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COMPARATIVE METABOLISM OF A DIELDRIN ANALOGUE: HEPATIC MICROSOMAL SYSTEMS AS MODELS FOR METABOLISM IN THE WHOLE ANIMAL

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Abstract—The metabolism of the liposoluble dieldrin analogue HCE† was studied *in vivo* and *in vitro* in the rat, rabbit, pigeon and Japanese quail. Comparisons were made between the metabolites formed by liver microsomal preparations and those released from conjugates in the bile and urine. Under both *in vitro* and *in vivo* conditions, metabolism was predominantly oxidative in all species with the formation of a major primary metabolite and the progressive conversion of this to two secondary metabolites. The pattern of oxidative metabolism after 30 min of microsomal incubation was broadly similar to that found in bile after 2 hr collection and in urine after 24 hr collection. A minor metabolic route involved hydration of HCE to a *trans*-diol by microsomal epoxide hydratase and this was found in all species except the pigeon both *in vivo* and *in vitro*. There were certain discrepancies between *in vitro* and *in vivo* studies with regard to the formation of minor metabolites and to the quantity of *trans*-diol formed relative to other metabolites. In the case of the rat, the rate of oxidative transformation by microsomes was much higher than the rate of excretion of the metabolites (both figures being expressed in terms of g liver), suggesting that the excretion rate was not limited by the metabolic rate.

It is relatively easy to study the metabolism of xenobiotics *in vitro* using preparations such as microsomes, high speed supernatants, and plasma. Under these conditions one can investigate more closely the nature and activity of the enzymes involved than is possible in whole animal experiments. Thus it is worthwhile considering the extent to which *in vitro* studies can predict events *in vivo*. Is it possible to replace expensive and time-consuming *in vivo* experiments by studies of this kind, in such fields as the testing of pesticides and drugs? If *in vitro* studies have some predictive value, then they may be used to identify major species differences in drug metabolising enzymes. The fact that correlations have been found between hepatic microsomal monooxygenase activities and the half life of certain drugs which are metabolised by this enzyme system tends to support this point of view [1].

In the present context variations in drug metabolising enzymes of the liver are of interest as factors affecting the persistence and selective toxicity of lipophilic chlorinated insecticides. Of particular interest are the hepatic microsomal enzymes monooxygenase and epoxide hydratase (EC 4.2.1.63) which play a dominant role in the initial degradation of dieldrin and its analogues. Comparative studies of microsomal monooxygenase activity and microsomal epoxide hydratase activity have been carried out using dieldrin analogues as substrates [2]. This work revealed striking interspecific variations in epoxide hydratase, mammals showing much higher activities than birds or fish. Microsomal monooxygenase was less variable than epoxide hydratase, although the fish-eating shag showed a much lower activity than the terrestrial species investigated [2].

The present work was undertaken to test the usefulness of microsomal systems for predicting *in vivo* metabolism in the case of the dieldrin analogue HCE.

MATERIALS AND METHODS

Animals. Mature male rats, rabbits, pigeons (*Columba livia*) and Japanese quail (*Coturnix coturnix japonica*) were used throughout. Wistar rats (200–350 g) were obtained from Charles River, Manston, Kent: Old English rabbits (2–2.5 kg) were from Goodchild Farms, Crawley, Sussex, while pigeons (350–450 g) were from Roebuck Farm, Welwyn, Herts. Japanese quail were bred in the Departmental Animal House and were used when between six and nine weeks of age at a weight of 90–110 g.

Substrates. The cyclodiene epoxide HCE was chemically synthesized as described previously [3]. [^{14}C]-HCE was prepared from [^{14}C]-hexachlorocyclopentadiene (supplied by the Radiochemical Centre, Amersham, Bucks) as described by Walker and El Zorgani [4]. The [^{14}C]-HCE substrate was employed for *in vitro* studies as an ethanolic solution (2 mg ml^{-1}) with a specific radioactivity of 19 μCi mmole^{-1}. For *in vivo* studies, animals received an intraperitoneal dose of 15 mg kg^{-1} [^{14}C]-HCE (9.2 μCi mmole^{-1}) in ethanolic solution (50 mg ml^{-1}). Animals were given intraperitoneal injections of isotonic saline (1 ml kg^{-1}) before and after the dose to

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

dilute the ethanol and to prevent inflammation of the gut. A sample of [^{14}C]-HHC (Fig. 1, Structure 3) was obtained by biosynthesis from [^{14}C]-HCE, as described elsewhere [5]. The [^{14}C]-HHC substrate was employed to study conjugating enzymes as a 2 mg ml^{-1} ethanolic solution, with a specific radioactivity of 13 μCi mmole^{-1}.

