Comparative Detoxication

13. DETOXICATION OF AROMATIC ACIDS IN ARACHNIDS: ARGININE, GLUTAMIC ACID AND GLUTAMINE CONJUGATIONS

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The arachnids are an important and biologically successful class of the arthropod phylum but, in comparison with the insects, little attention has been paid to their biochemistry. The most frequently quoted biochemical difference between insects and arachnids is related to their nitrogenous metabolism: a major end product in arachnid excreta is often guanine (Florkin, 1949; Mlodecki, 1958; Schmidt, Liss & Thannhauser, 1955; Rao & Gopalakrishnaireddy, 1962) whereas insects characteristically excrete uric acid.

We wished to see if the detoxication mechanisms of arachnids showed any similar class characteristic which would qualitatively distinguish them from insects and other arthropods. The peptide conjugation of aromatic acids was selected for initial study since it was known that considerable variation was possible in the amino acid used in this detoxication mechanism (Smith, 1964).

A second consideration was the hope that studies of the detoxication mechanisms of arachnids might provide data useful to designers of selective acaricides and might possibly throw light on some examples of insecticide resistance in arachnids. This consideration applied particularly to ticks, since many of these are disease vectors or agricultural pests and have been subjected to repeated insecticidal attack.

Preliminary accounts of some of this work have already been reported (Smith, 1962; Hitchcock & Smith, 1963).

EXPERIMENTAL

Reference compounds. All melting points are uncorrected. The benzoyl derivatives of the amino acids were prepared by standard methods from benzoyl chloride. *N*²-Benzoyl-L-arginine had m.p. 298°, $[\alpha]_D^{20} + 17^\circ$ (c 1 in *N*-NaOH). It formed a *picrate*, m.p. 190° [Found: mol.wt. 487 (Cunningham, Dawson & Spring, 1951); $C_{19}H_{21}N_7O_{10}$ requires mol.wt. 507]. *N*²-Benzoyl-L-glutamine had m.p. 154°, $[\alpha]_D^{20} + 16^\circ$ (c 1 in *N*-NaOH). Benzoyl-L-glutamic acid, m.p. 138°, had $[\alpha]_D^{20} + 18^\circ$ (c 1 in *N*-NaOH), and formed a *dibenzylamine salt*, m.p. 115° (Found: N, 9.2; $C_{26}H_{31}N_2O_5$ requires: N, 9.0%). *N*²-Benzoyl-L-citrulline had m.p. 175°, $[\alpha]_D^{20} + 20^\circ$

(c 1 in *N*-NaOH) (Found: C, 55.1; H, 6.0; N, 14.6. $C_{13}H_{17}N_3O_4$ requires C, 55.9; H, 6.1; N, 15.0%).

*N*²-*p*-Nitrobenzoyl-L-arginine was made by the mixed anhydride method from ethyl chloroformate and *p*-nitrobenzoic acid (see Albertson, 1962). It crystallized from methanolic 2*N*-HCl as the hydrochloride, m.p. 198°, $[\alpha]_D^{20} - 176^\circ$ (c 1 in *N*-NaOH) (Found: HCl, 9.8; H_2O , 2.3. Calc. for $C_{13}H_{17}N_5O_6 \cdot HCl \cdot \frac{1}{2}H_2O$: HCl, 9.8; H_2O , 2.4%).

Other *p*-nitrobenzoyl derivatives were prepared from *p*-nitrobenzoyl chloride and the amino acids. *p*-Nitrobenzoyl-L-glutamic acid had m.p. 112°, $[\alpha]_D^{20} + 16^\circ$ (c 1 in *N*-NaOH). *N*²-*p*-Nitrobenzoyl-L-glutamine had m.p. 183°, $[\alpha]_D^{20} + 18^\circ$ (c 1 in *N*-NaOH).

*N*²-*p*-Nitrobenzoyl-DL-citrulline had m.p. 193° (Found: C, 48.1; H, 4.5; N, 17.3. $C_{13}H_{16}N_4O_6$ requires C, 48.1; H, 5.0; N, 17.3%).

p-Aminobenzoyl derivatives were prepared from nitro compounds by reduction with H_2 and Adams platinum catalyst in methanol. *N*²-*p*-Aminobenzoyl-L-arginine sulphate crystallized from 2*N*- H_2SO_4 by addition of ethanol and had m.p. 138° (decomp.), $[\alpha]_D^{20} + 16^\circ$ (c 1 in *N*-NaOH) (Found: C, 34.8; H, 5.9; N, 15.7. $C_{13}H_{16}N_3O_6 \cdot H_2SO_4 \cdot 3H_2O$ requires C, 35.0; H, 6.1; N, 15.7%).

p-Aminobenzoyl-L-glutamic acid had m.p. 173° and $[\alpha]_D^{20} + 29^\circ$ (c 1 in *N*-NaOH). *N*²-*p*-Aminobenzoyl-L-glutamine had m.p. 210°, $[\alpha]_D^{20} + 23^\circ$ (c 1 in *N*-NaOH). *N*²-*p*-Aminobenzoyl-DL-citrulline had m.p. 185° (Found: C, 51.9; H, 6.0; H_2O , 2.8. $C_{13}H_{18}N_4O_4 \cdot \frac{1}{2}H_2O$ requires C, 51.5; H, 6.3; H_2O , 3.0%). *N*²-Substituted ornithine derivatives were samples prepared by Smith (1958). Glycine conjugates were commercial samples.

