Biochemical Pharmacology, Vol. 35, No. 21, pp. 3745–3752, 1986.
Printed in Great Britain.

0006–2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

GLUCOSIDE FORMATION AS A NOVEL METABOLIC PATHWAY OF PANTOTHENIC ACID IN THE DOG

KOZABURO NAKANO, YOICHI SUGAWARA, MOTOAKI OHASHI* and SHOICHI HARIGAYA
Biological Research Laboratory, Tanabe Seiyaku Co. Ltd., Saitama 335, Japan

(*Received* 9 *October* 1985; *accepted* 7 *March* 1986)

Abstract—Metabolism of pantothenic acid (PaA) in beagle dogs was investigated. The dogs excreted 12.3% of the dose in the urine within 24 hr after a single oral administration of [^{3}H]PaA (3 mg/kg). High performance liquid chromatographic analysis of the urine showed the presence of unchanged vitamin and a major metabolite, which accounted for 60.2 and 39.8% of the urinary radioactivity respectively. Although the metabolite was hydrolyzed by treatment with β-glucuronidase or acid phosphatase, it was found that this hydrolysis resulted from the actions of β-glucosidase contained as a contaminant in these enzyme preparations. β-Glucosidase completely hydrolyzed the metabolite to generate PaA and glucose. The metabolite was isolated and subjected to GC/MS and NMR analyses. It was identical to synthetic PaA β-glucoside, 4′-O-(β-D-glucopyranosyl)-D-pantothenic acid. It was shown by the use of dog liver microsomes that PaA underwent β-glucosidation in the presence of uridine diphosphate glucose (UDPG). It is proposed that β-glucosidation by UDP-glucosyltransferase is a novel metabolic pathway of PaA in the dog.

Despite extensive studies on PaA†, its metabolism in mammals is not understood completely. Nakamura and Tamura [1] and Pietrzik and Hornig [2] reported that rats excrete in urine free PaA and 4′-phosphopantothenic acid, which is the first intermediate in the biosynthesis of coenzyme A. However, Karnitz *et al.* [3] detected only free PaA.

Taylor *et al.* [4, 5] reported that in the dog a major metabolite is excreted in urine with a small amount of free PaA after administration of [^{14}C]PaA, and that the metabolite accounts for 40% of the dose in 7 days. The metabolite was hydrolyzed by treatment with β-glucuronidase (*Helix pomatia*). Therefore, they concluded that the metabolite was a glucuronide of PaA. Enzymatic analysis alone, however, may lead to an incorrect conclusion as a result of contamination by other enzymes in the preparation used.

In the present study, we isolated the urinary metabolite of PaA in beagle dogs by high performance liquid chromatography (HPLC) and, by spectral and chromatographic comparisons between the metabolite and the synthetic compound, determined that it was a β-glucoside of PaA which has been heretofore unreported in mammals. We also demonstrated that in dog liver microsomes PaA undergoes β-glucosidation in the presence of UDPG.

* Address all correspondence to: Dr. Motoaki Ohashi, Department of Drug Metabolism, Biological Research Laboratory, Tanabe Seiyaku Co. Ltd., 2-2-50 Kawagishi, Toda, Saitama 335, Japan.

† Abbreviations: PaA, pantothenic acid; TMS, trimethylsilyl; UDPG, uridine diphosphate glucose; PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

MATERIALS AND METHODS

Chemicals. [^{3}H]Calcium pantothenate was synthesized by the method of Nishizawa *et al.* [6] using calcium, [^{3}H]β-alanine and pantolactone. This compound was purified by preparative TLC with *n*-butanol–acetic acid–water (4:1:2, by vol.) as a developing solvent. The specific activity of the product obtained was 9.7 μCi/mg, and the radiochemical purity was 98% as determined by HPLC. [1-^{14}C]PaA (57.0 mCi/mmole, purity > 98.5%) was purchased from the New England Nuclear Co. TSIM (trimethylsilyl imidazole) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). β-Glucosidase (almonds, Grade II) was obtained from the Toyobo Co. (Osaka, Japan). Alkaline phosphatase (calf intestine, Type 1), acid phosphatase (wheat germ, Type 1), α-glucosidase (yeast, Type 1), and D-glucaric acid 1,4-lactone were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-Gluconic acid 1,5-lactone, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine for preparation of diazomethane, Triton X-100 and UDPG (sodium salt) were obtained from the Nakarai Kagaku Co. (Kyoto, Japan). Precoated silica gel TLC plates (0.25 mm of thickness, $60F_{254}$) were purchased from the Merck Co. (Darmstadt, West Germany). All other chemicals and solvents used were of the best grade commercially available.

