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DIFFERENTIAL SUBSTRATE SELECTIVITY OF MURINE HEPATIC CYTOSOLIC AND MICROSOMAL EPOXIDE HYDROLASES

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Abstract—The initial rates of hydration of sixteen epoxides in the presence of cytosolic and microsomal fractions of mouse liver were determined. 1,2-Disubstituted *trans*-epoxides were found to be excellent, selective substrates for the cytosolic epoxide hydrolase, while 1,2-*cis*-epoxides were poorly hydrated when one or more substituents was a phenyl moiety. Epoxides of cyclic systems including benzo[*a*]pyrene 4,5-oxide, and two cyclodiene analogs were hydrated almost exclusively by the microsomal epoxide hydrolase while monosubstituted epoxides were hydrated by both systems. Some epoxides which were mediocre substrates proved to be reasonable inhibitors of the cytosolic epoxide hydrolase, indicating that the structural requirements for substrate binding and turnover are different. Some reagents known to interact with sulfhydryl groups, including styrene oxide, proved to be good inhibitors. This work facilitates the design of radiochemical and spectrophotometric assays for both major forms of epoxide hydrolase as well as prediction of potential intrinsic substrates. Also such data may be meaningful in assessing the risk involved in human exposure to epoxidized xenobiotics.

It is now recognized that there are at least two forms of epoxide hydrolase in mammalian tissue (EC 3.3.2.3, syn epoxide hydrolase or hydratase). The most commonly studied form is largely attached to cell membranes (although it may appear as a cytosolic form) and is referred to here as the microsomal epoxide hydrolase [1–4]. The second form is largely present in the cytosol and mitochondrial lumen (although a small amount adheres tightly to cellular membranes) and is referred to here as the cytosolic epoxide hydrolase [5–8]. Both enzymes are of interest to pharmacologists and toxicologists because they hydrate epoxides which are present in the diet or air of humans or are formed *in vivo* from olefinic or aromatic precursers. Both enzymes add water to epoxides in a *trans* manner to yield 1,2-diols. However, the enzymes differ in their subcellular, tissue, sex and species distributions; they selectively hydrate different substrates; they are induced and inhibited by different compounds; and they have different biochemical and immunochemical properties [5, 9–14]. Although an *in vivo* role has not been established for either enzyme, both are undoubtedly involved in the detoxification of potentially mutagenic and/or carcinogenic epoxides. Since both enzymes are involved in xenobiotic detoxification, and they are induced and inhibited differentially, it is imperative for valid risk assessment that their substrate selectively is appreciated and that selective substrates are available for their analysis.

Studies on the substrate selectivity and occasionally the substrate specificity of the two epoxide hydrolases are scattered in the literature [1–5], and very few studies have involved a direct comparison of activities [6–10, 12, 13, 15, 16]. Thus, this work was undertake to compare the initial rates of hydration of a series of structurally simple epoxides by the cytosolic and microsomal epoxide hydrolases. These epoxides and several other compounds were then screened as potential enzyme inhibitors.

MATERIALS AND METHODS

Substrates. Styrene oxide, *p*-chlorophenoxyepoxypropyl ether and *trans*-stilbene oxide were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Limonene 1,2-oxide, *cis*-stilbene oxide, allylbenzene oxide and cyclohexene 1,2-oxide were synthesized by per acid oxidation of the commercially available olefins [17]. 1-Phenyl-1-butene and 1-pentene (from the Chemical Samples Co., Columbus, OH) were oxidized with *N*-bromosuccinimide to the corresponding bromohydrins which were cyclized in base to yield *trans*-β-ethyl- and propylstyrene oxides. The *cis*-β-ethyl- and propylstyrene oxides were synthesized stereospecifically from α-bromopropiophenone and buteriophenone by reduction with $NaBH_4$ and base catalyzed cyclization. The 7,8-epoxides of limonene and vinylcyclohexene were prepared by first protecting the 1,2-position by bromination, oxidation with *m*-chloroperbenzoic acid, and then removal of the bromines with zinc. In most cases the corresponding diols were made by hydrating the epoxides in acidic aqueous tetrahydrofuran (0.05 N H_2SO_4 in 40% aqueous THF). In the few cases where this procedure led to decomposition, the epoxide was hydrated in the dark in 0.2 M acetic acid buffer, pH 4.0. In the case of the limonene oxides, the *cis*-diols were also made from OsO_4 oxidation of the corresponding olefin [18]. All compounds were judged pure by thin-layer chromatography (TLC) using both a general charring spray

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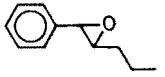
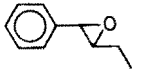
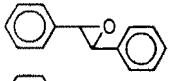
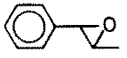
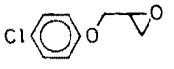
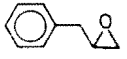
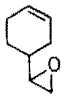
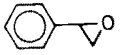
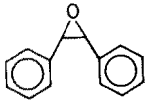
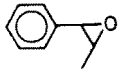
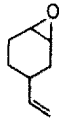
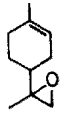
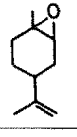
Table 1. GLC conditions, initial rates of hydration by the cytosolic and microsomal fractions, and inhibition of *trans*-β-ethylstyrene oxide hydration by thirteen sample aliphatic epoxides

