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3-HYDROXY- AND 3-KETO-3-PHENYLPROPIONIC ACIDS: NOVEL METABOLITES OF BENZOIC ACID IN HORSE URINE*

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Abstract—The metabolism of benzoic acid has been examined in the horse, using ^{14}C- and deuterium-labelled compounds. Chromatographic analysis of the urine showed the presence of hippuric acid, benzoyl glucuronide and benzoic acid and a discrete band which accounted for 2% of the dose administered. This material was isolated by solvent extraction and HPLC and, following treatment with diazomethane, examined by GC/MS.

The major component of this fraction was 3-hydroxy-3-phenylpropionic acid methyl ester, which was accompanied by very much smaller amounts of cinnamic acid methyl ester and acetophenone. The two latter minor components have been shown to be artefacts produced during workup and analysis. Cinnamic acid methyl ester arises by the thermal decomposition of 3-hydroxy-3-phenylpropionic acid methyl ester on the GC column. It is proposed that acetophenone has formed, during workup, by decarboxylation of 3-keto-3-phenylpropionic acid.

It is suggested that 3-hydroxy and 3-keto-3-phenylpropionic acids, which are also endogenous in horse urine, have arisen by an addition of a 2 carbon fragment to benzoyl CoA, in a sequence analogous to the reactions of fatty acid biosynthesis. Some implications of the metabolic interrelationships between xenobiotic acids and fatty acids are discussed.

The carboxylic acid group is commonly encountered in a variety of compounds to which animals and man may be exposed, including drugs, herbicides and pesticides. This functional group may be metabolized along one or more of a number of pathways, including decarboxylation, reduction, conversion to a amide or methyl ester and conjugation with an amino acid, glucuronic acid or another monosaccharide [1]. The relative extents of the major metabolic options of amino acid and glucuronic acid conjugation are a function of the structure of the acid and species in question [1, 2].

The amino acid conjugations are reactions of interest, since prior to the conjugation with the amino acid the carboxylic acid must be activated by conversion to a CoA‖ thioester [1–4]. This reaction is catalysed by an ATP-dependent acid:CoA ligase (AMP), the energy required being derived from the cleavage of ATP to AMP and pyrophosphate [4]. The enzyme activating aromatic and arylacetic acids in mammals is a relatively non-specific intermediate chain length fatty acyl-CoA synthetase, which is also involved in fatty acid biosynthesis [4].

The principal fate of the acyl CoAs of xenobiotics which are formed within the cell is to act as substrates for the amino acid acyl transferases, which form the amino acid conjugates with the liberation of CoA.SH [4]. However, since the acyl CoAs are formed by non-specific enzymes with a well-defined role in lipid metabolism, it would not be surprising if xenobiotic acyl CoAs shared some of the fates of endogenous fatty acyl CoAs, In the present paper, we show the formation by the horse of novel metabolites of benzoic acid, in which a 2-carbon fragment has been added to the carboxyl group, and suggest that these arise by a reaction sequence analogous to that of fatty acid biosynthesis.

MATERIALS AND METHODS

Compounds

[^{14}C-*Carboxyl*]-benzoic acid, sp. act. 56 mCi/mmole, radiochemical purity > 99%, was purchased from Amersham International, U.K. [Ring-d_5]-benzoic acid, isotope abundance > 99% and ethyl benzoylacetate were purchased from Aldrich Chem. Co. (Gillingham, U.K.). Acetophenone, *trans*-cinnamic acid and sodium borohydride were purchased from Sigma Chem. Co. (London, U.K.). All other reagents were of Analar quality and purified as needed prior to use.

3-Hydroxy-3-phenylpropionic acid

Ethyl benzoylacetate (5 g) was dissolved in 90% aqueous ethanol (25 ml) and sodium borohydride

* A preliminary account of part of this work has appeared in this journal [M. V. Marsh *et al.*, *Biochem. Pharmac.* **30**, 1879 (1981)].

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‖ Abbreviations used: CoA, coenzyme A; NMR, nuclear magnetic resonance; MS, mass spectrometry; GC/MS, gas chromatography–mass spectrometry; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography.

