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The Metabolism of Hexachlorocyclohexanes and Pentachlorocyclohexenes in Flies and Grass Grubs

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1. γ-Hexachlorocyclohexane, γ-pentachlorocyclohexene and δ-pentachlorocyclohexene were converted by houseflies and grass grubs into metabolites that had chromatographic properties identical with those of *S*-2,4-dichlorophenylglutathione. 2. The metabolism of γ-hexachlorocyclohexane and the pentachlorocyclohexene isomers was negligible in newly emerged blowflies, but increased over the next 10 days. 3. The metabolism of both γ-hexachlorocyclohexane and the pentachlorocyclohexene isomers was inhibited by simultaneous dosage with tetrabromophenolphthalein ethyl ester or Bromophenol Blue in both grass grubs and flies, but only the metabolism of pentachlorocyclohexenes in blowflies was stopped by simultaneous dosage with bis-(*N*-dimethylaminophenyl)methane. *NN*-Di-*n*-butyl-*p*-chlorobenzenesulphonamide had no effect on the metabolism of pentachlorocyclohexenes by blowflies. 4. The use of these inhibitors and colorimetric assays leads to the conclusion that a pentachlorocyclohexene is not a major intermediary metabolite of γ-hexachlorocyclohexane in these insects.

The insecticide γ-HCH* (gammexane) is metabolized more rapidly in some insecticide-resistant insects than in the corresponding susceptible strains (Oppenoorth, 1955; Busvine & Townsend, 1963; Sternburg & Kearns, 1956). In principle, inhibitors of the enzyme that catalyses the initial detoxication reaction could be used as synergists for the insecticide in the resistant strains, but this approach has been hampered by a lack of information about the metabolism of γ-HCH in insects, especially in its early stages. GSH is required by the enzymes involved, and Bradbury & Standen (1959) also showed that in flies γ-HCH forms a complex mixture of metabolites that on hydrolysis give rise to a mixture of dichlorothiophenols. These are probably derived from isomeric *S*-dichlorophenylglutathiones, which were detected by Clark, Hitchcock & Smith (1966) as major metabolites of γ-HCH in flies, ticks and locusts. The identification of this type of conjugated metabolite did not, however, show whether the primary detoxication reaction was conjugation with glutathione or dehydrochlorination of γ-HCH to a PCH isomer.

Some evidence for the formation of PCH was provided by Sternburg & Kearns (1956), who showed that resistant flies rapidly formed large amounts of material from γ-HCH that gave the colour reactions of PCH in the Schechter & Hornstein (1952) assay procedure. Radioactive-tracer studies (Bradbury & Standen, 1958; Bridges, 1959) showed that the chromogen was not γ-PCH itself and that several water-soluble or ether-soluble metabolites were present that could give the colour reaction. A number of GSH-dependent enzymes are known for which PCH is an excellent substrate (Grover & Sims, 1965; Ishida & Dahm, 1965), and it is possible that if PCH were first formed these enzymes could produce GSH derivatives as secondary metabolites that might give the Schechter & Hornstein (1952) colour reaction. These could eventually, by a series of dehydrochlorinations, be metabolized to the aromatic metabolite detected by Clark *et al.* (1966).

An alternative suggestion is that the primary detoxication reaction of γ-HCH yields a *S*-pentachlorocyclohexylglutathione, which might also be expected to give the PCH colour reaction measured by Sternburg & Kearns (1956). Subsequent dehydrochlorinations of this compound could also produce the aromatic GSH conjugate detected by Clark *et al.* (1966).

We have examined the metabolism in some insects of γ-HCH and δ-HCH with these two possibilities in mind, and it is shown below that PCH isomers are probably not major intermediary products in the metabolic degradation of γ-HCH.

* Abbreviations: HCH, hexachlorocyclohexane; PCH, pentachlorocyclohexene; DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane.

MATERIALS AND METHODS

Reference compounds and substrates. γ-HCH, m.p. 112° (British Drug Houses Ltd., Poole, Dorset), and δ-HCH, m.p. 130° (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) were commercial samples. [^{14}C]γ-HCH was obtained from The Radiochemical Centre, Amersham, Bucks., and its chemical purity was checked by dilution analysis.

γ-PCH, m.p. 35°, and δ-PCH, m.p. 68°, were prepared by treatment of the corresponding HCH isomers with alkali (Hughes, Ingold & Pasternak, 1953).