The *p*-anisidine salt of (*p*-aminobenzoyl β -D-glucosid)-uronic acid, m.p. 184°, was a sample prepared by Smith (1958).

p-Aminobenzoyl β -D-glucoside was prepared from the sodium salt of 4,6-benzylideneglucose (Zervas, 1931). The sodium salt (6.5 g.) and *p*-nitrobenzoyl chloride (4 g.) were shaken in 40 ml. of $CHCl_3$ for 10 min. and left at room temperature for 1 hr. The product was filtered, washed with $CHCl_3$ and water and crystallized from 250 ml. of aqueous 80% (v/v) acetone. The 4,6-benzylidene-1-(*p*-nitrobenzoyl)-glucose, (6 g.) had m.p. 235° (decomp.), $[\alpha]_D^{25} - 46^\circ$ (c 0.2 in acetone) (Found: C, 57.5; H, 4.6; N, 3.4. $C_{20}H_{16}NO_9$ requires C, 57.4; H, 4.6; N, 3.4%).

The nitro compound (2 g.) was dissolved in 100 ml. of aqueous 50% (v/v) methanol and reduced with H_2 and Adams platinum catalyst for 3 hr. at room temperature. Evaporation of the solvent gave 1.3 g. of needle-shaped crystals of 1-(*p*-aminobenzoyl)-4,6-benzylideneglucose, which had m.p. 242° (decomp.), $[\alpha]_D^{20} - 24^\circ$ (c 0.3 in acetone) (Found: C, 61.6; H, 5.3; N, 3.4. $C_{20}H_{21}NO_7$ requires C, 62.0; H, 5.5; N, 3.0%).

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The amino compound (1 g.) was stirred vigorously with 10 ml. of 0.1 N-H₂SO₄ at 60° until it dissolved (45 min.). The solution was neutralized with Ba(OH)₂, filtered and evaporated to dryness *in vacuo*. The residue was crystallized from ethanol to give needles, 0.3 g., of 1-*p*-aminobenzoyl β -D-glucoside, m.p. 212° (decomp.), [α]_D²⁰ -20° (c 0.3 in H₂O) (Found: C, 51.9; H, 5.6; N, 5.0. C₁₃H₁₇NO₇ requires C, 52.2; H, 5.7; N, 4.7%).

The glucoside (300 mg.) was treated with 1 ml. of acetic anhydride and 1 drop of 70% HClO₄. After cooling, water was added to precipitate the 1-(*p*-acetamidobenzoyl) tetra-acetyl glucoside, which on crystallization from ethanol had m.p. 196-198°, [α]_D²⁰ -36° (c 1 in CHCl₃) (Found: C, 53.7; H, 5.3; N, 2.8. C₂₃H₂₇NO₁₂ requires C, 54.2; H, 5.3; N, 2.8%). This compound was also prepared from *p*-acetamidobenzoic acid and tetra-acetyl- α -1-bromoglucose by the procedure of Glazer & Wulwek (1924). After crystallization from ethanol the *p*-acetamidobenzoyl tetra-acetyl glucoside had [α]_D²⁰ -34° (c 1 in CHCl₃) and m.p. 200°, not depressed by mixing with the previous sample.

The potassium salt of *p*-carboxyphenylsulphamic acid (*p*-aminobenzoic acid *N*-sulphate) was prepared from *p*-aminobenzoic acid and chlorosulphonic acid according to the procedure for synthesizing *O*-sulphates (Burkhardt & Lapworth, 1926).

Ring-labelled [¹⁴C]benzoic acid was obtained from The Radiochemical Centre, Amersham, Bucks. (specific activity approx. 1 mc/m-mole).

Animals. The cattle tick *Boophilus decoloratus* was provided by the Cooper Technical Bureau as engorged adult females weighing about 100 mg. They were dosed during the week before the onset of egg laying. Compounds were administered by injection of aqueous solutions (20 μ g. in 2 μ l.) into the body cavity at a point near the capitulum. With care it was possible to carry out injections without puncturing the enormous stomach, which accounted for most of the bulk of the animal, and in tests with [¹⁴C]benzoic acid it was found possible to inject 2 μ l. of the aqueous solutions with less than 2% volumetric error. Ticks were kept at 32-34° in beakers for the duration of the experiments.

Spiders (*Tegenaria* sp., a house spider; *Epeira* sp. and *Agelena* sp., garden spiders) were collected in the autumn and dosed immediately by subcutaneous injection on the dorsal side of the abdomen. Spiders were more easily killed by the injection process than insects or ticks but those surviving for a few hours normally survived for up to 2 weeks. Injections (0.5 mg./g. in water) were given with either a fine glass capillary or, with spiders weighing more than 0.5 g., a no. 20 hypodermic needle. After dosing they were left in separate beakers for periods of up to 2 weeks. Food and water were not required. Harvestmen (*Mitopus* sp. and *Phalangium* sp.) were also collected in the autumn and dosed similarly. These were kept in gauze-covered beakers and fed on small pieces of bread and butter with access to water.

Extraction of tissue or excreta. Bodies of spiders, harvestmen or ticks were ground separately in a glass homogenizer with 5 ml. of aqueous 80% (v/v) acetone and the mixture was centrifuged. The supernatant solution was concentrated to small bulk *in vacuo*, if necessary, before being used for chromatography in qualitative experiments.

