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Comparative Detoxication

6. THE METABOLISM OF 6-AMINO-4-NITRO-*o*-CRESOL AND 4:6-DINITRO-*o*-CRESOL IN LOCUSTS*

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4:6-Dinitro-*o*-cresol (DNOC) has been used for many years as an insecticide, especially against locust swarms in Africa (Rainey & Sayer, 1953). No chemical data on its metabolism in locusts are available, but it seems possible from toxicological experiments that it is detoxicated in these insects. McCuaig & Sawyer (1957) have shown that repeated sublethal doses of DNOC are not fully cumulative in locusts and point out that this can seriously diminish the effectiveness of field-spraying operations where locusts are sprayed over a period of several days.

Detoxication of DNOC in locusts could be accomplished by a reduction to 6-amino-4-nitro-*o*-cresol, such as is found in rabbits (Smith, Smithies & Williams, 1953), since it is known that locusts can reduce the aromatic nitro group (Friedler & Smith, 1954). We have therefore studied the fate of 6-amino-4-nitro-*o*-cresol in *Locusta migratoria* and *Schistocerca gregaria* as a preliminary to searching for this compound and its metabolites in locusts poisoned with DNOC.

Detoxication of a phenol like DNOC might also occur by conjugation reactions leading to either a β-glucoside or an arylsulphate (cf. Smith 1955*a*, *b*). Locust-crop fluid, however, contains an active β-glucosidase and aryl sulphatase (Robinson, Smith & Williams, 1953), and β-glucosidase has also been found in the intestinal wall of mid- and hind-gut (Robinson, 1956). If these enzymes were to mix with the excretions from the Malpighian tubes, which discharge into the posterior end of the mid-gut of the locust, an initial conjugation might be reversed. We have therefore examined the contents of the different parts of the gut for β-glucosidase and aryl sulphatase in an attempt to assess their possible effect on any *O*-conjugated metabolites before these are eliminated in the excreta.

* Part 5: Smith (1958).

MATERIALS AND METHODS

Reference compounds. DNOC and other phenolic compounds were samples used in earlier work (Smith *et al.* 1953) or prepared by the same methods.

Glucosides of phenols were prepared by the general method of Glazer & Wulwek (1924). Tetra-acetyl-α-glucosyl bromide (34 g.) in 200 ml. of acetone was mixed with 6-acetamido-4-nitro-*o*-cresol (24 g.) in 150 ml. of water containing 6·4 g. of NaOH. After 24 hr. the acetone was removed *in vacuo* at 40–50° and the acetylated glucoside precipitated by addition of water to the residue. The precipitate was crystallized from ethanol to give 5 g. of *6-acetamido-4-nitro-o-cresyl tetra-acetyl-β-D-glucoside* as pale-yellow needles, m.p. 169°; $[\alpha]_D^{25} = -1{\cdot}8°$ (*c*, 1 in $CHCl_3$) (Found C, 51·1; H, 5·0; N, 5·5. $C_{23}H_{28}O_{13}N_2$ requires C, 51·1; H, 5·2; N, 5·2%).

Deacetylation of this compound (5 g.) in 90 ml. of methanol with methanolic 2N-barium methylate (1·5 ml.) at 0° for 18 hr. gave a crystalline precipitate (3·5 g.) which was recrystallized from 90% (v/v) ethanol to give *6-acetamido-4-nitro-o-cresyl β-D-glucoside monohydrate*, m.p. 147–155°; $[\alpha]_D^{25} = -6{\cdot}1°$ (*c*, 0·4 in 50%, v/v ethanol) (Found C, 46·2; H, 6·1; N, 7·7. $C_{15}H_{20}O_9N_2,H_2O$ requires C, 46·2; H, 5·7; N, 7·2%). The water was not lost at 100°. The glucoside had $\lambda_{max.}$ 250, 291; $\epsilon_{max.}$ 11 700, 6900 in 0·1N-NaOH and $\lambda_{max.}$ 240, 289; $\epsilon_{max.}$ 12 500 and 7500 in 0·1N-HCl.