Phenol Red, Bromophenol Blue, tetrabromophenolphthalein ethyl ester, phenoltetrabromophthaleindisulphonate and bis-(*NN*-dimethylaminophenyl)methane were commercial samples (British Drug Houses Ltd.) and were used without further purification. WARF anti-resistant (*NN*-di-*n*-butyl-*p*-chlorobenzenesulphonamide), m.p. 37°, was a sample provided by the D.S.I.R. Entomology Division, Nelson, New Zealand.

The six isomeric *S*-dichlorophenyl-L-cysteines were prepared by the method of Parke (1959) and were conveniently purified by crystallization of their hydrochlorides from 5M-HCl. 2,3-Dichlorophenylcysteine hydrochloride had m.p. 187° (Found: Cl$^-$, 11·9%), 2,4-dichlorophenylcysteine hydrochloride had m.p. 184° (Found: Cl$^-$, 11·6%), 2,5-dichlorophenylcysteine hydrochloride had m.p. 180° (Found: Cl$^-$, 11·9%), 2,6-dichlorophenylcysteine hydrochloride had m.p. 170° (Found: Cl$^-$, 11·8%), 3,4-dichlorophenylcysteine hydrochloride had m.p. 185° (Found: Cl$^-$, 11·8%), 3,5-dichlorophenylcysteine hydrochloride had m.p. 185° (Found: Cl$^-$, 11·5%). $C_9H_9Cl_2NO_2$,HCl requires Cl$^-$, 11·6%.

S-2,4-Dichlorophenylglutathione, m.p. 215° (decomp.), was prepared as described by Clark *et al.* (1966). *S-2,5-Dichlorophenylglutathione* was prepared similarly and crystallized from aqueous ethanol, after isoelectric precipitation, with m.p. 215–217° (decomp.) (Found: C, 40·7; H, 4·3; N, 9·1; $C_{16}H_{19}Cl_2N_3O_6S,H_2O$ requires C, 40·9; H, 4·5; N, 8·9%).

Chromatography and electrophoresis. Paper chromatograms were run as described by Smith (1958). No solvent systems were found that were capable of separating the isomeric glutathione or cysteine conjugates, and the four solvents of Table 1 were used only to separate GSH conjugates from cysteine derivatives. T.l.c. on silica gel G (E. Merck A.-G., Darmstadt, Germany) was carried out as described by Hook & Smith (1967), and HCH and PCH were separated in cyclohexane–chloroform (4:1, v/v), in which γ-HCH and γ-PCH had R_F 0·5 and 0·7 respectively. Paper electrophoresis on Whatman no. 31 paper was carried out at pH 1·85 (200g. of acetic acid and 20g. of formic acid/l. of water) in a Shandon high-voltage apparatus at 200v/cm. for 1hr. (Atfield & Morris, 1961). Only the 2,6-dichlorophenylcysteine ($2·3 \times 10^{-3}$cm./v/hr.) could be separated from the other isomers, which all had mobilities of $1·9 \times 10^{-3}$cm./v/hr.

Gas–liquid chromatography. G.l.c. was carried out in a Perkin–Elmer 811 or 801 gas chromatograph with electron-capture detection and a 0·4% silicone oil (Embaphase; May and Baker Ltd., Dagenham, Essex) on a Celite column. For HCH isomers the oven temperature was 140°, and for PCH isomers 90°. Quantitative measurements were made by comparison of peak heights with those of similar standards, and flow rates of N_2 were adjusted so that retention times of all compounds measured were about 1min.

Insects and dosing. Houseflies were a DDT-resistant strain (LD_{50} 1·7μg. of DDT/female fly) (SP2AB, obtained from Dr D. Spiller, D.S.I.R., Auckland, New Zealand). These and blowflies (*Lucilia sericata*) were reared on a yeast–milk–agar medium and fed as adults on dried milk and sucrose. Houseflies were used 3 days, and blowflies 8 days, after emergence. Third-instar grass grubs were collected in the field by the D.S.I.R. Entomology Division, Nelson, New Zealand, from populations not exhibiting insecticide resistance and were stored at 3° in moist earth until used.

Chlorinated compounds were administered by injection, in 0·5μl. of ethanol or ethanol–water (1:1, v/v), or topically in 1μl. of acetone. The indicators and inhibitors were administered in 1μl. of water by injection or, if water-insoluble, topically in 1μl. of acetone.

Preparation of homogenates. Insects were ground in a Potter–Elvehjem homogenizer at 0° in phosphate buffer, pH 7·4 (0·1M-Na_2HPO_4–0·1M-KH_2PO_4), and the homogenate was centrifuged at 10000*g* for 30min. Supernatants were used for enzymic work and usually contained the equivalent of two grass grubs/ml. or ten flies/ml.

