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Detoxications in Peripatus

SULPHATE, PHOSPHATE AND HISTIDINE CONJUGATIONS

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Phenols were detoxified in the Onycophoran *Peripatoides novaezealandiae* by conjugation with sulphuric acid and phosphoric acid, but no evidence for a glycoside detoxication could be found. [^{14}C]Benzoic acid was metabolized in 24h to N^2-benzoyl-L-histidine, which was identified by electrophoresis, chromatography and dilution analysis. Similar conjugates were formed with *p*-aminobenzoic acid and *p*-nitrobenzoic acid. In longer-duration experiments further unidentified metabolites were formed, two of which appeared to result from the further metabolism of the histidine conjugate.

The phylum Onycophora contains animals popularly known as peripatus, which are of interest since they occupy a position in taxonomy intermediate between the Annelida and the Arthropoda and have in the past been classified either as primitive arthropods (Borradaile & Potts, 1963) or as aberrant annelids (Borradaile & Potts, 1963; Fox & Fox, 1964). Little biochemical work, which might be of taxonomic value, has been carried out with these invertebrates, though a comparative serological study of lactate dehydrogenase in invertebrates by Wilson & Kaplan (1964) suggested that peripatus was not allied to the Chelicerata, an arthropod group that includes the arachnids but not the insects, myriapods and crustaceans.

Morphologists have discussed the possible connexion of peripatus with the other major group of arthropods, the Mandibulata (Butt, 1960; Tiegs & Manton, 1958), and, though some phylogenies put peripatus on the myriapod–insect evolutionary line, Onycophora are usually regarded as a separate group that had already diverged from other invertebrates in Cambrian times.

We have previously examined some detoxication mechanisms of chelicerates (Hitchcock & Smith, 1964, 1966) and have shown their means of detoxifying aromatic acids to be different from those of insects and crustaceans (Smith, 1964, 1969) but similar in some respects to those of millipedes. The taxonomic value of the peptide conjugation is debatable since few species of arthropods in this area have been examined, but the availability of a peripatus species in New Zealand and its fascination to zoologists suggested that a study of its detoxication mechanisms would be of interest and possible value.

MATERIALS AND METHODS

Reference compounds. 8-Quinolyl dihydrogen phosphate and 4-methylumbelliferonyl dihydrogen phosphate were a gift from Dr M. P. Heenan. N^2-Benzoyl-L-histidine, m.p. 246°C and $[\alpha]_D^{20}$ −47.5±1° (Bauer, Adams & Tabor, 1965), and N^2-*p*-nitrobenzoyl-L-histidine, m.p. 238°C (Okano & Fukugawa, 1957), were prepared from the corresponding acid chlorides and the amino acid. N^2-*p*-Aminobenzoyl-L-histidine, m.p. 230°C, was prepared by reduction of the corresponding nitro compound with Adams platinum catalyst. N^2-p-*Nitrobenzoyl-L-histidine hydrochloride*, m.p. 206°C, was conveniently isolated from a solution in M-HCl by the addition of NaCl and crystallized from M-HCl (Found: Cl^-, 10.0; $C_{13}H_{12}N_4O_5$,HCl requires Cl^-, 10.4%).

N^1-Benzoylagmatine was prepared from the base and benzoyl chloride (Hitchcock & Smith, 1966) and was conveniently isolated by addition of a saturated solution of 2,4-dinitrobenzenesulphonic acid, with which it formed a *salt*, m.p. 154°C, from water (Found: C, 44.7; H, 4.4; N, 17.5; $C_{18}H_{22}N_6O_8S$ requires C, 44.8; H, 4.6; N, 17.4%). Addition of picric acid to a solution of this salt gave benzoylagmatine picrate, m.p. and mixed m.p. 116°C (Hitchcock & Smith, 1966).

Other conjugates of phenols and of *p*-nitrobenzoic acid, *p*-aminobenzoic acid and benzoic acid were samples used previously (Smith, 1958; Smith & Turbert, 1964; Hitchcock & Smith, 1964, 1966).

[^{14}C]Benzoic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., with specific radioactivity 2mCi/mmol.

