Comparative Detoxication

12. THE OXIDATION OF *p*-NITROTOLUENE AND *p*-NITROETHYLBENZENE IN INSECTS*

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(Received 21 January 1964)

Oxidation of a foreign molecule in the body usually yields a more polar metabolite which is more easily excreted and frequently lacks the biological activity of the non-polar precursor (Williams, 1959, pp. 717-739). In vertebrates, when compounds susceptible to oxidation either in an aromatic ring or in an alkyl side chain are metabolized, reaction occurs almost exclusively in the alkyl side chain (Williams, 1959, pp. 197-204). Insects can oxidize the aromatic ring (Smith, 1962) but little information is available on the relative ease of metabolism of alkyl side chains in these invertebrates.

The metabolism of the alkyl side chains is of interest since these are structural features of several useful insecticides such as Diazinon [*OO*-diethyl-*O*-(2-isopropyl-4-methylpyrimidin-5-yl)phosphorothionate], Ruelene [*O*-(4-*tert*.-butyl-2-chlorophenyl)-*N*-methyl-*O*-methylphosphoramidate] and a series of alkylaryl *N*-methylcarbamates (Metcalf, 1955).

We wished therefore to assess the ability of several insect species to oxidize aliphatic groups, and chose *p*-nitrotoluene for initial study as it carried the simplest aliphatic side chain and the oxidation product, *p*-nitrobenzoic acid, is capable of easy detection and estimation. The metabolism of this compound has been well studied in vertebrates (Williams, 1959, pp. 417, 418) and it has recently been used as a substrate for the microsomal oxidation system of rabbit liver (Gillette, 1959).

EXPERIMENTAL

Compounds. *p*-Nitrotoluene, m.p. 48°, *p*-nitrobenzoic acid, m.p. 240°, *p*-nitroethylbenzene, b.p. 245°/760 mm. Hg, n_D^{20} 1.5458, and *p*-nitrophenylacetic acid, m.p. 153°, were commercial samples and free of trace impurities by paper chromatography. 1-(*p*-Nitrophenyl)ethanol, b.p. 162°/16 mm. Hg, and 2-(*p*-nitrophenyl)ethanol, m.p. 56°, were prepared by sodium borohydride reduction of *p*-nitroacetophenone and methyl *p*-nitrophenylacetate respectively.

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Insects and dosing. Fifth-instar locust hoppers were obtained from the Anti-Locust Research Centre and kept and dosed as previously described (e.g. Cohen & Smith, 1964; Myers & Smith, 1953). Houseflies were a normal strain obtained from the Rothamsted Experimental Station as pupae and were used within 3 days of emergence. Other insects were also obtained from Rothamsted and dosed immediately.

Compounds were administered at 200 µg./g. by injection in 0.01-0.1 ml. of acetone, except to flies and mustard beetles which were dosed topically at 100 and 200 µg./g. respectively.

Paper chromatography and ionophoresis. Acidic metabolites were identified by ionophoresis as described by Smith (1958). Paper chromatography was carried out by downward displacement and approximate R_F values are quoted in Table 1. Separation of 1- and 2-(*p*-nitrophenyl)-ethanol was achieved by allowing solvent to run off the bottom of the chromatogram in solvent system A or E. Compounds were detected on paper as described by Cohen & Smith (1964).

Estimation of *p*-nitrobenzoic acid. Single large insects or groups weighing approx. 1 g. were homogenized in 5 ml. of water containing 0.5 ml. of 0.5 *N*-NaOH. Protein was removed by addition of 0.5 ml. of 10% (w/v) ZnSO₄, and 10 ml. of CCl₄ was added to extract unchanged *p*-nitrotoluene. After shaking and centrifugation, 3 ml. of the supernatant was acidified with 0.2 ml. of 2 *N*-HCl and reduced by shaking for 0.5 min. with 1 ml. of 1% (w/v) zinc amalgam. The *p*-aminobenzoic acid formed was then determined according to the Bratton & Marshall (1939) procedure. In some insects, particularly flies, large blank values were obtained from untreated flies but these were minimized by extracting the azo dye into 5 ml. of amyl alcohol in which the interfering azo-colour was not soluble.

Recovery of *p*-nitrobenzoic acid from aqueous solution was quantitative and recoveries of *p*-nitrobenzoic acid added to 1 g. of locust homogenate were 83.2 S.E.M. ± 1.9 (10).