Extraction of insects and enzymes. For measurement of external HCH or PCH, insects were washed with two 1ml. portions of toluene or hexane, which was then diluted to a volume suitable for g.l.c. assay or transferred to vials for scintillation counting. After being washed, separate insects were ground with 1ml. of acetone in a glass Potter–Elvehjem homogenizer; 1ml. of toluene (sulphur-free; May & Baker Ltd.) was added and then 25ml. of water. After centrifugation suitable volumes of solvent were used for

Table 1. *Paper chromatography of some possible HCH metabolites*

Solvents were run on Whatman no. 4 paper until the front had moved about 15in. The solvents used were: A, pyridine–water–butan-1-ol (1:1:1, by vol.); B, butan-1-ol–acetic acid–water (4:1:5, by vol.); C, butan-1-ol–benzene–aq. 2M-ammonia (1:1:2, by vol.); D, *o*-cresol saturated with water and with 2ml. of aq. ammonia (sp.gr. 0·88)/100ml.

	R_F values			
Solvent	A	B	C	D
S-2,4-Dichlorophenylglutathione	0·7	0·7	0	0·2
S-2,4-Dichlorophenylcysteine	0·8	0·9	0·5	0·7
S-2,5-Dichlorophenylglutathione	0·7	0·7	0	0·2
S-2,5-Dichlorophenylcysteine	0·8	0·9	0·5	0·7

g.l.c. or radioactivity analysis. Small volumes of enzymic reaction mixtures were directly extracted with toluene. Recovery of isomers of HCH or PCH immediately after administration by topical application or injection was quantitative.

In those experiments where a Schechter & Hornstein (1952) assay of the aqueous layer was required, spectroscopic-grade *n*-hexane was used as solvent for extraction.

Colorimetric assay of HCH and PCH isomers. This was carried out in the apparatus and under the conditions described by Schechter & Hornstein (1952) except that Zn dust and acetic acid–water (9:1, v/v) was used in the reduction stage. The dichlorophenylcysteines and dichlorophenylglutathiones listed above gave an insignificant colour in this reaction, corresponding to less than 5% of the colour due to an equivalent amount of γ-PCH or γ-HCH.

Measurement of radioactivity. A Packard 4312 scintillation spectrometer was used, as described by Binning, Darby, Heenan & Smith (1967).

RESULTS

Metabolism of [^{14}C]γ-*HCH*

In whole insects. Ten houseflies were dosed topically with 0·01 μg. of [^{14}C]γ-HCH in acetone and kept at 25° overnight. They were ground in a Potter–Elvehjem homogenizer with 10 ml. of water and 10 ml. of toluene, and the extent of metabolism was measured by counting the radioactivity remaining in the organic phase after centrifugation. The aqueous phase was deproteinized by a twice-repeated precipitation with ethanol followed by concentration *in vacuo* to a small volume, and the residue was applied to Whatman no. 31 paper as a line (30 cm. long × 1 cm. wide). The chromatogram was developed in solvent A (Table 1), and radioactivity was found only at R_F 0·5. The zone with R_F 0·4–0·8 was cut out, eluted with water and rechromatographed in the four solvents shown in Table 1 with dichlorophenylcysteines and 2,4-dichlorophenylglutathione added as references. The total water-soluble radioactivity corresponded to 35% of the dose and only radioactivity co-chromatographing with 2,4-dichlorophenylglutathione was detected on the chromatograms.

Similar experiments were done with three grass grubs, each dosed with 0·15 μg. of γ-HCH, which were kept for 18 hr. at 25° before homogenization and examination as above. Only material co-chromatographing with 2,4-dichlorophenylglutathione was detected in the aqueous fraction, and this corresponded to 25% of the dose of γ-HCH.

In homogenates. Homogenates of 100 flies or 20 grass grubs were prepared as described above in 10 ml. of 0·1 M-phosphate buffer, pH 7·4. These were made 2 mM with respect to GSH, and 145 μg. of γ-HCH was added to each in a small volume of acetone or on a small piece of filter paper from which the acetone solvent had evaporated. Control incubations were prepared with buffer in place of enzyme and both were kept at 37° for 6 hr. before extraction with 10 ml. of toluene.