Synthesis of benzyl 4′-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-D-pantothenate (I). This compound was synthesized according to the synthetic method for glucosides reported by Wulff *et al.* [7]. Benzyl-D-pantothenate (2.0 g, 6.5 mmoles) was dissolved in anhydrous ether (100 ml) and stirred in the dark at room temperature for 18 hr with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (6.7 g,

16.3 mmoles) and silver salicylate (4.4 g, 18.0 mmoles). The reaction mixture was filtered through celite. The solid residue was washed with chloroform. The combined filtrate and washing was evaporated to dryness under reduced pressure. The residue was dissolved in chloroform. The resultant solution was washed with ice-cold saturated sodium bicarbonate solution and water, and then dried over $MgSO_4$. The solution was concentrated to dryness *in vacuo*. Silica gel column chromatography of the residue using benzene–ethyl acetate (1:1, v/v) as an eluting solvent gave a white powder of I (1.1 g, 1.7 mmoles, yield 26%); m.p. 120–123°, IR (nujol): 3420 (OH), 1750 (C=O), 1660 (C—O), 1230, 760, 700 cm^{-1}; NMR ($CDCl_3$): 0.93, 1.01 (2 × s, 6H, 2 × —C—CH_3), 2.00–2.10 (12H, 4 × O—$COCH_3$), 2.66 (t, 2H, —CH_2—CH_2—, J = 6.3 Hz), 4.13 (d, 1H, —C*H*), 5.19 (s, 2H, —$COOCH_2$—), 7.40 (s, 5H, aromatic) ppm. Anal. Calc. for $C_{30}H_{41}NO_{14}$: C, 56.32; H, 6.46; N, 2.19. Found: C, 56.52; H, 6.50; N 2.25.

Synthesis of 4′-*O*-(2,3,4-6-*tetra-O-acetyl*-β-D-*glucopyranosyl*)-D-*pantothenic acid (II)*. A mixture of I (1 g, 1.6 mmoles) and palladium black (160 mg) in tetrahydrofuran (8 ml) was stirred vigorously under a hydrogen atmosphere (1 atm) at room temperature for 8 hr. After removal of the catalyst by filtration, the filtrate was evaporated under reduced pressure. The residue was chromatographed twice on silica gel columns using chloroform–methanol–acetic acid (95:4:1, by vol.) and chloroform–ethanol (95:5) as eluting solvents. II was obtained as a white powder (604 mg, 1.1 mmoles, yield 69%); m.p. 135–137°, IR (nujol): 3330 (OH), 2910, 1750 (C=O), 1720, 1655, 1625 cm^{-1}; NMR (D_2O): 0.90, 1.00 (2 × s, 6H, 2 × —C—CH_3), 1.82 (q, 2H, —CH_2—CH_2—, J = 7 Hz), 2.41 (t, 2H, —CH_2—CH_2—, J = 7 Hz) ppm. Anal. Calc. for $C_{23}H_{35}O_{14}N$: C, 50.25; H, 6.42; N, 2.55. Found: C, 50.15; H, 6.45; N, 2.62.

Synthesis of 4′-O-(β-D-glucopyranosyl)-D-*pantothenic acid (III)*. To a solution of II (550 mg, 1 mmole) in dichloromethane–methanol (1:3, v/v) (10 ml) was added 3 ml of a methanol solution of 0.5 N sodium methoxide with cooling in an ice bath. The mixture was allowed to stand at 4° for 18 hr. Water (5 ml) was added to the solution, and it was neutralized with 1 N HCl. After the solution was concentrated to a small volume under reduced pressure, the residue was purified by preparative TLC (*n*-butanol–acetic acid–water, 25:4:10, by vol.) to afford III (343 mg, 0.9 mmole, yield 90%) as a white hygroscopic powder.

Sample collection. Two male beagle dogs weighing about 13 kg were used. The animals were fasted for 18 hr and orally administered [^{3}H]PaA in 10 ml of water at a dose of 3 mg/kg (*ca.* 180 μCi/animal). They were placed in individual stainless steel metabolic cages. Urine and feces were collected over 24-hr periods, and the urine in containers was frozen with dry ice. Feces were homogenized in about 4 vol. of water, and aliquots of the urine and fecal homogenates were assayed for total radioactivity. Control urine was collected on the day before administration of the compound. The urine collected during 24 hr after administration was lyophilized, and the residue was extracted with 95% methanol, and centrifuged at 3000 rpm for 10 min. The supernatant fraction, to which all of the radioactivity transferred, was evaporated to dryness *in vacuo*. The residue was dissolved in a small amount of water and stored at −20° until used. A small portion of the solution was directly used for HPLC analysis. The 24-hr fecal homogenates were centrifuged at 5000 rpm for 20 min, and the aqueous layer containing all of the fecal radioactivity was analyzed by HPLC.

Isolation of the metabolite. Since the metabolite was acidic and insoluble in nonpolar organic solvents, the isolation was performed as follows. The urine (3.5×10^7 dpm) was saturated with ammonium sulfate, adjusted to pH 1 with concentrated HCl, and extracted three times with *n*-butanol. The aqueous layer (1.4×10^7 dpm) was neutralized with 4 N NaOH, and methanol was then added. After insoluble substances were removed by centrifugation, the solution was concentrated to a small volume, and placed on an Amberlite IR-120B (H^+ type) column. The effluent and washing were combined and then adsorbed to an Amberlite IRA-400 (OH^- type) column. After the column was washed with a sufficient amount of water, radioactive compounds were eluted with 0.5 N formic acid. The eluate (1.1×10^7 dpm) was lyophilized. The residue was dissolved in a small volume of water and subjected to preparative HPLC. Radioactive fractions were combined, treated with Amberlite IR-120B (H^+ type), and concentrated *in vacuo*. The residue was subjected to TLC with *n*-butanol–acetic acid–water (25:4:10, by vol.) as a developing solvent. The radioactive substance on the plate detected by autoradiography was eluted with methanol. After evaporation of the solvent, the purified metabolite (2.8×10^6 dpm) was obtained.

