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CYCLODIENE EPOXIDE RING HYDRATION BY MICROSOMES FROM MAMMALIAN LIVER AND HOUSEFLIES

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Abstract—Microsomes from houseflies and from the livers of rats, pigs and rabbits hydrate enzymically the epoxide rings of several cyclodiene type insecticides. The hydration reactions proceed without the addition of cofactors, but when $NADPH_2$ and oxygen are also present, oxidation products are formed in some cases, by the action of mixed function oxidases. Housefly and rat liver microsomes had least epoxide hydrase activity. Observed rates of hydration by pig and rabbit liver microsomes were very low with dieldrin and the heptachlor epoxide m.p. 160° (HE 160), intermediate with the heptachlor epoxide m.p. 89° (HE 90) and rapid with synthetic analogues of these epoxides. The enzymic hydration of certain asymmetrical epoxides was stereoselective, resulting in the hydration of mainly one enantiomeric form and permitting partial resolution of the racemates. The hydration of dieldrin by rabbit liver microsomes would account for the observed excretion of the hydration product by the dieldrin-treated rabbit.

EPOXIDES have long been assumed to be intermediates in the biological conversion of aromatic ring systems into the corresponding dihydrodiols[1] and Jerina *et al.*[2] recently demonstrated the intermediate formation of 1,2-naphthalene oxide in the oxidative metabolism of naphthalene by rat liver microsomes. Isolated double bonds in reduced polycyclic systems can behave similarly, since Breuer and Knuppen[3] found that the

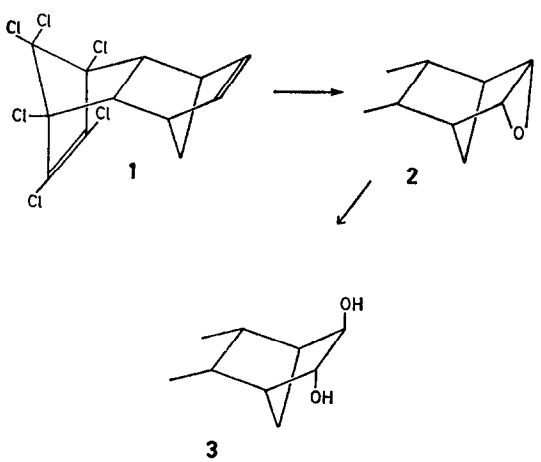

FIG. 1. Aldrin metabolism. 1, Aldrin; 2, dieldrin; 3, *trans*-6,7-dihydroxydihydroaldrin.

16α, 17α-epoxide was an intermediate in the conversion of oestra-1,3,5(10), 16-tetraene-3-ol into oestra-1,3,5(10)-triene-3,16β,17α-triol through oxidation of the olefinic double bond by rat liver slices.

In most cases, hydration of the intermediate epoxides is sufficiently rapid to make recognition of their existence difficult. However, in the case of the chlorinated polycyclic insecticide aldrin* (1, Fig. 1) and the related toxicants isodrin and heptachlor, epoxides that are rather stable in a number of living organisms are produced by biological oxidation.[4] The formation of toxic epoxides as the terminal products of metabolism of these compounds, in, for example, the housefly, is an important factor for their rapid action on this insect.[5] So far, the expected detoxication mechanism involving hydration of the epoxide rings of these products has been demonstrated only for dieldrin* (aldrin-6,7-epoxide; shown as the partial structure 2, Fig. 1), which is converted into the corresponding diol (3, Fig. 1) in the intact rabbit[6] and by intact larvae and adults of some species of mosquitoes.[7, 8]

We now show that the microsomal enzymes of some species can hydrate these epoxides, and their uniquely high stability is emphasised by the facile metabolism[9, 10] of related compounds whose biological hydration is also discussed.

EXPERIMENTAL

Materials. (*Melting points are not corrected*)

Dieldrin (1,2,3,4,10,10-hexachloro-*exo*-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene; 2, Fig. 1), m.p. 175° was obtained by recrystallisation of a Shell technical sample. *trans*-Dihydroxydihydroaldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene-*trans*-6,7-diol, DAD; 3, Fig. 1) m.p. 132°, was the gift of Shell Research Ltd. Heptachlor epoxide m.p. 160–161° (HE 160; Fig. 2, 1; X = Cl) and its epimeric epoxide m.p. 87–89° (HE 90) were analytical reference standards provided by the Velsicol Chemical Corporation, Chicago, U.S.A.

4,5,6,7,8,8-Hexachloro-*exo*-(*syn*)-2,3-epoxy-3a,4,7,7a-tetrahydro-4,7-methanoindane (chlordene epoxide, CE; Fig. 2, 1; X = H); 1,2,3,4,9,9-hexachloro-*exo*-5,6-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene (HCE; 3, Fig. 2) and 1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene (HEOM; 5, Fig. 2) were prepared as described previously.[11] 4,5,6,7,8,8-Hexachloro-3a,4,7,7a-tetrahydro-4,7-methanoindan-*exo*-1,*endo*-2-diol (*trans*-dihydroxydihydrochlordene or chlordene diol; Fig. 2, 2; X = H), m.p. 139–141° was prepared from 4,5,6,7,8,8-hexachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene (chlordene) by epoxidation and cleavage of the epoxide ring.[12]

4,5,6,7,8,8-*Hexachloro-endo*(*anti*)-2,3-*epoxy*-3*a*,4,7,7*a*-*tetrahydro*-4,7-*methanoindane* (*anti-chlordene epoxide, ACE; epimeric epoxide of* 1, *X* = *H*, *Fig.* 2). Chlordene (3 g) in glacial acetic acid (10·5 ml), treated at 75° with t-butyl hypochlorite (2 g) gave a chlorohydrin acetate which afforded the corresponding chlorohydrin (1·8 g) m.p. 104–106° on hydrolysis (methanol/HCl).[13] The product contained 5–10 per cent of an impurity believed to be a second chlorohydrin and cyclised nearly quantitatively to a corresponding mixture of epoxides when heated with sodium hydroxide in 80%

* The names aldrin and dieldrin are used for convenience although they are normally used, not for the pure compounds of Fig. 1, but for products containing 95% and not less than 85%, respectively, of these compounds.

aqueous dioxan.[14] After crystallisation from methanol, the major epoxide product had m.p. 221–222° and differed from the other known chlordene epoxide (Found: C, 34·2; H, 1·8; Cl, 59·4. $C_{10}H_6Cl_6O$ requires C, 33·8; H, 1·7; Cl, 59·9%).

The minor epoxide product clearly arose from the chlorohydrin impurity and its infrared spectrum showed it to be the heptachloro-epoxide HE 90, indicating that the process also results partly in allylic chlorination of chlordene followed by formation of the corresponding chlorohydrin containing eight chlorine atoms.[15]

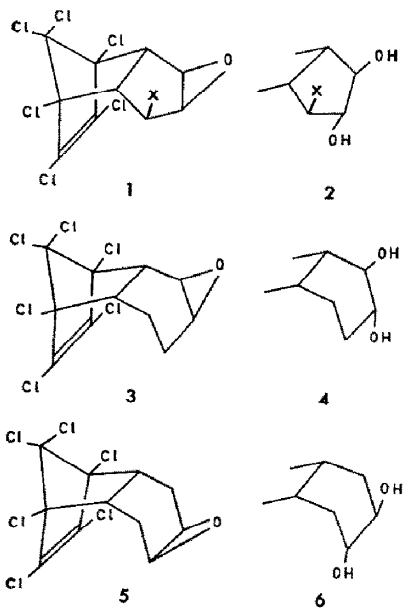

FIG. 2. Cyclodiene epoxides and their hydrolysis products. Chlordene epoxide (1, X = H); HE 160 (1, X = Cl); *trans*-dihydroxydihydrochlordene (2, X = H); HE 160-diol (2, X = Cl); 3, HCE; 4, HCE-*trans*-diol; 5, HEOM; 6, HEOM-*trans*-diol.

