Cyclodiene Insecticides as Inducers, Substrates, and Inhibitors of Microsomal Epoxidation

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Orally administered cyclodiene insecticides, aldrin and heptachlor, and their corresponding epoxides, dieldrin and heptachlor epoxide, are all active inducers of microsomal epoxidation. The "no effect" dietary dosage, determined from significant induction, is less than 5.0 p.p.m. but greater than 1.0 p.p.m. for each of these compounds when fed to young male rats for 2 weeks. The increase in rate of epoxidation may be correlated to the dietary dosage and concentrations of residual cyclodienes in the microsomes. Further microsomal metabolism of the cyclodiene epoxides was not found. Heptachlor and aldrin are cosubstrates of the same enzyme and are competitively inhibited by dieldrin and heptachlor epoxide, respectively.

The cyclodiene insecticides, aldrin, heptachlor, and isodrin, are converted by the NADPH-dependent microsomal oxidase system to their respective epoxides: dieldrin, heptachlor epoxide, and endrin (Wong and Terriere, 1965). Chlordan (Hart *et al.*, 1963), another cyclodiene insecticide, was the first chlorinated hydrocarbon insecticide found to enhance the metabolism of drugs by rat liver microsomes. Subsequently, Ghazal *et al.* (1964) demonstrated that dieldrin injected into rats produced a similar response. Since microsomal epoxidation is readily inducible by a number of chlorinated hydrocarbon insecticides (Gillett *et al.*, 1966), and the cyclodiene insecticides are also established as inducers of microsomal oxidases (Hart *et al.*, 1963), a more thorough study of the interaction of the cyclodienes with their own metabolism seemed necessary.

Unlike many of the drugs which are converted by microsomal mixed-function oxidases to highly polar or immediately conjugatable metabolites, aldrin and heptachlor are activated by epoxidation to the more lethal materials dieldrin and heptachlor epoxide. A concomitant induction of dieldrin metabolism and a decrease in tissue storage of dieldrin have been observed by Street (1964) in intact animals fed both dieldrin and DDT in amounts providing substantial increases in microsomal oxidation. Hence, a complex situation is apparent in the life of a cyclodiene insecticide in an exposed animal. The experiments reported herein consider the effects of feeding low levels of the various cyclodienes on hepatic microsomal epoxidation. Additionally, the nature of the epoxidative system was further characterized by cosubstrate and product inhibition studies. Finally, evidence was sought for the subsequent metabolism of dieldrin by the microsomal fraction. The results demonstrate that cyclodiene epoxidation is carried out by a single system which may be induced by both products and substrates and may be inhibited by reaction products, but that further metabolism of the epoxides was not demonstrable.

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EXPERIMENTAL

Treatment of Rats. Male white rats of the Corvallis-Wistar strain were weaned when 28 days old onto a semisynthetic ration (Tinsley, 1965) to which cyclodiene insecticides (analytical standard) could be added in corn oil solutions. The rats were caged individually with free access to food and water. To facilitate evaluation and reduce experimental error, only litter mate groups were used. Two rats of each litter were fed a control ration containing corn oil with no added insecticide. Each of the other rats received a ration containing one of the levels of insecticide. After 14 days on the diet, the litter was sacrificed under ether anesthesia, and microsomal preparations were made from the individual livers by the method previously described (Gillett *et al.*, 1966).

Analysis of Dietary and Microsomal Residue Levels. Sufficient ration was prepared to last for the whole experimental feeding period, and samples were taken and stored at $-20°$ C. for subsequent analysis (Gillett *et al.*, 1966). Periodically, the laboratory chows fed the mothers, and to which the pups had access during the last week prior to weaning, were ground and extracted for analysis.

A portion of the microsomal suspension was analyzed for cyclodienes by extraction with a 2-propanol–hexane mixture (2 to 3, v./v.) under conditions which allowed the amounts introduced and recovered in the assay for microsomal epoxidase to be corrected for tissue residues. This technique also provides the microsomal residue of cyclo-

diene insecticides. Recoveries by this procedure are 95± 5%, so no correction for recovery was applied in the calculations.

Determination of Microsomal Epoxidation. Three levels of microsomal protein (0.2 to 2.0 mg.) were incubated for 15 minutes with 20 μg. of aldrin and an NADPH-generating system at 37° C. and pH 7.6 as previously described (Gillett *et al.*, 1966). The reaction was stopped after 15 minutes by addition of the hexane–2-propanol mixture. Re-extraction of the aqueous phase twice with hexane was followed by gas chromatography of the combined non-aqueous extracts. Controls to measure endogenous conversions were included and the values of the sample were corrected thereby.

