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THE METABOLISM OF DIELDRIN AND TWO OF ITS ANALOGUES: THE RELATIONSHIP BETWEEN RATES OF MICROSOMAL METABOLISM AND RATES OF EXCRETION OF METABOLITES IN THE MALE RAT

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Abstract—Male rats receiving re-entrant bile duct cannulae were given [^{14}C]-dieldrin (HEOD)*, [^{14}C]-HCE† and [^{14}C]-HEOM‡ (15 mg/kg of each) by intraperitoneal injection. Bile collections were made without anaesthesia and nearly all ^{14}C excretion occurred by this route. Maximal ^{14}C excretion rates were observed between 20 and 40 min after dosing and these were much lower for dieldrin (3.17 nmoles/kg body wt/min) than for HCE or HEOM (204 and 298 nmoles/kg body wt/min respectively). Induction of liver enzymes by treatment with sodium phenobarbitone caused a significant increase in ^{14}C excretion in the case of dieldrin (threefold) but not in the case of HCE. The rates of ^{14}C excretion *in vivo* were compared with the rates of metabolism *in vitro* by liver microsomes for each compound.

The pattern of primary metabolism observed *in vivo* was similar to that found *in vitro* for both HCE and HEOM. Whereas HCE was metabolised predominantly by microsomal monooxygenase attack, both monooxygenase and epoxide hydratase were important in the degradation of HEOM. The rate of microsomal metabolism of HCE and HEOM by non-induced rat liver microsomes was over 100 times greater than that reported for dieldrin using induced rat liver microsomes. Using values expressed in terms of unit body weight, the maximal rates of microsomal metabolism of HCE and HEOM were 5–6 times greater than the corresponding maximal rates of biliary excretion of their metabolites in non-induced rats.

HCE concentrations were measured in liver microsomes from non-induced rats dosed with the chemical. The rates of hydroxylation by liver microsomes at these concentrations were of the same order as the corresponding rates of biliary excretion for the hydroxylated metabolites. The rate of microsomal hydroxylation of HCE increased approximately seventeen-fold in terms of liver weight, after phenobarbitone induction. These results suggest that the rate of metabolism can limit the rate of excretion of dieldrin metabolites in the male rat. With HCE and HEOM, however, the maximum metabolic rate is much faster and does not apparently influence the excretion rate. There may be a 'threshold metabolic rate' for these highly liposoluble compounds below which the rate of excretion is limited by the rate of metabolism.

The metabolism of many drugs and pesticides has been studied using microsomes and other liver preparations. In contrast to studies on whole animals this approach has the advantage of being rapid and relatively inexpensive whilst being particularly suitable for use in the characterisation of the enzyme systems involved. There are, however, difficulties in using data from such *in vitro* studies to predict events in the whole animal.

Reasonable correlations have been found between the activity of hepatic microsomal monooxygenase towards antipyrine and the half life of this compound in individual rabbits and dogs [1, 2]. In a survey of the literature a correlation was found between hepatic microsomal monooxygenase activity in different vertebrates, and the corresponding half lives for antipyrine, dieldrin, phenobarbitone and phenylbutazone [3]. Eleven different assay procedures were represented and ten different species were compared in this survey. These findings suggest that approximate monooxygenase activities *in vivo* can be predicted from data obtained *in vitro*. The compounds covered by this survey were all liposoluble, and might be expected to undergo most of their primary metabolic transformation by monooxygenase attack in the endoplasmic reticulum of the liver.

Dieldrin and its analogues (HCE and HEOM) are examples of extremely liposoluble compounds whose primary metabolism is largely effected by hepatic microsomal monooxygenase and hepatic microsomal epoxide hydratase (EC 4.2.1.63): see [4]. In a comparative study upon the dieldrin analogue HCE in the rat, rabbit, pigeon and Japanese quail [5], microsomal metabolism was predominantly oxidative, with the production of secondary metabolites as the substrate concentration declined. An essentially similar picture was found *in vivo*. The aim of the present work was to compare the rate of hepatic microsomal metabolism of HCE with the rate of excretion of its metabolites. Parallel studies were performed with dieldrin itself and HEOM.

* Dieldrin (HEOD) is 1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-epoxy-*exo*-1,4-*endo*-5,8-dimethanonaphthalene.

† HCE is 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-*exo*-7,8-epoxy-1,4-methanonaphthalene.

‡ HEOM is 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-epoxy-1,4-methanonaphthalene.