1,2,3,4,9,9-*Hexachloro*-1,4,4*a*,5,6,7,8,8*a*-*octahydro*-1,4-*methanonaphthalene*-*trans*-5,6-*diol* (4, *Fig.* 2). A solution of 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,8a-hexahydro-1,4-methanonaphthalene[11] (1·5 g) in acetic acid (50 ml) containing formic acid (5 ml), 100 vol. hydrogen peroxide (0·55 ml) and concentrated sulphuric acid (0·1 ml), heated at 45° for 18 hr, gave the crude *trans*-diol mono-acetate which was converted to the *trans*-diol when heated in refluxing methanol (100 ml) containing concentrated hydrochloric acid (0·5 ml). The crude product was chromatographed on neutral alumina (Woelm), with petroleum (b.p. 40–60°)/diethyl ether mixtures as eluants, and the eluted oil gave crystals m.p. 135–137° (1·2 g) from benzene/petroleum b.p. 60–80°. (Found: C, 34·3; H, 2·6; Cl, 55·3. $C_{11}H_{10}Cl_6O_2$ requires C, 34·1; H, 2·8; Cl, 55·0%). M = 384 (mass spectrum).

1,2,3,4,9,9-*Hexachloro*-1,4,4*a*,5,6,7,8,8*a*-*octahydro*-1,4-*methanonaphthalene*-*trans*-6,7-*diol* (6, Fig. 2). (a) The epoxide (5, Fig. 2; 32 mg) was hydrolysed in a mixture of dioxan (3·5 ml) and water (15 ml) containing sulphuric acid (10 N; 0·25 ml) at 100° for 2 hr. The mixture was diluted with water and extracted with diethyl ether. The ether

phase, washed free of acid, dried and evaporated, gave a solid m.p. 145–147° after recrystallisation from diethyl ether/petroleum b.p. 40–60°. (Found: C, 34·8; H, 2·8; Cl, 57·3. C_{11} H_{10} Cl_6 O_2 requires C, 34·1; H, 2·8; Cl, 55·0%) M = 384 (mass spectrum). (b) The *trans*-diol was also prepared from 1,2,3,4,9,9-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-methanonaphthalene[11] (1 g) by the method (above) used for compound 4 (Fig. 2). The diol (0·9 g) derived from the crude mono-acetate had m.p. 145–147° after recrystallisation and was identical with that obtained in (a).

Squalene 2,3-oxide and squalene 2,3-diol were prepared from squalene as described by Willett *et al.*[16] NADP, glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were purchased from Boehringer, London, and other chemicals used were A.R. grade. Benzylpenicillin B.P. and streptomycin sulphate B.P. were obtained from Glaxo Laboratories, Ltd., and *N*-ethylmaleimide (NEMI) from Koch-light Laboratories Ltd.

Analytical procedures

Electron-capture (100 mc tritium source) gas–liquid chromatography (GC) was conducted on a Pye Panchromatograph. For estimation of epoxides by measurement of peak height, 2 ft, 0·25 in. internal diameter glass columns packed with 100–120 mesh celite carrying 2·5% Apiezon L plus 0·25% Epikote 1001; carrier gas nitrogen; flow rate 150 ml/min; column temperature 160°, were used. These columns gave unsatisfactory peaks for the dihydroxy-derivatives, which could, however, be quantitated by peak height measurement at 180° on 12 in., 0·25 in. i.d. glass columns of 80–100 mesh acid washed DMCS treated chromosorb G (Perkin–Elmer Ltd.) carrying 2·5% SE.30 plus 0·5% Epikote 1001; carrier gas nitrogen; flow rate 120 (occasionally 170) ml/min. These conditions also permitted estimation of the epoxides.

For preparation of trimethylsilyl (TMS) ethers, authentic hydroxy-compounds (approx. 1 mg) or evaporated organic extracts of microsomal incubations were treated at room temperature (1 hr) with 0·4 ml of a mixture of dry pyridine (2·5 ml), hexamethyldisilazane (0·75 ml) and trimethylchlorosilane (0·5 ml).[17] The TMS-ethers were recovered by dilution to 10 ml with water, followed by rapid extraction of the aqueous phase with petroleum b.p. 40–60°, which was then quickly dried (Na_2SO_4). Extracts so prepared were concentrated for thin layer chromatography[18] (TC) or diluted for electron-capture GC. Although this technique was valuable for recognition of hydroxylated products, its effectiveness was often reduced by the presence in extracts of large amounts of unchanged substrates which were usually removed by small scale column chromatography before derivatisation.

The small amounts of DAD formed by the enzymic hydration of dieldrin were particularly difficult to detect by GC in the presence of large amounts of unchanged dieldrin: the ethereal extracts of incubation mixtures were evaporated to dryness in nitrogen, and the residues, redissolved in petroleum b.p. 40–60°, were chromatographed on small columns of alkaline alumina (Woelm, deactivated with 10% water; 12 × 5 mm) prewashed with diethyl ether and petroleum. Elution with the petroleum continued until the eluate (total 30–35 ml) contained only traces of dieldrin, when the columns were washed with diethyl ether/methanol (9:1, 2 × 10 ml portions). DAD was usually entirely removed in the first portion and was readily detected by GC following concentration of the solvent to 1–2 ml. These extracts were then treated with the silanes reagent as described. To exclude the possibility that other substances simulating DAD might be present in the final eluate, this procedure was followed with

microsomes and heat killed microsomes incubated alone, heat killed microsomes incubated with dieldrin, and microsomes plus dieldrin extracted without incubation. The final eluates were compared with those from the incubations with live microsomes. To test the recovery of DAD from incubation mixtures, dieldrin (500 μg) and DAD (2 μg) were added to pig liver microsomes and the DAD was recovered and estimated as usual (expt 24, Table 3).

Qualitative TC of diols and their TMS-ethers was on 3 × 2 in. microscope slides coated with basic alumina (Woelm; no binder) from a slurry in methanol, or with silica gel G or H according to Stahl (E. Merck) from a slurry in methanol/benzene (1:1), and dried at 110° for 30 min. Benzene/ethanol (4:1) (for alumina plates) and diethyl ether (for silica gel plates) were eluants for the diols, while *n*-hexane (alumina) and *n*-hexane/benzene (4:1) (silica gel) were used for the TMS-ethers (Table 2). Spot detection was by the phenoxyethanol/$AgNO_3$ test of Mitchell[19] for alumina plates but the ammoniacal $AgNO_3$ test of Morley and Chiba[20] was preferred for silica gel plates.

Extracts from housefly or pig liver microsomes incubated with squalene or squalene 2,3-oxide were examined for the unchanged substrates, squalene 2,3-diol or lanosterol by TC on silica gel G plates with the ethyl acetate/hexane mobile phases described by Willett *et al.*[16] and the zones were detected by exposing the plates to iodine vapour. Lanosterol formed by pig liver microsomes was further characterised by TC as the TMS-derivative. Infrared spectra were obtained for 2% solutions in CCl_4 using a Perkin–Elmer Infracord spectrophotometer and 0·5 mm NaCl liquid cells. Mass spectra were obtained by the direct insertion technique on a Perkin–Elmer Hitachi RMU 6E mass spectrometer and optical rotations were measured for solutions in methanol with a Bendix-NPL automatic polarimeter type 143.