(1 g) in 20% aqueous ethanol (10 ml) was added dropwise with stirring at room temperature. After 4 hr, the reaction mixture was cooled in an ice bath and 6M HCl added cautiously until the evolution of H_2 ceased. Molar NaOH (100 ml) was added and the mixture refluxed for 2 hr. After cooling, the solution was extracted with 2 vol. ether, which were discarded. The aqueous phase was adjusted to pH 1 (10 M HCl) and extracted with 4 × 2 vol. ether, the extracts pooled and the ether removed on the rotary evaporator. The residue was dissolved in a small volume of methanol and stored for 7 days at −20°, whereby white crystals of the title compound precipitated, which were harvested by filtration, m.p. 87–88°, yield 31%, $C_9H_{10}O_3$ requires C, 65.1; H, 6.0; found C, 65.5; H, 6.4. ^{1}H-NMR spectrum at 250 MHz in $CDCl_3$, δ ppm 2.8 (M, 2, $C\underline{H}_2$), 5.15 (dd, 1, on SDC at 2.8 coalesced to a singlet $C\underline{H}$), 6.2–7.0 (broad singlet, exchanges with D_2O—$O\underline{H}$, —$COO\underline{H}$), 7.3 (M, 5 $Ar\underline{H}$): MS (direct insertion E.I. at 70 eV) *m/z* (relative abundance) 166 (25, M^+), 148 (20), 131 (6), 120 (6), 107 (100), 106 (34), 105 (38), 103 (28), 79 (75), 78 (28), 77 (24), 60 (8), 52 (10), 51 (39), 50 (16), 44 (14), 39 (11).

Instrumentation

^{1}H-NMR. ^{1}H-NMR spectra were recorded at 250 MHz with a Bruker WM 250 spectrometer, with TMS as internal standard (University of London Intercollegiate NMR Service).

MS. MS was performed in the E.I. mode by direct insertion with a VG Micromass ZAB IF instrument, ionizing voltage 70 eV (University of London Intercollegiate Mass Spectrometry Service).

GC/MS. This used a Finnigan 4000 instrument with a Finnigan 6100 data system. The GC column was of glass 1.8 m × 3.2 mm i.d. packed with 3Q3%E30 (Pye Instruments, Cambridge, U.K.), carrier gas He 20 ml/min with oven temperature programmed to rise from 100° at 10°/min, with an upper limit of 280°. The injection port temperature was 240°, MS ionizing voltage 70 eV and source temperature 200°.

Chromatography

TLC employed Merck Silica gel F_{254} plates (Cat. No. 5554) 0.2 mm layer thickness on aluminium support. Solvents were (A) benzene/acetone/glacial acetic acid 6:2:1, (B) benzene/dioxan/glacial acetic acid 90:25:8; (C) benzene/acetone/glacial acetic acid 2:2:1 (all compositions by vol.). Plates were developed to 15 cm from the origin and compounds located under a u.v. lamp (254 nm).

HPLC used a Waters Associates U6K valve loop injector and Model 6000A pump, a Cecil 2012 u.v. dectector set at 235 nm, a 250 × 5 mm column packed with 5 μ ODS-Hypersil and an LKB RediRac fraction collector. The mobile phase was 40% aqueous methanol, flow rate 2 ml/min.

Chromatographic mobilities of benzoic acid and related compounds are given in Table 1.

Radiochemical techniques

^{14}C content of solutions and excreta was determined by liquid scintillation spectrometry using a Triton X-100-toluene based scintillant with a Packard TriCarb instrument, Model 3385. Efficiency of counting was determined by reference to an external standard. ^{14}C on chromatograms was located with a Packard radiochromatogram scanner, Model 7201 and quantitated by counting of 0.5 cm sections of adsorbent.

Animal and dosing

A gelded male horse, weight 374 kg, was given 2 g [ring-d_5]-benzoic acid containing 150 μCi [^{14}C]benzoic acid, dissolved in dilute $NaHCO_3$, by stomach tube, followed by 500 ml water. The total dose was 5.35 mg/kg. The urine was collected for 24 hr as described by Warwick [5].

