Journal of Chemical Ecology, Vol. 27, No. 6, 2001

DOES EXCRETION OF SECONDARY METABOLITES ALWAYS INVOLVE A MEASURABLE METABOLIC COST? FATE OF PLANT ANTIFEEDANT SALICIN IN COMMON BRUSHTAIL POSSUM, *Trichosurus vulpecula*

S. McLEAN,[1] G. J. PASS,[1] W. J. FOLEY,[2,*] S. BRANDON,[1] and N. W. DAVIES[3]

[1] *School of Pharmacy and*

[3] *Central Science Laboratory*
University of Tasmania
Hobart Tas 7001, Australia

[2] *Division of Botany & Zoology*
Australian National University
Canberra 0200, Australia

(Received October 26, 2000; accepted February 8, 2001)

Abstract—Salicin was administered orally to six brushtail possums by incorporation in food for six days at three dose levels (0.05, 0.5, and 1.5% wet weight), giving mean ± SD daily intakes of 0.31 ± 0.09, 2.76 ± 0.75, and 6.04 ± 1.12 mmol salicin. Metabolites were identified by mass spectrometry and assayed by HPLC. Salicyl alcohol glucuronide accounted for 56–64% of urinary metabolites over the three doses, salicyluric acid 15–26%, salicin 10–18%, and there were smaller amounts of free (2–4%) and conjugated (0–6%) salicylic acid. β,2-Dihydroxyphenylpropionic acid was a minor metabolite. The hydrolysis of dietary salicin enabled reconjugation of its aglycone, salicyl alcohol, with a more polar sugar, glucuronic acid, thus enhancing its renal excretion and resulting in little net loss of substrates for conjugation and a low measurable metabolic cost of excretion.

Key Words—Salicin, metabolism, glucose conjugate, plant secondary metabolite, common brushtail possum, detoxication, costs.

INTRODUCTION

Although there is clear evidence that some plant secondary metabolites (PSMs) influence foraging of herbivores (Foley et al., 1999), the mechanisms by which

*To whom correspondence should be addressed. e-mail: william.foley@anu.edu.au

0098-0331/01/0600-1077$19.50/0 © 2001 Plenum Publishing Corporation

these effects are exerted remain unclear. Some compounds exert clear antifeedant effects (Lawler et al., 1998) or have a demonstrable impact on the metabolism of the animal. Yet most mammalian herbivores ingest large concentrations of many PSMs that do not appear to have major effects on their choices of foods (Palo and Robbins, 1991). Most syntheses of mammal–plant interactions place some importance on the cost of neutralizing and excreting any PSMs that are ingested (Freeland and Janzen, 1974; Foley and McArthur, 1994; Foley et al., 1999). The difficulty with assigning costs to the neutralization and excretion of PSMs is that only rarely are the pathways of metabolism known with any certainty (Freeland, 1991). Available information is generally restricted to laboratory species, and it is hard to place these studies in an ecological context. Costs that might be important in an ecological context could include the cost of maintaining enzyme systems, the cost of conjugates for excretion and the opportunity costs of lost foraging time because of the pharmacokinetic constraints of neutralizing and disposing of metabolites (Foley et al., 1999).

Although all these avenues could potentially be important, only the cost of excreted conjugates has been quantified. For example, Cork (1981) showed that glucuronic acid excretion comprised up to 25% of the fasting glucose entry rate of koalas (*Phascolarctos cinereus*). Lowry et al. (1993) showed that sheep fed diets rich in organic acids excreted 17% of their digestible N intake as benzoyl glycine (hippuric acid). Nonetheless, we think that some compounds, in particular phenolic glycosides, could be excreted with very little cost either because they are excreted unconjugated (Scheline, 1991) or because the glycoside is used as a source of metabolizable energy to compensate for the excretion of a carbohydrate-based conjugate such as a glucuronide.

We evaluated this possibility by studying the metabolism of salicin in common brushtail possums. Salicin [salicyl alcohol glucoside; 2-(hydroxymethyl)-phenyl-β-D-glucopyranoside] is a bitter-tasting phenolic glycoside found in the bark and leaves of various species of *Salix* and *Populus* (Trease and Evans, 1972). Earlier studies had implicated salicin as a cause of selective feeding on *Salix* and *Populus* by common brushtail possums (Markham, 1970; Edwards, 1978) and mountain hares (*Lepus timidus*) (Tahvanainen et al., 1985), but little was known of how such an antifeedant effect could occur. Pass and Foley (2000) examined the effect of isolated salicin on feeding in common brushtails but could find no evidence of postingestive effects and attributed the antifeedant effects of high concentrations of salicin to preingestive effects such as taste. Therefore, we undertook this study of the metabolic disposition of salicin to provide further evidence of the nature of any postingestive effects.