Experiments with houseflies and with microsomal preparations

The strain of insecticide susceptible houseflies (*Musca domestica* L.) used and the methods for determination of insecticide toxicity were those described previously.[11] Laboratory animals were the gift of Glaxo Research Ltd.

Preparation of microsomes. Pig liver microsomes were prepared as described previously.[21] Housefly microsomes were prepared from mixed-sex dieldrin-resistant flies[11] (*Musca domestica vicina*) by a method similar to that described by Ray,[22] but with centrifugation conditions as for pig microsomes,[21] the microsomal pellet being finally suspended in 1·15% KCl. These preparations contained 20–25 mg of microsomal protein per ml as measured by the Biuret method.[23] For rat liver microsomes, male rats of the Carworth Farm—Charles River strain (wt. about 200 g) were killed by a blow on the head and their livers homogenised in ice-cold 1·15% KCl (1:3 w/v) in a Waring-Blender. After initial centrifugation at 20,000 g_{max} ($\frac{1}{2}$ hr) to remove heavier particles, the microsomal pellet obtained by further centrifugation (1 hr) at 104,000 g_{max} was resuspended and resedimented in the same medium and manner and the washed pellet finally suspended in cold 1·15% KCl with a hand-operated PTFE pestle, to give 15–20 mg microsomal protein per ml. Microsomes were prepared in the same way from livers of freshly killed female New Zealand White rabbits (wt. 2 kg). For these preparations, the livers were homogenised in cold 1·15% KCl (1:4 w/v). All operations were conducted at 0–4° and preparations stored at 0°.

Incubation procedures. In small scale experiments with microsomes the appropriate cyclodiene epoxide (50–500 μg), squalene 2,3-oxide (250 μg) or squalene (250 μg), each

in ethanol (50–100 μl), was normally added to a mixture of the microsomal suspension (0·1–0·5 ml) and 0·1 M Tris-phosphate buffer (4·5 ml) pH 8·4, with 1·15% KCl added when necessary to give a total volume of 5 ml. When determining the optimal conditions for the reactions, Tris-phosphate buffers of pH from 7·4–9·5 were used. In some experiments (Fig. 7) the reaction medium (pH 7·4 or pH 8·4) of Lewis *et al.*[21] replaced the usual buffer and was used with or without the addition of NADP, and, for houseflies only, contained KCN at 2×10^{-4} M. Incubations were conducted in open conical flasks for various times at 30°, 37° or 45°, the reactions were stopped by addition of acetone (7 ml) and the products recovered by three extractions with diethyl ether (4 ml portions). Variations of this extraction procedure were sometimes used, as indicated, in time course studies. The combined organic phase was dried (Na_2SO_4) and adjusted to a volume suitable for GC determination of the diols and unchanged epoxides. Long-term incubations such as those with the heptachlor epoxides and dieldrin were conducted at 37° or 45° in closed tubes. In these cases, control incubations were included in which water (1·25 ml) containing benzylpenicillin (3 mg; 5000 units) and streptomycin sulphate[32] (0·54 mg; 400 units) was added to each 5 ml incubation mixture. All experiments also included controls with heat denatured microsomes.

Isolation of unchanged epoxides and metabolites after incubation of chlordene epoxide, HCE, HE 90 and HEOM with pig liver microsomes

Pig liver microsomal suspension (5 ml; 25 mg protein/ml), was incubated at 37° in 0·1 M Tris-phosphate buffer pH 8·4 (90 ml) and 1·15% KCl (5 ml) with the appropriate epoxide (20 mg) added in ethanol (2 ml). GC-analysis indicated the formation of a single, more polar metabolite in each case and this was confirmed by TC. When conversion of the epoxides had ceased, as indicated by GC, the mixture was extracted with diethyl ether (3×100 ml than 1×50 ml) with centrifugation to separate the phases when necessary, and the extract dried (Na_2SO_4) and evaporated in nitrogen. The residual oil, containing much lipid material, was added to a column (14×100 mm) of alkaline alumina (Peter Spence type H) in petroleum b.p. 40–60° (2 ml) and elution continued with this solvent to remove microsomal lipids, as indicated by visual examination of evaporated fractions. Further elution with diethyl ether/40–60° petroleum (1:9) effected the removal of unchanged epoxide. This eluate, containing unchanged epoxide, was concentrated to 2 ml (fraction I). Continued elution with diethyl ether, then diethyl ether/methanol (9:1) removed the polar metabolite (fraction II). Fraction I was re-chromatographed on an identical column and the recovered epoxide (either chlordene epoxide or HCE) was resublimed (130°/0·1 mm) and recrystallised (methanol); 7–8 mg of each epoxide was recovered in this manner. Recovered chlordene epoxide had m.p. 223–226° (sublimes), and was slightly laevorotatory ($[\alpha]_D^{19}$ in methanol approx. −2°); recovered HCE had m.p. 220–225° (sublimes) and $[\alpha]_D^{23}$ $-20 \pm 2°$ (*c* 0·5 in methanol).

The metabolite (fraction II) from each epoxide was further purified by column (14×100 mm) chromatography on silica gel using 40–60° petroleum/diethyl ether mixtures, followed by diethyl ether, as eluants. Finally, the metabolites were short-path distilled (140–150°/0·1 mm) to give colourless oils which were compared with the authentic diols 2 and 4 (Fig. 2) by GC and TC (both as the free diols and as their trimethylsilyl ethers) and by infrared and mass spectroscopy. The diol from chlordene

epoxide had $[\alpha]_D^{23} + 10 \pm 1°$ and that from HCE, $[\alpha]_D^{23} + 15 \pm 1{\cdot}5°$ (*c* 1 in methanol).

HE 90 (20 mg), added in ethanol (2 ml), was incubated with pig liver microsomes (10 ml; 30 mg protein/ml) in 0·1 M Tris-phosphate (90 ml) containing NEMI (12·5 mg; final concentration 10^{-3} M) at 45°. GC analysis at intervals indicated that hydration had ceased after 20 hr, and about 9 mg of the epoxide remained. The latter, and the hydration products, were isolated as for chlordene epoxide and HCE and their metabolites. Recovered HE 90 was a colourless oil having an infrared spectrum identical with that of original epoxide and was laevorotatory ($[\alpha]_D^{23} - 101 \pm 10°$, *c* 0·7 in methanol). According to GC-analysis, the metabolites consisted of a main product and two minor products of shorter retention time (t_R). TC-analysis showed the main product to consist of two similar compounds; the recovered mixture was an oil which showed —OH absorption at 3510 cm^{-1} (sharp) and 3300 cm^{-1} (broad) in the infrared and had $[\alpha]_D^{23} + 17{\cdot}2 \pm 1{\cdot}7°$ (*c* 0·7 in methanol). M = 404 (m.s.).

HEOM (20 mg) was added in ethanol (2 ml) to 0·1 M Tris-phosphate buffer pH 8·4 (96 ml) containing pig liver microsomal suspension (2 ml, 25 mg protein/ml) and the mixture was incubated at 37° until conversion of HEOM into a single more polar metabolite appeared complete (2½ hr), as indicated by GC-analysis of 1 ml samples of the mixture removed at 30-min intervals. The metabolite was extracted from the mixture as before and the crude material chromatographed on a column (6 × 40 mm) of neutral alumina (Woelm) deactivated with 6% water. Elution with petroleum b.p. 40–60° and then diethyl ether removed lipid materials and diethyl ether/methanol (1:1) eluted the metabolite. The latter (17 mg), resublimed (150°/0·1 mm) and recrystallised from diethyl ether/40–60° petroleum, had m.p. 144–146° and $[\alpha]_D^{23} + 13 \pm 1{\cdot}3°$ (*c* 1 in methanol) and was compared with the authentic diol 6 (Fig. 2) by the methods previously described.

