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# METABOLISM OF *trans*-CINNAMIC ACID IN THE RAT AND THE MOUSE AND ITS VARIATION WITH DOSE

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**Abstract**—The metabolism of [3-$^{14}$C/phenyl-$^{2}H_5$] cinnamic acid was investigated in rats and mice at a dose level of 2.5 mmol/kg body weight. Recoveries of the $^{14}$C dose were between 92 and 98% with most (82–90%) present in the 0–24 hr urine samples. Urinary metabolites were identified by their chromatographic properties and mass spectra. In both species the major metabolite was hippuric acid, which is also an endogenous urinary component. Several minor metabolites, 3-hydroxy-3-phenylpropionic acid, benzoic acid and benzoyl glucuronide, were found in both species. Two, acetophenone and cinnamoylglycine, the glycine conjugate of cinnamic acid, could be positively identified only in mouse urine. The effect of dose size on the urinary excretion of $^{14}$C-cinnamic acid metabolites was studied over the dose range 0.0005 to 2.5 mmol/kg. In the rat the pattern of metabolite excretion was very similar over the whole dose range with slight increases in the proportion of the dose excreted as minor metabolites as dose size increased. In the mouse the excretion of cinnamoylglycine was more important at the lowest dose level and decreased in relative importance as dose size increased. Changes in the other metabolites were similar to those seen in the rat.

## INTRODUCTION

*trans*-Cinnamic (3-phenyl-2-propenoic) acid, hereinafter named cinnamic acid, is ubiquitous in the plant kingdom and is essential in lignin formation. It is derived from the action of L-phenylalanine ammonia-lyase (EC 4.3.1.5) on L-phenylalanine forming ammonia and cinnamic acid (Goodwin and Mercer, 1972). Plants convert cinnamic acid to *p*-hydroxycinnamic (*p*-coumaric) acid, one of the more important precursors of lignin that is converted to polyphenolic alcohols which readily polymerize to form lignin.

Cinnamic acid is used in flavour compositions and may reach 40 ppm in ice cream (Arctander, 1969). FEMA (Flavor and Extract Manufacturers' Association) have given cinnamic acid GRAS (Generally Recognized As Safe) status (FEMA, 1965) and it is also approved for food use by the Food and Drug Administration of the United States. Cinnamic acid is included in the Council of Europe (1981) Acceptable Daily Intake (ADI) of 1.25 mg/kg body weight for total cinnamyl compounds.

Under normal circumstances, dietary exposure to cinnamic acid generally arises from cinnamate esters, which are readily hydrolysed in the organism. These esters can be either of natural origin, such as methyl cinnamate, or synthetic, such as isoamyl cinnamate; they are widely used in flavour and fragrance mixtures as they can provide either 'spicy' or 'fruity' notes. Because of the complex nature of most flavours and fragrances, the esters are rarely used in isolation, and mixtures of these compounds and other aromatic agents are commonplace.

To date there have been two reviews of the pharmacology, toxicology and metabolism of cinnamic acid (Hoskins, 1984; Opdyke, 1978) and readers are referred to these for further information. Cinnamic acid was the first compound shown to undergo the now generally recognized biphasic sequence of xenobiotic metabolism and the work of Ure in the early 1840s (see Hutt and Caldwell, 1990) showed that there was an increase in urinary excretion of hippuric acid after ingestion of this compound. This result was confirmed by Dakin (1909) with more extensive studies in which dogs and cats were given large doses of ammonium cinnamate. In addition to hippuric acid, Dakin was able to identify 3-hydroxy-3-phenylpropionic acid and acetophenone in urine. Cinnamoylglycine was also tentatively identified in cat urine but the significance of this finding was uncertain.

Since these early experiments various workers have studied the metabolism of cinnamic acid in a number of species (rat: Fahelbum and James, 1977; Teuchy

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*Abbreviations*: ADI = acceptable daily intake; FEMA = Flavor and Extract Manufacturers' Association of the USA; GC-MS = gas chromatography–mass spectrometry; GRAS = generally recognized as safe; NMR = nuclear magnetic resonance.

and van Sumere, 1971; rabbit: El Masry *et al.*, 1956; Fahelbum and James, 1977; dog: Raper and Wayne, 1928; human: Hoskins *et al.*, 1984; Snapper *et al.*, 1940). In all cases, hippuric acid was the major metabolite but in most reports identification of other metabolites was only tentative. Information about the other metabolites has been difficult to obtain, the major reason for this being the nature of the analytical techniques used. Most of the work has involved the use of either classical chemistry or paper and thin-layer chromatography. Additionally, several of the metabolites, notably hippuric acid, are also normal urinary components, which has hampered attempts at quantitative analysis.

There is now increased concern about the safety-in-use of cinnamyl compounds in general, triggered largely by cinnamaldehyde (WHO, 1989) and cinnamyl anthranilate (Caldwell *et al.*, 1985; *Federal Register*, 1985). There is thus a need for comprehensive metabolic studies with up-to-date techniques. To underpin the evaluation of this whole series of compounds, we have investigated the fate of cinnamic acid in rats and mice, using $^{14}C$ labelling for quantification combined with $^{2}H$ labelling to allow unequivocal identification of those cinnamic acid metabolites that also occur as endogenous urinary components.

Current toxicological wisdom relies on the use of very high dose animal studies as predictors of the human situation. However, at the high doses of chemicals generally used for such studies the metabolic fate of these compounds can differ from the pattern seen at lower levels of exposure in the same species. Such a situation arises when the capacities of the various primary mechanisms involved in the absorption, distribution, metabolism and excretion of a compound are overwhelmed and secondary mechanisms come into play. The combined effect of overwhelming these processes has been referred to as 'metabolic breakthrough' by Feron and Kroes (1986). The outcome is that high to low dose extrapolation for a given species can be complicated by any process that does not have a linear dose-response relationship over the dose range of interest. To provide information about cinnamic acid metabolism in rodents over a range of doses, $^{14}C$-cinnamic acid was administered to rats and mice at a number of dose levels within the range 0.0005 to 2.5 mmol/kg body weight (0.074–370 mg/kg). The top of this dose range was the maximum dose tolerated without apparent discomfort to the animals. The lowest dose approximates to human exposure to cinnamic acid as calculated by FEMA, who estimated the per capita intake of cinnamic acid to be about 4 mg/day, equivalent to 0.0003 mmol/kg body weight for a 70-kg individual (FEMA, 1978). The Council of Europe ADI for all cinnamyl compounds has been provisionally set at 1.25 mg/kg (Council of Europe, 1981) and is therefore covered by the range of dose levels chosen for these studies.

