BENZOYLGLUTAMIC ACID, A METABOLITE OF BENZOIC ACID IN INDIAN FRUIT BATS

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1. Introduction

It was reported from this laboratory [1] that the Indian fruit bat did not excrete hippuric acid after the administration of [^{14}C]benzoic acid. This was confirmed by Bababunmi et al. [2] and later by Ette et al. [3] who showed that phenylacetic acid, unlike benzoic acid, did conjugate with glycine in this bat. However, we suspected that the urine of fruit bats dosed with [^{14}C]benzoic acid did contain a conjugate other than the major one, benzoylglucuronide, since thin-layer chromatography of the urine revealed a small ^{14}C peak of the same R_F as hippuric acid in the solvents used [3], but contained no hippuric acid on isotope dilution. The nature of this peak has now been elucidated and the conjugate shown to be benzoyl-L-(+)-glutamic acid.

2. Materials and methods

[Carboxy-^{14}C]benzoic acid (49.9 m Ci/mmol; Radiochemical Centre, Amersham, UK), cyclohexanecarboxylic acid (Aldrich Chemical Company, Wembley, UK), benzoylglutamic acid, benzoyl-α-alanine, benzoyl-β-alanine, benzoylarginine and benzoylvaline (Sigma Chemical Company Ltd., Kingston-upon-Thames, UK) were purchased. Benzoic acid, 4-hydroxybenzoic acid, hippuric acid, 4-hydroxyhippuric acid, benzoylglucuronide, benzoyltaurine and N^1, N^5-dibenzoylornithine were available in this laboratory. R_F values (t.l.c.) of these compounds are given in table 1.

Indian fruit bats (*Pteropus giganteus*) were maintained as described by Bababunmi et al. [2]. Sodium [^{14}C]benzoate in water (2–3 ml) was injected intraperitoneally into the bats, each one of which was kept in a cage mounted on a stainless steel funnel for urine collection. The urine was collected for 24 h and filtered together with washings through glass wool. It was then frozen and kept at −20°C until used. The output of ^{14}C was determined by scintillation counting (Packard Tri-Carb scintillation spectrometer, model 3320). The urine was chromatographed on thin layer (see table 1) and the chromatograms scanned in a Packard Radiochromatogram scanner (Model 7200).

Table 1
R_F values of benzoic acid and possible metabolites

Compound	R_F values in solvent A	B	C
Cyclohexanecarboxylic acid	0.83	0.65	–
Benzoic acid	0.79	0.60	0.97
4-Hydroxybenzoic acid	0.75	0.53	0.93
Benzoyl-D, L-valine	0.71	0.47	0.87
Benzoyl-β-alanine	0.63	0.36	0.82
Benzoyl-D, L-α-alanine	0.63	0.35	0.77
N^1, N^5-Dibenzoyl-L-(+)-ornithine	0.57	0.23	0.69
Hippuric acid	0.51	0.23	0.61
Benzoyl-L-(+)-glutamic acid	0.51	0.18	0.57
4-Hydroxyhippuric acid	0.46	0.11	–
Benzoylglucuronide	0.16	0.01	0.10
Benzoyltaurine	0.13	0.01	0.07
Benzoyl-L-(+)-arginine	0.05	0.00	0.01

Solvents: A, benzene–acetone–acetic acid (2:2:1); B, benzene–acetone–acetic acid (6:2:1); C, acetone–benzene–pentan-1-ol–acetic acid (5:4:2:1) with proportions by vol. The t.l.c. system consisted of Kieselgel 60F_{254} aluminium backed plates (E. Merck, Darmstadt, West Germany). The solvent was allowed to run 150 mm from the origin. All compounds, except cyclohexanecarboxylic acid, could be located on the fluorescent plates by their quenching of U.V. light. Cyclohexanecarboxylic acid was located by the chromosulphuric acid spray of Bertetti [6].

