Enzymic Oxidation of some Alkylbenzenes in Insects and Vertebrates

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(Received 8 August 1966)

1. Oxidation rates of alkylbenzenes have been measured in 10000g supernatants of vertebrate livers, locust fat bodies and housefly abdomens. 2. Activity per g. of insect was greater in fly than locust preparations but both were of the same order as a range of vertebrate species. 3. Methyl groups of toluene and *p*-nitrotoluene were oxidized more rapidly than the side chains of higher homologues. 4. In the higher homologues hydroxylation occurred most readily at the α -methylene group and less readily at penultimate methylene and terminal methyl groups. 5. Oxidations in both vertebrates and insects were inhibited by piperonylbutoxide and similar synergists. 6. Oxidation activity was stimulated by pretreatment of rats, but not locusts, with phenobarbitone or 3,4-benzopyrene.

Selectivity of action is a desirable property of insecticides and the selective destruction of the insecticide by detoxication mechanisms in different species is one way of achieving this effect. The best established examples of this are found among those organophosphates that contain carboxy ester or amide groups (Kreuger & O'Brien, 1959; Uchida, Dauterman & O'Brien, 1964; Smith, 1964) where low mammalian toxicity results from the more extensive hydrolysis of ester or amide links in these species. The term 'selectophore' has been applied to those organic functional groups that offer a point of selective attack for a detoxication mechanism and a knowledge of such groups would be of value in the design of insecticides. So far, little attempt has been made to find selectophoric groups that depend for their action on metabolic processes other than hydrolysis.

Some vertebrates can oxidize the alkyl chain in alkylbenzenes with great ease (El Masry, Smith & Williams, 1956) and it was shown that a range of insects varied considerably in their ability to oxidize the methyl group in nitrotoluene (Chakraborty & Smith, 1964). This suggested to us that methyl and other alkyl groups might function as selectophores, and since little is known of their fate in insects we have studied the metabolism of a series of alkylbenzenes in insects and enzyme preparations and have compared these results with those from similar experiments with vertebrate material.

EXPERIMENTAL

Reference compounds. Alkylbenzenes were commercial samples that contained variable amounts of alcohols and

ketones. They were distilled and left over powdered sodium for a week and then redistilled through an efficient fractionating column with a variable reflux head. Before use in an incubation mixture they were checked by gas-liquid chromatography for absence of alcohols or ketones.

Aromatic alcohols and acids were redistilled commercial samples used previously or were prepared by methods then described (El Masry *et al.* 1956; Smith, Smithies & Williams, 1954; Robinson, Smith & Williams, 1955; Robinson & Williams, 1955) and were homogeneous by gas-liquid chromatography.

Piperonylbutoxide (3,4-methylenedioxy-6-propylbenzyl-*n*-butyl diethylene glycol ether), sulphoxide [1-methyl-2-(3,4-methylenedioxyphenyl)ethyloctyl sulphoxide], *n*-propylisome (di-*n*-propyl 6,7-methylenedioxy-3-methyl-1,2,3,4-tetrahydronaphthalene-1,2-dicarboxylate), sesamex [2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxundecane] and D.M.C. (4,4'-dichloro- α -methylbenzhydrol) were a gift from Dr B. C. Fine; SKF 525A (diethylaminoethyldiphenyl-*n*-propyl acetate hydrochloride) was a sample provided by Professor R. T. Williams.

Insects. Locusts (*Schistocerca gregaria*) were obtained as fifth-instar hoppers from the Anti-Locust Research Centre and enzymes were prepared from mid-instar specimens. Resistant strains of houseflies were obtained from the London School of Hygiene and Tropical Medicine and a non-resistant strain from the Rothamsted Experimental Station. Houseflies were obtained as pupae and after emergence the adults were fed on sucrose and water for periods of up to a week. They were normally used for enzyme preparation the day after emergence.

Vertebrate species. These were obtained from the same sources as those described by Creaven, Parke & Williams (1965).