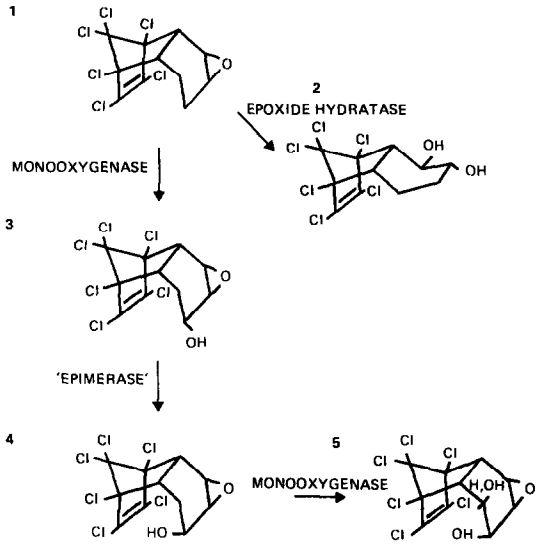

Fig. 1. Major pathways of HCE metabolism in the rat [5]. (1) HCE. (2) HCE *trans*-diol. (3) Monohydroxy-HCE (HHC). (4) *endo*-HHC. (5) Dihydroxy-HCE.

METHODS

Animals. Mature male Wistar rats (ASH/W), weighing 250–350 g, were obtained from Charles River Ltd., Manston, Kent. Rats were maintained on diet 86 (E. Dixon and Sons (Ware) Ltd., Herts).

Materials. HCE and HEOM were synthesised as described by Brooks and Harrison [6]. [^{14}C]-HCE and [^{14}C]-HEOM were prepared from [^{14}C]-hexachlorocyclopentadiene supplied by The Radiochemical Centre, Amersham, Bucks. ^{14}C-dieldrin was obtained from The Radiochemical Centre and purified by thin layer chromatography on Kieselgel GF_{254} using hexane: acetone (95:5 v/v) (R_f 0.5). The glucuronide conjugate of hydroxy-HCE (HHC) (Structure 3, Fig. 1) was synthesised by incubating HHC with washed rat liver microsomes (200 μg HHC/g liver equivalent) supplemented by uridine 5′-diphosphoglucuronic acid (UDPGA) (10 mg/g liver). The conjugate was extracted from the microsomal preparation with ethyl acetate after bringing the pH to 2 with 2M H_2SO_4. The conjugate was not formed when UDPGA was excluded from the incubation medium. All three [^{14}C]-organochlorine substrates were administered (15 mg/kg) to rats by intraperitoneal injection, as ethanolic solutions (50 mg/ml). For *in vitro* studies ^{14}C-HCE and ^{14}C-HEOM were employed as ethanolic solutions (0.5 to 10 mg/ml). Substrates for both *in vivo* and *in vitro* studies were of the following specific radioactivities: 9.20 μCi/mmole (HCE), 9.02 μCi/mmole (HEOM) and 239 μCi/mmole (dieldrin).

Technical hexane and acetone were redistilled and the purity of the products was checked by gas chromatography. Diethyl ether and standard laboratory reagents were of AnalaR grade from Fisons. NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were supplied by the Boehringer Corporation Ltd. Sodium phenobarbitone was obtained from BDH Chemicals Ltd. and was administered to rats by intraperitoneal injection (75 mg/kg) in saline, for three successive days. Animals were then used on the fourth day. Aryl sulphatase (type III, limpet) and β-glucuronidase (type B1, bovine or type 1, bacterial) were supplied by the Sigma Chemical Company. The liquid scintillator used for assessing radioactivity was "Cocktail D" (Fisons) or a scintillant based on toluene (Fisons) consisting of 40 per cent by vol. methoxyethanol (Fisons) and 6 g/l of 2-(4′-tert-butylphenyl)-5-(4″-biphenylyl)-1.3,4-oxadiazole (Sigma Chemical Company). Bile samples (0.1 ml) or organic extracts (0.5 ml) were added to 10 ml aliquots of scintillation fluid for analysis on a Nuclear Enterprise Scintillation Counter (8310). A standard sample of [^{14}C] hexadecane supplied by The Radiochemical Centre was used to construct a quench curve.

Incubation procedure. The preparation of washed liver microsomes and the incubation procedure are described elsewhere [7]. Flasks containing microsomes and the reaction medium were preheated for 90 sec in a water bath at 37°C before addition of substrate, and incubation was for periods ranging from 30 sec to 5 min. Reactions were terminated by partitioning with diethyl ether. Microsomal protein concentrations were determined by the method of Lowry *et al* [8].

Analysis of metabolites. Metabolites in aqueous systems were extracted into diethyl ether and converted into their trimethyl silyl (TMS) derivatives [7]. Analysis of extracts was by gas liquid chromatography (Perkin Elmer F11 chromatograph) with electron capture detection. The following stationary phases were used in glass columns: (1) 2.5% SE52 and 0.5% epikote 1001 resin on 80–100 mesh acid-washed DMCS treated chromosorb W operated at 165°; and (2) 2.5% Apiezon L and 0.5 epikote 1001 resin on 80–100 mesh chromosorb W operated at 180°.