In the only detailed study of the metabolism of salicin, Fotsch et al. (1989a) found that rats excreted oral doses of salicin mostly as free salicylic acid and unchanged salicin. However, after a subcutaneous dose, salicin was excreted virtually unchanged (Fotsch and Pfeifer 1989). In vitro experiments showed that salicin was

hydrolyzed by bacteria in the cecum and colon of rats, indicating that salicin is extensively hydrolyzed in the rat gastrointestinal tract.

METHODS AND MATERIALS

Materials. Salicin (min. 99% purity) was purchased from Sigma. All other chemicals were of good commercial quality. Reference metabolites (salicyl alcohol, salicylic acid, salicyluric acid) were chromatographically pure. Methanol and acetonitrile were HPLC grade (Waters Associates, Sydney, Australia) and acetone was an analytical reagent grade (May & Baker).

Animals and Urine Collection. Animal work was carried out at James Cook University, Townsville, and was approved by that university's ethics committee (Pass and Foley, 2000). Six common brushtail possums were trapped in the wild and kept in an air-conditioned animal house maintained at 22 ± 2°C and a 12-hr light–dark cycle. The animals were adult males weighing 1.97–2.50 kg (mean = 2.27 kg). They had been living in a mangrove forest and had not been previously exposed to willows or poplars. The animals were fed on a diet of fruit and cereals (Pass and Foley, 2000). In feeding experiments, salicin was added to the dry ingredients of this diet and mixed to give a final uniform distribution of salicin 0.05, 0.5, or 1.5% wet weight of feed. Animals were housed individually and fed each of the three salicin diets for eight days, using a balanced Latin-square design. During each eight-day diet period, 24-hr urine samples were taken on day 6 for metabolite analyses. Urine was collected into bottles immersed in solid carbon dioxide and stored frozen until analyzed. Food intake was measured, by weight, on day 5.

Identification of Metabolites. Most metabolites were identified by mass spectrometry (MS) after separation by gas chromatography (GC). After hydrolysis of conjugates with β-glucuronidase/arylsulfatase (see below), urine samples were acidified to pH 1 with 5 M HCl and extracted three times with ethyl acetate. An aliquot of the extract was then placed into an ice bath in a fume hood, ethereal diazomethane added, and the reaction allowed to stand for 30 min. Excess diazomethane was removed with a gentle stream of nitrogen. The methylated metabolites were dissolved in ethyl acetate and analyzed by GC. The instrument was a Hewlett-Packard 5890 gas chromatograph with 5970B mass-selective detector using HP 59970A Chemstation software (Hewlett-Packard, Melbourne, Australia). GC conditions were: Hewlett-Packard HP1 capillary column (0.52 μm methylpolysiloxane), 25 m × 0.32 mm ID; oven 100–190°C at 5°C/min, then 190–200°C at 20°C/min; carrier He at 12 psi; injector 250°C; detector 300°C.

Salicyl alcohol glucuronide was isolated by liquid chromatography to enable its identification by high-resolution MS. Salicin and salicyl alcohol glucuronide were separated from urine on a column of Amberlite XAD-4 resin (Serva) using

a method adapted from that of White and Schwartz (1980). The resin (10 g) was washed successively with methanol, water, acetone, acetone–water, methanol, and water. An aliquot of urine (50 μl) was diluted in 75 ml water and passed through the column at 3 ml/min. The column was then washed with 80 ml water followed by successive 30 ml volumes of acetone–water (1:1). Eluted fractions were concentrated to approximately 1–2 ml by rotary evaporation at 40°C and analyzed by HPLC. Salicyl glucuronide eluted with the aqueous wash and gave no HPLC peak for salicin or salicyl alcohol, but after enzyme hydrolysis, produced salicyl alcohol. Salicin eluted with the acetone–water fractions, and enzyme hydrolysis gave salicyl alcohol. The structures of urinary salicyl glucuronide and salicin were confirmed by solid probe mass spectrometry. The instrument (Kratos Concept ISQ, Manchester, UK) used a Cs ion gun at 10 kV for FAB mass spectrometry in a glycerol matrix.