When 6-amino-4-nitro-*o*-cresol was treated as above with acetobromoglucose, two glucosidic derivatives were formed, the major product being an *N*-glycoside. Tetra-acetyl-α-glucosyl bromide (30 g.) in 150 ml. of acetone and 6-amino-4-nitro-*o*-cresol (18 g.) in 50 ml. of water containing 3·2 g. of NaOH were worked up as described above for the acetamido compound and the dark-brown gum was crystallized twice from ethanol to give 5 g. of pale-orange plates: N-(2-*hydroxy*-3-*methyl*-5-*nitrophenyl*) *tetra-acetyl*-β-D-*glucosylamine monohydrate* had m.p. 106°; $[\alpha]_D^{25}$ −69°→ −30° (4 days in $CHCl_3$, *c*, 1) (Found C, 48·5; H, 5·6; N, 5·2. $C_{21}H_{26}O_{12}N_2,H_2O$ requires C, 48·8; H, 5·5; N, 5·4%). This compound could be extracted from solution in $CHCl_3$ into ice-cold 0·1 N-NaOH, as a dark-red solution from which it could be re-extracted with $CHCl_3$ on immediate re-acidification. Treatment with methanolic barium methoxide as described above gave a dark-red amorphous precipitate which was probably the free *N*-glucoside. This was free from barium and very soluble in water but could not be purified without decomposition to the corresponding aminocresol.

The ethanolic mother liquors from the crystallization of the orange-acetylated derivative were diluted with 300 ml. of $CHCl_3$ and washed repeatedly with 0·1 N-NaOH till no further red colour was extracted. The $CHCl_3$ was washed with water, dried with $CaCl_2$ and evaporated *in vacuo* to leave a pale-brown gum which could not be crystallized. This was treated in 50 ml. of methanol with 2 ml. of N-barium methylate for 18 hr. at 0°. On partial evaporation of the methanol, a crop of yellow crystals was obtained which were recrystallized from water to give 300 mg. of 6-*amino*-4-*nitro*-o-*cresyl* β-D-*glucoside hemihydrate* as long pale-yellow needles, m.p. 215°; $[\alpha]_D^{25}$ +32·4° (*c*, 0·08 in water); $[\alpha]_D^{25}$ −16·6° (*c*, 0·5 in 0·4 N-HCl) (Found C, 45·5; H, 5·4; N, 8·0; H_2O, 2·8. $C_{13}H_{18}O_8N_2,0{\cdot}5H_2O$ requires C, 46·0; H, 5·6; N, 8·3; H_2O, 2·7%). It had $\lambda_{max.}$ 227, 246, 300, $\epsilon_{max.}$ 11 200, 8700, 5400 in 0·1 N-NaOH and $\lambda_{max.}$ 274, $\epsilon_{max.}$ 7750 in 0·1 N-HCl. Hydrolysis with purified emulsin (L. Light and Co. Ltd.) or boiling N-HCl gave glucose (Myers & Smith, 1954) and 6-amino-4-nitro-*o*-cresol (Table 1) which were identified chromatographically. Acetylation with pyridine and acetic anhydride as described by Kamil, Smith & Williams (1951) gave 6-acetamido-4-nitro-*o*-cresyl tetra-acetyl-β-D-glucoside, m.p. and mixed m.p. 169°.

6-Acetamido-4-nitro-*o*-cresol treated with chlorosulphonic acid and pyridine according to Burkhardt & Lapworth (1926) gave 6-*acetamido*-4-*nitro*-o-*cresyl sulphate*, which was isolated as the potassium salt. It formed pale-yellow needles from water (Found C, 33·0; H, 2·7; N, 8·3; S, 10·0; K, 12·1. $C_9H_9O_7N_2SK$ requires C, 32·9; H, 2·8; N, 8·5; S, 9·8; K, 11·9%). It had $\lambda_{max.}$ 240, 287, $\epsilon_{max.}$ 12 400, 7500 in 0·1 N-NaOH and $\lambda_{max.}$ 240 and 285, $\epsilon_{max.}$ 11 100, 7100 in 0·1 N-HCl.

The ethereal sulphate of 6-amino-4-nitro-*o*-cresol was prepared in small yield by the persulphate oxidation of 3-methyl-5-nitroaniline (cf. Boyland, Manson & Sims, 1953). Trial experiments were first carried out with *m*-nitroaniline. *m*-Nitroaniline (10 g.), potassium persulphate (10 g.) and KOH (2 g.) were stirred in a mixture of 100 ml. of acetone and 100 ml. of water for 8 hr. and left overnight. Unchanged *m*-nitroaniline (9·8 g.) was filtered off and the filtrate concentrated to small bulk. The inorganic salts were precipitated by addition of acetone and the filtrate was evaporated *in vacuo* to give a dark-brown residue which was crystallized from ethanol and water. The *potassium* 2-*amino*-4-*nitrophenyl sulphate* (20 mg.) was obtained as dark-yellow needles, very soluble in water, sparingly soluble in ethanol (Found C, 26·9; H, 2·0; K, 14·6. $C_6H_5O_6N_2SK$ requires C, 26·5; H, 1·9; K, 14·4%). It had $\lambda_{max.}$ 270, $\epsilon_{max.}$ 7600 in 0·1 N-HCl, and $\lambda_{max.}$ 225, 245, 288, 355, $\epsilon_{max.}$ 12 200, 10 700, 5300, 2300 in 0·1 N-NaOH.