In quantitative experiments with [¹⁴C]benzoic acid the homogenates in acetone were diluted to 25 ml. and stirred under reflux for 0.5 hr. on a steam bath with added diluent

reference compounds if required. After removal of the solvent, the extraction was repeated until no further radioactivity could be extracted. Normally, three or four such extractions were required.

The cattle ticks used did not produce any excreta and excretion by the spiders was erratic and usually slow. If any small specks of excreta were available, they were collected by suspending in a few microlitres of water and transferred to filter-paper strips for chromatography. Harvestmen produced excreta freely and this was examined separately from the bodies.

Measurement of radioactivity. Solid and liquid samples were measured in aluminium planchets under a thin end-window counter tube as described by Gessner & Smith (1960). Chromatographic or paper-ionophoretic strips (Whatman no. 4 paper) were attached to a pen-recorder chart with adhesive tape and drawn between two opposed thin end-window tubes which were connected to a Panax RM 202 rate meter supplying the pen recorder. Radioactivity on the strips was estimated by reference to a calibration curve, relating radioactivity and peak areas, prepared from samples of [¹⁴C]benzoic acid also chromatographed on no. 4 paper. No significant difference was found between the response after chromatographic or ionophoretic separation and known amounts of radioactive compounds were recovered within $\pm 5\%$ when 0.001-0.01 μ C zones were assayed.

Measurements of fluorescence. An Aminco-Bowman spectrophotofluorimeter was used and fluorescence-pH curves were prepared from reference compounds (1 μ g./ml.) by adding small amounts of 10N- or 1N-HCl or 1N-NaOH to 100 ml. of solution followed by immediate determination of pH and fluorescence intensity. Similar curves for eluted chromatograms of extracts were made by diluting the eluates into 0.01M-citrate buffer or into 0.1-0.01N-HCl. With the instrument used all the compounds were excited by light at 300 m μ and fluoresced at 360 m μ in the more alkaline pH range. No fluorescence was exhibited in strongly acid solution.

The curves had the sigmoid form of a simple titration curve and apparent pK₁ values for a number of possible aminobenzoyl conjugates were determined by noting the pH at which fluorescence was half the maximum. The glycosidic conjugates and *p*-aminobenzoic acid itself were clearly distinguished from the amino acid conjugates, which all had pK₁ near 3 (Table 1).

Paper chromatography and ionophoresis. This was done as described by Smith (1958) and separations achieved are shown in Tables 2-5. Unless stated differently, all identifications were carried out in all of the solvent and buffer systems and unknowns and reference samples were run side by side on the same paper.

Colour reactions. Reactions for nitro and aromatic amino compounds were those used by Cohen & Smith (1964). *p*-Carboxyphenylsulphamic acid reacted only slowly with 1% dimethylaminobenzaldehyde and 2N-acetic acid but reaction was more rapid if the reagent contained 0.1N-HCl or if the papers were heated or exposed to ultraviolet light. Arginine derivatives were detected by the Sakaguchi reaction (Jepson & Smith, 1953). Glycine conjugates were detected by the orange-coloured azlactones (El Masri, Smith & Williams, 1956). Urea and citrulline conjugates gave lemon-yellow colours with the dimethylaminobenzaldehyde reagent used for aromatic amines.

RESULTS

Metabolism of [¹⁴C]benzoic acid

Dilution analyses. Six ticks were dosed by injection with [¹⁴C]benzoic acid (0.1–0.5 μ l., 2–10 μ g.) and after 2–4 days at 32° they were extracted with aqueous acetone as described above. Benzoic acid, N²-benzoyl arginine and benzoylglutamic acid (0.5 g. each) were added to the aqueous acetone during the extraction. The extract was concentrated *in vacuo* to 25 ml. and extracted with 3 × 50 ml. of ether to remove benzoic acid. The ether solution was evaporated to dryness and the residue crystallized twice from water, when it had constant specific activity and m.p. 121°.

The aqueous layer, containing benzoylglutamic acid and benzoylarginine, was extracted with

3 × 50 ml. of ethyl acetate. The ethyl acetate was concentrated to small bulk and treated with benzylamine to give the dibenzylamine salt of benzoyl-L-glutamic acid. This was crystallized from ethanol to constant specific activity and m.p. 115°.

The aqueous layer containing the benzoyl-arginine was treated with charcoal, filtered and concentrated to a syrup *in vacuo*. N²-Benzoyl-L-arginine was precipitated by adding acetone and recrystallized from aqueous acetone to constant specific activity. It was then converted into its picrate, m.p. 190°, which had the same equivalent specific activity.

Dilution analysis for benzoylornithine in tick extracts was carried out in a separate experiment in which 0.5 g. of N²-benzoyl-L-ornithine was added to the homogenized bodies in aqueous acetone. After concentration of the extract, and extraction with ether to remove benzoic acid, as above, the aqueous layer was concentrated to small bulk and acidified to pH 3 with hydrochloric acid. The precipitated benzoyl-L-ornithine was recrystallized twice from ethanol to give a sample, m.p. 222°, without entrained radioactivity.

