Biochemical Pharmacology, Vol. 35, No. 3, pp. 499–503, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

THE HYDRATION OF STERICALLY HINDERED EPOXIDES BY EPOXIDE HYDROLASE OF THE RAT AND RABBIT

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(*Received* 9 *May* 1985; *accepted* 23 *August* 1985)

Abstract—The hydration of the sterically-hindered epoxides dieldrin (HEOD) (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) and MME (1,2,3,4,9,9-hexachloro-1,4,4a,5,8,8a-hexahydro-6-methyl-6,7 epoxy 1,4-methanonaphthalene) was studied in pig and rabbit liver microsomes, and in apparently homogenous preparations of epoxide hydrolase purified from liver microsomes of the rat and rabbit. A non-hindered substrate, HEOM (1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro exo-6,7 epoxy-1,4 methano naphthalene) was used to assay both the enzyme preparations and the microsomes for epoxide hydrolase activity. The purified enzymes had more stable activity when incorporated into suspensions of phospholipid derived from rat liver microsomes than when used alone, so this preparation was used for long-term incubation with HEOD and MME. Activity towards the three substrates followed the sequence HEOM ⟫ MME ⟫ HEOD, in the approximate ratios $1.5 \times 10^6 : 10^4 : 1$, the activity towards dieldrin being only 0.0065–0.052 pmol/mg prot/min. The sole product of MME hydration was identified as a *trans* diol. The only product identified in the case of HEOD hydration by microsomes or enzyme preparations was the *trans* diol so there was no evidence for a significant metabolic pathway through the *cis* diol which is mediated by epoxide hydralase.

Hepatic microsomal epoxide hydrolase can hydrate to *trans* diols many potentially toxic epoxides including certain carcinogens, mutagens and insecticides [1]. Usually hydration is rapid, but there are certain sterically hindered epoxides which are metabolised very slowly; notable amongst these are some dieldrin analogues which were some of the first substrates to be used in the discovery of epoxide hydrolase [2, 3]. The formation of both a *trans* and a *cis* diol has been reported when the sterically hindered epoxide dieldrin (HEOD) is added to hepatic post mitochondrial supernatants of the rat [4, 5]. The *cis* diol was found to be converted to the *trans* diol by an NADPH-dependent epimerase. It was suggested, on the basis of this and other evidence, that the initial product in microsomes is a *cis* diol, but that this is largely converted to a *trans* diol, the latter being the only hydration product of HEOD reported by other workers [2, 6]. In another study Forrest *et al.* [7] reported the slow formation of a diol from the sterically-hindered dieldrin analogue MME (1,2,3,4,9,9-hexachloro 1,4,4a,5,8,8a, hexahydro-6-methyl-6,7 epoxy 1,4-methanonaphthalene) in pig liver microsomes; the NMR spectrum of this diol suggested a *cis* rather than *trans* configuration.

To clarify the enzymic processes responsible for these slow hydrations, the metabolism of HEOD and MME was studied in pure preparations of hepatic microsomal epoxide hydrolase. The activities of these preparations were initially determined using another dieldrin analogue, HEOM (1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro exo-6,7 epoxy-1,4 methano naphthalene), which is rapidly metabolised [3, 8].

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (*ca.* 200 g) were supplied by Versuchstierzuchtanstalt Sulzfeld, F.R.G. Male New Zealand White rabbits were supplied by a private breeder (*ca.* 3 kg). Samples of pig liver came from Reading Abbatoir.

Preparation of epoxide hydrolase

Microsomes were prepared as described [9]. Epoxide hydrolase was prepared from rat liver by the method of Bentley and Oesch [10]. Epoxide hydrolase was prepared from rabbit liver by DEAE-Sepharose chromatography and chromatofocusing [11, 12].

Preparation of lipid extract

Liver microsomes from phenobarbitone-treated rats were extracted with 3:1 chloroform:methanol. After drying over anhydrous sodium sulphate, the volume of the extract was reduced to give a concentration of 15 mg lipid/ml. This was stored in a dark bottle at 0–4° until needed. Aliquots were blown down under nitrogen, and suspensions prepared by sonicating at 0–4° with a phosphate buffer at pH 7.4.