In a similar experiment 3-methyl-5-nitroaniline (2·5 g.) (Haworth & Lapworth, 1923), potassium persulphate (2·5 g.) and KOH (1 g.) yielded 25 mg. of *potassium* 6-*amino*-4-*nitro*-o-*cresyl sulphate* as orange needles, very soluble in water (Found C, 29·6; H, 3·2; K, 14·1. $C_7H_7O_6N_2SK$ requires C, 29·4; H, 2·5; K, 13·7%). In 0·1 N-HCl it had $\lambda_{max.}$ 269, $\epsilon_{max.}$ 6600; in 0·1 N-NaOH it had $\lambda_{max.}$ 225, 293, $\epsilon_{max.}$ 11 700, 4900.

Both ethereal sulphates were rapidly hydrolysed by 0·1 N-HCl at 100° to the corresponding phenols, which were identified by paper chromatography (Table 1).

Locusts. Immature adult *Schistocerca gregaria* or *Locusta migratoria* were obtained from the Anti-Locust Research Centre and kept as previously described (Myers & Smith, 1953). They were fed on grass except while being used for experiment, when they were given water and rice paper.

Compounds were administered to the insects by intrathoracic injection as their sodium salts dissolved in 0·1–0·2 ml. of water. All metabolic experiments described below were carried out on both species and no qualitative difference was observed in the results.

Preparation of extracts. Excreta were roughly ground in a mortar with twice their bulk of aq. 0·1 N-NH_3 soln., then filtered off through cheese cloth and washed with a further equal volume of water. The dark-green filtrate was evaporated to a syrup *in vacuo* at 50° and treated with ethanol until precipitation was complete. The ethanolic filtrate was evaporated to small bulk and a further small amount of insoluble material was thrown down with ethanol. The filtrate was concentrated and used for large-scale chromatography.

Locust tissues were ground with sand in a mortar under ethanol, or, when whole locusts were extracted, macerated in an Ato-Mix Blender with ethanol. An equal volume of water was added and the mixture filtered through cheese cloth. The filtrate was concentrated and the residue precipitated with ethanol as described for excreta extracts, before application to chromatograms.

Paper chromatography. This was carried out as described earlier (Smith *et al.* 1953) and approximate R_F values are quoted in Table 1 to indicate degrees of separation. Identifications were confirmed in all of the solvent systems quoted by running reference compound and unknown on the same paper.

Large-scale chromatography. Crude ethanolic extracts were purified on large-scale paper chromatograms (cf. Smith, 1958), since in most cases the metabolites were associated with large amounts of interfering material and initial chromatograms did not usually give well-defined bands. Zones were located by the yellow or orange colours produced in NH_3 vapour, and cut out and eluted chromatographically with aq. 0·1 N-NH_3 soln. Separation of these initial elutes on further large-scale chromatograms gave a number of well-defined bands, one of which usually predominated. These were eluted for identification.

Paper electrophoresis. This was carried out as described earlier (Smith, 1958), and for identifications the 'unknowns' were run side by side with reference compounds at three or more of the pH values quoted. No significance could be attached to the absolute directions of migration since no allowance was made for electro-osmotic effects. Figures in Table 2 are therefore quoted only to show the degree of separation achieved in a particular electrolyte.

Detection of compounds on papers. All the compounds in Table 1 prevented the fluorescence of paper in ultraviolet light from a filtered low-pressure mercury arc (Hanovia Chromatolite) and showed as dark-purple spots on a lighter background. All nitro compounds except 6-acetamido-4-nitro-*o*-cresyl glucoside gave yellow or orange colours when exposed to NH_3 fumes. Glucosides were detected by hydrolysis to their aglycones on spraying with a 1:5 (v/v) dilution of snail gastric juice, which contains a β-glucosidase, and leaving for 5 min. at room temp. and treating with NH_3 fumes. Ethereal sulphates were detected by hydrolysis to the coloured nitrophenols by spraying with 0·1 N-HCl and heating to 60° for 10 min.

Spectroscopic measurements. These were made in a Unicam SP. 500 spectrophotometer. Eluates of paper chromatograms were measured against controls prepared from a section of paper chromatogram treated as far as possible in the same way as the metabolite spot.