## MATERIALS AND METHODS

### *Chemicals*

Benzoic acid, hippuric acid, acetophenone, *trans*-cinnamic acid and *p*-hydroxycinnamic acid were purchased from Aldrich Chemical, Gillingham, Dorset, UK. [2,3,4,5,6-$^{2}H_5$]-Benzaldehyde (98 atom% deuterium) was purchased from MSD Isotopes (Cambrian Gases, Croydon, UK). Benzoyl glucuronide and 3-hydroxy-3-phenylpropionic acid were samples prepared in this laboratory (Baldwin *et al.*, 1959; Marsh *et al.*, 1982). All other common laboratory reagents were of analytical grade.

[3-$^{14}C$]Cinnamic acid, specific activity 2.34 mCi/mmol, was purchased from CEA (Gif-Sur-Yvette, France). The commercial material was a mixture of *cis*- and *trans*-isomers; the *trans*-isomer was isolated by HPLC using system 1 (see below). The fraction of eluant co-eluting with *trans*-cinnamic acid was collected, and extracted with diethyl ether; the ether was dried with anhydrous $Na_2SO_4$ and evaporated under reduced pressure. The residue was taken up in hexane and stored at −20 C until used. This material was shown by radio-HPLC in solvent systems 1 and 2b to be more than 99.5% pure.

### *[phenyl-$^{2}H_5$]Cinnamic acid*

The reaction used for the synthesis of [$^{2}H_5$]cinnamic acid was adapted from Murray and Williams (1958). [*phenyl*-$^{2}H_5$]Benzaldehyde (2 ml) was mixed with malonic acid (2.18 g), anhydrous $Na_2SO_4$ (1 g), piperidine (0.25 ml) and pyridine (5 ml) and the whole heated for 2 hr at 100 C. The temperature was then increased to 120 C for a further 2 hr. After cooling, the reaction mixture was poured into 100 ml iced water and 13 ml concentrated HCl added slowly, with stirring. A white precipitate of the title compound formed, which was filtered and recrystallized twice from boiling water. After drying the yield was 2.09 g (73% of theory) m.p. 131–132 C (literature value 133 C for protonated cinnamic acid).

Gas chromatography–mass spectrometry of [$^{2}H_5$]cinnamic acid methylated with diazomethane gave a spectrum with the major fragmentation ions 5 atomic mass units (amu) higher than the equivalent ions in the spectrum of methylated unlabelled cinnamic acid:

*Methyl cinnamate*: m/z (relative abundance) 162 (37, $M^+$); 161 (15, M-$H^+$); 132 (10); 131 (100, $B^+$); 103 (65); 102 (14); 77 (42); 51 (35); 50 (10).

*Methyl [$^{2}H_5$]cinnamate*: m/z (relative abundance) 167 (56, $M^+$); 165 (12, M-$D^+$)" 137 (10); 136 (100, $B^+$); 108 (46); 107 (9); 81 (11); 54 (10).

The $^{1}H$-NMR spectrum of [$^{2}H_5$]cinnamic acid in $CD_3OD$ shows the loss of the coalesced signals at 7.4–7.6 ppm from the phenyl ring protons seen with unlabelled cinnamic acid. Other chemical shifts due

Table 1. Retention times of cinnamic acid and related compounds used as standards in various HPLC systems

| Standard | HPLC $R_t$ values (min) | | |
|---|---|---|---|
| | System 1 | System 2a | System 2b |
| Hippuric acid | 2.6 | 6.4 | 4.2 |
| Benzoyl glucuronide | 2.8 | 9.0 | 4.4 |
| 3-Hydroxy-3-phenylpropionic acid | 3.8 | 11.6 | 7.0 |
| Cinnamoylglycine | 5.9 | 29.2 | 12.6 |
| Benzoic acid | 7.0 | 24.0 | 14.0 |
| Acetophenone | 7.6 | 32.0 | 15.6 |
| Cinnamyl alcohol | 10.5 | >90.0 | 23.6 |
| Cinnamaldehyde | 11.3 | >90.0 | 26.8 |
| Cinnamic acid | 15.8 | >90.0 | 30.2 |

to protons in the side-chain are virtually identical for both analogues.

*Cinnamic acid*: $\delta$ ppm 7.67 (d, 1, Ar-CH = CH); 7.6–7.4 (m, 5, Ar); 6.47 (d, 1, CH = CH-$CO_2$H); 4.98 (broad singlet, -$CO_2$H).

*[$^2H_5$]Cinnamic acid*: $\delta$ ppm 7.67 (d, 1, Ar-CH = CH); 6.47 (d, 1, CH = CH-$CO_2$H); 5.04 (broad singlet, -$CO_2$H).

*Cinnamoylglycine*

*trans*-Cinnamoylglycine was synthesized from purified *trans*-cinnamic acid and glycine ethyl ester. Glycine ethyl ester hydrochloride (14 g) was dissolved in water (50 ml) and shaken with diethyl ether (500 ml) and 20% NaOH (25 ml). After separating the layers the aqueous phase was discarded, the ether layer dried with anhydrous $Na_2SO_4$ and the ether evaporated under reduced pressure. The crude glycine ethyl ester (3.3 g) was dissolved with *trans*-cinnamic acid (4.8 g) in 75 ml 1,4-dioxan. *N,N*-Dicyclohexylcarbodiimide (6.6 g) was then added and the reaction mixture stirred for 24 hr. Glacial acetic acid (5 ml) was added to precipitate the substituted urea by-product, which was removed by filtration. After evaporation of the 1,4-dioxan, the residue was dissolved in 0.2 M NaOH (100 ml) and heated in a water-bath at 60 C for 1 hr. The solution was acidified with 10 ml concentrated HCl and the precipitated cinnamoylglycine collected by filtration. The product was washed twice with cold diethyl ether (2 × 20 ml) and twice recrystallized from hot ethanol. Melting point was 192 C (literature value 193 C), yield 1.03 g (17%) of theory. Mass spectral and NMR data were in agreement with the proposed structure.

MS (direct insertion at 70 eV) m/z (relative abundance) are: 205 (3, $M^+$); 161 (31; M-$COO^+$); 132 (13); 131 (100, $B^+$; $C_6H_5CH$ = CHCO); 104 (9); 103 (17); 77 (51); 51 (28).