RESULTS

The 0–24 hr urine of the horse was examined by TLC in systems A–C and by HPLC. Details of these studies are reported elsewhere [6], and these analyses revealed the presence of hippuric acid, benzoic acid and benzoyl glucuronide in the urine. In addition to these compounds, an unknown metabolite fraction was detected accounting for 2% of the administered dose, with chromatographic mobility between that of benzoic and hippuric acids.

The unknown metabolite fraction was isolated by solvent extraction and preparative HPLC. A sample (25 ml) of the 0–24 hr urine of the horse was adjusted to pH 1 (6 M HCl) and extracted with 2 × 25 ml diethyl ether. The pooled ethereal extracts were dried (anhyd. Na_2SO_4) and the ether removed by rotary evaporation. The residue was taken up in 2 ml methanol and this injected in 100 μl aliquots on to the HPLC column. Radioactivity eluting from the column between 3 and 4 min retention was collected and the pooled fractions concentrated *in vacuo*. On

Table 1. Chromatographic characteristics of benzoic acid and related compounds

	TLC R_f in system			HPLC Retention time
	A	B	C	(min)
Benzoic acid	0.70	0.73	0.89	7.2
Hippuric acid	0.25	0.16	0.63	2.4
Acetophenone	0.80	0.81	0.91	12.7
trans-Cinnamic acid	0.74	0.73	0.91	7.8
3-Hydroxy-3-phenylpropionic acid	0.57	0.45	0.80	3.5
3-Keto-3-phenylpropionic acid	0.55*	—	—	—

* Accompanied by large amounts of acetophenone, R_f 0.80 (see text).

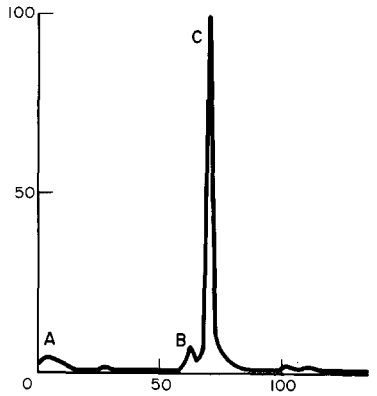

Fig. 1. Total ion chromatogram obtained by GC/MS of the unknown metabolite fraction isolated from urine, after methylation with diazomethane. Vertical axis shows relative peak intensity and horizontal axis gives scan number (1 scan/4 sec).

TLC in system A, scanning revealed a single radioactive band whose centre had R_f 0.56. The residue was treated with ethereal diazomethane and examined by GC/MS. The total ion chromatogram of this is given in Fig. 1, which shows the presence of one major and two minor peaks.

The MS of the first peak (A, Fig. 1) contains fragment ions diagnostic of acetophenone, and those ions containing the intact aromatic ring were accompanied by fragment ions 5 amu higher in a ratio of 2.3:1. This shows that as well as being derived from the administered [ring-d_5]-benzoic acid, acetophone was also present as an endogenous component of horse urine. The MS of the metabolic sample, together with that of an authentic sample of acetophenone which had the same GC retention time, is shown in Fig. 2.

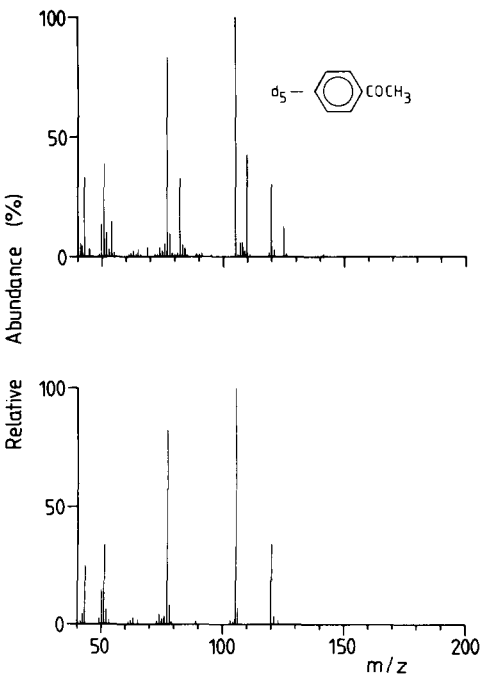

Fig. 2. Mass spectra of acetophenone, obtained by GC/MS. Upper panel shows the MS of peak A of Fig. 1, and lower panel gives that of authentic acetophenone.