HPLC analysis. HPLC was performed using a set of Waters Associates instruments including a model 6000A pump, a Radial Pak C_{18} column (10 cm × 8 mm i.d.), a model 440 UV detector (254 nm filter), an RCM-100 radial compression module, and a Shimadzu SIL-IA injector. The mobile phase was made by mixing methanol with 19 vol. of water containing 0.1% triethylamine which was adjusted to pH 2.9 with formic acid. The flow rate was 2.0 ml/min. For quantitative analyses, the eluates from the column were collected directly into scintillation vials every minute using an automatic fraction collecter. For preparative purposes, 800 μl each of the urine samples was injected. Fractions of 1.0 ml each were collected under the same conditions as above, and 10 μl of each was used for measurement of the radioactivity. The same procedures were repeated to collect a sufficient amount of the sample for isolation of the urinary metabolite.

Enzymatic treatment of the metabolite. The metabolite (*ca.* 5000 dpm) was dissolved in 1 ml of the appropriate buffer system (one of three) shown in Table 2. The resulting solution was treated with β-glucuronidase, alkaline phosphatase, acid phosphatase, β-glucosidase, or α-glucosidase. In some experiments, enzymatic hydrolysis was conducted in the presence of D-glucaric acid 1,4-lactone or D-gluconic acid 1,5-lactone. After incubation at 37° for 18 hr, the solution was adjusted to pH 1 with

concentrated HCl and extracted twice with 3 ml of ethyl acetate. The radioactivity in an aliquot of each extract was measured. The remainder of the extract was evaporated to dryness, and the residue was subjected to HPLC and/or GC/MS. Blanks were treated identically but for the absence of the enzymes.

Enzyme preparation. Male mongrel dogs were used. They were anesthetized by an i.v. injection of sodium pentobarbital (30 mg/kg) and their livers were perfused *in situ* with 0.9% NaCl at 37°. Portions of liver (*ca.* 40 g) were homogenized in 5 vol. of 1.15% (w/v) KCl. Homogenization was carried out by six to eight passes in a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle at 1500 rpm. The homogenates were filtered through gauze and then centrifuged at 9,000 *g* for 20 min. The supernatant fraction was centrifuged at 105,000 *g* for 60 min. The microsomal pellets were suspended in 1.15% KCl and centrifuged at 105,000 *g* for 60 min. The washed microsomes were resuspended by homogenization in a volume of 1.15% KCl equivalent to the original wet weight of liver. Microsomal suspensions were immediately placed in liquid nitrogen and stored at −20°. The microsomes could be stored up to 3 weeks without loss of the enzyme activity. All the preparation procedures were performed at 4°.

Incubation procedure. Except when otherwise stated, incubations were performed as follows. The reaction mixture contained 0.1 M Tris–HCl (pH 7.4), 1 mM [^{3}H]PaA (or [^{14}C]PaA), 10 mM UDPG, 5 mM $MgCl_2$ and 1–2 mg of microsomal protein in a total volume of 0.5 ml. After 2 min of preincubation at 37°, the reaction was started by the addition of UDPG. The reaction was terminated 60 min later by the addition of 1 ml of chloroform, and the tubes were shaken vigorously. Proteins were precipitated by centrifugation at 3000 rpm for 10 min. Under these conditions, the pellets retained no radioactivity. The supernatant fraction was filtered through, and 100 μl of the filtrate was used for analysis of the conjugated metabolite of PaA by HPLC. The HPLC system consisted of a 655-15 pump (Hitachi), a Rheodyne 7125 injector and a Radial Pak C_{18} column (10 cm × 8 mm i.d.) in a Z compression separation system (Waters Associates) with a spheri-10 pre-column (RP-10, Brounlee Laboratories). The solvent mixture was 1% triethylamine, adjusted to pH 2.9 with formic acid–methanol (95:5, v/v) at a flow rate of 2 ml/min. The eluates from the column were collected directly into scintillation vials every minute using an automatic fraction collector. The enzyme activity was determined from the production rate of the conjugate from [^{14}C]PaA. Blanks were performed in the absence of either microsomes or UDPG. Protein concentration was determined by the method of Lowry *et al.* [8] using bovine serum albumin as a standard.

Identification of the metabolite in vitro *experiments.* The incubation mixture contained [^{3}H]PaA (100 μmoles, *ca.* 20 μCi), dog liver microsomal suspension (30 ml), 50 mM $MgCl_2$ (5 ml), 100 mM UDPG (5 ml), 1 M Tris–HCl (pH 7.4, 5 ml) in a total volume of 50 ml. After 14 hr at 37°, the incubation mixture was extracted with chloroform. The aqueous layer obtained after centrifugation was passed through an Amberlite IR-120B (H^+ type) column. The residue was submitted to preparative HPLC. Radioactive fractions were combined and treated with Amberlite IR-120B (H^+ type). The effluent was concentrated to dryness. The residue was methylated with diazomethane, followed by treatment with TSIM for trimethysilylation. The TMS derivatives of methyl ester of the metabolite and authentic PaA β-glucoside were analyzed by GC/MS.

Derivatization for GC/MS. The metabolite (*ca.* 40 μg equiv. of PaA) was dissolved in 20 μl of methanol, and was treated with about 500 μl of an ethereal solution of diazomethane. The resulting solution was allowed to stand at room temperature for 30 min and evaporated to dryness. The residue was dissolved in 20 μl of pyridine and silylated with 10 μl of TSIM at room temperature for 30 min.