Structures of conjugates were confirmed by liquid chromatography–mass spectrometry (LC-MS) with a Waters Alliance HPLC and Finnegan LCQ mass spectrometer using LCQ Navigator software. Diluted urine samples were chromatographed on a reverse-phase C-18 Nova-Pak column (3.9 mm × 150 mm; Waters Associates) with a mobile phase starting with 90% water–10% methanol containing 2% acetic acid in each and programmed to 100% methanol after 40 min at 0.8 ml/min. Peaks were detected by negative electrospray ionization (ESI). MS-MS analysis confirmed the origin of daughter ions. Retention times were: salicyl alcohol glucuronide, 4.89 min; salicylic acid glucuronide, 6.49 min; and salicyluric acid, 9.93 min. Salicin does not produce a negatively charged ion under the conditions used and was not detected.

Quantification of Metabolites by HPLC. A Varian 9010 solvent delivery system with Rheodyne injector model 7161 was connected to a Varian 9050 UV-VIS absorbance detector and Star workstation (Varian Instruments, Melbourne, Australia). The column was a reverse-phase C-18 Nova-Pak (3.9 mm × 150 mm; Waters Associates) and solvents were pumped at 0.9 ml/min. The injection volume was 10 μl.

Salicin and salicyl alcohol were analyzed with methanol–water (25:75) at a wavelength of 213 nm (HPLC system 1). Calibration curves were prepared using 400 μl predose urine, to which were added known amounts of salicin and salicyl alcohol in methanol (0–500 μl), internal standard (1.32 mg benzyl alcohol in 1 ml 25% aqueous methanol), and water to 5 ml. Elution times were: salicin, 2.34 min; salicyl alcohol, 3.59 min; and benzyl alcohol, 6.49 min. Calibration curves were constructed using the concentration of the final solution and peak areas and were linear for salicin (0–1.76 μmole/ml; $r^2 = 0.998$) and salicyl alcohol (0–4.12 μmol/ml; $r^2 = 1.000$). Urinary concentrations were calculated using dilution factors to account for the varying volumes of urinary aliquots (200–500 μl) that were required to accommodate the large range of metabolite concentrations found at the different doses.

Salicylic acid and salicyluric acid were analyzed with acetonitrile (27%)–acetic acid (2%) in water (73%) at a wavelength of 313 nm (HPLC system 2). Calibration curves were prepared as before using predose urine spiked with standards. The internal standard was *o*-anisic acid (600 μg in 100 μl 25% aqueous methanol), urine volumes were 100–900 μl, and the final volume was made to 1 ml with water. Elution times were: salicyluric acid, 2.30 min; *o*-anisic acid, 2.85 min; and salicylic acid, 4.29 min. Calibration curves were linear for salicylic acid (0–3.62 μmoles/ml; $r^2 = 1.000$) and salicyluric acid (0–2.56 μmoles/ml; $r^2 = 0.999$), using the final concentration as above.

Hydrolysis of Conjugates. To analyze for conjugates of salicyl alcohol, a 500-μl aliquot of urine was mixed with 250 μl water, 200 μl acetate buffer (1.1 M, pH 5.2), and 50 μl of *Helix pomatia* extract (5.5 units β-glucuronidase and 2.6 units arylsulfatase in 1 ml) (Boehringer Mannheim, Germany). After overnight incubation at 37°C, an aliquot was transferred to a vial, 500 μl of internal standard solution (benzyl alcohol, as above) added, and the volume made to 2.5 ml with water before analysis by HPLC system 1. To analyze for conjugates of salicylic acid, 200–600 μl of urine hydrolysate was transferred to a vial with 100 μl internal standard solution (*o*-anisic acid, as above) and made to 1.0 ml with water before analysis by HPLC system 2.

RESULTS

Identification of Metabolites. Metabolites were identified by their chromatographic behavior and comparison with standards. Their structures were confirmed by mass spectrometry. Initial analysis of unhydrolyzed urine using HPLC system 1 showed a small peak for salicin, but no salicyl alcohol. Acid hydrolysis (pH 1, boiling water bath, 30 min) converted the salicin into an equivalent amount of salicyl alcohol. However, after enzymatic hydrolysis, the salicin disappeared, and there was a very large amount of salicyl alcohol, more than could be accounted for by the hydrolysis of salicin alone. This indicated that there was another conjugate of salicyl alcohol that was not detected by HPLC analysis. A glucuronide conjugate subsequently was isolated from urine using a column of XAD-4 resin (see Methods and Materials for details). FAB MS showed a protonated molecular ion (MH^+) at 301.0 and several adducts with Na^+ and K^+ whose structures were supported by high-resolution data, confirming the formula $C_{13}H_{16}O_8$ that corresponds to salicyl glucuronide (Table 1). Salicin, which was found in later fractions eluting from the XAD-4 column, did not give a molecular ion, but high-resolution analysis of ions with masses corresponding to the mono- and di- Na^+ adducts agreed closely with expected values, confirming the formula of $C_{13}H_{18}O_7$.