Chromatographic and ionophoretic analysis. Ticks were dosed by injection with [¹⁴C]benzoic acid and extracted repeatedly with aqueous acetone as described above. When all the radioactivity had

Table 1. Apparent pK₁ values for p-aminobenzoyl conjugates determined spectrophotofluorimetrically

	pK ₁
p-Aminobenzoic acid	4.5
p-Aminohippuric acid	3.2
p-Aminobenzoylglutamic acid	3.2
N ² -p-Aminobenzoylglutamine	2.9
N ² -p-Aminobenzoylornithine	3.0
N ² -p-Aminobenzoylarginine	2.7
N ² -p-Aminobenzoylcitrulline	2.9
1-p-Aminobenzoyl glucoside	1.9
(1-p-Aminobenzoyl glucosid)uronic acid	1.9

Table 2. R_F values of some aromatic acids and their amino acid conjugates

Solvents were run on Whatman no. 1 or 4 paper until the front had moved about 15 in. Solvents were: A, butan-2-one saturated with 2N-ammonia; B, ethanol-water (4:1, v/v); C, propan-1-ol-ammonia (sp.gr. 0.88) (7:3, v/v); D, butan-2-one-water-2N-acetic acid (200:100:1, by vol.); E, butan-1-ol-acetic acid-water (4:1:5, by vol.); F, butan-1-ol-ammonia (sp.gr. 0.88)-water (4:1:5, by vol.); G, propan-2-ol-ammonia (sp.gr. 0.88)-water (8:1:1, by vol.).

Solvent	...	...	...	A	B	C	D	E	F	G
Benzoic acid				0.1	0.8	0.9	0.1	0.8	0.4	0.7
N ² -Benzoylarginine				0.1	0.7	0.8	0.1	0.5	0.5	0.6
Benzoylglutamic acid				0	0.9	0.6	0.9	0.8	0.1	0.3
N ² -Benzoylglutamine				0.1	0.7	0.6	0.4	0.7	0.3	0.6
Benzoylglycine				0.1	0.8	0.7	0.9	0.9	0.6	0.7
N ² -Benzoylcitrulline				0.1	0.7	0.6	0.9	0.6	0.4	0.7
p-Nitrobenzoic acid				0.3	0.8	0.8	0.9	0.9	0.6	—
N ² -p-Nitrobenzoylarginine				0.1	0.6	0.7	0.4	0.5	0.5	—
N ² -p-Nitrobenzoylglutamine				0.1	0.6	0.6	0.5	0.3	0.3	—
p-Nitrobenzoylglutamic acid				0	0.4	0.3	0.8	0.8	0.1	—
N ² -p-Nitrobenzoylornithine				0.5	0.8	0.9	0.9	0.9	0.8	—
p-Nitrobenzoylglycine				0.2	0.7	0.6	0.9	0.8	0.4	—
N ² -p-Nitrobenzoylcitrulline				0.3	0.8	0.8	0.9	0.9	0.7	—
Urea				0.2	0.7	0.6	0.4	0.6	0.4	—
p-Aminobenzoic acid				0	0.8	0.3	0.9	0.9	0	—
N ² -p-Aminobenzoylarginine				0	0.6	0.3	0.1	0.5	0.2	—
N ² -p-Aminobenzoylglutamine				0	0.6	0.2	0.2	0.6	0.1	—
p-Aminobenzoylglutamic acid				0	0.5	0.1	0.3	0.7	0	—
N ² -p-Aminobenzoylornithine				0	0.5	0.3	0.1	0.3	0.1	—
p-Aminobenzoylglycine				0	0.6	0.3	0.4	0.7	0.2	—
p-Aminobenzoyl glucoside				0.7	0.8	0.6	0.5	0.5	0.5	—
(p-Aminobenzoyl glucosid)uronic acid				0	0.5	0.3	0.1	0.4	0.1	—
N ² -p-Aminobenzoylcitrulline				0	0.5	0.4	0.2	0.6	0.1	—
p-Carboxyphenylsulphamic acid				0	0.4	0.1	0.1	0.3	0	—

been extracted the solution was reduced to small bulk *in vacuo* and portions were chromatographed in solvent systems C, E and F (Table 2).

Radioactive areas corresponding to reference benzoylarginine and benzoylglutamic acid were present; these were measured by the strip-scanning procedure described above (Table 6).

Extracts of excreta and bodies from harvestmen or spiders dosed with [^{14}C]benzoic acid were also scanned for radioactive metabolites after ionophoretic separation at pH 6, which allowed the separation of benzoylglutamic acid, benzoylarginine and benzoic acid. Benzoylglutamine and benzoyl-citrulline ran close together at this pH and zones which could have contained either of these compounds were eluted with water and rechromatographed in solvent system F. A single zone was

present having R_F identical with reference benzoyl-L-glutamine. This was eluted with 10 ml. of water and 0.5 g. of unlabelled benzoylglutamine was added. Recrystallization of the benzoyl-L-glutamine yielded a sample, m.p. 154° , containing radioactivity equivalent to all the eluted material.

No radioactive benzoyl-L-citrulline was recovered from the filtrate after addition and recrystallization of 0.5 g. of reference benzoyl-L-citrulline. Results of the ^{14}C assays are summarized in Table 6.