Animals. Peripatoides novaezealandiae weighing about 0.5g were collected from decayed logs in the bush near Wellington. They were kept until used in rotted wood and moss, and could be maintained for about 3–4 weeks in the laboratory. Phenols (0.5mg/g of animal) were injected into the animals in 1μl of acetone. Aqueous solutions of *p*-aminobenzoic acid or *p*-nitrobenzoic acid were injected at a dose of 0.5mg/g after the solution had

been adjusted to pH 5–7 with $NaHCO_3$. After being dosed, the peripatus were kept in separate test tubes at 25°C in a moist environment for 1–3 days before homogenization and extraction. Excreta were not normally produced in this period, but in the occasional experiments where it was voided this was separately extracted as described for the tissues. [^{14}C]Benzoic acid diluted to 4.8 μCi/g was similarly administered, and in some experiments 2 μg of [^{14}C]benzoic acid (2 mCi/mmol) was injected or applied topically in 0.2 μl of benzene with a Hamilton microlitre syringe.

Extraction procedures. Only surviving animals were used for extraction of metabolites, and these were ground with aq. 80% (v/v) acetone (2 ml/g) in a glass Potter–Elvehjem homogenizer and the suspension was kept at 60–70°C on a water bath for 2 h. The homogenate was then centrifuged at 2000*g* for a few minutes, and the supernatant removed and evaporated *in vacuo* to small bulk. Ethanol (4 vol.) was added, the small precipitate centrifuged off and the supernatant again concentrated *in vacuo* and used for chromatography and ionophoresis.

In some experiments this extract was separated on large-scale chromatograms, and zones, located by their radioactivity or colour reactions, were eluted and examined further. In most experiments with the acids, however, it was more convenient to clean up the extract by high-voltage electrophoresis in 0.1 M-phosphate buffer, pH 6 (0.1 M-KH_2PO_4 adjusted to pH 6 with 10 M-NaOH), before elution of the zones of metabolites.

Chromatography and electrophoresis. Chromatography was carried out as described previously (e.g. Hitchcock & Smith, 1964; Hook & Smith, 1967). Electrophoresis was carried out on Whatman no. 3MM paper in a Shandon high-voltage electrophoresis apparatus and most separations were adequately achieved by using 200 V for 20 min. The buffers used were: pH 1.85, 75 ml of acetic acid and 20 ml of formic acid in 1 litre of water; pH 6, 0.1 M-KH_2PO_4 adjusted to pH 6 with 10 M-NaOH; pH 8, 0.1 M-KH_2PO_4 adjusted to pH 8 with 10 M-NaOH; pH 12.3, 0.05 M-NaOH. The order of separations are shown in Tables 1–7; all the chromatographic and electrophoretic identifications were made on mixed chromatograms or electrophoretic runs, which showed no separation of the appropriate reference compound and the metabolite applied as a mixture.

Colour reactions. The colour reactions used to detect conjugates were those used previously (Smith, 1955, 1958; Smith & Turbert, 1964; Hitchcock & Smith, 1964, 1966). Histidine derivatives were also located by the red colour given by spraying with 1% diazotized sulphanilic acid in M-HCl followed by 5% (w/v) Na_2CO_3 solution after 5 min. Peptide links were detected by standing plates or papers in an atmosphere of Cl_2 for 20 min, airing the plates or papers for 20 min and spraying with 0.03% *o*-tolidine in M-acetic acid containing KI (0.1%). Yellow–orange colours were obtained.

Radioactivity measurements. Radioactivity measurements on paper strips were made by scintillation counting in a Packard model 4312 scintillation spectrometer as described by Binning, Darby, Heenan & Smith (1967). Radioactivity of aqueous solutions (1 ml/vial) was counted by using the same scintillation chemicals in a solvent in which one-third of the toluene was replaced by Triton X-100 (Rohm and Haas, Philadelphia, Pa., U.S.A.).

RESULTS

Phenol conjugations. Peripatus were dosed with 4-methylumbelliferone and extracted as described after 4 days at 25°C. The extract, which contained some unchanged material, was separated on Whatman no. 31 paper as a large-scale chromatogram in solvent *A* (Table 1). After the spraying of test strips with snail-crop fluid to hydrolyse any

Table 1. *Separation of some phenolic conjugates on paper*

Solvents were run on Whatman no. 4 paper until the front had moved about 38 cm. Solvents were: *A*, butan-1-ol–acetic acid–water (4:1:5, by vol.); *B*, propan-1-ol–aq. ammonia (sp.gr. 0.88) (7:3, v/v); *C*, butan-1-ol–aq. ammonia (sp.gr. 0.88)–water (4:1:5, by vol.); *D*, butan-2-one–water–2 M-acetic acid (200:100:1, by vol.); *E* and *F* show electrophoretic migrations (in cm) to anode (+) or cathode (−) at 10 V/cm for 1 h in 0.05 M-HCl and 0.05 M-NaOH respectively.