Salicyl alcohol, salicylic acid, and salicyluric acid were identified in urine by GC-MS analysis of their methylated derivatives (Table 1). Some urine samples

TABLE 1. MASS SPECTRAL DATA OF METABOLITES FOUND IN BRUSHTAIL POSSUM URINE.

Metabolite	Derivative[a]	R_t (min)[b]	Significant EI ions [m/z (% *abundance*)]				
Salicyl alcohol		6.72	124 (M^+)	106	78		
			(27)	(42)	(100)		
Salicylic acid	Methyl	5.75	152 (M^+)	120	92		
			(*42*)	(*100*)	(*87*)		
Salicyluric acid	Methyl	13.27	209 (M^+)	121	92		
			(*16*)	(*100*)	(*24*)		
β,2-Dihydoxy-phenyl propionic acid	Methyl	12.64	196 (M^+)	178	123	121	95
			(*66*)	(*3*)	(*100*)	(*34*)	(*90*)
		Significant FAB ions (m/z)[c]					
Salicin			309.09541 $[M + Na]^+$		331.1 $[M + 2 Na - H]^+$		
Salicyl alcohol glucuronide		301.0 $[M + H]^+$	323.07365 $[M + Na]^+$		339.04778 $[M + K]^+$		
			345.0 $[M - H + 2 Na]^+$		361.0 $[M - H + Na + K]^+$		

[a] Methyl ester.
[b] Retention time in GC-MS analysis (see Methods and Materials for chromatography conditions).
[c] FAB MS using probe insertion.

also showed traces of gentisic acid by GC-MS. All urine samples contained small amounts of β,2-dihydroxyphenylpropionic acid, which gave a mass spectrum similar to that of β-hydroxyphenylpropionic acid but with ions 16 amu heavier. β-Hydroxyphenylpropionic acid is a urinary metabolite of benzoic acid (Marsh et al., 1982; Awaluddin and McLean, 1985) and β,2-dihydroxyphenylpropionic acid is the analogous metabolite of salicylic acid, containing one extra oxygen atom. β,2-Dihydroxyphenylpropionic acid was not quantified because of its small amount and interference from the hippuric acid peak.

Conjugates were confirmed by LC-MS. Salicyl alcohol glucuronide formed a dimer at m/z 599 (a feature of negative ESI) and showed a deprotonated molecular ion $[M\text{-}H]^-$ at 299. MS-MS showed significant daughter ions at m/z 175 and 113 (characteristic of glucuronic acid) and 123 (the anion from salicyl alcohol). Salicylic acid glucuronide showed an $[M\text{-}H]^-$ ion at m/z 313, glucuronic acid ions at m/z 175 and 113 and a salicylic acid anion at m/z 137. Salicyluric acid gave ions at m/z 194 $[M\text{-}H]^-$ and 150 $[M\text{-}H\text{-}CO_2]^-$.

Quantification of Metabolites. Predose urine gave no interfering HPLC peaks. The 24-hr recovery of urinary metabolites of salicin for the three consumption levels of salicin is shown in Table 2. The dose of salicin was calculated from the food intake on day 5 and the percentage salicin in the food. Only two thirds of the estimated dose of salicin was recovered in urine as salicin and its metabolites, except at the lowest intake of salicin when recovery was complete. Excretion of individual metabolites is expressed as the percentage of total recovered metabolites in order to enable direct comparisons between animals and doses. The proportion found as unchanged salicin varied from 10% to 18% of total recovered metabolites, while the major metabolites were salicyl alcohol glucuronide (56–64%) and salicyluric acid (15–26%). There were only traces of free salicyl alcohol and small amounts of free and conjugated salicylic acid. There was a significantly greater proportion of salicin excreted as salicyluric acid at the lowest consumption rate compared to the higher doses, but no other significant dose-related differences were found.

DISCUSSION

The major urinary metabolite of salicin in the common brushtail possum was the glucuronide of its aglycone, salicyl alcohol, while very little unchanged salicin was excreted. Thus after an oral dose the glucoside of salicyl alcohol is excreted largely as its glucuronide. The metabolic pathways are shown in Figure 1.

