Inhibitors of Microsomal Oxidations in Insect Homogenates

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1. Homogenates of insect tissues were assayed for enzymes capable of oxidizing *p*-nitrotoluene to *p*-nitrobenzoic acid. 2. Locust fat-body homogenate 10000*g* supernatant was an effective enzyme and required no added cofactors. 3. Homogenates of other insects or locust organs and 10000*g* sediment from locust fat-body were not active and inhibited microsomal oxidations carried out by locust fat-body or rabbit liver enzyme. 4. Inhibitory power was high in homogenates of whole flies and of fly heads or thoraces. 5. Inhibition appeared to involve both irreversible inactivation of enzyme and the removal of essential cofactors.

In vertebrates the methyl groups in toluene and substituted toluenes are readily oxidized to the corresponding aromatic acids and the enzymology of this process has been studied by Gillette (1959) with liver microsomal enzyme. Alkyl side chains of barbiturates and other drugs are also oxidized by liver microsomal enzymes (Cooper & Brodie, 1957). Some preliminary enzymic studies with locust fat-body homogenate as the source of the enzyme were made (Chakraborty & Smith, 1964) and similar centrifugal preparations have been used in the study of the metabolism of naphthalene and a variety of insecticides (Arias & Terriere, 1962; Agosin, Michaeli, Miskus, Nagasawa & Hoskins, 1961; Fenwick, 1958; Nagatsugawa & Dahm, 1962, 1965). In this work the activity of the insect enzymes has usually been weaker than similar preparations from rat or rabbit liver and we have therefore examined some insect preparations for endogenous inhibitors that might account for the low activity.

EXPERIMENTAL

Insects. Fifth-instar locusts (*Schistocerca gregaria*) were from the Anti-Locust Research Centre, London, and enzymes made from mid-instar specimens. Other insects from previously used strains (Chakraborty & Smith, 1964) were obtained from the Insecticide Department, Rothamsted, Herts. Flies, unless otherwise stated, were used within 2 days of emergence as adults. At a later stage of the work an insecticide-sensitive strain of housefly was obtained from Wallaceville Animal Research Station, New Zealand, and reared in the laboratory.

Estimation of p-nitrobenzoic acid. This was carried out by the method used by Chakraborty & Smith (1964).

Estimation of p-acetamidobenzoic acid. This was carried out by the technique described by Hook & Smith (1967).

Preparation of vertebrate enzymes. Liver from rabbit or mouse was homogenized in 4 vol. of 0.25*M*-sucrose with a Teflon pestle in a glass Potter-Elvehjem type grinder. The homogenate was centrifuged at 10000*g* for 10 min. and the supernatant ('10000*g* supernatant') used as enzyme. All operations were carried out below 3°. Incubations were in 50 ml. beakers in a shaking water bath in air at 37° and in a total volume of 5 ml. containing, in most experiments, enzyme equivalent to 0.5*g*. of rabbit liver or 0.25*g*. of mouse liver. Rabbit liver incubations were fortified with 0.5 mg. each of NADPH₂ and NAD and 6 mg. each of MgCl₂ and nicotinamide. Mouse enzyme was fortified with 0.2 mg. each of NADP and NAD, 5 mg. of glucose 6-phosphate, 1 mg. of MgCl₂ and 6 mg. of nicotinamide. Each 5 ml. of incubation mixture was at pH 7.4 and contained 1 ml. of 0.05*M*-tris buffer, pH 7.4.

p-Nitrotoluene (1 mg.) or *p*-acetamidotoluene (0.5 mg.) was added to the incubation mixtures in 0.05 ml. of acetone to start the reaction. Reaction rates were linear with time for up to 1 hr. and in inhibition experiments incubations were stopped after 0.5 hr.

Insect homogenates. These were normally prepared in 0.25*M*-sucrose, as described above, except in the few cases noted below where 0.15*M*-KCl was used. Where centrifugal fractions were used the whole homogenate was centrifuged at 3° at 10000*g* for 10 min. to give a '10000*g* supernatant' and '10000*g* sediment' fraction.

Clear-cut separation of a 'nuclei and debris' fraction by centrifugation at 900*g* was difficult because of the nature of the sediment and in most cases the 10000*g* sediment contained this debris as well as the 'mitochondrial fraction'. 'Microsomal' pellets were prepared by sedimentation of the 10000*g* supernatant at 90000*g* for 1 hr. ('90000*g* sediment') for some experiments.