$^1$H-NMR in dimethyl sulfoxide (DMSO)-$d_6$ gave the following chemical shifts: $\delta$ ppm 8.4 (t, 1, -CO-NH-$CH_2$); 7.4 (d, 1, Ar-CH = CH coalesced with signals due to aromatic protons at 7.45 (m, 5, Ar); 6.7 (d, 1, -CH = CH-CO-); 3.9 (d, 2, -NH-$CH_2$-CO).

*Animals and dosing*

Male Fischer 344 rats weighing 150–200 g (Oxford Laboratory Animal Co., Oxford, UK) and male CD-1 mice weighing 30–35 g (Charles River UK, Ltd, Margate, UK) were maintained on Oxoid 41B pellets and tap water *ad lib.* during the experiments.

For the metabolite identification studies, dose solutions were prepared by dilution of the radioactive material with [$^2H_5$]cinnamic acid. The animals were dosed with 2.5 mmol/kg body weight equivalent to 383 mg/kg for [$^2H_5$]cinnamic acid. Doses were administered as aqueous solutions after neutralization with 5 M NaOH; rats were dosed orally and mice ip; rats received 10–20 $\mu$Ci and mice 1–5 $\mu$Ci.

Table 2. Recovery of radioactivity after administration of [$^{14}$C]- or [$^{14}$C/$^2H_5$]cinnamic acid at various dose levels to male Fischer 344 rats

| Compound and dose (mmol/kg) | Percentage of administered dose recovered in period | | | | | |
|---|---|---|---|---|---|---|
| | 0–24 hr | | 0–72 hr | | | |
| | Urine | Faeces | Urine | Faeces | Carcass | Total* |
| [$^{14}$C]Cinnamic acid (0.0005) | 74.1 ± 2.3 | 0.5 ± 0.4 | 78.3 ± 0.8 | 0.9 ± 0.3 | 0.4 ± 0.2 | 84.0 ± 3.4 |
| [$^{14}$C]Cinnamic acid (0.005) | 73.0 ± 5.8 | 1.0 ± 0.5 | 79.0 ± 6.5 | 2.0 ± 0.4 | 0.2 ± 0.1 | 84.6 ± 5.7 |
| [$^{14}$C]Cinnamic acid (0.05) | 79.8 ± 6.8 | 0.9 ± 0.7 | 85.5 ± 3.7 | 1.6 ± 1.2 | 0.3 ± 0.1 | 92.5 ± 2.4 |
| [$^{14}$C]Cinnamic acid (0.5) | 72.5 ± 5.8 | 0.5 ± 0.2 | 79.0 ± 4.8 | 0.9 ± 0.4 | 0.3 ± 0.1 | 83.5 ± 3.7 |
| [$^{14}$C]Cinnamic acid (2.5) | 87.8 ± 3.9 | 0.8 ± 0.2 | 92.9 ± 3.6 | 1.3 ± 0.4 | 0.7 ± 0.2 | 96.7 ± 2.8 |
| [$^{14}$C/$^2H_5$]Cinnamic acid (2.5) | 82.4 ± 6.9 | 0.9 ± 0.7 | 88.1 ± 6.5 | 1.1 ± 0.9 | 1.4 ± 0.4 | 91.7 ± 5.6 |

*Total results include cage washings.
Values are means ± SD, n = 4.

Table 3. Recovery of radioactivity after administration of [$^{14}$C]- or [$^{14}$C/$^2H_5$]cinnamic acid at various dose levels to male CD-1 mice

| Dose and level (mmol/kg) | Percentage of administered dose recovered in period | | | | | |
|---|---|---|---|---|---|---|
| | 0–24 hr | | 0–72 hr | | | |
| | Urine | Faeces | Urine | Faeces | Carcass | Total* |
| [$^{14}$C]Cinnamic acid (0.0005) | 77.9 ± 7.4 | 2.3 ± 3.5 | 84.9 ± 5.7 | 2.7 ± 3.7 | 0.2 ± 0.2 | 89.4 ± 5.4 |
| [$^{14}$C]Cinnamic acid (0.005) | 88.0 ± 8.5 | 1.5 ± 2.0 | 92.2 ± 2.9 | 1.7 ± 2.1 | 0.2 ± 0.2 | 95.3 ± 1.9 |
| [$^{14}$C]Cinnamic acid (0.05) | 83.6 ± 5.9 | 2.4 ± 3.6 | 87.2 ± 3.6 | 2.9 ± 3.5 | 1.1 ± 1.6 | 91.8 ± 1.2 |
| [$^{14}$C]Cinnamic acid (0.5) | 85.0 ± 7.1 | 3.1 ± 2.2 | 88.9 ± 6.3 | 3.4 ± 2.2 | 0.9 ± 0.4 | 94.6 ± 4.4 |
| [$^{14}$C]Cinnamic acid (2.5) | 93.3 ± 4.4 | 3.2 ± 1.9 | 95.6 ± 3.8 | 3.5 ± 2.3 | 0.7 ± 0.3 | 100.6 ± 1.7 |
| [$^{14}$C/$^2H_5$]Cinnamic acid (2.5) | 90.0 ± 3.9 | 3.4 ± 0.5 | 92.9 ± 4.1 | 4.0 ± 0.4 | 0.5 ± 0.4 | 98.4 ± 3.5 |

*Total results include cage washings
Values are means ± SD, n = 4

For the dose-ranging studies, aqueous solutions of [$^{14}$C]cinnamic acid were used. Rats were given 5–10 $\mu$Ci and mice 0.4–2 $\mu$Ci. Cinnamic acid was administered at the following dose levels: 2.5, 0.5, 0.05, 0.005 and 0.0005 mmol/kg body weight equivalent to 370, 74, 7.4, 0.74 and 0.074 mg/kg, respectively. For all these experiments, rats were dosed orally and mice ip.

The animals were housed in glass metabolism cages equipped for the separate collection of urine and faeces. Excreta were collected at 24 hr intervals; depending on the initial volume of urine produced, samples were adjusted to a standard 10 or 20 ml with distilled water. After 3 days the cages were washed with methanol–water (1:1, v/v) 500 ml per rat cage and 100 ml per mouse cage. Urine, faeces, cage washes and carcasses were stored at −20°C until analysed.