GC/MS and NMR analysis. The GC/MS analyses were performed on a Hitachi M-80A gas chromatograph-mass spectrometer interfaced with a Hitachi M-003 data processing system. A coiled glass column (1 m × 3 mm i.d.) packed with 3% OV-1 on 100/120 mesh GAS-CHROM Q was used. The column temperature was programmed to rise from 240 to 280° at 5°/min. The helium gas flow rate was 40 ml/min. Other conditions were as follows: injection port temperature, 250°; ion source temperature, 180°; separator temperature, 280°; ion accelerating voltage, 3 kV; total emission current, 100 μA; and ionizing voltage, 20 eV.

NMR spectrum of the purified metabolite (*ca.* 400 μg equiv. of PaA) was obtained using a JEOL-FX-100S spectrometer in D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate monohydrate (DSS) as an internal standard.

Measurement of radioactivity. Each liquid sample was dissolved in Triton X-100-toluene based scintillator (PPO, 4 g; dimethyl POPOP, 0.1 g; toluene, 660 ml; and Triton X-100, 330 ml). The radioactivities were counted in a Packard TRI-CARB 460CD liquid scintillation spectrometer. The efficiency of quenching and counting were determined by external standardization.

RESULTS

Urinary and fecal excretion of radioactivity. Figure 1 shows the excretion of radioactivity in the urine and feces after oral administration of 3 mg/kg of [^{3}H]PaA to two beagle dogs. Dog 1 and Dog 2 excreted 11.7 and 12.9% of the dose in 24-hr urine respectively. The excretion rate of radioactivity became very low from day 2. Within 9 days, 23.2 and 27.1% of the administered radioactivity were excreted in the urine of Dog 1 and Dog 2 respectively. The fecal excretion rates of radioactivity of Dog 1 and Dog 2 were 32.5 and 28.5% of the dose in 4 days respectively.

HPLC analysis. All the radioactivity excreted in the urine was extracted with 95% methanol. The extracts were analyzed by HPLC. The recovery of radioactivity in the analysis was quantitative. Figure 2 shows the elution profile of radioactivity of 24-hr urine of Dog 1. Two radioactive peaks (Peak 1 and

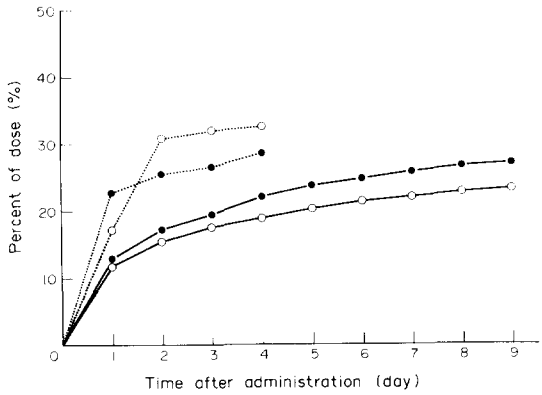


Fig. 1. Cumulative excretion of radioactivity in urine (—○— for Dog 1 and —●— for Dog 2) and feces (···○··· for Dog 1 and ···●··· for Dog 2) after oral administration of [^{3}H]PaA (3 mg/kg) to two beagle dogs. The results are expressed as dose percent. The radioactivity in the feces was not measured from day 5.

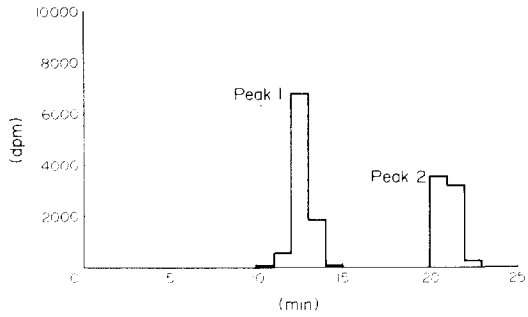


Fig. 2. HPLC profile of radioactivity of the 24-hr urine sample from a beagle dog after oral administration of [^{3}H]PaA (3 mg/kg).

Peak 2) are seen. A similar profile was obtained with the urine of Dog 2. The ratio of the mean radioactivities in Peak 1 and Peak 2 was 60.2:39.8. The retention time of Peak 1 was 13 min, which was identical to that of authentic [^{3}H]PaA. R_f values on TLC of Peak 1 and authentic PaA were the same (Table 1). These results indicate that Peak 1 is PaA. Finally, it was identified as PaA on GC/MS analysis by comparison of the retention times and mass spectra of the TMS derivatives of methyl esters between the compound in Peak 1 and authentic PaA (data not shown). Peak 2 at 21–22 min was not detected in the control urine spiked with [^{3}H]PaA. Therefore, the component of Peak 2 was assumed to be a urinary metabolite of [^{3}H]PaA. As shown in Table 1, in three solvent systems the R_f values of Peak 2 were identical to those of the metabolite which Taylor had proposed as a β-glucuronide of PaA.

On the other hand, the HPLC profile of fecal extract showed no radioactive peak other than [^{3}H]PaA (peak 1 in Fig. 2).

