Comparative Detoxication

THE DETOXICATION OF AROMATIC ACIDS BY INVERTEBRATES: DETECTION OF AGMATINE CONJUGATES IN SCORPIONS

By MARGARET HITCHCOCK AND J. N. SMITH

*Department of Biochemistry, St Mary's Hospital Medical School, London, W.2,
and Department of Chemistry, Victoria University of Wellington, New Zealand*

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1. Benzoic acid and *p*-nitrobenzoic acid were converted in the scorpion *Palamnaeus* into *N*¹-benzoylagmatine and *N*¹-*p*-nitrobenzoylagmatine. 2. Some benzoyl- and *p*-nitrobenzoyl-arginine were also formed and these compounds were probable precursors of the agmatine derivatives. 3. *p*-Nitrobenzoyl- and *p*-aminobenzoyl-arginine were formed in millipedes dosed with the aromatic acids. 4. Woodlice excreted *p*-amino- and *p*-nitro-benzoic acid as their glycine conjugates and no conjugation could be found in the crab *Gecarcinus* and a freshwater crayfish *Astacus*.

A wide variety of amino acids are used in the peptide detoxication of aromatic acids (Smith, 1964) and it is possible that the identification of a particular conjugation mechanism may be of some taxonomic value. Conjugations of aromatic acids with arginine have been shown to occur in a number of arachnids (Hitchcock & Smith, 1964) and may be characteristic of this Class, though a preliminary report (Smith, 1962) suggests that myriapods may also use this arginine detoxication.

Scorpions are usually classified as an arachnid Order, but when the Indian scorpion *Palamnaeus* sp. was examined by techniques used with spiders and ticks no arginine conjugates were found and the major metabolites of aromatic acids were unidentified basic compounds. We have now identified these scorpion metabolites as the agmatine derivatives of the administered aromatic acids. It is shown below that these are probably formed from arginine conjugates by a secondary decarboxylation reaction and that, as with other arachnids, scorpions form arginine conjugates as the primary detoxication products of aromatic acids.

EXPERIMENTAL

Reference compounds. All melting points are uncorrected. Agmatine sulphate (2g.) was dissolved in the minimum amount of water and stirred with 10g. of NaHCO₃ and 6g. of benzoyl chloride for 2hr. The mixture was acidified with 2*N*-HCl and the benzoic acid filtered off; the filtrate was evaporated to dryness *in vacuo* and extracted with 3 × 50 ml. of acetone. Addition of ether to the acetone solution precipitated a small quantity of a salt of benzoylagmatine,

which was recrystallized from methanol by the addition of ether as *N*¹-benzoylagmatine carbonate, m.p. 192° (decomp.) [Found: C, 56.4; H, 7.4; N, 20.9. (C₁₂H₁₈N₄O)₂.H₂CO₃ requires C, 56.6; H, 7.2; N, 21.1%]. The carbonate was very soluble in water and on the addition of aqueous picric acid formed a sparingly soluble *picrate*, m.p. 218° [Found: C, 46.6; H, 5.0; mol.wt. (Cunningham, Dawson & Spring, 1951), 450. C₁₈H₂₁N₇O₈ requires C, 46.7; H, 4.6%; mol.wt. 463].

