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METABOLIC FATE OF DIETARY TERPENES FROM *Eucalyptus radiata* IN COMMON RINGTAIL POSSUM (*Pseudocheirus peregrinus*)

STUART McLEAN,[1,*] WILLIAM J. FOLEY,[2] NOEL W. DAVIES,[3] SUSAN BRANDON,[1] LI DUO,[1] and ADRIAN J. BLACKMAN[4]

[1]*School of Pharmacy, University of Tasmania*
PO Box 252 C, Hobart, Tasmania, Australia

[2]*Department of Zoology, James Cook University*
Townsville, Queensland, Australia

[3]*Central Science Laboratory*

[4]*Department of Chemistry*
University of Tasmania
Hobart, Tasmania, Australia

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Abstract—Arboreal marsupials consume terpenes in quantities that are toxic to other mammals, indicating that they possess special detoxification mechanisms. The metabolic fate of dietary terpenes was studied in the common ringtail possum (*Pseudocheirus peregrinus*). Three animals were fed *Eucalyptus radiata* leaf for 10 days. Leaf consumption increased over three days to an average steady state of about 10–15 mmol total terpenes per day. GC-MS analysis identified six urinary terpene metabolites, which were dicarboxylic acids, hydroxyacids, or lactones. Another nine metabolites could only be shown to be terpene-derived but of unknown structure. The amounts excreted were estimated by GC-FID, using response factors based on carbon content. Total 24-hr excretion of terpene-derived metabolites increased to 6.2–7.6 mmol on days 5–10, while glucuronic acid excretion remained constant at about 1.5 mmol. No other conjugates of terpene metabolites were found. The strategy used by the possum to detoxify dietary terpenes seems to be to polyoxygenate the molecules forming highly polar, acidic metabolites that can be readily excreted. Conjugation is minimal, perhaps to conserve carbohydrate and amino acids.

Key Words—Terpene, possum, *Pseudocheirus peregrinus*, *Eucalyptus radiata*, metabolism, foliage, diet, urinary acids, glucuronic acid.

*To whom correspondence should be addressed.

INTRODUCTION

Although *Eucalyptus* is the dominant genus of trees in 90% of Australian forests, few vertebrate herbivores eat the foliage. This is largely due to a combination of a low nutritional quality and the presence of significant quantities of allelochemicals, in particular, polyphenols and volatile oils (Foley and Hume, 1987; Foley, 1987; Cork, 1986; Cork and Foley, 1991). *Eucalyptus* leaf contains up to 22% dry weight of volatile oil (Morrow and Fox, 1980), a complex mixture of mainly C_{10} terpenoids whose composition varies with species of *Eucalyptus*, leaf age, locality, and season (Eberhard et al., 1975). Nonetheless, some species of arboreal marsupials, including koalas (*Phascolarctos cinereus*), greater gliders (*Petauroides volans*), common brushtail possums (*Trichosurus vulpecula*), and common ringtail possums (*Pseudocheirus peregrinus*) do eat *Eucalyptus* foliage in varying amounts, and the high terpene content of *Eucalyptus* leaf presents them with a considerable toxic challenge. The koala, for example, can ingest several milliliters of *Eucalyptus* oil daily (Eberhard et al., 1975), a dose that has been fatal in humans despite their 10-fold larger body weight (Martindale, 1989). The different extent to which arboreal marsupials feed on *Eucalyptus* leaf may be due to their varying abilities to handle this potentially toxic load.

There is evidence that the terpenes of *Eucalyptus* oil are well absorbed from the gut and require metabolism before excretion in the urine, as would be expected for lipophilic substances. Eberhard et al. (1975) found that only 15% of the ingested oil from *E. punctata* appeared in the feces of koalas, and 1% was excreted unchanged in the urine. Administration of pure *Eucalyptus* terpenes to the brushtail possum (*Trichosurus vulpecula*) resulted in only trace amounts being excreted unchanged in urine and feces (Southwell et al., 1980). In the greater glider (*Petauroides volans*) and brushtail possum, *Eucalyptus* oils are almost completely absorbed before reaching the hindgut, which is the principal site of microbial activity in these species (Foley et al., 1987). Thus the gut flora are unlikely to be important in terpene metabolism in these animals.

Early reports of glycosuria in koalas and possums suggested that *Eucalyptus* terpenes are ultimately excreted as conjugates with glucuronic acid; sulfate conjugation was found to be very low (Hinks and Bollinger, 1957a,b; Roy, 1963). Southwell (1975) reported some novel monoterpenoid lactones in the urine of the koala fed *E. punctata*. The author considered that these metabolites were probably formed during isolation by cyclization of hydroxycarboxylic acids released as hydrolysis products of labile urinary glucuronides. The hydroxyacids were thought to be derived from α- and β-pinene, two of the major leaf terpenes of *E. punctata*. When the brushtail possum was dosed with pure α- or β-pinene, different metabolites were found: a carboxylic acid (myrtenic acid) and an alcohol (*trans*-verbenol), whereas the koala can apparently carry out both oxidations

to produce the hydroxyacid metabolites and subsequently the lactones (Southwell et al., 1980). Thus, it may be that terpene oxidation is more extensive in the koala which, unlike the brushtail possum, is entirely dependent on a *Eucalyptus* leaf diet.