Designation	Structure	GLC conditions			Initial rates† [pmoles·min^{-1}·(mg tissue equivalent)$^{-1}$]		Percent inhibition of *trans*-β-ethylstyrene oxide hydration‡		
							Epoxide		Diol
		Column temp (°)	Retention time of diol	Internal standard*	Cytosol	Microsomes	10^{-4} M	10^{-3} M	2×10^{-5} M
A		170	2.96	SG	11,200	9	38.4		<10
B		170	2.30	SG	5,700	5	21.3		<10
C		200	3.31	CPPD	350	ND§	56.4		11.6
D		170	1.72	ABG	97	ND	<10		<10
E		180 200	4.78 2.47	ABG ABG	59 59	88 88	10.3	32.9	<10
F		170 180	2.12 1.60	SG SG	53 53	140 140	<10	14.8	<10

G		170	1.54	ABG	30	29	<10	52.5	11.5
H		170	1.56	ABG	25	19	<10	35.8	<10
I		200	3.92	CPPD	36	52	<10		
J		170	1.61	ABG	8	4	<10		<10
K		170	1.54	ABG	ND	ND	<10	12.8	14.7
L		170	2.27	SG	14	210	<10	46.4	<10
M		170	2.27	SG	ND	134	10.0	52.0	<10

* Peak areas where determined were based upon the peak area of an internal standard. SG, CPPD, and ABG stand for styrene glycol, 1-(*p*-chlorophenoxy)propane-2,3-diol, and allylbenzene glycol.

† Initial rates of hydration were determined using 5×10^{-5} M substrate solution at 37° and pH 7.4 for the cytosolic fraction and 9.0 for the microsomal fraction. For conversion of rates to a mg protein basis, a 1% original wet weight/volume solution of cytosolic fraction averaged 0.6 mg/ml and that of microsomal fraction 0.12 mg/ml.

‡ A substrate concentration of 5×10^{-4} M *trans*-β-ethylstyrene oxide was used.

§ Not determined.

and/or ultraviolet detection as well as epoxide and diol selective sprays and by gas–liquid chromatography (GLC). Structures were verified at least by their nuclear magnetic resonance and infrared spectra. Radiolabeled, isomerically pure *cis*- and *trans*-β-ethylstyrene oxides and stilbene oxides were radiolabeled as described earlier [7, 19].

Radiolabeled benzo[*a*]pyrene 4,5-oxide was provided by F. Oesch (Mainz, West Germany). HCE and HEOM are isomeric epoxides of compounds from the cyclodiene group of insecticides were provided by G. T. Brooks (Brighton, England). HCE (1,2,3,4,9,9-hexachloro-*exo*-5,6-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonapthalene) is slowly converted to its corresponding *trans*-diol (1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene-*trans*-5,6-diol) while the less sterically hindered HEOM (1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene) is rapidly hydrated to its corresponding *trans*-diol (1, 2, 3, 4, 9, 9 - hexachloro- 1 - 4, 4a, 5, 6, 7, 8, 8a-octahydro - 1,4-methanonaphthalene-*trans*-6,7-diol) by microsomal epoxide hydrolases from several species.

Chemicals. Tris (hydroxyamino) methane (gold label) was purchased from the Aldrich Chemical Co. The geranylphenyl ethers were synthesized as previously described [11, 18, 20], while the trifluoromethyl ketones were prepared by modified Grignard or Collman procedures [21]. Other compounds were from established commercial sources.

Enzymes. Male Swiss–Webster mice (Simonsen Laboratories, Gilroy, CA), about 10 weeks old, were killed by cervical dislocation. The gall bladder was removed intact and discarded before removal of the liver which was immediatedly immersed in chilled potassium phosphate buffer (I = 0.2 M, pH 7.4). The livers were dabbed dry, weighed, and then perfused with chilled buffer. The perfused livers were minced and homogenized in enough phosphate buffer to give a 20% weight to volume solution. The liver homogenate was centrifuged at 10,000 *g* (20 min, 0–4°). The supernatant fraction was centrifuged at 100,000 *g* (60 min, 0–4°). The resulting pellet (microsomes) was washed by resuspending in Tris buffer (I = 0.1 M, pH 9.0) and then centrifuged again at 100,000 *g* (60 min, 0–4°). The microsomal pellet was resuspended in a solution of 50% glycerol in Tris buffer (pH 9.0) so that the microsomal suspension was 50% (original liver weight to volume).

The 50% microsomal fraction was used immediately or stored at −70° and thawed immediately before each use. The cytosolic fraction (100,000 *g* supernatant) was stored in ice for immediate work or at −70°. Protein concentrations were determined by the Warburg–Christian method [22].