	R_F				Migration (cm)	
System ...	*A*	*B*	*C*	*D*	*E*	*F*
8-Hydroxyquinoline	0.9	0.9	0.9	1.0	−4.9	+2.4
8-Quinolyl hydrogen sulphate	0.5	0.8	0.4	0.4	0	+4.0
8-Quinolyl dihydrogen phosphate	0.3	0.4	0.0	0.0	0	+5.7
8-Quinolyl glucoside	0.5	0.8	0.6	0.4	−2.8	−1.3
(8-Quinolyl glucosid)uronic acid	0.4	0.6	0.1	0.3	−3.0	+1.5
4-Methylumbelliferone	0.9	0.8	0.9	1.0	0	+2.4
4-Methylumbelliferonyl hydrogen sulphate	0.6	0.7	0.5	0.9	+4.4	+3.1
4-Methylumbelliferonyl dihydrogen sulphate	0.5	0.3	0.6	0.0	+2.8	+5.4
4-Methylumbelliferonyl glucoside	0.6	0.7	0.7	0.5	0	+1.5
(4-Methylumbelliferonyl glucosid)uronic acid	0.4	0.5	0.4	0.1	0	+2.2

conjugates, additional fluorescent zones appeared at R_F 0.4 and 0.7. The corresponding zones on the unsprayed areas were eluted in 0.1 M-ammonia and examined in each of the separation systems of Table 1. The zone at R_F 0.7 was identical in all systems with the ethereal sulphate of 4-methylumbelliferone, which was quickly hydrolysed at room temperature after spraying with cold 0.1 M-hydrochloric acid. The zone at R_F 0.4 was not hydrolysed by 0.1 M-hydrochloric acid at 100°C, but gave the characteristic 4-methylumbelliferone fluorescence when treated with alkaline phosphatase (Sigma Chemical Co., St Louis, Mo., U.S.A.). It behaved identically with the phosphate ester of 4-methylumbelliferone in each of the systems of Table 1.

No other conjugates were detected, though the snail-enzyme procedure was adequate to detect conjugates with glucose or glucuronic acid (Smith, 1955, 1958; Smith & Turbert, 1964).

Similar experiments were carried out with 8-quinolinol, and the large-scale chromatograms of the extracts in solvent system *A* (Table 1) had two green-fluorescent zones at R_F 0.2 and 0.5 as well as a zone containing unchanged material at R_F 0.9 and a light-blue-fluorescent zone at R_F 0.8. The band with R_F 0.5, after elution as above, was identical in the systems of Table 1 with 8-quinolyl hydrogen sulphate, and was rapidly hydrolysed to 8-quinolinol by 0.1 M-hydrochloric acid and by the snail-crop fluid. The zone at R_F 0.2 was not hydrolysed by 0.1 M-hydrochloric acid at 100°C, but treatment with alkaline phosphatase (Sigma Chemical Co.) or with snail-crop fluid discharged the green fluorescence of the conjugate, and 8-quinolinol could then be detected by colour reagents. This conjugate was identical with 8-quinolyl dihydrogen phosphate in each of the separation systems of Table 1.

No conjugates of glucose or glucuronic acid were detected by the green fluorescence characteristic of 8-quinolinol *O*-conjugates or by treatment with glycosidases.

Fluorescence intensity–pH curves for 8-quinolyl glucoside, 8-quinolyl hydrogen sulphate and 8-quinolyl dihydrogen phosphate were measured by using reference compounds in the Aminco–Bowman spectrophotofluorimeter as described by Hitchcock & Smith (1964). The shapes of the curves from the glucoside and sulphate were identical between pH 3.5 and 6.5, and had the form of a simple sigmoid titration curve corresponding to a pK value of 4.5. The material in the zone at R_F 0.5 had a fluorescence–pH curve that superimposed exactly on this.