The only other published data on oxidative metabolism of terpenes in marsupials are also from the brushtail possum: *p*-cymene was reported to be oxidized to *p*-cresol and cumic acid; and 1,8-cineole was oxidized at the 9-methyl group to the corresponding alcohol and carboxylic acid (Flynn and Southwell, 1979; Southwell et al., 1980). Recently Carman and Klika (1992) have shown that the hydroxycineole and cineolic acid metabolites of 1,8-cineole are excreted by the brushtail possum as partially racemic mixtures, with a sex difference in the enantiomeric ratio. The authors have made an interesting proposal that the racemate may act as a pheromone in this species.

The present study has examined the metabolic fate of the terpene component of *E. radiata* in the common ringtail possum (*Pseudocheirus peregrinus*).

METHODS AND MATERIALS

Animals and Urine Collection. The study was approved by the Animal Experimentation Ethics Committee of the University of Tasmania and conforms with the Australian Code of Practice for the care and use of animals for scientific purposes. Three common ringtail possums were maintained on an artificial diet (fruit and cereals), then offered *E. radiata* foliage ad libitum for 10 days. All foliage was collected from a single *E. radiata* tree at one cutting. Food intake was measured daily. All urine produced was collected from galvanized iron trays into plastic bottles sitting in flasks of liquid nitrogen and thereafter stored at $-20°C$ or below. Urine collection began on the last day of the artificial diet (day 0) and continued for the 10 days of the *Eucalyptus* diet.

In a separate experiment, possums fed the artificial diet were gavaged with doses of individual terpenes (*p*-cymene, α-phellandrene) at 0915–0930 hr and urine collected as above for 24 hr. The terpenes were obtained commercially and were redistilled before use. All were $>90\%$ pure by GC.

Analysis of Eucalyptus Oil. The oil was obtained by steam distillation of a sample of the *E. radiata* leaf and analyzed by gas chromatography–mass spectrometry (GC-MS). The instrument used was a Hewlett-Packard 5890 gas chromatograph and 5970 series mass-selective detector with 59970A Chemstation for programming and data processing (Hewlett-Packard Australia Ltd., Melbourne, Australia). *Eucalyptus* oil was dissolved in hexane (5 μl/ml) and a 1-μl aliquot injected on to a 50-m fused silica capillary column (0.3 mm ID) coated with a polyethylene glycol bonded phase (BP 20; Scientific Glass Engineering Pty. Ltd., Melbourne, Australia). Operating conditions were: helium flow 2 ml/

min; injection split ratio 10:1; oven temperature 50°C for 1 min, then increasing by 6°C/min to a final temperature of 220°C; injector 260°C; open–split interface 240°C. Accurate mass and ammonia CI data were obtained on a Kratos Concept IH mass spectrometer.

Analysis of Urinary Metabolites. Glucuronic acid (free and conjugated) in urine was measured colorimetrically by the method of Fishman and Green (1955). Urine samples were diluted 50- or 500-fold before analysis. Other metabolites were analyzed by gas chromatography of urine extracts, using the following general method. To 0.25 ml urine in a 10-ml tapered centrifuge tube was added the internal standard solution (0.5 mg 2,5-dimethylbenzoic acid dissolved in 0.25 ml aqueous base) and 0.20 ml distilled water. For hydrolysis of conjugates, 0.2 ml acetate buffer (1.1 M, pH 5.2) was added, then 0.050 ml extract of *Helix pomatia* (β-glucuronidase plus aryl sulfatase; Boehringer, Mannheim, Germany) and overnight incubation at 37°C. This step was omitted for analysis of unconjugated metabolites. The mixture was next acidified to pH 1 with 5 M hydrochloric acid and extracted with ethyl acetate (1 × 2 ml followed by 2 × 1 ml), using a vortex mixer and centrifugation to separate the phases. A 1-ml aliquot of the combined ethyl acetate extracts was placed in a clean tube and concentrated to about 0.2 ml at 30°C under a gentle stream of nitrogen. The acid metabolites were then methylated by the addition of 0.5 ml of ethereal diazomethane (Vogel, 1956). After standing in a stoppered tube in ice for 30 min, the excess diazomethane was evaporated and the sample concentrated to about 0.2 ml at 20–25°C under nitrogen before GC analysis.

Metabolites were identified by GC-MS using the instrument described above for oil analysis. Quantitative analyses were made on another Hewlett-Packard 5890 gas chromatograph with flame ionization detection (FID). For all GC analyses of metabolites a fused silica capillary column was used [25 m × 0.32 mm ID, coated with 0.52-μm cross-linked 5% phenylmethylsilicone (HP 5); Hewlett Packard, Melbourne, Australia]. Operating conditions for quantification were: carrier gas He 2 ml/min; injector 250°C; detector 300°C; oven program 60°C for 1 min, then increasing at 10°C/min to 140°C, 5°C/min to 250°C, 30°C/min to 290°C; 1 μl injected with split ratio 20:1. Similar conditions were used for GC-MS.

The efficiency of extraction of metabolites from urine was examined by separately analyzing each of the three ethyl acetate extracts and two additional extractions, and comparing the amounts of metabolites found in each.

To facilitate metabolite identification, in some experiments acidic and phenolic metabolites were separated from neutrals before methylation and GC analysis. A 3-ml aliquot of the ethyl acetate extract was extracted with 2 × 3 ml aqueous sodium bicarbonate (5%) to remove carboxylic acids, then with 2 × 3 ml aqueous sodium hydroxide (5%) to remove phenolics, leaving neutral metabolites in the ethyl acetate. The acidic and phenolic extracts were then brought

to pH 1 with 5 M HCl and extracted with ethyl acetate and methylated as usual before GC analysis.