Conjugates in bile were treated with β-glucuronidase and aryl sulphatase as described later and were also investigated by thin layer chromatography using a modification of the procedure described by Bedford *et al.* [9] for studying endrin * conjugates in rabbit urine. Isolation of the intact conjugates was attempted by adjusting the pH of bile to 2 using 2M H_2SO_4 and extracting three times with diethyl ether: ethanol (3:1 v/v) then twice with ethyl acetate. Extracts were examined by Kieselgel GF_{254} thin layer chromatography using ethyl acetate: 90% formic acid: water (70:4:4 v/v) as a mobile phase (system A). Chromatographic data are given in Table 1. Radioactive areas on thin layer plates were identified by scanning with a Panax instrument. A second system (B) (hexane:acetone 7:3 v/v) was used to chromatograph dieldrin metabolites following hydrolysis of conjugates with sodium metaperiodate following a method by Bedford, C. T. & Baldwin, M. K. (personal communication). Bile samples (2 ml) were added to a saturated sodium metaperiodate solution (5 ml) and heated to 70° for 45 min. A solution of potassium carbonate and potassium bicarbonate (0.1 M with respect to each) was then added (5 ml) and the sample was heated to 70°for a further 15 min. On cooling, the deconjugated metabolites were extracted into hexane.

Residues of HCE in liver homogenates were extracted with 20:80 v/v acetone: hexane. The livers were washed in 1.15% KCl solution prior to homogeniza

* 1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*, *endo*-5,8-dimethanonaphthalene.

Table 1. Chromatographic characteristics of HCE, HEOM, dieldrin and their metabolites

Compound	Number of compound or underivatised metabolite in Figures 1–3	Gas-liquid chromatography‡ *relative retention time	
		SE52	*Apiezon*
HCE	1	70	73
Monohydroxy-HCE (HHC)	3	199	250
†TMS derivative of HHC		140	125
HEOM	9	86	87
TMS derivative of hydroxy-HEOM	10	208	154
TMS derivative of HEOM *trans*-diol	11	169	97
Dieldrin	6	100	100
9-Hydroxy-dieldrin	7	227	160
		Thin layer chromatography‡ System A (R_f)	
Conjugate(s) of HCE from bile		0.62	
Monohydroxy-HCE (HHC)	3	0.95	
Glucuronide conjugate of HHC		0.62	
Conjugate(s) of dieldrin from bile		0.45	

* Gas liquid chromatographic relative retention times are calculated with respect to dieldrin (= 100).
† TMS = Trimethyl silyl derivative.
See analytical techniques for details of columns and t.l.c. system.

tion. Total radioactivity in liver subcellular fractions was measured using a Packard sample oxidiser.

Collection of bile and urine. Rats received a reentrant bile duct cannula under anaesthesia following a procedure described elsewhere [10]. A minimum postoperative recovery period of 10 days was allowed before administration of organochlorine substrates. Bile from unanaesthetized rats kept under minimal restraint was collected in 1 ml tubes kept on ice. When, during the operation, the sampling tube was not sealed with a spigot, bile flowed into the sampling arm with no observable flow into the reentrant arm. Hence it was assumed that virtually all of the bile passed down the sampling arm during collection without any significant amounts flowing to the duodenum. Rat urine was collected separately from faeces in a metabowl cage.

RESULTS

Following the administration of [^{14}C]-HCE, [^{14}C]-HEOM and [^{14}C]- dieldrin, continuous bile collections were made over a period of 4 hr to measure the first pass elimination from the liver without the possibility of enterohepatic circulation of metabolites. Nearly all of the ^{14}C excreted after injection was found in the bile, the urinary excretion of metabolites being a low percentage of total excreted metabolites (< 4% for HCE, < 5% for HEOM [11] < 10% for dieldrin [12]). Typical biliary excretion patterns for the three compounds are given in Fig. 4. Following HCE or HEOM administration the rate of excretion reached a maximum between 20 and 40 min after dosing, and then fell away rapidly with time. After dieldrin administration the rate of excretion reached a maximum at about same time, but this was less than 2 per cent of that found for HCE or HEOM, and did not show as great a tendency to decline with time.

Analysis of the bile samples showed that only very small quantities of ^{14}C were extractable by diethyl ether (Table 2), and no more than 1 per cent of the total ^{14}C

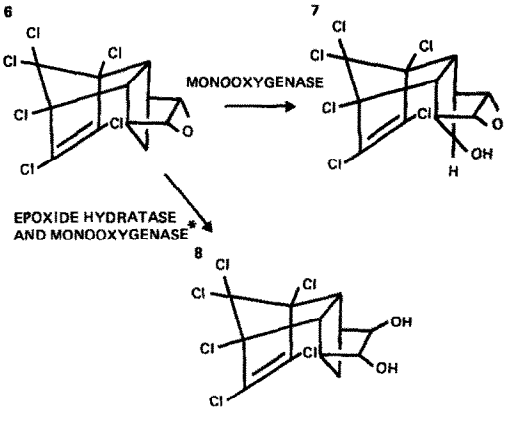

Fig. 2. Major pathways of dieldrin metabolism in the rat [14]. (6) Dieldrin. (7) 9-Hydroxy-dieldrin. (8) Aldrin *trans*-diol. * It has been suggested that monooxygenase as well as epoxide hydratase can effect this conversion [see 23].