Metabolism of p-nitrobenzoic acid

Separation of metabolites. When extracts of spiders, harvestmen or ticks were chromatographed in solvent system F (cf. Table 2), zones containing nitro compounds could, in general, be

Table 3. *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 0.02N-NaOH or -HCl at 10 v/cm. for 2 hr.

pH	...	...	...	...	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

Table 4. *Apparent migration to anode (+) or cathode (-) of some conjugates of p-aminobenzoic acid*

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

pH	...	...	...	...	...	1.3	2.5	3.9	7.5	9.0	12.7
<i>p</i> -Aminobenzoic acid						0	0	0	0	0	0
<i>p</i> -Aminohippuric acid						0	0	+3.2	-0.9	-0.2	-0.1
<i>p</i> -Aminobenzoylglutamic acid						+1.6	+1.0	+3.5	+2.0	+3.0	+1.7
<i>N</i> ² - <i>p</i> -Aminobenzoylglutamine						+1.6	+1.0	+2.8	-2.0	+0.3	-2.8
<i>N</i> ² - <i>p</i> -Aminobenzoylcitrulline						+1.8	+1.0	+2.2	-2.2	-0.2	-3.7
<i>N</i> ² - <i>p</i> -Aminobenzoylarginine						-4.4	-3.7	-2.2	-7.7	-5.4	-10.6
<i>N</i> ² - <i>p</i> -Aminobenzoylornithine						-5.4	-4.9	-1.5	-6.9	-4.8	-5.2
<i>N</i> ⁵ - <i>p</i> -Aminobenzoylornithine						-3.4	-4.2	-1.2	-6.6	-4.8	-4.9
<i>N</i> ² <i>N</i> ⁵ -Bis-(<i>p</i> -aminobenzoyl)ornithine						-2.4	-1.7	+1.0	-3.6	-3.1	-5.5
<i>p</i> -Aminobenzoyl glucoside						+4.1	+4.4	-1.3	-6.5	-4.8	-10.8
(<i>p</i> -Aminobenzoyl glucosid)uronic acid						+4.8	+6.3	+3.3	-2.4	-1.7	-4.8
<i>p</i> -Carboxyphenylsulphamic acid						+15.8	+13.1	+6.0	+5.8	+5.8	+4.9

Table 5. *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	...	...	...	...	1.3	4	6	12.7
Benzoic acid					0	0	0	0
<i>N</i> ² -Benzoylarginine					-5.6	-7.1	-8.9	-11.5
Benzoylglutamic acid					+20.0	+1.6	+2.1	+4.0
<i>N</i> ² -Benzoylglutamine					+1.2	-0.9	-3.0	+2.2
<i>N</i> ² -Benzoylcitrulline					0	-1.1	-0.6	+1.4
<i>N</i> ² -Benzoylornithine					-4.3	0	-8.4	+2.0
Duration of ionophoresis (hr.)	...	...	...	...	3.5	2.5	4.0	2.5

detected at R_F values 0.6 (I), 0.5 (II), 0.3 (III) and 0.1 (IV). The relative intensity of these bands varied considerably with the source of the extract (see Table 7).

The extracts of bodies or excreta were first separated on a suitably large-scale chromatogram in this solvent and the zones eluted with aqueous 0.1N-ammonia for further examination.

Identification of material in band I. *p*-Nitrobenzoic acid was identified by paper chromatography in the solvents of Table 2. Conjugation was usually extensive and in extracts made from bodies 24 hr. after dosing, or from excreta, only a small amount of unchanged *p*-nitrobenzoic acid was found.

Identification of p-nitrobenzoylarginine in band II. In each chromatographic system of Table 2 and after ionophoresis at each pH value of Table 3 single spots were obtained from the eluate of band II. These were identical in each system or pH with reference *p*-nitrobenzoylarginine, and gave the same coloured Sakaguchi reaction but no reaction with ninhydrin.

A portion of the eluate of band II from *Tegenaria* extracts was subjected to ionophoresis at pH 12.7 for 2 hr. and the Sakaguchi-positive zone eluted with water. The eluate was evaporated to dryness *in vacuo* and heated with 50 mg. of hydrazine sulphate and 0.5 ml. of dry hydrazine at 60° for 16 hr. The solution was evaporated and treated with dinitrofluorobenzene and sodium hydrogen carbonate and examined for amino acid hydrazides as described by Bradbury (1956). Only the ornithine derivative was detected; since no ornithine or other free amino acid was present in the original eluate, this was taken to indicate the presence there of an arginine conjugate (Bradbury, 1956).

A portion of the eluate of band II, purified by ionophoresis as above, was eluted with 0.1M-borate buffer, pH 9, and incubated overnight at 25° with Mn^{2+} ions and arginase (L. Light and Co. Ltd.). Chromatography of the incubation mixture in solvent systems E and F showed the presence of N^2 -*p*-nitrobenzoylornithine and urea. No *p*-nitrobenzoylarginine remained.

Identification of p-nitrobenzoylglutamine in band III. The eluate of this zone from *Epeira* excreta was identical with reference *p*-nitrobenzoylglutamine in the solvents of Table 2 and also on ionophoresis at pH 12.7 and 1.3. The spots gave no Sakaguchi or ninhydrin reactions. Only small amounts of this material were found in *Tegenaria* extracts and it was not detected as a metabolite in harvestmen or ticks (Table 7).

Identification of p-nitrobenzoylglutamic acid in band IV. Chromatography of the eluate of this band in the solvent systems of Table 2 and at each pH of Table 3 showed it to consist of a single compound identical in behaviour with reference *p*-nitrobenzoylglutamic acid.