This suggests that there is little measurable metabolic cost in the excretion of metabolites of salicin because, although the compound has been transformed, there is little net loss of substrate in the conjugation reactions. We recognize that the enzyme systems and intermediate reactions leading to the hydrolysis of salicin and the formation of the glucuronide require energy to initiate and maintain. Similarly,

TABLE 2. URINARY METABOLITES OF SALICIN EXCRETED IN 24 HOURS AFTER THREE DIFFERENT DIETARY CONCENTRATIONS

			Urinary metabolites as % total recovered (*mean* ± *SD*)					
Salicin in diet (%)	Salicin intake (μmol)[a]	Fraction recovered in urine (mean ± SD)	Salicin	Salicyl alcohol glucuronide	Salicyluric acid	Free salicyl alcohol	Free salicylic acid	Conjugated salicylic acid
1.5	6037 ± 1115	0.64 ± 0.13	9.9 ± 3.7	63.7 ± 7.0	16.7 ± 5.4	0.2 ± 0.5	3.5 ± 2.5	5.9 ± 3.0
0.5	2756 ± 745	0.68 ± 0.25	17.8 ± 10.9	63.0 ± 9.6	14.8 ± 2.4	0.9 ± 1.4	3.5 ± 2.4	0.1 ± 0.6
0.05	303 ± 85	1.02 ± 0.47	16.5 ± 12.3	56.3 ± 19.1	25.8 ± 10.5	0	1.9 ± 0.9	0
P^b		0.129	0.077	0.43	0.008			

[a]Calculated from the food consumed during the 24 hr prior to urine collection. ($N = 6$).
[b]Significance of differences between doses, repeated measurements ANOVA.

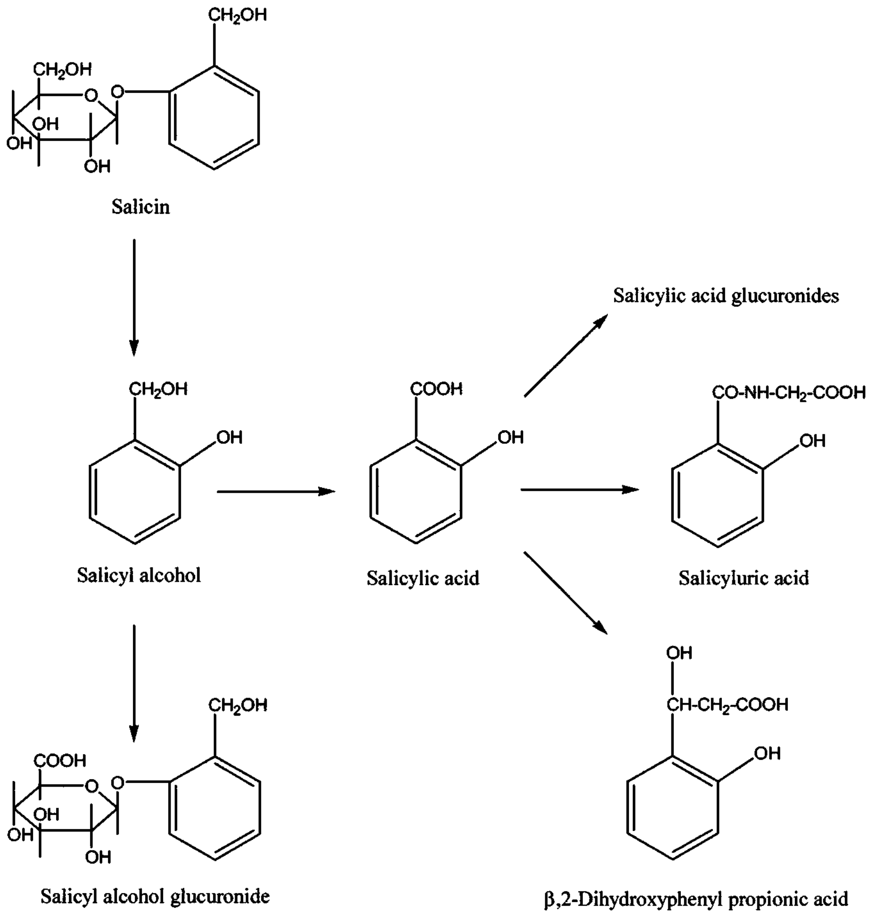

FIG. 1. Metabolic pathways of salicin in the brushtail possum.

there may be unmeasured cell damage or other metabolic disruptions that could constitute a cost, but this seems unlikely in view of the findings of Pass and Foley (2000) that ingestion of salicin did not cause measurable postingestive effects on possums. In an ecological context, the net result is that the phenolic moeity is ingested with a simple sugar derivative and excreted with a simple sugar derivative and the net cost is minimal.