Initial rates. Initial rates of epoxide hydration by cytosolic and microsomal fractions were assayed at enzyme concentrations of 0.25% original wet weight/v (0.15 mg/ml protein) in Tris buffer (I = 0.2 M, pH 7.4, 10^{-4} M EDTA) and 1% (0.12 mg/ml protein) in Tris buffer (I = 0.1 M, pH 9.0, 10^{-4} M EDTA), respectively, unless otherwise noted. The appropriate substrates, 50 nmoles, were added in 10 μl of ethanol to 1 ml of the enzyme solution which had been preincubated at 37° for 5 min. The reaction mixture was vortexed gently and incubated at 37° for various times. An internal standard, (Table 1), 10 nmoles, was added in 10 μl of ethanol just prior to the end of the incubation period. The reaction was immediately stopped by addition of approximately 250 mg NaCl. The sample was extracted twice with 1 ml of ether, and the organic phase was dried over anhydrous Na_2SO_4. The ether was evaporated under a gentle stream of nitrogen, and the residue was derivatized by addition of 50 μg *n*-butylboronic acid (Aldrich gold label), in 50 μg of ethyl acetate; then 1–2 μl of the derivatized sample was analyzed by GLC.

Rates were determined from a minimum of three time points each in triplicate which yielded a regression line passing through the origin when the data were corrected for a nonenzymatic hydration. Each substrate was also incubated in both boiled enzyme and in 1% (w/v) bovine serum albumin solutions to determine background rates of hydration. These rates varied from undetectable with *trans*-stilbene oxide to very high with styrene oxide. Nonenzymatic rates were subtracted from enzymatic rates to correct for spontaneous hydrolysis.

Generally four or five compounds were assayed on one day with a single enzyme fraction. Styrene oxide was always run with the microsomal enzyme and *trans*-stilbene oxide with the cytosolic enzyme. The rates of hydration of each compound on each day were then compared with that of the standard compound run simultaneously. Compounds with similar initial rates of hydration were then run simultaneously for direct comparison. Ultimately each compound was run on at least 3 separate days with enzyme preparations from at least two separate liver pools. At least once, each compound was run with the substrates which were hydrated more and less rapidly than itself. In the case of compounds which were very rapidly or very slowly hydrated, decreased or increased enzyme concentrations were used. The hydration rates were then compared with other compounds run under both the modified and routine enzyme concentrations.

The hydration of benzo[*a*]pyrene 4,5-oxide was measured using a modification of the method of Jerina *et al.* [23]. The substrate, 7.5 nmoles, was added in 2 μl of acetonitrile to 150 μl of the enzyme solution, and the mixture was incubated for various times at 37°. The reaction was quenched by addition of 100 μl of ether–ethanol solution (2:1) and a supersaturating amount of NaCl (*ca.* 30 mg). After vigorous vortexing, another 100 μl of the ether–ethanol solution was layered on top and the emulsion was broken by centrifugation. The aqueous phase was extracted three times, and the extracts were pooled and spotted on the cellulose prelayer of a channeled TLC plate (Whatman LKSDF). The plates were developed in toluene–methanol (9:1) and the epoxide and diol visualized under short-wave u.v. light. The spots, including 1 cm above and below, were scraped into scintillation vials and 3 ml of counting solution was added (3 Amersham OCS:1 Triton X-100). Radioactivity was monitored by liquid scintillation counting after the samples were held for 24 hr in the dark. Spraying the plate lightly with water

after development was found to reduce the chemiluminescence more rapidly.

[^{3}H]*trans*-Stilbene oxide hydration was measured by addition of 10.2 nmoles of substrate in 4 μl of ethanol to 200 μl of enzyme solution. The reaction was stopped and extracted as described above using ether instead of the ether–ethanol mixture. Nonradiolabeled epoxide and glycol (500 nmoles each) were added to each channel for u.v. visualisation. The plates were developed in toluene–propanol (20:1) and then scraped and counted as described above.

Hydration of HEOM and HCE was monitored in a fashion similar to the other nonradiolabeled substrates in that the enzyme mixtures were extracted with ether and the ether dried over Na_2SO_4. However, the extracts were then analyzed by electron capture GLC without derivatization.

Inhibition studies. To screen for the effects of different inhibitors, the previously reported radiometric partition assay with *trans*-β-ethylstyrene oxide was used with a substrate concentration of 5×10^{-4} M [19]. The inhibitor (0.1 or 1.0 nmole) was added in 1 μl of ethanol 15 sec prior to addition of the substrate (also in 1 μl of ethanol) to 50 μl of 0.5% (w/v) cytosolic fraction (0.3 mg/ml protein). After addition of the substrate the mixture was gently vortexed and incubated at 37° for 10 min. The reaction was quenched with 100 μl isooctane, and vigorous vortexing partitioned the unreacted epoxide into the organic phase. A 20 μl aliquot of the aqueous phase was monitored for radioactivity by liquid scintillation counting. Nonenzymatic hydration was monitored by incubation of the substrate with boiled enzyme.