In other experiments, the effectiveness of enzymatic hydrolysis of conjugated metabolites was compared with acid hydrolysis of the same urine samples. Urine samples for acid hydrolysis were prepared without the acetate buffer and enzyme, as for analysis of unconjugated metabolites, except that sufficient 5 M HCl was added to bring the pH to 1. The sample was then either left overnight at 26–28°C, or placed in a boiling water bath for 2 hr.

Calibration Curves. Reference compounds were not available for most of the urinary metabolites, so an indirect method was devised to estimate their amounts in urine. The method is based on the observation that the molar FID response to a substance is proportional to the number of carbon atoms present (Jorgensen et al., 1990). GC-MS analyses showed that the terpene metabolites typically retained the 10-carbon skeleton and gained two, three, or four oxygen atoms to form hydroxyacids or mono- or dicarboxylic acids. Methylation added another carbon atom for each carboxylic acid group. Accordingly, GC calibration curves were prepared for a series of mono- and dicarboxylic acids added to blank ringtail possum urine (obtained from animals not eating *Eucalyptus* leaf). The acids used were hydrocinnamic, *n*-decanoic, camphoric, phenylsuccinic, 3,4-dimethoxyphenylacetic, 3-(3,4-dimethoxyphenyl)-propionic, 3-hydroxybenzoic, and hippuric. These acids were chosen because they had 10–13 carbons after methylation or, for the last two, had been found in the urine. Analysis was by GC-FID, using 2,5-dimethylbenzoic acid as internal standard, as described above for urinary terpene metabolites. The acids were dissolved together in methanol and different volumes added to urine to give eight concentrations ranging from 0 to 4.5 mg/ml. The relationship between the slopes of the calibration curves and the number of carbons in each compound was used to estimate the slope of the calibration curve for metabolites that were not available as pure substances. For some terpenoid metabolites for which the molecular formula, and therefore the number of carbon atoms, was not known, the slope for C_{12} (corresponding to a methylated dicarboxylic acid metabolite) was used.

RESULTS

Consumption of Terpenes. Steam distillation of the *E. radiata* leaf yielded 3.3% oil by wet weight (the leaf contained 59% water). Analysis of the oil showed that eight major components accounted for 85% of terpenes in the oil: α-phellandrene, 15.8%; β-phellandrene, 5.8%; *p*-cymene, 5.3%; *trans-p*-menth-2-en-1-ol, 16.9%; terpinen-4-ol, 3.5%; *cis-p*-menth-2-en-1-ol, 15.8%; *cis*-piperitol, 6.6%; and *trans*-piperitol, 15.6%. The mean daily consumption of

leaf is shown in Figure 1A. It can be seen that the consumption of leaf increased considerably during the experiment, from about 20 g wet wt on the first day of *E. radiata* diet to nearly 80 g on days 7–9.

Excretion of Glucuronic Acid. Total glucuronic acid excretion was low for ringtail possums fed the artificial diet, but increased rapidly when they were switched to *E. radiata* (Figure 1B). The proportion of urinary glucuronic acid that was conjugated varied from 0.49 (day 4) to 0.95 (day 0), with a mean of 0.64 (SE = 3.9, $N = 11$). There was a good correlation between mean food consumption and mean total glucuronic acid excreted ($R^2 = 0.862$, $P < 0.001$, $df = 9$).

Identification of Leaf-Derived Metabolites. GC-MS analyses showed that a large number of urinary metabolites were excreted by possums feeding on *E. radiata* leaf (Figure 2). Virtually none were present when the animals were fed the artificial diet (Figure 2 inset), indicating that all the metabolites were

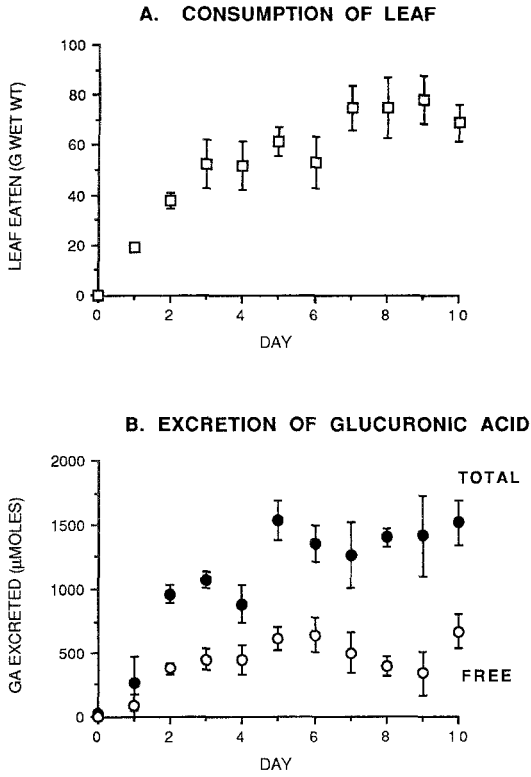

FIG. 1. (A) Consumption of leaf (g wet wt) per day. Mean (±SE) of three animals. (B) Excretion of glucuronic acid (μmoles) per day. Mean (±SE) of three animals.