Reagents and enzymes. 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. NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were supplied by Boehringer Corporation Ltd. Aryl sulphatase (Type III, limpet) and β-glucuronidase (Type B1, bovine) were supplied by the Sigma Chemical Co. Washed microsomes were prepared as described elsewhere [4] and resuspended in isotonic KCl solution (1.15%) to give the equivalent of 0.5–4 g of liver per ml of enzyme preparation. Microsomal protein was determined by the method of Lowry *et al.* [6].

Incubation procedure. 0.5 ml aliquots of microsomal preparations (equivalent to 0.8–1.5 g liver) were added to 4.2 ml of phosphate buffer (0.11 M) at pH 7.4 in 25 ml Erlenmeyer flasks. To this were added 0.2 ml of NADPH generating system (20 μmoles of glucose 6-phosphate, 20 μmoles of NADPH and 1.8 units of glucose 6-phosphate dehydrogenase) and 20 μl of 20 mg ml^{-1} [^{14}C]-HCE in ethanol [4]. Incubations were carried out at 37° for the rat and rabbit, but at 42° for the quail and pigeon (see Table 3 for further details).

Extraction and analytical techniques. Reactions were stopped by partitioning the contents of the incubation flasks with 3 ml diethyl ether. Extraction was completed by two further ether partitions. Trimethyl silyl (TMS) derivatives of metabolites were prepared by the method previously described [4]. TMS derivatives were examined on a Perkin Elmer F11 Gas chromatograph fitted with an electron capture detector and glass columns. The following stationary phases were used: (1) 2.5% w/w SE52 and 0.5% w/w epikote 1001 resin on 80–100 mesh acid-washed DMCS-treated chromosorb W. (2) 2.5% w/w Apiezon L and 0.2% w/w epikote 1001 resin on 100–120 mesh acid-washed DMCS-treated chromosorb W. (3) 3.0% w/w OV225 and 0.25% w/w epikote 100–120 mesh acid-washed DMCS-treated chromotreated chromosorb W.

The ^{14}C-content of the ether extracts and the residual aqueous mixtures was determined by scintillation counting.

Collection of bile and urine. Rats received a re-entrant bile duct cannula from which intermittent bile collections could be made without anaesthesia, as described by Chipman and Cropper [7]. The bile ducts of the rabbit, pigeon and quail were cannulated by a similar technique. Both bile ducts of the quail were cannulated, but the relatively small right bile duct of the pigeon was not cannulated.

Following [^{14}C]-HCE administration, bile was collected in tubes kept on ice. Rat urine was separated from faeces in metabowl cages. Rabbit cages were fitted with plastic mesh floors which held the faeces but allowed the urine to pass through to a collection tray below. Urine samples were obtained from colostomized pigeons, using the technique of Fussell [8] with the following modifications:-Vinyl tubing (3 mm i.d./4 mm o.d.) was used as a semi-permanent cannula for the rectum, replacing the temporary glass cannula used by Fussell in the chicken. This was necessary to prevent stenosis of the relatively small lumen of the pigeon rectum. A postoperative liquid diet, consisting of 10 per cent ground diet 509 (BOCM Silcock) and a mineral–vitamin mix (see Table 2) suspended in a 50 per cent chicken egg yolk solution, was administered (5 ml twice daily) until replacement with diet 509 alone was possible without causing rectal blockage and subsequent impaction of faeces (normally about 4 days). Pigeons were given at least

Table 1. Chromatographic characteristics of HCE and its oxidative metabolites

Compound	Compound number in Fig. 1	Gas–liquid chromatography * relative retention time		
		2·5% Apiezon	2.5% SE52	3% OV225
HCE	1	73	70	60
Monohydroxy-HCE (HHC)	3	250	199	n.d.
TMS derivative of HHC	3	125	140	95
endo-HHC	4	294	234	n.d.
TMS derivative of *endo*-HHC	4	145	222	110
Dihydroxy-HCE (DHHC)	5	445	390	n.d.
TMS derivative of DHHC	5	254	305	150
TMS derivative of HCE *trans*-diol	2	178	197	100
TMS derivative of metabolite X	unknown structure	213	222	254

* Gas–liquid chromatographic relative retention times are calculated with respect to HEOD (dieldrin) = 100.

See extraction and analytical techniques for details of columns.

n.d. = not detectable.