Aryl sulphatase assays. Potassium *p*-nitrophenyl sulphate was used as substrate as described earlier (Robinson, Smith, Spencer & Williams, 1952). A final substrate concentration of 0·033 M in 0·033 M-sodium succinate–succinic acid buffer, pH 5·4, was used for routine assays at 37°.

β-Glucosidase assays. Enzyme solutions (1 ml.) were incubated at 37° with 2 ml. of 0·1 M-phosphate–citrate buffer (prepared from 0·1 M-Na_2HPO_4 and citric acid), pH 7, and 2 ml. of 0·01 M-*p*-nitrophenyl β-glucoside for 0·5 hr.; 5 ml. of 0·2 N-NaOH was then added and the nitrophenol liberated calculated from the absorption at 402 mμ as described for the aryl sulphatase method. Controls were prepared similarly except that the enzyme was added after incubating substrate and buffer, immediately before the addition of NaOH.

RESULTS

Metabolism of 6-amino-4-nitro-o-cresol

Fifty *Schistocerca gregaria*, which were each given 0·4 mg. of 6-amino-4-nitro-*o*-cresol, were decapitated 24 hr. later. The intestinal tract and any adhering organs were removed and an ethanol extract of these was prepared as described above. The final extract was separated on a large-scale chromatogram in solvent *A* (Table 1) in which five yellow or orange zones could be distinguished. The paper was cut into five sections, I–V, corresponding to these zones with approximate R_F values 0–0·15, 0·15–0·3, 0·3–0·5, 0·5–0·7 and 0·7–0·9, and each zone was eluted chromatographically with aq. 0·1 N-NH_3 soln.

Fraction I was chromatographed in solvent *D* and gave a main band with R_F 0·1. This yellow

Table 1. *R_F values of some nitro compounds*

Whatman no. 1 or 4 paper was used and the solvent run until the front had moved 10–12 in. Solvent systems: *A*, Benzene–butanol–aq. NH_3 soln. (sp.gr. 0·88), 2:5:2 (by vol.); *B*, benzene–acetic acid–water, 1:1:2 (by vol.); *C*, butanol–acetic acid–water, 4:1:5 (by vol.); *D*, butanol saturated with water; *E*, ethyl methyl ketone saturated with water; *F*, ethyl methyl ketone–aq. 2 N-NH_3 soln., 1:1 (v/v); *G*, propanol–aq. NH_3 soln. (sp.gr. 0·88), 7:3 (v/v).

	A	*B*	*C*	*D*	*E*	*F*	*G*
6-Amino-4-nitro-*o*-cresol	0·25	0·33	0·91	0·86	0·32	0·47	0·63
6-Amino-4-nitro-*o*-cresyl sulphate	0·49	0·0	0·57	0·51	0·80	—	0·75
6-Amino-4-nitro-*o*-cresyl glucoside	0·70	0·0	0·77	0·60	0·80	—	0·75
6-Acetamido-4-nitro-*o*-cresol	0·53	0·78	0·91	0·82	0·92	0·57	0·75
6-Acetamido-4-nitro-*o*-cresyl sulphate	0·65	0·0	0·56	0·64	0·83	—	0·84
6-Acetamido-4-nitro-*o*-cresyl glucoside	0·70	0·0	0·82	0·69	0·83	—	0·75
3-Amino-5-nitrosalicylic acid	0·06	0·07	0·69	0·26	0·74	0·67	—
3-Acetamido-5-nitrosalicylic acid	0·06	0·01	0·62	0·33	0·56	0·55	—
4:6-Dinitro-*o*-cresol	0·75	0·95	0·95	—	—	0·85	—
2-Amino-4-nitrophenol	0·13	—	—	—	0·95	0·33	—
2-Amino-4-nitrophenyl sulphate	0·25	—	—	—	0·37	0·51	—

Table 2. *Ionophoresis of some nitro compounds*

Apparent migration (in cm.) to anode (+) or cathode (−) on Whatman no. 1 paper in 2 hr. at 12 v/cm.