The statistical relationships were derived by the methods of analysis of variance, as shown by Li (1964), for comparisons by Student's *t* test of the effects of dietary cyclodienes on microsomal epoxidation, using $P < 0.05$ as the test of significance. Comparison of the microsomal residue data to activity utilized Li's methods of linear regression.

RESULTS AND DISCUSSION

Induction of Microsomal Epoxidation. All rats appeared normal by gross anatomical examination and showed normal growth patterns during the course of treatment with insecticide. Although both conventional and axenic rats of the caesarean-derived Fischer strain and conventional rats of the Corvallis-Wistar strain consuming as little as 0.31 p.p.m. of dieldrin for several months have been found by Harr and Bone (1967) to have marked histopathic anomalies in epiphyseal plates and arterial walls, their findings indicate that the dietary levels and feeding periods used in these experiments cause negligible changes. Since 20 p.p.m. was the highest level of cyclodiene tested, no acute or chronic toxic symptoms were anticipated or found. Liver weights and amounts of isolated microsomes were not significantly higher in the treated animals compared to controls receiving no additional insecticide (Table I). The diets all contained the stated formulation in approximately the amounts intended, although small (2 to 3%) contaminations of epoxides occurred in all aldrin and heptachlor diets.

The increase in aldrin epoxidation is highly significant for all of the cyclodiene insecticides, except aldrin, fed at the level of 5 p.p.m. Aldrin was the least effective at this concentration; the increase in specific activity (Table I) is almost significant at $P < 0.05$, but the increase in activity per gram of liver was significant only at $P < 0.1$. None of the cyclodienes fed at the 1.0-p.p.m. level were effective in increasing the level of epoxidase activity above that of the controls, and, with the exception of aldrin, the differences between the effects of 1.0 and 5.0 p.p.m. were significant. The rats on a 5.0-p.p.m. diet, eating about 20 grams per day, received a cumulative daily dosage of about 1 to 2 mg. per kg. The dietary "no effect" level for these rats is considerably above the levels of the cyclodiene residues recently reported from a market basket sample taken in the Baltimore, Md., area (Cummings, 1967).

The epoxides dieldrin and heptachlor epoxide are significantly more effective in inducing aldrin epoxidation than are aldrin or heptachlor, when these are fed at 5.0 p.p.m. The differences between aldrin and heptachlor or between dieldrin and heptachlor epoxide, however, are negligible. A "no effect" dosage for the induction of epoxidation by dietary DDT has been found to be between 2.0 and 2.5 p.p.m. for this same strain of male rats tested at the same age (Gillett, 1968). Although cyclodiene epoxides appear to be somewhat more active in the induction of microsomal epoxidation than is DDT for the same dietary dosage, the no effect levels are practically the same.

The residues of cyclodienes in the microsomal fraction were measured to correct the total epoxides determined in the epoxidase assay. Figure 1 shows that these residues may be correlated to epoxidase activity in the microsomes and to the diet fed the rat. The linearity of this relation-

Table I. Effect of Dietary Cyclodiene Insecticides on Aldrin Epoxidation

Compound Added to Diet	Concentration, P.P.M. Formulated	Concentration, P.P.M. Found[a]	No. of Rats	Liver Wt.[b]	Microsomal Protein[c]	Units[d] × 10^3 per Mg. Protein	Units per G. Liver
None	...	<0.20	8	4.54	17.20	92.2	1.68
Aldrin	1.0	0.92	3	5.25	18.37	110.3	1.80
	5.0	4.7	3	5.05	16.07	213.3[e]	3.363[e]
Dieldrin	1.0	1.16	4	5.14	17.88	147.5	2.63
	5.0	5.20	3	5.58	15.00	406.7[f]	5.983[f]
Heptachlor	1.0	1.05	2	5.41	14.05	52.5	0.70
	5.0	5.05	3	5.20	19.13	261.7[f]	4.683[f]
Heptachlor epoxide	1.0	0.87	3	4.70	19.57	114.7	2.50
	5.0	4.93	2	5.45	22.90	313.0[f]	7.365[f]
Error mean square (22 d.f.)				0.2369	20.8272	7459.1952	1.8971

[a] Total cyclodienes.
[b] Grams per 100 grams of live body weight.
[c] Mg. of biuret protein per gram of whole, washed liver.
[d] Mμmoles dieldrin formed per minute.
[e] $P < 0.10$, relative to controls.
[f] $P < 0.01$, relative to controls.

