intestinal microflora due to the administration of antibiotics sometimes causes serious enteritis.[7,8] So far the development of a simple, reliable and noninvasive method to evaluate intestinal microflora in humans is a subject of great importance. Therefore, we newly synthesized a conjugate of 7-hydroxy-3-*O*-phosphonocholic acid (monophosphated ursodeoxycholic acid) with 5-aminosalicylic acid (5-ASA–UDCA monophosphate), with the intention that this compound could solve the above-described problem. The aim of this article is first to describe the method of synthesis of 5-ASA–UDCA monophosphate, second, to reveal its basic characteristics, and third, to investigate whether this compound is suitable for the evaluation of microbial overgrowth in rats.

## MATERIALS AND METHODS

Melting point was measured on a Yanagimoto micro-melting point apparatus and its uncorrected value used. Mass spectra (MS) were recorded with a Shimadzu GCMS-9020DF. Proton nuclear magnetic resonance ($^1$H-NMR) spectra were determined on a JEOL PMX 60Si spectrometer using phosphoric acid as an internal standard.

**Chemicals and Reagents** The following compounds were used: ursodeoxycholic acid (UDCA), 5-aminosalicylic acid (5-ASA), cobalt(II) choride hexahydrate ($CoCl_2 \cdot 6H_2O$), α-chymotrypsin (from bovine pancreas), pancreatin (from porcine pancreas), and Triton X-100 (polyoxyethylene octylphenyl ether) were purchased from Wako Pure Chemical Ind., Ltd., Japan. Hippuryl-L-arginine (Bz–Gly–L-Arg) and benzoyl-L-arginine-*p*-nitroanilide hydrochloride (Bz–L-Arg–PNA) were purchased from Peptide Institute, Inc., Osaka, Japan. *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (Bz–Tyr–PABA) was purchased from Eisai Co., Ltd., Japan. Hippuryl-L-phenylalanine (Bz–Gly–L-Phe), carboxypeptidase A (type I, from bovine pancreas), carboxypeptidase B (type I, from porcine pancreas), trypsin (type I, from bovine pancreas), 4-amino-2-hydroxybenzoic acid and glycocholic acid cholylglycine hydrolase (from clostridium perfringens) were purchased from Sigma Chemical Co., U.S.A. All other chemicals and solvents were of analytical grade.

**Statistical Analysis** The Student's *t*-test was applied to evaluate the significance of difference between the experimental groups.

**Synthesis of 5-ASA–UDCA Monophosphate** The 5-ASA–UDCA monophosphate was synthesized according to the modified method of Bergström and Norman.[9] Ursodeoxycholic acid (1 g, 2.55 mmol) was dissolved in 5 ml of dioxane containing 0.57 ml of tri-*n*-butylamine. The solution was cooled to 10 °C and 0.33 ml of ethylchlorocarbonate was added. After 15 min at this temperature, a solution of 0.46 mg of 5-ASA in 3.75 ml of 1 N sodium hydroxide was added and the mixture was rapidly mixed. After 15 min, 60 ml of distilled water was added to the reaction mixture. Following acidification with HCl, extraction was carried out with 60 ml of ethyl acetate 3 times. The extracts were washed with distilled water and dehydrated with anhydrous sodium sulfate. Then the organic solvent was gradually evaporated under reduced pressure to obtain crystals of the synthesized product. 1.21 g of this product was dissolved in a mixture of 20 ml of acetic acid and 6 ml of chloroform under ice cooling, and 1.92 ml of tetrachloropyrophosphate (TCPP) was dropped for a period of 5 min. The solution was stirred overnight at 15 to 20 °C. Subsequently, 3.76 ml of distilled water was dropped under stirring and ice cooling. The reaction mixture was stirred overnight. The mixture was evaporated under reduced pressure (< 40 °C). 20 ml of distilled water was added to the solution, and the whole was cooled to 0 °C under stirring. The precipitates were filtered and washed with distilled water. 1 g of this prod-