	0·02 N-HCl	pH 3·9*	pH 6·0*	pH 8·3*	0·02 N-NaOH
6-Amino-4-nitro-*o*-cresol	−3·0	−0·2	+0·1	+0·2	+5·0
6-Amino-4-nitro-*o*-cresyl sulphate	−0·5	+4·3	+4·3	+4·7	+5·2
6-Amino-4-nitro-*o*-cresyl glucoside	−1·5	−0·2	−0·7	−0·1	−1·0
6-Acetamido-4-nitro-*o*-cresol	−0·5	0·0	+0·1	0·0	+4·9
6-Acetamido-4-nitro-*o*-cresyl sulphate	+2·6	+4·6	+4·0	+4·2	+5·0
6-Acetamido-4-nitro-*o*-cresyl glucoside	−0·5	0·0	0·0	0·0	−1·0
4:6-Dinitro-*o*-cresol	−0·5	—	—	—	+5·0

* Britton & Robinson (1931) buffer (British Drug Houses Ltd.).

Table 3. *Absorption spectra in* 0·1 N-*sodium hydroxide of metabolites of* 6-*amino*-4-*nitro*-o-*cresol in locusts*

~, Indicates point of inflexion.

	Reference compounds		Metabolites	
	$\lambda_{max.}$	$\epsilon_{max.}$	$\lambda_{max.}$	$E_{1\,cm.}$
6-Acetamido-4-nitro-*o*-cresol	285	6 700	285	0·90
	425	13 900	425	1·52
6-Amino-4-nitro-*o*-cresyl sulphate	225	11 700	225	3·20
	~245	8 500	~245	2·40
	293	4 900	295	1·28
6-Amino-4-nitro-*o*-cresyl glucoside	227	11 200	230	3·52
	247	8 700	~245	3·05
	295	5 400	295	1·68

material, which was also present in extracts of untreated locusts, was not identified. No 3-amino-5-nitrosalicylic acid or 3-acetamido-4-nitrosalicylic acid, which have R_F values near 0·1 in solvent *A*, was detected in this extract. Small amounts of material corresponding to the main bands of fractions II–V were also present.

Fraction II was chromatographed in solvent *D* and gave two main zones. A pink band of R_F 0·4, which on elution had light-absorption maxima at 460 and 270 mμ in 0·1 N-NaOH and at 320 and 270 mμ in 0·1 N-HCl, was not identified. A second major, orange band in solvent *D*, with R_F 0·8, was identified as 6-amino-4-nitro-*o*-cresol by paper chromatography, absorption spectrum in 0·1 N-NaOH and electrophoresis. From the extinctions in 0·1 N-NaOH it was calculated that 5% of the dose was present in this form and in other experiments corresponding values ranged from 5 to 8%.

Fraction III was chromatographed in solvent *D* and gave a pale-yellow main band of R_F 0·5, which was eluted with aq. 0·1 N-NH_3 soln. and identified as the ethereal sulphate of 6-amino-4-nitro-*o*-cresol by paper chromatography, ionophoresis and comparison of absorption spectra (Table 3). It was rapidly hydrolysed by boiling 0·1 N-HCl to 6-amino-4-nitro-*o*-cresol. Calculation from the absorption spectrum in 0·1 N-NaOH showed that 0·5% of the dose was present as this ethereal sulphate.

Fraction IV was chromatographed in solvent *D* and gave a major yellow band which after elution was identified as 6-acetamido-4-nitro-*o*-cresol by chromatography, ionophoresis and measurement of absorption spectrum (Table 3). From the absorption spectrum in 0·1 N-NaOH it was calculated that 0·2% of the dose was present in this form and in other experiments the corresponding values ranged from 0·2 to 5·0% of the dose. A small amount of material of R_F 0·6 was pooled with fraction V.

Fraction V was chromatographed in solvent *D* and gave a main, pale-yellow band of R_F 0·6, which was eluted and identified as 6-amino-4-nitro-*o*-cresyl glucoside by paper chromatography, ionophoresis and measurement of absorption spectrum in 0·1 N-NaOH. From this spectrum it was calculated that 0·7% of the dose was present as the glucoside.

Extracts of *Locusta migratoria* dosed with 6-amino-4-nitro-*o*-cresol were fractionated as described above and 6-acetamido-4-nitro-*o*-cresol, 6-amino-4-nitro-*o*-cresol, its glucoside and its ethereal sulphate were identified as with *Schistocerca*.

These four compounds were also separated and identified in extracts of *Schistocerca* and *Locusta* made 4 hr. after dosing, and at this time the hind-gut of some locusts was full of a red liquid in which these compounds could be directly demonstrated by paper chromatography.

In some experiments excreta were collected for 48 hr. after dosing and extracts made as described above. The same metabolites were present in these extracts but the amounts of *O*-conjugates, especially the glucoside, were much smaller. This is probably attributable to the variable but usually large amounts of β-glucosidase in the hind-gut and excreta (see below).