TMS = Trimethyl silyl derivative.

Table 2. Composition of mineral–vitamin mix given post-operatively to pigeons undergoing colostomy

	Amount per kg diet
Cupric Carbonate	18.5 mg
Sodium fluoride	4.4 mg
Potassium iodate	0.8 mg
Ferrous sulphate	130.5 mg
Magnesium carbonate	1.6 g
Manganese carbonate	245 mg
Sodium molybdate	2.5 mg
Sodium selenite	0.2 mg
Zinc carbonate	138.0 mg
Calcium orthophosphate	25.0 mg
Sodium chloride	5.0 g
Potassium chloride	5.0 g
Calcium hydrogen orthophosphate	8.3 g
Rovimix AD3 vitamins (type 500/100) (Roche Products Ltd. London)	20 mg

7 days postoperative recovery period prior to [^{14}C]-HCE administration followed by collections of urine and faeces for up to a further 10 days.

Breakdown of conjugates in bile and urine. Conjugates in bile were hydrolysed by treatment with β-glucuronidase in the presence of sodium dodecyl sulphate (0.1 %) in 0.05 M phosphate buffer at pH 7. Conjugates in the urine were hydrolysed by acidification to 2 M with respect to HCl and incubation at 75° for 30 min. In the case of both bile and urine, the released metabolites were extracted by three partitions with diethyl ether.

RESULTS

The metabolic fate of HCE in vertebrate liver microsomes reinforced with NADPH and O_2 is shown in Fig. 1 (HCE = structure No. 1). The dominant conversion in all species studied is hydroxylation to HHC (Fig. 1, Structure 3). When the substrate is reduced to relatively low concentrations, secondary oxidation products are formed from HHC. Firstly, an epimer of HHC (*endo*-HHC, Fig. 1, Structure 4) is formed and this is subsequently hydroxylated to give a dihydroxy-epoxide (DHHC, Fig. 1, Structure 5) [4]. This reaction sequence is observed in microsomal preparations given relatively low loadings of HCE, as detailed in Fig. 2(B). Where higher concentrations of HCE are used, the secondary metabolism does not occur and the rate of formation of HHC under standard conditions may be determined [2]. In a second line of metabolic attack, epoxide hydratase converts HCE to its *trans*-diol (Fig. 1, Structure 2). This was a minor metabolic pathway which was most developed in the rabbit and was not found at all in the pigeon.

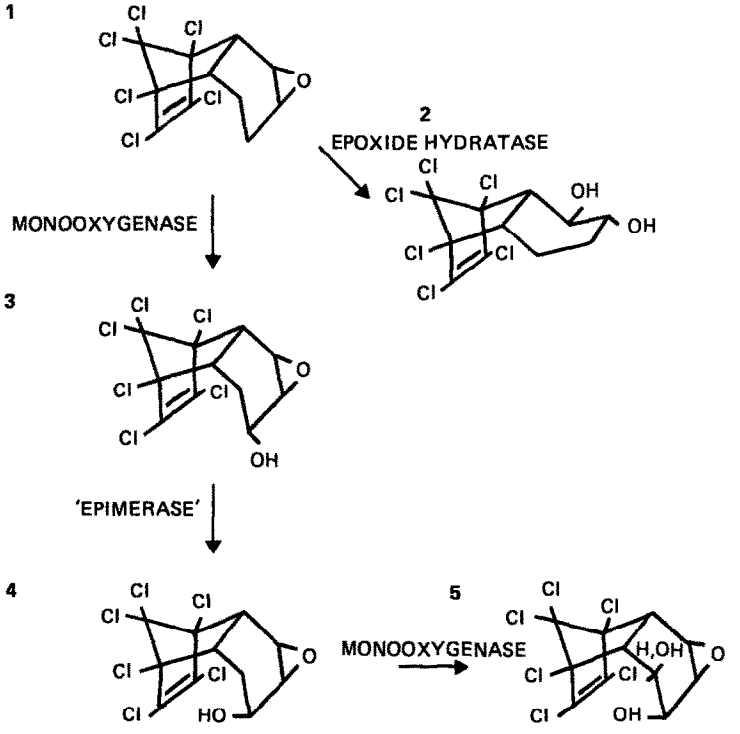

Fig. 1. The metabolism of HCE. (1) HCE (2) HCE *trans*-diol (3) monohydroxy-HCE (HHC) (4) *endo*-HHC (5) dihydroxy-HCE (DHHC). The metabolic pathway shown was found *in vitro* and *in vivo* for the rat, rabbit and Japanese quail. The pigeon carried out all the oxidative conversions but did not produce measurable quantities of HCE *trans*-diol.