Agmatine hydrochloride (2g.) was stirred in 20 ml. of acetone-water (1:1, v/v) with 10 ml. of *N*-KOH. *p*-Nitrobenzoyl chloride (1.9g. in 10 ml. of acetone) was added and a further 10 ml. of *N*-KOH added dropwise to maintain the pH at 7–8. The solution was filtered and the yellow solution concentrated *in vacuo* to remove acetone. The crystalline precipitate was removed and extracted with 10 ml. of boiling water. Insoluble material was filtered off and the filtrate cooled to precipitate the impure *p*-nitrobenzoic acid salt of *p*-nitrobenzoylagmatine. The crude salt (300 mg.) was acidified with *N*-HCl and extracted with ether to remove *p*-nitrobenzoic acid. The aqueous layer was evaporated to dryness and the residue crystallized from ethanol. The crude *p*-nitrobenzoylagmatine hydrochloride was converted into its *p*-nitrobenzoic acid salt, which was recrystallized from water. The *p*-nitrobenzoylagmatine salt of *p*-nitrobenzoic acid formed fine needles, m.p. 187–189° after losing water at 100° (Found: C, 45.9; H, 5.7. C₁₉H₂₂N₆O₇.3H₂O requires C, 45.6; H, 5.6%). It was converted into the hydrochloride as described above and the *p*-nitrobenzoylagmatine hydrochloride had m.p. 188° (Found: C, 40.6; H, 6.4. C₁₂H₁₈ClN₅O₃ requires C, 41.0; H, 6.3%). It was very soluble in water and on the addition of picric acid formed the sparingly soluble *dipicrate*, m.p. 187–189° (Found: C, 38.6; H, 3.7; mol.wt. 381. C₂₄H₂₃N₁₁O₁₇.H₂O requires C, 38.2; H, 3.3%; mol.wt. 378). The *p*-nitrobenzoylagmatine was identical in its chromatographic and ionophoretic behaviour with the product present in solution obtained by incubating *N*²-*p*-nitrobenzoyl-L-arginine over-

night with L-arginine decarboxylase (Sigma Chemical Co., St Louis, Mo., U.S.A.).

Other aroylamino acids were samples prepared previously (Hitchcock & Smith, 1964; Smith, 1958) or were commercial products.

Ring-labelled [¹⁴C]benzoic acid was obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Scorpions were obtained from India (*Palamnaeus* sp., 10–20 g.) or from France (*Euscorpius* sp., 0.5–1 g.) and were kept in closed clear-plastic boxes with access to water. They were fed on mealworms or other small insects but survived readily without food for several months.

Millipedes were obtained from India (*Thyropygus* sp., 10–20 g.) or France (a species of Schizothyllinae, 0.5–1 g.) and kept in moist grass or moss without food.

Woodlice (*Ligia* sp., *Armadillidium* sp.) were collected on the sea shore and from gardens near London.

Land crabs (*Gecarcinus ruricola*, 150–250 g.) were kept on damp moss without food. Freshwater crayfish (*Astacus fluviatilis*) were kept in a shallow layer of freshwater and fed on earthworms.

Ticks (*Boophilus decoloratus*) were obtained from the Cooper Technical Bureau, Berkhamstead, Herts.

Compounds were normally administered as their sodium salts in aqueous solution, at 0.2 mg./g. by injection into the haemocoel, but woodlice were conveniently dosed by allowing them to feed on rice paper that had been soaked in an ether solution of the aromatic acid and dried.

Assay of nitrobenzoyl derivatives. This was carried out by TiCl₃ reduction and coupling with *p*-dimethylaminobenzaldehyde as described by Cohen & Smith (1964) for *p*-nitrobenzoic acid. Zones of chromatograms that contained nitro compounds were left to soak in the reagent mixture for 0.5 hr. and colours measured as described previously.

Table 1. *R_F* values of some aromatic acids and their amino acid conjugates

Solvents were run on Whatman no. 1 or no. 4 paper until the front had moved about 15 in. Solvents were: A, butan-2-one saturated with 2*N*-NH₃; B, ethanol-water (4:1, v/v); C, propan-1-ol-aq. NH₃ (sp.gr. 0.88) (7:3, v/v); D, butan-2-one-water-2*N*-acetic acid (200:100:1, by vol.); E, butan-1-ol-acetic acid-water (4:1:5, by vol.); F, butan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (4:1:5, by vol.).