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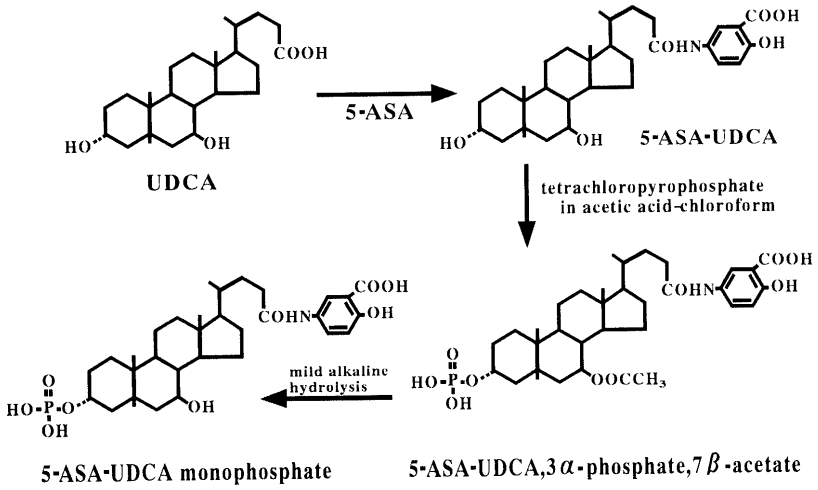


Fig. 1. Synthesis of 5-ASA–UDCA Monophosphate

uct was dissolved in 5 ml of 10% NaOH. Following acidification with HCl, the product was filtered and washed well with distilled water. A pale red powder was obtained. The precipitates were purified by column chromatography (AcOEt : MeOH = 2 : 1) (yield, 68.6%), The chemical structure of 5-ASA–UDCA monophosphate is shown in Fig. 1. 5-ASA–UDCA monophosphate; mp 174—177 °C (dec.). MS *m/z*: 606 $[M-H]^-$. $^1$H-NMR ($D_2O$): 0.60 (3H, s, 18 or 19-$CH_3$), 0.85 (3H, s, 18 or 19-$CH_3$), 0.93 (3H, d, 21-$CH_3$), 0.85—2.50 (26H, m, ring $CH_2$ proton; 20-H; 22-$CH_2$; 23-$CH_2$), 3.95 (1H, br s, 7-CH), 6.86 (1H, d, 6′-H), 7.46 (1H, dd, 5′-H), 7.89 (1H, d, 3′-H). *Anal.* Calcd for $C_{31}H_{46}NO_9P$: C, 61.27; H, 7.63; N, 2.30. Found: C, 61.54; H, 7.57; N, 2.28.

The purity of 5-ASA–UDCA monophosphate was investigated according to high performance thin-layer chromatography (HPTLC) (Kieselgel 60 $F_{254}$ plate, Merck) using a solvent system of ethanol–ammonia solution (3 : 1, v/v). The 5-ASA and its conjugate appeared on HPTLC as apparent spots under short-wave ultraviolet light (254 nm). UDCA and 5-ASA–UDCA monophosphate were further identified by spraying with 5% phosphomolybdate in ethanol. The *Rf*s of these compounds were as follows: 5-ASA, 0.77; UDCA, 0.73; and 5-ASA–UDCA monophosphate, 0.367. HPTLC revealed that the synthesized material was practically pure; the material showed less than 5% contamination by free UDCA and no contamination by 5-ASA. Figure 2 shows the thin-layer chromatography (TLC) of 5-ASA–UDCA monophosphate, UDCA, and 5-ASA.

***In Vitro* Studies** Incubation Experiment of 5-ASA–UDCA Monophosphate with Cholylglycine Hydrolase (CGH): 5-ASA–UDCA monophosphate was incubated with 60 U of CGH in accordance with the method that Nair described.[10] Solutions with four different concentrations of 5-ASA–UDCA monophosphate were studied: solutions A, B, C, and D contained 2, 1.5, 1.0 and 0.5 mM of 5-ASA–UDCA monophosphate, respectively. The reaction solutions were incubated at 37 °C for 6 h. The amount of 5-ASA released was determined using high performance liquid chromatography (LC-10AS, Shimadzu Corporation, Japan) with the method of Devos.[11] 4-Amino-2-hydroxybenzoic acid (4-ASA) was used as an internal standard.