The fluorescence–pH plot of the reference 8-quinolyl dihydrogen phosphate did not have the form of a simple titration curve, but fell between pH 1 and pH 6 with a half-intensity point at pH 2. Nevertheless the metabolite with R_F 0.2 had an identical form of curve, which could be superimposed exactly on this reference curve between pH 1 and 6.

Chromatograms of the body extracts from experiments with both phenols showed that considerable amounts of unchanged phenols were present, but some animals dosed with 8-quinolinol voided small yellow faecal pellets that contained only the two conjugates and no unchanged quinolinol.

Eluates of the blue-fluorescing band at R_F 0.8 from the 8-quinolinol experiments contained a

Table 2. *Paper chromatography of benzoic acid conjugates*

Solvents were run on Whatman no. 1 paper until the fronts had moved about 30 cm. Solvents were: *A*, butan-1-ol–acetic acid–water (4:1:5, by vol.); *B*, butan-1-ol–aq. ammonia (sp.gr. 0.88)–water (4:1:5, by vol.); *C*, propan-1-ol–aq. ammonia (sp.gr. 0.88) (7:3, v/v); *D*, butane-2-one–water–2 M-acetic acid (200:100:1, by vol.); *E*, ethanol–water (4:1, v/v). N.T., Not tested.

	R_F				
System ...	*A*	*B*	*C*	*D*	*E*
Benzoic acid	0.95	0.40	0.70	0.95	0.90
Benzoylglycine	0.90	0.25	0.65	0.80	0.70
Benzoylglutamic acid	0.90	0.05	0.35	0.85	0.80
N^2-Benzoylglutamine	0.80	0.20	0.60	0.45	0.60
N^2-Benzoylarginine	0.75	0.35	0.65	0.15	0.65
N^2-Benzoylagmatine	0.70	0.75	0.85	0.90	0.80
N^2-Benzoylornithine	0.95	0.70	0.85	0.90	0.85
N^5-Benzoylornithine	0.65	0.20	0.65	0.10	0.65
N^2N^5-Dibenzoylornithine	0.95	0.65	0.85	0.90	0.90
N^2-Benzoylcitrulline	0.85	0.20	0.65	0.45	0.65
N^2-Benzoylhistidine	0.65	0.30	0.70	0.10	0.57
(Benzoyl glucosid)uronic acid	0.70	0.15	N.T.	0.25	0.60

Table 3. *Paper electrophoresis of some benzoic acid conjugates*

Whatman no. 3MM paper was used at 60 V/cm for 20 min in the following buffers: pH 1.85, 75 ml of acetic acid and 20 ml of formic acid/l; pH 6.0, 6.8 g of KH_2PO_4 and 0.27 g of NaOH/l; pH 8.0, 6.8 g of KH_2PO_4 and 1.86 g of NaOH/l; pH 12.3, 0.02 M-NaOH. Migration distances are measured relative to benzoic acid (in cm) towards anode (+) or cathode (−).

	Migration (cm)			
	pH 1.85	pH 6	pH 8	pH 12.3
Benzoic acid	0	0	0	0
Benzoylglycine	0	−0.5	−1.2	−1.6
Benzoylglutamic acid	0	+1.2	+1.4	+1.4
N^2-Benzoylglutamine	0	−2.0	−2.8	−3.4
N^2-Benzoylarginine	−4.4	−6.6	−7.6	−9.2
N^2-Benzoylagmatine	−5.0	−9.6	−11.5	−11.4
N^2-Benzoylcitrulline	0	−2.0	−3.2	−4.0
N^2-Benzoylhistidine	−4.1	−6.2	−4.6	−4.4
N^5-Benzoylornithine	−3.0	−6.1	−7.4	−4.4
N^2N^5-Dibenzoylornithine	0	−1.8	−4.0	−4.4
(Benzoyl glucosid)uronic acid	0	−2.4	−3.2	−3.6

metabolite that was present only in body extracts and not in those from excreta. This gave an intense colour with 2,6-dichloroquinonechloroimide in sodium hydrogen carbonate and reduced ammoniacal silver nitrate. Its fluorescence was not affected by the hydrolases in snail-crop fluid and it may have been a hydroxylated derivative of 8-quinolinol.