Solvent	...	<i>R_F</i>					
		A	B	C	D	E	F
Benzoic acid		0.1	0.8	0.9	0.1	0.8	0.4
<i>N</i> ² -Benzoylarginine		0.1	0.7	0.8	0.1	0.5	0.5
Benzoylglutamic acid		0	0.9	0.6	0.9	0.8	0.1
<i>N</i> ² -Benzoylglutamine		0.1	0.7	0.6	0.4	0.7	0.3
Benzoylglycine		0.1	0.8	0.7	0.9	0.9	0.6
<i>N</i> ² -Benzoylcitrulline		0.1	0.7	0.6	0.9	0.6	0.4
<i>N</i> ¹ -Benzoylagmatine		0.2	0.8	0.4	0.1	0.3	0.7
<i>p</i> -Nitrobenzoic acid		0.3	0.8	0.8	0.9	0.9	0.6
<i>N</i> ² - <i>p</i> -Nitrobenzoylarginine		0.1	0.6	0.7	0.4	0.5	0.5
<i>N</i> ² - <i>p</i> -Nitrobenzoylglutamine		0.1	0.6	0.6	0.5	0.3	0.3
<i>p</i> -Nitrobenzoylglutamic acid		0	0.4	0.3	0.8	0.8	0.1
<i>N</i> ² - <i>p</i> -Nitrobenzoylornithine		0.5	0.8	0.9	0.9	0.9	0.8
<i>p</i> -Nitrobenzoylglycine		0.2	0.7	0.6	0.9	0.8	0.4
<i>N</i> ² - <i>p</i> -Nitrobenzoylcitrulline		0.3	0.8	0.8	0.9	0.9	0.7
<i>N</i> ¹ - <i>p</i> -Nitrobenzoylagmatine		0.9	0.9	0.9	—	0.8	0.7
Urea		0.2	0.7	0.6	0.4	0.6	0.4

Table 2. Apparent migration to anode (+) or to cathode (–) of some conjugates of *p*-nitrobenzoic acid

Distances were measured in cm. relative to *p*-nitrobenzoic acid. Ionophoresis was in Britton & Robinson buffer (British Drug Houses Ltd.) or 0.02*N*-NaOH or -HCl at 10 v/cm. for 2 hr.

pH	...	Migration (cm.)						
		12.7	10	8	6	4	3	1.3
<i>p</i> -Nitrobenzoic acid		0	0	0	0	0	0	0
<i>N</i> ² - <i>p</i> -Nitrobenzoylarginine		–9.0	–3.2	–2.8	–3.8	–6.0	–3.8	–1.5
<i>N</i> ² - <i>p</i> -Nitrobenzoylglutamic acid		+2.8	+0.5	+0.9	+1.0	–1.5	–1.0	0
<i>N</i> ² - <i>p</i> -Nitrobenzoylglutamine		–2.7	–1.2	–1.1	–1.5	–2.0	–1.0	0
<i>N</i> ² - <i>p</i> -Nitrobenzoylornithine		–5.2	–1.5	–1.7	–2.0	–3.5	–1.5	0
<i>p</i> -Nitrobenzoylglycine		–1.0	–0.5	–1.2	–0.4	–0.5	–0.1	0
<i>N</i> ² - <i>p</i> -Nitrobenzoylcitrulline		0	0	0	0	–0.1	0	0
<i>N</i> ¹ - <i>p</i> -Nitrobenzoylagmatine		–15.2	–5.4	–4.5	–7.8	–8.4	–6.0	–2.3

Table 3. *Apparent migration to anode (+) or cathode (-) of conjugates of benzoic acid*

Distances were measured in cm. relative to benzoic acid. Ionophoresis was in Britton & Robinson buffer (British Drug Houses Ltd.) or 0.02N-NaOH or -HCl at 10 v/cm.

	pH	Migration (cm.)			
		1.3	4	6	12.7
Benzoic acid	0	0	0	0	0
N ² -Benzoylarginine	-5.6	-7.1	-8.9	-11.5	
Benzoylglutamic acid	+20.0	+1.6	+2.1	+4.0	
N ² -Benzoylglutamine	+1.2	-0.9	-3.0	+2.2	
N ² -Benzoylcitrulline	0	-1.1	-0.6	+1.4	
N ² -Benzoylornithine	-4.3	0	-8.4	+2.0	
N ¹ -Benzoylagmatine	-8.3	-14.7	-11.4	-13.7	
Duration of ionophoresis (hr.) ...	3.5	2.5	4.0	2.5	

Table 4. *Conjugation of [¹⁴C]benzoic acid in Palamnaeus*

Duration of experiment (days)	Extracted radioactivity (μC)	Percentage of dose recovered				
		Unchanged	N ² -Benzoyl-glutamic acid	N ² -Benzoyl-glutamine	N ² -Benzoyl-arginine	N ² -Benzoyl-agmatine
2	0.65	69*	—	—	0.87*	—
2	0.63	58*	2.8*	0.0*	5.7*	—
2	0.89	44*	0.93*	0.0*	0.68*	—
2	0.91	71	0.0*	—	0.71*	29
2	0.86	62	0.74*	—	0.30*	38

* Assay by dilution analysis; other assays were by paper-strip counting.