Reagents

Reagents were of Analar grade or better. Solvents were of chromatographic grade. Their purity was checked by gas chromatography.

Authentic samples of HEOD, aldrin *cis*-diol and aldrin *trans*-diol were gifts from Shell Research Laboratories, Sittingbourne, Kent. MME was a gift from

Dr K. A. Hassall. HEOM and MME were synthesised as described elsewhere [13, 7]. All of these compounds were >99% pure. Solutions containing 2 mg/ml of HEOM, HEOD and MME in ethanol were used as substrates. Samples of MME diol were obtained by incubating MME with pig and rabbit liver microsomes as described elsewhere [7].

Assay procedures

Microsomal assays were performed as previously described [7, 8]. Appropriate aliquots of preparations of purified enzymes (1–5.3 mg protein/ml) were added to lipid suspensions (15 mg/ml) in the proportion 1:10 protein:lipid. Samples of these preparations were heat-denatured by holding for 10 min at 100°. Between 20 μg and 40 μg of MME and HEOM were added to 2 ml volumes of incubation media containing 2.5–10 μg of enzyme protein. In the case of HEOD, 20 μg was added to 5 ml aliquots of incubation medium each containing 0.5 mg of enzyme protein. Incubations with these enzyme/lipid preparations, and with enzyme alone were performed for varying periods of time in 15 ml glass stoppered tubes held in a shaking water bath at 37°, and were terminated by extracting with 3 × 3 ml diethyl ether. For long incubations, sterile precautions were observed, and in no case was bacterial growth observed. Trimethyl silyl derivatives of extracted metabolites were prepared, and their concentrations determined by electron-capture gas chromatography (GC) using 5 ft all-glass columns packed with 2.5% SE52 and 0.2% Epikote on 80/100 Chromosorb W. Confirmatory runs were done using glass columns packed with 2.5% SE30 and 0.2% Epikote on 80/100 Chromosorb W [14]. Assays of the enzyme with styrene oxide were done by a radiometric method [10].

Certain extracts were examined by thin-layer chromatography (TLC). Silica G plates of 250 μm thickness were prepared in the laboratory and stored in a desiccator prior to use. They were run in 1:1 and 2:1 benzene:ethyl acetate and the spots were located by spraying with 1% $AgNO_3$ in ethanol, and then irradiating with 254 nm u.v. light (see [14]).

Preparations of derivatives of MME diol

(i) *Borate.* A saturated solution of boric acid in acetone was allowed to migrate up thin-layer plates. The plates were then removed and dried. Concentrated samples of extracts and of authentic diol standards were applied to the plates. The plates were then run in 1:1 benzene:ethyl acetate before development and the spots located as described above.

(ii) *Acetonide.* Twenty micrograms of diol was added to 10 ml of acetone and 100 mg of freshly prepared anhydrous $CuSO_4$, in a 100 ml round bottomed flask with two side arms. The mixture was refluxed under argon for 4 hr, cooled and filtered. Subsamples of filtrate were reduced to dryness and examined on GC both before and after silylation. MME diol and the *cis* and *trans* diols of aldrin were all treated in this way.

RESULTS

Hydration of HEOD and MME by microsomes

Both HEOD and MME were hydrated by pig and rabbit liver microsomes. In both species a single product was formed from MME (Table 1) which corresponded to the diol prepared previously [7]. The initial rates of formation of the diol in nmols/g liver/min were 1.2 in the pig and 0.4 in the rabbit. After 930 min the rate of formation of diol was still 75% of the initial rate in the rabbit. In the case of HEOD the only product identified corresponded to the *trans* diol on TLC and GC.

Hydration of HEOM, HEOD and MME by epoxide hydrolase purified from rat and rabbit liver microsomes

The activity of apparently homogenous preparations of rat and rabbit epoxide hydrolase incorporated into lipid suspension was assayed using HEOM (Table 2; Fig. 1). The preparations tested showed constant activity up to 10 min and >76% of initial activity at 40 min. One rabbit preparation and one rat preparation both showed 61% of initial activity at 360 min. By contrast, preparations of pure enzyme without lipid showed lower activities than these for both species; initially the difference was small, but was much more marked at 40 min and 360 min (Table 2). After heat treatment activity was usually non detectable, and in no case exceeded 3% of initial activity in preparations of enzyme and lipid. In view of the above findings the enzyme was incorporated into the lipid suspension in all subsequent long term incubations with MME and HEOD.