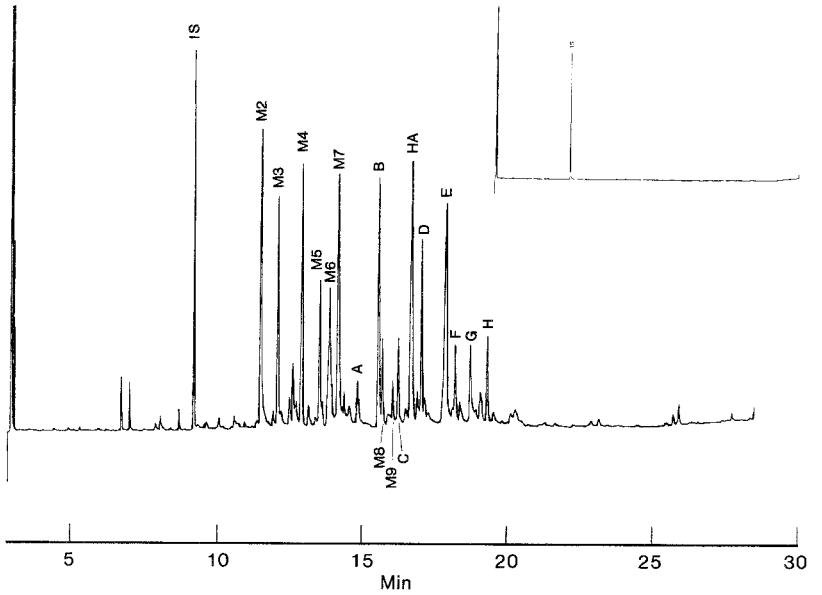

FIG. 2. GC-FID trace showing urinary metabolites (possum 1, day 5). Inset shows a GC-FID trace obtained under identical conditions using urine collected from the same animal on day 0, before the *Eucalyptus* diet. IS = internal standard, HA = hippuric acid.

leaf-derived. Analysis of the mass spectra showed the presence of hippuric acid (HA) and 3-hydroxybenzoic acid (M2), which were confirmed by comparison with the retention times and mass spectra of authentic materials (Table 1).

Other significant metabolites showed ions characteristic of the C_{10} terpenoid structure, as outlined below. Although authentic reference materials were not available, two known terpene metabolites (M5 and M8) were found and a number of other terpenoid metabolites (M3, M4, M6, M7, M9, D-F, H) were at least partially identified. For most metabolites, mass spectral analysis gave an accurate molecular ion (confirmed by low resolution CI-MS), which provided the molecular formulae (Table 1). Not all metabolites gave a molecular ion under electron impact conditions: for M6, B, and G only the CI molecular ion (MH^+) was found, and not even that was obtained for A and C.

The neutral fraction showed only two metabolites (M3 and M4), which had mass spectra similar to those of the two unsaturated lactones found by Southwell (1975) in the urine of koalas fed *E. puctata* leaf (Figure 3). All showed loss of methyl and the lactone ring to give ions at *m/z* 151 $[C_9H_{11}O_2]^+$ and *m/z* 93 $[C_7H_9]^+$, respectively. However, M3 and M4 differed in the relative abundances of ions, both from each other and from Southwell's lactones (which

TABLE 1 GC-MS DATA ON METABOLITES

Metabolite[a]	Empirical formula and calc. FW	M^+ found	Retention time (min)[b]	Significant EI ions (*m/z*) and relative abundance (%)								
M1	$C_{10}H_{11}O_3N$	193	16.65	193	161	134	105	77	51			
	193			8	8	17	100	44	18			
M2	$C_8H_{10}O$	152	11.44	152	123	121	93	65	43			
	152			45	11	100	44	28	21			
M3	$C_{10}H_{14}O_2$	166.1010	12.00	166	151	107	93	77	55			
	166.0994			22	100	28	58	27	41			
M4	$C_{10}H_{14}O_2$	166.1002	12.84	166	151	107	93	79	55			
	166.0994			28	93	40	100	59	50			
M5	$C_{11}H_{14}O_3$	194.0954	13.45	194	179	137	77	59	43			
	194.0943			1	100	24	16	30	94			
M6	$C_{11}H_{16}O_3$	197[c]	13.78		178	105	91	88	79	57		
	196				7	15	95	100	75	49		
M7	$C_{11}H_{18}O_3$	198.1279	14.09	198	183	166	155	139	128	96	69	59
	198.1256			13	17	24	17	15	11	100	33	21
M8	$C_{12}H_{14}O_4$	222.0898	15.61	222	191	163	131	103	77	59		
	222.0892			29	15	100	15	16	14	18		
M9	$C_{12}H_{16}O_4$	224.1054	15.96	224	193	165	137	105	91	88	77	59
	224.1048			31	11	15	45	100	24	20	25	28
A			14.77	163	152	137	93	91	59			
				6	15	17	77	48	100			
B		195[c]	15.47	194	164	163	137	105	88	77	59	
				7	39	55	58	71	100	56	56	
C			16.16	151	119	107	105	91	88	59		
				98	77	27	23	90	100	50		

D	$C_{13}H_{18}O_4$[d]	238.1204	16.96	238	223	151	137	119	91	88	59
	238.1205			2	3	47	32	73	100	26	26
E	$C_{11}H_{18}O_4$	214.1211	17.80	214	196	181	137	127	79	59	
	214.1205			1	5	10	64	100	45	62	
F	$C_{11}H_{14}O_4$	210.0881	18.10	210	195	179	151	105	91	79	59
	210.0888			59	100	53	39	32	54	52	41
G		273[c]	18.66		182	123	77	69	59	55	
					60	100	5	10	8	8	
H	$C_{11}H_{14}O_4$	210.0873	19.22	210	195	179	151	107	91	79	59
	210.0888			68	100	18	31	48	82	77	66

[a] Acids as their methyl ester.
[b] GC-FID conditions.
[c] MH^+ by CI-MS.
[d] One extra CH_2 probably from methylation of a double bond.