The only other study of the metabolic fate of salicin used rats that were given repeated oral doses (1000 μmol/kg daily) (Fotsch et al., 1989a). Only about half (0.52) of the dose was recovered in urine, somewhat less than found in the present study after comparable doses (Table 2). The major urinary metabolites in the rat were free salicylic acid (57% of total urinary metabolites) and salicin (27%), with

smaller amounts of conjugated salicylic acid (10%), gentisic acid (4%), salicyluric acid (0.2%), and salicyl alcohol (0.2%). Apart from salicin, no urinary conjugates of salicyl alcohol were found. Thus, the metabolic fate of salicin appears to be quite different in the two species, with large amounts of salicyl alcohol glucuronide and salicyluric acid being excreted in the possum whereas salicylic acid was the major rat urinary metabolite. However, this comparison must be treated cautiously because other factors, such as diet, can influence the extent of metabolism (Mulder et al., 1990; Hutt and Caldwell, 1990).

The absorption and excretion of intact salicin may seem surprising, since it is well known that substances must be lipophilic to cross cell membranes, except where specific transport systems exist (Pratt, 1990). Salicin does not appear to interact with rabbit Na^+/glucose cotransporters (Lostao et al., 1994), whereas some other phenylglycosides (e.g., arbutin) are transported. However, Matsumoto et al. (1993) showed that salicin and other monosaccharides are able to permeate the cell membranes of human erythrocytes, although they do so more slowly than the more lipophilic aglycones. Whether this occurs by a paracellular pathway is not known.

After subcutaneous administration of salicin to rats, most of the dose was recovered unchanged in urine, indicating that the hydrolysis of oral doses occurs in the gut (Fotsch and Pfeifer, 1989). In vitro experiments by these authors showed that salicin was hydrolysed by rat caecum or colon contents, but not by liver homogenates.

A broad-specificity β-glucosidase has been found in guinea pig liver that is capable of hydrolyzing various plant glucosides including salicin (LaMarco and Glew, 1986; Gopalan et al., 1992). These authors postulated that the hepatic glucosidase may have evolved as a response of herbivores to the presence of toxic plant glucosides, such as the cyanogenic glycosides amygdalin and prunasin, and vicine, which can cause hemolytic anemia. Gopalan et al. (1992) showed that these three plant glucosides were able to cross segments of guinea pig jejunum in vitro without hydrolysis. Since lipid-soluble substances are reabsorbed from the renal tubules (Pratt, 1990), glucosides with sufficient hydrophobic character to be absorbed from the gut are also liable to be reabsorbed from the renal tubules, resulting in a low renal clearance. However, after hydrolysis, the aglycone can be conjugated with a more polar sugar, glucuronic acid, enabling more efficient renal excretion. Although cecal microorganisms can hydrolyze glucosides, any that are absorbed from the small intestine will escape microbial hydrolysis (unless secreted in the bile). The hepatic glucosidase would be in position to hydrolyze absorbed glucosides, enabling their reconjugation with glucuronic acid. Our data showing the hydrolysis of salicin and reconjugation of its aglycone to salicyl alcohol glucuronide are consistent with this proposed mechanism.

A simple oxidation reaction could convert salicin directly to the corresponding glucuronide, but there is no evidence that this reaction occurs. The glucuronic

acid in conjugates is formed by a specific enzymatic oxidation of UDP-glucose (Mulder et al., 1990), indicating that other glucosides would not be suitable substrates. It is also unlikely that salicyl alcohol is reconjugated with glucose, as β-glucoside formation is very uncommon in mammals, although an unusual α-glucoside formation has been reported in rat liver (Kamimura et al., 1992). When 1-naphthol-β-glucuronide was administered orally to mice, no glucoside was excreted, but administration of 1-naphthol-β-glucoside resulted in the excretion of both the glucoside and glucuronide (Chern and Dauterman, 1983).

Although little has been published on the fate of salicyl alcohol or salicin, there is a vast literature on the metabolism of salicylic acid, the oxidation product of salicyl alcohol (Scheline, 1991). There is considerable variability between and within species in the urinary metabolites of salicylic acid (Scheline, 1991). In the brushtail possum, only traces of free salicyl alcohol appeared in the urine, with small amounts of free and conjugated salicylic acid (presumably the glucuronide), while the major salicylate was the glycine conjugate, salicyluric acid (Table 2). There was relatively less salicyluric acid excreted after the higher doses of salicin, suggesting that the glycine conjugation pathway may be readily saturable in the brushtail possum, as in other species (Hutt and Caldwell, 1990). Other factors including diet and gut microorganisms also can be important determinants of glycine conjugation (Phipps et al., 1998).