*High-performance liquid chromatography*

HPLC used a Waters M6000A pump, Rheodyne 7215 valve loop injector and Waters 441 UV detector equipped with a 254 nm filter. The various systems were as follows:

1. Stainless-steel column (100 × 5 mm); column packing ODS-Hypersil 5$\mu$; mobile phase 40% aqueous methanol adjusted to pH 3 with concentrated HCl; flow rate 1 ml/min.
2. Stainless-steel column (250 × 5 mm); column packing ODS-Hypersil 5$\mu$; with one of the following mobile phases:
   (a) Methanol–glacial acetic acid–water (15:3:82, by vol); flow rate 2 ml/min;
   (b) Methanol–glacial acetic acid–water (30:3:67, by vol); flow rate 2 ml/min.

Retention times of standards are given in Table 1.

*Radiochemical techniques*

Radioactivity in all samples was determined by liquid scintillation spectrometry (LSC) with a Packard Tricarb 4640 instrument. Counting efficiency was assessed by reference to an external standard after production of a quench correction curve. All samples were counted using Scintran Cocktail T (BDH Chemicals, Poole, Dorset).

Urine and cage wash samples were analysed directly and faecal samples were homogenized with water (1:10, v/v) and bleached with hydrogen peroxide before analysis. Carcasses were digested with 10 M NaOH (1 ml/g tissue) and homogenized; weighed aliquots of the digest (0.2 ml) were mixed with water (0.4 ml) and 0.4 ml Triton X-405–methanol (1:3, v/v) in glass scintillation vials. The samples were heated at 70°C with shaking in a water-bath for 1 hr to solubilize the digest. When cool, the solution was neutralized with 4.4 M $HNO_3$ (0.5 ml) before addition of scintillant and analysis.

For radio-HPLC, sequential 0.5 or 1.0 ml fractions of HPLC eluant were collected using a LKB Redirac fraction collector, scintillant was added and the samples were analysed.

*Metabolite isolation and characterization*

Metabolites were isolated from rat and mouse urine by the repeated collection of HPLC eluant fractions corresponding to the various radioactive peaks identified by radio-HPLC. The pooled fractions for each metabolite were then acidified, extracted with diethyl ether and evaporated to dryness. The residues were taken up in appropriate solvents for gas chromatography–mass spectrometry (GC–MS) as such and after derivatization, and nuclear magnetic resonance (NMR) characterization.

*Gas chromatography–mass spectrometry.* Gas chromatographic separations took place on a fused silica capillary column (25 m × 0.3 mm), coated with SE52 or OV 1701 stationary phase, housed in a Carlo Erba Mega HRGC 5160 gas chromatograph. Samples were injected using on-column or split/splitless injectors and columns were temperature programmed from 100 C to 270 C at 10 C/min. Mass spectrometric analyses used a VG 70-70 analytical mass spectrometer linked to a VG 2035 data system. Electron impact mass spectra were obtained at an ionization energy of 70 eV and a source temperature of 180–200 C.

Samples were methylated at room temperature by dissolving them in a saturated ethereal solution of diazomethane with a reaction time of 10 min.

*Nuclear magnetic resonance spectroscopy.* $^1$H-NMR spectra were recorded at 250 MHz using the Bruker WM 250 instrument of the University of London Intercollegiate NMR Service with TMS as internal standard.

## RESULTS

*Excretion of radioactivity*

Table 2 summarizes the excretion of radioactivity after dosing rats with [$^{14}$C/$^{2}$H$_5$]cinnamic acid over the dose range 0.0005 to 2.5 mmol/kg. The mean total recovery of radioactivity varied from 84 to 97%, with most (73–88%) in the 0–24-hr urine. The amount of radioactivity recovered in this time was not systematically related to the administered dose. After 3 days only trace amounts of radioactivity were present in the carcasses, indicating that cinnamic acid was readily and quantitatively excreted at all dose levels.

Comparable results were obtained in the mouse (Table 3). Overall, the total amount of dose recovered was slightly higher, ranging from 89 to 101%, with the 0–24-hr urine accounting for 78–93% of the dose. A further 1.7–3.5% of dose was excreted in the 0–72-hr faeces, but there did not appear to be any dose-related pattern. Trace amounts of radioactivity (0.2–1.1%) were detected at all dose levels in the carcasses 72 hr after dosing.