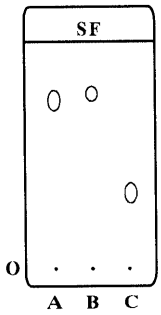


Fig. 2. TLC of the Compounds

Plate: Kieselgel 60 $F_{254}$; solvent system; ethanol–ammonia solution (3 : 1, v/v). SF, solvent front; A, 5-ASA; B, UDCA; C, 5-ASA–UDCA monophosphate; O, origin.

Incubation of 5-ASA–UDCA Monophosphate with Human Plasma, Pancreatic Enzymes and Homogenates of Liver and Intestinal Mucosa from Rats: Plasma: Human plasma was incubated with 5-ASA–UDCA monophosphate in accordance with the method of Erdos.[12] Activation of plasma carboxypeptidase A and B was assured by adding 2 mg of activated trypsin per milliliter of plasma carboxypeptidase N and was increased by adding 0.1 mM $CoCl_2$. This solution was preincubated at 37 °C for 60 min. 0.25 ml of the activated plasma was added to 0.75 ml of reaction mixtures, with a final concentration of 1 mM 5-ASA–UDCA monophosphate, 0.025 M Tris–HCl buffer (pH 7.6) containing 0.1 M NaCl. The reaction solutions were incubated at 37 °C for 12 h.

The incubation of 5-ASA–UDCA monophosphate with pancreatic enzymes and homogenates of liver and intestinal mucosa from rats was performed according to the method of Huijghebaert and Hofmann.[13]

Pancreatic Enzymes: The effect of pancreatic enzymes on 5-ASA–UDCA monophosphate was studied *in vitro*. 5-ASA–UDCA monophosphate was dissolved, with the final concentration of 1 mM in 0.1 M phosphate buffer. We used pancreatin from porcine pancreas, carboxypeptidase A[14] from bovine pancreas, carboxypeptidase B[15] from porcine pancreas, α-chymotrypsin[16] from bovine pancreas and trypsin[17] from bovine pancreas. One milliliter of each reaction mixture was prepared as follows: 0.05 M

3-morpholinopropropane-sulfonate (MOPS) buffer, pH 7.0, 1.0 mg enzyme in the case of pancreatine; 0.025 M Tris–HCl buffer, pH 7.5, 0.5 M NaCl, 22.7 unit/ml of enzyme in carboxypeptidase A; 0.025 M Tris–HCl buffer, pH 7.65, 0.1 M NaCl, 20 unit/ml of enzyme in carboxypeptidase B; and 0.05 M Tris–HCl buffer, pH 7.8, 1 mM $CaCl_2$, 500 unit/ml of enzyme in α-chymotrypsin, respectively. Trypsin was dissolved in 1 mM HCl and 300 U was added to a mixture with a final concentration of 0.05 M Tris–HCl buffer, pH 8.1, containing 11.5 mM $CaCl_2$. In addition, 5-ASA–UDCA monophosphate, Bz–Gly–L-Phe, Bz–Gly–L-Arg, Bz–Tyr–PABA, and Bz–L-Arg–PNA were assayed as substrate compounds in these systems. The reaction solutions were incubated at 37 °C for 12 h.

Intestine[18,19]: For preparation of the homogenates, Wistar rat (weighing approximately 240 g) was killed by exposure to diethyl ether. The small intestine was isolated and divided between the proximal and distal small intestine at the midpoint between the stomach and the cecum. The intestinal contents were squeezed out and the intestine was then washed out with sterile saline. It was then opened and the intestinal mucosa was scraped off with a stainless steel spatula, suspended in 3-fold, ice-cold 1.15% KCl–0.01 M phosphate buffer (pH 7.4) and homogenized with a Polytron homogenizer (Kinematica GmbH, Kriens-Luzern, Switzerland) for 20 s. After centrifugation at 3000 rpm for 10 min, the supernatant fraction was adjusted to 8 mg/ml of protein with 0.1 M phosphate buffer (pH 7.4) by the method of Lowry *et al.*[20] The reaction mixtures consisted of 1 ml of 0.01 M potassium phosphate buffer (pH 7.0) containing 0.5% v/v Triton X-100 (Sigma) and 2 mg/ml of bovine serum albumin (Sigma), and 5-ASA–UDCA monophosphate solution (final concentration 1 mM). The reaction solutions were incubated at 37 °C for 12 h.