Metabolism of 6-*acetamido*-4-*nitro*-o-*cresol*

Extracts were made as described above after dosing 100 locusts with 0·25 mg. each of 6-acetamido-4-nitro-*o*-cresol. After fractionation of visceral extracts as described above, 2% of unchanged material was recovered from the chromatograms. An additional 12% of the dose was recovered by similarly extracting the eviscerated bodies, but no *O*-conjugates could be detected in this or a number of other experiments with *Locusta* and *Schistocerca*.

Metabolism of 4:6-*dinitro*-o-*cresol*

Two hundred *Locusta migratoria* which had received 4 μg. each of DNOC were decapitated 24 hr. later and an ethanol extract was prepared from the combined viscera and excreta. The final extract was separated on a large-scale chromatogram in solvent *A*. A feeble-yellow band at R_F 0·7 was eluted and rechromatographed in solvent *F* to

free it from a large amount of fast-running brown waxy material. The yellow band with R_F 0·2 was eluted with aq. 0·1 N-NH_3 soln. and identified as 6-acetamido-4-nitro-*o*-cresol by chromatography, ionophoresis in 0·02 N-NaOH and comparison of absorption spectra (Fig. 1). The extinction in 0·1 N-NaOH corresponded to a recovery of 1 % of the dose. When boiled with N-HCl for 1 hr. the metabolite was converted into 6-amino-4-nitro-*o*-cresol, which was extracted with ether and identified chromatographically. 6-Acetamido-4-nitro-*o*-cresol was similarly identified in parallel experiments with *Schistocerca gregaria*.

In another experiment 22 μg. of DNOC was given to each of 24 *Schistocerca* in two divided doses with a 3 hr. interval, and an extract of viscera made 3 hr. later. At this time a trace of DNOC could still be detected in the haemolymph by paper chromatography.

Separation of the visceral extracts on an initial chromatogram in solvent *A* gave, as before, a yellow band at R_F 0·7. On rechromatographing this in solvent *F* it separated into a minor band identified, as above, as 6-acetamido-4-nitro-*o*-cresol (< 1 % of dose) and a more definite band which was identified by paper chromatography and measurement of absorption spectra as DNOC (4 % of dose).

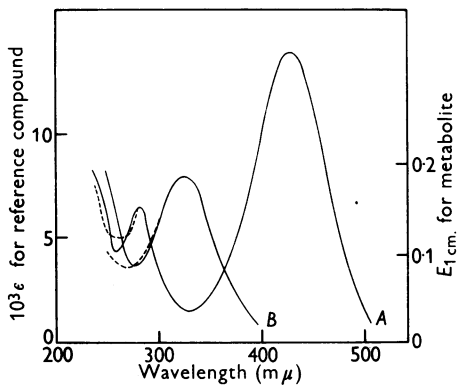


Fig. 1. Absorption spectra of 6-acetamido-4-nitro-*o*-cresol (continuous lines) and of metabolite from locusts poisoned with DNOC (broken lines). For wavelengths between about 300 and 500 mμ the curves for reference compound and for metabolite coincided. Curves *A*, in 0·1 N-NaOH; curves *B*, in 0·1 N-HCl.

Enzyme experiments

Distribution of glucosidase and sulphatase in gut. The heads and terminal abdominal segments of locusts were cut off and the intestinal tracts removed. The gut was divided immediately anterior to the attachment of the gastric caecae and at the point of attachment of the Malpighian tubes. The three sections, designated fore-gut, mid-gut and hind-gut, were opened, and the dark-coloured contents weighed and diluted to measured volume with water. The mid-gut solution included the contents of the gastric caecae. Freshly voided, moist excreta were also weighed, and suspended in a measured volume of water; all solutions were diluted to a volume containing enough activity to give spectrophotometer readings of 0·2–1·0 and were filtered through cotton wool before use.

(*a*) Arylsulphatase activity in each section of gut showed optimum activity in succinate buffer at pH 5·4. Optimum substrate concentration was not reached with saturated solutions of potassium *p*-nitrophenylsulphate. Plots of $1/V$ against $1/S$ (cf. Alberty, 1956) for the range 0·5–5·0 mM were linear and crossed the $1/S$ axis close to the origin at values corresponding to K_s values of not less than 0·05 M.

(*b*) β-Glucosidase activity in each section of the gut showed a broad maximum between pH 5 and 6 and there was little loss of activity at pH 7 in phosphate–citrate buffer. Measurements were carried out at pH 7 since this was the pH of moist excreta, and the optimum substrate concentration determined empirically at this pH was 4 mM-*p*-nitrophenyl glucoside. Plots of $1/V$ against $1/S$ for the range 0·4–4·0 mM were linear and the intercept on the $1/S$ axis corresponded to values of K_s of 0·9 mM for each section of the gut.