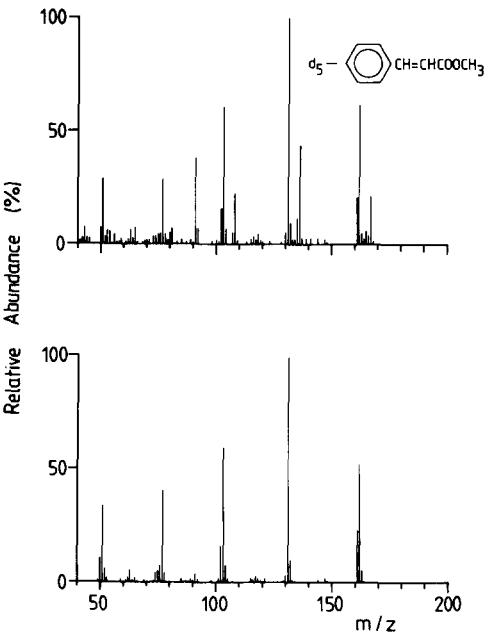

Fig. 3. Mass spectra of *trans*-cinnamic acid methyl ester, obtained by GC/MS. Upper panel shows the MS of peak B of Fig. 1, and lower panel gives that of the methyl ester of authentic *trans*-cinnamic acid.

The second minor peak (B, Fig. 1) in the GC trace gave the MS of the methyl ester of cinnamic acid, again with those ions containing the intact aromatic ring accompanied by ions 5 amu higher in a ratio of 2.3:1. Figure 3 shows the MS of metabolic and

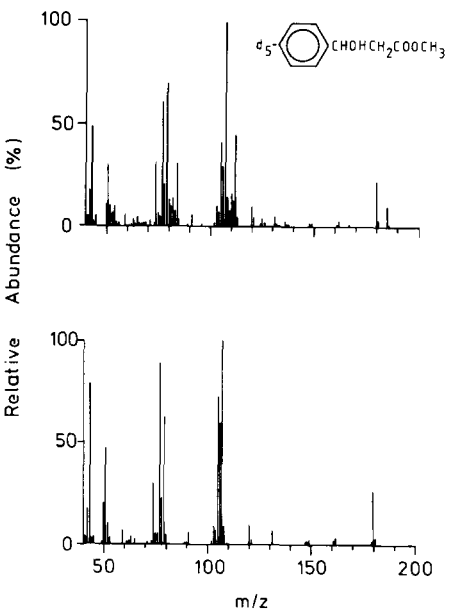

Fig. 4. Mass spectra of 3-hydroxy-3-phenylpropionic acid methyl ester, obtained by GC/MS. Upper panel shows the MS of peak C of Fig. 1, and the lower panel shows that of the methyl ester of authentic 3-hydroxy-3-phenylpropionic acid.

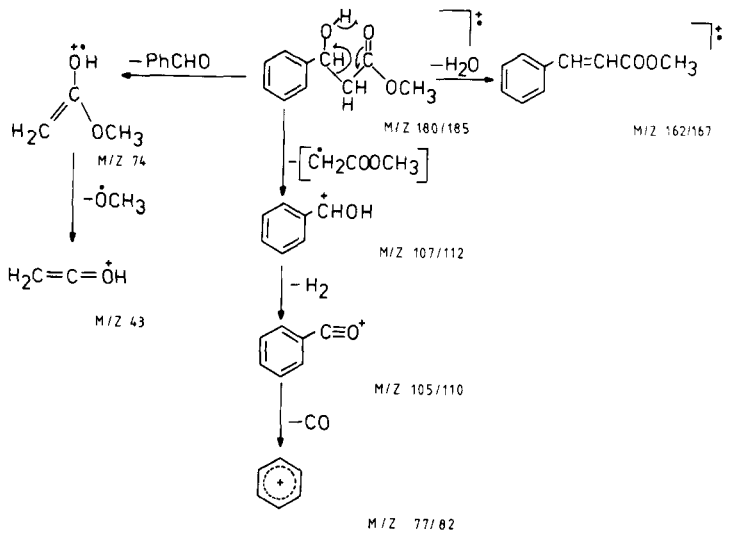

Fig. 5. Proposed fragmentation scheme for the formation of the diagnostic ions in the mass spectrum of 3-hydroxy-3-phenylpropionic acid methyl ester.