When MME was incubated with rat or rabbit enzyme in lipid, a single product was identified on

Table 1. Chromatographic characteristics of diols

Diol	Relative retention times (HEOD = 100) SE52 Column: Original diol	Relative retention times (HEOD = 100) SE52 Column: Trimethyl-silyl (TMS) derivative	R_f value on silica gel G plates 1:1 benzene: ethyl acetate
HEOM *trans* diol	450	155	
MME diol	375	170	0.54
Aldrin *cis* diol	147	232	0.69
Aldrin *trans* diol	150	185	0.52

The relative times are for a freshly prepared column. As the column aged these values tended to increase.

Table 2. Stability of epoxide hydrolase preparations

Preparation		Substrate	Activity nmoles/mg protein/min [% Initial activity] 10 min	40 min	360 min	1700 min
Rat	Enzyme 1 alone	HEOM	12.8 [100]	9.1 [71]	4.4 [34]	
	Enzyme 1 + lipid	HEOM	13.2 [100]	12.3 [93]	8.1 [61]	
	Enzyme 1 + lipid	MME	0.17 [100]			0.064 [35]
Rabbit	Enzyme 1 + lipid	HEOM	31 [100]	24 [77]	18 [61]	
	Enzyme 1 + lipid	MME	0.21 [100]			0.084 [40]
	Enzyme 2 alone	HEOM	150 [100]	63 [49]		
	Enzyme 2 + lipid	HEOM	167 [100]	144 [86]		

In assay systems containing lipid, pure enzyme was incorporated into suspensions of lipid from rat liver microsomes at the rate of 1 μg enzymic protein to 10 μg of lipid. Rat enzyme 1 and rabbit enzyme 1 had been stored at −20° for 3–6 months. Rabbit enzyme 2 was freshly prepared.

Individual values represent means based on at least three replicate assays.

Standard errors for replicate determinations did not exceed ±4% in any case.

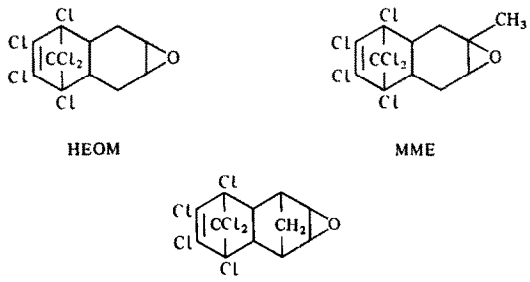

Fig. 1. Structures of organochlorine substrates.

two TLC systems and on GC before and after silylation; this corresponded to the diol formed by pig and rabbit liver microsomes (Table 1). The rates of formation in nmoles/mg protein/min were 0.17 and 0.21 for the rat and the rabbit enzymes respectively. Between 35 and 40% of the initial activity was found at 1700 min, and no detectable product was formed in preparations which had been heat-treated. No problems with bacterial growth were encountered in this or any other long term incubation (Table 2).

Incubations of the most active rat and rabbit enzyme/lipid preparations with HEOD for periods <2160 min yielded very small amounts of product. Accordingly extracts of each incubation were run on TLC alongside standards of aldrin *cis* diol and aldrin *trans* diol. Before irradiating the plates, the tracks containing the products of dieldrin metabolism were covered with metal strips to protect them from the action of u.v. light. After development of colour, the positions on the plate which should contain *cis* and *trans* diols originating from extracts were marked out. These areas were carefully removed and extracted with acetone and methanol. After blowing the extracts to dryness under nitrogen, the residues were examined by GC in the usual way. The presence of the *trans* diol was established (Table 3) in two separate runs for both rat and rabbit preparations whilst no *cis* diol was found. Any significant con-

Table 3. The activity of purified epoxide hydrolase preparations towards different substrates