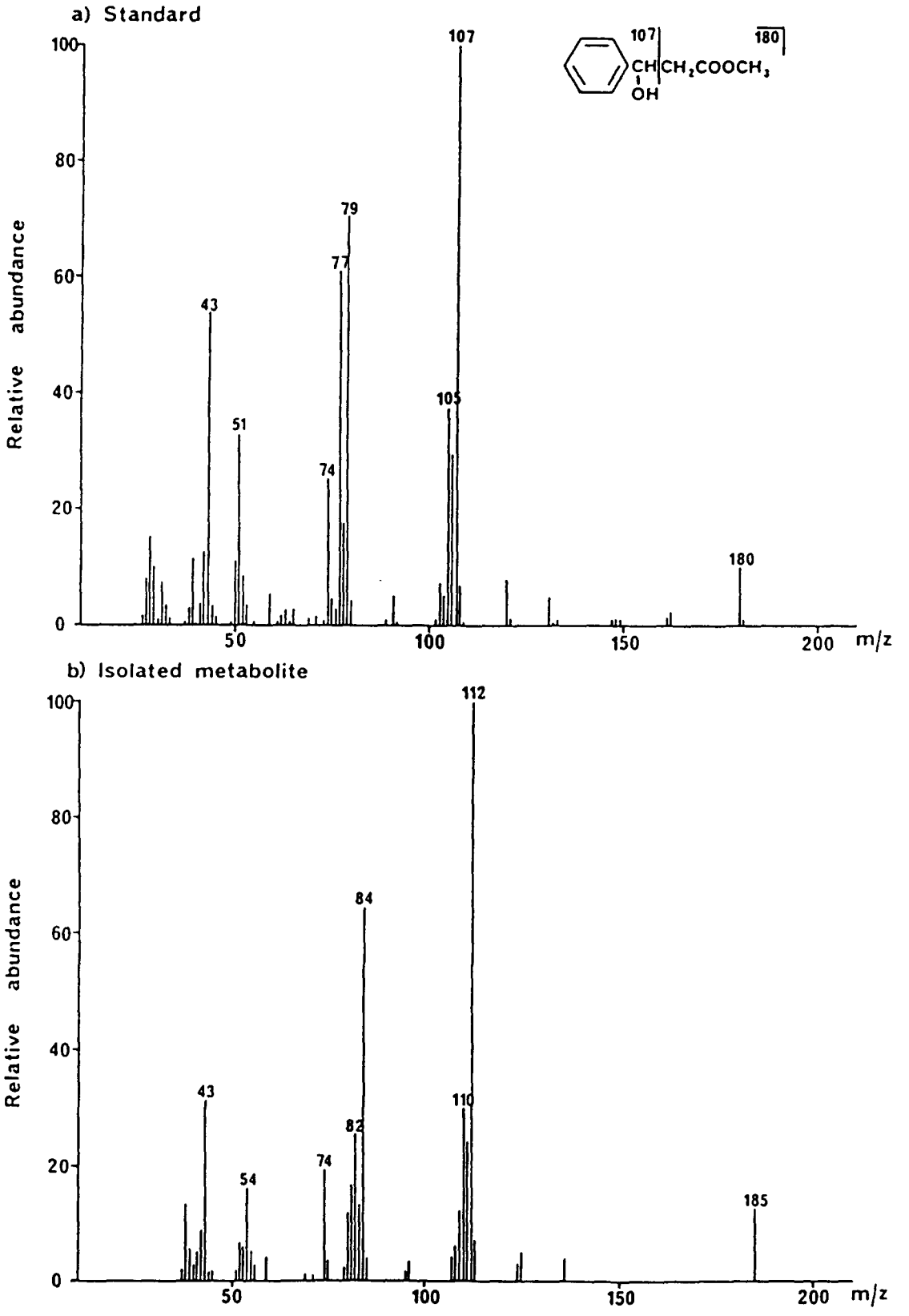


Fig. 1. Mass spectrum of (a) authentic methyl 3-hydroxy-3-phenylpropionate (standard) and (b) its phenyl-$^2H_5$ analogue in a methylated extract of rat urine after administration of [$^2H_5$]cinnamic acid.

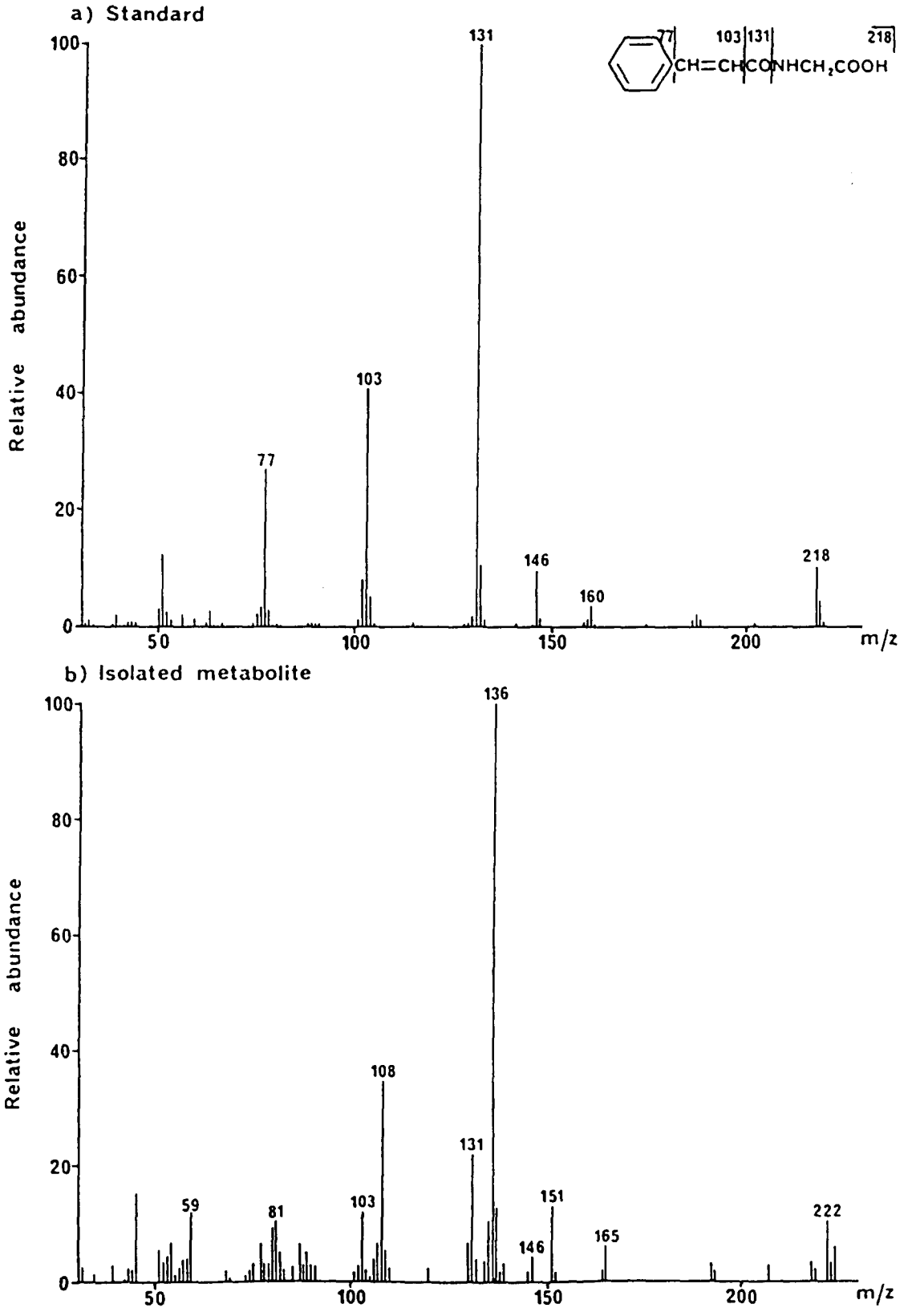


Fig. 2. Mass spectrum of (a) authentic cinnamoylglycine methyl ester (standard) and (b) cinnamoylglycine methyl ester isolated from mouse urine.

*Identification of metabolites*

HPLC analysis of 0–24-hr urine samples from rats and mice dosed with [$^{14}C/^{2}H_5$]cinnamic acid showed the presence of several radiolabelled metabolites. These metabolites were isolated from the urine, as described in Materials and Methods, and derivatized for GC–MS analysis with diazomethane. The following discussion thus refers to the methyl esters of the metabolites and standards unless otherwise stated.

*Hippuric acid.* The major fragment ions of methyl hippurate standard were at m/z 77, 105 ($B^+$), 134 and 193 ($M^+$). In the methylated rat or mouse urine extract a peak with the same retention time as the standard gave fragment ions with m/z values of 82, 110 ($B^+$), 139 and 198 ($M^+$), that is, all 5 amu higher than the standard owing to the presence of five deuterium atoms, but with similar relative intensities to the analogous fragments obtained with the standard. Less intense ions at m/z 77, 105 and 134, presumably arising from endogenous urinary hippuric acid, were also present.