Enzymatic hydrolysis. Table 2 shows the results of the enzymatic hydrolyses of the metabolite of PaA. When the metabolite was incubated with β-glucuronidase from *H. pomatia*, it was completely hydrolyzed to release PaA as reported by Taylor *et al.* [4]. However, the metabolite was not hydrolyzed with β-glucuronidase from bovine liver. The former reaction was not affected by D-glucaric acid 1,4-lactone which is an inhibitor of β-glucuronidase. This suggests that the metabolite is not a glucuronide. Acid phosphatase hydrolyzed the metabolite in contrast to the report by Taylor *et al.* [4]. Alkaline phosphatase, however, did not hydrolyze the metabolite. This suggests that the metabolite is not the phosphate of PaA that has been found in rat urine [1, 2]. The metabolite was completely hydrolyzed with β-glucosidase, and the generated PaA and glucose were identified by GC/MS analyses. The hydrolysis was inhibited by the addition of D-gluconic acid 1,5-lactone, an inhibitor of β-glucosidase [9]. The metabolite was not attacked by α-glucosidase. These results indicate that the metabolite is the β-glucoside of PaA. Since GC/MS analysis also gave evidence that the metabolite was a glucose conjugate of PaA as described below, the hydrolysis of the metabolite with β-glucuronidase from *H. pomatia* or acid phosphatase possibly occurred by the action of β-glucosidase contained in those enzyme preparations as a contaminant.

Structural identification by GC/MS and NMR analysis. The mass chromatograms of the total ion and the specific fragment ions of the TMS derivative of methyl ester of the metabolite showed one peak at a retention time of 3 min (not shown). The mass spectrum of this peak is shown in Fig. 3. The molecular ion peak was not seen in this figure. The peak at m/z 740 was assumed to be $[M\text{-}15]^+$. A series of ions at m/z 361, 217 and 204 appeared. They are

Table 1. R_f values of radioactive components isolated by HPLC, authentic PaA and PaA metabolite

	R_f values in solvent system		
	A	B	C
Peak 1	0.29	0.65	0.38
Peak 2	0.15	0.33	0.21
PaA	0.29 (0.23)	0.65 (0.65)	0.38 (0.40)
PaA metabolite	(0.13)	(0.32)	(0.20)

The two components isolated by HPLC (peak 1 and 2) and authentic PaA were developed by TLC in the following solvent systems: (A) *n*-butanol–isopropanol–28% aqueous ammonia–water (20:10:1:9 by vol.); (B) *n*-butanol–acetic acid–water (25:4:10, by vol.); and (C) absolute ethanol.

Radioactive spots were detected by autoradiography. R_f values in parentheses for PaA and PaA metabolite are those reported by Taylor *et al.* [4].

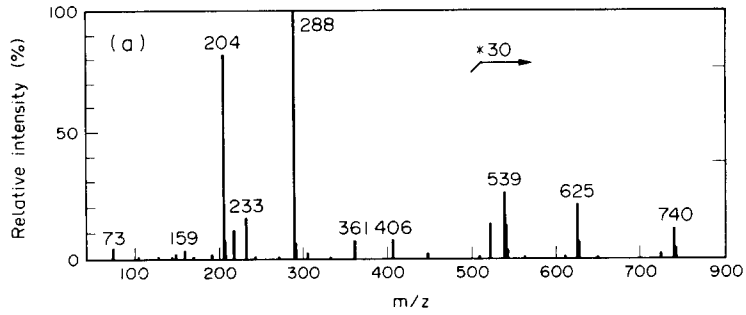
Table 2. Effects of β-glucuronidase, acid phosphatase, alkaline phosphatase, β-glucosidase, and α-glucosidase on the release of PaA from the metabolite of PaA

Enzyme	Source	Buffer	Release of PaA
β-Glucuronidase	Bovine liver	A	–
	H. pomatia	A	+
	H. pomatia	A*	+
Acid phosphatase	Wheat germ	A	+
Alkaline phosphatase	Calf intestine	B	–
β-Glucosidase	Almonds	A	+
	Almonds	A†	–
α-Glucosidase	Yeast	C	–

The metabolite was hydrolyzed with the enzyme listed above in 0.15 M acetate buffer (pH 5.0) (A), 0.15 M carbonate bicarbonate buffer (pH 10.2) (B) or 0.15 M acetate buffer (pH 6.0) (C) in the absence and presence of glucaric acid 1,4-lactone (*) or gluconic acid 1,5-lactone (†).

Production of PaA was analyzed by HPLC and/or GC/MS.

characteristic ions in the mass spectra of TMS derivatives of carbohydrates [10, 11]. Therefore, the presence of a sugar moiety was suggested in the molecule of the PaA metabolite. The sugar was identified as glucose by GC/MS analysis of the hydrolysate treated with β-glucosidase (data not shown). Fragmentations in the mass spectrum of the TMS derivative of the methyl ester of the PaA metabolite are proposed in Fig. 3. The base peak ion, m/z 288, indicates the presence of a PaA moiety in its structure. This fragment could be formed directly by cleavage of the aglycone C—O bond with charge retention on C-4′ of PaA in the same manner as has been reported for some ether type glucuronides of aliphatic compounds [12–16]. The fragment at m/z 233 which is probably formed via McLafferty rearrangement indicates that the glucose conjugation occurs at the C-4′ hydroxy group, not at the C-2′ one. The retention time and mass spectrum of the TMS derivative of the methyl ester of authentic 4′-O-(β-D-glucopyranosyl)-D-pantothenic acid are identical with those of the corresponding derivative of the metabolite. The ^{1}H-NMR spectrum of the purified metabolite is shown in Fig. 4. The coupling constant in the anomeric proton (H_1) of the glucopyranosyl group at 4.42 ppm is 7.3 Hz (doublet).



