Purification and Specificity of a Human Microsomal Epoxide Hydratase

By FRANZ OESCH

Biocenter of the University of Basel, CH-4056 Basel, Switzerland

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Epoxide hydratase was solubilized from human liver microsomal fractions and purified to an extent where the specific activity was 40-fold greater than that of the liver homogenate. Combination of homogenate and purified preparation showed that the increase in activity was not due to the removal of an inhibitor. Monosubstituted oxiranes with a lipophilic substituent larger than an ethyl group (isopropyl, *t*-butyl, *n*-hexyl, phenyl) readily interacted as substrates or inhibitors with this purified human epoxide hydratase, whereas those with a small substituent (methyl, ethyl, vinyl) were inactive, probably reflecting greater affinity of the former epoxides owing to lipophilic binding sites near the active site of the enzyme. In a series of oxiranes having a lipophilic substituent of sufficient size (styrene oxides), monosubstituted as well as 1,1- and *cis*-1,2-disubstituted oxiranes readily served as substrates or inhibitors of the enzyme, but not the *trans*-1,2-disubstituted, tri- or tetra-substituted oxiranes. *trans*-Substitution at the oxirane ring apparently prevents access of the oxirane ring to the active site by steric hindrance. Epoxide hydratase was also solubilized from microsomal fractions of rat and guinea-pig liver and purified by the same procedure. Structural requirements for effective interaction of substrates, inhibitors and activators were qualitatively identical for epoxide hydratase from the three sources. However, several quantitative differences were observed. Thus human hepatic epoxide hydratase seems to be very similar to, although not identical with, the enzyme from guinea pig or rat. Studies with epoxide hydratase from the latter two species therefore appear to be significant with respect to man. In addition, knowledge of structural requirements for epoxides to serve as substrates for human epoxide hydratase may prove useful for drug design. Compounds which need aromatic or olefinic moieties for their desired effect would not be expected to lead to accumulation of epoxides if their structure was such as to allow for a metabolically produced epoxide to be rapidly consumed by epoxide hydratase.

Arene oxides and alkene oxides (formalistically resulting from the epoxidation of one of the double bonds of an aromatic nucleus or of an olefinic double bond respectively) are formed from aromatic and olefinic foreign compounds by liver microsomal mono-oxygenases (Jerina *et al.*, 1968*a*, 1970; Leibman & Ortiz, 1970; Maynert *et al.*, 1970; Selkirk *et al.*, 1971; Grover *et al.*, 1971*a*, 1972; Oesch *et al.*, 1972). These epoxides are reactive electrophiles, and it has been clearly established that several of them bind to macromolecular tissue constituents (Grover & Sims, 1970; Lawley & Jarman, 1972) and exert mutagenic (Loveless, 1966; Huberman *et al.*, 1971; Ames *et al.*, 1972) and carcinogenic (Van Duuren *et al.*, 1967*a,b*; Grover *et al.*, 1971*b*; Marquardt *et al.*, 1972) effects. Epoxide hydratase (EC 4.2.1.63) converts such epoxides into far less reactive vicinal diols (Oesch *et al.*, 1971*a,b*; for a complete review see Oesch, 1973). To date no information about epoxide hydratase in man is available. Since these enzymes may be of critical importance to the extent of accumulation of reactive

epoxides and therefore to carcinogenic or hepatotoxic effects caused by such reactive metabolites of aromatic or olefinic compounds it was decided to investigate the existence and specificity of human epoxide hydratase. Epoxide hydratase was solubilized from human liver microsomal fractions and purified, and the specificity of the enzyme with respect to substrates, inhibitors and activators was investigated. By using the same procedure, epoxide hydratase was also solubilized and purified from rat and guinea-pig liver microsomal fractions and the specificity of the enzyme from these three sources was compared to clarify whether studies performed with epoxide hydratases from the latter two species are relevant to the situation in man.

Materials and Methods

Chemicals

[7-³H]Styrene oxide (Oesch *et al.*, 1971*c*), [3,4,5,6-³H]cyclohexene oxide (Oesch *et al.*, 1971*b*),

benzene oxide (Jerina *et al.*, 1968b) and naphthalene 1,2-oxide (Vogel & Klärner, 1968) were synthesized as described. Other compounds were gifts or were prepared by standard procedures essentially as described for the synthesis of [³H]-styrene oxide (Oesch *et al.*, 1971c) or obtained from commercial sources. Compounds were purified until homogeneous as judged by t.l.c. (benzene-chloroform-ethyl acetate, 1:1:1, by vol.). No resolution of the racemic mixtures has been attempted.