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Hippuric acid [1] and benzoylglutamic acid were determined by reverse isotope dilution. The urine (10 ml) was saturated with NaCl and then benzoyl-L-(+)-glutamic acid (1 g) added, dissolution being facilitated with a little $NaHCO_3$. The solution was brought to pH 1 with 11.6 M HCl and kept for 30 min. It was then extracted with ethyl acetate (3 × 50 ml). The extracts were dried by filtering through Whatman Phase Separating (P.S.) paper and then reduced to 1–2 ml at 40°C. Boiling ethanol (25 ml) followed by benzylamine (3 ml) was added. The solution was cooled to −10°C and treated with acetone (30 ml) followed by light petroleum, b.p. 40–60°C (100 ml). A white precipitate separated which was filtered off, triturated with light petroleum and dried in vacuo. The *benzylamine salt* of benzoyl-L-(+)-glutamic acid was recrystallised to constant specific activity from ethanol/water (20:1, v/v). It had m.p. 182–184°C (Found:C, 63.65; H, 6.2; N, 7.9% $C_{19}H_{22}N_2O_5$ requires C, 63.7; H, 6.2; N, 7.8%).

3. Results

Radiochromatogram scans in solvent A (table 1) showed three peaks of R_F 0.16, 0.51 and 0.79. The first was the major peak and corresponded to benzoylglucuronide. It gave a strong positive naphtharesorcinol test for glucuronic acid and disappeared on incubating with β-glucuronidase (Ketodase) or on warming in 2 M-NaOH, the ^{14}C reappearing at R_F 0.79 which corresponds to benzoic acid.

The peak of R_F 0.51 corresponded to hippuric acid and benzoylglutamic acid (see table 1), but reverse isotope dilution for hippuric acid eliminated this acid and the peak was proved to be due to benzoylglutamic acid.

The benzoylglutamic acid was further identified as follows. The urine (5 ml) saturated with NaCl was brought to pH 1 with 11.6 M-HCl and extracted with ether (3 × 20 ml). The extract was dried with Whatman P. S. paper and taken to dryness at room temperature. The white residue dissolved in methanol (1 ml) was chromatographed on Kieselgel $60F_{254}$ aluminium backed t.l.c. plates (200 × 200 mm). A strip (10 × 200 mm) of the plate was scanned for ^{14}C. In the samples (3) from the urine of the female bats, three ^{14}C peaks were found of R_F in solvent A of 0.16 (benzoylglucuronide), 0.51 (benzoylglutamic acid) and 0.79 (benzoic acid). In the sample from the male bat but not in females an additional minor peak (< 1% of the dose of ^{14}C) was found at R_F 0.63 which corresponded to benzoyl-α or β-alanine but this was not further investigated. In the sample from one (No. 1; see table 3) female bat another minor ^{14}C peak (4% of the urinary ^{14}C; 3% of dose of ^{14}C) was found at R_F 0.30, which did not correspond to any of the reference compounds listed in table 1.

The band of R_F 0.51 which corresponded to 5–16% (in 4 urines) of the ^{14}C excreted was cut from the chromatograms and eluted with methanol (50 ml). The methanol was removed at 40°C and the white residue dissolved in water (0.5 ml). T.l.c. of a sample (10 μl) of the aqueous solution showed that the material ran as a single substance with R_F 0.51 in solvent A, 0.18 in B and 0.60 in C, which correspond to benzoylglutamic acid (see table 1). The aqueous solution was hydrolysed in a sealed tube with 11.6 M HCl (0.5 ml) by heating for 18 h at 110–120°C. The contents of the tube were dried over NaOH pellets in vacuo and the residue dissolved in water (0.1 ml). Paper chromatography in 4 solvents showed it to contain glutamic acid (table 2). The amount of glutamic acid in the hydrolysate was determined by a quantitative

Table 2
Paper chromatography of the amino acid from the conjugate of R_F 0.51 (solvent A)