RESULTS

Microsomal metabolism of the chlordene epoxides, HCE and HEOM

Hydration products. Small scale incubations in buffer solutions without other additions indicated that microsomes from whole houseflies, and from pig, rabbit and rat liver, converted each of chlordene epoxide, HCE and HEOM into a corresponding diol. The epoxides were not hydrolysed by boiling 50% aqueous acetone, or when incubated in buffers containing microsomes previously held for 1 min at 100°.

For each species, GC- and TC-examination of the metabolite from a particular epoxide indicated it to be the same *trans*-diol as that produced by chemical hydrolysis of the epoxide ring. Some GC- and TC-characteristics of the diols and their TMS-ethers are indicated in Tables 1 and 2. The metabolites isolated following incubation of these epoxides with pig liver microsomes had infrared (Fig. 3) and mass spectra identical with those of the corresponding chemically prepared *trans*-diols but were optically active and in two cases had different physical properties from the authentic compounds.

The microsomes of all species examined readily converted most of added HEOM into the *trans*-diol, but conversion of HCE (Fig. 5) or chlordene epoxide by pig or rabbit liver microsomes proceeded only slowly beyond 50% under optimal conditions, an effect not due to substrate depletion, loss of enzyme activity or product inhibition. At pH 9·0, 37°, and with other conditions optimal, the rates of hydration of chlordene epoxide and HCE by rat liver microsomes were about eleven and thirty

TABLE 1. GAS CHROMATOGRAPHY OF CYCLODIENE EPOXIDES, DIOLS AND DIOL-TRIMETHYLSILYL (TMS) ETHERS*

Compound	Retention time (t_R) relative to dieldrin† (=1)	
	Underivatised	Trimethylsilyl ether
Chlordene epoxide (Fig. 2; 1, X = H)	0·39	—
ACE (epoxide epimer of chlordene epoxide)	0·64	—
HE 160 (Fig. 2; 1, X = Cl)	0·64	—
HE 90 (epoxide epimer of HE 160)	0·65	—
HCE (3, Fig. 2)	0·70	—
HEOM (5, Fig. 2)	0·90	—
trans-Dihydroxydihydrochlordene (Fig. 2; 2, X = H)	3·1	1·3
ACE-diol	4·4	1·3
HE 160-diol	4·5	1·8
HE 90-'diol'‡	4·5	1·8
HCE-*trans*-diol (4, Fig. 2)	2·9	1·9
HEOM-*trans*-diol (6, Fig. 2)	6·7	1·5
trans-Dihydroxydihydroaldrin (3, Fig. 1)	11·0	1·6

Gas-chromatography was conducted at 170° on 12 in., 0·25 in. internal diameter glass columns packed with 80–100 mesh acid washed, DMCS treated chromosorb G (Perkin–Elmer Ltd.) carrying 2·5% SE.30 plus 0·5% Epon 1001; carrier gas nitrogen; flow rate 120 ml/min. Detection by electron capture (100 mC tritium source).

* The chromatographic behaviour of these compounds indicates that they are bis-trimethylsilyl ethers.

† Retention time of dieldrin = 5·7 min.

‡ Mixture of two compounds according to TC.

TABLE 2. THIN-LAYER CHROMATOGRAPHY OF SOME CYCLODIENE-DIOLS AND THEIR TRIMETHYLSILYL (TMS) ETHERS*

	R_f values			
	underivatised		TMS ether	
Adsorbent:	I†	II	I	II
Mobile phase:	A‡	B	C	D
Compound:§				
trans-Dihydroxydihydrochlordene (2, Fig. 2; X = H)	0·38	0·34	0·29	0·34
HCE-*trans*-diol (4, Fig. 2)	0·44	0·32	0·29	0·42
HEOM-*trans*-diol (6, Fig. 2)	0·32	0·23	0·39	0·42
trans-Dihydroxydihydroaldrin (3, Fig. 1)	0·38	0·34	0·46	0·53
HE 90-'diol'	0·36 0·48	— —	0·49	— —
HE 160-diol	0·36	—	0·49	—
ACE-diol	0·42	—	0·30	—

* bis-Trimethylsilyl ethers.

† I, basic alumina without binder for TC (M. Woelm); II, silica gel G according to Stahl (E. Merck).

‡ A, benzene/ethanol (4:1), B, diethyl ether; C, *n*-hexane; D, *n*-hexane/benzene (4:1).

§ Free diols remained at point of application with systems I.C and II.D; TMS ethers ran with solvent front with systems I.A and II.B.

times lower, respectively, and those for housefly microsomes three hundred and two hundred times lower, respectively, than the corresponding rates found with rabbit liver microsomes, which were somewhat less active than pig liver microsomes toward these epoxides.

Each of the microsomal preparations converted epoxide ACE,[27] epimeric with chlordene *syn*-epoxide (Fig. 2, 1; X = H), into a trace of a product resembling (GC and TC) the known chlordene *trans*-diol (Fig. 2, 2; X = H) together with much larger