All epoxides gave a positive reaction with the epoxide spray reagent (Oesch *et al.*, 1971c). The reaction is based on the formation of alkali on heating an epoxide with NaI (Ross, 1950), detected by a redox indicator. All compounds synthesized were checked by mass spectrometry (AEI MS 30 instrument). The parent peak, identified by its increasing intensity relative to other peaks at lowered energy of the bombarding electron beam to near the appearance potential (Silverstein & Bassler, 1968), and fragmentations were compatible with the structures expected from the synthetic procedure for each compound prepared. Where enough synthetic material was available, n.m.r. data were also obtained. In the following compilation δ values with respect to tetramethylsilane at 60 MHz (Varian A-60 instrument) in CCl₄ (except where stated) are given, and these abbreviations are used: s = singlet, d = doublet, q = quartet, m = multiplet, dist. = distorted, sl. = slightly. Benzene oxide (equilibrium mixture with oxepine) (100 MHz, Varian HA-100): CH α (2H, 5.30, d, dist. 5 Hz), CH β (2H, 5.57–5.69, m), CH γ (2H, 6.06–6.14, m). *m*-Bromostyrene oxide: CH₂ oxirane [H *cis* with respect to bromophenyl: 1H, 2.62, 2d, sl. dist., 6 Hz (*gem*), 2.4 Hz (*trans*); H *trans* with respect to bromophenyl: 1H, 3.03, 2d, dist. 6 Hz (*gem*), 4 Hz (*cis*)], CH oxirane and benzylic [1H, 3.72, 2d, 4 Hz (*cis*), 2.4 Hz (*trans*)], aromatic (4H, 7.09–7.51, m). *p*-Bromostyrene oxide: CH₂ oxirane [H *cis* with respect to bromophenyl: 1H, 2.60, 2d, sl. dist., 6 Hz (*gem*), 2.4 Hz (*trans*); H *trans* with respect to bromophenyl: 1H, 3.03, 2d, 6 Hz (*gem*) 4 Hz (*cis*)], CH oxirane and benzylic [1H, 3.70, 2d, 4 Hz (*cis*), 2.4 Hz (*trans*)], aromatic (2H *ortho* to epoxyethyl, 7.0–7.28, heavily dist. d, 8 Hz; 2H *ortho* to bromo, 7.33–7.53, heavily dist. d, 8 Hz). [³H]Cyclohexene oxide: CH₂ (4H, 1.12–1.57, broad and complex; 4H, 1.67–2.02, broad and complex), CH oxirane (2H, 2.88–3.00, broad) (spectrum of commercial cyclohexene oxide identical). 3,3-Dimethylbutene oxide (in C²HCl₃): CH₃ (9H, 0.92, s), CH and CH₂ oxirane (3H, 2.57–2.79, m). 2,2-Dimethyl-1-phenyloxirane: CH₃ (*cis*: 3H, 1.01, s; *trans*: 3H, 1.43, s), CH oxirane (1H, 3.70, s), aromatic (5H, 7.22, s, sl. dist.). *cis*-1,2-Dimethyl-1-phenyloxirane: β -CH₃ (3H, 1.39, d, 6 Hz), α -CH₃ (3H, 1.59, s), CH oxirane (1H, 2.78, q, sl. dist., 6 Hz), aromatic (5H, 7.22, s, dist.). *trans*-1,2-Dimethyl-1-