Recovery of 10–100 μg. of *p*-nitrobenzoic acid, after paper chromatography, was quantitative.

Tissue homogenates containing nitro compounds were mixed with 5 vol. of acetone and centrifuged at 1000g. The supernatant was concentrated in a rotary evaporator and transferred quantitatively to a 7.5 cm.-wide strip of Whatman no. 1 paper, which was developed in solvent F. Zones containing nitro compounds were eluted and rechromatographed in solvent E before assaying as described above.

Extraction of metabolites. Animals were immobilized, if necessary, by freezing to -20° and the integuments removed. The soft internal parts were scraped out and ground with acetone-water (4:1, v/v) in a Potter-Elvehjem homogenizer. After centrifugation at 1000g, the supernatant was concentrated to small bulk for chromatographic or ionophoretic experiments.

In [¹⁴C]benzoic acid experiments with scorpions integument and legs were cut in small pieces and added to the acetone homogenate of internal organs (100 ml./20g. of scorpion), which was refluxed on a water bath for 1 hr. The insoluble part was centrifuged off and refluxed for a further 1 hr. with 15 ml. of water. Most of the radioactivity injected was found in the acetone extract and the rest in the aqueous extract.

Radioactive assays and scanning. These were carried out as described previously (Gessner & Smith, 1960; Hitchcock & Smith, 1964).

Paper chromatography and ionophoresis. These were carried out under conditions used previously (Hitchcock & Smith, 1964; Smith, 1958), with the same methods for detection of spots. Agmatine derivatives gave no ninhydrin reaction but with the Sakaguchi reagents gave a colour similar to that of the arginine conjugates. The degree of separation between conjugates is shown in Tables 1, 2 and 3.

RESULTS

Metabolism of [¹⁴C]benzoic acid. Scorpions (*Palamnaeus* sp.) were injected with 2 mg. of [¹⁴C]benzoic acid and kept for 2 days in closed plastic boxes. They were extracted as described above and the box was rinsed with water, which was added to the extract. The extract was divided into portions and analysed by dilution analysis for benzoic acid, benzoylglutamic acid, benzoylglutamine and benzoylarginine as described by Hitchcock & Smith (1964).

Other portions of the extract containing about 0.01 μC of radioactivity were applied to chromatograms or ionophoretic strips with 100 μg. of the reference *N*-benzoylamino acids. In quantitative experiments, strips were scanned for radioactivity after ionophoretic separation at pH 6.

Trace amounts of benzoylglutamic acid and benzoylarginine were detected (Table 4) in addition to the major metabolite, benzoylagmatine. Other conjugates of benzoic acid were not observed.

Metabolism of *p*-nitrobenzoic acid and *p*-aminobenzoic acid. Scorpions or millipedes were dosed, left for 48 hr. at room temperature and extracted as described above. Initial large-scale chromatograms, as described by Hitchcock & Smith (1964), were run in solvent F and bands, after location, were eluted in aq. 0.1N-ammonia for further characterization.

Millipedes (*Thyropygus* and *Schizothyllinae*) normally yielded an extract giving two zones at R_F 0.5 and 0.6. The zone at R_F 0.6 was identified as unchanged *p*-nitrobenzoic acid, and the zone at R_F 0.5 gave the colour reactions of *p*-nitrobenzoylarginine and was chromatographically and ionophoretically identical with this compound in each of the solvent systems or buffers of Tables 1, 2 and 3. In some experiments traces of *p*-nitrobenzoylglutamic acid were present in *Thyropygus* extracts. When *p*-aminobenzoic acid was injected into *Thyropygus* and the animals were extracted a week later, only *p*-aminobenzoylarginine was detected by its chromatographic and ionophoretic properties.