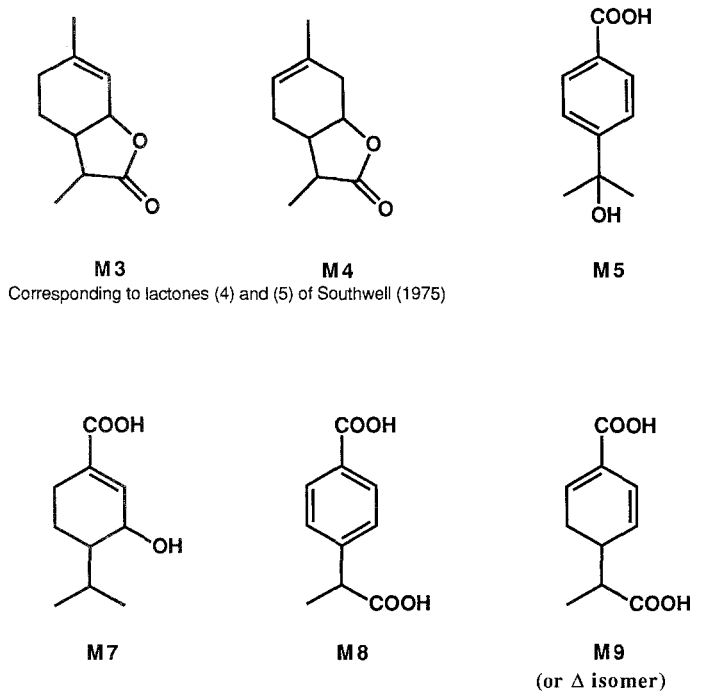

FIG. 3. Terpene metabolites found in urine. M3 and M4 probably differ from the lactones shown in the position of the double bond and in their stereochemistry. The position of the double bond in M7 is uncertain. See Results for details.

had identical mass spectra). M3 and M4 were partially purified by thin-layer chromatography, and NMR spectra indicated that M3 gave the same shifts as Southwell's lactone (4), while M4 corresponded to lactone (5). It is possible that M3 and M4 differ in the position of the double bond and in stereochemistry, as there are three chiral centers that could give rise to a number of possible diastereomers.

All other metabolites appeared in the carboxylic acid fraction and were analyzed as their methyl esters. Metabolites M5 and M8 have been reported previously as metabolites of *p*-cymene in the rat and guinea pig (Walde et al., 1983) and were also detected (by GC-MS) after administration of pure α-phellandrene or *p*-cymene to possums. Their mass spectra agreed with those found by Walde et al. (1983) and were consistent with the structures shown in Figure 3. Accurate mass data on M5 identified the following fragments: *m/z* 179 $C_{10}H_{11}O_3$ $[M\text{-}CH_3]^+$, *m/z* 137 $C_8H_9O_2$ $[M\text{-}C_3H_5O]^+$, and *m/z* 77 C_6H_5 (the ring structure). Major fragmentations for M8 were to *m/z* 191 $[M\text{-}CH_3O]^+$, *m/z*

163 $[M\text{-}COOCH_3]^+$, *m/z* 77 C_6H_5 (the ring), and *m/z* 59 $C_3H_3O_2$ (the carboxymethyl group).

Based on their molecular formulae, metabolites M6 and M7 are likely to be unsaturated hydroxy acids. The structure of M7 (Figure 3) is proposed to account for ions *m/z* 183 $C_{10}H_{15}O_3$ $[M\text{-}CH_3]^+$, *m/z* 166 $C_{10}H_{14}O_2$ $[M\text{-}CH_3OH]^+$, *m/z* 155 $C_8H_{11}O_3$ $[M\text{-}C_3H_7]^+$, *m/z* 139 $C_9H_{15}O$ $[M\text{-}COOCH_3]^+$, and *m/z* 128 $[C_6H_8O_3]^+$ formed by the retro-Diels-Alder elimination of C_5H_{10}, followed by the loss of CH_3OH to form an unsaturated lactone $C_5H_4O_2$ at *m/z* 96.

The mass spectrum of M9 was similar to that of M8 except that the molecular ion and several others were heavier by two hydrogens (Table 1). The same metabolite was found in urine of a possum administered α-phellandrene (a terpene with two double bonds), and M9 has been assigned a corresponding structure. The mass spectrum showed ions at *m/z* 193 $[M\text{-}OCH_3]^+$, *m/z* 165 $[M\text{-}COOCH_3]^+$, *m/z* 137 $[M\text{-}C_4H_7O_2]^+$, and *m/z* 105 $[M\text{-}COOCH_3\text{-}COOCH_3]^+$, and the formation of phenyl (*m/z* 77 C_6H_5) and carboxymethyl (*m/z* 59 $C_2H_3O_2$) ions. The ion at *m/z* 88 $C_4H_8O_2$ was also found for M6, B, C, and D and may arise from a McLafferty rearrangement of a hydrogen in the cyclohexane ring to the carbonyl on the isopropyl side chain.

Metabolites A–C could not be characterized from their mass spectra, but the ions were those expected for acidic terpene metabolites (as outlined above): *m/z* 59 ($C_2H_3O_2$), *m/z* 77 (C_6H_5), *m/z* 88 ($C_4H_8O_2$), *m/z* 91 (C_7H_9), and *m/z* 93 (C_7H_{11}). There is less evidence that metabolite G is terpenoid, but it is leaf-derived and has a similar molecular weight and so was provisionally grouped with the others.