phenyloxirane: β -CH₃ (3H, 0.94, d, 6 Hz), α -CH₃ (3H, 1.58, s), CH oxirane (1H, 3.03, q, sl. dist., 6 Hz), aromatic (5H, 7.23, s, dist.). 1,1-Diphenyloxirane: CH₂ oxirane (2H, 3.13, s), aromatic (10H, 7.26, s). 1-Methylcyclohexene oxide (90 MHz in C²HCl₃, Bruker HX-90): CH₃ (integration not feasible since peak sits on top of broad CH₂ absorption, 1.28, s), CH₂ (together with CH₃ accounting for 11H, 1.10–1.42, broad; 1.60–1.89, broad), CH oxirane (1H, 2.89–2.98, broad). 3-Methylcyclohexene oxide: CH₃ (integration accounting for 1.13H, 1.00, s; integration accounting for 1.88H, 1.12, s, 3:5 mixture of *cis* and *trans* isomers?), CH and CH₂ (3H, 1.20–1.48, m; 4H, 1.61–1.97, broad and complex), CH oxirane (2H, 2.62–3.04, m). 1-Methyl-1-phenyloxirane: CH₃ (3H, 1.67, s), CH₂ oxirane (H *cis* with respect to phenyl: 1H, 2.61, d, dist., 6 Hz; H *trans* with respect to phenyl: 1H, 2.85, d, 6 Hz), aromatic (5H, 7.24, s, sl. dist.). *cis*-2-Methyl-1-phenyloxirane: CH₃ (3H, 1.06, d, 5 Hz), CH oxirane (1H, 3.02–3.39, m), CH oxirane and benzylic (1H, 3.92, d, 4 Hz), aromatic (5H, 7.24, s, sl. dist.). *trans*-2-Methyl-1-phenyloxirane: CH₃ (3H, 1.42, d, 5 Hz), CH oxirane (1H, 2.78–2.92, m), CH oxirane and benzylic (1H, 3.42, d, 2 Hz), aromatic (5H, 7.19, s, sl. dist.). Naphthalene-1,2-oxide: CH oxirane (1H, 3.85–4.01, m; 1H, 4.30, d, 4 Hz), vinyl (2H, 6.19–6.75, m), aromatic (4H, 7.18–7.62, m). *m*-Nitrostyrene oxide (90 MHz in C²HCl₃, Bruker HX-90): CH₂ oxirane [H *cis* with respect to nitrophenyl: 1H, 2.80, pair of doublets, 6 Hz (*gem*), 2.4 Hz (*trans*); H *trans* with respect to nitrophenyl: 1H, 3.22, 2d, 6 Hz (*gem*), 4 Hz (*cis*)], CH oxirane and benzylic [1H, 3.97, 2d, dist. 4 Hz (*cis*), 2.4 Hz (*trans*)], aromatic (4H, 7.36–8.18, m). *p*-Nitrostyrene oxide: CH₂ oxirane [H *cis* with respect to nitrophenyl: 1H, 2.66, 2d, 6 Hz (*gem*), 2.4 (*trans*); H *trans* with respect to nitrophenyl: 1H, 3.14, 2d, dist., 6 Hz (*gem*), 4 Hz (*cis*)], CH oxirane and benzylic [1H, 3.89, 2d, dist., 4 Hz (*cis*), 2.4 Hz (*trans*)], aromatic (2H *ortho* to epoxyethyl, 7.44, d, heavily dist., 8 Hz; 2H *ortho* to nitro, 8.16, d, heavily dist., 8 Hz). 3-Phenylpropene oxide: CH₂ benzylic, CH₂ and CH oxirane (5H, 2.32–3.09, m), aromatic (5H, 7.20, s, sl. dist.). *p*-Phenylstyrene oxide: CH₂ oxirane [H *cis* with respect to biphenyl: 1H, 2.67, 2d, 6 Hz (*gem*), 2.4 Hz (*trans*); H *trans* with respect to biphenyl: 1H, 3.05, 2d, dist., 6 Hz (*gem*), 4 Hz (*cis*)], CH oxirane and benzylic [1H, 3.78, 2d, 4 Hz (*cis*), 2.4 Hz (*trans*)], aromatic (9H, 7.16–7.64, m). *cis*-Stilbene oxide: CH oxirane (2H, 4.21, s), aromatic (10H, 7.08, s). [³H]Styrene oxide: CH₂ oxirane [H *cis* with respect to phenyl: 1H, 2.62, 2d, sl. dist., 6 Hz (*gem*), 2.4 Hz (*trans*); H *trans* with respect to phenyl: 1H, 3.00, 2d, sl. dist., 6 Hz (*gem*), 4 Hz (*cis*)], CH oxirane and benzylic [1H, 3.72, 2d, 4 Hz (*cis*), 2.4 Hz (*trans*)], aromatic (5H, 7.22, s, sl. dist.) (identical with spectrum from commercial

styrene oxide). 1,2,2-Trimethyl-1-phenyloxirane: β -methyl, *cis* with respect to phenyl (3H, 0.91, s), β -methyl, *trans* with respect to phenyl (3H, 1.41, s), α -methyl (3H, 1.57, s), aromatic (5H, 7.22, s, sl. dist.).

Assay of epoxide hydratase activity

The radiometric assay based on conversion of [^3H]styrene oxide (1-phenyl-1-[^3H]ethylene oxide) into [^3H]styrene glycol (1-phenyl-1-[^3H]ethylene glycol) was used (Oesch *et al.*, 1971c). Standard incubation mixtures contained 50 μmol of Tris-HCl (pH 9), 0.8 μmol of [^3H]styrene oxide (280000 d.p.m.), 0.8 μmol of inhibitor or activator, and enzyme preparation, in a final volume of 0.4 ml. Substrate and inhibitor were each added in 20 μl of acetonitrile. After incubation at 37°C for 5 min, unchanged substrate was removed by two extractions with 10 ml of light petroleum (b.p. 30–60°C). The product was then extracted into 2 ml of ethyl acetate for liquid-scintillation counting (Oesch *et al.*, 1971c). Product formation was found to be linear with respect to time and protein concentration at each step in the purification. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The method of least squares was used to calculate regression lines for the determination of kinetic parameters, the Student's *t* test to establish the significance of differences between means (Snedecor & Cochran, 1967). The measure of variation in this study is the s.e.m. Determinations were performed at least in triplicate.