The scorpion *Euscorpis* yielded two bands in solvent system F, one of which consisted of unchanged *p*-nitrobenzoic acid, at R_F 0.6, and the second, at R_F 0.1, gave the colour reactions of *p*-nitrobenzoylglutamic acid and behaved identically in each of the solvent systems and buffers of Tables 1 and 2.

The scorpion *Palamnaeus*, in most experiments, yielded two bands in solvent F, one of which, at R_F 0.6, was unchanged *p*-nitrobenzoic acid, and the second, at R_F 0.7, gave a positive Sakaguchi reaction. After elution it behaved identically with *p*-nitrobenzoylagmatine in each of the systems and buffers of Tables 1 and 2. Some *Palamnaeus* specimens gave extracts that contained very weak zones in solvent system F with R_F 0.1, and large amounts of this compound were found in extracts of a scorpion that died during the experiment. This zone was identified chromatographically and ionophoretically as *p*-nitrobenzoylglutamic acid.

Scorpions (*Palamnaeus* sp.) were also dosed with *p*-nitrobenzoic acid after preliminary treatment with neomycin. This was injected daily for 3 days (10 µg./scorpion) or provided as a 1% solution in water during the week before injection of *p*-nitrobenzoic acid. No significant change was seen in the nature of the metabolites.

The terrestrial crustaceans *Ligia* sp. and *Armadillidium* sp. were dosed with *p*-aminobenzoic acid or *p*-nitrobenzoic acid as described above and the

excreta were ground with a few drops of water and chromatographed in solvent F. Only one zone was found that differed from that of the aromatic acid administered.

These bands were eluted with 0.1N-ammonia and the eluate was examined in each of the systems of Tables 1 and 2. In each system the eluate behaved identically with the glycine conjugate of the aromatic acid used.

In experiments with the land crab *Gecarcinus* and the freshwater crayfish *Astacus*, no evidence of amino acid conjugations could be found.

Metabolism of some conjugates in arachnids. Twelve ticks were injected with 20 µg. each of the conjugates of *p*-amino- or *p*-nitrobenzoic acid dissolved in 2 µl. of water. They were kept at 32° for 2 days and extracted as described by Hitchcock & Smith (1964), and the extracts were examined ionophoretically and chromatographically for metabolites. Small amounts of unchanged conjugates were detected and some hydrolysis to the aromatic acids occurred, but the conjugates of *p*-nitrobenzoic acid with L- and DL-arginine, L-citrulline, L-ornithine and L-glutamine were all converted into *p*-nitrobenzoylglutamic acid. No other conjugates were detected. Similar results were obtained when the *p*-aminobenzoyl conjugates were administered, *p*-aminobenzoylglutamic acid being formed in each case.

When scorpions (*Palamnaeus* sp.) were dosed with 1 mg. of *p*-nitrobenzoyl-L-arginine in 0.2 ml. of water and extracted after 2 days, *p*-nitrobenzoylagmatine was found along with traces of *p*-nitrobenzoic acid and unchanged *p*-nitrobenzoylarginine.

Metabolism of conjugates in tissue homogenates. Mixed tissues from the scorpion body were ground with aq. 0.8% sodium chloride in a Potter-Elvehjem homogenizer to give 3 ml. of homogenate/scorpion. The homogenate (1 ml.) was incubated for 2 hr. at 37° with 0.5 mg. of the N^2 -*p*-nitrobenzoyl derivative of L-arginine, L-ornithine or L-citrulline and 5 ml. of buffer solutions ranging from pH 3 to 10. McIlvaine's (1921) citrate-phosphate buffer (0.1 M) was used between pH 3 and 8 and Sørensen's (1912) glycine buffer (0.1 M) between pH 8.5 and 10.

The solutions were deproteinized by the addition of 30 ml. of acetone, concentrated to small bulk and examined in each of the chromatographic systems of Table 1. Different products were found at different pH values (see Table 5), and no reaction occurred in the control tubes containing boiled homogenate.

In similar quantitative experiments with *p*-nitrobenzoyl-L-arginine as substrate rates of formation of metabolites (in µg./scorpion/hr.) were 50, 81, 82 and 68 for *p*-nitrobenzoylagmatine, 112, 150 and 75 for *p*-nitrobenzoylglutamic acid, and 92, 95 and 100 for *p*-nitrobenzoylornithine.