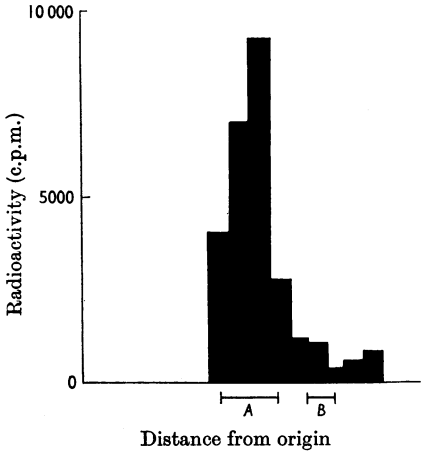

Fig. 1. Chromatogram of water-soluble products after incubation of 290 μc of [^{14}C]γ-HCH and GSH with a homogenate of 1000 houseflies in 50 ml. of 0·1 M-phosphate buffer, pH 7·4. Co-chromatographed reference samples of *S*-2,4-dichlorophenylglutathione (*A*) and isomers of *S*-dichlorophenylcysteines (*B*) are indicated. Descending paper chromatography in solvent A (Table 1) was used.

In three experiments with grass grubs the mean metabolism of the substrate to water-soluble products was 52%. In seven experiments with flies the mean value for the metabolism of the substrate was 84% in 6 hr. Experiments were also done with homogenates of flies and grass grubs to which 0·1 mM-Bromophenol Blue or 0·1 mM-bromosulphophthalein were also added. In these experiments less than 5% of the γ-HCH was converted into water-soluble products by the enzyme prepared from either insect.

Aqueous layers from these experiments with both inhibited and uninhibited enzymes were deproteinized with ethanol, as above, and the concentrated solutions were examined chromatographically in each of the solvents of Table 1. In each case only trace amounts of material other than that co-chromatographing with 2,4-dichlorophenylglutathione was found (Fig. 1).

In the presence of γ-PCH. A homogenate of 100 flies was prepared as above and a solution of 2·5 mg. of γ-PCH in egg lipoprotein (Lipke & Kearns, 1960) was added with 145 μg. of [^{14}C]γ-HCH. Control solutions contained buffer in place of enzyme and the tubes were incubated for 1 hr. Extraction with toluene followed by radioactivity and g.l.c. measurements showed that 20% of the ^{14}C-labelled material was now water-soluble and that 0·6 mg. of γ-PCH remained. The toluene-soluble

materials were separated on thin-layer plates with the cyclohexane–chloroform system. The major peak of radioactivity corresponded to γ-HCH and only 0·1% of the radioactivity on the plate co-chromatographed with γ-PCH.

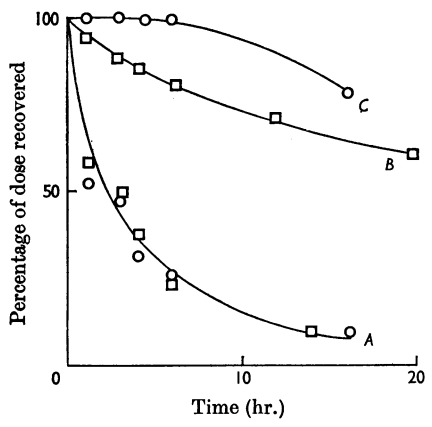

Fig. 2. Metabolism of a 10 μg. topical dose of δ-HCH by grass grubs after injection with 65 μg. of tetrabromophenolphthalein ethyl ester. □, Grubs dosed with δ-HCH only; ○, grubs dosed with δ-HCH and inhibitor. Curve *A* shows the external δ-HCH for both sets. Curves *B* and *C* show total δ-HCH for grubs dosed with δ-HCH without and with inhibitor respectively.

Metabolism of non-radioactive γ-HCH and δ-HCH

Grass grubs and houseflies were dosed topically with δ-HCH in 1 μl. of acetone and groups of six were analysed separately at hourly intervals, up to 15 hr., for external and internal δ-HCH by g.l.c. Maximum internal concentrations of HCH were reached after 10–12 hr. in grass grubs and after 8–10 hr. in flies. Similar experiments were done in which the grubs or houseflies had been treated topically or by injection with tetrabromophenolphthalein ethyl ester 15 min. before being dosed with HCH. Absorption of HCH was the same in both groups, but metabolism of it was slow in the group dosed with the phthalein (Fig. 2). Phenol Red and Bromophenol Blue produced similar results but the effect of these, and of tetrabromophenolphthalein ethyl ester, was limited to the initial few hours (Table 2).