Conditions for the use of locust preparations as an oxidizing enzyme were those used by Chakraborty & Smith (1964) in which *p*-nitrotoluene was used as substrate.

Oxidation of NADPH₂. This was followed by direct spectrophotometric measurement at 340 mμ in either the Beckman DB spectrophotometer or the recording Unicam SP.700. Concentrated fly homogenates rapidly became

anaerobic below the surface layer so that the amount of homogenate had to be restricted to maintain the aerobic conditions in the cuvette. Normally a concentration equivalent to 2 flies/ml. was suitable. The reaction was started by adding 0.5 mg. of NADPH₂ in 0.01 ml. of water to the 0.5 cm. cuvette that contained the enzyme diluted into 0.05 M-tris buffer, pH 7.4, to give a total volume of 1.5 ml.

RESULTS

Preparation and properties of the locust fat-body enzyme. Homogenates of locust fat body usually showed a low capacity to convert *p*-nitrotoluene into *p*-nitrobenzoic acid, which was not increased by fortification with 0.2 mg. of oxidized or reduced NAD or NADP or by addition of 6 mg. of nicotinamide or magnesium chloride to the standard 5 ml. of reaction mixture.

The locust homogenate was similar to vertebrate liver enzyme (Gillette, 1959) in that centrifugation at approx. 10000g for 10 min. yielded an active enzyme in the supernatant and the 'microsomal' sediment of this supernatant at 90000g for 1 hr. was inactive unless combined with 90000g supernatant. Samples of fat-body homogenate prepared as described above were centrifuged at various g values for 10 min. and activities of the supernatants plotted graphically. The curve showed a broad maximum at the region 10000–12000g and 10000g for 10 min. was used as a routine thereafter.

The effect of adding cofactors was tested at different times on a variety of batches of locusts but no increase in activity was produced in the locust 10000g supernatants by the addition of 0.2 mg. of NAD, NADH₂ or NADPH₂ or 6 mg. of either nicotinamide or magnesium chloride. The activity of this preparation with *p*-nitrotoluene as substrate was in the range 5–10 μ moles of *p*-nitrobenzoic acid/min./locust (Chakraborty & Smith, 1964).

The enzymic reaction of preparations containing enzyme from five locusts' fat-bodies in 5 ml. of incubation mixture was linear with time for 1 hr. and measurements were therefore carried out for 0.5 hr.

The optimum substrate concentration for *p*-nitrotoluene was 1.5 mM and a double-reciprocal plot of substrate concentration and reaction velocity gave an apparent K_m value 6×10^{-4} M.

When the pH was varied in tris buffer and reaction velocity measured between pH 6.5 and 8.5, a narrow pH-activity curve was obtained with a maximum at pH 7.4.

The enzyme lost about a fifth of its activity when stored at 0° for 16 hr. or if left at room temperature for 3 hr. When the homogenate was left at room temperature for 3 hr. before centrifugation no activity was found in the 10000g supernatant.

No oxidation occurred if the reaction was carried out in a nitrogen atmosphere and incorporation of mm-piperonylbutoxide or SKF 525A (diethylamino-ethylidiphenylpropyl acetate) into the standard reaction mixture decreased the activity by 40%.

Inhibition by sedimentable fractions of locust fat-body. The 10000g supernatant enzyme from locust fat-body was inhibited by sedimented fractions made from crude homogenate and the inhibitory power of the 10000g sediment was not destroyed by heating in 0.25 M-sucrose solution for 15 min. at 100° (Table 1).

The nature of the inhibition by fat-body 10000g sediment, resuspended in 0.05 M-sucrose, was tested with rabbit enzyme prepared as described above and inhibitor equivalent to sediment from 0.5–1.25 locusts' fat-bodies at a range of substrate concentrations. The results at each substrate concentration indicated an irreversible inhibition in which inhibitor from 1.5 locusts was sufficient to completely inhibit the 10000g supernatant enzyme from 0.75 g. of rabbit liver in an incubation mixture containing the standard cofactors (Fig. 1).

Attempts were made to measure the rate of the inhibition by leaving resuspended locust fat-body 10000g sediment in contact with rabbit enzyme at 0° for various periods, centrifuging off the inhibitor sediment and assaying the remaining supernatant. A small decrease in activity was found after leaving fat-body sediment in contact with rabbit enzyme,

Table 1. *Inhibition of locust fat-body enzyme by sedimented fractions*

Supernatant (10000g for 10 min.) equivalent to fat bodies of six locusts was incubated with 1 mg. of *p*-nitrotoluene at 37° in a total volume of 5 ml. in air at pH 7.4. Sediments equivalent to three locusts' fat bodies were used as inhibitors.