Both the monooxygenase and 'epimerase' conversions occured in microsomes in the presence of NADPH and O_2. It is not known whether they are mediated by the same enzyme system [5].

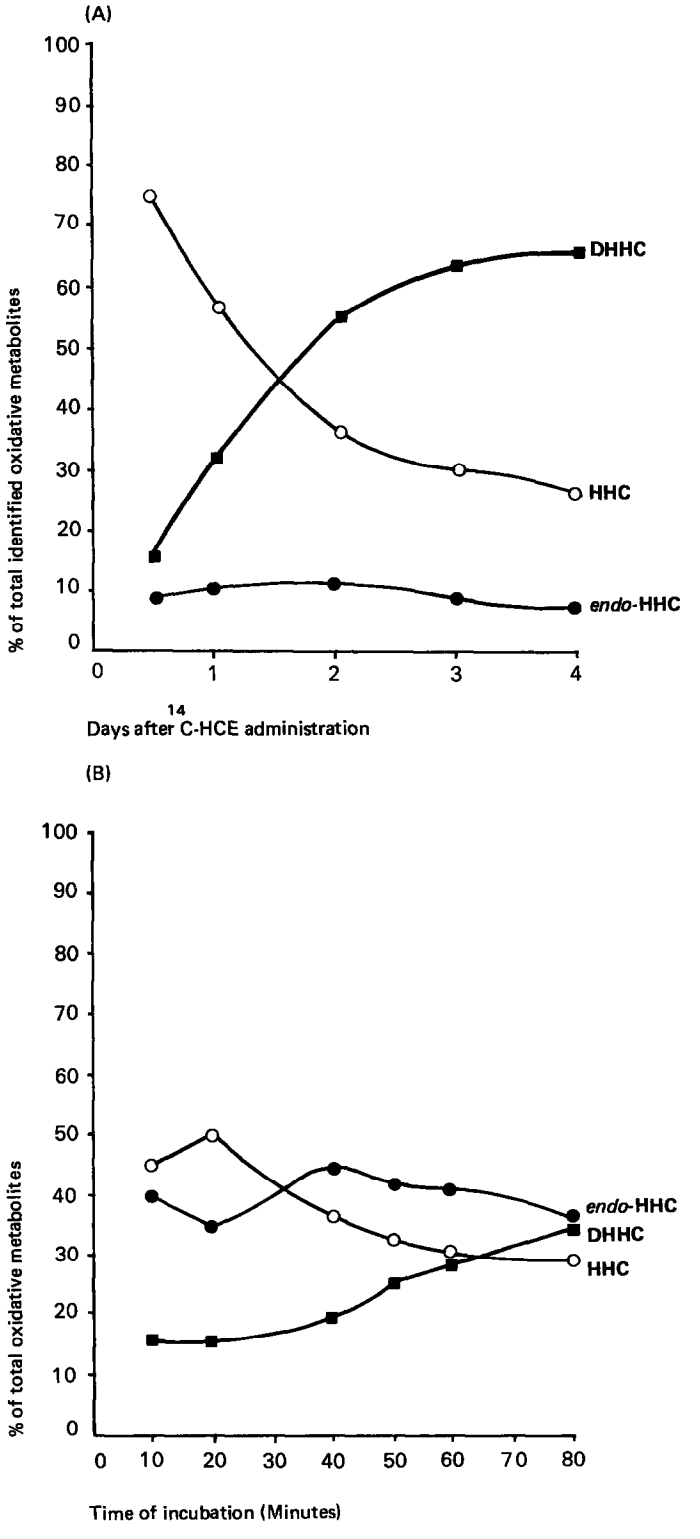

Fig. 2. Pattern of HCE oxidative metabolism observed in the rat with respect to time.

(A) *In vivo*: metabolites found in bile with intermittent collections. Each point represents a mean value from four rats. 37 per cent of the ^{14}C dose (15 mg kg^{-1}) was excreted into the bile within 10 hours of administration of [^{14}C]-HCE. The oxidative metabolites HHC, *endo*-HHC and DHHC shown in this figure accounted for 82 per cent of the total excreted radioactivity. (See Fig. 1 for structures).