*Benzoic acid.* Authentic benzoic acid methylated with diazomethane showed major ions at m/z 51, 77, 105 ($B^+$) and 136 ($M^+$). Ions for methyl [$^{2}H_5$]benzoate, isolated from rat or mouse urine, are found at m/z 82, 110 ($B^+$) and 141 ($M^+$), that is, 5 amu higher than the standard, whereas those ions at m/z 77 and 105 are from endogenous methyl benzoate.

*Cinnamic acid.* Major ions of methyl cinnamate are found at m/z 77, 103, 131 ($B^+$) and 162 ($M^+$) whereas the corresponding ions for methyl [$^{2}H_5$]cinnamate isolated from rat or mouse urine are found at m/z 82, 108, 136 ($B^+$) and 167 ($M^+$).

*3-Hydroxy-3-phenylpropionic acid.* Figure 1 shows the mass spectrum of (a) authentic methyl 3-hydroxy-3-phenylpropionate and (b) its phenyl-$^{2}H_5$ analogue identified in a methylated extract of rat urine after administration of [$^{2}H_5$]cinnamic acid. The comparable GC peak from an identically treated extract of mouse urine gave the same mass spectrum. The standard shows characteristic ions at m/z 77, 79, 107 ($B^+$) and 180 ($M^+$) whereas the metabolite has ions at m/z 82, 84, 112 ($B^+$) and 185 ($M^+$), all 5 amu higher than those for the synthetic standard.

*Cinnamoylglycine.* Cinnamoylglycine was identified in mouse urine and the mass spectra of (a) the standard and (b) the isolated metabolite as methyl esters are shown in Fig. 2. Cinnamoylglycine methyl ester gave major ions at m/z 103, 131 ($B^+$), 132, 146, 160, 218 and 219 ($M^+$); for the metabolite the analogous ions were at m/z 108, 136 ($B^+$), 137, 151, 165, 222 and 224 ($M^+$). Ions also present at m/z 103, 131, 132, 146 and 218 of lower intensity were due to endogenous cinnamoylglycine.

*Acetophenone.* The presence of [$^{2}H_5$]acetophenone in an ethereal extract of urine from mice dosed with [$^{14}C/^{2}H_5$]cinnamic acid was shown by GC–MS without derivatization. Fragment ions of acetophenone occur principally at m/z 77, 78, 105 ($B^+$) and 120 ($M^+$) and in the spectrum obtained from the urine extract they are found at m/z 82, 83, 110 ($B^+$) and 125 ($M^+$).

*Benzoyl glucuronide.* It was not possible to obtain a mass spectrum of benzoyl glucuronide, so other means were used to confirm the identity of this metabolite, which co-chromatographed with an authentic sample of benzoyl glucuronide in both HPLC systems 2a and 2b. The disappearance of this peak upon treatment of the urine with β-glucuronidase indicated the presence of a glucuronide, and the increase in the peak attributable to benzoic acid showed that this was the aglycone (data not shown). In this way it was possible to show that benzoyl glucuronide was a minor urinary metabolite in both the rat and mouse.

*Quantitation of urinary metabolites*

Metabolites were quantified by radio-HPLC in solvent system 2a.

Tables 4 and 5 show the metabolites in the 0–24-hr urine samples from rats dosed orally and mice dosed ip with [$^{14}C/^{2}H_5$]cinnamic acid. For both species, hippuric acid is the major metabolite, accounting for 67–72% of the administered radioactivity: more than 97% of $^{14}C$ injected onto the HPLC column was recovered in the discrete peaks reported in the tables. Four minor metabolites, totalling some 8%, were quantified in rat urine, none of which accounted for more than 5% of the dose. Six minor metabolites were quantified in mouse urine, none of which accounted for more than 9% individually but which together made up 18% of the dose.

Table 4. Metabolites present in 0–24-hr urine after administration of [$^{14}C$]- or [$^{14}C/^{2}H_5$]cinnamic acid to male Fischer 344 rats at various doses

| Metabolite | Percentage of dose recovered as metabolite after administration of cinnamic acid at $x$ mmol/kg | | | | | |
|---|---|---|---|---|---|---|
| | $x$ = 0.0005 | 0.005 | 0.05 | 0.5 | 2.5 | 2.5 ($^{2}H_5$) |
| Hippuric acid | 71.4 ± 2.1 | 69.3 ± 5.3 | 77.0 ± 6.5 | 69.3 ± 5.9 | 76.2 ± 3.4 | 71.8 ± 6.6 |
| Benzoyl glucuronide | 0.4 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.5 ± 0.1 | 4.7 ± 0.6 | 4.2 ± 1.0 |
| 3-Hydroxy-3-phenyl propionic acid | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.2 |
| Benzoic acid | 0.4 ± 0.2 | 0.6 ± 0.7 | 0.4 ± 0.2 | 0.4 ± 0.1 | 2.3 ± 0.9 | 1.8 ± 0.4 |
| Cinnamic acid | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.3 ± 0.1 | 0.5 ± 0.5 |
| Total | 72.7 ± 2.4 | 70.5 ± 5.8 | 78.1 ± 6.7 | 70.7 ± 5.8 | 84.9 ± 3.7 | 79.7 ± 5.9 |

Values are means ± SD, n = 4.

Table 5. Metabolites present in 0–24-hr urine after administration of [$^{14}$C]- or [$^{14}$C/$^{2}$H$_5$]cinnamic acid to male CD-1 mice at various doses