Inhibitor added	<i>p</i> -Nitrobenzoic acid formed in 0.5 hr. (μ g.)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
No inhibitor	36	31	12	36
10000g sediment added after incubation	—	—	12	—
1000g sediment	6.4	—	—	0
10000g sediment	1.5	10.4	2.8	0
10000g sediment heated for 15 min. at 100°	—	10.4	4.8	—
Washings of 10000g sediment after heating for 15 min. at 100°	—	—	10.4	—

but most of the inhibitory activity could be centrifuged away (Table 2). The sediment did not appear to interfere with the determination of *p*-nitrobenzoic acid (Tables 1 and 2).

Prolonged homogenization of fat bodies (2min.) gave a progressively less active enzyme, activity

Fig. 1. Inhibition of rabbit enzyme by locust fat-body 10000g sediment. Each incubation mixture contained 10000g supernatant from a homogenate of 0.75g. of rabbit liver with the addition of 10000g sediment from locust fat-body homogenate equivalent to the stated number of locusts in a total volume of 5ml. containing 0.5mg. of NADPH₂, 0.5mg. of NAD, 2mg. of MgCl₂, 3mg. of nicotinamide and 0.5ml. of 0.1M-tris buffer, pH 7.4. Incubations were carried out at 37° in air, with shaking for 0.5hr. Curves A, B and C had 0.25, 0.5 and 1.0mg. respectively of *p*-nitrotoluene added to 5ml. of incubation mixture and oxidation of *p*-nitrotoluene to *p*-nitrobenzoic acid was measured as described in the text.

being zero after 6min., and the possibility of an inhibitor being released from the cells on protracted grinding was therefore considered. A sample of 10000g sediment equivalent to 0.5 locust fat body from a preparation that had been homogenized for 0.5min. was resuspended in sucrose and ground at 0° for various times. This quantity of locust inhibitor decreased the rate of oxidation of a rabbit enzyme preparation from 144 μmoles to 100 μmoles of *p*-nitrobenzoic acid/min., but no increased inhibition was found if the homogenization was prolonged to 6min. and no water-soluble inhibitor was released.

In experiments of this type and in those shown in Table 2 the locust sediment rendered only a portion of the rabbit activity ineffective and could be centrifuged away, but if assays were carried out while the suspended sediment was present, along with the added cofactors, no oxidation occurred.

Attempts to prepare an oxidizing enzyme from locust gut were not successful though *in vitro* locust gut forms more *p*-nitrobenzoic acid from *p*-nitrotoluene than the fat body (Chakraborty & Smith, 1964). Assays were therefore made with rabbit enzyme with the equivalent of homogenized parts of the gut of one locust added. Whole homogenate of mid-gut was markedly inhibitory (Table 3).

Inhibition of vertebrate enzyme by other insects. The 10000g supernatant equivalent to 4g. of flies from homogenates made in either 0.25M-sucrose or 0.15M-potassium chloride had no oxidizing activity with *p*-nitro- or *p*-acetamido-toluene as substrate when fortified with the amounts of NADP, NAD, magnesium chloride and nicotinamide used with rabbit enzyme. Fly microsomes (90000g sediment) from 4g. of flies were also inactive when 90000g supernatant from 0.5g. of rabbit liver was added with the cofactors used in the rabbit assay system.

Table 2. *Inhibition of rabbit microsomal oxidation by insect preparations*

Inhibitors were added to the enzyme at 0° and centrifuged off at 10000g after the stated contact times. Cofactors were then added and assays carried out at 37° for 0.5hr. as described in the text with *p*-nitrotoluene as substrate. Inhibitors were 0.25M-sucrose homogenates of: A, fat body from 0.5g. of cockroach; B, fat body from 1g. of cockroach; C and D, 10000g sediment from fat body of 0.5g. of locust; E, 0.25g. of mustard beetles; F, 0.5g. of mustard beetles; G, 0.5g. of cabbage caterpillar.