Blowflies were injected with 5 μg. of δ-HCH or with 0·05 μg. of γ-HCH and analyses were made at 30 min. intervals for 3 hr. The rate of metabolism of γ-HCH and δ-HCH was negligible in flies less than 1 day after emergence, and increased up to a maximum about 8–10 days later (Fig. 3). Flies from the same batch were also injected, 8–10 days after emergence, with 5 μg. of δ-HCH or 0·05 μg. of γ-HCH after previously having been dosed topically with bis-(*NN*-dimethylaminophenyl)methane or injected with Bromophenol Blue.

Table 2. *Effect of inhibitors on the metabolism of γ-HCH and δ-HCH in insects*

Groups of insects were dosed with HCH 15 min. after being injected with solutions of the stated inhibitors. HCH was measured by g.l.c. in six separate insects taken at intervals thereafter and results were plotted as in Fig. 2. Abbreviations: PR, Phenol Red; BPB, Bromophenol Blue; TBE, tetrabromophenolphthalein ethyl ester; TM, bis-(*NN*-dimethylaminophenyl)methane.

Insect	Dose (μg.)	Isomer of HCH	Inhibitor	% of dose metabolized: 10 hr. after topical dose	% of dose metabolized: 2 hr. after injection
Grass grub	10	δ	—	11	—
	1	δ	—	20	—
	10	δ	—	10	—
	10	δ	35 μg. of PR	7	—
	10	δ	65 μg. of BPB	7	—
	10	δ	—	12	—
	10	δ	60 μg. of TBE	5	—
Housefly	1	δ	—	15	—
	1	δ	60 μg. of TBE	7	—
Blowfly	5	δ	—	—	12
	5	δ	50 μg. of TM	—	10
	5	δ	30 μg. of BPB	—	5
	0·05	γ	—	—	60
	0·05	γ	50 μg. of TM	—	58
	0·05	γ	30 μg. of BPB	—	30

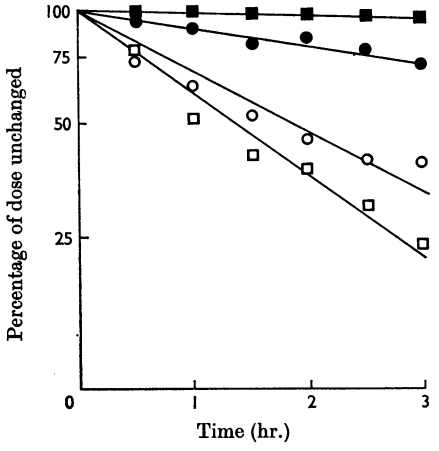

Fig. 3. Effect of age on metabolism of injected γ-HCH by the blowfly. Flies were injected with 0·05 μg. of γ-HCH and unchanged insecticide was assayed by g.l.c. at intervals. ■, Freshly emerged adults; ●, 3 days old; ○, 6 days old; □, 10 days old.

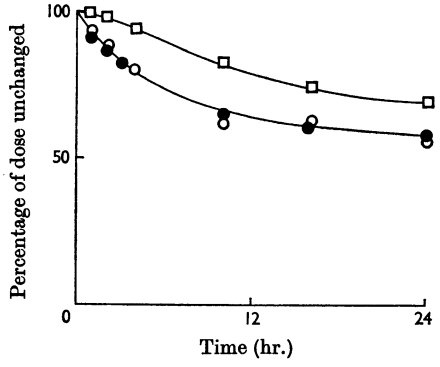

Fig. 4. Effect of inhibitors on metabolism of injected δ-HCH by the blowfly. Flies were injected with 1 μg. of δ-HCH 30 min. after treatment with inhibitors as described in the text. ●, δ-HCH only; ○, δ-HCH and 50 μg. of bis-(*NN*-dimethylaminophenyl)methane; □, δ-HCH and 30 μg. of Bromophenol Blue.

Bis-(*NN*-dimethylaminophenyl)methane was without effect on the rate of metabolism of the HCH isomers, but Bromophenol Blue appeared to inhibit their metabolism for a short time after injection (Figs. 4 and 5). The coloured phthalein and sulphonphthaleins (Table 2) were rapidly excreted and could be seen through the integument of grass grubs and blowflies to have accumulated in the hind gut after 1–3 hr.