(B) *In vitro*: metabolism by hepatic microsomes reinforced with NADPH and O_2. Each point represents a mean value from duplicate incubations (8 μg [^{14}C]-HCE g liver equivalent^{-1}). The oxidative metabolites HHC, *endo*-HHC and DHHC shown in this figure accounted for 95 per cent of the total metabolites found. (See Fig. 1 for structures).

Table 3. HCE metabolites found *in vitro* and *in vivo*

(a) *In vivo*

Species	Number of animals	% Total ^{14}C in bile or urine which was extractable	Individual metabolites as a % total metabolites extracted (compound number in Fig. 1 given in parentheses) HHC (3)	*endo*-HHC (4)	DHHC (5)	HCE *trans*-diol (2)	X	Others
		In bile						
Rat	7	89	61	7	14	4	8	6
Pigeon	4	91	50	46	4	0	0	
Japanese quail	4	57	63	19	5	0†	13	
		In urine*						
Rabbit	3	87	68	10	19	3		
Pigeon	4	95	89	6.4	4.6	0		

(b) *In vitro* (Microsomes)‡

Species	Number of experiments	% Substrate converted	g Liver represented per incubation	Individual metabolites as a % total metabolites HHC (3)	*endo*-HHC (4)	DHHC (5)	HCE *trans*-diol (2)	others
Rat	6	60	1.5	41	33	20	5	0
Rabbit	3	75	0.8	41	4	10	30	15
Pigeon	3	55	1.0	45	31	20	0	4
Japanese quail	3	25	1.0	70	12	8	7	0

Only traces of radioactivity were found in rat urine and rabbit bile. All urine and bile collections were made during the first 24 hr and two hours, respectively, following dosing (15 mg kg^{-1} [^{14}C]-HCE). Concentrations of metabolites were determined by gas–liquid chromatography.

* Urine samples were adjusted to 2 M with respect to HCl before incubating for 30 min at 75° to break down conjugates. Material so released was extracted with various organic solvents as detailed in the text.

† The *trans*-diol has, however, been isolated from quail droppings [4].

‡ Microsomal incubations were at 37° for the mammals, but at 42° for the two birds. 40 μg of HCE was added to 5 ml of incubation medium containing microsomal material derived from the stated quantity of liver.

Clearly the time of incubation is important in determining the pattern of metabolites found *in vitro*. The picture after 30 min incubation with a low substrate concentration is shown in Table 3(b).

The emphasis here has been upon initial metabolic attack. It should be mentioned, however, that HHC is converted to a glucuronide in the presence of liver microsomes reinforced with uridine 5′-diphosphoglucuronic acid (UDPGA), and to a sulphate in liver 105,000 *g* supernatants containing 3′-phosphoadenosine 5′-phosphosulphate [5]. All four species were capable of effecting these conversions.

Turning now to *in vivo* metabolism, [^{14}C]-HCE dissolved in ethanol was given to rats, rabbits, pigeons and quail by intraperitoneal injection. In all species the bile collections were made after fitting re-entrant cannulae. Urine collections were made from rats, rabbits and colostomized pigeons. Nearly all of the excreted radioactivity was found in the bile of the rat, but in the urine of the rabbit. In the pigeon, the urine and the bile contained similar quantities of radioactivity.

The ^{14}C in bile and urine existed largely as conjugates, less than 3 per cent being extractable by ether at pH 7. After treatment of bile with β-glucuronidase [9], the following percentages of ^{14}C became ether-extractable: rat (89 ± 5.2%), pigeon (91 ± 4.3%) and quail (57 ± 4.0%). Incubation of bile samples, with aryl sulphatase [9] did not result in hydrolysis of conjugated metabolites for any of the species. The conjugates in urine were broken down by acid hydrolysis and the following percentages of ^{14}C then became extractable by ether: 87 per cent (rabbit) and 95 per cent (pigeon). The metabolites released from bile and urine are given in Table 3(a) and in Fig. 2(A). HHC and the two secondary oxidative metabolites *endo*-HHC and DHHC are formed in all four species *in vivo*. The increase, with time, of the secondary metabolites at the expense of HHC parallels the situation found *in vitro* (Fig. 2(B)).

With the rat, the biliary excretion rate reached a maximum between 20 and 30 min after dosing with HCE. The maximum excretion rate was much lower than the *in vitro* metabolic rate under conditions of high substrate concentration, when both values are expressed in terms of g liver [9].