Preparation of enzymes. Fat bodies from fifth-instar locusts were homogenized and centrifuged to give a 10000g supernatant as previously described (Chakraborty & Smith, 1964). When *p*-nitrotoluene or toluene was substrate, enzyme from 5-10 locusts was used in a total

volume of 5 ml. of incubation mixture. For higher alkylbenzenes, fat-body enzyme from 30–50 locusts was necessary and a total incubation solution of 20–25 ml. was used.

Abdomens of adult houseflies were similarly homogenized in a glass Potter–Elvehjem homogenizer with Teflon pestle and centrifuged at 0–3° for 10 min. at 10000g. When *p*-nitrotoluene was used as substrate, the supernatant fraction from 2 g. of flies in 5 ml. of incubation mixture was used, and when higher alkylbenzenes were substrates, abdomens from 5–15 g. of flies were used in an incubation volume of 20–25 ml.

In fly enzyme experiments each 5 ml. of incubation mixture contained 0.5 mg. of NADP, 0.5 mg. of NAD, 2 mg. of glucose 6-phosphate, 0.5 mg. of $MgCl_2$, 1 mg. of nicotinamide and 0.5 ml. of 0.1 M-tris buffer, pH 7.4.

Vertebrate livers were homogenized in 5 vol. of 0.25 M-sucrose at 0° and centrifuged for 10 min. at 10000g. Incubations contained the supernatant fraction equivalent to 1 g. of liver in a total volume of 5 ml. containing 0.5 mg. of NADP, 0.5 mg. of NAD, 2 mg. of glucose 6-phosphate, 2 mg. of $MgCl_2$, 3 mg. of nicotinamide and 0.5 ml. of 0.1 M-tris buffer, pH 7.4.

Substrates, 1 mg. in 0.05 ml. of acetone, were added to test and enzyme-free control mixtures to start the reaction. Controls were also prepared from separately incubated enzymes that were mixed with the other components immediately before assay. Incubations of 5 ml. samples were for 1 hr. in 50 ml. beakers in a shaking water bath at 37° in air. Other incubations requiring 25–30 ml. volumes were in 250 ml. beakers. The oxidation rate, per g. of liver or per insect, of the standard substrate, *p*-nitrotoluene, was the same whether measured in a 5 ml. or 25 ml. incubation mixture, and oxidation rates were linear with time for at least 60 min. but routine assays were normally carried out for 30 min.

Extraction of metabolites of alkylbenzenes from incubation mixtures. Incubation mixtures, 5–25 ml., were acidified with 1.5 ml. of 10 N- H_2SO_4 to each 5 ml. and extracted with 3 × 7 ml. of ether. After drying over Na_2SO_4 the ether was evaporated at 0° in a stream of N_2 . The residue was dissolved in 100 μ l. of *n*-hexane and 2–5 μ l. portions were used for gas-liquid chromatography (see below).

In the determination of acidic metabolites the incubation mixture was extracted with ether as above and acidic compounds were transferred into 5 ml. of 0.5 N-NaOH, which was acidified with 2 N-HCl and extracted with 2 × 5 ml. of ether. The ether was dried and evaporated as above and treated with a freshly distilled ethereal solution of diazomethane, to give a permanent yellow colour (approx. 1 ml.). After 1 hr. this solution was evaporated in a stream of N_2 at 0° and the residue dissolved in 100 μ l. of *n*-hexane for gas-liquid chromatography.

Recovery experiments in which 5–50 μ g. of the different alcohols or acids studied were added to 15–25 ml. volumes of 10000g supernatants from liver, locust fat body or houseflies, showed a constant loss of 5–7 μ g. from each amount of alcohol or methyl ester added. This loss occurred during the evaporation of the ether extract and was more significant at higher temperatures. The volume of ether were therefore standardized as above and evaporation was carried out at 0°. Under these conditions linear correlation curves relating alcohol or acid added and amounts recovered were obtained. By using these calibrations and standardized extraction conditions the recovery of known quantities of alcohols or

Table 1. *Gas-liquid chromatography of metabolites of alkylbenzenes*

Retention times are given in minutes on A: 3% (w/w) polyethylene glycol adipate on Celite, 6 ft., 100°; B: 3% (w/w) Carbowax 4000 on Celite, 6 ft., 125°; C: 2% (w/w) Carbowax 4000 on Celite, 6 ft., 120°; D: 1.5% (w/w) Carbowax 4000 on Celite, 6 ft., 90°. The flow rate of N_2 was 100 ml./min.