Conjugation of p-*aminobenzoic acid.* Peripatus were dosed and extracted as described above and the initial extract was run on a large-scale chromatogram in the top layer of benzene–acetic acid–water (1:1:2, by vol.) to separate unchanged *p*-aminobenzoic acid (R_F 0.9). After being dried, the paper was run again in solvent system *A* (Table 2). Three bands were revealed by colour reagents for amino compounds with R_F 0.9 (unchanged *p*-aminobenzoic acid) and at R_F 0.4 and 0.45, the last band being very weak and sometimes absent from extracts made at 24 h.

After elution with ammonia the material in the band at R_F 0.4 was examined in each of the systems of Tables 4 and 5. The material was homogeneous and was not separable from *p*-aminobenzoylhistidine in these solvents or electrophoretic systems. The zone gave the pink reaction with diazotized sulphanilic acid characteristic of histidine compounds.

A portion of the eluate was rechromatographed in solvent *C* (Table 2). The zone giving colour reactions for aromatic amino compounds was free of compounds responding to ninhydrin. This zone was eluted with 0.1 M-ammonia, refluxed with 6 M-hydrochloric acid for 2 h and then evaporated to dryness *in vacuo*. The residue, on chromatography in solvents *A* and *B* (Table 2), now contained spots corresponding to authentic *p*-aminobenzoic acid (R_F 0.9 and 0.45 respectively) and histidine (R_F 0.1 and 0.3 respectively).

Conjugation of p-*nitrobenzoic acid.* Peripatus were dosed and extracted as described above and initial extracts were run on a large-scale chromatogram in benzene–acetic acid–water (1:1:2, by vol.) to separate the unchanged nitrobenzoic acid (R_F 0.9) from the conjugates, which remained at the origin. After being dried, the chromatogram was run again in solvent *A* (Table 2), when metabolite bands having the colour reactions of aromatic amines were detected at R_F 0.8 and 0.4 in extracts made 24 or 48 h after the dosing. Extracts made from longer-duration experiments also contained a material chromatographing to give a faint band at R_F 0.55 that was not identified.

The bands at both R_F 0.8 and 0.4 gave colour reactions for the aromatic amino group, and the weak band at R_F 0.55, when present, gave colour reactions of a nitro compound. The material in the band at R_F 0.8 was identified as *p*-aminobenzoic acid by chromatography in the solvents given in Table 6 and by electrophoresis (Table 7). The material in the band at R_F 0.4 also gave colour reactions for the imidazole ring. In the solvents given in Table 2 and on electrophoresis (Tables 4 and 5) it was indistinguishable from *p*-aminobenzoylhistidine.

In some experiments the initial extracts were cleaned up by high-voltage electrophoresis at pH 1.85, when three bands were located. One with a small positive migration was identified chromatographically and by electrophoresis as *p*-nitrobenzoic acid, one with a small negative migration was identified as above as *p*-aminobenzoic acid and a third with a large negative migration was identified as above as *p*-aminobenzoylhistidine.

Table 4. *Paper chromatography of some* p-*aminobenzoic acid conjugates*

Conditions and solvents were the same as in Table 2.

	R_F			
System ...	*A*	*B*	*C*	*D*
p-Aminobenzoic acid	0.90	0.10	0.40	0.95
p-Aminobenzoylhistidine	0.65	0.10	0.45	0.30
p-Aminobenzoylglutamic acid	0.70	0.0	0.20	0.25
N^2-*p*-Aminobenzoylglutamine	0.55	0.05	0.40	0.15
N^2-*p*-Aminobenzoylarginine	0.50	0.15	0.45	0.05
N^2-*p*-Aminobenzoylornithine	0.35	0.05	0.40	0.0
N^5-*p*-Aminobenzoylornithine	0.35	0.05	0.40	0.05
N^2N^5-Di-*p*-aminobenzoylornithine	0.70	0.15	0.60	0.50
N^2-*p*-Aminobenzoylcitrulline	0.60	0.05	0.45	0.10
N^2-*p*-Aminobenzoylhistidine	0.40	0.05	0.45	0.05
p-Carboxyphenylsulphamic acid	0.40	0.0	0.15	0.15
p-Aminobenzoyl glucuronide	0.50	0.1	0.40	0.05

Table 5. *Paper electrophoresis of some* p-*aminobenzoic acid conjugates*

Migration distances are measured relative to *p*-aminobenzoic acid (in cm) towards the anode (+) or the cathode (−). Other conditions were the same as in Table 3.