This is the first report of the formation of β,2-dihydroxyphenylpropionic acid as a salicylate metabolite. It was presumably formed from the acyl-CoA of salicylic acid, in an analogous fashion to the postulated formation of β-hydroxyphenylpropionic acid from benzoic acid (Marsh et al., 1982). This may represent a general reaction of acyl-CoAs, as it has now been seen for benzoic acid in the horse and rabbit (Marsh et al., 1982), as well as in rats, seven marsupials (including the brushtail possum), and the echidna (a monotreme) (Awaluddin and McLean, 1985). Acyl-CoAs are intermediates in the formation of amino acid conjugates (Hutt and Caldwell, 1990), and the β-hydroxy metabolite has always been seen in animals that form acylglycine conjugates.

Although alcohols and phenols can form sulfate conjugates, there was no evidence of this occurring. Sulfate conjugates have not been reported for salicylic acid, and the brushtail possum is considered to have a low capacity for sulfatation (Baudinette et al., 1980).

Both Fotsch et al. (1989a) and we, in the present study, could recover only part of the salicin consumed. In the present study, some errors may be due to uncertainty in estimating the dose consumed and loss of some of the urine. Some metabolites may have been excreted in bile and eliminated in the feces. There is also the possibility of the formation of unknown metabolites. Chern and Dauterman (1983), using a ^{14}C label, found that oral administration of 1-naphthol-β-glucoside to mice resulted in the urinary excretion of a considerable amount (15–33% dose) of unidentifiable polar material, suggesting the formation of unknown metabolites.

This was not seen after administration of 1-naphthol or its glucuronide. As in the present study, they also found an inverse relationship between the dose of glucoside and both the fraction of dose recovered, and the proportion excreted unchanged in the urine.

Acknowledgments—We thank Mr Robert Gegg for assistance with maintenance of captive animals.

REFERENCES

AWALUDDIN, A. B., and MCLEAN, S. 1985. Conjugation of benzoic acid in marsupials. *Aust. J. Zool.* 33:693–698.

BAUDINETTE, R. V., WHELDRAKE, J. F., HEWITT, S., and HAWKE, D. 1980. Metabolism of [^{14}C] phenol by native Australian rodents and marsupials. *Aust. J. Zool.* 28:511–520.

CHERN, W. H., and DAUTERMAN, W. C. 1983. Studies on the metabolism and excretion of 1-naphthol, 1-naphthyl-β-D-glucuronide, and 1-naphthyl-β-D-glucoside in the mouse. *Toxicol. Appl. Pharmacol.* 67:303–309.

CORK, S. J. 1981. Digestion and metabolism in the koala *(Phascolarctos cinereus* Goldfuss): An arboreal folivore. PhD thesis. University of New South Wales, Australia.

EDWARDS, W. R. N. 1978. Effect of salicin content on the palatability of *Populus* foliage to opossum (*Trichosurus vulpecula*). *N. Z. J. Sci*. 21:103–106.

FOLEY, W. J., and MCARTHUR, C. 1994. The effects and costs of ingested allelochemicals in mammals: An ecological perspective, pp. 370–391, *in* D. J. Chivers and P. Langer (eds.). The Digestive System in Mammals: Food, Form and Function. Cambridge University Press, Cambridge, UK.

FOLEY, W. J., IASON, G., and MCARTHUR, C. 1999. Role of plant secondary metabolites in the nutritional ecology of mammalian herbivores—how far have we come in 25 years? pp. 203–274, *in* H.-J. G. Jung and G. C. Fahey, Jr. (eds.). Nutritional Ecology of Herbivores; Vth International Symposium on the Nutrition of Herbivores. American Society of Animal Science, Savoy, IL.

FOTSCH, G., and PFEIFER, S. 1989. Die Biotransformation der Phenolglycoside Leiocarposid und Salicin-Beispiele für Besonderheiten von Absorption und Metabolismus glycosidischer Verbindungen, *Pharmazie* 44:710–712.

FOTSCH, G., PFEIFER, S., BARTOSZEK, M., FRANKE, P., and HILLER, K. 1989. Biotransformation der Phenolglycoside Leiocarposid und Salicin. *Pharmazie* 44:555–558.