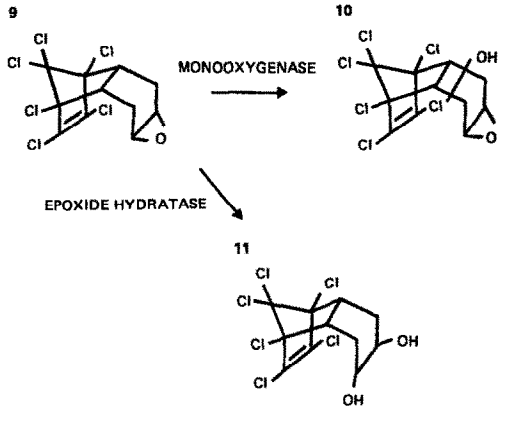

Fig. 3. Major pathways of HEOM metabolism in the rat [13]. (9) HEOM (10) Hydroxy-HEOM. (11) HEOM *trans*-diol.

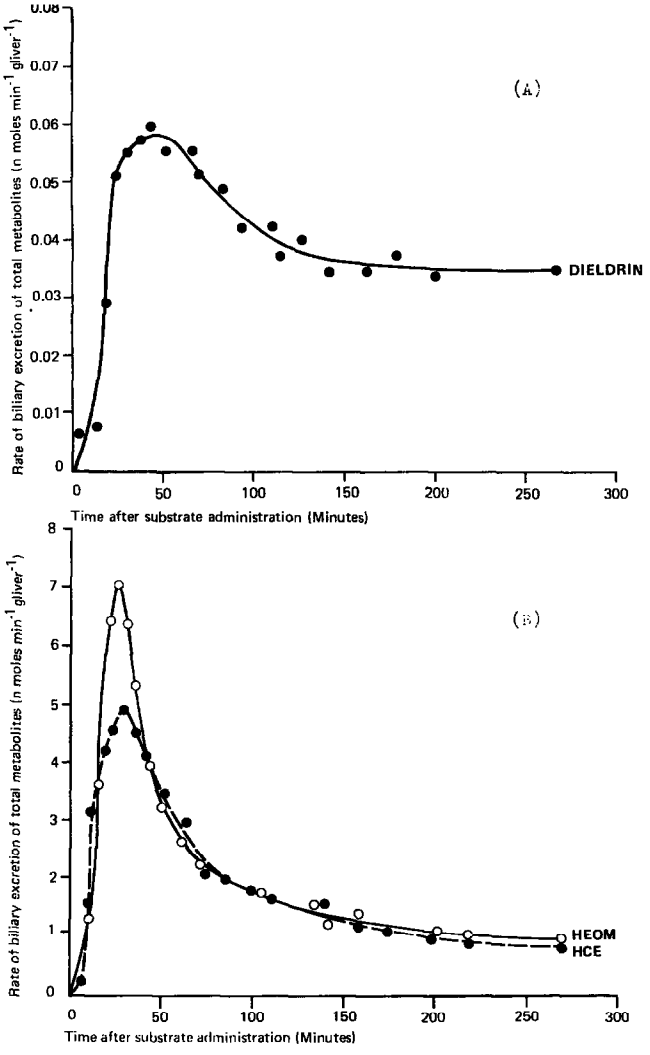

Fig. 4. Typical biliary excretion patterns of total metabolites of dieldrin, HCE and HEOM in the rat. Substrates were administered as ethanolic solution by intraperitoneal injection at a dose rate of 15 mg/kg.

was present as HCE, HEOM or dieldrin in this or any other extract of the sample. The nature of the ^{14}C in bile was investigated in two ways.

Firstly, the samples were treated with β-glucuronidase or sodium metaperiodate so that released aglycones could be extracted into diethyl ether. With β-glucuronidase treatment, samples were incubated at neutral pH to avoid acid hydrolysis of conjugates which would occur at the low pH value required for optimal enzyme activity. Early experiments with HCE metabolites showed that 60 per cent of the ^{14}C was extractable from bile after prolonged incubation with the enzyme. A further 29 per cent became extractable if the bile was treated with 0.1 per cent sodium dodecyl sulphate (SDS) before incubation with β-glucuronidase (Table 2). This improvement could not be attributed to the effect of SDS alone and was thought to result from the release of conjugated material from bile salt–phospholipid micelles. 78 and 36 per cent of the radioactivity in bile was extractable after treatment with SDS and β-glucuronidase from HEOM- and dieldrin-treated rats respectively. Only small amounts of ^{14}C were released from control incubations or from incubations with aryl sulphatase in the case of all three compounds. Approximately 95 per cent of the dieldrin metabolites in bile were extractable with hexane following treatment with sodium metaperiodate. From this and other evidence (see on) it was concluded that most of the ^{14}C excreted following dosing with HCE, HEOM and dieldrin, was in the form of conjugates which were predominantly glucuronides in the case of HCE and HEOM.