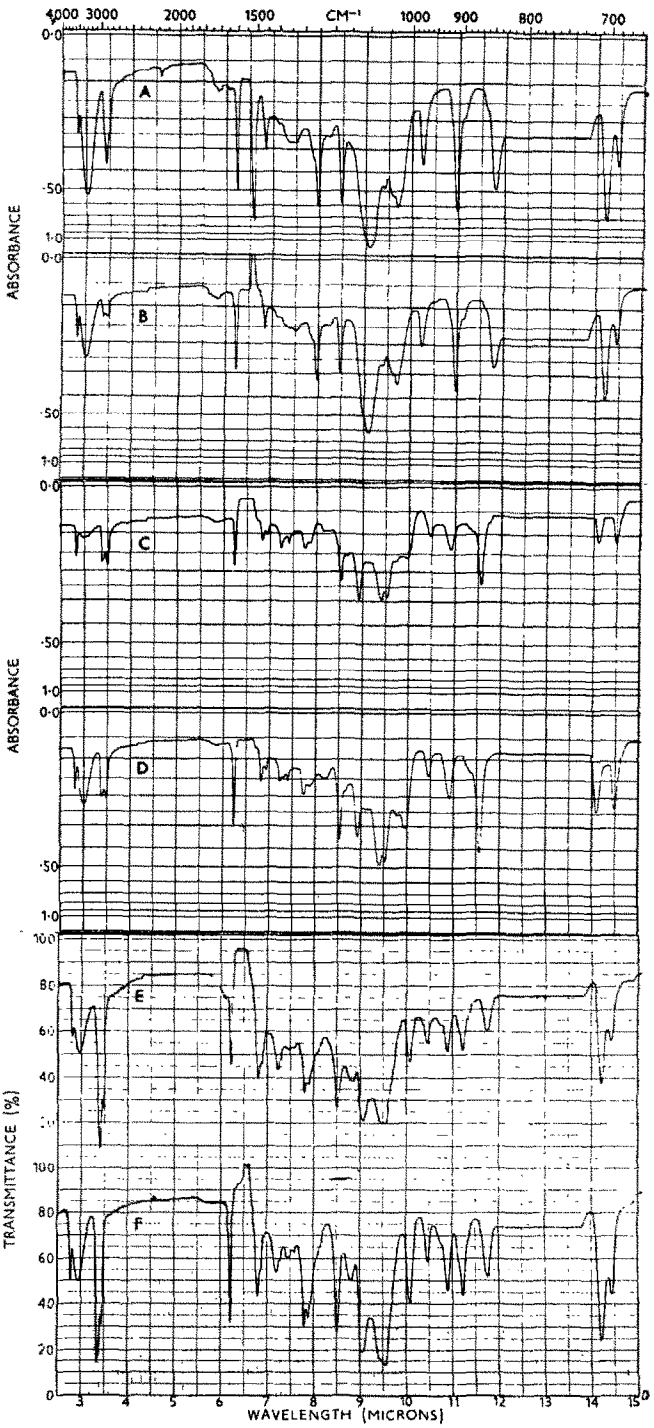

FIG. 3. Infrared spectra of cyclodiene epoxide metabolites and authentic *trans*-diols measured for 2% solutions in CCl_4 with NaCl cells of light path 0·5 mm. 12–14 μ is the solvent absorption region. Peaks from impurities probably of biological origin appear in places, as in A at 2900 cm^{-1} and 1520 cm^{-1}. A, chlordene epoxide metabolite; B, *trans*-dihydroxydihydrochlordene; C, HEOM-metabolite; D, HEOM-*trans*-diol; E, HCE-metabolite; F, HCE-*trans*-diol.

amounts of a different diol (Table 1). Similar results were obtained when ACE was heated at 45° (24 hr) in aqueous tetrahydrofuran containing perchloric acid, but not by incubation with heat denatured microsomes, and the main product must be either the *trans*-diol epimeric with 2 (Fig. 2, X = H) or, less probably, a *cis*-diol. Again, least activity was found in rat liver and housefly microsomes. The enzymic hydration of this epoxide by pig and rabbit liver microsomes appeared to be less stereoselective than for chlordene epoxide or HCE, since with comparable incubation conditions, more than half of the added ACE was hydrated fairly rapidly before the conversion rate fell off.

Stereoselectivity of microsomal hydration

The unmetabolised chlordene epoxide or HCE recovered from experiments with pig liver microsomes in which enzymic hydration had ceased (i.e. 50% conversion) had

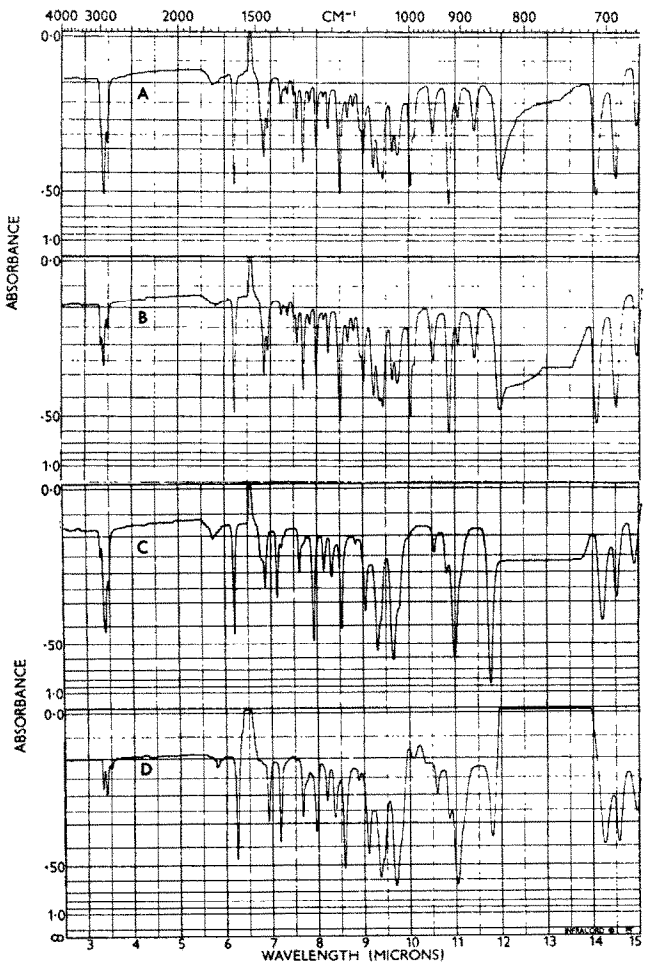

Fig. 4. Infrared spectra of racemic cyclodiene epoxides and of the enantiomers recovered from pig liver microsomes after stereoselective hydrolysis of the racemates. Measurements as in Fig. 3. A, recovered HCE; B, racemic HCE; C, recovered chlordene epoxide; D, racemic chlordene epoxide.

infrared (Fig. 4) and mass spectra identical with those of the epoxide originally added. Optical rotatory dispersion (ORD) measurements indicated that the specific rotation of recovered chlordene epoxide, small and negative between 589 and 340 mμ, changed sign at 339 mμ and increased progressively thereafter ($[\alpha]^{19}_{256}$ + 91°), while the negative rotation of recovered HCE increased rapidly between 589 and 250 mμ ($[\alpha]^{19}_{250}$ − 795°).

Recovered chlordene epoxide was hydrated by pig liver microsomes at about 1/20th the rate of its enantiomer as judged from experiments with the racemate and typical

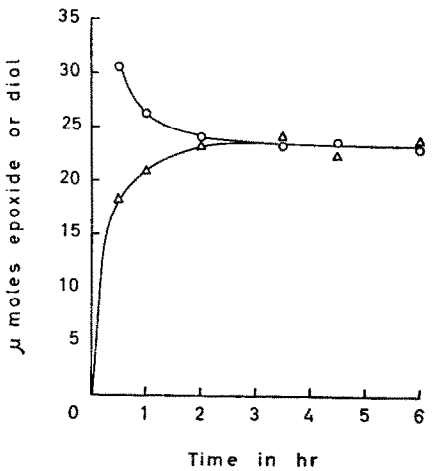

FIG. 5a

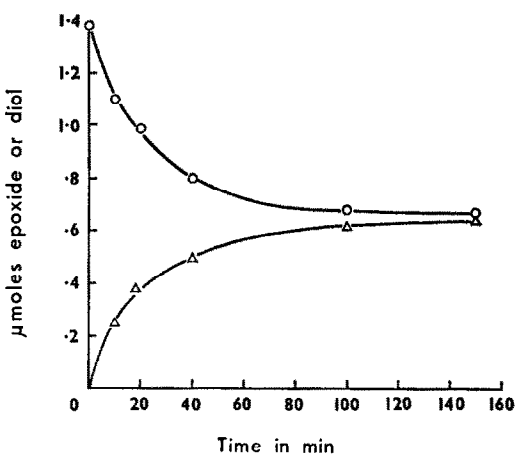

FIG. 5b

FIG. 5. Time course of racemic HCE hydration showing approach to half conversion. (a) Pig liver microsomes (5 ml; 25 mg protein/ml) incubated at 37° with HCE (20 mg; 54·2 μmoles, added in 2 ml ethanol) in 0·1 M Tris-phosphate buffer pH 8·4 (90 ml) plus 1·15% KCl (5 ml). For GC analysis, 1 ml samples taken at intervals into acetone (7 ml) plus water (4 ml) and mixture extracted with 3 × 4 ml diethyl ether. (b) Rabbit liver microsomes (0·5 ml; 30 mg protein/ml) incubated at 37° with HCE (500 μg; 1·36 μmoles, added in 100 μl ethanol) in 0·1 M Tris-phosphate pH 8·4 (9·5 ml). Analysis as in (a). Epoxide remaining —○—; diol produced—△—.

time courses are compared in Fig. 6. In a comparative experiment with recovered chlordene epoxide and recovered HCE in which hydration of these enantiomers by this preparation was approximately linear for 2 hr, the initial hydration rate for the HCE was about half that for the chlordene epoxide and was barely measurable in comparison with the hydration rate of racemic HCE. After 70 hr the HCE was 40% hydrated and the chlordene epoxide 70% hydrated, so that the low initial rates were not attributable to contamination by traces of the labile enantiomers.