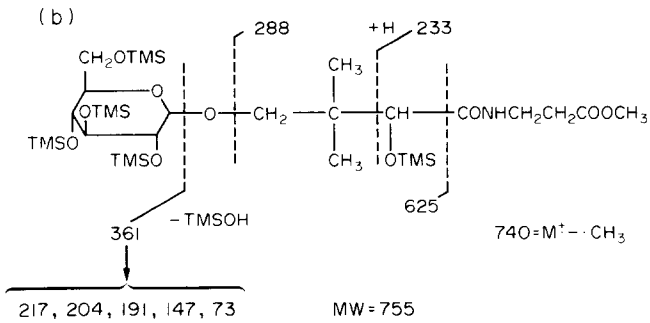


Fig. 3(a). Mass spectra of TMS derivatives of the methyl ester of the metabolite of PaA, methyl 4′-*O*-(β-D-glucopyranosyl)-D-pantothenate. (b) Proposed fragmentations of the TMS derivative of methyl 4′-*O*-(β-D-glucopyranosyl)-D-pantothenate.

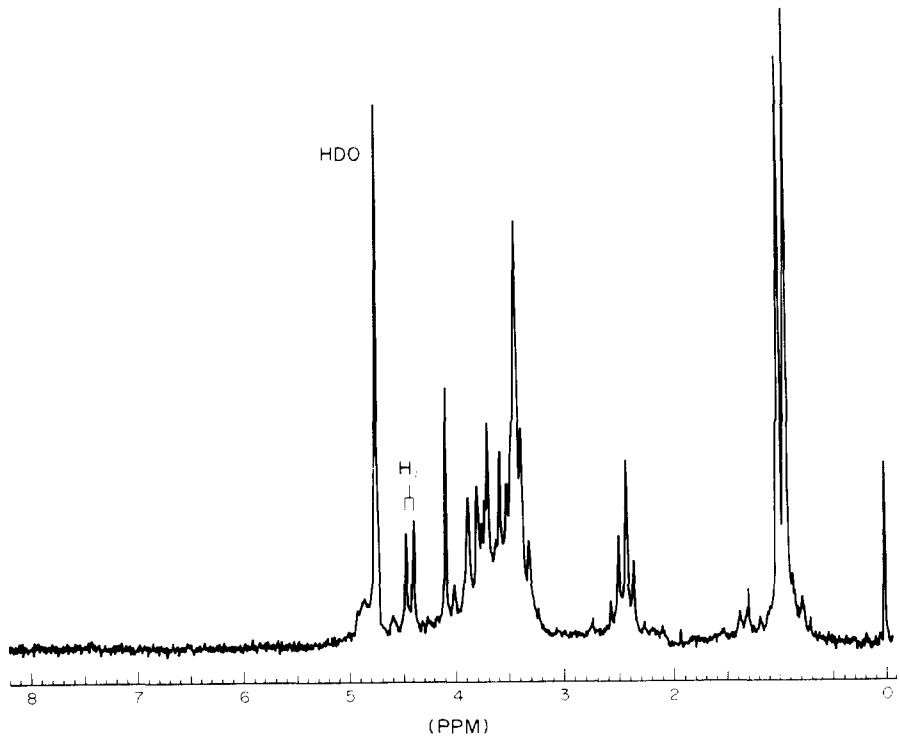


Fig. 4. One hundred MHz proton NMR spectrum of isolated PaA metabolite in D_2O. Chemical shifts are referenced to internal standard DSS. The doublet at 4.42 ppm is assigned to the anomeric proton (H_1) of the glucopyranosyl group.

This is in accordance with the coupling constant of the β-anomeric proton of glucosides [17]. Other proton signals of the PaA moiety are assigned to two *tert*-methyl groups at 0.93 and 1.00 ppm, a methylene at 2.42 ppm, and a methine at 4.08 ppm.

From these results, the urinary metabolite of PaA is identified as 4′-*O*-(β-D-glucopyranosyl)-D-pantothenic acid, a β-glucoside of PaA.

β-Glucoside formation in dog liver microsome preparations. The formation of PaA β-glucoside in dog liver microsomes was examined using 2 mM [^{3}H]PaA in the presence of 100 mM UDPG and 5 mM $MgCl_2$. After 14 hr of incubation at 37°, the aqueous portion of the incubation mixture after the extraction with chloroform was subjected to HPLC analysis; no radioactivity was found in the chloroform layer. Two radioactive peaks appeared at the retention times identical to those of authentic PaA (11 min) and PaA β-glucoside (19 min) in the same manner as in the *in vivo* experiment described previously. PaA β-glucoside accounted for 15% of the total radioactivity. The peak components at 11 and 19 min were isolated and subjected to GC/MS analysis. A mass spectrum of the TMS derivative of the methyl ester of the component at 19 min was identical to those of the PaA β-glucoside shown in Fig. 3. The mass spectrum of the component at 11 min was the same as that of authentic PaA. No PaA β-glucoside was produced in the absence of UDPG.

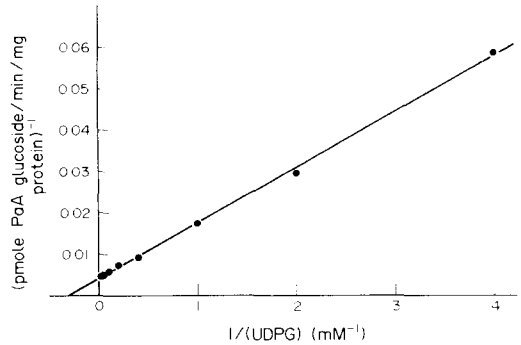


Fig. 5. Double-reciprocal plots of formation of PaA β-glucoside (pmoles/min/mg protein) vs concentration of UDPG. Incubations were carried out in the presence of 5 mM $MgCl_2$, 1 mg microsomal protein, 1 mM [^{14}C]PaA, and 100 mM Tris–HCl (pH 7.4) for 60 min.