Estimation of 1-(*p*-nitrophenyl)ethanol in enzyme mixtures. The reaction mixture (5 ml.) was acidified with 3 ml. of 10 *N*-HCl and extracted with 3 × 5 ml. of ether. The ether layer was evaporated to small bulk and transferred to a paper chromatogram and run in solvent system A. The zone corresponding to 1-(*p*-nitrophenyl)ethanol was eluted with methanol and this was determined by the procedure described above for *p*-nitrobenzoic acid. Colour development was allowed to proceed for 3 hr. before measurement and calculation was made by reference to a calibration curve prepared from known amounts of a methanol solution of 1-(*p*-nitrophenyl)ethanol. Recoveries of known amounts

Table 1. R_f values of some nitro compounds

Chromatograms were run on Whatman no. 4 paper until solvent fronts had moved 12 in. Solvents: A, *n*-hexane-di-isopropyl ether (5:1, v/v) run on paper treated with formamide-saturated ether and dried; B, butan-2-one-2*N*-NH₃ (1:1, v/v); C, butan-1-ol-ammonia (sp.gr. 0.88)-water (4:1:5, by vol.); D, ethanol-water (7:3, v/v) on paper treated with 5% (v/v) olive oil in ether and dried; E, *n*-hexane-di-isopropyl ether (5:1, v/v) on paper treated with a saturated solution of Carbowax 4000 in ether-ethanol (50:1) and dried.

Solvent	...	...	A	B	C	D	E
1-(<i>p</i> -Nitrophenyl)ethanol			0.20	0.92	0.93	0.92	0.30
2-(<i>p</i> -Nitrophenyl)ethanol			0.15	0.91	0.90	0.90	0.25
<i>p</i> -Nitrobenzoic acid			0.01	0.71	0.43	0.81	0.32
<i>p</i> -Nitrophenylacetic acid			0.02	0.60	0.42	0.81	0.34
<i>p</i> -Nitrotoluene			0.92	0.93	0.90	0.72	0.91
<i>p</i> -Nitroethylbenzene			0.91	0.92	0.91	0.70	0.90

of material added to 5 ml. of incubation mixture were 71 s.e.m. $\pm$ 3.2 (6).

Tissue incubations. Organs were removed from locusts and incubated in saline as described by Cohen & Smith (1964) at 37° in air. Organs from six hoppers were used in each experiment in 2 ml. of saline to which 1.0 mg. of *p*-nitrotoluene in 0.02 ml. of acetone had been added. After a suitable time, usually 30 min., the mixture was ground in a Potter-Elvehjem homogenizer and the *p*-nitrobenzoic acid estimated as above.

Centrifuged fractions were prepared from 25 locust fat bodies by homogenizing in 20 ml. of 0.25*M*-sucrose for 1 min. at 0-3°. The homogenate was then centrifuged at 10000*g* for 10 min. at 3° and the supernatant used for enzymic experiments.

RESULTS

Metabolism of *p*-nitrobenzoic acid

Insects (1-5 g.) were dosed as described above and homogenized and deproteinized after 5 hr. The supernatant was concentrated to small bulk *in vacuo* and examined by paper chromatography and ionophoresis. Only *p*-nitrobenzoic acid was detected as a metabolite, except in *Periplaneta* where traces of *p*-aminobenzoic acid were found.

Quantitative assays made on four large insects or 1 g. batches of small insects at intervals of 5 hr. after dosing showed a linear relationship between time and amount of *p*-nitrobenzoic acid formed (Table 2).

Oxidation also occurred in intact isolated tissues of the locust in saline (Table 3), but homogenization of any of these tissues for 1 min. in a Potter-Elvehjem homogenizer gave a preparation devoid of oxidizing power. Active preparations could be made from locust fat-body homogenate, but not from other insects or organs, by centrifugation at 10000*g* for 10 min. at 0-3°.

The 10000*g* supernatant of five locusts in 5 ml. of 0.25*M*-sucrose was incubated at 37° in air with 1 mg. of *p*-nitrotoluene. Examination of the mixture after deproteinization showed *p*-nitrobenzoic acid to be the only metabolite and quantitative assays showed the rate of formation to be

Table 2. Rates of formation of *p*-nitrobenzoic acid from insects dosed with 200 µg. of *p*-nitrotoluene/g.

	Formation of <i>p</i> -nitrobenzoic acid (µg./g. of insect/hr.)
Locust (<i>Schistocerca</i>)	10
Cockroach (<i>Periplaneta</i>)	9
Cockroach (<i>Blattia</i>)	12
Cricket (<i>Gryllus</i>)	12
Flour beetle (<i>Tenebrio</i>)	8
Mustard beetle (<i>Phaedon</i>)	20
Housefly (<i>Musca</i>)	25
Cotton stainer (<i>Dysdercus</i>)	5
Caterpillar (<i>Pieris</i>)	8

Table 3. Rates of formation of *p*-nitrobenzoic acid from *p*-nitrotoluene by locust organs in vitro

For conditions see the text.