Secondly, intact conjugates in bile from HCE- and dieldrin-treated rats were extracted at pH 2 with diethyl ether: ethanol (3:1 v/v) and ethyl acetate prior to examining them by thin layer chromatography using system A. 88 and 91 per cent of the ^{14}C was extracted in this way for HCE and dieldrin metabolites respectively.

The nature of the conjugates in bile was investigated for each substrate. Considering HCE, the metabolites extracted after treatment with β-glucuronidase were examined by gas chromatography (Table 1). Seventy-five per cent of the material released (67 per cent of the

Table 2. Enzymatic hydrolysis of biliary conjugates

Treatment to 0.2–0.5 ml bile. All incubations were carried out at 37° with 3 ml phosphate buffer (0.05 M Ph 7) in stoppered flasks for 72 hours. Enzyme additions (~ 2000 units) were 12 hourly	% of Total ^{14}C extracted into ether after treatment (Mean ± S.E.M.) (n = no. of samples)	
A. Bile from rats injected with [^{14}C]-HCE (15 mg/kg i.p.)		
No treatment to bile	0.44 ± 0.04	n = 8
Incubation alone	5.0 ± 0.62	n = 6
Incubation with SDS 0.1%	6.8 ± 1.4	n = 6
Incubation with β-glucuronidase (bovine)	60.8 ± 5.0	n = 6
Incubation with SDS (0.1%) + β-glucuronidase (bovine)	89.0 ± 3.2	n = 12
Incubation with aryl sulphatase (~ 200 units)	2.2 ± 0.09	n = 3
B. Bile from rats injected with [^{14}C] Dieldrin (15 mg/kg i.p.)		
No treatment to bile	4.0 ± 0.32	n = 3
Incubation alone	9.0 ± 0.91	n = 3
Incubation with β-glucuronidase (bacterial ~ 500 units) + SDS (0.1%)	36.0 ± 3.6	n = 7
Incubation with aryl sulphatase (200 units)	9.5 ± 0.76	n = 3
C. Bile from rats injected with [^{14}C]-HEOM (15 mg/kg i.p.)		
No treatment to bile	1.2 ± 0.04	n = 3
Incubation alone	6.2 ± 0.2	n = 3
Incubation with β-glucuronidase (bovine) + SDS (0.1%)	78.0 ± 3.8	n = 4
Incubation with aryl sulphatase (~ 200 units)	5.9 ± 0.31	n = 3

SDS = sodium dodecyl sulphate

^{14}C in bile) was identified as HHC (structure 3 Fig. 1) while 9 per cent (8 per cent of the ^{14}C in bile) was as secondary metabolites [5]. When extracted conjugates were run on thin layer plates, 84 per cent of the applied radioactivity (74 per cent of the total ^{14}C in bile) was located in one spot (R_f 0.62), which coincided in position with an authentic sample of the glucuronide of HHC. A further 9 per cent of the applied radioactivity was identified as unconjugated HHC. Thus it was concluded that the major primary metabolite *in vitro* (HHC) accounted for not less than 67% of the total excreted ^{14}C, and this was mainly in the form of the glucuronide. With dieldrin, the metabolites extracted after treatment with sodium metaperiodate were examined by gas chromatography and thin layer chromatography. Not less than 74 per cent of the ^{14}C in bile was accounted for as 9-hydroxy-dieldrin (Structure 7 Fig. 2). The R_f value for 9-hydroxy-dieldrin using system B for thin layer chromatography was 0.59. Gas chromatographic values are given in Table 1. Thin layer chromatography of extracted conjugates showed that 86 per cent of the applied material (78 per cent of the ^{14}C in bile) was located in a spot with an R_f value of 0.45. Thus, the main primary metabolite *in vivo* was identified as 9-hydroxy-dieldrin, accounting for not less than 74 per cent of the total excreted ^{14}C in both induced and non-induced rats: nearly all of this was present as a conjugate. Gas chromatographic analysis (Table 1) of HEOM metabolites extracted from bile following β-glucuronidase treatment (Table 2) showed two major metabolites to be present: hydroxy-HEOM (Structure 10 Fig. 3) (23 per cent of extracted material) and HEOM *trans*-diol (Structure 11 Fig. 3) (75 per cent of extracted material). Hence in this case not less than 76 per cent of the total excreted radioactivity was accounted for as the oxidative product hydroxy-HEOM and the hydrative product HEOM *trans*-diol. Both appeared to be in the form of glucuronide conjugates.

The maximal rates of biliary excretion for total metabolites are given in Table 3. These are comparable for HCE (204 nmoles/kg body wt/min) and HEOM (298 nmoles/kg body wt/min) in control rats and are very much larger than the corresponding value for dieldrin (3.17 nmoles/kg body wt/min). Phenobarbital pretreatment had little effect upon the rate of excretion of HCE metabolites, there being a slight increase in terms of the total body weight, but a slight decrease with reference to liver weight. Neither of these changes was statistically significant. The increase in overall excretion following induction was less than the increase in liver weight and in the rate of bile flow. With dieldrin, on the other hand, phenobarbital pretreatment caused a significant increase in the excretion rate in terms of body weight (3 fold) and in terms of liver weight (2.5 fold).