synthetic samples of the methyl ester of cinnamic acid, this latter being produced by treatment of authentic *trans*-cinnamic acid with ethereal diazomethane and having the same GC retention time as the metabolic sample.

The MS of the major peak in the GC trace (C, Fig. 1) is shown in the upper panel of Fig. 4. The fragmentation pattern shown in Fig. 5 proposes identities for the major ions in the mass spectrum. Diagnostic ions are seen at *m/z* 74, arising from the elimination of a neutral molecule of benzaldehyde from the molecular ion *m/z* 180 by a γ-hydrogen

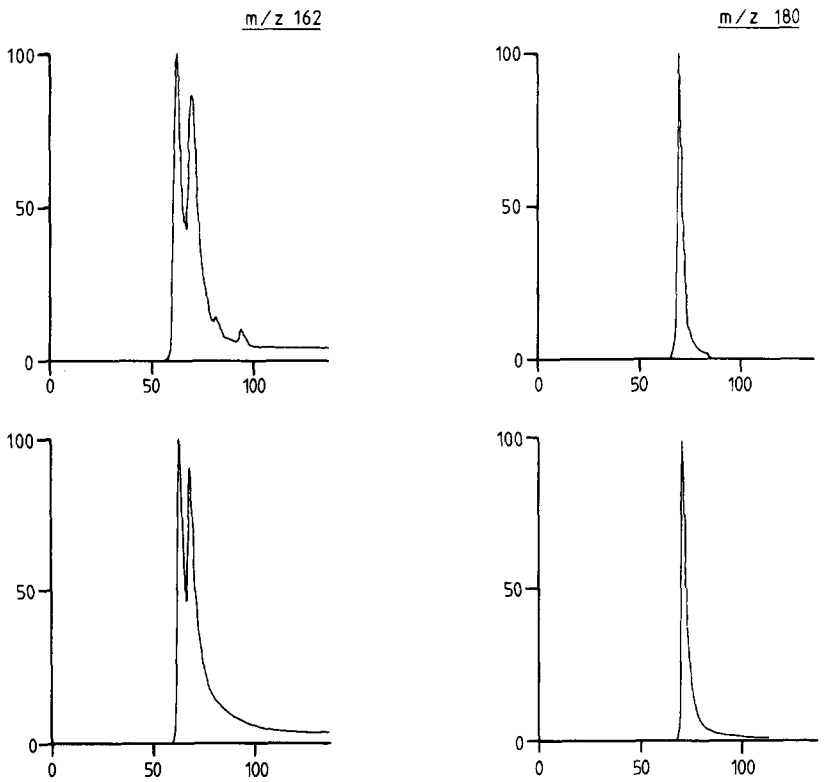

Fig. 6. GC/MS/SIM traces of 3-hydroxy-3-phenylpropionic acid methyl ester. Left-hand side traces show monitoring at *m/z* 162, right-hand traces show *m/z* 180. The upper two traces are from the metabolic sample and the lower two from authentic 3-hydroxy-3-phenylpropionic acid. Vertical axes show relative peak intensities and horizontal axes give scan numbers (1 scan/4 sec).