GLC. A Hewlett–Packard 5710A gas–liquid chromatograph with a flame ionization detector was used to determine initial rates of hydration of most substrates. The columns were silanized glass spirals (1.5 m × 1.5 mm i.d.) containing 2.5% OV 101 on Gas Chrom Q (100/200 mesh). The injector port and detector temperatures were both 300°. The column temperature for quantitation of diols varied depending on the compound (see Table 1, 170°, 180°, 200°). Nitrogen (carrier gas), hydrogen air flow rates were 20, 30, and 240 ml/min respectively. *n*-Butylboronic esters of the internal standards and the authentic diols (10 nmoles each) were used to calibrate the Hewlett–Packard 3380A integrator based upon peak area.

Hydration of HEOM and HCE was monitored on a Hewlett–Packard 5710A electron capture gas–liquid chromatograph using a silane-treated 1.5 mm × 2 m glass column packed with 2% OV 101 on Gas Chrom Q (100/120 mesh), the column temperature was 250° while the injector and detector were held at 300°. The carrier gas flow rate of 5% methane in argon was 14 ml/min. Quantitation by peak area using a Hewlett–Packard 3380A integrator was based upon the ratio between the epoxide and diol peak areas [11, 20].

RESULTS

GLC methodology. To increase the accuracy and precision of the GLC assays, an internal standard method was employed in much of this work, and for the analysis of HEOM and HCE hydration was based upon the ratio of the diol and the epoxide substrate [11, 20]. Most of the epoxide substrates used in this study were so volatile that large and varying amounts of the epoxides were lost during work-up. This problem was avoided only when samples were carefully concentrated using a small Snyder column. Even relatively high molecular weight compounds such as the stilbene oxides readily evaporated during work-up or from cellulose pre-layer TLC plates if extraordinary care was not taken. In contrast, all of the diols were nonvolatile, stable compounds. Thus, it was decided to use them as internal standards.

As shown in Table 1, one of three internal standards was chosen for each compound which gave a sharp peak near but distinct from the product diol. Experiments in which diol mixtures were extracted with a variety of different solvents and then the diol ratio was analyzed by GLC demonstrated that the polarities of the diols were so similar that the internal standards also served to correct for extraction efficiency. Styrene oxide and *trans*-stilbene oxide were analyzed by monitoring total diol produced, by monitoring the diol/epoxide ratios following careful work-up, and by the internal standard method just described. All methods gave similar results, but the internal standard method was used for subsequent studies because it gave the least variability and was less laborious.

Standard substrates. *trans*-Stilbene oxide was chosen as the standard substrate against which other epoxides were compared in preliminary studies which indicated that this epoxide was an excellent substrate for the cytosolic epoxide hydrolase [12]. There was little substrate loss due to volatility during the course of the reaction, and *trans*-stilbene oxide was found stable in ethanol solution at room temperature and showed negligible nonenzymatic hydration. The compound is not highly soluble in that a 1.25×10^{-4} M solution in distilled water is visibly turbid. Thus, all studies were run at 5×10^{-5} M to facilitate direct comparisons of rates.

Conversion of *trans*-stilbene oxide to its diol at a substrate concentration of 5×10^{-5} M and an enzyme concentration of 1% (w/v) was out of the linear region in less than 10 min. At 0.25% the hydration rate was linear for more than 30 min, and approximately 40% of the epoxide was converted to diol.

Styrene oxide lacked the numerous attributes of *trans*-stilbene oxide as a substrate; however, it was used as the standard substrate for studying the mouse microsomal epoxide hydrolase because it has been very commonly employed by numerous previous workers [1, 2].

Initial rates of hydration. The initial rates of hydration of the thirteen alkyl epoxides by the cytosolic and microsomal hepatic epoxide hydrolases are shown in Table 1. All *trans*-epoxides opened to *erythro*-diols and all *cis*-epoxides opened to *threo*-diols with both the microsomal and cytosolic epoxide hydrolases. The *trans*-disubstituted epoxides of the substituted styrene oxide series were excellent substrates for the cytosolic enzyme. The initial rates of hydration of these epoxides were propyl>ethyl>>phenyl>>methyl (A>B>C>D).

Table 2. Hydration of benzo[*a*]pyrene 4,5-oxide by the cytosolic fraction*

% Cytosolic fraction (w/v equivalent)	Incubation time (min)	Amount of diol (nmole)	
		Boiled enzyme	Normal enzyme
1	45	0.075 ± 0.054	0.130 ± 0.032
	90	0.043 ± 0.12	0.150 ± 0.012
5	45	0.170 ± 0.050	0.361 ± 0.038
	90	0.079 ± 0.049	0.627 ± 0.006
10	30	0.051 ± 0.026	0.317 ± 0.030
	60	0.087 ± 0.065	0.764 ± 0.032

* A 5×10^{-5} M solution of benzo[*a*]pyrene 4,5-oxide was incubated in Tris buffer (pH 7.4, I = 0.2 M, with 1×10^{-4} M EDTA) with or without enzyme.