	Contact time (min.)	Expt. ...	p-Nitrobenzoic acid formed in 0.5hr. (μg.)						
			A	B	C	D	E	F	G
No inhibitors	—		81	81	79	48	81	81	115
Inhibitor added after incubation	—		88	—	—	46	81	—	—
Inhibitor not removed before incubation	—		42	—	—	0	44	—	109
Inhibitor alone	—		0	0	0	0	0	0	0
Inhibitor centrifuged off before assay	4		81	100	58	28	76	64	—
	14		80	102	51	34	73	64	—
	24		75	100	51	31	70	66	—
	34		—	—	51	—	—	—	—

Whole-fly homogenates were also effective inhibitors of the rabbit oxidizing enzyme (Table 4), and the inhibition by homogenate of 0.05 g. or 0.075 g. of flies was only offset by the addition of very large amounts of NADPH₂ or by the use of a regenerating system for NADPH₂.

Cockroach fat body, mustard beetles and caterpillars (Table 2) contained much less inhibitor than flies but nevertheless their 10000g supernatants were inactive when tested for *p*-nitrotoluene-oxidizing power.

The inhibition by fly preparations of mouse liver oxidation of 2mm-*p*-acetamidotoluene was measured with both whole-fly homogenate and fly 10000g supernatant. The results followed the pattern of the locust inhibition (Fig. 1) with a negative linear relation between amount of inhibitor and reaction velocity, and in three experiments with whole-fly homogenates complete inhibition of the standard mouse liver preparation was reached with 10 flies. When 10000g supernatant from a 0.15M-potassium chloride homogenate of houseflies was used as source of inhibitor in eight

similar experiments, complete inhibition of the mouse enzyme was reached with material from 40–50 flies per incubation. Distribution of inhibitory activity in flies is shown in Table 5.

The stability of NADPH₂ in some diluted fly homogenates was measured (Tables 6 and 7). Loss of NADPH₂ was rapid in homogenates containing fly heads and the loss was stimulated by addition of menadione or catechol. However, when glucose 6-phosphate (1mg./ml. of reaction mixture) was added the extinction at 340m μ was restored to its initial level. In every experiment the rate of recovery of the extinction was higher than its fall had been and it was concluded that this amount of glucose 6-phosphate was adequate to maintain the NADPH₂ in its reduced form even in the presence of polyphenols or quinones.

DISCUSSION

The oxidation by an insect of an insecticide is often of primary importance in deciding its effectiveness since this reaction can lead to either activation

Table 3. *Inhibition of rabbit oxidation system by homogenates of locust organs*

Whole homogenates equivalent to organs of one locust in 0.25M-sucrose were incorporated in the standard rabbit enzyme mixture and the oxidation of *p*-nitrotoluene was measured as described in the text.

Addition to rabbit enzyme	<i>p</i> -Nitrobenzoic acid formed in 0.5 hr. (μ g.)
No addition	123
Fat-body 10000g sediment	83
Mid-gut homogenate	67
Hind-gut homogenate	125
Fore-gut homogenate	105
Gastric-cecae homogenate	147
Malpighian-tube homogenate	127

Table 5. *Inhibition of mouse liver enzyme by housefly homogenates*

Mouse liver enzyme and cofactors were incubated for 0.5 hr. as described in the text with *p*-acetamidotoluene as substrate. The 10000g supernatant of fly organs (equivalent to 20 flies) was incorporated in the standard mouse assay mixture.

Age of flies ...	<i>p</i> -Acetamidobenzoic acid formed (μ g.)	
	2 days	8 days
Additions to mouse enzyme		
No addition	136	136
Fly-head 10000g supernatant	98	103
Fly-thorax 10000g supernatant	68	40
Fly-abdomen 10000g supernatant	94	65

Table 4. *Inhibition of rabbit oxidation enzyme by housefly preparations*

Fly homogenates were incorporated in the standard rabbit assay preparation along with the extra cofactors quoted and the mixtures assayed with *p*-nitrotoluene as described in the text.

Addition to rabbit system	<i>p</i> -Nitrobenzoic acid formed in 0.5 hr. (μ g.)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
No addition	133	115	103	103
Fly homogenate (0.25 g. of fly)	0	—	—	—
Fly 90000g supernatant (0.25 g. of fly)	74	—	—	—
Fly homogenate (0.1 g. of fly)	—	0	—	—
Fly homogenate (0.075 g. of fly)	—	—	71	76
Fly homogenate (0.075 g. of fly) + NADPH ₂ (8mg.)	—	—	83	74
Fly homogenate (0.05 g. of fly)	—	62	—	—
Fly homogenate (0.05 g. of fly) + NADP (0.2 mg.) + glucose 6-phosphate (5mg.)	—	81	—	—
Fly homogenate (0.01 g. of fly)	—	117	—	—

Table 6. *Oxidation of NADPH₂ in housefly homogenates*

Cuvettes contained 10000g supernatant equivalent to 2 flies/ml. in 0.05 M-tris buffer, pH 7.4. Reactions were started by adding 0.5 mg. of NADPH₂/ml. to the test cuvette. Whole homogenate was used in Expts. 2, 5 and 7 and 10000g supernatant in others.