Table 5. *Formation of metabolites from some conjugates of p-nitrobenzoic acid*

Homogenate from scorpions was incubated at 37° in buffer with 0.5 mg. of substrate.

Substrate	pH range for optimum formation				
	<i>p</i> -Nitrobenzoyl- agmatine	<i>p</i> -Nitrobenzoyl- ornithine	<i>p</i> -Nitrobenzoyl- citrulline	<i>p</i> -Nitrobenzoyl- glutamic acid	<i>p</i> -Nitrobenzoyl- arginine
<i>p</i> -Nitrobenzoylarginine	4.5-6.0	8.0-10.0	Not found	6.5-8.5	—
<i>p</i> -Nitrobenzoylornithine	Not found	—	5.5-6.5	Not found	Not found
<i>p</i> -Nitrobenzoylcitrulline	Not found	4.5-6.5	—	7.0 (trace)	5.5-8.5

Scheme 1.

Values for the rate of conversion of *p*-nitrobenzoyl-L-ornithine into *p*-nitrobenzoylcitrulline were 95 and 70 $\mu\text{g.}/\text{scorpion}/\text{hr.}$; *p*-nitrobenzoyl-L-citrulline was converted into the ornithine derivative at 70 $\mu\text{g.}/\text{scorpion}/\text{hr.}$ and into *p*-nitrobenzoylarginine at a maximum rate of 30 $\mu\text{g.}/\text{scorpion}/\text{hr.}$

Assays of the arginase activity in the homogenates from six scorpions by using the urease micro-diffusion technique (Glick, 1963) were in the range 0.7-3.1 μmoles of urea/min. at 25°.

DISCUSSION

The small amount of arginine conjugation detected in *Palamnaeus* dosed with aromatic acids and the demonstration that injected *p*-nitrobenzoylarginine was rapidly decarboxylated *in vitro* by scorpion homogenates supports the view that scorpions use arginine as the amino acid in the peptide detoxication as do the other arachnids. These observations reinforce the suggestion made by Hitchcock & Smith (1964) that the arginine conjugation is a biochemical characteristic of the arachnid Class.

Although all the arachnids so far studied have formed arginine conjugates a notable feature of the metabolism of aromatic acids in this group has been the multiplicity of other conjugated forms of aromatic acids found. Thus *Palamnaeus* extracts always contained small amounts of glutamic acid conjugates and these were major metabolites in *Euscorpis*. Extracts of ticks had both arginine and glutamic acid conjugates, and extracts of spiders and harvestmen usually contained glutamine

and glutamic acid conjugates as well as the primary arginine conjugates. It seems probable that all of these may have been derived from a primary detoxication product, which in each case was the arginine conjugate of the aromatic acid administered (see Scheme 1), and it is these rather than the glutamic acid conjugates that merit the trivial name of arachnuric acids.

The mechanism by which the arginine conjugates were converted into the secondary metabolites is not clear. In experiments with homogenates the major product of enzyme action on *p*-nitrobenzoylarginine depended on the pH of the reaction mixture. The major metabolite *in vivo*, *p*-nitrobenzoylagmatine, was formed at about pH 5 *in vitro* but at pH 9 the major product was *p*-nitrobenzoylornithine, and at pH 7 was *p*-nitrobenzoylglutamic acid. The decarboxylation of *p*-nitrobenzoylarginine in homogenates or intact scorpions occurred equally well in the presence of neomycin and, though bacterial arginine decarboxylases have pH optima near 5 (Gale, 1946), it seems unlikely that the formation of agmatine conjugates is due to bacterial contamination.

Palamnaeus homogenates also catalysed reactions analogous to those of the normal urea cycle and contained an active true arginase, but *in vivo* neither *p*-nitrobenzoylornithine nor -citrulline could be identified as an intermediate in the metabolism of *p*-nitrobenzoic acid or *p*-nitrobenzoylarginine. The *p*-nitrobenzoylglutamic acid, which was formed by all arachnids to some extent *in vivo*, was only formed from *p*-nitrobenzoylarginine in homogenates.

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