	Formation of <i>p</i> -nitrobenzoic acid (µg./locust/hr.)		
	Males	Females	Mixed sexes
Fat body	5.1	6.2	7.9
Gastric cecae	2.8	4.8	4.0
Foregut	0.7	1.0	1.8
Midgut	0.7	1.0	1.9
Hindgut	0.3	0.3	0.8
Malpighian tubes	0.3	0.6	1.9
Total	9.9	13.9	18.3

linear up to 90 min. In a series of more than 50 experiments the rate of formation of *p*-nitrobenzoic acid in this type of preparation ranged from 5 to 10 µg./hr./locust and was not affected by addition of NADPH, NADP, NAD, NADH, Mg²⁺ ions, nicotinamide or glucose 6-phosphate.

Assays of the oxidizing activity of the 10000*g* supernatant from the fat body were made at intervals on a large group of fifth-instar hoppers. Rates of 3.6, 4.5, 4.5 and 6.4 µg./hr./locust were obtained 2, 4, 7 and 11 days after collection. Moulting started in the group at 11 days and

enzyme from adults assayed on the fourteenth and eighteenth days had rates of 1.5 and 2 μ g. of *p*-nitrobenzoic acid/hr./locust.

Oxidation of p-nitroethylbenzene

The 10000g supernatant from 25 locusts was incubated for 60 min. with 5 mg. of *p*-nitroethylbenzene at 37° in air. After acidification with 3 ml. of 10N-hydrochloric acid and extraction with ether, the ether extract was examined by paper chromatography and ionophoresis. The major metabolite found was 1-(*p*-nitrophenyl)ethanol together with a trace of *p*-nitrophenylacetic acid. No 2-(*p*-nitrophenyl)ethanol was found.

In quantitative assays with 10000g supernatant from five locusts the rate of formation of the alcohol was 2 μ g./locust/hr.

DISCUSSION

The oxidative metabolism of *p*-nitrotoluene is similar in insects and vertebrates, the major product being *p*-nitrobenzoic acid. Oxidation of the ethyl side chain in *p*-nitroethylbenzene occurred mainly at the α -methylenic group, and similarly methylphenylcarbinol is the main oxidation product of ethylbenzene in rabbits (Smith, Smithies & Williams, 1954). The locust oxidizing system is also similar to that of rabbit in that it can be located in the 10000g supernatant of fat body or liver. Some activity was also found in the gut of the insect and good agreement was found between rates of oxidation in whole insect, isolated organ and 10000g supernatant from the fat body.

With the high doses used in the present work the rate of oxidation of *p*-nitrotoluene was constant over the experimental period and the values quoted in Table 2 probably show the maximum oxidative capacity of the insects for aliphatic centres. These would not necessarily show the same ratios with the much lower doses absorbed if insecticides were being oxidized, but they suggest that different rates of oxidation could be a factor in different rates of detoxication of insecticides having aliphatic side chains.

The rates of oxidation found are considerably lower than has been found for other detoxication reactions such as *O*-conjugation (Smith & Turbert, 1964) or glutathione conjugations (Cohen, Smith & Turbert, 1964), but oxidation is frequently the first biochemical attack made on a foreign molecule,

and in molecules having an alkylaryl grouping small differences in the oxidative detoxication could be the basis of differential toxicity.

Oxidation of a methylene group adjacent to an aromatic ring is a feature of the metabolism of DDT [2,2,2-trichloro-1,1-di-(*p*-chlorophenyl)ethane] in some insects. This α -methylenic oxidation yields Kelthane [2,2,2-trichloro-1,1-di-(*p*-chlorophenyl)ethanol] as the metabolite and an intensification of this reaction under DDT pressure can give rise to insecticide-resistant strains (Agosin, Michaeli Miskus, Nagasawa & Hoskins, 1961; Tsukamoto, 1959). The present results suggest that it would be worth while to investigate the possibility of alkyl-chain oxidation in other insecticides containing alkylaryl structures.

SUMMARY

1. *p*-Nitrotoluene was converted into *p*-nitrobenzoic acid in nine species of insects.
2. The most rapid oxidation occurred in houseflies.
3. In isolated locust organs, most oxidation occurred in fat body and gut.
4. *p*-Nitroethylbenzene was converted into 1-(*p*-nitrophenyl)ethanol by locust fat-body homogenates; traces of *p*-nitrophenylacetic acid were also formed.

This work has been supported by the Agricultural Research Council and we are also grateful for supplies of insects from the Anti-Locust Research Centre and the Insecticide Department, Rothamsted.

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