As shown in Fig. 5, the β-glucosidation of PaA was dependent on the concentration of UDPG. The K_m and V_{max} values for UDPG determined from double-reciprocal plots were 3.2 mM and 241.5 pmoles/min/mg protein respectively. The K_m and V_{max} values for PaA determined from double-reciprocal plots shown in Fig. 6 were 0.2 mM and 122.4 pmoles/min/mg protein.

DISCUSSION

We have investigated the metabolism of PaA in beagle dogs using [^{3}H]PaA. The radioactivity excreted in 24-hr urine was 12% of the dose, and reached about 25% of the dose within 9 days. Only one metabolite was found in the urine. The metabolite accounted for about 40% of the 24-hr urinary

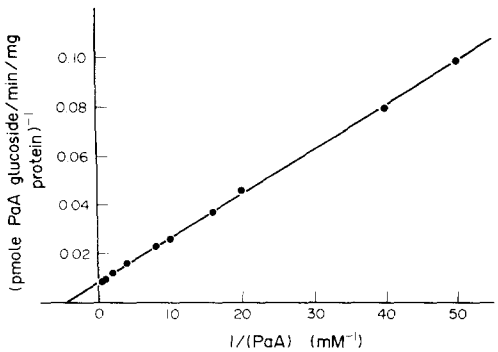


Fig. 6. Double-reciprocal plots of formation of PaA β-glucoside (pmoles/min/mg protein) vs concentration of PaA. Incubations were carried out in the presence of 5 mM $MgCl_2$, 1 mg microsomal protein, 10 mM UDPG and 100 mM Tris–HCl (pH 7.4) for 60 min.

radioactivity. The metabolite was hydrolyzed with β-glucuronidase from *H. pomatia*. These findings are coincident with those reported by Taylor *et al.* [4]. They proposed β-glucuronide of PaA for the identity of the metabolite. In the present study, however, it is clearly shown that the metabolite of PaA in dog urine is a β-glucoside of PaA, 4′-*O*-(β-D-glucopyranosyl)-D-pantothenic acid. Taylor *et al.* probably drew an incorrect conclusion on account of the contamination by β-glucosidase in the preparation of β-glucuronidase (*H. pomatia*). We suggest here the presence of β-glucosidase not only in β-glucuronidase (*H. pomatia*) but also in the acid phosphatase preparations. The metabolite was hydrolyzed by the treatment with β-glucuronidase (*H. pomatia*) in the presence of D-glucaric acid 1,4-lactone, an inhibitor of β-glucuronidase, whereas the hydrolysis was completely blocked by D-gluconic acid 1,5-lactone, an inhibitor of β-glucosidase. Taylor *et al.* have reported that after oral administration of [^{14}C]PaA to dogs no metabolite of PaA is found in the feces, and that the urinary metabolite of PaA is detected in the dog plasma 1 hr after intravenous administration of [^{14}C]PaA [4]. We also could not observe any metabolite in the feces of beagle dogs. These findings indicate that the metabolite is not formed by microflora in the gastrointestinal tract.

Glucoside formation is a common metabolic pathway in plants, bacteria, insects, and molluscs. In mammals, however, glucoside formation is rare compared to glucuronide formation [18–21]. Williamson *et al.* [22] found small amounts of 17α-estradiol 17β-glucopyranoside in the β-glucuronidase-treated urine of a rabbit injected with 17β-estradiol. In *in vitro* experiments steroid glucoside is produced when a steroid is incubated with uridine diphosphate glucose (UDPG) and microsomal fractions from rabbit liver [23], mouse liver [24], or human liver [25]. Fevery *et al.* [26] reported that bilirubin glucoside was detected in dog bile, and UDP-glucosyltransferase activity toward bilirubin was also shown in rat liver [27]. In addition, the formation of glucosides of vitamins such as riboflavin [28–30] and pyridoxine [31] has been reported. The enzymatic reaction forming riboflavinyl glucoside was studied in rat liver [28], pig liver [29], and cat liver [30]. Recently, Ohkawa *et al.* [32] reported the occurrence of riboflavinyl glucoside in rat urine after oral administration of [^{14}C]riboflavin. The physiological significance of glucosidation in drug metabolism has not yet been established.

The formation of the β-glucoside of PaA has not been reported in animals, although it has been observed in plants and microorganisms. PaA β-glucoside was first isolated by Amachi *et al.* [33–35] from tomato juice as a growth factor for a malolactic fermentation strain WNB-75 bacterium. Its growth-promoting activity was reported to be about 100 times higher than that of PaA. Kawai *et al.* [36] reported that PaA β-glucoside is formed from PaA and β-glucosyl donors such as cellobiose, phenyl-β-D-glucoside and salicin in the presence of a β-glucosidase from a plant or a microorganism. They also reported the formation of α-glucoside of PaA from PaA and maltose, sucrose or phenyl-α-D-glucoside in the presence of a microorganism or α-glucosidase [37, 38]. They considered glucosyl transferase actions of α- and β-glucosidase as responsible for the formation of PaA-glucosides.