Product formation from epoxides

Substrate (5 μmol) was dissolved in 0.1 ml of acetonitrile and added dropwise, with swirling, to a mixture of 0.85 ml of 0.1 M-Tris-HCl buffer (pH 9.0) containing 0.2% (w/v) Tween 80 and 50 μl of the purified human epoxide hydratase at 0°C. After incubation at 37°C for 20 min, products were extracted with 2 $\times$ 3 ml of ethyl acetate. The combined extract was dried over Na_2SO_4 , concentrated under a stream of Ar at 40°C and chromatographed on thin-layer silica gel 60 F₂₅₄ plates (E. Merck, Darmstadt, Germany) with benzene-chloroform-ethyl acetate (1:1:1, by vol.). Compounds were detected with u.v. light, and the product was extracted from the silica gel with 1.0 ml of methanol and determined by u.v. spectroscopy [molar extinction coefficient, ϵ , of *trans*-1,2-dihydroxyindane 650 litre $\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at λ_{max} , 265 nm (Oesch *et al.*, 1971b); ϵ for the glycol products from the styrene oxide derivatives were assumed to be similar to those of the corresponding oxides (214 and 225 litre $\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at λ_{max} , 258 nm for 1-methyl-1-phenyloxirane and *trans*-2-methyl-1-phenyloxirane respectively),

since ϵ for styrene oxide and styrene glycol (218 litre $\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at λ_{max} , 258 nm) were indistinguishable]. Product formation from [^3H]styrene oxide and from [^3H]cyclohexene oxide was determined as described (Oesch *et al.*, 1971b, c).

Solubilization and purification of a human hepatic epoxide hydratase

A sample (80 g) of human liver was obtained 10 h after death from a healthy 43-year-old Caucasian woman. No gross lesions were observed on pathological examination. In rats post-mortem activity of epoxide hydratase (with [^3H]styrene oxide as substrate) remained virtually unchanged for 16 h after death (decrease less than 10% compared with fresh liver). This does not, of course, strictly exclude post-mortem changes in the human enzyme, since the body temperature decreases more rapidly in the rat than in the human.

All the steps in the following procedure were done at 0–5°C. The liver sample was homogenized in 200 ml of 0.25 M-sucrose containing 0.01 M-sodium phosphate buffer (pH 7.4). The postmitochondrial supernatant (8500 g, 15 min) was centrifuged at 100000 g for 1 h and the resulting pellet was resuspended in 100 ml of 10 mM-sodium phosphate buffer (pH 7.4). A detergent (Cutscum; Fisher Scientific Co., Pittsburgh, Pa., U.S.A.) in aqueous solution was added dropwise, with gentle stirring, to give a final concentration of 1% (w/v). The preparation was then centrifuged at 100000 g for 1 h and the upper layer, containing a whitish material, was removed by aspiration and the remainder of the supernatant was filtered through glass wool. $(\text{NH}_4)_2\text{SO}_4$ was added to 10% saturation and the precipitated protein removed by centrifugation at 27000 g for 10 min. $(\text{NH}_4)_2\text{SO}_4$ was then added to the supernatant to 20% saturation. On centrifugation at 27000 g for 20 min some of the precipitate settled at the bottom of the tube, but most rose to the surface and was collected, dissolved in 10 ml of 10 mM-sodium phosphate buffer (pH 7.4) and desalted by Sephadex G-25 chromatography, the same buffer being used for elution. Active fractions were combined and frozen in solid CO_2 -acetone. After storage for 12 h at –70°C, the sample was thawed and the precipitated protein removed by centrifugation at 27000 g for 20 min. The supernatant (protein concentration 9.76 mg/ml) was divided into 1 ml portions, frozen in solid CO_2 -acetone and stored at –70°C. This preparation lost less than 20% of its activity in 3 months. Purified epoxide hydratase preparations from the liver of male guinea pigs (300–400 g, undetermined strain) and male Sprague-Dawley rats (200–250 g) (protein concentrations of these purified preparations were 8.94 and 9.91 mg/ml respectively) were obtained by the same procedure.

Results and Discussion

Previous studies have shown that epoxide hydratase was localized exclusively in microsomal fractions of guinea-pig liver (Oesch *et al.*, 1971c). Microsomal fractions were therefore isolated from human liver, and epoxide hydratase was solubilized from the microsomal membranes by using Cutscum, a non-ionic detergent. After precipitation and filtration steps (summarized in Table 1) an overall 40-fold increase in specific activity was achieved, with a recovery of 14% of the epoxide hydratase originally present in liver homogenate. Cutscum was added to a portion of the original homogenate (final concentration, 1%, w/v) and this mixture was stirred for 5 min at 0°C. Combination of this suspension with the purified preparation in various proportions always resulted in an activity that was the sum of the activities in the two components (results not shown). Thus the increase in specific activity during purification is not likely to be due to the removal of an endogenous inhibitor. Moreover, epoxide hydratase activity was linear with respect to protein concentration at each step in the purification. Recovery of more than 100% of the epoxide hydratase activity present in the liver homogenate after Cutscum treatment (Table 1) possibly reflects exposure of active sites by the action of the detergent.

Epoxide hydratase was purified from guinea-pig and rat liver microsomal fractions by the same procedure. The ratio of the specific activity of the purified preparation to that of the homogenate was 39 for the guinea pig (107 versus 2.78 nmol of styrene glycol/min per mg of protein) and 32 for the rat enzyme (36.4 versus 1.14 nmol of styrene glycol/min per mg of protein).