Liver[21]: For preparation of the homogenates, Wistar rat (weighing approximately 240 g) was killed by exposure to diethyl ether. The liver was perfused through the liver artery with ice-cold 1.15% KCl solution and excised. The liver was homogenized with a Polytron homogenizer (Kinematica GmbH, Kriens-Luzern, Switzerland) for 20 s. After centrifugation at 1000 rpm for 10 min, the supernatant fraction was adjusted to 8 mg/ml of protein with 0.1 M phosphate buffer (pH 7.4) by the method of Lowry *et al.*[20] The reaction mixtures consisted of a NADPH-generating system (6.25 mM $MgCl_2$, 0.25 mM glucose-6-phosphate (G-6-P), and 3/75 mM nicotinamide), the enzyme preparation (2 mg protein/ml), 5-ASA–UDCA monophosphate solution (final concentration 1 mM), and sodium–potassium phosphate buffer (pH 7.4). The reaction solutions were incubated at 37 °C for 12 h.

All reaction solutions were incubated at 37 °C, and the extent of hydrolysis of the compounds was determined by sampling at 1, 3, 6 and 12 h in each experiment. Measurements of the hydrolysis rates were determined using TLC with a solvent system of ethanol–28% ammonia water (3 : 1, v/v).

Everted Gut Sac Experiment: Male Wistar rats weighing 200 to 250 g were fasted overnight before being used in the experiment. Under ether anesthesia, laparotomy was performed and the intestine was rinsed with ice-cold 0.9% NaCl solution. The entire small intestine was then removed and divided equally into four segments (segments of jejunum I, jejunum II, ileum I, and ileum II from proximal to distal). The forthcoming procedures were carried out according to the method of Wilson and Wiseman.[22,23] The concentration of 5-ASA–UDCA monophosphate was prepared to 0.1 mM both inside and outside of the everted gut sac, and in each intestinal sac was placed an incubation flask which was incubated at 37 °C for 1 h under gentle oxygenation (95% $O_2$–5% $CO_2$). At the end of the incubation period, the concentration of 5-ASA–UDCA monophosphate in the serosal and mucosal compartments was measured using a fluorescence depolarization analyzer (TDX; Dainabot Co., U.S.A.).

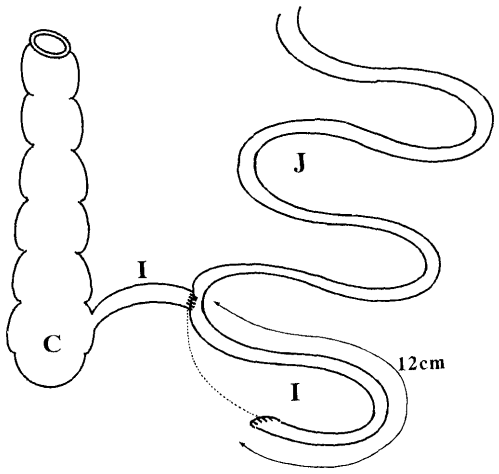


Fig. 3. Sketch of the Surgical Manipulation to Produce Rats with Intestinal Stagnant Loop

C, cecum; I, ileum; J, jejunum.

***In Vivo* Studies** Biliary Recovery of 5-ASA–UDCA Monophosphate in Rats: Male Wistar rats weighing 250 to 300 g were fasted overnight before surgery. Under ether anesthesia, cannulation into the bile duct was carried out for continuous bile collection after the laparotomy. 15 mg of 5-ASA–UDCA monophosphate was injected into the jejunal lumen or intravenously, and bile was collected for 4 h. Biliary recovery of the compounds was determined using TLC and TDX.