| Metabolite | Percentage of dose recovered as metabolite after administration of cinnamic acid at $x$ mmol/kg | | | | | |
|---|---|---|---|---|---|---|
| | $x$ = 0.0005 | 0.005 | 0.05 | 0.5 | 2.5 | 2.5 ($^{2}$H$_5$) |
| Hippuric acid | 44.2 ± 4.5 | 54.0 ± 5.1 | 63.5 ± 3.0 | 65.9 ± 6.2 | 56.8 ± 6.2 | 66.9 ± 6.9 |
| Benzoyl glucuronide | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 1.7 ± 1.4 | 1.0 ± 0.6 |
| 3-Hydroxy-3-phenyl propionic acid | 0.6 ± 0.9 | 0.3 ± 0.1 | 0.5 ± 0.3 | 2.0 ± 0.5 | 9.8 ± 1.0 | 4.9 ± 2.3 |
| Cinnamoylglycine | 28.6 ± 8.1 | 27.0 ± 3.5 | 13.6 ± 6.3 | 8.2 ± 1.9 | 2.4 ± 0.9 | 2.4 ± 0.9 |
| Benzoic acid | 0.8 ± 0.4 | 0.7 ± 0.4 | 2.1 ± 1.1 | 2.5 ± 1.9 | 8.6 ± 6.5 | 8.6 ± 6.5 |
| Acetophenone | — | — | — | 0.9 ± 0.4 | 0.8 ± 0.2 | 0.4 ± 0.1 |
| Cinnamic acid | — | — | 0.2 ± 0.1 | 0.3 ± 0.1 | 2.3 ± 1.0 | 0.7 ± 0.3 |
| Total | 74.4 ± 6.5 | 82.3 ± 8.5 | 80.2 ± 6.8 | 80.1 ± 6.5 | 82.4 ± 4.1 | 82.0 ± 3.9 |

Values are means ± SD, n = 4.

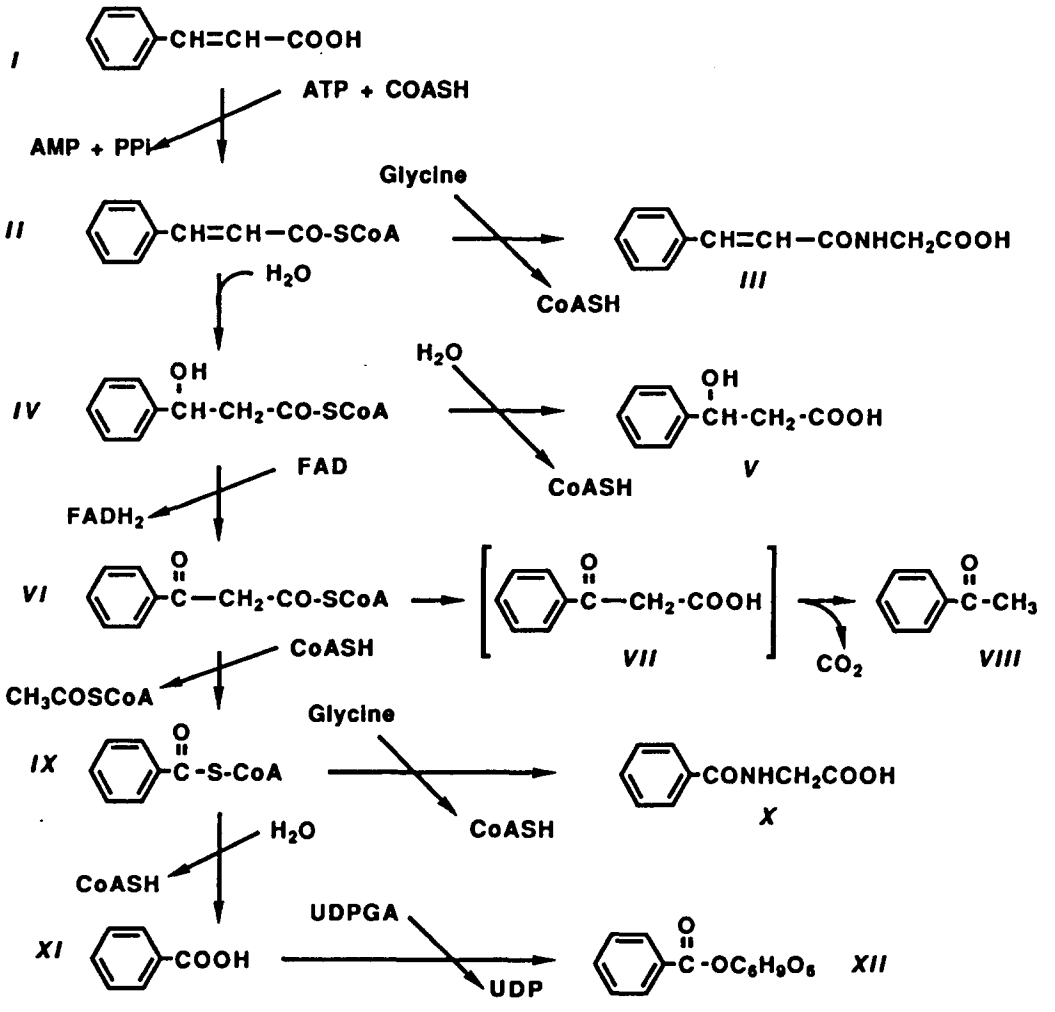


Fig. 3. Cinnamic acid metabolism.

All metabolites that were identified in rat urine at the 2.5 mmol/kg dose level were detected in urine at all doses. The levels of these metabolites in the 0–24-hr urine varied very little over the dose range 0.0005 to 0.5 mmol/kg. However, at the highest dose given (2.5 mmol/kg) the proportion of dose excreted as the minor metabolites increased. The major increases are seen with 3-hydroxy-3-phenylpropionic acid, benzoic acid and benzoyl glucuronide, which all increased four- to five-fold compared with the lower doses.

Urinary metabolites showed much greater variation with dose in the mouse. A number of interesting relationships can be seen between the levels of particular metabolites excreted and the administered dose. As the dose indicated from 0.0005 to 2.5 mmol/kg, the proportion excreted as hippuric acid increased from 44% to a maximum of 66% at

a dose of 0.5 mmol/kg, but then decreased to 57% at the highest dose. At the same time the levels of cinnamoylglycine decreased from 29% at the lowest dose to 2% at the highest dose. All other metabolites identified accounted for greater proportions of the dose as the dose increased. Compared with the lowest dose, benzoyl glucuronide, 3-hydroxy-3-phenylpropionic acid and benzoic acid show increases ranging from 11- to 17-fold. Acetophenone and cinnamic acid, which were not detected at the lowest dose, show a dose-related increase to 0.8 and 2.3% of the dose, respectively, at 2.5 mmol/kg.

## DISCUSSION

The literature contains only fragmentary information on the metabolism and disposition of cinnamic acid and the work described here constitutes the most comprehensive study to date.