rearrangement [7], and this ion fragments further by loss of a methoxy radical giving an ion at *m/z* 43. Fragment ions at *m/z* 107 (base peak) and 105 indicate the presence of an hydroxyl group on the benzylic carbon atom, *m/z* 107 being due to an hydroxylated tropylium ion and *m/z* 105 arising by the loss of a hydrogen molecule to give the benzoyl cation, which further fragments by expulsion of CO giving an ion at *m/z* 77. A minor fragmentation route involves the elimination of water from the molecular ion, giving the ion of methyl cinnamate (*m/z* 162, *ca.* 2% relative intensity) which further fragments to ions at *m/z* 131 (loss of methoxy radical) and *m/z* 103 (loss of CO from *m/z* 131). All the ions and fragments with an intact aromatic ring system were paired with peaks of 5 amu greater mass derived from [ring-d_5]-benzoic acid, again in a ratio of 2.3:1. From this interpretation of the MS of the methylated sample, the major GC peak was identified as 3-hydroxy-3-phenylpropionic acid methyl ester. This acid was synthetized and characterized as described, and after methylation had identical GC characteristics with the metabolic sample. It gave the MS shown in the lower panel of Fig. 4.

The GC/MS results would suggest that the unknown metabolite fraction isolated by HPLC contains 3-hydroxy-3-phenylpropionic acid, acetophenone and cinnamic acid. However, comparison of the TLC and HPLC properties of these compounds (Table 1) with those of the unknown metabolite suggest very strongly that both acetophenone and cinnamic acid are artefacts produced during the workup of this fraction.

Acetophenone is proposed to arise by the decarboxylation of 3-keto-3-phenylpropionic acid. Such β-keto acids are known to undergo facile decarboxylation to the corresponding ketones [8] and this has been invoked to explain the formation of acetophenone during the metabolism of phenylpropionic acid [9] and of *p*-methoxyacetophenone from the β-keto acid metabolites of estragole and anethole [10]. Attempts to prepare 3-keto-3-phenylpropionic acid by the saponification of its ethyl ester, ethyl benzoylacetate, resulted in the formation of acetophenone in far greater quantities than the acid. On TLC in system A, the unknown metabolite fraction had R_f 0.55–0.57, which corresponds to both 3-keto- and 3-hydroxy-phenylpropionic acids (Table 1), indicating that the proposed precursor of acetophenone would have been isolated from urine during the procedure used.

That cinnamic acid has been produced artefactually is suggested by Fig. 6, which shows the traces obtained by single ion monitoring (SIM) at *m/z* 180 and at *m/z* 162 of the unknown metabolite fraction and of authentic 3-hydroxy-3-phenylpropionic acid methyl ester.

SIM at *m/z* 180, the molecular ion of 3-hydroxy-3-phenylpropionic acid methyl ester, of the metabolic sample showed a single peak at scan number 71. Two peaks of *m/z* 162 are seen at scan numbers 63 and 71, that at scan number 71 being due to the formation of methyl cinnamate during the fragmentation of the 3-hydroxy-3-phenylpropionic acid methyl ester (see above) and that at scan number 63 being the molecular ion of the methyl ester of cinnamic acid (see above). The traces obtained with the methyl ester of an authentic sample of 3-hydroxy-3-phenylpropionic acid are identical with those given by the unknown metabolite. This shows that the cinnamic acid methyl ester present in the GC/MS of the unknown metabolite fraction is produced by the thermal dehydration of 3-hydroxy-3-phenylpropionic acid methyl ester in the GC prior to entry into the MS.

DISCUSSION

This paper prevents evidence for the formation of two novel metabolites of benzoic acid, excreted in horse urine, which both involve the addition of a two-carbon fragment to the carboxyl group. The first of these is 3-hydroxy-3-phenylpropionic acid, which has been identified by comparison of its TLC, HPLC and GC/MS properties with those of an authentic sample. The second is a precursor of acetophenone, the presence of which has been shown by GC/MS, and chemical and TLC evidence has been presented suggesting that this precursor is 3-keto-3-phenylpropionic acid, which undergoes spontaneous decarboxylation during workup. Cinnamic acid has been shown to be an artefact produced by thermal dehydration of 3-hydroxy-3-phenylpropionic acid on the GC column. Both the 3-keto- and 3-hydroxy-3-phenylpropionic acids are endogenous compounds in horse urine, as the ions in the MS arising from the deuterated aromatic ring of the administered ring [ring-d_5]-benzoic acid are accompanied by ions 5 amu lower from the endogenous protonated compounds.