	Migration (cm)		
	pH 1.85	pH 6.0	pH 12.3
p-Aminobenzoic acid	0	0	0
p-Aminobenzoylglycine	0	0	0
p-Aminobenzoylglutamic acid	+0.8	+2.2	+2.0
N^2-*p*-Aminobenzoylglutamine	+1.0	−0.4	−1.6
N^2-*p*-Aminobenzoylarginine	−2.2	−5.4	−6.6
N^2-*p*-Aminobenzoylornithine	−2.8	−5.0	−3.8
N^5-*p*-Aminobenzoylornithine	−1.8	−4.8	−3.2
N^2N^5-Di-*p*-aminobenzoylornithine	−1.2	−2.2	−3.2
N^2-*p*-Aminobenzoylcitrulline	+1.2	−0.8	−2.4
N^2-*p*-Aminobenzoylhistidine	−1.8	−4.8	−3.0
p-Carboxyphenylsulphamic acid	+9.6	+5.8	+6.0

Conjugation of benzoic acid. Peripatus were injected with 0.2–0.4mg of low-specific-radioactivity [^{14}C]benzoic acid and extracted as described above after 24h at 25°C. Extracts were chromatographed in solvent systems *A*, *B*, *C*, *D* and *E* (Table 2) with reference samples of benzoylhistidine and as mixed chromatograms with benzoylhistidine. No separation of the compound giving the diazo reaction from reference benzoylhistidine was observed. Radioactive scans of these chromatograms showed that all the radioactivity was associated with the benzoylhistidine zone or with unchanged benzoic acid and that 25% of the dose had been converted into the metabolite.

Animals were also injected or topically treated with high-specific-radioactivity benzoic acid (0.05μCi, 2μg) in 0.2μl of benzene. The homogenates were prepared after 3, 6, 24 or 72h as described above except that 100mg of diluent benzoic acid and 1mg each of the N^2-benzoyl derivatives of histidine, arginine, agmatine and glutamic acid were added, and the aqueous-acetone extract was then extracted with 5ml of ether. The ether was removed with most of the residual [^{14}C]benzoic acid, and the aqueous layer was used for chromatography and electrophoresis. Only [^{14}C]benzoic acid was found in the ether extracts when these were examined by paper electrophoresis. Conjugation of the small dose was extensive; in seven experiments water-soluble radioactivity corresponded to 74–100% of the dose in homogenates made after 24h.

The distribution of water-soluble metabolites was examined by using paper electrophoresis at the pH values of Table 3. Radioactive zones on these coincided with the reference diluent conjugates of benzoic acid with histidine, arginine, agmatine and glutamic acid at each of the pH

Table 6. *Paper chromatography of* p-*nitrobenzoic acid conjugates*

Conditions and solvents were the same as in Table 2.

	R_F			
System ...	*A*	*B*	*C*	*D*
p-Nitrobenzoic acid	0.95	0.50	0.80	0.90
p-Nitrobenzoylglycine	0.90	0.30	0.70	0.65
p-Nitrobenzoylglutamic acid	0.90	0.0	0.50	0.40
N^2-*p*-Nitrobenzoylarginine	0.80	0.20	0.65	0.30
N^2-*p*-Nitrobenzoylagmatine	0.70	0.35	0.60	0.20
N^5-*p*-Nitrobenzoylornithine	0.60	0.60	0.70	0.20
N^2N^5-Di-*p*-nitrobenzoylornithine	0.95	0.70	0.90	0.90
N^2-*p*-Nitrobenzoylcitrulline	0.90	0.40	0.80	0.30
N^2-*p*-Nitrobenzoylhistidine	0.65	0.35	0.70	0.15

Table 7. *Paper electrophoresis of some* p-*nitrobenzoic acid conjugates*

Migration distances are measured relative to *p*-nitrobenzoic acid (in cm) towards the anode (+) or cathode (−). Other conditions were the same as in Table 3.