Quantification of Other Leaf-Derived Metabolites. For each reference compound, an excellent linear relationship was found between peak area ratio (compound/internal standard) and amount added to urine ($R^2 \geq 0.999$) and the intercept was close to zero. There was a reasonably good correlation between the slopes of the individual calibration curves and the number of carbon atoms in each compound, from eight to twelve for the methylated acids ($R^2 = 0.729$, $P < 0.05$, $df = 6$). The equation of the line of fit was slope ($\times 10^2$) $= -1.55 + 0.85$ (no. carbon atoms). When the amounts of 3-hydroxybenzoic acid and hippuric acid excreted by possum 1 on days 0–10 were calculated using both the actual calibration curve for each compound and that estimated from the general slope versus carbon number relationship, there was no significant difference (paired *t* test: for M2, $P = 0.001$, $df = 7$; for HA, $P = 0.002$, $df = 8$). The unknown terpenoid metabolites A–C and G were assumed to have 12 carbons (i.e., to be methylated dicarboxylic acids) for the purpose of estimating their concentrations in urine.

Recovery experiments showed that very little of each metabolite remained after the third extraction with ethyl acetate. Three extractions removed 95–100% of most metabolites, except for metabolite C for which 93% was recovered.

Comparison of different methods of hydrolysis showed that enzymatic hydrolysis yielded as much or more of each free metabolite as either of the acid hydrolyses.

Time Course of Excretion of Leaf-Derived Metabolites. The daily excretion of hippuric acid (HA) and total 3-hydroxybenzoic acid (M2) is shown in Figure 4. The mean excretion of each increased with mean food consumption (for HA, $R^2 = 0.805$, $df = 9$, $P < 0.01$; for M2, $R^2 = 0.765$, $df = 9$, $P < 0.01$). The excretion of the terpenoid metabolites M3–M9 (after hydrolysis of conjugates) is shown in Figure 5. This also increased with daily food consumption ($R^2 = 0.826$, $df = 9$, $P < 0.01$). The major terpenoid metabolite was the hydroxyacid M7, followed by approximately equal amounts of M3, M4, M5, and M6, and smaller amounts of M8 and M9.

The excretion of the unknown metabolites A–H also increased over the first few days of the *Eucalyptus* diet, and then stabilized in amounts ranging from approximately 100 to 1000 μmol/day. The mean daily excretion of metabolites A–H was highly correlated with dietary leaf consumption ($R^2 = 0.775$, $P < 0.01$, $df = 9$). The A–H excretion data are not shown separately but are included with M3–M9 as total terpenoid metabolites in Figure 6.

Conjugated Metabolites. Only a minor proportion of the leaf metabolites was excreted as conjugates. Figure 6 shows this for the total terpenoid metabolites (i.e., M3–M9 plus A–H), where the amounts found after hydrolysis were only slightly greater than those found in unhydrolyzed urine ($t = 2.262$, $P = 0.047$, $df = 10$). The same pattern applied to the individual terpenoid metabolites and to 3-hydroxybenzoic acid (M2), which, while lacking the terpene skeleton, can also be conjugated with glucuronic acid.

By the second day of the terpene diet, the excretion of total conjugatable metabolites (Figures 4 and 6) far exceeded the total urinary glucuronic acid

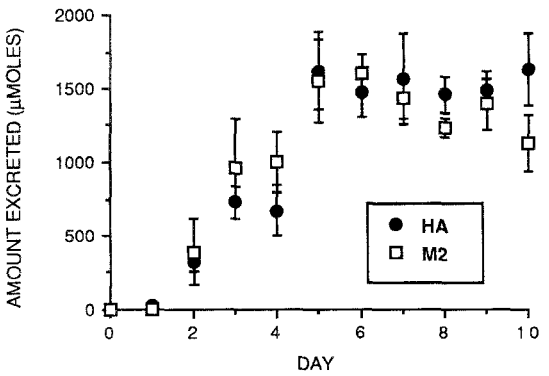

FIG. 4. Daily excretion of hippuric acid (HA) and total (free plus conjugated) 3-hydroxybenzoic acid (M2). Data are the mean (±SE) of three animals.

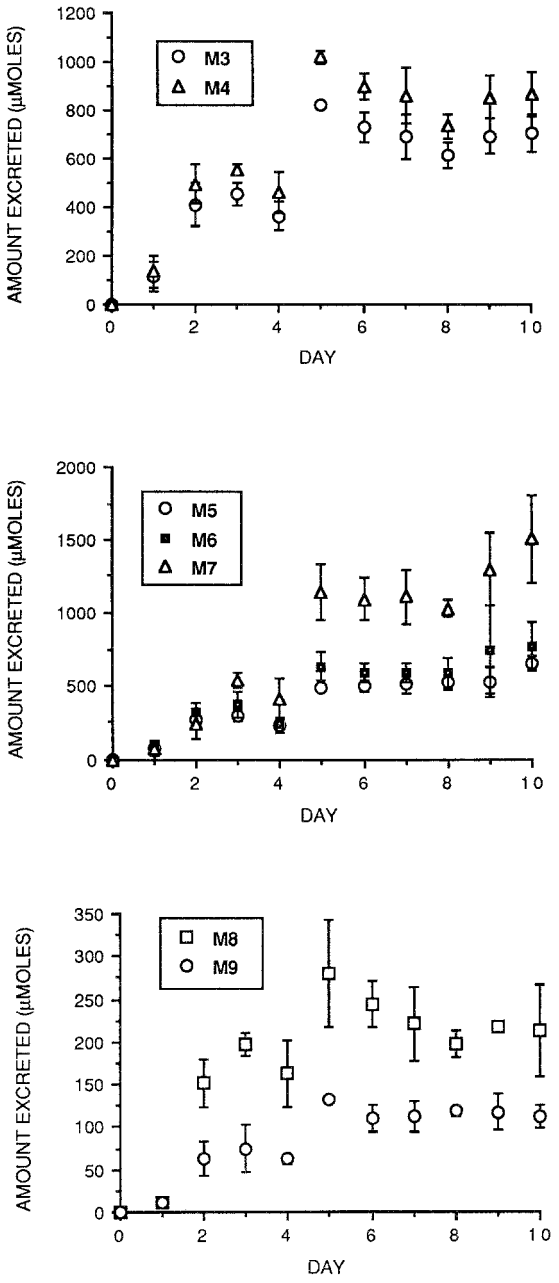

FIG. 5. Daily excretion of metabolites M3–M9. The total amount of each metabolite (free plus conjugated) is shown, as the mean (±SE) of three animals.