Metabolism of γ-PCH and δ-PCH

In whole insects. The PCH isomers were injected into grass grubs (10 μg./grub) or blowflies (5 μg./fly)

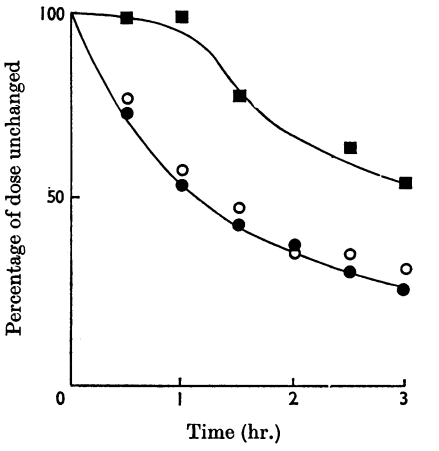

Fig. 5. Effect of inhibitors on metabolism of topical γ-HCH by the blowfly. Flies were dosed topically with 0·05 μg. of γ-HCH 30 min. after treatment with inhibitors as described in the text. ●, γ-HCH only; ○, γ-HCH and 50 μg. of bis-(*NN*-dimethylaminophenyl)methane; ■, γ-HCH and 30 μg. of Bromophenol Blue.

and separate insects were analysed at intervals for unchanged material. Experimental points on the plotted results (Figs. 6–8) are mean values from six individual flies or grubs. In *Lucilia* the rate of metabolism of both γ- and δ-isomers was negligible in newly emerged flies and increased to a maximum after about 8–10 days.

Assays were also done on insects that had been injected with possible enzyme inhibitors 30 min. before being dosed with PCH. Inhibition of metabolism of PCH by the phthaleins was effective for only a few hours, although the effect was more prolonged in grass grubs than in flies (Figs. 6–8). In both insects the end of the inhibition was associated with the excretion of the dyes, which could be seen to have accumulated in the hind guts of the flies after 2 hr. and in those of grass grubs after 6 hr. Inhibition of PCH metabolism by bis-(*NN*-dimethylaminophenyl)methane was prolonged in flies (Figs. 6 and 7), but no effect was observed when grass grubs were dosed topically with this compound (Fig. 8).

Grass grubs were also injected with 50 μg. of bis-(*NN*-dimethylaminophenyl)methane in aqueous solution 30 min. before injection of 5 μg. of γ-PCH. Assays for PCH made at intervals showed that there was no significant decrease in the rate of removal of PCH in comparison with that in a control group receiving PCH only. The DDT dehydrochlorinase inhibitor, WARF, had no effect on the metabolism of PCH in blowflies when administered in doses of either 1 μg. or 5 μg./fly (Fig. 6).

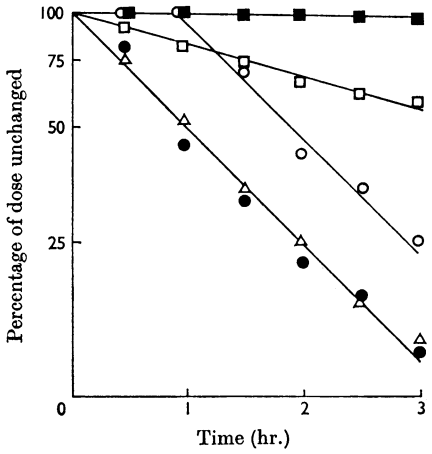

Fig. 6. Effect of inhibitors on metabolism of injected γ-PCH by the blowfly. All flies were injected with 5μg. of PCH and inhibitors were administered 30min. before dosing. ●, γ-PCH only; ○, γ-PCH and 30μg. of Bromophenol Blue; ■, γ-PCH and 50μg. of bis-(*NN*-dimethylaminophenyl)methane; □, γ-PCH and 5μg. of bis-(*NN*-dimethylaminophenyl)methane; △, γ-PCH and 1μg. of WARF antiresistant.

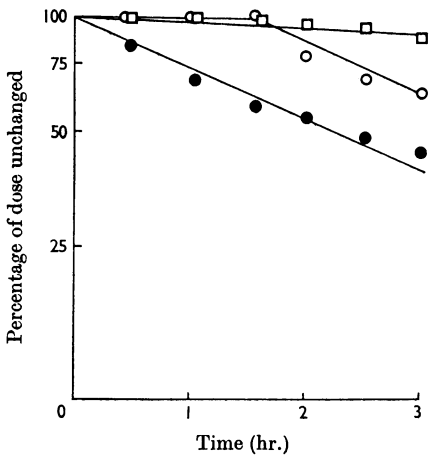

Fig. 7. Effect of inhibitors on metabolism of injected δ-PCH by the blowfly. All flies were dosed by injection with 5μg. of PCH and inhibitors were given 30min. before dosing. ●, δ-PCH only; ○, δ-PCH with 30μg. of Bromophenol Blue; □, δ-PCH with 50μg. of bis-(*NN*-dimethylaminophenyl)methane.