	R_F *in solvent*			
	D	E	F	G
Amino acid	Whatman No.4		Whatman No.1	
Unknown	0.27	0.13	0.32	0.27
Glutamic acid	0.29	0.14	0.33	0.29
Aspartic acid	0.20	0.14	0.24	0.16
Glycine	0.25	0.38	–	–
Taurine	0.17	0.48	–	–
Alanine	0.34	0.50	–	–

The conjugate of R_F 0.51 (solvent A, table 1) present in fruit bat urine following the administration of [^{14}C]benzoic acid was hydrolysed and the hydrolysate chromatographed. Solvents systems: D, butan-1-ol–acetic acid–water (4:1:1); E, propan-1-ol–ammonia (sp.gr. 0.88) (7:3); F, butan-1-ol–acetic acid–water (12:3:5) with proportions by volume; G, phenol–water (4:1, w/v). Solvents D and E were used with Whatman No. 4 and F and G with No. 1 paper. The amino acids were visualised with a ninhydrin spray (0.1% in acetone)

Table 3
Urinary metabolites of benzoic acid in the Indian fruit bat

Fruit bat No.	Sex	^{14}C excreted in 24 h % dose	% of 24 h excretion of ^{14}C as Unchanged acid	Benzoyl-glucuronide	Benzoyl-glutamic acid		Benzoyl-glycine
1	F	69[a]	0	88	8	(9)[b]	0[b]
1	F	78	3	86	11	–	0[b]
2	F	92	0	95	5	(5)[b]	0[b]
3	M	96	tr.	84	16	(15)[b]	0[b]

[a]A small unknown peak appeared in this urine amounting to 4% of the urinary ^{14}C.
[b]Determined by reverse isotope dilution.
The Indian fruit bats (M = male, 0.81 kg; F = female, 0.37 and 0.39 kg body weight) were injected intraperitoneally with [^{14}C]benzoic acid (100 mg/kg and 11 μCi/bat) and the urine collected for 24 h. The metabolites were separated by t.l.c. and the ^{14}C estimated by scintillation counting and where indicated by reverse isotope dilution. Values are given to the nearest whole number and 0 means not detected (< 0.5% of the ^{14}C excreted). tr. means trace (0.5 – 1% of the ^{14}C excreted).

ninhydrin reaction [4] against a standard curve prepared with L-(+)-glutamic acid. The amount of glutamic acid found was 1.86 μmol/ml. On t.l.c. of the hydrolysate a single ^{14}C peak was found with R_F 0.79 in solvent A corresponding to benzoic acid. On scintillation counting the amount of benzoic acid present was found to be 2.20 μmol/ml. This demonstrates that the metabolite of R_F 0.51 in solvent A contained benzoic acid and glutamic acid in the molecular ratio 1:1.

The quantitative results for three fruit bats, one (No.1) of which was given [^{14}C]benzoic acid on two occasions are shown in table 3. This table shows that the major metabolite of benzoic acid in the fruit bat is benzoylglucuronide which amounts to nearly 90% of the ^{14}C excreted (74% of dose) in 24 h.

The bats also excrete a minor metabolite which on average is about 10% of the ^{14}C excreted and this metabolite is benzoyl-L-(+)-glutamic acid.

4. Discussion

Many animals excrete the glycine conjugate, hippuric acid, as a major metabolite of benzoic acid [1]. Several birds and reptiles also excrete hippuric acid but often the ornithine conjugate is the major metabolite [5]. Insects have also been shown to form hippuric acid. Conjugation with glutamic acid as distinct from glutamine has so far been reported only in arachnids and millipedes [5].

The Indian fruit bat therefore seems to be the first vertebrate in which a glutamic acid conjugate, albeit a relatively minor one, has been reported. Although the fruit bat does not conjugate benzoic acid with glycine, it does have a glycine conjugation system since the major metabolite of phenylacetic acid in the fruit bat is phenacetylglycine [3].

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