A similar trend was noted earlier for a series of terpenoid epoxides [11]. The four monosubstituted epoxides (E, F, G, H) were all hydrated at reasonalble rates. It should be noted that, under these conditions in the mouse, styrene oxide (H) was hydrated faster in the cytosolic than in the microsomal fraction. As indicated earlier [24], at the high substrate concentrations often used in previous studies styrene oxide inhibits the cytosolic epoxide hydrolase. The *cis*-stilbene oxide (I) (investigated earlier with the microsomal enzyme [25]) and *cis*-β-methylstyrene oxide (J) were each hydrated at rates approximately 10-fold lower than their corresponding *trans*-isomers. Interestingly, hydration of the *cis*-β-ethyl- and propylstyrene oxide derivatives (data not shown) was negligible by either fraction. The 1,1-disubstituted 7,8-limonene oxide (L) was hydrated at a very slow but detectable rate by the cytosolic enzyme.

Negligible hydration was observed for the epoxides on cyclic systems including vinylcyclohexene 1,2-oxide (K), limonene 1,2-oxide (M), cholesterol 5,6-epoxide (data not shown), HEOM, HCE and, as shown in Table 2, benzo[*a*]pyrene 4,5-oxide. The initial rate of hydration of benzo[*a*]pyrene 4,5-oxide by the cytosolic fraction was estimated to be about 0.1 pmole·min^{-1}·(mg tissue equivalent)$^{-1}$ or about 3000 times slower than the hydration of *trans*-stilbene oxide. HEOM and HCE have no detectable diol when incubated in buffer for up to 90 min. During this same period no hydration of the substrates appeared to occur with up to 1% (w/v) of the cytosolic fraction under conditions where 0.01 pmole diol·min^{-1}·(mg tissue equivalent)$^{-1}$ could have been detected.

As indicated earlier, the microsomal fraction was very poor at hydrating the *trans*-β-alkylstyrene oxides (A, B, D) and *trans*-stilbene oxide (C) [1, 5, 6, 12, 19]. The monosubstituted epoxides examined were hydrated at similar rates in both fractions. Interestingly, allylbenzene oxide (F) was hydrated seven times faster than styrene oxide in the microsomal fraction and *cis*-stilbene oxide (I) was hydrated three times faster. Vinylcyclohexene 1,2-oxide (K) is interesting in that it was very poorly hydrated by both fractions. Possibly it could be a good inhibitor of the microsomal enzyme. Both the 1,2 and the 7,8-limonene oxides (L, M) were good substrates for the microsomal epoxide hydrolase.

Inhibition of cytosolic hydrolase. The thirteen epoxides and their respective diols, which were previously examined as substrates, were also screened as potential inhibitors of the hydration of *trans*-β-ethylstyrene oxide (Table 1). The only epoxides which caused significant inhibition at one-fifth substrate concentration were those which were also good substrates for the cytosolic epoxide hydrolase. For instance, the best inhibitor, *trans*-stilbene oxide, has a K_m of 1×10^{-5} M compared to the K_m of *trans*-β-ethylstyrene oxide of 7×10^{-5} M. At two times substrate concentration, vinylcyclohexane 7,8-oxide (G) and the two limonene oxides (L, M) gave approximately 50% inhibition. As expected, the inhibition caused by high concentrations of styrene oxide increased with preincubation time. None of the compounds caused inhibition when the juvenoid R 20458 ($K_m = 2.0 \times 10^{-6}$ M) was used as a substrate as described earlier [5]. Inhibition by the corresponding diols was very low.

A wide variety of compounds were then screened as potential inhibitors of *trans*-β-ethylstyrene oxide hydration by the cytosolic epoxide hydrolase (Table 3). *p*-Nitro-2-bromoacetophenone (**1**) was earlier reported to inhibit the microsomal epoxide hydrolase presumably by alkylating a catalytically important histidine [26], and it caused good inhibition of cytosolic epoxide hydrolase even in crude form. *p*-Nitro-2-bromoacetophenone is known to modify a number of different amino acid residues, but its selectivity, for instance with serine proteases, is thought to stem from formation of a "transition-like" intermediate at the active side following enzymatic attack on the polarized carbonyl. Thus, a variety of carbonyl containing compounds was screened as potential inhibitors (**12–15**, **20–30**). 5,5′-Dithiobis (2-nitrobenzoic acid) (**2**) also caused significant inhibition at one-fifth and twice substrate concentration. Thus, compounds **1** and **2** were used as standard compounds for the comparison of other inhibitors. A variety of moderately water soluble amino acid modifiers failed to cause significant inhibition of the cytosolic epoxide hydrolase activity. However, the sulfhydryl modifying organomercurials (**9**, **10**) were excellent inhibitors. Chalcone and 4-hydroxychalcone (**12**, **14**), but not a closely related compound (**15**), were found to be good inhibitors as could be anticipated for a sulfhydryl containing enzyme. As covered in greater detail elsewhere [24],