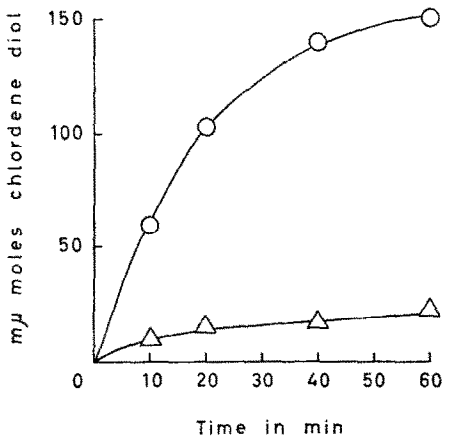

Fig. 6. Hydration by pig liver microsomes of racemic chlordene epoxide (—○—) and the chlordene epoxide enantiomer recovered from pig liver microsomes (—△—). Epoxides (100 μg; 282 mμmoles, added in 100 μl ethanol) incubated at 37° with microsomes (0·5 ml; 6 mg protein) in 0·1 M Tris-phosphate buffer pH 8·4 (4·5 ml). GC-analysis as in Fig. 5.

Rat or rabbit liver microsomes hydrolysed these enantiomers recovered from the pig preparation at less than 1/10th the rate observed for the corresponding racemates under the same conditions, and with housefly microsomes hydration of the enantiomers was negligible. The gas chromatograms of Fig. 7 illustrate the relatively slow hydration of the recovered chlordene epoxide and HCE enantiomers by pig liver microsomes and show also that mixed function oxidases attacked the epoxides only when a source of $NADPH_2$ was present.[9] The recovered enantiomers showed the same pattern of oxidative metabolism as the racemates with all the microsomal preparations tested.

In the reaction medium of pH 7·4, containing $NADPH_2$, rabbit liver microsomes effected 90 per cent conversion of added HCE (100 μg HCE, 0·5 ml microsomal suspension = 16 mg protein; incubation 60 min at 37°) into oxidation products, with little diol formation. The hydrolytic and oxidative processes appeared to provide alternative pathways for metabolism; diol formation became significant when the pH of the medium was increased to that optimal for diol formation (pH 8·4–9·4), when the amount of substrate was substantially increased, or when oxidation was prevented by omitting NADP from the incubation medium.

The toxicity to the housefly of optically active HCE or chlordene epoxide was not significantly different from that of the corresponding racemate[11] when the LD_{50}'s were compared by topical application in acetone.

Experiments with dieldrin and the heptachlor epoxides

Dieldrin. Pig and rabbit liver preparations were used in these investigations since the pig preparation appeared to be most active in regard to epoxide hydration and since *trans*-dihydroxydihydroaldrin (DAD) has been found in the urine of rabbits treated with dieldrin.[6] In early experiments with microsomes and liver slices at 30° or 37° for relatively short incubation periods (1–3 hr), hydration of dieldrin was not detected. Later experiments, summarised in Table 3, showed low but consistent diol

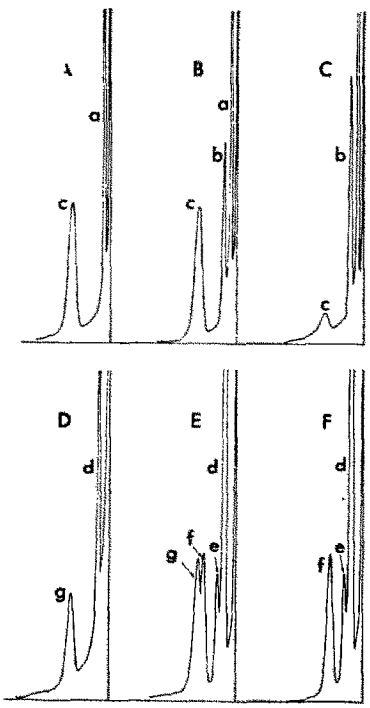

FIG. 7. Gas chromatograms of chlordene epoxide and HCE metabolites recovered from pig liver microsomes. Top row: 100 μg racemic chlordene epoxide incubated 30 min at 37°, A in reaction medium[21] pH 7·4 *minus* NADP and G-6-P dehydrogenase; B, as in A but *with* $NADPH_2$ generating system. C, recovered chlordene epoxide enantiomer (100 μg) incubated as in B. a, unchanged chlordene epoxide; b, oxidation product; c, *trans*-dihydroxydihydrochlordene. Bottom: D, 100 μg racemic HCE incubated 30 min at 37° as in A; E, as in D *with* $NADPH_2$. F, recovered HCE enantiomer (100 μg) incubated as in E. d, unchanged HCE; e and f, oxidation products; g, HCE-*trans*-diol. GC at 180° on 12 in., 0·25 in. i.d. glass column of 80–100 mesh acid-washed DMCS treated Chromosorb G carrying 2·5% SE30 plus 0·5% Epikote 1001; carrier gas N_2; Flow 170 ml/min.

formation that was not observed with heat denatured preparations and still occurred when antibiotics were included in the incubation mixture to eliminate bacteria.

Diol (DAD) formation increased slowly with time and more was recovered from rabbit than from pig preparations under comparable conditions. Direct comparison, by GC or GC after TMS-derivatisation, with heat denatured controls given the full extraction treatment, confirmed that the product was not an artefact of the isolation

procedure and recovered dieldrin, freed from the diol produced after long incubation periods, was hydrated in the same way on reincubation (expts 10–19, Table 3). There was some indication that NEMI, which stimulated some of the more rapid enzymic hydrations,[31] also stimulated production of DAD from dieldrin (expts 7 and 8, Table 3).

Heptachlor epoxide m.p. 160°. The metabolism of HE 160 (Fig. 2, 1; X = Cl) by pig and rabbit liver microsomes could be detected by GC without prior removal of the unchanged epoxide. Conversion progressed very slowly during long periods of incubation at 37° or 45° with other conditions as used for the investigations with dieldrin.