Species enzyme prep.	Incubation time (min)	Activity towards various substrates (pmoles/mg prot./min) HEOM	MME	HEOD	Styrene oxide (SO)*	Ratio of activities to different substrates HEOM SO	HEOM MME	HEOM HEOD
Rat (1)	5	13.2×10^3			333×10^3	0.040		
	60	13.2×10^3	110				120	
Rat (2)	5	27×10^3			840×10^3	0.032		
	2160			0.0065				
	1700	10×10^3						1.5×10^6
Rabbit (1)	5	31×10^3			252×10^3	0.12		
	180	21×10^3	150				140	
	1700		69					
Rabbit (2)	60	136×10^3						
	1700	67×10^3						
	2160			0.052				1.3×10^6

All values obtained with preparations containing 1:10 protein:microsomal lipid except for the styrene oxide assay (*) which was performed with the enzyme alone. Activity ratios were calculated from values obtained with two substrates incubated for the same length of time, except in the case of dieldrin, where the activity measured at 2160 min was compared with the activity for HEOM at 1700 min. The enzyme preparations corresponded to those referred to in Table 2 (Rat 1, Rabbit 1 and Rabbit 2).

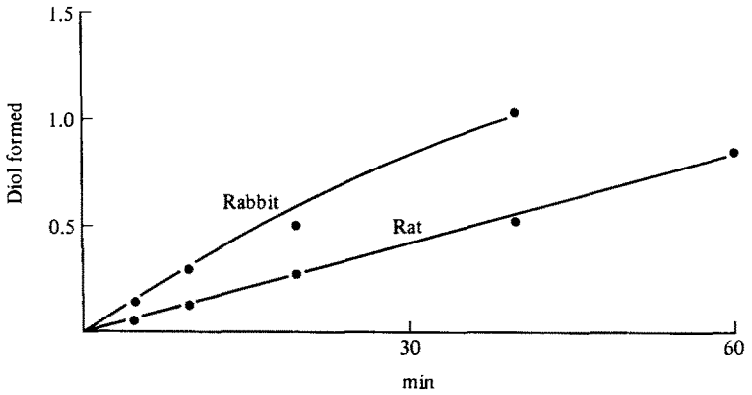

Fig. 2. Hydration of HEOM by epoxide hydrolase preparations in lipid suspension. The enzyme preparations correspond to "rat 1" and "rabbit 1" cited in Table 2. All points represent mean values for at least three assays. Diol formation, μmols/mg protein.

tribution by chemical hydration can be discounted as no products were found when the incubations were carried out with heat-denatured preparations of rat enzyme + lipid. This is in agreement with the finding of Brooks *et al.* [2], who were unable to show any conversion in heat-denatured microsomes. Thus the *trans* diol was found in these enzyme preparations, and the *cis* diol was not found down to the limits of detection (approximately 10% of the lowest level of *trans* diol).

The results of chemical tests upon the MME diol prepared from rabbit liver microsomes were as follows. The R_f value of this compound on TLC was not affected by the presence of boric acid on the plate; on the same plate aldrin *cis* diol was held back at the point of application whereas the migration of the *trans* diol was unaffected. After attempting synthesis of acetonide, products were examined by GC before and after TMS derivatisation. Of the three diols tested, only the aldrin *cis* diol was converted into an acetonide derivative. Neither aldrin *trans* diol nor MME diol were affected by acetone treatment. Thus both of these tests clearly indicated that MME diol has a *trans* and not a *cis* configuration.

DISCUSSION

The hydrative capacity of preparations of microsomes and/or of epoxide hydrolase in lipid suspensions towards the three substrates is heat-sensitive, indicating that this is an enzymic reaction. Microsomes and enzyme preparations in lipid suspensions showed more stable activities than did enzyme preparations without lipid, and when MME and HEOM were used as substrates, assays of enzyme preparations in lipid suspension still showed 35–40% of the initial activity at 1700 min (Table 2).