Oral Administration Test of 5-ASA–UDCA Monophosphate in Rats: Male Wistar rats (weighing 210 to 300 g) were used in this experiment. Rats were divided into a control group (group A; $n=6$) and a group with intestinal bacterial overgrowth (group B; $n=6$). In the rats of group B, stagnant loops were constructed surgically as shown in Fig. 3 to produce intestinal bacterial overgrowth.[24] After 20 mg of 5-ASA–UDCA monophosphate was orally administered to each rat using a Mark needle, each rat was placed in an individual stainless steel metabolic cage, and urine specimens were collected for 24 h. Urinary excretions of 5-ASA and its metabolite (*N*-acetyl-5-ASA) were determined by HPLC.[11,25] Detection was performed with a fluorescence monitor (Shimadzu, Model RF-10A, Kyoto, Japan), with an excitation wavelength of 315 nm and an emission wavelength of 430 nm. The mobile phase consisted of 0.1 M acetic acid–acetonitrile–triethylamine (920 : 80 : 2, v/v). 4-ASA was used as an internal standard.

## RESULTS

***In Vitro* Studies** Hydrolysis of 5-ASA–UDCA Monophosphate by CGH: Figure 4 shows the time course of enzymatic hydrolysis of the compound over 6 h at 4 different concentrations of substrate. 5-ASA–UDCA monophosphate was hydrolyzed to release 5-ASA by CGH, revealing that this new compound was capable of being a substrate to the enzyme as glycine- or taurine-conjugated bile acids.[26–29]

Stability of 5-ASA–UDCA Monophosphate to Other Organic Enzymes: 5-ASA–UDCA monophosphate was completely resistant to deconjugation by pancreatic and human plasma, intestinal mucosal enzymes and liver homogenate (Table 1).

Everted Gut Sac Experiment: 5-ASA–UDCA monophosphate was revealed to be a compound which was not actively absorbed from any part of the small intestine because the serosal/mucosal concentration ratios were below 1.0 in the everted gut sac experiment (Fig. 5).

***In Vivo* Studies** Biliary Recovery of the Compound: In the experiment with the intravenous administration of 5-ASA–UDCA monophosphate, 75% of the compound was recovered in the bile without any degradation for 4 h after administration. In contrast, 5-ASA–UDCA monophosphate was not detected in bile in the experiment involving intrajejunal administration of the compound (data not shown).

5-ASA–UDCA Monophosphate Administration Test: Figure 6 shows the results of the urinary excretion of *N*-acetyl-5-ASA (Ac-5ASA) for 24 h after the oral administration of 20 mg of 5-ASA–UDCA monophosphate to rats. The rats of the control group (group A) excreted 276.3 ± 89.0 μg (mean ± S.E.) of Ac-5ASA into urine. Sig-

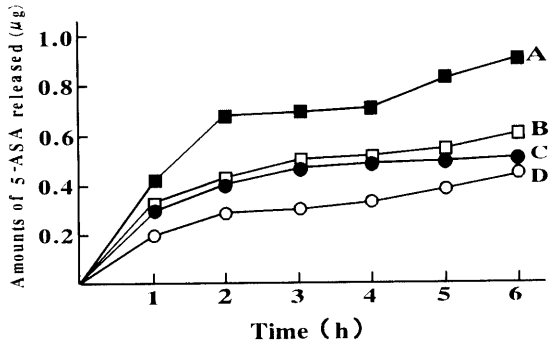


Fig. 4. Time Course of Enzymatic Hydrolysis of 5-ASA–UDCA Monophosphate by CGH

Incubation was carried out in an acetate buffer (pH 5.6) containing 2 mM (A), 1.5 mM (B), 1 mM (C), 0.5 mM (D) 5-ASA–UDCA monophosphate.