Hydration of [^3H]styrene oxide by human epoxide hydratase was not inhibited by monosubstituted oxiranes if the substituent was a methyl, ethyl or vinyl group, indicating that these epoxides either do not bind to the active site of the enzyme or have a much lower affinity for this site than styrene oxide,

since the concentration of all epoxides used was the same. However, the enzyme did interact with monosubstituted oxiranes if the substituent was an isopropyl, *t*-butyl, *n*-hexyl, phenyl or an even larger lipophilic group, presumably reflecting lipophilic binding sites near the active site of the enzyme (Table 2). In a series of oxiranes having such substituents (styrene oxides), human epoxide hydratase readily interacted with mono-, 1,1- and *cis*-1,2-disubstituted oxiranes, whereas *trans*-1,2-disubstituted oxiranes, tri- and tetra-substituted oxiranes did not inhibit the hydration of [^3H]styrene oxide (Table 3). Table 4 shows that the potency of five styrene oxides as inhibitors was paralleled by their ability to serve as substrate for purified human epoxide hydratase: styrene oxide and 1-methyl-1-phenyloxirane were good inhibitors and also good substrates, whereas *trans*-2-methyl-1-phenyloxirane, 2,2-dimethyl-1-phenyloxirane and 1,2,2-trimethyl-1-phenyloxirane did not serve as either inhibitors or substrates. The topography around the active site of the enzyme appears to be such that a *trans*-1,2-disubstitution at the oxirane ring sterically prevents the access of the oxirane ring to the active site, even if the substituent at the 2-position is as small as a methyl group. With 1,1-disubstituted oxiranes effective interaction with the enzyme appeared to decrease with increasing bulk of the two substituents (Table 3). Results obtained with purified epoxide hydratase from human, guinea-pig and rat liver appeared qualitatively identical (Tables 2 and 3). On the other hand, several quantitative differences became apparent. Thus *n*-octene 1,2-oxide was a stronger inhibitor of epoxide hydratase from rat, 3-phenylpropene 1,2-oxide of the enzyme from man and *cis*-2-methyl-1-phenyloxirane of the enzyme from guinea pig and rat compared with the enzyme from the other species, suggesting that the epoxide hydratases purified from man, guinea-pig and rat liver are very similar, but not completely identical.

Table 5 shows that the ability of several styrene oxides with different substituents on the aromatic

Table 1. *Purification of a human hepatic epoxide hydratase*

For details see the text.

Fraction	Protein recovery (%)	Activity recovery (%)	Specific activity (μmol of styrene glycol/min per mg of protein)
Liver homogenate	100	100	0.00294
Microsomal fraction	15.1	52.8	0.0106
High-speed supernatant after treatment with a detergent, Cutscum	12.9	131.3	0.0300
Supernatant after removal of top layer and filtration	7.1	121.1	0.0503
10–20%-satd.-(NH_4) $_2\text{SO}_4$ precipitate after Sephadex G-25 chromatography	1.40	40.6	0.0854
Supernatant after removal of protein precipitated after freezing and thawing	0.34	13.6	0.118

Table 2. *Monosubstituted oxiranes as inhibitors of hydration of [³H]styrene oxide*

Substrate ([³H]styrene oxide) and inhibitor concentrations were 2mM. The inhibitors were added in 20 μ l of acetonitrile at zero time with no preincubation. The same amount of acetonitrile was added to the controls; 50 μ l of purified epoxide hydratase preparation was used per assay. All effects were significant at $P < 0.001$, except for those marked ns.

Inhibitor		Percentage inhibition of epoxide hydratase purified from liver of		
Name	Structure	Man	Guinea pig	Rat
Propene oxide		ns	ns	ns
<i>n</i> -Butene 1,2-oxide		ns	ns	ns
Butadiene monoxide		ns	ns	ns
3-Methylbutene 1,2-oxide		12 $\pm$ 1.2	9 $\pm$ 2.3	17 $\pm$ 2.1
3,3-Dimethylbutene oxide		37 $\pm$ 5.2	39 $\pm$ 2.1	43 $\pm$ 6.0
<i>n</i> -Octene 1,2-oxide		59 $\pm$ 4.4	50 $\pm$ 3.1	82 $\pm$ 3.2
3-Phenylpropene 1,2-oxide		56 $\pm$ 2.3	28 $\pm$ 1.3	40 $\pm$ 2.1
<i>p</i> -Chlorophenyl 2,3-epoxypropyl ether		37 $\pm$ 5.4	38 $\pm$ 6.2	28 $\pm$ 2.7
Styrene oxide		51 $\pm$ 2.1	49 $\pm$ 1.5	52 $\pm$ 2.2
<i>p</i> -Phenylstyrene oxide		68 $\pm$ 4.3	74 $\pm$ 9.2	79 $\pm$ 2.3

* Incubation in sealed tubes at 22°C; for all other compounds in open tubes at 37°C.

ring to inhibit hydration of [³H]styrene oxide was very similar to purified epoxide hydratase from the liver of man, guinea pig and rat. In a more extensive study on structure-activity relationships in a purified preparation from guinea-pig liver (Oesch & Daly, 1971), no correlations between the electronic effects of

substituents and the potency of the unsubstituted styrene oxides as inhibitors were apparent (Oesch *et al.*, 1971a).