Activity towards different substrates followed the order HEOM⟫MME⟫HEOD in enzyme/lipid preparations of both rat and rabbit, the ratios being, in round figures, $1.5 \times 10^6 : 10^4 : 1$ (Table 3). This provides striking evidence for the influence of steric hindrance upon the rate of hydrolysis by epoxide hydrolase, and is in general agreement with the conclusions of Brooks *et al.* [2] and Oesch *et al.* [15], who drew attention to the effects of steric hindrance upon rates of hydration by epoxide hydrolase.

These results throw some light on the stereochemistry of hydration of sterically-hindered substrates. The NMR spectrum of the diol of MME suggested that it might have a *cis* configuration [7], but the present study clearly indicates that it is a *trans* diol. It is clearly desirable to repeat the NMR study with a more powerful spectrophotometer, and including a sample of MME *cis* diol produced by chemical synthesis (e.g. by osmylation of the olefine precursor of MME). In the case of HEOD, the only product identified after long term microsomal incubation in the absence of NADPH was the *trans* diol, in agreement with the findings of Brooks *et al.* [2]. The product formed by pure rat and rabbit epoxide hydrolase in lipid was also the *trans* diol.

Working on HEOD metabolism by rat liver post mitochondrial supernatants reinforced with NADPH, Matthews and McKinney [4] produced evidence suggesting that the *cis* diol is formed first and is then converted to the *trans* diol by an epimerase which is NADPH-dependent. However, in agreement with earlier studies by Brooks *et al.* [2], only the *trans* diol was identified when pig and rabbit liver microsomes were incubated with HEOD in the absence of NADPH, suggesting that epoxide hydrolase does not form significant quantities of the *cis* diol. Likewise, only the *trans* diol was identified when purified epoxide hydrolases of the rat and rabbit were incubated with HEOD. Thus *cis* diol formation by epoxide hydrolase attack does not appear to be a significant metabolic route in the species investigated, although such a conversion may be affected by NADPH-dependent oxidation; Bedford and Hutson [16] have suggested this mechanism for *trans* diol formation. However, the formation of *cis* diol *in vivo* has yet to be established.

Acknowledgements—One of us (CHW) was supported by a travel grant from I.C.R.E.T.T. The authors are grateful to Drs. A. Seidel, K. L. Platt, C. T. Bedford and P. M. Dansette for valuable discussion

REFERENCES

1. F. Oesch, in *Progress in Drug Metabolism*, Vol. 3 (Eds. J. W. Bridges and L. F. Chasseaud). John Wiley, Chichester (1979).
2. G. T. Brooks, A. Harrison and S. E. Lewis, *Biochem. Pharmac.* **19** 225 (1970).
3. G. T. Brooks, *World Rev. Pest Contr.* **5**, 62 (1966).
4. H. B. Matthews and J. D. McKinney, *Drug Metab. Dispos.* **2**, 333 (1974).
5. J. D. McKinney, H. B. Matthews and N. K. Wilson, *Tetrahedron Lett.* **21**, 1895 (1973).
6. F. Korte and H. Arent, *Life Sci.* **4**, 2017 (1965).
7. T. J. Forrest, C. H. Walker and K. A. Hassall, *Biochem. Pharmac.* **28**, 859 (1979).
8. A. C. C. Craven, C. H. Walker and G. T. Brooks, *Pestic. Biochem. Physiol.* **6**, 132 (1976).
9. J. K. Chipman and C. H. Walker, *Biochem. Pharmac.* **28**, 1337 (1979).
10. P. Bentley and F. Oesch, *FEBS Lett.* **59**, 291 (1975).
11. F. Oesch, C. W. Timms, C. H. Walker, T. M. Guenthner, A. Sparrow, T. Watabe and C. R. Wolf, *Carcinogenesis* **5**, 7 (1984).
12. C. W. Timms, manuscript in preparation.
13. G. T. Brooks and A. Harrison, *Biochem. Pharmac.* **13**, 827 (1964).
14. C. H. Walker and G. A. El Zorgani, *Arch. Env. Cont. Toxicol.* **2**, 97 (1974).
15. F. Oesch, D. M. Jerina and J. Daly, *Biochim. biophys. Acta* **227**, 685 (1971).
16. C. T. Bedford and D. H. Hutson, *Chem. Ind.* 440 (1976).