DISCUSSION

The *in vitro* work described here is concerned only with liver enzymes and does not attempt to account for metabolism in other organs. This approach was considered to be justified on two grounds. Firstly, when liposoluble compounds are given by intraperitoneal injection [10] (or, for that matter, orally) it is usual for most of the dose to be taken directly to the liver by the hepatic portal system. Secondly, most of the microsomal monooxygenase [11] and epoxide hydratase [12] activity of mammals is located in the liver. In birds, this is also true of microsomal monooxygenase, but not of epoxide hydratase where the kidney and the liver may make similar contributions to the total activity within the body [2]. On the other hand, epoxide hydratase does not appear to attack HCE to any important extent in birds. Indeed epoxide hydratase activity towards dieldrin analogues is generally much lower in birds than in mammals on present evidence [2].

The formation of the *trans*-diol in microsomal preparations occurred most readily in the rabbit. Formation was relatively slow in quail and rat liver microsomes, and no detectable amounts were formed by pigeon liver microsomes. This is in accordance with the order of hepatic microsomal epoxide hydratase activities found in studies with the cyclodiene epoxide HEOM* (rabbit > rat > quail > pigeon [2]). A similar picture was found *in vivo* except that the quantities of *trans*-diol relative to other metabolites were similar in the rat and the rabbit. Small quantities of *trans*-diol were found in quail droppings [4] although this metabolite was not found in quail bile. It should be noted, however, that the recovery of metabolites from quail bile was rather poor. The failure to detect the *trans*-diol in the bile, urine or droppings [4] of the pigeon is in agreement with the failure to demonstrate its formation *in vitro*.

One unidentified metabolite (X) isolated during *in vivo* studies in the rat and the quail was not found *in vitro*.

In all four species, metabolism was predominantly oxidative both *in vivo* and *in vitro*. In all cases HHC was the principal primary metabolite, and *endo*-HHC and DHHC were identified as secondary metabolites. With microsomes, the secondary oxidative metabolites were formed from HHC in the later stages of incubation [13]. It is interesting to compare the patterns of oxidative metabolites found at different times *in vitro* with the patterns found after collection for specified times *in vivo*. There is a reasonable correspondence between the *in vitro* and *in vivo* patterns for each species when the incubations have proceeded for 30 min (Fig. 2 and Table 3). Studies with the rat showed that the tendency for the ratio of secondary: primary metabolites to increase with time is found *in vivo* as well as *in vitro* (Fig. 2). The main discrepancies found between the *in vitro* and *in vivo* situations were (i) the occurrence of certain minor metabolites in one situation but not in the other (see Table 3), such metabolites being found in the rabbit and pigeon *in vitro* and in the rat *in vivo*, and (ii) the difference in relative amounts of *trans*-diol produced in the two situations in the rabbit. It should be noted, however, that unidentified minor metabolites may be included in material which was not extractable from urine or bile. With the rat, minor metabolites were found in the bile which were not found *in vitro*. These may have arisen from conversions carried out by extrahepatic enzymes such as those in the intestine. Thus it may be concluded that the *in vitro* system employed here gives a reasonable prediction of the major primary and secondary oxidative metabolites excreted by the four different species, when the dependence of the metabolic pattern on time is taken into account.

A glucuronide conjugate of HHC was found in the bile of the rat, quail and pigeon, and the formation of this was expected from the results of microsomal assays for glucuronyl transferase using HHC as substrate [5].

The fact that the observed excretion rate for the rat was well below the metabolic rate determined *in vitro* requires further discussion. This should favour the production of secondary metabolites, as indeed proves to be the case. Furthermore, it is not a situation in which the rate of metabolism is likely to limit the rate of excretion or the length of half life of the compound. The rate at which HCE reaches the endoplasmic reticulum may well be the principal factor determining the rate of excretion here. This raises important issues for the persistent liposoluble chlorinated compounds in general. Compounds like HCE, which are readily biodegradable, may be expected to have relatively short half lives, dependent upon the degree of availability to enzymes rather than upon metabolic rate: species differences in metabolic activity are unlikely to be important here. On the other hand such species differences may result in corresponding differences in the patterns of metabolites formed. This may be the basis for selective toxicity where biological activity is associated with the metabolites rather than the insecticide.

Future investigation into the relationship between *in vivo* and *in vitro* metabolism will be concerned with further elucidation of the change in metabolic pattern with time in the whole animal, and with the comparison of the rates of key metabolic steps in the two situations. Of particular interest regarding biological half lives, will be the relationship, if any, between the metabolic rates *in vitro* and the excretion rates and half lives of liposoluble compounds (including pollutants) which are degraded only slowly by enzyme systems.

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

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