Table 3. Percent inhibition of the hydration of *trans*-β-ethylstyrene oxide (5×10^{-5} M) by a variety of compounds of diverse structure

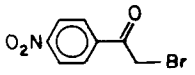
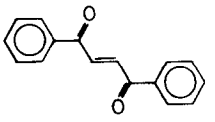
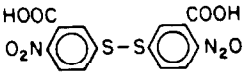
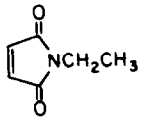
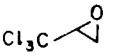
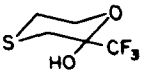
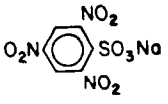
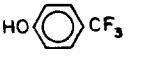
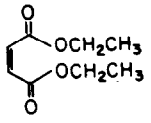
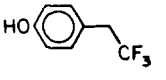
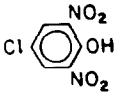
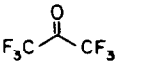
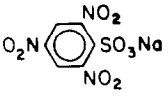
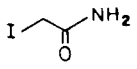
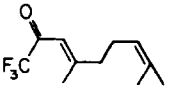
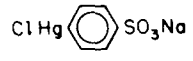
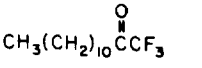
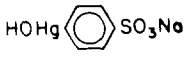
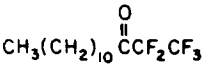
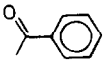
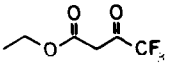
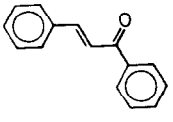
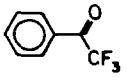
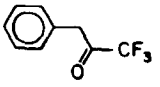
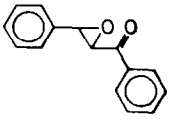
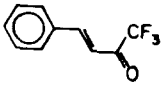
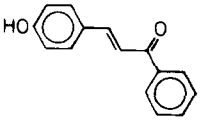
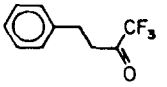
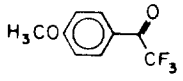
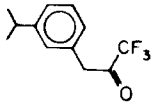
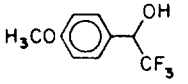
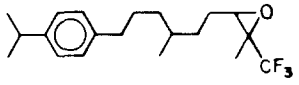
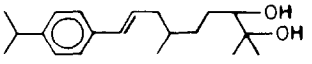
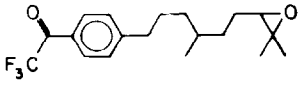
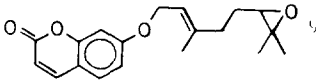
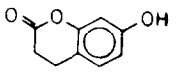
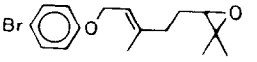
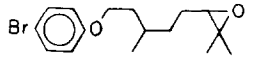
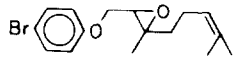
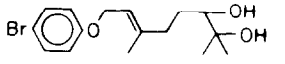
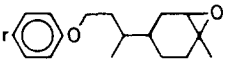
No.	Inhibitor structure	Inhibitor concentration 10^{-4} M	Inhibitor concentration 10^{-3} M	No.	Inhibitor structure	Inhibitor concentration 10^{-4} M	Inhibitor concentration 10^{-3} M
1		37	75	15		<10	
2		26	61				
3		<10	29	16		42	91
				17		<10	<10
4		<10					
				18		<10	37
5		<10	<10	19		<10	<10
6		<10	<10	20		<10	<10
7		<10	27				
8		<10	<10	21		12	17
9		98.2		22		13	17
10		97.3		23		<10	<10
11		<10	<10	24		<10	<10
12		28	46	25		<10	<10
				26		10	24
13		84	98				
				27		15	45
14		73	89	28		11	35

Table 3 *cont.* Percent inhibition of the hydration of *trans*-β-ethylstyrene oxide (5×10^{-5} M) by a variety of compounds of diverse structure

No.	Inhibitor structure	Inhibitor concentration 10^{-4} M	Inhibitor concentration 10^{-3} M
29		16	19
30		7.2	42
31		10	22
32		85	
33		38	51
34		82	95
35		91	
36		10	12
37		82	
38		90	
39		19	
40		10	
41		54	

the corresponding chalcone oxide proved to be an excellent inhibitor. Trichloropropene oxide (**16**) has been widely used as an inhibitor of the microsomal epoxide hydrolase. Although it fails to inhibit the activity of the cytosolic epoxide hydrolase on R 20458 [5], it is a good inhibitor of *trans*-β-ethylstyrene oxide hydration. A variety of fluorinated compounds failed to give significant inhibition with the possible exceptions of **26–28** and **30**.