It was demonstrated here that PaA undergoes β-glucosidation in the presence of UDPG with dog liver microsomes. This gives additional proof of the formation of PaA β-glucoside in the dog, not in microflora. The glucosidation of PaA follows Michaelis–Menten kinetics with respect to both PaA and UDPG.

It is of interest that β-glucoside of PaA is formed not only in plants but also in a mammal. The present study suggests that in the dog a glucosyltransfer pathway by UDP-glucosyltransferase plays an essential part in the metabolism of PaA.

Acknowledgements—The authors wish to express their deep gratitude to Dr. H. Nakajima, Director of this Research Laboratory, for his interest and encouragement, and to Miss N. Suzuki for her assistance in preparing the manuscript.

REFERENCES

1. H. Nakamura and Z. Tamura, *Chem. pharm. Bull., Tokyo* **20**, 2008 (1972).
2. K. Pietrzik and D. Hornig, *Int. J. Vit. Nutr. Res.* **50**, 283 (1980).
3. L. M. Karnitz, C. J. Gross and L. M. Henderson, *Biochim. biophys. Acta* **769**, 486 (1984).
4. T. Taylor, D. R. Hawkins, D. E. Hathway and H. Partington, *Br. vet. J.* **128**, 500 (1972).
5. T. Taylor, B. D. Cameron, D. E. Hathway and H. Partington, *Res. vet. Sci.* **16**, 271 (1974).
6. Y. Nishizawa, T. Kodama, T. Meshi and Y. Sato, *J. Vitamin.* **15**, 1 (1969).
7. G. Wulff, W. Krüger and G. Röhle, *Chem. Ber.* **104**, 1387 (1971).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
9. J. Conchie and G. A. Levy, *Biochem. J.* **65**, 389 (1957).
10. D. C. Dejongh, T. Radford, J. D. Hribar, S. Hanessian, M. Bieber, G. Dawson and C. C. Sweeley, *J. Am. chem. Soc.* **91**, 1728 (1969).
11. K. Jankowski and D. Gaudin, *Biomed. Mass Spectrom.* **5**, 371 (1978).
12. S. Billets, P. S. Lietman and C. Fenselau, *J. med. Chem.* **16**, 30 (1973).

13. G. L. Larsen and J. E. Bakke, *Biomed. Mass Spectrom.* **5**, 391 (1978).
14. C. Fenselau and L. P. Johnson, *Drug Metab. Dispos.* **8**, 274 (1980).
15. J. E. Bakke, V. J. Feil and C. Struble, *Biomed. Mass Spectrom.* **9**, 246 (1982).
16. P. C. C. Feng, C. Fenselau, M. E. Colvin and J. A. Hinson, *Drug Metab. Dispos.* **11**, 103 (1983).
17. J. M. Van der Veen, *J. org. Chem.* **28**, 564 (1963).
18. J. B. Pridham, *Phytochemistry* **3**, 493 (1964).
19. G. J. Dutton and A. M. Duncan, *Biochem. J.* **77**, 180 (1960).
20. J. N. Smith and H. B. Turbert, *Nature, Lond.* **189**, 600 (1961).
21. G. J. Dutton, *Archs Biochem. Biophys.* **116**, 399 (1966).
22. D. G. Williamson, D. C. Collins, D. S. Layne, R. B. Conrow and S. Bernstein, *Biochemistry* **8**, 4299 (1969).
23. D. C. Collins, D. G. Williamson and D. S. Layne, *J. biol. Chem.* **245**, 873 (1970).
24. R. S. Labow and D. S. Layne, *Biochem. J.* **142**, 75 (1974).
25. D. G. Williamson, D. S. Layne and D. C. Collins, *J. biol. Chem.* **247**, 3286 (1972).
26. J. Fevery, G. P. Van Hees, P. Leroy, F. Compernolle and K. P. M. Heirwegh, *Biochem. J.* **125**, 803 (1971).
27. K. P. Wong, *Biochem. J.* **125**, 929 (1971).
28. L. G. Whitby, *Biochem. J.* **50**, 433 (1952).
29. K. Uchida and Y. Suzuki, *Agric. biol. Chem., Tokyo* **38**, 195 (1974).
30. S. Kasai, S. Isemura, M. Masuoka and K. Matsui, *J. Vitamin.* **18**, 17 (1972).
31. K. Yasumoto, H. Tsuji, K. Iwami and H. Mitsuda, *Agric. biol. Chem., Tokyo* **41**, 1061 (1977).
32. H. Ohkawa, N. Ohishi and K. Yagi, *J. Nutr. Sci. Vitamin.* **29**, 515 (1983).
33. T. Amachi and H. Yoshizumi, *Agric. biol. Chem., Tokyo* **33**, 139 (1969).
34. T. Amachi, S. Imamoto, H. Yoshizumi and S. Senoh, *Tetrahedron Lett.* **56**, 4871 (1970).
35. T. Amachi, S. Imamoto and H. Yoshizumi, *Agric. biol. Chem., Tokyo* **35**, 1222 (1971).
36. F. Kawai, H. Yamada and K. Ogata, *Agric. biol. Chem., Tokyo* **38**, 831 (1974).
37. F. Kawai, K. Maezato, H. Yamada and K. Ogata, *Biochim. biophys. Acta* **286**, 91 (1972).
38. F. Kawai, K. Maezato, H. Yamada and K. Ogata, *Agric. biol. Chem., Tokyo* **42**, 1675 (1978).