In insect homogenates. A homogenate of blowflies was prepared as described above and 5ml. was made 5mM with respect to GSH and 0·02mM with respect to γ-PCH. It was incubated at 37° and 50μl. portions were removed at 10min. intervals and shaken with 2ml. of *n*-hexane each. After centrifugation, the hexane solution was analysed for γ-PCH. In control incubations in which enzyme was replaced by buffer, recovery of γ-PCH from the system was quantitative up to the end of the experimental period.

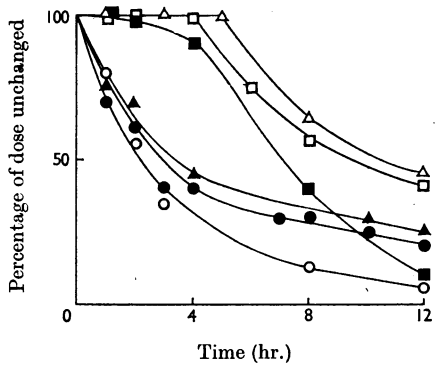

Fig. 8. Effect of enzyme inhibitors on metabolism of injected γ-PCH and δ-PCH by grass grubs. Grubs were injected with 10μg. of γ-PCH or δ-PCH 30min. after a topical dose of 50μg. of bis-(*NN*-dimethylaminophenyl)-methane or injection with 60μg. of Bromophenol Blue or tetrabromophenolphthalein ethyl ester. ●, γ-PCH only; ○, δ-PCH only; □, γ-PCH with 30μg. of Bromophenol Blue; ■, δ-PCH with 30μg. of Bromophenol Blue; △, γ-PCH with 30μg. of tetrabromophenolphthalein ethylester; ▲, γ-PCH with 50μg. of bis-(*NN*-dimethylaminophenyl)-methane.

The results were plotted graphically and the initial rates of metabolism were estimated from the slopes of the curves obtained. Homogenates prepared from newly emerged blowflies had negligible metabolic activity, but this increased up to a maximum after 8–10 days, when reaction rates under the conditions described were in the range 0·3–0·5nmole/min./fly equivalent. Incubations were also carried out in similar reaction mixtures that were made 5μM with respect to Bromophenol Blue or bis-(*NN*-dimethylaminophenyl)methane. Reaction rates in both of these solutions were in the range 0·01–0·03nmole/min./fly.

Detection of a GSH conjugate. A homogenate of houseflies prepared as described above was dialysed against the buffer used for homogenization for 24hr. to remove amino acids. A volume of the dialysis residue corresponding to 100 flies (10ml.) was made 2mM with respect to GSH and incubated with 25μg. of γ-PCH (added in 0·02ml. of acetone) at 37° for 6hr. One control incubation mixture contained 10ml. of buffer with 25μg. of γ-PCH in

2mM-GSH and a second control contained enzyme and GSH only.

After 6hr. the three mixtures were extracted with 10ml. of toluene and the extracts analysed by g.l.c. No PCH was found in the test mixture, but 24μg. was recovered from the non-enzymic control. The three aqueous layers were concentrated *in vacuo* and protein was removed by ethanol precipitation and centrifugation. The ethanolic supernatant and washings were again concentrated *in vacuo* and the residue was chromatographed on paper in solvent system A (Table 1). The zone with R_F 0·4–0·7 was eluted chromatographically with water and examined in solvent systems A, B, C and D along with cysteine and GSH conjugates as reference compounds.

In each of these systems a single ninhydrin-positive spot was found from test incubations that ran identically with the reference substance *S*-2,4-dichlorophenylglutathione. This spot did not appear in the two control preparations.

Similar experiments were carried out with an amount of enzyme corresponding to 20 grass grubs and both experiments were repeated with δ-PCH in place of the γ-isomer. In all cases a ninhydrin-positive spot was found only on the test chromatograms and in each case this had chromatographic properties identical with those of *S*-2,4-dichlorophenylglutathione in the four solvent systems used.

Water-soluble Schechter–Hornstein-positive metabolites. Blowflies were injected with 5μg. of γ-PCH in 0·4μl. of ethanol and left at room temperature for 80min. Batches of 40 flies were homogenized in 4ml. of hexane, 30ml. of water was added to each batch and the mixture was centrifuged at 2000*g* for 10min. A 20ml. portion of the aqueous layer was evaporated to 1ml. at 45° *in vacuo* and assayed by the Schechter & Hornstein (1952) procedure. Similar analyses were done on undosed flies and no difference was observed between these and flies that had received γ-PCH.