	Migration (cm)		
	pH 1.85	pH 6.0	pH 12.3
p-Nitrobenzoic acid	0	0	0
p-Nitrobenzoylglycine	0	0	−0.8
p-Nitrobenzoylglutamic acid	0	+2.0	+2.0
N^2-*p*-Nitrobenzoylglutamine	0	−1.4	−2.2
N^2-*p*-Nitrobenzoylarginine	−4.2	−5.8	−7.6
N^2-*p*-Nitrobenzoylagmatine	−8.0	−8.4	−7.8
N^5-*p*-Nitrobenzoylornithine	−3.0	−5.8	−3.2
N^2N^5-Di-*p*-nitrobenzoylornithine	−0.2	−2.6	−3.6
N^2-*p*-Nitrobenzoylcitrulline	0	−2.0	−2.6
N^2-*p*-Nitrobenzoylhistidine	−4.0	−5.6	−3.2

Table 8. *Distribution of water-soluble metabolites of* [^{14}C]*benzoic acid in peripatus*

Water-soluble metabolites obtained from homogenized animals as described in the text were separated at pH 8 and pH 1.85 by high-voltage electrophoresis on paper and assayed by scintillation counting as described in the text. Results are given as percentages of the total radioactivity present on the electrophoresis strips (with ranges in parentheses).

		% of water-soluble radioactivity with electrophoretic behaviour identical with			
Duration of experiment (days)	No. of animals	Benzoylglutamic acid	Benzoyl-arginine	Benzoyl-agmatine	Benzoyl-histidine
1	3	1	4 (3–6)	10 (7–13)	47 (42–55)
1	3	5 (0–14)	13 (8–22)	5 (0–10)	66 (53–80)
3	4	1	8 (6–11)	33 (19–40)	33 (25–46)
3	4	7 (3–15)	19 (8–30)	29 (24–36)	23 (22–32)

values used. Extracts from animals made after a longer period contained more of the apparent conjugates with arginine and agmatine and less benzoylhistidine (Table 8).

Zones from electrophoretic experiments at pH 8 that corresponded to the benzoic acid conjugates of arginine, agmatine and histidine were eluted with water and, after the total radioactivity in each eluate had been counted, dilution analyses were made for the appropriate conjugate. In three experiments similar to those in Fig. 1, 86, 80 and 81% respectively of the radioactivity of the

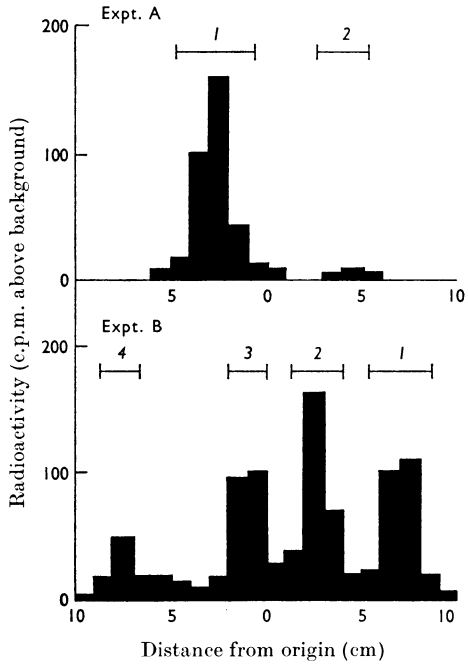

Fig. 1. Paper electrophoresis of water-soluble metabolites of benzoic acid. Electrophoresis was carried out as described in the text on Whatman no. 3MM paper in 0.1 M-phosphate buffer, pH 8, for 30 min at 60 V/cm, and radioactivity was assayed by counting the dried paper in 1 cm sections. Bars indicated the position taken up by added reference compounds, which were detected by their colour reactions: *1*, benzoylhistidine; *2*, benzoylarginine; *3*, benzoylagmatine; *4*, benzoylglutamic acid. Expt. A was from peripatus extracted 1 day after being dosed topically and Expt. B from animals extracted after 3 days.

benzoylhistidine zones was shown to consist of N^2-benzoyl-L-histidine, but of the 6000, 8000 and 4000 c.p.m. in the apparent benzoylarginine zones only 5, 6 and 2% respectively was carried by the added N^2-benzoyl-L-arginine. A 'benzoylagmatine' zone was only found in 2- or 3-day experiments, but of the 16000 and 10000 c.p.m. concerned only 10 and 6% respectively was entrained by the diluent.