The most potent inhibitor of the epoxide hydratase purified from human liver was 3,3,3-trichloropropene oxide (Table 6). At a concentration of only 0.4 mM,

Table 3. *Effect of the substitution pattern of the oxirane ring on the inhibitory potency of various styrene oxides towards hydration of [³H]styrene oxide*

Substrate ([³H]styrene oxide) and inhibitor concentrations were 2mM. For incubation conditions see Table 2. All effects were significant at $P < 0.001$, except for those marked ns.

Inhibitor		Percentage inhibition of epoxide hydratase purified from liver of		
Name	Structure	Man	Guinea pig	Rat
Styrene oxide		51 ± 2.1	49 ± 1.5	52 ± 2.2
1-Methyl-1-phenyloxirane		32 ± 4.1	30 ± 3.1	40 ± 4.3
1-Isopropyl-1-phenyloxirane		24 ± 3.8	22 ± 3.4	29 ± 5.0
1,1-Diphenyloxirane		19 ± 2.7	18 ± 2.1	16 ± 3.2
cis-2-Methyl-1-phenyloxirane		46 ± 3.0	74 ± 6.1	73 ± 4.2
cis-Stilbene oxide		21 ± 8.1	16 ± 3.2	14 ± 2.3
trans-2-Methyl-1-phenyloxirane		ns	ns	ns
trans-Stilbene oxide		ns	ns	ns
trans-1,2-Dimethyl-1-phenyloxirane		ns	ns	ns

Table 3.—*continued.*

Inhibitor		Percentage inhibition of epoxide hydratase purified from liver of		
Name	Structure	Man	Guinea pig	Rat
<i>cis</i> -1,2-Dimethyl-1-phenyloxirane		ns	ns	ns
2,2-Dimethyl-1-phenyloxirane		ns	ns	ns
1,2,2-Trimethyl-1-phenyloxirane		ns	ns	ns

inhibition was almost complete. Both the bulk and the strong electron-withdrawing effect of the trichloromethyl group appeared essential, since 3,3,3-trifluoropropene oxide and 3,3,3-trimethylpropene oxide (3,3-dimethylbutene oxide) were much weaker inhibitors. Again hepatic epoxide hydratase preparations from man, guinea pig and rat acted similarly, with some quantitative differences especially with respect to the inhibitory effect of the 3,3,3-trifluoropropene oxide in the three preparations.

Oxiranes fused to rings also appeared to interact with epoxide hydratase from the three sources to a generally similar extent (Table 7). Indene oxide proved to be both a good inhibitor (Table 7) and an excellent substrate (Table 4) for human epoxide hydratase. Cyclohexene oxide, on the other hand, had been previously shown to be a good inhibitor, but a very poor substrate for guinea-pig hepatic epoxide hydratase, and kinetic analysis showed the inhibition to be of the non-competitive type (Oesch *et al.*, 1971a). Cyclohexene oxide was also a poor substrate for the enzyme from human liver (Table 4), but was much less potent an inhibitor than for guinea-pig epoxide hydratase (Table 7). Inhibition of epoxide hydratase from human liver by cyclohexene oxide might therefore be expected to be of the mixed type. However, kinetic analysis showed the inhibition of the human enzyme to be non-competitive (Fig. 1). The K_m values obtained by calculation of regression

Fig. 1. Inhibition by cyclohexene oxide of a purified hepatic epoxide hydratase from man with [3 H]styrene oxide as substrate

Double-reciprocal plot of velocity (nmol of [3 H]styrene glycol/min per mg of protein) versus concentration of substrate (M). Inhibitor [1 mM (●), 0.5 mM (▲) or zero (■)] was added in 20 μ l of acetonitrile at zero time with no preincubation; 20 μ l of the purified epoxide hydratase preparation was used per incubation. Points represent single determinations.

Table 4. *Diol formation from some epoxides with purified human epoxide hydratase*

Substrate (5 μ mol) was incubated with 50 μ l of the purified human epoxide hydratase at 37°C for 20 min in 0.85 ml of 0.1 M-Tris buffer (pH 9) containing 0.2% Tween 80.