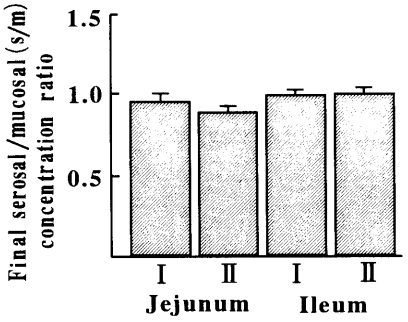


Fig. 5. Active Transport of 5-ASA–UDCA Monophosphate by Everted Gut Sacs Prepared from Various Segments of the Rat Intestine

Initial concentration was prepared to 0.1 mM of 5-ASA–UDCA monophosphate in both the serosal and mucosal compartments. Each bar represents the mean ± S.E. of three incubations.

Table 1. Hydrolysis of 5-ASA–UDCA Monophosphate by Pancreatin, Carboxypeptidase A and B, α-Chymotrypsin, Trypsin, Plasma, Small Intestinal Homogenates, and Liver Homogenates

| Substrate | Extent of hydrolysis (%) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pancreatine | | | | CPA | | | | CPB | | | | α-Chymotrypsin | | | |
| | 1 h | 2 h | 3 h | 12 h | 1 h | 2 h | 3 h | 12 h | 1 h | 2 h | 3 h | 12 h | 1 h | 2 h | 3 h | 12 h |
| 5-ASA–UDCA monophosphate | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bz–Gly–L-Phe | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | — | — | — | — | — | — | — | — |
| Bz–Gly–L-Arg | 100 | 100 | 100 | 100 | — | — | — | — | 100 | 100 | 100 | 100 | — | — | — | — |
| Bz–Try–PABA | 100 | 100 | 100 | 100 | — | — | — | — | — | — | — | — | 100 | 100 | 100 | 100 |
| Bz–L-Arg–PNA | — | 75 | 100 | 100 | — | — | — | — | — | — | — | — | — | — | — | — |

| Substrate | Extent of hydrolysis (%) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Trypsin | | | | Human plasma | | | | Intestinal mucosal homogenate | | | | Liver homogenate | | | |
| | 1 h | 2 h | 3 h | 12 h | 1 h | 2 h | 3 h | 12 h | 1 h | 2 h | 3 h | 12 h | 1 h | 2 h | 3 h | 12 h |
| 5-ASA–UDCA monophosphate | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bz–Gly–L-Phe | — | — | — | — | 0 | 0 | 0 | 0 | 100 | 100 | 100 | 100 | 0 | 0 | 14 | 22 |
| Bz–Gly–L-Arg | — | — | — | — | 0 | 6 | 14 | 25 | 66 | 75 | 83 | 100 | 0 | 0 | 0 | 10 |
| Bz–Try–PABA | — | — | — | — | 0 | 0 | 0 | 0 | 0 | 0 | 14 | 35 | 0 | 0 | 0 | 10 |
| Bz–L-Arg–PNA | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 11 | 20 | 32 | 84 | 0 | 0 | 0 | 0 |

CPA, carboxypeptidase A; CPB, carboxypeptidase B.

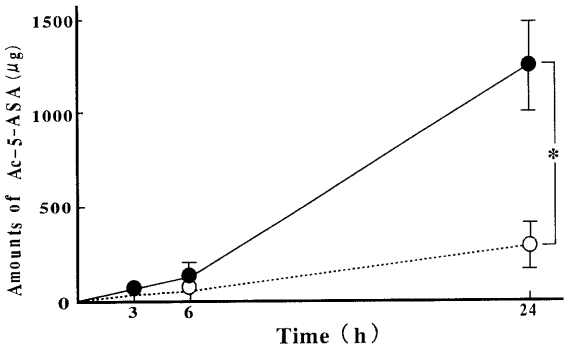


Fig. 6. Urinary Excretions of 5-ASA after Oral Administration of 20 mg of 5-ASA–UDCA Monophosphate to Rats

Each point and vertical bar represents the mean ± S.E. * Statistically significant difference from the control group ($p<0.01$). ●, intestinal bacterial overgrowth group; ○, control group.

nificant elevations of urinary Ac-5ASA excretion ($p<0.01$) were found in the group with intestinal bacterial overgrowth (group B; 1224.1 ± 231.5 μg) compared with the control group. On the other hand, urinary excretions of 5-ASA within 24 h were 6.26 ± 0.033 and 4.47 ± 0.047 μg in group A and B, respectively.