Metabolism of *p*-aminobenzoic acid

Separation of metabolites. In general, when extracts of spiders or ticks were chromatographed in solvent system E, amino compounds were detected at R_F 0.9 (V), 0.75 (VI), 0.55 (VII), 0.5

Table 6. Conjugation of [^{14}C]benzoic acid in some arachnids

	Duration of experiment (days)	Extracted activity (μC)	Percentage of dose recovered			
			Unchanged	As N^2 -benzoyl conjugates		
				Of glutamic acid	Of glutamine	Of arginine
<i>Boophilus</i>						
Body	2*	2.88	17	—	—	—
	2*	2.83	6	44	—	40
	2*	0.93	1	49	—	42
	4*	0.94	2	93	—	2
	2	3.00	<5	62	<5	23
<i>Epeira</i>						
Body	1	0.56	<5	<5	<5	96
Excreta	16	0.52	<5	<5	<5	106
			<0.2	2	<0.2	<0.2
<i>Tegenaria</i>						
Body	1	0.57	<5	<5	<5	96
Excreta	10	0.61	<5	<5	<5	103
			0.2	2	<0.2	<0.2
<i>Mitopus</i>						
Body	2	0.45	<5	<5	<5	92
Excreta			<0.2	<0.2	4	4
Body	2	0.19	<5	<5	<5	<5
Excreta			<0.2	6	24	36
<i>Phalangium</i>						
Excreta	2	0.07	<1	<1	<1	64

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

Table 7. *Species variations in the conjugation of p-nitrobenzoic acid and p-aminobenzoic acid*

+, Metabolite detected; ++, intense spot detected; -, not detected. Methods of identification: ¹ paper chromatography and colour reactions; ² ionophoresis and colour reactions; ³ ultraviolet spectra; ⁴ fluorescence-pH curves; ⁵ hydrolysis; ⁶ arginase; ⁷ hydrazinolysis.

	<i>p</i> -Aminobenzoyl-			
	- <i>N</i> -sulphate	-glutamic acid	-glutamine	-arginine
<i>Tegenaria</i>				
Excreta	+ ^{1, 2, 5}	+ + ^{1, 2, 3, 4, 5}	- ^{1, 2}	+ ^{1, 2}
Bodies	- ¹	Trace ^{1, 2}	Trace ^{1, 2}	+ + ^{1, 2, 4, 5, 7}
<i>Epeira</i>				
Excreta	+ ^{1, 2, 5}	+ ^{1, 2, 5}	+ ^{1, 2}	+ ^{1, 2}
Bodies	- ¹	+ ^{1, 2}	+ + ^{1, 2}	+ ^{1, 2}
<i>Agelena</i>				
Excreta	+ ^{1, 2}	+ + ^{1, 2}	- ¹	Trace ^{1, 2}
Bodies	- ¹	+ ^{1, 2}	+ ²	+ + ^{1, 2, 5}
<i>Boophilus</i>				
Bodies	- ^{1, 2}	+ ^{1, 2}	- ^{1, 2}	+ + ^{1, 2, 6}
<i>Phalangium</i> *				
Excreta	- ^{1, 2}	- ^{1, 2}	- ^{1, 2}	+ + ^{1, 2}
Bodies	- ²	- ²	+ ^{2*}	+ ^{2*}
	<i>p</i> -Nitrobenzoyl-			
	-glutamic acid	-glutamine	-arginine	
<i>Tegenaria</i>				
Excreta	Trace ^{1, 2}	Trace ^{1, 2}	+ ^{1, 2}	
Bodies	- ¹	+ ¹	+ + ^{1, 2}	
<i>Epeira</i>				
Excreta	+ ^{1, 2}	+ + ^{1, 2}	+ ¹	
Bodies	Trace ¹	+ + ^{1, 2}	+ + ^{1, 2}	
<i>Boophilus</i>				
Bodies	+ + ^{1, 2}	- ^{1, 2}	+ + ^{1, 2}	
<i>Phalangium</i> *				
Bodies	- ²	+ + ²	+ ²	

* Ionophoresis at pH 1.3 and 12.9 only.

(VIII) and 0.3 (IX). As with *p*-nitrobenzoic acid, the related intensity of the various zones depended on the source of the extract (Table 7). Band IX was not observed in tick experiments and bands VII and VIII were not usually clearly separated. These two last-named zones were eluted together but subsequent examination by paper ionophoresis allowed the separation of acidic and basic constituents.

Identification of p-aminobenzoic acid in band V. This band was invariably weak if extracts were made of excreta or bodies 24 hr. after dosing. It was identified by comparison with reference *p*-aminobenzoic acid on paper chromatograms and by ionophoresis.

Identification of p-aminobenzoylglutamic acid in band VI. This material gave a negative azlactone reaction for hippuric acids. By paper chromatography and ionophoresis the eluate of this band was shown to contain a single compound moving identically with synthetic *p*-aminobenzoylglutamic acid.

A sample of this eluate from *Tegenaria* excreta was rechromatographed in solvent system C and the zone containing the aromatic amino compound eluted. This eluate was dissolved in 0.1N-hydro-

Fig. 1. Ultraviolet-absorption spectra of *p*-aminobenzoylglutamic acid. —, From reference compound; ---, from spider metabolite. (A) and (B), in 0.1N-NaOH; (C) and (D), in 0.1N-HCl.

chloric acid and 0.1N-sodium hydroxide and its ultraviolet absorption measured in a Unicam SP. 500 spectrophotometer (Fig. 1). This was similar to that of reference *p*-aminobenzoylglutamic acid and

other *p*-aminobenzoyl conjugates (cf. Friedler & Smith, 1954).