In order to facilitate comparison with *in vitro* data, livers were removed from animals at different times after dosing [^{14}C]-HCE. The total radioactivity and the total HCE content were measured in the whole liver and, in certain cases, in the microsomal fraction (Table 4). With control animals injected with HCE (15 mg/kg), a maximum concentration of 26.4 μg HCE/g liver was reached within 3 min of dosing, and 21 per cent of this was estimated to be associated with liver microsomes. (Total microsomal protein was assumed to be 45 mg/g liver, see Discussion). Thirty minutes after dosing, the hepatic levels in two phenobarbital-pretreated rats (0.83 and 0.44 μg/g) were much lower than that found in a control rat (10.7 μg/g).

In parallel *in vitro* experiments, the rates of metabolism of HCE and HEOM were measured in microsomal preparations, reinforced with NADPH from control and phenobarbital treated rats (Table 5). UDPGA was not included in the medium. This was because UDPGA was not found to stimulate the hydroxylation of HCE by microsomal preparations, in contrast to the situation

Table 3. The excretion of HCE and dieldrin metabolites from control and phenobarbital-treated male rats

Intraperitoneal dose of organochlorine substrate (15 mg/kg)	Treatment	Maximum values obtained for total metabolite excretion into bile (nmoles/kg body wt/min)	(nmoles/g liver/min)	(nmoles/ml bile)	Bile flow rate (ml/kg/hr)	Liver wt. (% body wt)
[^{14}C]-HCE ($n = 9$)	Control	203.6 ± 6.5	4.58 ± 0.13	3013 ± 263	4.23 ± 0.37	4.47 ± 0.17
[^{14}C]-HCE ($n = 6$)	Phenobarbital*	217.4 ± 16.4 ns	4.34 ± 0.29	2545 ± 130	5.15 ± 0.14^{I}	5.00 ± 0.09^{II}
[^{14}C]-Dieldrin ($n = 5$)	Control	3.17 ± 0.55	0.073 ± 0.012	49.2 ± 9.8	4.0 ± 0.33	4.41 ± 0.05
[^{14}C]-Dieldrin ($n = 7$)	Phenobarbital*	9.36 ± 1.43^{I}	0.18 ± 0.025^{III}	117.6 ± 18.5^{III}	4.91 ± 0.2^{II}	5.2 ± 0.1^{II}
[^{14}C]-HEOM ($n = 4$)	Control	298.0 ± 10.6	6.60 ± 0.19	—	—	—

Figures are means ± S.E.M.
Significantly higher than control values (Students t-test):-
I – $P < 0.1$; II – $P < 0.05$; III – $P < 0.02$; IV – $P < 0.01$.
ns = not significant.
The maximum excretion rates were observed within 1 hr of substrate administration to rats.
* Phenobarbital was administered (75 mg/kg) for three successive days prior to the organochlorine injection.

Table 4. Concentrations of HCE in the liver of rats following HCE administration (15 mg/kg i.p.)

Time after injection	Concentration of HCE μg/total liver	μg/g liver	% of total liver HCE associated with the microsomal fraction (assuming 45 mg microsomal protein per g liver)
0.5 min	237 ($n = 2$)	16.9	
1.5 min	323	21.4	
3 min	396 ± 37 (S.E.M.) ($n = 4$)	26.4	21%
5 min	390	25.7	
10 min	161	12.4	21.5%
30 min	140	10.7	28.9%
1 hr	120	9.3	
2 hr	82	5.9	
5 hr	80	5.9	
10 hr	61	4.8	
15 hr	82	5.6	
	Phenobarbital-pretreated rats		
30 min	14.1	0.83	
	7.5	0.44	

HCE was extracted from liver homogenates into 20% acetone: hexane and analysed by gas chromatography.

found with dieldrin [14]. Metabolism of HCE was predominantly oxidative, the dominant metabolite being HHC. In the longer term, secondary oxidative metabolites of HCE can be produced as the substrate concentration falls [15] (see Fig. 1), but this situation was avoided here by ensuring that the substrate/product ratio remained sufficiently high to prevent this occurring.

Using HCE as substrate, a Lineweaver–Burk plot [16] was constructed employing incubation times of 30 sec, 3 and 5 min. The activity of hepatic microsomal monooxygenase remained constant over this period.