	A	B	C	D
Benzyl alcohol	—	—	4.5	—
Methyl benzoate	2.0	—	1.5	—
Acetophenone	—	—	2.0	—
1-Phenylethanol	2.5	3.0	3.5	—
2-Phenylethanol	5.0	4.5	6.0	—
Methyl phenylacetate	3.5	—	3.0	—
<i>p</i> -Ethylphenol	—	—	20.0	—
1-Phenylpropan-1-ol	4.5	5.0	—	5.5
1-Phenylpropan-2-ol	2.0	3.8	—	5.5
1-Phenylpropan-3-ol	9.0	9.5	—	11.0
2-Phenylpropan-1-ol	5.0	5.5	—	7.5
2-Phenylpropan-2-ol	2.5	2.3	—	3.0
Methyl phenylpropionate	6.0	—	—	—
Methyl 2-phenylpropionate	3.0	—	—	—
1-Phenylbutan-1-ol	6.5	6.8	—	10.5
1-Phenylbutan-2-ol	5.0	5.0	—	8.5
1-Phenylbutan-3-ol	7.5	7.8	—	10.5
1-Phenylbutan-4-ol	13.5	11.5	—	—
Methyl phenylbutyrate	9.5	—	—	—
2,2-Dimethylphenylethanol	—	7.8	—	—

acids from incubation mixtures were $100 \text{ s.e.m.} \pm 1.5$ (30) over the range 10–50 μ g. Quantities of methyl esters or alcohols smaller than 5 μ g. were not detectable.

Gas-liquid chromatography. Copper tubes, 6 ft. in length and $\frac{1}{8}$ in. internal diam., packed with stationary phases on 100-mesh Celite were used. Nitrogen was used as carrier with flow rate of 100 ml./min. and compounds were detected by flame ionization. Stationary phases and temperatures of operation are shown along with retention times in Table 1. With the apparatus used, the minimum detectable quantity of an alcohol or ester was 0.01 μ g. and normally 2 μ l. volumes of *n*-hexane solutions were used in injections.

Quantitative measurements were made by comparison with the peak height from an injection of a standard solution of the compound being measured, and the values were corrected for losses on extraction by reference to the appropriate correlation curve.

Identification of metabolites was made on the columns (Table 1) by comparison of the retention times of metabolites and reference compounds and by demonstration of identity of retention times in mixed chromatograms. Phenols had high retention times on the columns used and no peaks were found in the experimental extracts in this region.

Determination of p-nitrobenzoic acid. The method previously described was used (Chakraborty & Smith, 1964). In experiments where *p*-nitrobenzyl alcohol was the substrate, ether was used instead of CCl_4 to extract excess of substrate and the diazo colours were measured within 10 min. of coupling. *p*-Nitrobenzyl alcohol gave the same intensity of diazo colour in the procedure for assaying *p*-nitrobenzoic acid but the speed of azo coupling was

considerably slower, the intensity reaching its maximum only after several hours.

RESULTS

With normal animals. Incubation mixtures were set up as described above with vertebrate livers, housefly abdomens or locust fat bodies as sources of enzymes and alkylbenzenes as substrates. In all insect experiments, where the enzyme was derived from large numbers of insects (30–50 locusts or up to 1000 flies), the preparations were assayed with *p*-nitrotoluene as substrate to confirm the activity of the enzyme.

The oxidation of *p*-nitrobenzyl alcohol by fly enzyme was measured under the conditions used for the oxidation of *p*-nitrotoluene and over the concentration range 0.2–1 mM. The double-reciprocal plot of these results was linear and gave a K_m value 2.8×10^{-3} M. The rate of oxidation of *p*-nitrobenzyl alcohol at low concentrations ($3 \mu\text{g./g.}$ of flies/hr. at 0.2 mM) was considerably lower than the rate of formation of *p*-nitrobenzoic acid from *p*-nitrotoluene in the routine assay.