Substrate		Percentage conversion into diol
Name	Structure	
Styrene oxide	*	36
1-Methyl-1-phenyloxirane	†	31
<i>trans</i> -2-Methyl-1-phenyloxirane	†	Not detectable
2,2-Dimethyl-1-phenyloxirane	†	Not detectable
1,2,2-Trimethyl-1-phenyloxirane	†	Not detectable
Indene 1,2-oxide	†	84
Cyclohexene oxide	*	2.6

* Radioactive substrate was used. Product was isolated and determined as described (Oesch *et al.*, 1971b,c).

† Product was isolated by t.l.c. and determined quantitatively by u.v. spectroscopy.

lines by the method of least squares were not significantly different ($0.3 > P > 0.2$) in the presence and in the absence of the inhibitor, cyclohexene oxide. Thus even where quantitatively marked differences became apparent, basically similar mechanisms seem to operate in purified epoxide hydratase preparations from human, guinea-pig and rat liver.

Since intermediate arene and alkene oxides which are formed by the action of microsomal monooxygenases (Jerina *et al.*, 1968a, 1970; Leibman & Ortiz, 1970; Maynert *et al.*, 1970; Selkirk *et al.*, 1971; Grover *et al.*, 1971a, 1972; Oesch *et al.*, 1972) may produce disturbance of normal cell functions (Loveless, 1966; Van Duuren *et al.*, 1967a,b;

Huberman *et al.*, 1971; Grover *et al.*, 1971b; Ames *et al.*, 1972; Marquardt *et al.*, 1972) owing to their alkylating ability (Grover & Sims, 1970; Lawley & Jarman, 1972), and since epoxide hydratase transforms such epoxides to much less reactive diols (Oesch *et al.*, 1971a,b; Oesch, 1973, and references therein), activators of human epoxide hydratase appear to be of special interest. Such activators could serve as tools to provide insight into the mechanism of carcinogenesis, mutagenesis and cytotoxicity produced by aromatic or olefinic compounds, and may even be useful in alleviating or preventing these effects. This action would be even more pronounced if the compound was at the same time an inhibitor

Table 5. *Effect of some substituents of the aromatic ring on the potency of styrene oxides as epoxide hydratase inhibitors*

Substrate ($[^3\text{H}]$ styrene oxide) and inhibitor concentrations were 2mM. For incubation conditions see Table 2. All effects were significant at $P < 0.001$.

Inhibitor		Percentage inhibition of epoxide hydratase purified from liver of		
Name	Structure	Man	Guinea pig	Rat
<i>m</i> -Bromostyrene oxide		85 ± 1.7	85 ± 2.5	82 ± 3.0
<i>p</i> -Bromostyrene oxide		74 ± 6.8	75 ± 2.0	78 ± 7.1
Styrene oxide		51 ± 2.1	49 ± 1.5	52 ± 2.2
Methyl- <i>p</i> -epoxymethylbenzoate		32 ± 2.8	45 ± 4.3	40 ± 1.9
<i>m</i> -Nitrostyrene oxide		37 ± 2.8	33 ± 3.1	39 ± 1.9
<i>p</i> -Nitrostyrene oxide		11 ± 2.1	12 ± 1.8	18 ± 2.3

of microsomal mono-oxygenases, thus counteracting accumulation of epoxides by inhibiting their formation and facilitating their degradation. Table 8 shows that some mono-oxygenase inhibitors, namely two imidazoles (Johnson *et al.*, 1969; Leibman, 1971) and metyrapone (2-methyl-1,2-di-(3-pyridyl)propan-1-one) (Netter *et al.*, 1967; Leibman, 1969) were activators of human hepatic epoxide hydratase *in vitro*. Aminogluthetimide (3-(*p*-aminophenyl)-3-ethyl-2,6-dioxopiperidine) which, with respect to mono-oxygenases, has several properties in common with metyrapone (Dexter *et al.*, 1967), had no effect on epoxide hydratase. Ethanol, which has been reported to induce mono-oxygenases (Lieber &

De Carli, 1971), at high concentrations (2.7M) enhanced the activity of human epoxide hydratase *in vitro*, whereas acetone, which stimulates activity of mono-oxygenases *in vitro* (Anders, 1968; Daly *et al.*, 1969; Daly, 1970; Vainio & Hänninen, 1972) had no effect on epoxide hydratase. Changes of activity of epoxide hydratase *in vitro* produced by these compounds are qualitatively very similar with respect to epoxide hydratase purified from the liver of man, guinea pig or rat. Quantitatively, the most striking difference was a considerably weaker effect of 1-(2-isopropylphenyl)-imidazole on the rat liver enzyme as compared with the preparations from guinea pig and man (Table 8).

Table 6. *Effect of electronegativity and bulk of substituents on the potency of propene oxides as epoxide hydratase inhibitors*

Substrate ($[^3\text{H}]$ styrene oxide) concentration was 2mM. For incubation conditions see Table 2. All effects were significant at $P < 0.001$, except for those marked ns.