HCE has an extremely low solubility in water, and a correspondingly high solubility in oil. Because of this, practically all of the HCE added to incubation media is found in the microsomal membranes, with negligible concentrations in the aqueous phase. Thus, the concen-

Table 5. The metabolism of HCE, HEOM and dieldrin by rat liver microsomes

Substrate dose	Mode of metabolism	Control nmoles of substrate metabolised/min per mg microsomal protein	Control nmoles of substrate metabolised/min per g liver	Phenobarbital Treated nmoles of substrate metabolised/min per mg microsomal protein	Phenobarbital Treated nmoles of substrate metabolised/min per g liver*
HCE ($n = 6$) 16 μg/mg microsomal protein	hydroxylated	0.53 ± 0.05	24.00 ± 2.3	5.54 ± 0.45	404.3 ± 32.8
HEOM ($n = 2$) 17 μg/mg microsomal protein	hydroxylated	0.58 ± 0.04	26.1 ± 1.8	—	—
Dieldrin ($n = 1$) 1.3 μg/mg microsomal protein	hydrated	0.36 ± 0.03	16.2 ± 1.4	—	—
	hydroxylated	—	—	0.0028+	0.204

Microsomal preparations from male Wistar rats were preheated for 90 seconds with reaction medium prior to addition of HCE and HEOM. Incubations were then carried out for 30 sec or 3 min, and terminated by partitioning with diethyl ether. Figures are means ± S.E.M.

* Total microsomal protein content per g liver was assumed to equal 45 mg (control rats) and 73 mg (phenobarbital-treated rats) (see Discussion). Microsomal protein yields were 18.7 ± 1.08 mg g liver^{-1} ($n = 6$) (control) and 30.2 ± 2.08 mg/g liver^{-1} ($n = 6$) (phenobarbital-treated).

+ Data for dieldrin metabolism from Hutson [14] where hepatic microsomes were obtained from a male CFE rat and incubation was for 30 min. Sodium phenobarbitone was administered in the diet (100 ppm for 2 weeks).

Sodium phenobarbitone was administered (75 mg/kg i.p.) once a day for three successive days prior to preparing hepatic microsomes.

trations of HCE have been expressed in terms of nmoles per mg protein, where protein is taken to represent a constant proportion of the weight of the microsomal membrane. Under the experimental conditions used here the initial velocities of enzymic transformation were dependent upon the concentration of HCE per mg microsomal protein, and were independent of the concentration of microsomal protein in the incubation medium. The enzyme activity was therefore linear with respect to both time and microsomal protein concentration. The resulting line gave a correlation coefficient of 0.964 when subjected to linear regression analysis (Fig. 5). From this an apparent V_{max} value of 0.48 nmoles. mg protein/min was estimated. The plot was used to estimate the metabolic rate at the highest substrate concentration encountered *in vivo*.

HEOM was metabolized both oxidatively and hydratively. For a 30 sec incubation at a substrate concentration of 17 μg HEOM/mg microsomal protein, 60 per cent of the observed conversion was oxidative, and 40 per cent was hydrative (Table 5). The rate of degradation of HEOM (0.94 nmoles/mg protein/min) was more rapid than that measured for HCE under similar conditions (0.53 nmoles/mg protein/min).

DISCUSSION

In attempting to relate *in vitro* to *in vivo* results, it was necessary to estimate the total microsomal protein per gram liver. The total microsomal protein content in the case of control male rat liver has been estimated as 45 mg/g liver using epoxide hydratase as a marker [17] and 47 mg/g liver using cytochrome *P*-450 as a marker [18]. Values of 45 mg/g liver (control) and 73 mg/g liver (phenobarbital-pretreated [18]) were used in the present study to convert *in vitro* enzyme activities expressed per milligram of microsomal protein to activities per gram liver. Thus, in the case of non-induced rats, the maximum microsomal concentration

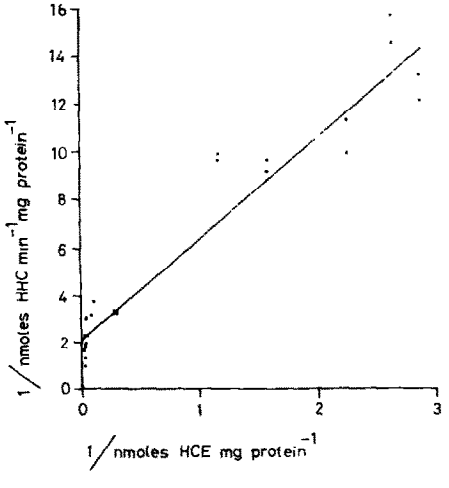

Fig. 5. Lineweaver–Burk plot of HCE hydroxylase activity. Collated data from 10 rats (No. of observations = 30). Correlation coefficient of regression analysis = 0.964. Incubation times were 0.5, 3 or 5 min. For structure of HCE and HHC see Fig. 1.

of HCE was taken to be 0.35 nmoles/mg protein, and the expected rate of hydroxylation from Fig. 5 is 0.0702 nmoles/mg protein/min, which is equivalent to 3.16 nmoles/g liver/min. This figure is well below the maximum capacity of the microsomes to hydroxylate HCE (approx. V_{max} = 21.6 nmoles/g liver/min) and is comparable to the maximum excretion rate of HHC (3.07 nmoles/g liver/min) and to the maximum excretion rate of HHC plus secondary metabolites derived from HHC (3.44 nmoles/g liver/min). The reasonably close agreement between *in vivo* and *in vitro* hydroxylation rates is encouraging in view of the difficulty in making conditions *in vitro* exactly comparable with those operating *in vivo*, e.g with regard to the concentration and distribution of HCE in microsomes.