Small groups of *Schistocerca* were obtained from the Anti-Locust Research Centre at different times and the glucosidase and sulphatase activity of the sections of the gut measured. The results are summarized in Table 4. Locusts in these experi-

Table 4. *Enzymic liberation of* p-*nitrophenol from conjugates by gut contents and excreta of* Schistocerca gregaria

For conditions see Methods. Ranges of values are given in parentheses.

	Fore-gut	Mid-gut	Hind-gut	*Excreta
From *p*-nitrophenyl sulphate				
Total μg./hr.	590 (70–2700)	210 (16–860)	11 (0–40)	3 (0–10)
μg./mg. of gut contents/hr.	20 (2–40)	7 (1–17)	0·5 (0·1–1)	0·2 (0–0·9)
From *p*-nitrophenyl glucoside				
Total μg./hr.	510 (220–710)	390 (80–980)	330 (60–650)	767 (114–1600)
μg./mg. of gut contents/hr.	30 (8–45)	32 (2–180)	24 (14–130)	120 (12–220)

* Freshly voided faecal pellets, total wt. 2–10 mg. were used.

ments were fed, as normally, on grass but no significant diminution of activity occurred in one experiment where a group of locusts was starved for a week before assay. Similar high β-glucosidase activity was found in *Locusta* gut contents.

Hydrolysis rates of glucosides

Solutions of 6-amino-4-nitro-*o*-cresyl glucoside and 6-acetamido-4-nitro-*o*-cresyl glucoside were rapidly hydrolysed to the corresponding phenols when ground at room temperature with dry excreta from the stock cage. On the other hand, *m*-aminophenyl glucoside has been separated fairly readily from excreta (Myers & Smith, 1954) and was therefore presumably fairly stable. We have therefore measured the hydrolysis rates of some of the glucosides with enzyme from excreta.

Solutions of glucosides, 3 ml. of 0·01 M in 0·02 M-phosphate buffer (prepared from 0·02 M-Na_2HPO_4 and 0·02 M-KH_2PO_4), pH 7, and enzyme (1 ml.), prepared by grinding 5 g. of dry excreta with 25 ml. of water and centrifuging the debris, were incubated at 37°. Samples of this solution and similar controls prepared with boiled enzyme were withdrawn at intervals and analysed for the aglycones. Control samples were used as instrument blanks in the spectrophotometer. Nitrophenols were estimated by their characteristic absorption in 0·1 N-NaOH (see above). *m*-Aminophenol was estimated, after ether extraction, by the dimethylaminobenzaldehyde method used earlier for *m*-aminophenyl sulphate (Smith, 1955*b*).

With one sample of enzyme the values of $t_{\frac{1}{2}}$ calculated from the unimolecular velocity constant were 31 min. for 6-amino-4-nitro-*o*-cresyl glucoside, 33 min. for *p*-nitrophenyl glucoside, 85 min. for 6-acetamido-4-nitro-*o*-cresyl glucoside and 700 min. for *m*-aminophenyl glucoside.

DISCUSSION

The results quoted in Table 4 show that the β-glucosidase activity which occurs in locust-crop fluid is found in all the other parts of the intestine (cf. Robinson, 1956) and is also present in substantial amounts in the excreta. It is possible that the activity is due to the crop enzyme being carried along with the food. The hind-gut in a number of locust species has a pH of 7–8 (Uvarov, 1948), and at this value the β-glucosidase is still active. It is probable that a considerable part of any β-glucoside excreted from the Malpighian tubes is hydrolysed before elimination in the excreta, but the extent of this hydrolysis will depend on the nature of the glucoside as well as on the amount of activity present in the gut. 6-Amino-4-nitro-*o*-cresyl glucoside is hydrolysed about 20 times as rapidly as *m*-aminophenyl glucoside. It is likely, however, that sufficient glucosidase is always present in hind-gut to hydrolyse completely any glucoside produced from the very small doses of DNOC used, even if all the insecticide had been converted into its β-glucoside.