Name	Inhibitor Structure	Concentration	Percentage inhibition of epoxide hydratase purified from liver of		
			Man	Guinea pig	Rat
3,3,3-Trichloro- propene oxide		2mM	99 ± 0.9	99 ± 0.6	98 ± 1.4
3,3,3-Trichloro- propene oxide		0.4mM	95 ± 1.2	97 ± 1.5	90 ± 2.1
3,3,3-Trifluoro- propene oxide	*	2mM	30 ± 5.4	60 ± 5.1	32 ± 4.8
3-Chloropropene 1,2-oxide		2mM	14 ± 2.3	14 ± 1.9	24 ± 3.7
3-Fluoropropene 1,2-oxide		2mM	ns	ns	ns
3,3-Dimethylbutene oxide		2mM	37 ± 5.2	39 ± 2.1	43 ± 6.0
Propene oxide	*	2mM	ns	ns	ns

* Incubation was in sealed tubes at 22°C; for all other compounds in open tubes at 37°C.

In conclusion, the epoxide hydratase purified from human liver microsomal fraction appeared to be qualitatively identical with the enzyme from rat and guinea pig with respect to specificity towards substrates, inhibitors and activators. Epoxides with various substitution patterns around the oxirane ring, monosubstituted oxiranes with various sizes of substituents, styrene oxides with different substituents on the aromatic ring, propene oxides with substituents of different bulk and electronegativity in the 3-position, and oxiranes fused to rings, displayed the full range of 0–100% inhibition towards hydration of $[^3\text{H}]$ styrene oxide by human epoxide hydratase. Epoxide hydratase from guinea pig or rat proved qualitatively sensitive and insensitive to the same compounds respectively. The same was true for several activators. However, some quantitative differences were noted, the most striking being the action of cyclohexene oxide on epoxide hydratase from human

as compared with guinea-pig liver. With both preparations this compound was a very poor substrate, but its inhibitory activity was potent with hepatic epoxide hydratase from guinea pig but much weaker with the hepatic enzyme from man. However, kinetic analysis showed the inhibition to be purely non-competitive in both preparations, indicating basically similar mechanisms even in those cases where quantitative differences were observed.

It therefore appears that human epoxide hydratase is probably very similar to, although not identical with, the guinea-pig and rat enzyme, and that studies on epoxide hydratase from the latter species are important with respect to the human enzyme. The structural features required for an epoxide to interact with the human epoxide hydratase as delineated in Tables 2–6 may allow us to predict from what kind of aromatic or olefinic compounds accumulation of metabolically produced epoxides would not occur,

Table 7. *Oxiranes fused to rings as inhibitors of epoxide hydratase*

Substrate ($[^3\text{H}]$ styrene oxide) and inhibitor concentrations were 2mM. For incubation conditions see Table 2. All effects were significant at $P < 0.001$, except for those marked ns.

Inhibitor		Percentage inhibition of epoxide hydratase purified from liver of		
Name	Structure	Man	Guinea pig	Rat
Alicyclic oxiranes				
Cyclohexene oxide		22 ± 0.3	63 ± 2.1	43 ± 2.1
1-Methylcyclohexene oxide		27 ± 2.2	25 ± 3.4	—
3-Methylcyclohexene oxide		75 ± 1.9	83 ± 9.3	71 ± 4.1
Indene 1,2-oxide		36 ± 2.9	39 ± 3.0	35 ± 2.8
Arene oxides				
Benzene oxide		ns	ns	ns
Naphthalene 1,2-oxide		30 ± 2.8	32 ± 4.0	25 ± 3.4

Table 8. *Activators of epoxide hydratase*

Substrate ($[^3\text{H}]$ styrene oxide) concentration was 2mM. Activator was added in $20\mu\text{l}$ of either acetonitrile or water, or neat (ethanol), at zero time with no preincubation. Controls received the same amount of solvent. A $50\mu\text{l}$ portion of purified epoxide hydratase preparation was used per assay. All effects were significant at $P < 0.001$, except for those marked ns.

Activator	Percentage activation of epoxide hydratase purified from liver of		
	Man	Guinea pig	Rat
1-(2-Isopropylphenyl)-imidazole (2mM)	174 ± 2.7	162 ± 1.5	76 ± 6.0
1-(2-Cyanophenyl)-imidazole (2mM)	58 ± 1.6	61 ± 7.4	57 ± 5.6
Metirapone (2mM)	50 ± 8.1	74 ± 7.8	87 ± 4.3
Aminogluthethimide* (2mM)	ns	ns	ns
Ethanol (2.7M)	91 ± 5.9	54 ± 8.2	70 ± 2.2
Acetone (2–800mM)	ns	ns	ns

* Used as phosphate salt.

especially in view of the fact that both enzyme systems, mono-oxygenases responsible for the formation of epoxides and epoxide hydratases, are localized in the membrane of the endoplasmic reticulum, and moreover appear to be architecturally and functionally closely coupled (Oesch & Daly, 1972; Oesch *et al.*, 1972). These delineations may therefore prove useful for the design of drugs which require an aromatic or olefinic moiety for their intended pharmacological effect.

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