The effect on the oxidation of *p*-nitrotoluene of some compounds used as insecticide synergists or drug extenders was investigated and inhibition was found with both types of compound (Table 2).

Oxidation rates of alkylbenzenes other than toluene were lower than that of *p*-nitrotoluene and large numbers of insects were needed to obtain sufficient activity to permit detection of the metabolites. Toluene and *p*-nitrotoluene were converted into benzoic acid or *p*-nitrobenzoic acid by enzymes from all sources and no alcoholic metabolites were detected, whereas with the other alkylbenzenes from ethylbenzene onwards alcohols were the major metabolites.

After extraction with ether to remove metabolites, as described, above the incubation mixtures were

boiled with N-sulphuric acid for 10 min. to hydrolyse any conjugates of the alcohols. No further metabolites were extracted from these solutions and it was concluded that the low activity was not due to loss of alcoholic metabolites as glucosides or glucosiduronates. No phenolic metabolites or ketones were found in the extracts. In rabbit liver enzyme the optimum pH for the oxidation of isopropylbenzene to dimethylphenylcarbinol was at pH 7.4 and the cofactor requirements were as described above, both NAD and NADP being required for optimum yield in the presence of the NADPH₂-generating system.

Results of quantitative experiments are summarized in Tables 2, 3 and 4.

In some experiments with locusts and flies the enzyme was tested with lower substrate concentrations of *n*-propylbenzene and isopropylbenzene. The concentration used as a routine, 0.2 mg./ml. or about 2 mM, appeared to be optimum but lower concentrations considerably decreased the rates of oxidation.

With pretreated animals. Male rats weighing 50 g. were injected intraperitoneally with aqueous sodium phenobarbitone (35 mg./kg.) twice daily for 4 days. Control animals were similarly dosed with water. A second group were given a single dose of 3,4-benzopyrene (25 mg./kg.) in arachis oil with corresponding controls. On the day after the final dose, liver-homogenate 10000 g supernatant enzymes were prepared and the oxidizing enzyme was assayed as described above with *p*-nitrotoluene.

Female locust hoppers were given the same dose rate of phenobarbitone and benzopyrene for 3 days and 1 day respectively before preparations of fat-body enzyme. Fat-body 10000 g-supernatant was prepared from these and from the appropriate controls which had received solvent injections only.

The LD₅₀ of *m*-isopropylphenyl *N*-methylcarbamate was also determined on normal locusts and

Table 2. Inhibition of *p*-nitrotoluene oxidation by insecticide synergists

The 10000 g supernatant enzyme from rabbit or rat liver or from fly abdomen homogenates was incubated with *p*-nitrotoluene under conditions described in the text and *p*-nitrobenzoic acid formed in 0.5 hr. compared in normal preparations with those containing inhibitors.

Inhibitor concn. ... Inhibitor	Percentage inhibition of enzyme					
	From rabbits		From rats	From flies		
	0.5 mM	0.1 mM	0.5 mM	0.1 mM	0.01 mM	
Piperonylbutoxide	27	6	22	100	51	
Sulphoxide	44	18	33	100	48	
<i>n</i> -Propylisome	22	7	19	100	39	
Sesamex	44	20	27	—	—	
D.M.C.	8	—	3	—	—	
SKF 525A	32	11	38	—	—	

Table 3. *Oxidation of alkylbenzenes by rabbit, fly and locust enzyme*

Enzymes from rabbit liver, fly abdomen and locust fat body were prepared and incubations carried out as described in the text.