## DISCUSSION

An excellent recovery of 5-ASA–UDCA monophosphate in bile after intravenous administration of the compound can conceivably be attributed to the existence of a bile acid moiety in the 5-ASA–UDCA monophosphate molecule, because it is well accepted that the rate of the transfer of serum bile acids to hepatocytes is quite high, resulting in good recovery of serum bile acids in bile.[29,30] In the experiment involving the intraduodenal administration of 5-ASA–UDCA monophosphate, the compound was not recovered in bile, though it was a bile-excreted type of substance. This means that 5-ASA–UDCA monophosphate is not absorbed passively from the intestine. Similarly, in the active transport experiment using everted gut sac, it was demonstrated that 5-ASA–UDCA monophosphate was not a compound to be actively absorbed by the intestine. This lack of intestinal absorption of the compound is attributable to the increase in water-solubility by the presence of a phosphate group in the molecule. The enzymes which were contained in pancreatic juice and intestinal mucosal homogenate had no effect on the compound. Thus, 5-ASA–UDCA monophosphate is proven to be unabsorbable, and to be a single pass type of substance in the intestine without degradation when it is given orally.

Bile acids are synthesized through many steps of enzymatic reactions from cholesterol in the liver, resulting in the production of primary bile acids such as cholic and chenodeoxycholic acids. These primary bile acids are further conjugated in the liver with glycine or taurine, and are excreted in bile as a conjugated form.[26] In the intestine, microorganisms hydrolyze the conjugated bile acids by the action of CGH, leading to the formation of deconjugated bile acids.[27] Many strains of anaerobic microbes such as *Clostridium*, *Bacteroides*, *Bifidobacterium*, and *Eubacterium* have been reported to produce CGH, which is capable of bile salt deconjugation.[28,31,32] In our experiment, 5-ASA–UDCA monophosphate was deconjugated by CGH to release free 5-ASA.

When 5-ASA is given orally, it is rapidly absorbed from the small intestine, acetylated and excreted in urine. Only small amounts of 5-ASA are found in urine following the oral administration of 5-ASA.[33–35] Similarly, in our experiment with the oral administration of 5-ASA–UDCA monophosphate, 5-ASA that was absorbed from the intestine was excreted in urine as an acetylated form, whereas negligibly small amounts of 5-ASA were detected in urine. Urinary excretions of Ac-5ASA were significantly elevated in rats with a stagnant intestinal loop as a result of intestinal bacterial overgrowth.

The basic characteristics of 5-ASA–UDCA monophosphate and the kinetics of 5-ASA as described above led us to entertain the working hypothesis that the measurement of urinary 5-ASA metabolites after oral administration of the compound could be useful for the evaluation of intestinal microflora. Thus, an oral administration test of 5-ASA-UDCA monophosphate to rats was carried out to clarify whether the hypothesis is correct.

In our experiment, the result strongly supports that 5-ASA–UDCA monophosphate a possible suitable diagnostant for the evaluation of intestinal microflora.

In clinical practice, the diagnosis of bacterial overgrowth is difficult and laborious. Bacteriological analysis of the intestinal fluid is the most reliable method but is limited by the need for sophisticated equipment, high costs, and the need to intubate the patient. In 1971, Fromm and Hofmann[36] reported on a test called the breath test for human use, utilizing $^{14}$C-labeled glycocholate. King *et al.*[37,38] proposed the [$^{14}$C]D-xylose breath test, which had good specificity and sensitivity for the detection of bacterial overgrowth. However, the major drawback of these two breath tests is the need for a radioactive tracer. Metz *et al.*[39] developed the breath hydrogen test, but again, this required special, expensive equipment for detecting hydrogen.

The oral administration test of 5-ASA–UDCA monophosphate that we described here is expected to solve these problems. Moreover, another interesting aspect concerning 5-ASA–UDCA monophosphate is that this compound could theoretically be a prodrug in the treatment of ulcerative colitis by oral use. Such investigations are advertently under way.

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