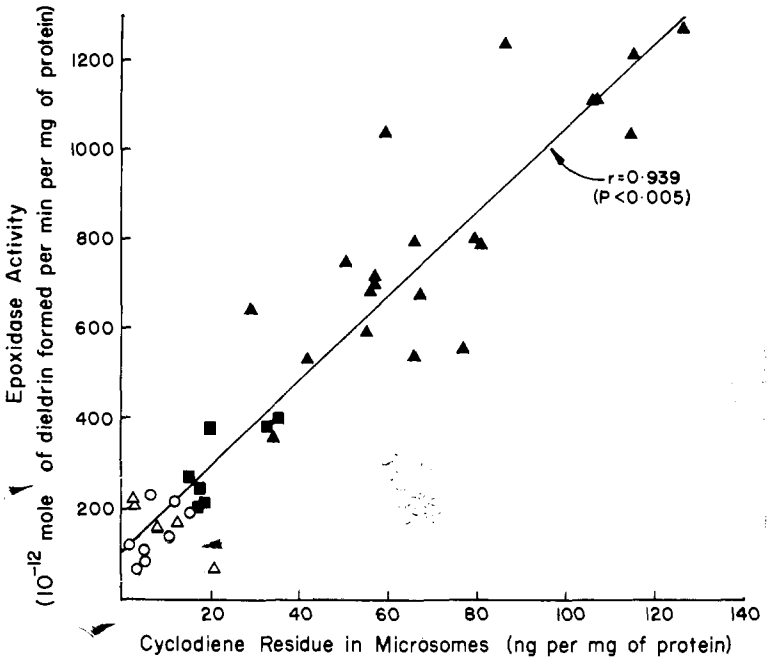

Figure 1. Cyclodiene epoxidation, in microsomal preparations from rats fed various concentrations of cyclodiene insecticides, compared to microsomal cyclodiene residue

○ Control (no added insecticide)
△ 1.0 p.p.m. of aldrin, dieldrin, heptachlor, or heptachlor epoxide
■ 5.0 p.p.m. of cyclodiene insecticides
▲ 20 p.p.m. of dieldrin

ship is only approximate, since we do not know the location of the residues in the intact cell and isolation of the microsomes may have resulted in a redistribution of cyclodienes. Further investigation is required to determine to what extent these residues reflect exposure of the rat to dietary (or other environmental) cyclodienes and to what extent, if any, microsomal concentrations of cyclodienes affect the specific activity of microsomal epoxidation.

Interaction of Cyclodienes as Substrates and Inhibitors. Preliminary experiments indicated that the rates of epoxidation of aldrin, heptachlor, and isodrin and of hydroxylation of naphthalene are comparable, yet variable between preparations from different organisms (Schonbrod *et al.*, 1965). The extent of increase in microsomal epoxidation in rats fed various chlorinated hydrocarbon insecticides was found to depend partially on the substrate selected to test for the activity (Gillett *et al.*, 1966). To establish whether or not this might be due to the presence of "epoxidases" with divergent affinities for the substrates, heptachlor and aldrin were incubated simultaneously in the same flask and the resultant amounts of individual epoxides were measured. With both normal and DDT-induced microsomes, the total amounts of epoxides were less than would have been expected from incubations containing only a single cyclodiene. Computation of the inhibition constants of heptachlor for aldrin epoxidation and aldrin for heptachlor epoxidation showed that these inhibition constants were about the same as the Michealis constants for each substrate (Table II), as is expected for two substrates competing for a single site. Although dieldrin and heptachlor epoxide could be established (Figure 2) as strictly competitive inhibitors of the epoxidation of heptachlor and aldrin, respectively, analytical problems prevented accurate determination of small changes in the rate of product formation in the presence of large concentrations of that product. Since epoxidation of aldrin and heptachlor occurs at a single site, the action of dieldrin and heptachlor epoxide should, therefore, be similarly competitive in inhibiting aldrin and heptachlor epoxidation, respectively, at this indicated site.