TABLE 3. ENZYMIC HYDRATION OF DIELDRIN BY LIVER PREPARATIONS

Expt. (1–9, Pig 10–23, Rabbit)	Incubation time (hr) (any additions)	diol recovered (μg)	Expt.	Incubation time (hr) (any additions)	diol recovered (μg)
1	15	0·5	13	72 (+ 1·25 ml water)	1·8
2	48	0·7	14	72 (+ antibiotics)	2·1
3	48	None	15	72 (+ antibiotics)	1·7
4	87	None	16	72 (+ 1·25 ml water)	None
5	50 (+ 1·25 ml water)	0·6	17	72 (+ 1·25 ml water)	None
6	50 (+ antibiotics)	0·5	18	39	0·8
7	60 (+ 50 μl water)	0·8	19	39	0·6
8	60 (+ NEMI, 10^{-3}M)	1·1	20	21	0·3
9	60 (+ 50 μl water)	None	21	21	None
10	48	1·4	22	48	1·4
11	48	1·2	23	48	None
12	48	0·8	24*	0	1·8

Dieldrin (500 μg; 400 μg in 7 and 8 only) incubated with 0·5 ml pig (1–9) or rabbit liver (10–19) microsomal suspension or 3 ml rabbit liver homogenate (20–23) plus Tris-phosphate buffer pH 8·4 (4·5 ml with microsomes, 7 ml with homogenate) with additions when shown (50 μl of 0·1M aqueous *N*-ethylmaleimide per 5 ml incubation mixture: antibiotics; 1·25 ml water containing 5000 units benzylpenicillin and 400 units streptomycin sulphate added to each 5 ml incubation mixture). Microsomal protein: 1–6, 30 mg/ml; 7–9, 28·0 mg/ml; 10–12 and 18–19, 32 mg/ml; 13–17, 29 mg/ml. Rabbit liver homogenate (20–23) equivalent to 0·7 g liver (wet wt.) or 0·25 ml microsomal suspension (32 mg/ml). Incubations at 45° except 18–21 at 37°. 3, 4, 9, 16, 17, 21 and 23 used preparations held at 100° for 1 min. In 10–12 and 18–19, the dieldrin recovered from 15, 13, 16 and 14, 17, respectively, was reincubated as indicated.

* Dieldrin (500 μg) and DAD (2 μg) added to microsomes to test recovery of DAD.

There was no metabolism with heat denatured microsomes but the conversion was not affected by addition of antibiotics or when the HE 160 was further recrystallised. Moreover, HE 160 incubated with pig liver microsomes at 45° for 60 hr, freed from the metabolite by chromatography, and then re-incubated, was converted at the same rate as the original HE 160. The product behaved as a diol and gave a TMS-derivative (Table 1) that appeared as a sharp, single peak on GC. This diol was separated from the bulked HE 160 recovered from several incubations, using the method employed for separating DAD from dieldrin. According to TC (Table 2), it consisted of a single compound, with possibly a trace of a second product with higher R_f. In a parallel experiment, about 1/6th as much of a chromatographically similar product was produced in 60 hr by rabbit liver microsomes, which also consistently afforded a second product having a shorter GC-retention time.

Heptachlor epoxide m.p. 90°. In contrast with HE 160, HE 90 (the epoxide epimer

of HE 160) was metabolised relatively rapidly by both pig and rabbit liver microsomes, although the latter were six times less active under comparable conditions (pH 8·4, 45°). Half conversion of added HE 90 could be effected with pig liver microsomes at 45° and the recovered, laevorotatory epoxide was hydrated much more slowly (Fig. 8) when re-incubated in the same manner. Figure 8 also shows the much greater hydration rate found with ACE, the corresponding epoxide lacking a chlorine atom adjacent to the epoxide ring. According to GC, the reaction products from either preparation consisted of one major component (HE 90 'diol' in Table 1), and two minor components which were not further investigated. None of these were

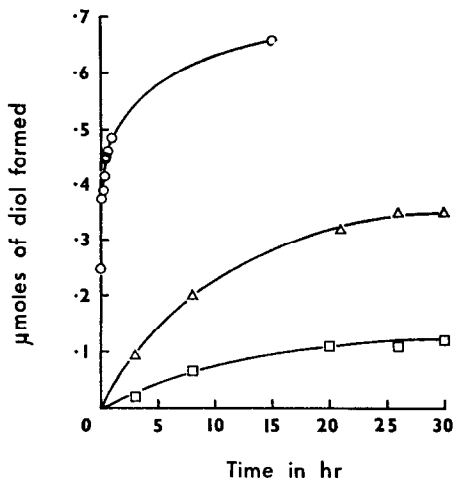

FIG. 8. Hydration by pig liver microsomes of racemic chlordene *anti*-epoxide (ACE, —○—), racemic HE 90 (—△—) and HE 90 recovered from pig liver microsomes (—□—). ACE (250 μg; 0·70 μmoles, added in 50 μl ethanol) incubated at 45° with microsomes (0·2 ml; 6 mg protein) in 0·1 M Tris-phosphate buffer pH 8·4 (4·5 ml) plus 1·15% KCl (0·3 ml). For GC-analysis 0·5 ml samples added to acetone (0·5 ml) at intervals and mixture shaken with diethyl ether (10 ml). Racemic and recovered HE 90 (250 μg; 0·64 μmoles, added in 50 μl ethanol) were each incubated similarly.

produced by heat denatured microsomes. TC (Table 2) showed the major peak to consist of two compounds, apparently the same for each species; each spot, excised from the plate and re-chromatographed, gave a single spot, so that the resolution was not due to decomposition on the alumina. The mixture gave TMS-ethers which ran together as a single but asymmetric peak on GC and their chromatographic behaviour indicated the products to be dihydroxy-compounds, rather than monohydroxy-compounds such as might result from hydrolytic removal of the odd chlorine atom with retention of the epoxide ring. When heated at 100° for 50 hr in aqueous tetrahydrofuran containing perchloric acid, HE 90 gave a small amount of a product which had the same t_R as the mixed metabolites and was similarly resolved into two compounds by TC. These compounds were not isolated but are likely to be the *trans*-diol 2 (Fig. 2; X = Cl) and the epimeric *trans*-diol formed by *trans*-opening of the epoxide ring in the alternative direction.

The GC-retention times of the metabolite from HE 160 and its TMS-derivative

were the same as the corresponding values for the HE 90 products (Table 1) and TC indicated this metabolite to be identical with the compound of lower R_f which was the predominant component in the HE 90 metabolite mixture (Table 2).

The toxicity to houseflies of the laevorotatory HE 90 was not significantly different from that of the corresponding racemate[11] when the LD_{50}'s were compared by topical application in acetone.

DISCUSSION

When the chlordene epoxides, HCE and HEOM are incubated with microsomes from different animals in the presence of $NADPH_2$ and air, various products are formed (Fig. 7), some by microsomal mixed function oxidase action[9] and others by epoxide hydrase enzymes that are also present in the microsomes. The present discussion is mainly concerned with epoxide hydration, which is enzymic but does not require $NADPH_2$ or oxygen.

Dieldrin and analogues. Of the above epoxides, the symmetrical molecule HEOM (5, Fig. 2) is structurally closest to dieldrin and is the compound most readily hydrated by the microsomes of all species examined, possibly because approach to its epoxide ring is least hindered by the bulky hexachloronorbornene nucleus. In dieldrin and several other molecules that result from insertion of a methylene or other bridge into the epoxycyclohexane ring of HEOM, this cyclohexane ring has an enforced boat conformation, and such molecules, in contrast to HEOM, are remarkably resistant to enzymic hydrolysis, although the present investigation demonstrates the slow hydration of dieldrin by preparations from pig and rabbit liver (Table 3). It may be that for these molecules, an association between enzyme and epoxide ring that normally precedes epoxide hydration is prevented by hindrance due to the presence of the methylene bridge and the consequent rigidity of the system.

There appears to be a missing link between the observed excretion of dieldrin metabolites by various mammalian species and the apparent inertness of this compound toward *in vitro* preparations from their livers, although Matthews and Matsumura[24] reported that rat liver microsomes produced unidentified metabolites from dieldrin, and rats are known to excrete oxidative metabolities of this compound in both urine and faeces.[25] Since rabbits treated with dieldrin excrete DAD,[6] and rabbit liver microsomes hydrate other cyclodiene epoxides fairly readily, their apparent inactivity toward dieldrin was unexpected. It is now clear that epoxide hydrase activity toward this compound is present but is quantitatively very different from the activity toward most of the other epoxides investigated. Surprisingly, rabbit liver microsomes appeared more active toward dieldrin than pig liver microsomes, although the latter had generally higher epoxide hydrase activity toward all the other compounds. Unfortunately, the fate of dieldrin in the intact pig is unknown.