There are few reports in the literature of such 2-carbon elongations occurring in the metabolism of xenobiotic carboxylic acids. Miyazaki *et al.* [11] found that 5-(4-chloro-*N*-butyl)-picolinic acid was converted by rats to the corresponding β-keto, α,β-unsaturated and saturated propionic acids by addition of a 2-carbon fragment to the carboxyl group. Quistad *et al.* [12–14] showed that cyclopropylcarboxylic acid, produced by the hydrolysis of the miticide Cycloprate (hexadecyl cyclopropylcarboxylate), gave rise to various ω-cyclopropyl fatty acid residues in the triglycerides of the adipose tissue of rats. These residues arose from the successive addition of 6 or 7 2-carbon units to cyclopropylcarboxylic acid. It is also interesting to recall the early finding of Jaffé and Levy [15] that furfural was converted to furylacryloylglycine by the dog, but later workers have not been able to substantiate this [16].

It is important to consider possible routes by which the 2-carbon fragment may be added to the carboxyl group of benzoic acid and other xenobiotic acids. It is likely that this proceeds via the acyl CoA of the xenobiotic acid which could react with acetyl or malonyl CoA to give the β-ketopropionyl CoA, as occurs in fatty acid biosynthesis [17]. This β-ketopropionyl CoA could give rise to the β-keto acid excreted in the urine and undergo metabolic reduction to the corresponding β-hydroxy acid, which is the major component of the unknown metabolite fraction isolated from horse urine. A scheme suggesting the likely routes of formation of the various urinary metabolites of benzoic acid in the horse is given in Fig. 7.

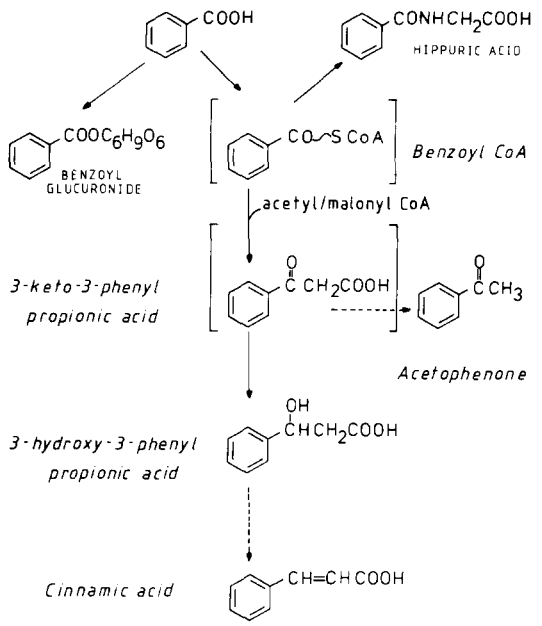

Fig. 7. Scheme showing the likely routes of formation of the urinary metabolites of benzoic acid in the horse. Compounds in square brackets are intermediates not isolated in this study. Broken lines represent chemical changes occurring during analysis.

These findings suggest that important links may occur between the metabolism of xenobiotic acids and the reactions of fatty acid synthesis. As well as the examples quoted above, it is noteworthy that 3-phenoxybenzoic acid, a major metabolite of pyrethroid insecticides, is found in the skin of rats in the form of mixed triglycerides, 2- and 3-(3-phenoxybenzoyl)-dipalmitin [18]. These are proposed to arise from the incorporation of 3-phenoxybenzoyl CoA by the enzymes of triglyceride synthesis, which normally use fatty acyl CoAs as their substrates. Additionally, a major metabolite of cyclopropylcarboxylic acid in dog urine and the milk of cows was identified as *O*-(cyclopropylcarbonyl)-carnitine [13, 14]. Carnitine is the carrier of long chain fatty acids through the mitochondrial membrane [17]. It thus appears that xenobiotic acids may participate in a number of aspects of fatty acid metabolism, giving rise to a variety of novel products in the tissues and excreta. These phenomena may be responsible for the accumulation of long lasting tissue residues of xenobiotic acids, and have the potential to alter membrane structure and cause disturbances of fatty acid biochemistry.

It is interesting to note that preliminary studies in this laboratory indicate that the formation of these novel metabolites of benzoic acid also occurs in the more commonly encountered laboratory species, rat and rabbit.

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