Further Metabolism of Cyclodiene Epoxides. Korte and Arent (1965) and Ludwig and Korte (1965) have reported aldrin and dieldrin to be excreted in rabbit urine as free or conjugated (−)-6,7-dihydro-6,7-*trans*-dihydroxyaldrin, commonly referred to as aldrin glycol. Aldrin

Table II. Kinetic Constants of Cyclodiene Insecticides as Substrates and Inhibitors of Microsomal Epoxidation

	Half-Maximal Concentrations[a]			
	Control Microsomes		DDT-Treated[b] Microsomes	
Compound	As substrate	As inhibitor	As substrate	As inhibitor
Aldrin	21.4	16.4	2.7	3.5
Heptachlor	4.9	4.8	0.7	1.3
Dieldrin	...	20.1	...	2.7
Heptachlor epoxide	...	14.9	...	2.5

[a] As mμmoles per flask, each containing about 0.2 mg. of microsomal protein in 6.0 ml.
[b] Isolated from litter mates of controls, but which had received 25 p.p.m. of DDT for 2 weeks.

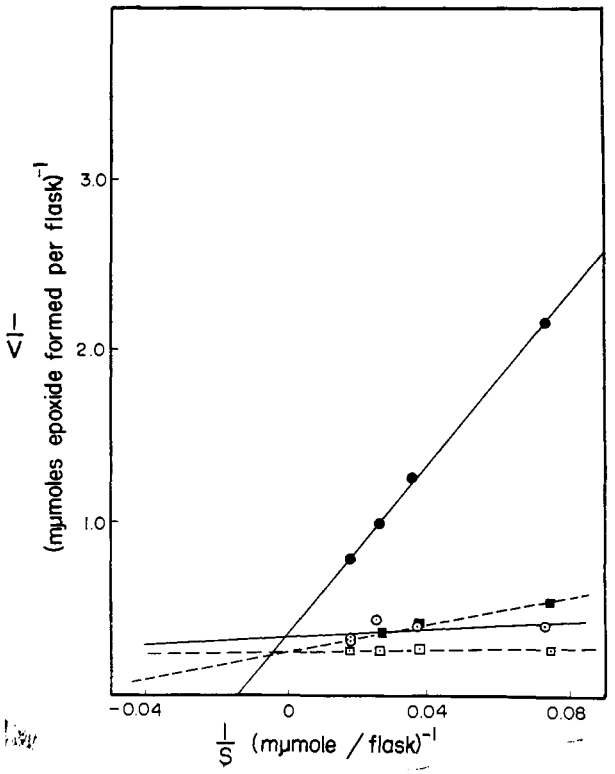

Figure 2. Competitive inhibition of cyclodiene epoxidation by epoxides

- ■ Heptachlor as substrate, 55.4 mμmoles of dieldrin as inhibitor
- ⊡ Heptachlor as substrate
- ● Aldrin as substrate, 53.5 mμmoles of heptachlor epoxide as inhibitor
- ⊙ Aldrin as substrate

glycol also has been tentatively identified as a metabolite of dieldrin in mosquito larvae (*Culex quintefaciatus*) by Oonnithan and Miskus (1964). When any of several animal species are fed dieldrin and/or DDT, decreased dieldrin storage and increased urinary excretion of metabolites have been observed by Street and Chadwick (1967) for animals receiving both compounds. However, experiments with barbituates and inhibitors of protein synthesis have not fully clarified the involvement of proliferation of the endoplasmic reticulum in the increased excretion and metabolism of dieldrin. No direct evidence of metabolism of dieldrin by the microsomes has been reported so far (Lewis *et al.*, 1967).

Gillett *et al.* (1966) found small, but insignificant, decreases in recovered dieldrin incubated with microsomes and only NADPH added as cofactor. Because of the interest in the role that cyclodienes might be playing in promoting their own metabolism and the interaction of that metabolism with other drugs and insecticides, a more thorough examination of this area was undertaken. Heptachlor epoxide and dieldrin were incubated for up to 2 hours with combinations of the microsomal and supernatant fractions from normal and DDT-induced rat livers and with combinations of Mg^{+2}, UDPGA, ATP, NADP, NADPH, reduced glutathione [suggested by the experiments of Sims (1965)], *N*-acetyl cysteine, and cysteine. No significant decrease in recoverable epoxide occurred at any level of tissue or substrate with any of these combinations. The recovery of dieldrin was uniformly 95 ± 5% from incubations containing 4 to 400 mμmoles per flask.

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