Expt. no.	Age of flies (days after emergence)	Homogenate ...	$10^3 \times \Delta E_{340}^{1\text{cm.}}$ in 10 min.		
			Fly heads	Fly thoraces	Fly abdomens
1	—		123	18	10
2	12		112	36	17
3	2		310	0	0
4	3		113	0	0
5	1		93	7	
6	4		93	0	
7	1		98	0	

Table 7. *Stimulation of NADPH₂ oxidation in fly homogenates*

The 12000g supernatant of whole flies used contained the equivalent of 2 flies/ml. in 0.05 M-tris buffer, pH 7.4.

Addition to cuvette	$10^3 \times \Delta E_{340}^{1\text{cm.}}$ /10 min.
None	26, 25, 27.6
Cytochrome <i>c</i> (50 μM)	95, 93.5, 95
Haemoglobin (50 μM)	22, 20, 25
Menadione (1 mM)	877, 877
Catechol (1 mM)	750, 700, 730

or deactivation of the molecule. In practical insecticides the more active detoxication mechanisms such as glucosidation can usually act only after the oxidation enzymes have introduced a suitable functional group capable of conjugation.

The study of the feeble oxidation systems in insects is difficult, particularly when low doses of toxic compounds are used, and the use of 'microsomal' enzyme preparations rather than whole insects could ease the problem of separating metabolites from irrelevant tissue material. Insect 'microsomal' preparations are usually made from homogenates of whole insects and are therefore more complex than vertebrate liver preparations. The diversity of tissues in the homogenate may introduce a variety of endogenous inhibitors and it is significant that, of nine species used to investigate the oxidation of *p*-nitrotoluene *in vivo*, a satisfactory enzyme extract was only obtained from locusts, where it was possible to work with a fat-body homogenate rather than one made from whole insects (Chakraborty & Smith, 1964). Homogenates of locust gut, which oxidizes *p*-nitrotoluene as actively *in vitro* as the fat body, were inactive and contained significant amounts of material that inhibited other microsomal oxidation systems.

A variety of natural inhibitors of microsomal oxidations have been reported in different liver preparations (Gillette, 1963) including enzymes hydrolysing the cofactors, but the nicotinamide nucleosidases can probably be excluded as possible inhibitors in locust or fly preparations since the addition of nicotinamide has little effect on the oxidizing activity of locust or fly microsomal enzymes (Chakraborty & Smith, 1964; Schonbrod, Philleo & Terriere, 1965). In the NADPH₂ oxidation experiments described above it was possible to restore the extinction at 340m μ to its initial value by adding glucose 6-phosphate 1 hr. after it had fallen to zero. This also suggests that the cofactor had not been attacked by any nucleosidase.

At least two inhibitory mechanisms appear to be acting in insect 10000g sediment. A heat-stable factor irreversibly inactivated a proportion of rabbit enzyme activity, and a second insoluble factor that further decreased the activity if left in the assay mixture could be removed by centrifugation (Tables 1 and 2). It is possible that the second mechanism removed some cofactor from the assay system.

The removal of NADPH₂ by oxidation is a factor in this facet of the fly inhibition and this oxidation was especially active in fly heads. The NADPH₂ oxidase was stimulated by cytochrome *c* and menadione and the presence of quinone-linked NADPH₂ oxidation is therefore a possible cause of loss of activity in insect preparations that may contain polyphenols and their oxidases. However, fly homogenates have adequate glucose 6-phosphate-dehydrogenase activities and the incorporation of glucose 6-phosphate into the enzyme mixture maintained the NADPH₂ in the reduced form even when high menadione-linked oxidation was present.

Fly head and thorax contained substantial amounts of inhibitor (Table 5) and the fat body,

which is a probable source of *p*-nitrotoluene-oxidizing enzyme in flies, was mainly in the abdomen. Homogenates prepared from fly abdomens had therefore a more favourable ratio of enzyme to inhibitor. For this reason in subsequent work with flies the oxidation enzyme has been prepared from abdomen homogenates and an NADPH₂-regenerating system has been used in preference to the addition of NADPH₂ as cofactor (Chakraborty & Smith, 1967).

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