The optical activity of the dieldrin metabolite (3, Fig. 1) isolated from rabbit urine[26] indicates that the epoxide ring opening *in vivo* involves mainly only one of the chemically equivalent carbon atoms carrying this ring, since non-selective hydration would give the racemic *trans*-diol. That pig liver microsomal enzymes can similarly distinguish between the corresponding carbon atoms of HEOM is shown by the optical activity of the *trans*-diol produced.

Heptachlor epoxides and analogues. Chemical hydrolysis of the epoxide ring of chlordene epoxide or HCE produces in each case only one (2; X = H and 4, respect-

ively, Fig. 2) of the theoretically possible racemic diols, presumably because ring opening in the other direction involves approach of OH^- ion to a strongly sterically hindered position. Enzymic hydrolysis proceeds in the same direction as chemical hydrolysis and the optical activity of the products is expected from the observed selective hydration of mainly only one enantiomer from each racemic epoxide. The same enantiomers are selected for hydration by microsomes of all the species examined, since in all cases hydration rates observed for the enantiomers of chlordene epoxide and HCE recovered from pig liver microsomes were much lower than those observed with the corresponding racemates.

The experiments with HE 90 (Fig. 8) and HE 160 show the remarkable stabilisation toward enzymic hydration that results from the introduction of a chlorine atom adjacent to the epoxide ring in ACE and chlordene syn-epoxide, respectively. Previous results[27] indicated that the additional chlorine atoms appear to protect these molecules from oxidative metabolism by occupying a position vulnerable to oxidative attack, but the effect on the hydration process may be a steric one similar to that produced by the introduction of a methylene or other bridge into HEOM. Hydration of ACE to give a *trans*-diol can occur in two possible ways involving attack of OH^- from the least hindered side of the molecule. Both possible products are formed by both chemical and enzymic hydration, although attack on C_2 of the cyclopentane ring is evidently favoured, this point being further from the hexachloronorbornene nucleus than the alternative position (C_3). There is evidence that besides stabilising ACE, the additional chlorine atom directs epoxide ring hydration in the alternative direction, since the preponderant hydration product in the metabolite mixture from HE 90 appears identical with the single product from HE 160 (Table 2). HE 160 (Fig. 2, 1; X = Cl) is analogous to chlordene syn-epoxide (Fig. 2, 1; X = H) and for the same reasons would be expected to give a single *trans*-diol (2; X = Cl) that should also be one of the hydration products of HE 90, as is observed. If, therefore, the structure assigned to HE 160 -diol is correct, the hydration of HE 90 favours attachment of $-OH$ at C_3, rather than at C_2 as in the hydration of ACE. Since HE 160 is asymmetric, its enzymic hydration is likely to be selective toward one enantiomer, but this cannot be demonstrated at present. The susceptibility of HE 90 to epoxide hydrase attack is evidently intermediate between that of the highly resistant epoxides such as dieldrin and HE 160 on the one hand and the simpler, readily hydrated epoxides on the other.

In relation to the foregoing observations, it is interesting that rabbit liver microsomes were found to convert racemic 1,2-naphthalene oxide into (−)-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene in 35–40% yield,[2] indicating that one enantiomeric form of the oxide is selectively hydrated. Also, pig liver microsomes converted squalene 2,3-oxide into lanosterol under conditions that we also found to be effective for hydration of the cyclodiene epoxides. Compared with the mammalian ones, the epoxide hydrases of housefly microsomes are relatively inactive toward cyclodiene epoxides, except HEOM, and they did not apparently convert squalene 2,3-oxide into either the corresponding diol or lanosterol. Since the juvenile hormones of several insect species now appear to be shorter carbon chain epoxides of the same type and, as such, are vulnerable to cyclisation or epoxide hydration reactions, the possession of active epoxide cyclising or cleaving enzymes might, indeed, be detrimental to insects, except as a possible means for disposal of unwanted hormone. In

these circumstances, squalene 2,3-oxide might be expected to accumulate if it was formed from squalene by the mixed function oxidases of housefly microsomes. However, no squalene 2,3-oxide could be detected when squalene was incubated with these microsomes and $NADPH_2$ at pH 7·4 or 8·4, although insects are evidently able to synthesise the similar epoxides of the juvenile hormone type.

Comparative metabolism and structure-activity considerations. The question as to whether the mixed function oxidases are also stereoselective has not been answered for pig liver microsomes, since for chlordene epoxide and HCE, with $NADPH_2$ present, enzymic hydration appears to compete very favourably with oxidative metabolism at pHs favourable for the latter. Hence, it seems likely that the hydrolytically labile enantiomers of these compounds are hydrated before their oxidation can occur and that the oxidation products formed arise mainly from the stable enantiomers. For housefly and rat liver microsomes also, it is unclear whether the normal oxidation pattern observed with the 'stable' enantiomers isolated from pig microsomes extends to the enantiomers that have not been isolated. With rabbit liver microsomes, however, the poor hydration of racemic HCE occurring at pH 7·4 apparently permits nearly complete oxidation of the racemate when $NADPH_2$ is present, thereby demonstrating non-selective oxidative attack on both enantiomers.

Houseflies appear to detoxify chlordene epoxide and HCE mainly by oxidative mechanisms, as indicated by their enhanced toxicity in the presence of synergists of the 1,3-benzodioxole type (inhibitors of microsomal oxidation).[11, 28] The results *in vitro* indicate that the oxidative pathways are qualitatively similar for all four species but the pig or rabbit, and possibly the rat, might be afforded some protection against synergised combinations that is not available to the housefly, by virtue of their additional ability to hydrate these toxicants:[10] the hydration process *in vitro* is unaffected by the low concentrations of inhibitors normally required to suppress oxidation.

The *trans*-6,7-dihydroxydihydroaldrin (DAD) isolated from rabbit urine is the only previously reported example of an optically active cyclodiene compound, since although a number of these molecules such as heptachlor, heptachlor epoxide, α-dihydroheptachlor[29] and the chlordane isomers are asymmetrical, there is no simple way of effecting their optical resolution. Büchel *et al.*[29] pointed out that a number of the most highly insecticidal cyclodiene molecules are symmetrical and indicated that this principle appears valid for the dihydroheptachlor isomers. In this series, however, the apparent differences in toxicity between symmetrical and asymmetrical molecules are not so marked, at least for the housefly, when allowance is made for metabolic detoxication.[15]

Since the stable, recoverable enantiomers of chlordene epoxide, HCE or HE 90 are as toxic to the housefly as the respective racemates, the two enantiomeric forms of each epoxide are about equitoxic to this insect. This could be expected from the low epoxide hydrase activity *in vitro*, even toward those enantiomers of chlordene epoxide and HCE that are hydrated by the mammalian microsomes, indicating that selective detoxication of these enantiomers should not be a factor influencing the observed toxicity of the racemates to this species. However, the intrinsic toxicities of the enantiomeric forms of each epoxide might have been very different for stereochemical reasons. That this is apparently not the case is in accord with other results[9, 30] with houseflies which indicate that in these molecules the positioning of the epoxide rings

and electro-negative groups generally is not too critical for toxicity provided that the molecules do not become too large.

Further details of the epoxide cleaving enzymes will be reported in a later paper.

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