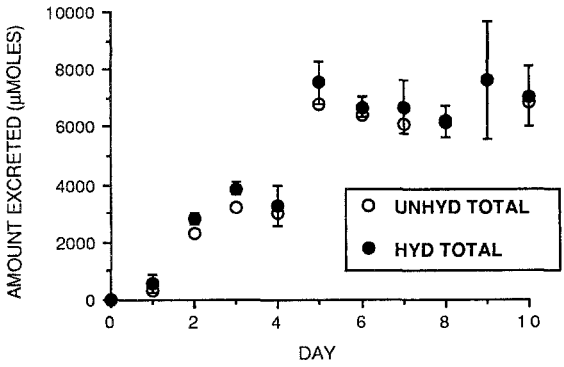

FIG. 6. Daily excretion of total of all terpene metabolites (M3–M9 plus A–H). UNHYD = before hydrolysis, HYD = after hydrolysis. Data are the mean (±SE) of three animals, except that the SE values have been omitted from the unhydrolyzed data for clarity.

(Figure 1B), supporting the observation that the metabolites are excreted mostly in the free form (Figure 6). Hippuric acid is the glycine conjugate of benzoic acid, and a GC-MS search was made for other possible glycine conjugates of carboxylic acids (especially M2), but none was found.

The proportion of total metabolites excreted as glucuronides was greatest (60%) on the first day of the *Eucalyptus* diet and declined thereafter (to 15–30%), although the total excretion of glucuronic acid peaked on day 5 (Figure 1B).

Accounting for Terpenes Consumed. The principal dietary terpenes had formula weights of 154 (the menthenols and piperitols, $C_{10}H_{18}O$), 134 (*p*-cymene, $C_{10}H_{14}$), and 136 (the phellandrenes, $C_{10}H_{16}$). In order to estimate the recovery of dietary terpenes as urinary metabolites, they were assigned a formula weight of 150 and the daily intake of *Eucalyptus* oil (in grams) was expressed as micromoles terpenes per day. This rose from a mean of 3.8 (SE 0.2) μmoles on the first day to a maximum of 15.6 (SE 1.9) μmol on day 9. The excretion of total terpene-derived metabolites (M3–M9 and A–H) is shown as a percentage of the dietary consumption of terpenes in Figure 7. Both M2 and hippuric acid were excluded as they lack the 10-carbon terpene skeleton. The proportion of dietary terpenes accounted for in the urine increased to an approximate steady state of one half after five days.

DISCUSSION

Eucalyptus radiata foliage was used in these experiments because it is an important natural diet item of common ringtail possums, and it is known to be rich in terpenes (Foley et al., 1987), a finding confirmed in this study. Although

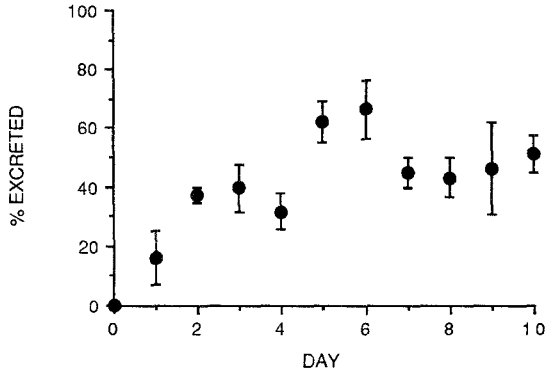

FIG. 7. Percentage of dietary terpenes found as urinary metabolites each day. Data are the mean (±SE) of three animals.

Boland et al. (1991) found cineole and piperitone to be major components of *E. radiata*, Foley et al. (1987) reported a different (and variable) composition. There may be up to six chemotypes in this species (Boland et al., 1991).

When offered the *E. radiata* leaf diet, the possums consumed small amounts at first, increasing their intake over several days, as has been reported previously (Foley, 1992). Glucuronic acid excretion increased with leaf consumption, to a maximum steady-state level of about 1.5 mmol/day. Dash (1988) reported a similar excretion rate of glucuronic acid in brushtail possums fed *E. melliodora* leaf. The free glucuronic acid is most likely the result of glucuronide conjugates being hydrolyzed in the bladder or in the urine after collection. Marsh (1969) found evidence of considerable β-glucuronidase activity in the urine of the brushtail possum. There was negligible glucuronic acid excreted by possums on the non-*Eucalyptus* diet, indicating that it is excreted as part of the process of eliminating *Eucalyptus* metabolites as glucuronide conjugates.