As well as the metabolites quoted in Table 8 two other electrophoretic zones could be recognized that accounted for about 20 and 15% of the water-soluble radioactivity respectively in both 1- and 3-day experiments. One of these behaved like benzoic acid but was not extracted by ether. The other behaved like a strong acid and migrated towards the anode at all pH values up to that of M-hydrochloric acid.

DISCUSSION

The ability to form peptide links between foreign aromatic acids and suitable amino acids is widespread in Nature. It is found in the vertebrates, invertebrates and plants (Smith, 1964, 1969) and also in some micro-organisms (Hutzinger & Kusuge, 1968; Towers, 1964; Corbin & Bulen, 1969). In many animals, both vertebrate and invertebrate, a variety of different amino acids may be detected in the conjugates formed from foreign aromatic acids if suitably sensitive assays (Esaac & Casida, 1968) or tissue preparations or dosages (Smith, 1964; Williams, 1963) are used. However, one of these mechanisms is usually predominant when whole-animal experiments are carried out with relatively large doses of aromatic acids and this particular peptide conjugate is usually characteristic of a taxonomic group.

The glycine conjugation is of little value for characterization since glycine is used by diverse groups that include most vertebrates, most insects and some crustaceans (Smith, 1964, 1969). On the other hand the ornithine conjugation appears to be confined to a few families of birds and reptiles (Baldwin, Robinson & Williams, 1960; Smith, 1958).

Among the invertebrates the arginine conjugation appears to be characteristic of the arachnids, though it is also found in some myriapods (Hitchcock & Smith, 1964, 1966). The use of a histidine detoxication in peripatus is in agreement with those classifications that show this group to be only distantly connected with either insect or arachnid phylogeny.

In the experiments described above when small doses were used several other amino acid conjugates were detected in addition to the major histidine derivative, though the amounts of these were somewhat variable. These always included a small amount of material identical in behaviour with benzoylglutamic acid and four as yet unidentified metabolites, one of which could be a conjugate of a neutral amino acid since it had the same electrophoretic behaviour as benzoic acid or hippuric acid. A second unidentified metabolite, which accounted for about 10–15% of a 2 μg dose, was a strong acid. Its behaviour in acid buffers and in M-hydrochloric acid suggested that it was unlikely to be either a phosphate or taurine conjugate, since these would be expected to have lost their ionic charge at pH 0.

Two basic metabolites were also present with electrophoretic behaviour very similar to that of conjugates of arginine and agmatine. These appeared more important in experiments carried on for longer times, and it seemed unlikely that the increased amounts of these in 3-day experiments represented further conjugation of benzoic acid,

since the amount of unconjugated acid in most 1-day experiments was small. The increase in these metabolites seemed to be at the expense of the histidine conjugate, and it is possible that this was metabolized to these strongly ionic compounds by secondary reactions.

Such secondary reactions have previously been observed to take place in invertebrates when cysteine or arginine conjugates are the primary detoxication products (Cohen & Smith, 1964; Hitchcock & Smith, 1966), and associated gut or tissue micro-organisms have been suspected as the agents.

In contrast with the complexity of the conjugation pattern at low doses, high doses of aromatic acids produced only a single detectable conjugate. This was the histidine derivative and only occasional and trace amounts of other metabolites were found when doses in the 500 μg/g range were given. The histidine conjugation may therefore be a taxonomic characteristic of peripatus as arginine conjugation is characteristic of arachnids.

The presence of the sulphate conjugation in the two ancient groups of peripatus and scorpions, which had already reached their present forms in the earliest fossil remains, argues for the primitive nature of the sulphate detoxication and thus for its ubiquitous distribution. The lack of an effective glycoside conjugation in peripatus and scorpions (Hitchcock & Smith, 1966) may also be significant. Most vertebrates and insects have well-developed detoxication mechanisms using glucuronic acid or glucose that are often able to detoxify large amounts of xenobiotics and may function as an overload mechanism when the older sulphate detoxication is saturated. Less than a dozen species from the non-insect arthropod groups have been examined as yet, but all of these have lacked detectable glycoside conjugations, and the lack of such a mechanism suggests that in this region the use of glucose in detoxication is a relatively late innovation of the insect evolutionary line.

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