FREELAND, W. J. 1991. Plant secondary metabolites: Biochemical coevolution with herbivores, pp. 61–68, *in* R. T. Palo and C.T. Robbins (eds.). Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

FREELAND, W. J., and JANZEN, D. H. 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. *Am. Nat.* 108:269–287.

GOPALAN, V., PASTUSZYN, A., GALEY, W. R., and GLEW, R. H. 1992. Exolytic hydrolysis of toxic plant glucosides by guinea pig liver cytosolic β-glucosidase. *J. Biol. Chem.* 267:14027–14032.

HUTT, A. J., and CALDWELL, J. 1990. Amino acid conjugation, pp. 273–305, *in* G. J. Mulder (ed.). Conjugation Reactions in Drug Metabolism. Taylor and Francis, London.

KAMIMURA, H., OGATA, H., and TAKAHARA, H. 1992. α-Glucoside formation of xenobiotics by rat liver α-glucosidases. *Drug Metab. Dispos.* 20:309–315.

LAMARCO, K. L., and GLEW, R. H. 1986. Hydrolysis of a naturally occurring β-glucoside by a broad-specificity β-glucosidase from liver. *Biochem. J.* 237:469–476.

LAWLER, I. R., FOLEY W. J., ESCHLER B., PASS D. M., and HANDASYDE, K. 1998. Intraspecific variation in secondary metabolites determines food intake by folivorous marsupials. *Oecologia* 116:160–169.

LOSTAO, M. P., HIRAYAMA, B. A., LOO, D. D. F., and WRIGHT, E. M. 1994. Phenylglucosides and the Na^{+}/glucose co-transporter (SGLT1): Analysis of interactions. *J. Membr. Biol.* 142:161–170.

LOWRY, J. B., SUMPTER, E. A., MCSWEENEY, C. S., SCHLINK, A. C., and BOWDEN, B.1993. Phenolic acids in the fibre of some tropical grasses; effect on feed quality and their metabolism by sheep. *Aust. J. Agric. Res.* 44:1123–1133.

MARKHAM, K. R. 1970. A chemotaxonomic approach to the selection of opossum resistant willows and poplars for use in soil conservation. *N. Z. J. Sci.* 14:179–186.

MARSH, M. V., CALDWELL, J., HUTT, A. J., SMITH, R. L., HORNER, M. W., HOUGHTON, E., and MOSS, E. 1982. 3-Hydroxy- and 3-keto-3-phenylpropionic acids: novel metabolites of benzoic acid in horse urine. *Biochem. J.* 31:3225–3230.

MATSUMOTO, Y., OHSAKO, M., TAKADATE, A., and GOTO, S. 1993. Reduction of erythrocyte membrane permeability and protein binding of low-molecular-weight drugs following glucoside derivitization. *J. Pharm. Sci.* 82:399–403.

MULDER, G. J., COUGHTRIE, M. W. H., and BURCHELL, B. 1990. Glucuronidation, pp. 51–105, *in* G. J. Mulder (ed.). Conjugation Reactions in Drug Metabolism. Taylor and Francis, London.

PALO, R. T., and ROBBINS, C. T. 1991. Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

PASS, G. J., and FOLEY, W. J. 2000. Plant secondary metabolites as mammalian feeding deterrents: Separating the effects of the taste of salicin from its post-ingestive consequences in the common brushtail possum (*Trichosurus vulpecula*). *J. Comp. Physiol. B.* 170:185–192.

PHIPPS, A. N., STEWART, J., WRIGHT, B., and WILSON, I. D. 1998. Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between the diet, gut microflora and substrate specificity. *Xenobiotica* 28:527–537.

PRATT, W. B. 1990. The entry, distribution and elimination of drugs, pp. 201–296, *in* W. B. Pratt and P. Taylor (eds.). Principles of Drug Action. Churchill, New York.

SCHELINE, R. R. 1991. CRC Handbook of Mammalian Metabolism of Plant Compounds. CRC Press, Boca Raton, Florida.

TAHVANAINEN, J., HELLE, E., JULKUNEN-TIITTO, R., and LAVOLA, A. 1985. Phenolic compounds of willow bark as deterrents against feeding by mountain hare. *Oecologia* 65:319–323.

TREASE, G. E., and EVANS, W. C. 1972. Pharmacognosy. Balliere Tindall, London.

WHITE, J. D., and SCHWARTZ, D. P. 1980. Evaluation of Amberlite XAD-4 for recovery of glucuronides and sulfates from aqueous solution. *J. Chromatogr.* 196:303–308.