Samples of the eluate were diluted with suitable amounts of 0.01M-citrate buffer and the fluorescence-pH curve was determined between pH 1 and 7. This corresponded closely to the curve of reference *p*-aminobenzoylglutamic acid and was different from the reference curves obtained with *p*-aminobenzoic acid or *p*-aminobenzoyl glucoside (Table 1).

A sample of the eluate of band VI from *Tegenaria* having a fluorescence corresponding to 10 μ g. of *p*-aminobenzoic acid, and free of ninhydrin-reacting material, was refluxed with 2N-hydrochloric acid for 2 hr. The hydrochloric acid was removed by evaporation with water *in vacuo* and the residue examined chromatographically in solvent systems B and E (Block, Durrum & Zweig, 1958, p. 110) and also by ionophoresis at pH 6, which distinguishes acidic from neutral and basic amino acids, and at pH 4.5 where glutamic acid and aspartic acid are separable (Block *et al.* 1958, p. 508). Only glutamic acid and *p*-aminobenzoic acid were found in these systems.

Identification of material in bands VII and VIII. The two compounds present in these zones could not be separated clearly in the paper-chromatographic systems used but during ionophoresis one behaved as an acid and the other as a base. The basic component, which was the major constituent in *Tegenaria* or tick extracts, was ionophoretically identical with *p*-aminobenzoylarginine at all the pH values of Table 4 and also at pH 14, when it migrated towards the anode. In extracts of *Agelena* or *Tegenaria* bodies, but not of ticks, a minor component was also present which was ionophoretically identical with *p*-aminobenzoylglutamine at each of the pH values of Table 4.

On paper chromatograms, when the Sakaguchi reagent was used to detect the compounds, the eluate of zones VII and VIII contained material identical with reference *p*-aminobenzoylarginine.

Ionophoretic or paper-chromatographic examination of these zones from extracts of *Epeira* bodies or excreta showed that these contained more of the component having ionophoretic properties identical with *N*²-*p*-aminobenzoylglutamine together with small amounts of *N*²-*p*-aminobenzoylarginine (Table 7).

The eluate of zones VII and VIII from *Tegenaria* was subjected to ionophoresis at pH 12.7 for 2 hr. to free it from acidic metabolites and the zone corresponding to *p*-aminobenzoylarginine eluted and rechromatographed in solvent system C. The eluate of the zone giving colour reactions for the aromatic amino group was eluted with water and the relation between fluorescence and pH measured. The pH-fluorescence curve was similar to that of

reference *p*-aminobenzoylarginine and other amino acid conjugates and corresponded to an apparent pK 2.8.

A sample of the mixed eluates of bands VII and VIII from *Tegenaria* was refluxed for 3 hr. with 2N-hydrochloric acid. Free acid was removed by evaporation *in vacuo* and the residue examined as above in solvent systems B and E and at pH 6 (Block *et al.* 1958, p. 110). Arginine, glutamic acid, *p*-aminobenzoic acid and some unchanged material were detected on these systems.

Since *p*-aminobenzoyl glucoside and (*p*-aminobenzoyl glucosid)uronic acid also had *R_F* near 0.5, a sample of the combined VII-VIII eluate from *Epeira* or *Tegenaria* extracts was incubated at 25° overnight with purified emulsin (L. Light and Co. Ltd.) and with locust-crop fluid, which contains β -glucosidase and β -glucuronidase (Robinson, 1956, 1957). No hydrolysis was detectable by paper chromatography. Samples of eluate from *Tegenaria* or *Agelena* were also incubated overnight at 25° with a homogenate of *Tegenaria* in a few drops of water. No significant hydrolysis or conversion of the eluate of *p*-aminobenzoylglutamic acid could be detected by paper chromatography or ionophoresis.

A sample of the eluate from a tick experiment was purified by ionophoresis at pH 12.7 and the Sakaguchi-positive zone eluted with 0.1M-borate buffer, pH 9. Arginase (L. Light and Co. Ltd.) and Mn²⁺ ions were added and the mixture was left overnight. Chromatography of the mixture in solvent systems E and F showed the presence of *p*-aminobenzoylornithine and urea. No *p*-aminobenzoylarginine remained but a trace of *p*-aminobenzoylarginine was found in the mixture.

Identification of carboxyphenylsulphamic acid in zone IX. The eluate of this band was identical on paper chromatography and ionophoresis with the reference *N*-sulphate of *p*-aminobenzoic acid. It gave the slow reaction with dimethylaminobenzaldehyde, characteristic of arylsulphamic acids, but reacted as a normal aromatic amine on diazotization and coupling with 1-naphthol.

Excreta from six *Tegenaria* yielded a zone IX which was eluted in aqueous 0.1N-ammonia (50 μ l.). The eluate was mixed with 10 μ l. of 2N-hydrochloric acid and a crystal of barium chloride added. The clear solution, after solution of the crystal, was heated for 0.5 min. at 90–100°, when a precipitate of barium sulphate formed. Paper chromatography of the hydrolysate showed that only *p*-aminobenzoic acid was present.

DISCUSSION

The results obtained show that representatives of three Orders of the Arachnida were able to metabolize aromatic acids by conjugation and that

aroyl derivatives of arginine, glutamic acid or glutamine were detectable in either excreta or bodies. Arginine conjugates were found in all species but the amount of glutamine or glutamic acid conjugates varied with the organism tested.