Hippuric acid (HA) has been found in many species that have dietary sources of benzoic acid (Bridges et al., 1970). It is the major metabolite of benzoic acid in the brushtail possum and six other marsupials, although small amounts were also glucuronidated (Awaluddin and McLean, 1985). Benzoyl glucuronide was not specifically tested for in the present study, and small amounts may have escaped detection. 3-Hydroxybenzoic acid (M2) might also have been expected to be conjugated with glycine, as its 2-isomer (salicylic acid) is extensively metabolized by this pathway in humans (Levy, 1965). However, no glycine conjugates were found for this or any of the other carboxylic acid metabolites. Salicylic acid is excreted largely unchanged by the rabbit (Williams, 1959b) and horse (Marsh et al., 1981), although both species quantitatively convert benzoic acid to its glycine conjugate. Separate enzymes may catalyze

these two reactions (Marsh et al., 1981). Only a small fraction of M2 was excreted in conjugated form each day (the mean ranged from 3 to 14%), except on day 2 when 53% was excreted as the conjugate (of a low total of 389 μmol).

Metabolites with a 10-carbon skeleton are more clearly derived from terpenes, and six were structurally identified and another five assigned a molecular formula (Table 1 and Figure 3). The pattern of formation of these metabolites involved oxidation of methyl groups to the corresponding carboxylic acids, and oxidation of other carbons to secondary or tertiary alcohols. Several metabolites were hydroxyacids (M5–M7) or their lactone derivatives (M3 and M4), the latter presumably formed in urine or during the work-up of samples. Being lipophilic, the lactones would be expected to be only slowly excreted by mammalian kidneys.

Early work on monoterpene metabolism in sheep and rabbits found oxidation to monoalcohols and monocarboxylic acids (Williams, 1959a). The rabbit oxidized *p*-cymene and other monoterpenes predominantly at a single carbon, producing the corresponding alcohol or monocarboxylic acid (Ishida et al., 1977, 1979, 1981, 1989; Asakawa et al., 1988). The guinea pig and rat similarly formed monoalcohol or monoacid metabolites of *p*-cymene, while the rat also produced some hydroxyacids (including M5) and the dicarboxylic acid M8 (16% dose) (Walde et al., 1983). Other major metabolites were cumic acid (19% dose in the rat) and its glycine conjugate (31% in guinea pig), neither of which was found in the ringtail possum. Menthol was found to be oxidized in rats successively by C-8 hydroxylation and C-1 carboxylation (Madyastha and Srivatsan, 1988).

In comparing these findings with previous reports, the extent of oxygenation of terpenes appears to be greater in ringtail possums than eutherians or even brushtail possums. Given that ringtail possums are more dependent on a *Eucalyptus* leaf diet than brushtail possums (Cork and Foley, 1991), it would not be surprising for this species to develop better systems for detoxification of terpenes.

Only a small proportion of metabolites were found as conjugates, and it is unlikely that there was significant conjugation that escaped detection. Sulfate conjugates would have been hydrolyzed by the enzyme mixture used, and in any case appear to be not formed in the possum (Roy, 1963). Acyl glucuronides are known to undergo rearrangements to isomers that are resistant to enzymatic hydrolysis (Faed, 1984), but this possibility was excluded by the demonstration that acid hydrolysis produced no more free metabolites than the enzymatic method.

The relatively low ceiling for glucuronide excretion is puzzling and may be a mechanism to conserve energy, by minimizing the loss of carbohydrate. For example, Cork (1986) calculated that excretion of glucuronic acid in koalas represented some 20% of the fasting glucose production. However, *Eucalyptus*

leaves contain significant amounts of ascorbate that could also serve as a precursor for glucuronic acid (Dash, 1988), and so the magnitude of the energy saving is not clear. It is also striking that benzoic acid was the only acid conjugated with glycine, although eutherians use this pathway extensively for elimination of carboxylic acids, and in this case the strategy may be to conserve nitrogen. Although *Eucalyptus* leaf is a poor source of nitrogen (Cork, 1986; Foley and Hume, 1987), ringtails possess effective systems for recycling and conserving nitrogen and again the potential saving is hard to estimate.

Ringtails fed *E. radiata* foliage excrete an acid urine (pH 5.7) that is rich in ammonium ion, and the speed of acid disposal may limit the rate at which animals can consume leaf (Foley, 1992). If this is true, the low level of conjugation could be disadvantageous because the renal clearance of glucuronide and other conjugates is generally much faster than that of the unconjugated metabolites (Caldwell, 1982; Moller and Sheikh, 1983), which presents the ringtail possum with the potential problem of accumulation of the carboxylic acid precursors. The ringtail possum may have a particularly efficient system to secrete organic acids across the renal tubular epithelium, and perhaps the extensive oxidation of the terpenes facilitates the renal clearance of unconjugated metabolites. Although a study of renal transport using cortical slices from the brushtail possum concluded that overall it was not significantly different from eutherians, it may be significant that penicillin G did not interact with the acid uptake system in the possum as it does in eutherians (Miller and Morris, 1982). There may also be quantitative differences, and these should be investigated.

After a few days of the leaf diet, over half of the ingested terpenes could be accounted for as urinary metabolites indicating that, despite the considerable uncertainties in estimating the levels of unknown terpenoid metabolites, the metabolite excretion data are approximately correct. The gradual increase in consumption of leaf over several days may reflect the possum's need to increase its capacity to detoxify the terpenes. Some terpenes are able to induce the synthesis of hepatic drug-metabolizing enzymes (Madyastha and Srivatsan, 1988), and substrates for renal tubular secretion can induce this transport system (Moller and Sheikh, 1983). A requirement for such adaptation may explain the slow increase in leaf consumption by possums offered the *Eucalyptus* diet. The interactions between pathways of metabolism of allelochemicals, nutrient requirements, and animal feeding have been little considered in previous studies of the interactions between mammals and woody plants. Clearly, such studies can help to explain the different effects of plant chemical defences on closely related herbivores.

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