Substrate	Products	Rate of formation of products (μ moles/min./g. of whole)						
		Rabbits				Locusts	Flies	
		1	2	3	4			
Toluene	Benzoic acid	—	—	—	—	0.40, 0.30, 1.10, 0.90	—	
p-Nitrotoluene	p-Nitrobenzoic acid	—	0.80	0.30	—	0.50	2.40, 1.70, 4.00, 1.50	
Ethylbenzene	1-Phenylethanol	—	—	—	0.26	—	—	
	2-Phenylethanol	—	—	—	<0.01	—	—	
	Phenylacetic acid	—	—	—	0.03	—	—	
n-Propylbenzene	1-Phenylpropan-1-ol	0.20	0.30	0.26	—	0.06	0.12, 0.22	
	1-Phenylpropan-2-ol	0.10	0.11	0.06	—	<0.01	<0.01	
	1-Phenylpropan-3-ol	<0.01	<0.01	<0.01	—	<0.01	<0.01	
	3-Phenylpropionic acid	0.04	0.05	0.06	—	<0.01	<0.01	
Isopropylbenzene	2-Phenylpropan-1-ol	0.04	0.04	0.07	—	<0.01	<0.02	
	2-Phenylpropan-2-ol	0.17	0.25	0.22	0.35	0.05	0.27	
	2-Phenylpropionic acid	0.04	0.03	0.05	—	<0.01	<0.02	
n-Butylbenzene	1-Phenylbutan-1-ol	0.09	0.15	0.11	—	—	—	
	1-Phenylbutan-2-ol	<0.01	<0.01	<0.01	—	—	—	
	1-Phenylbutan-3-ol	0.04	0.09	0.07	—	—	—	
	1-Phenylbutan-4-ol	<0.01	<0.01	<0.01	—	—	—	
	4-Phenylbutyric acid	0.04	0.04	0.04	—	—	—	
tert.-Butylbenzene	2,2-Dimethylphenylethanol	0.06	0.10	0.09	—	—	—	
	2,2-Dimethylphenylacetic acid	<0.01	<0.01	<0.01	—	—	—	

those which had been given phenobarbitone for 3 days.

The oxidizing power of rat liver enzyme was roughly doubled by pretreatment but no stimulation of locust enzyme or change in the sensitivity of locusts to *m*-isopropyl *N*-methylcarbamate was found (Table 5).

DISCUSSION

The pattern of oxidation of the alkylbenzenes is qualitatively similar in rabbit and the two insect species used and confirms earlier studies on the rabbit where the metabolites were isolated from urine (Robinson & Williams, 1955; Robinson *et al.* 1955; El Masry *et al.* 1956). The major oxidation product of the alkylbenzene, where this was possible, was the secondary alcohol formed by hydroxylation at the α -methylene group of the side chain. Preferential oxidation of the methylene group nearest to the benzene ring was observed in *p*-nitroethylbenzene (Chakraborty & Smith, 1964). The oxidation of the penultimate methylene group of straight chains, e.g. in *n*-butylbenzene, was somewhat slower and occurred to about the same extent as the oxidation of terminal methyl groups to carboxyl. Where no α - or penultimate methylene

Table 4. *Oxidation of p-nitrotoluene and isopropylbenzene in flies and vertebrates*

Liver enzyme or fly homogenates were prepared and incubated as described in the text and assays performed for *p*-nitrobenzoic acid or 2-phenylpropan-2-ol.

	Rate of oxidation (μ g./g. of liver or whole fly/hr.)	
	<i>p</i> -Nitrotoluene	<i>m</i> -Isopropylbenzene
Rabbit	200	56, 60, 70
Coypu	180	85, 94
Hamster	160	75
Guinea pig	120	41, 60
Cat	90	61, 52
Rat	50	32
Flies*		
Normal	14	3
FC	20	5
SKA	8	4
P	19	4
A	12	4
R	12	4

* Insecticide resistance of strains was: FC, 5 $\times$ to carbamates; SKA, 220 $\times$ to diazinon, 1100 $\times$ to DDT; A, 44 $\times$ to parathion, 14 $\times$ to malathion; P, pyrethrin-resistant.

Table 5. *Effect on oxidation enzyme of pretreatment of rats and locusts with phenobarbitone and 3,4-benzopyrene*

Rat liver or locust fat-body enzymes were prepared and incubated at 37° in air with the addition of cofactors as described in the text. Animals were dosed with phenobarbitone for 4 days or with benzopyrene for 1 day before preparation of the enzymes or measurement of toxicity of *m*-isopropyl *N*-methylcarbamate (IPMC).