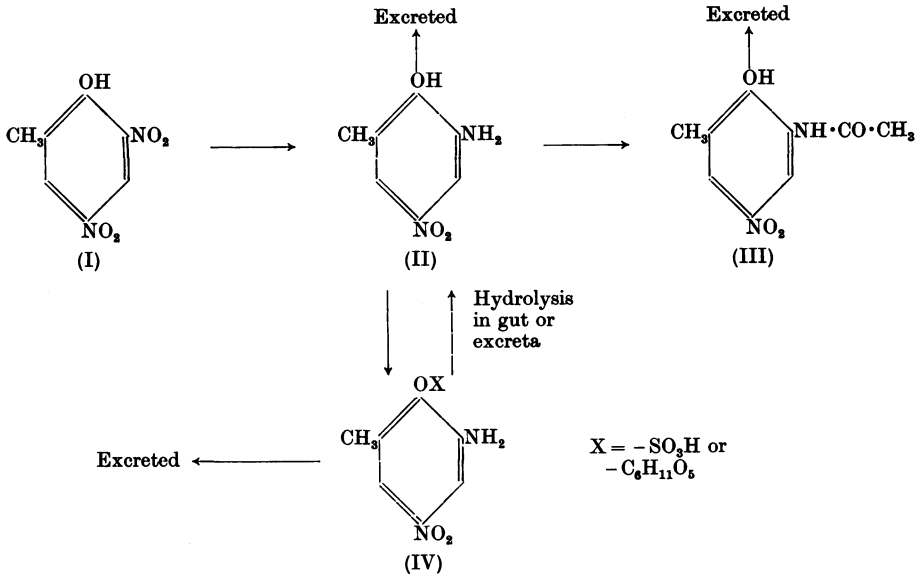
It has already been pointed out that a glucuronic acid conjugation would be of small value to a locust because of the presence of β-glucuronidase (Smith, 1955*b*), and the same observation must now be applied to the β-glucoside conjugation. The importance to the locust of this hydrolysis of conjugates in the gut must largely depend on how well the liberated phenols can be reabsorbed from the small intestine; it is possible that locusts recycle excreted phenols after gut hydrolysis of the initial conjugate. A similar situation occurs in mammals, where some compounds, e.g. chloromycetin (Glazko, Dill & Wolf, 1952), are excreted as conjugates in the bile, and hydrolysed in the intestine and reabsorbed into the system. This situation is more serious for the locust, however, as no alternative (urinary) route of excretion is available. If this recycling of phenols does occur in locusts the toxicity of phenols might be expected to be greater in non-feeding insects since the gut contents would not then be so rapidly expelled (cf. Uvarov, 1948).

The association of β-glucoside conjugation with active β-glucosidase activity may be fairly general. The glucoside conjugation appears fairly general in insects (Smith, 1955*a*) and β-glucosidase activity has been demonstrated in a variety of insects (Robinson, 1956, 1957), and in a number of species this is localized in the intestine (Newcomer, 1954; Evans, 1956; Koike, 1954).

Distribution of arylsulphatase activity in the locust intestine differs from that of β-glucosidase in that much less is found in hind-gut than in crop and mid-gut. Moreover, *p*-nitrophenyl sulphate requires a high substrate concentration for optimum activity. It may be for this reason that we were able to detect 6-amino-4-nitro-*o*-cresyl suphate in extracts of locust excreta after dosing with the cresol, and in many experiments the corresponding glucoside was not detectable.

The presence of these two enzymes in the gut makes it unlikely that any effective detoxication of DNOC occurs by *O*-conjugations and no such metabolites could be detected in the poisoned locusts. Reduction of a nitro group appears to be the effective detoxication in locusts as in rabbits. The metabolism of DNOC (I) and 6-amino-4-nitro-*o*-cresol (II) is shown in scheme 1.

When 6-amino-4-nitro-*o*-cresol was injected, the acetyl derivative (III) and the two *O*-conjugates (IV) were identified, but when DNOC was injected, necessarily at a lower dose level, only 6-acetamido-4-nitro-*o*-cresol (III) was found as a metabolite.



Scheme 1

SUMMARY

1. 6-Amino-4-nitro-*o*-cresol is conjugated in the locusts *Schistocerca gregaria* and *Locusta migratoria* to 6-amino-4-nitro-*o*-cresyl glucoside and 6-amino-4-nitro-*o*-cresyl hydrogen sulphate, which have been identified in the gut and excreta by chromatographic, ionophoretic and spectroscopic methods.

2. Small amounts of 6-acetamido-4-nitro-*o*-cresol were similarly identified in extracts of locusts poisoned with 4:6-dinitro-*o*-cresol.

3. β-Glucosidase activity is present in the contents of locust fore-, mid- and hind-gut, and in locust excreta.

4. Aryl sulphatase activity is present in locust fore-gut and mid-gut contents, and to a much smaller extent in hind-gut and excreta.

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