A variety of previously described terpenoid compounds [11, 20], some of which are shown in Table 3, were also tested as possible inhibitors. As exemplified by compounds **33** and **40**, all of the diols tested proved to be poor inhibitors. The epoxides were reasonable inhibitors and their inhibitory potency generally correlated with their apparent affinity for the enzyme when tested as substrates. For instance, the 2,3-epoxide (**39**) is a poor substrate and a poor inhibitor when compared with the corresponding 6,7-epoxides (**37**, **38**). As discussed earlier, substitution about the epoxide moiety increases the apparent affinity of the substrate for the enzyme but substitutions greater than 1,2-disubstituted epoxides decrease rather than increase the rates of hydration. The 6,7-epoxygeranyl coumarin (**35**) was a surprisingly good inhibitor. The lack of inhibition by the free phenol (**36**) indicated that the terpenoid moiety is important for inhibition. Interestingly, this compound is the most abundant coumarin in grapefruit oil [27]. Based upon the known inhibition of the microsomal epoxide hydrolase by trichloropropene oxide and cyclohexene oxide, compounds **32** and **41** were prepared. They proved to be only mediocre inhibitors.

DISCUSSION

An appreciation of the substrate selectivity and ultimately substrate specificity (k_{cat}/K_m) of the enzymes involved in the metabolism of epoxidized compounds is important for a variety of reasons. Some epoxidized xenobiotics are potentially dangerous toxins, carcinogens and mutagens. On the other hand, epoxides occur on a wide variety of natural and industrial products and intermediates. It may be desirable to limit human exposure to some of these materials, but large scale restriction of human exposure to epoxides and their precursors would be socially expensive, personally unpleasant and restrictive, and probably not toxicologically valid. Thus, regulatory agencies need information on the potential affinity of such epoxides for DNA and their reactivity with biological nucleophiles as well as their metabolism.

Based upon this and previous studies with the cytosolic and microsomal epoxide hydrolases, the enzyme systems in the species studied appear to be complementary. The cytosolic enzyme is very poor at hydrating epoxides on a variety of cyclic systems possibly due to steric hindrance. The marginal hydration noted for the cytosolic epoxide hydrolase on benzo[*a*]pyrene 4,5-oxide could be due, in part, to a small amount of the microsomal epoxide hydrolase present in the cytosolic fraction. *trans*-1,2-

Disubstituted and tri- and tetrasubstituted epoxides appear to be hydrated almost exclusively by the cytosolic epoxide hydrolase in mouse liver. *cis*-1,2-Disubstituted compounds in the β-alkylstyrene oxide series are very poorly hydrated by either system. Fortunately, they are metabolized by conjugation with glutathione. An interesting observation is that the guinea pig [19] and human [16] do hydrate *cis*-β-ethylstyrene oxide, indicating the potential importance of comparative studies. Other disubstituted compounds such as *cis*-stilbene oxide and *cis*-epoxysteric acid [10] are hydrated by both fractions.

The monosubstituted epoxides are hydrated by both the microsomal and cytosolic epoxide hydrolases. Such rapid hydration is potentially important because many monosubstituted epoxides will react with DNA. For instance, styrene oxide and allylbenzene oxide are mutagens in the Ames' system while the corresponding 1,2-disubstituted compounds are not. With an epoxide of given reactivity the mutagenic potency of a compound then depends, in part, upon its affinity for nucleic acid as shown for a series of glycidyl ethers [28]. Allylbenzene oxide provides a nice model for a variety of allyl containing drugs and other xenobiotics such as allylbarbital and safrole which are likely to be epoxidized *in vivo*. The glycidyl ether of *p*-chlorophenol provides a nice model substrate for several major industrial products. For instance, over 260 million pounds of the diglycidyl ether *bis*-phenol A is produced annually in the United States while phenylglycidyl ether is also a major ingredient in many epoxy-resin systems. The lack of hydration of the 1,2-epoxide of vinylcyclohexene by either fraction may indicate why vinylcyclohexene dioxide was an effective radiometic compound *in vivo*. The lack of hydration of benzo[*a*]pyrene 4,5-oxide, HEOM, HCE, and other epoxides on cyclic systems indicated that the cytosolic epoxide hydrolase will not be found important in the metabolism of most arene oxides, cyclodiene epoxides, and other such compounds.

A second value of such substrate selectivity studies is to indicate potentially useful model substrates for use in routine assays. *trans*-β-Methyl-, ethyl- and propylstyrene oxides are good, discriminating substrates for the cytosolic epoxide hydrolases of rodents. The compounds are somewhat volatile, but they are reasonably water soluble and, especially *trans*-β-ethyl- and propylstyrene oxides, are very rapidly turned over by the cytosolic enzyme. *trans*-Stilbene oxide is also a good substrate for the cytosolic enzyme. It has the advantage of being less volatile and very stable to hydration in buffer. The diols of each of these compounds differ greatly in polarity from the epoxide so that epoxide hydration can be monitored by a simple partition method. Several of these compounds have been radiolabeled at 52 mCi/mmole and all of the compounds (A–D) were radiolabeled at >10 Ci/mmole in quantitative yield using [^{3}H]$NaBH_4$ [7, 19, unpublished information]. The difference in the ultraviolet spectrum of *trans*-stilbene oxide and its corresponding diol also allowed the development of a continuous spectrophotometric assay [29].