Pretreatment	Rate of oxidation (mμmoles/min./g. liver or locust)			LD ₅₀ of IPMC (μg./locust)
	Isopropylbenzene	<i>p</i> -Nitrotoluene		
	By rat liver	By rat liver	By locusts	
Water control	4.4	6.5, 7.0	0.6	12
Arachis oil control	4.4	5.9, 7.8	—	—
Phenobarbitone	8.6	15.0, 15.0, 12.0	0.4	10
Benzopyrene	6.7	11.0, 14.0, 9.0	0.3	—

group was available the oxidation of the molecule was much slower and *tert*.-butylbenzene was oxidized nearly ten times slower than toluene or *p*-nitrotoluene by the same enzyme preparation.

The terminal oxidation of alkyl groups to primary alcohols and thence to carboxylic acids has been thought to involve the soluble alcohol dehydrogenase (Gillette, 1959) in vertebrate liver but this seems unlikely to have occurred in the insect enzymes. The very low concentrations of *p*-nitrobenzyl alcohol that would have been produced in the oxidation of *p*-nitrotoluene by fly enzyme would have been oxidized too slowly to account for the measured yields of *p*-nitrobenzoic acid. Moreover, at these low rates of oxidation some unchanged alcohol would have been expected to occur in the final assays, whereas no product with the slow colour development characteristic of *p*-nitrobenzyl alcohol was ever detected. It seems more likely therefore that the oxidation of a methyl group via the alcohol to a carboxyl group occurs in or on the microsomal particles in the 10000g supernatant without the intermediate stages appearing free in solution. In this way the soluble alcohol dehydrogenase would not be involved in the process.

The values quoted for microsomal oxidations of rabbit liver may be taken as a fair guide to the behaviour of these compounds in the intact organism since the liver is virtually the only organ carrying out this metabolic reaction. The values quoted for the insects, on the other hand, are probably minimal since the oxidation of *p*-nitrotoluene occurs in other organs of the locust and the fat body accounts for only about half of the oxidizing capacity of the insect (Chakraborty & Smith, 1964). Similar considerations probably apply to the fly abdomen preparation used in the present work, and the results quoted in Table 3 suggest that there is no great difference between the oxidation rates of alkylbenzenes in whole vertebrates and whole insects if the results are expressed in terms of

activity/g. of animal. On the other hand, the difference in oxidizing activity between individual insect or vertebrate species may be considerable and it may be possible to take advantage of this in modifying a toxic molecule to increase its selective action by increasing its susceptibility to oxidation.

The induction of increased amounts of microsomal oxidation enzymes in vertebrates by a variety of compounds is now well-known (Remmer, 1962; Conney & Burns, 1962) and evidence of a similar induction in insects has been reported (Morello, 1964). Pretreatment of rats with phenobarbitone or benzopyrene roughly doubled the rate of oxidation of *p*-nitrotoluene by the 10000g supernatant enzyme but in the present work neither compound stimulated the oxidation by locusts and pretreatment with phenobarbitone had no effect on the toxicity of an insecticidal carbamate towards locusts.

Species differences in rates of oxidation may, however, affect the toxicity of some carbamates since the LD₅₀ of *m*-isopropylphenyl *N*-methylcarbamate in the locusts used in this work was about 10 μg./g. whereas in flies the LD₅₀ is 90 μg./g. (Metcalf & Fukuto, 1965). Oxidation of the carbamates is generally believed to result in their detoxication (Metcalf & Fukuto, 1965) and it is significant that the enzyme preparations from several strains of fly studied in the present work were about three times more active in oxidizing isopropylbenzene than those from locusts (Tables 3 and 4).

Oxidation of the alkyl groups in alkylphenyl *N*-methylcarbamates is a feature of their metabolism along with the oxidation of the *N*-methyl group (Hook & Smith, 1967). The introduction of a hydroxyl group in the alkyl side chain would enable conjugation and elimination to take place and would be expected to result in a non-toxic product. If such a process accounted for a significant amount of the metabolic degradation of a

carbamate, the present results obtained suggest that a methyl group in a carbamate molecule might give more rapid detoxication than, e.g., a *tert.*-butyl group.

This work has been supported by the Agricultural Research Council. We are also grateful for supplies of insects from Professor J. R. Busvine, from the Anti-Locust Centre, and from the Insecticide Department, Rothamsted Experimental Station.

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