After phenobarbital induction the microsomal protein yield per g liver was increased by about 65 per cent, the liver weight by about 10 per cent (Table 3), and the rate of HHC formation from HCE per gram liver by about 17 fold. On the other hand the rate of excretion of HHC was not significantly altered either per unit body weight or per gram liver. The HCE content of microsomes fell dramatically compared with the control (< 10 per cent of control value). Thus the very small apparent increase in excretion rate per unit body weight is more than offset by the increase in liver size and of bile flow, and if it is real, may be explicable in terms of increased hepatic blood flow [19] and an attendant small increase in the rate of uptake of HCE by the liver.

The metabolites of HEOM were also excreted at a rate very much lower than the rate of metabolism observed *in vitro* (16 per cent of this rate), suggesting that the metabolism of HEOM *in vivo*, like that of HCE, does not approach the maximum capacity of the enzymes involved. HEOM contrasted with HCE and dieldrin in having as its major excreted metabolite a hydrative rather than an oxidative metabolite.

Dieldrin, like HCE and HEOM, has marked liposolubility, but is only slowly metabolised to 9-hydroxy dieldrin which is the major metabolite in the rat. Hutson [14] reports that 0.0028 nmoles of 9-hydroxy-dieldrin were formed per mg microsomal protein per minute during a 30 min incubation of microsomes from a phenobarbital-pretreated male CFE rat, in the presence of UDPGA. This would give a value of 0.204 nmoles/g liver/min assuming linearity with time and a total microsomal content of 73 mg protein/g liver. This was probably an underestimate since this conversion is unlikely to be linear over 30 minutes due to lipid peroxidase activity [20]. However it is of a similar order to the maximum excretion rate for total dieldrin metabolites in phenobarbital pretreated rats (0.18 nmoles/g liver/min), at least 74 per cent of the biliary metabolites being accounted for as conjugated 9-hydroxy-dieldrin.

The observation that the rate of excretion of dieldrin metabolites in terms of gram liver increased 2.5 fold ($P < 0.02$) after induction, is in line with the findings of Engebretson and Davison [21] who observed an enhanced excretion of dieldrin metabolites from rats following phenobarbital treatment. Cook [22] has shown that phenobarbital treatment reduced the dieldrin concentration in rat adipose tissue. So there is good evidence that the excretion rate of dieldrin metabolites is affected by the metabolic capacity. It might be argued that the rate limiting step was the conjugation of 9

hydroxy-dieldrin. However, this is unlikely since the rate of initial oxidative metabolism is extremely slow at a relatively high substrate concentration and appears to approximate to the excretion rate. 9-hydroxy-dieldrin was not excreted in the bile to any appreciable extent in the unconjugated form. Furthermore, in the case of the dieldrin analogue HCE, which is hydroxylated far more rapidly than is dieldrin, there was no evidence for the build up of primary metabolites in the liver.

It is interesting to note that endrin, which is metabolised relatively rapidly by microsomes, is excreted into bile more rapidly than dieldrin in the case of rats [12]. Endrin metabolites displayed a similar excretion pattern to that observed for HCE and HEOM metabolites in the present study.

The maximum rates of microsomal metabolism of HCE and HEOM (> 24 nmoles/g liver/min) are considerably greater than the maximum excretion rate of the metabolites. Since increasing the enzyme activity by treatment with a microsomal inducing agent does not increase the excretion rate of HCE per gram liver, the latter is evidently determined by the availability of the compounds to the liver. This contrasts with the situation for dieldrin which is slowly metabolised and where the excretion rate evidently depends on metabolic capacity.

These conclusions refer to the male rat which has a relatively high hepatic microsomal monooxygenase activity, and an unusually short half life for dieldrin (approximately 10 days compared with 47 days for the pigeon and 270 days for man [see 3]). The dependence of the excretion rate of dieldrin upon induction may be greater in species which metabolise the compound relatively slowly and have correspondingly long half lives for it.

All three of the substrates studied here have high liposolubility coupled with extremely low water solubility. At the dose levels encountered here, it seems likely that there is a 'threshold' metabolic rate below which the rate of excretion may be limited by the rate of metabolism. Considering the maximal rates of breakdown measured *in vitro*, it may be suggested that this threshold is in the region of 0.5–5 nmoles/g liver/min, under our conditions. Such a threshold would depend upon the rate at which the substrate reached the liver and could therefore depend upon the partition coefficient of any particular compound. In this context it is interesting to note that HEOM metabolites are excreted 44 per cent more rapidly than HCE metabolites at the same dose rate. Since both compounds were degraded at rates well above the excretion rates, metabolism is unlikely to be rate limiting. It therefore seems probable that HEOM is taken up by the liver more rapidly than HCE, perhaps because it is the more polar compound of the two. It may therefore be suggested that compounds which are more polar than dieldrin and its analogues, and have thereby a better balance between lipid and water solubility, will be taken up by the liver relatively rapidly, and will therefore have relatively high threshold metabolic rates.

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