After oral administration to rats, [$^{14}$C]cinnamic acid is well absorbed from the gastro-intestinal tract, in accord with the results of Fahelbum and James (1977). In 72 hr, more than 90% of administered $^{14}$C is excreted by rats given the acid orally and mice receiving it by ip injection, with most of this in the 0–24-hr urine. Only traces remained in the carcasses after 72 hr. The excretion balance achieved is markedly superior to that reported by Teuchy and van Sumere (1971) who gave [$^{14}$C]cinnamic acid ip to rats. These authors found up to 25% of the dose in the 0–24-hr faeces, suggesting contamination with urine, and some 10% was present in the carcasses after 72 hr. In the present study, cinnamic acid was given to rats orally and to mice by ip injection. The different routes were chosen to avoid the problem of unreliable oral dosimetry to mice, in which species a quantitative urinary recovery can only rarely be achieved. Comparable studies with cinnamaldehyde (Peters and Caldwell, 1994), furfural (Parkash and Caldwell, 1994) and benzoic acid (Nutley, 1989) have failed to show any route of administration-related differences in metabolism of these compounds, which have a low hepatic extraction after oral administration.

Figure 3 presents a metabolic map for cinnamic acid consistent with the data reported here. Like the great majority of xenobiotic carboxylic acids that enter the cell (Caldwell, 1984), cinnamic acid is converted to its acyl CoA and it is this high-energy intermediate that is the key to the fate of cinnamic acid. Cinnamoyl CoA undergoes either glycine conjugation by a glycine *N*-acyl transferase, a reaction only seen in the mouse, or the addition of water across the double bond to yield 3-hydroxy-3-phenylpropionyl CoA in the first step of $\beta$-oxidation. This CoA may be cleaved to yield the free acid as a very minor metabolite, but most of it loses two protons to FAD to give 3-keto-3-phenylpropionyl CoA. If this CoA is cleaved, the free $\beta$-keto acid is chemically unstable and decarboxylates to acetophenone, a minor urinary metabolite. 3-Keto-3-phenylpropionyl CoA then loses a two-carbon acetyl moiety by reaction with acetyl CoA, yielding benzoyl CoA and malonyl CoA. Benzoyl CoA is then in turn conjugated with glycine, giving hippuric acid, or hydrolysed to free benzoic acid, excreted as such or after glucuronic acid conjugation. The reactions in this sequence are of great historical importance in biochemistry, since it was studies on cinnamic and other $\omega$-phenyl fatty acids that revealed the pivotal $\beta$-oxidation pathway of fatty acid catabolism.

After administration of [$^{14}$C]cinnamic acid to rats over the dose range 0.0005 to 2.5 mmol/kg the excretion of radioactivity is rapid (see Table 2). At all dose levels, most of the dose (73–88%) is present in the 0–24-hr urine. These results confirm that, in the rat, cinnamic acid is readily excreted: after 72 hr only trace levels of radioactivity were detected in the carcasses, which suggests that little, if any, accumulation of radioactivity occurred. A total of 84–97% of the dose was recovered in these studies.

Over the dose range 0.0005 to 2.5 mmol/kg, hippuric acid is the major metabolite excreted in 0–24-hr urine, accounting for 69–77% of the dose. For doses in the range 0.0005 to 0.5 mmol/kg, minor metabolite levels are very similar. Individually, these metabolites account for no more than 0.5% of the dose in the 0–24-hr urine and collectively account for 1–1.2%. However, at a dose of 2.5 mmol/kg the minor metabolites account for 8.2% of the dose. From these results one can conclude that cinnamic acid metabolism is not influenced by dose size up to a dose of 0.5 mmol/kg. At the highest dose administered, there is an increase in the excretion of 3-hydroxy-3-phenylpropionic acid (the major metabolic intermediate observed in the degradation pathway of cinnamic acid in the rat) from 0.2 to 0.9% of the dose, indicating that in the rat the $\beta$-oxidation pathway is not capacity-limited up to the maximum tolerated dose.

The administration of cinnamic acid above 0.5 mmol/kg does result in the saturation of the ability of glycine *N*-acyl transferase to conjugate benzoyl CoA with glycine. There is a greater proportion of the dose excreted as benzoyl glucuronide and free benzoic acid and less as hippuric acid. This increasing role of glucuronic acid conjugation relative to glycine conjugation as dose size increases has been reported many times in metabolic studies of carboxylic acids (Caldwell *et al.*, 1980).

The excretion balance of $^{14}$C after administration of [$^{14}$C]cinnamic acid to mice over the dose range 0.0005 to 2.5 mmol/kg is similar to that in the rat. Excretion of radioactivity is rapid, with 78–93% of the dose present in 0–24-hr urine samples. Carcass residue levels range from 0.2 to 1.1% of the dose, with total recoveries of radioactivity of 89–101%. However, the pattern of metabolites in the 0–24-hr urine is markedly more variable, with an interesting change in the pattern of glycine conjugates excreted.

At the lowest dose, some 44% of the dose is excreted as hippuric acid with 29% as cinnamoylglycine. As the dose of cinnamic acid increases, the proportion of the dose excreted as cinnamoylglycine falls progressively to a minimum of some 2% at 2.5 mmol/kg. At the same time, the proportion of dose excreted as hippuric acid (i.e. after initial metabolism through the $\beta$-oxidation pathway and subsequent conjugation) increases to about 66% at 0.5 mmol/kg and falls slightly to 57% at the highest dose level given.

These results suggest that mouse glycine *N*-acyltransferase has a high affinity but low capacity for cinnamic acid and, as the dose increases, the higher capacity of the $\beta$-oxidation pathway leads to increased formation of benzoic acid. At the higher doses, especially 2.5 mmol/kg, the rate of benzoic acid production exceeds the capacity of the relevant glycine *N*-acyltransferase, so that the excretion of free benzoic acid increases from 2.5% of the dose at 0.5 mmol/kg to 8.6% at 2.5 mmol/kg. At all dose levels, the mouse excretes a small proportion of the dose as benzoyl glucuronide (maximum 1.7%), which suggests that this conjugation reaction is of minimal importance in this species.

An increase in excretion of 3-hydroxy-3-phenylpropionic acid from 2% of dose at 0.5 mmol/kg to 9.8% at 2.5 mmol/kg suggests that some saturation may occur within the $\beta$-oxidation pathway leading to hydrolysis of the intermediate CoAs. This view is supported by the excretion of acetophenone, which arises from the spontaneous decarboxylation of the $\beta$-keto acid 3-keto-3-phenylpropionic acid (Dakin, 1909; Marsh *et al.*, 1982) and was not detected below 0.05 mmol/kg but increased to 0.8% of the dose at 2.5 mmol/kg.

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