In ticks and in the house spider *Tegenaria*, it is possible that the glutamic acid conjugates were secondary products, since with longer experimental times more of these were found in the ticks and much larger amounts of the glutamic acid conjugates were found in *Tegenaria* excreta than in the bodies. Moreover, in preliminary experiments (Hitchcock & Smith, 1963), injected L- or DL-nitrobenzoylarginine was completely converted into nitrobenzoylglutamic acid.

The cattle tick *Boophilus decoloratus* does not eliminate any significant volume of excreta in the adult stage and the nitrogenous end products of its metabolism accumulate in the malpighian tubules in the few weeks between maturity and the end of egg laying. It seems possible therefore that the conversion of arginine conjugates into glutamic acid derivatives occurs in the malpighian tubes and this supposition is strengthened by the finding that *Tegenaria* bodies contain few glutamic acid conjugates even when the arginine conjugates were present in large amounts. After a meal this species readily produces a semi-solid mixture of faeces and urine and this invariably contained almost wholly the glutamic acid conjugate when freshly voided. Attempts to detect an enzyme converting arginine conjugates into the glutamic acids in fresh wet spider excreta were not successful.

In those species which produced large amounts of glutamine conjugates (harvestmen and *Epeira*) glutamic acid conjugates were either not detected or were only minor metabolites. It is possible that in these species both arginine and glutamine conjugates are primary metabolites which suffer little further metabolism. This is now being investigated in enzyme preparations.

A possible metabolic Scheme (1) relating the conjugates in the different arachnids is shown. This assumes the presence of enzymes which have been found in other organisms, but attempts to detect [^{14}C]benzoylornithine or [^{14}C]benzoylcitrulline in arachnids metabolizing [^{14}C]benzoic acid were not successful. The present results, e.g. Tables 6 and 7, suggest a direct relation between the glutamic acid conjugates and their presumed pre-

cursors, the arginine derivatives. However, when *p*-nitrobenzoyl-ornithine or -glutamine was given to ticks they were also converted into the glutamic acid conjugates (Hitchcock & Smith, 1963).

Amino acids used in the peptide conjugation vary considerably in different natural groups (Smith, 1964). The common conjugation is with glycine, as in most mammals, fish and pigeons, though some aromatic acids in some species are exceptional. A serine conjugate of xanthurenic acid is found in rats (Rothstein & Greenberg, 1957), and a glycyltaurine conjugate of quinaldic acid in cats (Kaiharu & Price, 1961). The glutamine conjugation of phenylacetic acid in primates has been known for many years (Williams, 1959). Some birds (Baldwin, Robinson & Williams, 1960) and reptiles (Smith, 1958) excrete ornithine conjugates after dosage with aromatic acids. In plants aromatic acids may be conjugated with aspartic acid (Andrae & Good, 1957, 1955).

Among the arthropods glycine conjugations have been frequently observed in insects, and those crustaceans (*Ligia*, *Armadillidium*) which conjugate aromatic acids also use glycine (Smith, 1962, 1964, and unpublished work). Millipedes and centipedes (Smith, 1962) appear to use arginine as the arachnids do.

The enzymic mechanism of this conjugation has as yet only been satisfactorily worked out with vertebrates but if it is similar in other organisms the wide variations in the end product are understandable. The initial reaction, in vertebrates, is the formation of benzoyl-CoA and the reaction of this to give benzoylglycine depends on the presence of the amino acid and glycine acylase (Williams, 1963). In organisms having an abundance of another amino acid acylase and another amino acid, other peptide detoxications could be expected. The identification of a particular peptide detoxication should therefore be an indication of the importance of the amino acid in the biochemical economy of each natural group.

Arginine and other guanidine compounds are plentiful in many arthropods (Roche & Robin, 1962; Thoai & Roche, 1960) and though not all arthropods use arginine in detoxication the appearance of this in a detoxication process is not unexpected (Roche & Robin, 1962).

This indication of the abundance of an amino acid and its acylase may be of some value in understanding the biochemical evolution of the arthropods. Differing opinions are held on arthropod evolution, some believing them to be monophyletic (Butt, 1960) and others that they have separate origins (Manton, 1960; Tiegs & Manton, 1958). A generalized opinion of the phylogenetic relationships held by many biologists and described by Stafford (1960) places myriapods close to the

Scheme 1.

Scheme 2. Classification of arthropods according to Stafford (1960).

origins of the insects and separate from arachnids (Scheme 2).

Consideration of the amino acids used in the peptide detoxication suggests that myriapods are less closely associated with the origins of the insects than Scheme 2 shows and that they are biochemically similar to the arachnids. The distribution of peptide detoxications within the arthropod phylum, however, is not necessarily incompatible with multiple origins for the members of this group.

SUMMARY

1. Conjugation of *p*-nitrobenzoic acid, *p*-amino-benzoic acid and [^{14}C]benzoic acid has been studied in representatives of three arachnid Orders.

2. N^2 -Aroylarginines were formed in each species.

3. In *Boophilus*, *Tegenaria*, *Agelena* and *Epeira* aroylglutamic acids were also found.

4. In *Tegenaria*, *Epeira*, *Agelena*, *Mitopus* and *Phalangium*, N^2 -aroylglutamines were found.

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