DISCUSSION

In the metabolism of HCH isomers the likely initial metabolic step is either the removal of hydrogen chloride from the molecule to form a pentachlorocyclohexene or the condensation of GSH with HCH to form a pentachlorocyclohexylglutathione. The results described above appear to eliminate the first of these possibilities as a major step in the metabolism of γ-HCH or of δ-HCH in blowflies, since the metabolism of both of these isomers is unaffected by pretreatment of the flies with bis-(*NN*-dimethylaminophenyl)methane. Similar doses of this enzyme inhibitor are able to stop almost entirely the metabolism of γ-PCH or δ-PCH and it is probable that the small amounts of PCH isomers that have been detected by dilution analysis (Bradbury & Standen, 1958) or g.l.c. (Reed & Forgash, 1968) in insects metabolizing γ-HCH represent minor routes of detoxication rather than residual quantities of an intermediate stage.

A similar conclusion can be drawn from experiments on houseflies with [^{14}C]γ-HCH where attempts have been made to trap any γ-PCH formed as an intermediate in a pool of diluent material (cf. Bridges, 1959). Difficulties arise in this type of experiment if the radioactive intermediate is prevented from mixing with the pool of diluent. This could conceivably happen in intact insects, but is less likely to be a hazard in the enzymic experiment described above.

A third conclusion from our results is that in blowflies PCH isomers do not give rise to water-soluble products that can give the Schechter–Hornstein colour reaction. This makes it more probable that the water-soluble metabolite of γ-HCH found by Bridges (1959) that gave this reaction was a primary metabolite of γ-HCH. Both γ-PCH and δ-PCH are rapidly converted by homogenates of flies or grass grubs into material that is chromatographically indistinguishable from *S*-2,4-dichlorophenylglutathione. Neither this compound nor the dichlorophenylcysteines, which are possible hydrolysis products, give significant responses in the Schechter & Hornstein (1952) procedure.

These results support the assumption made by Bradbury & Standen (1959) that a pentachlorocyclohexylglutathione is the initial metabolite of γ-HCH, and suggest that the detoxication enzyme belongs to the group of glutathione *S*-transferases that include aryltransferase (Clark, Darby & Smith, 1967), organophosphate methyltransferase (Fukami & Shishido, 1966), PCH-metabolizing enzymes (Ishida & Dahm, 1965; Ishida, 1968) and probably DDT dehydrochlorinase (Lipke & Kearns, 1960). That three of these are involved in detoxication and at least two of them in resistance to widely used insecticides makes their inhibition of considerable interest. Insect glutathione *S*-aryltransferase (Clark *et al.* 1967) and DDT dehydrochlorinase (Balabaskaran, Clark, Cundell & Smith, 1968) are inhibited by phthaleins and sulphonphthaleins, and these dyes also inhibit the metabolism of γ-HCH and δ-HCH (Figs. 2 and 5). Ishida & Dahm (1965) also showed that Bromophenol Blue inhibits the enzymes in houseflies that detoxify γ-HCH and DDT. Though these dyes are excellent inhibitors of the GSH-dependent enzymes, they would not be expected to be effective *in vivo* since they are ionized at tissue pH values and are readily excreted. The duration of inhibition of HCH and PCH metabolism corresponds to the lapse of time before

the dyes are concentrated into the hind guts of the insects. On the other hand, bis-(*NN*-dimethylaminophenyl)methane is not anionic at tissue pH values and might be expected to be excreted less rapidly than are the strongly acidic phthaleins or sulphonphthaleins, and to exert its inhibition for a longer period. This compound is also more readily absorbed through the cuticle of flies than are the phthaleins and may be used as an insecticide synergist in topical application.

No synergism of γ-HCH was observable with the possible enzyme inhibitors used. This would have been expected from the results of the metabolic studies and the lack of resistance to this insecticide in the strains of fly used. The SP2AB houseflies, however, had a low DDT resistance, and bis-(*NN*-dimethylaminophenyl)methane has been shown to be a good synergist for DDT in this strain and to be an excellent inhibitor of the DDT dehydrochlorinase in homogenates of these flies (S. Balabaskaran & J. N. Smith, unpublished work). This housefly enzyme can therefore be distinguished from the blowfly enzyme that detoxifies γ-HCH. WARF, which also inhibits housefly DDT dehydrochlorinase (Metcalf, 1967), had no effect on the PCH-metabolizing system of blowflies, so that these enzymes may also be distinguished.

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