Since a variety of glycidyl ethers can be readily radiolabeled in a one-step procedure, they may also represent good model substrates. Substantial spectral differences have been demonstrated for some glycidyl ethers and their corresponding diols, also raising the possibility of continuous assays. Allylbenzene oxide might be considered as a potential replacement for styrene oxide as a microsomal epoxide hydrolase model substrate. Not only is it hydrated seven times faster, but it shows greater specificity of hydration by the microsomal enzyme, it is less volatile, less mutagenic, and much more stable both in buffer and upon storage. This single substrate can be used to monitor both the microsomal and cytosolic enzymes if one takes advantage of their different subcellular distribution and/or pH optima.

Two other compounds appear to be useful substrates for the microsomal epoxide hydrolase. In this laboratory *cis*-stilbene oxide radiolabeled at 50 mCi/mmole or 10 Ci/mmole has proven to be an excellent model substrate for the microsomal epoxide hydrolase of a variety of species including rhesus monkey and man. It is relatively nonvolatile, stable and rapidly hydrated by the microsomal enzyme. At a pH of 9.0 very little hydration of this substrate is catalyzed by the cytosolic epoxide hydrolase, but it is slowly hydrated by the cytosolic enzyme at neutral pH. The 1,2-epoxide of limonene is hydrated seven times faster than styrene oxide by the microsomal epoxide hydrolase, while its hydration by the cytosolic enzyme was nondetectable. It could be readily radiolabeled by the selective reduction of the 7,8-olefin with tritium gas.

A third value of such substrate selectively studies is that they may indicate potential intrinsic substrates. The large variations in cytosolic epoxide hydrolase activity ovserved with sex and age and the failure of attempts to induce the cytosolic epoxide hydrolase with variety of compounds including phenobarbital, 3-methylcholanthrene, butylated hydroxyanisole, stilbene oxide and others may indicate that the cytosolic epoxide hydrolase has a constitutive role in addition to its ability to metabolize xenobiotics. The high affinity of the enzyme for terpenoid epoxides [11] suggested that it could be a scavenger enzyme for such by-products of steroid biosynthesis as squalene dioxide and lanosterol 24,25-epoxide. These compounds are substrates for the cytosolic epoxide hydrolase and the high affinity but low tunrover of these substrates would be appropriate for compounds produced in small amounts as biosynthetic by-products [5]. These epoxidized compounds have been shown to be quite potent as angiotoxic agents following injection into rabbits [30].

The rapid hydration of *cis*- and *trans*-1,2-disubstituted lipophilic epoxides indicated that lipid epoxides could be good substrates for the cytosolic epoxide hydrolase [11]. When subsequently tested, the *cis*- and *trans*-9,10-epoxides of stearic acid as well as their methyl esters were rapidly hydrated [10, 13], such epoxides are known to form in lung tissue following exposure to smog components [31], and they may also form as a result of lipid oxidation and peroxidation occurring in normal tissue. Both the cytosolic and microsomal epoxide hydrolase as well as glutathione *S*-transferases are probably involved in such catabolism since it was previously found in

this laboratory that cholesterol 5,6-epoxide is an exceptionally poor substrate for the cytosolic epoxide hydrolase while a number of steroid epoxides are good substrates for the microsomal enzyme [2–4, 32]. The increased rate of hydration observed in compounds such as *trans*-β-propylstyrene oxide or allylbenzene oxide indicate that lipid epoxides in which the epoxide is α or β to an olefin or conjugated olefinic system should be excellent substrates for the cytosolic epoxide hydrolase. Such substrates could be the epoxides of arachidonic acid or leukotriene A_4. The hydration of the later compound to the diol leukotriene B_4 is not inconsistent with the proposed mechanism of epoxide hydration by the cytosolic epoxide hydrolase.

A knowledge of inhibitors of the cytosolic epoxide hydrolase again has a variety of applications. If man is exposed to such inhibitors in addition to a potentially toxic epoxide, risk is enhanced. The high affinity and low turnover of some terpenoid epoxides as well as chalcone oxides [11, 24] could present such a risk. Additionally, inhibitors can provide mechanistic information on the enzyme. The potency of a variety of amino acid modifiers indicates that a sulfhydryl group may be catalytically important. Potent inhibitors may be of use in eliminating the role of epoxide hydrolase in short-term mutagenicity assays and in determining the pharmacological or biochemical role of epoxide hydration in hepatocytes or *in vivo*. To this end a variety of potent inhibitors have been synthesized, based upon the chalcone oxide structure [24].

Thus, the cytosolic microsomal epoxide hydrolases appear complementary in that between them they hydrate a wide variety of epoxidized xenobiotics. Although both the cytosolic and microsomal epoxide hydrolases have been found in all vertebrate species examined, the substrate selectivity studies described here should be applied cautiously to other species.

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