MOLECULAR BASIS OF ENVIRONMENTAL TOXICITY

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CHAPTER 10

COMPARISON OF EPOXIDE HYDRASES IN THE SOLUBLE AND MICROSOMAL FRACTIONS OF MAMMALIAN LIVER

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INTRODUCTION

Epoxides (also termed oxiranes or alkene oxides) are three-membered cyclic ethers. In some but not all cases, epoxides are electrophilically reactive and may be mutagenic, carcinogenic or cytotoxic, and they have thus received intense attention from chemists, environmentalists and biochemists.[1-3] Not only are they present naturally in our diet from both natural and man-made products, but they are formed *in vivo* both as intermediates in vital biosynthetic pathways and as metabolites of xenobiotics.[4-12]

Although epoxidized compounds may be biologically reactive, they are often important intermediates in the lipophile-to-hydrophile conversions in many organisms. Thus, the processes by which epoxides are further degraded, usually to more polar compounds, are of interest. Epoxides can rearrange or react with biological nucleophiles with or without enzymatic catalysis by glutahione-S-epoxide transferases.[7,13-21] Aliphatic epoxides can be converted to olefins apparently by microbes in the rumen of cattle[22,23] or in the large intestine of mice, rats and man (Hammock, unpublished), or

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arene oxides can be enzymatically reduced.[24,25] However, most research has dealt with the enzymes known as epoxide hydrases. These enzymes add water to or hydrate epoxides to yield 1, 2-diols or glycols which are generally quite hydrophilic and which can be further metabolized by conjugation.[4-12,26] These enzymes have been discussed in numerous reviews, some of which are referenced above, and most recently Chapter 13 in this text.[12]

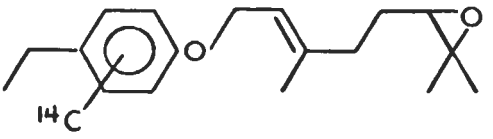
Several studies indicated that for the substrates investigated epoxide hydrase activity is membrane-bound in mammals.[8,27-30] The 100,000-g pellet or "microsomal" fraction was probably chosen for initial studies because it represented the most homogenous active subcellular fraction and because there was good evidence that the hydrase activity in other membrane fractions was due to microsomal contamination, at least for the substrate examined. Subsequent reviews indicated that epoxide hydrase activity was microsomal, and many investigations seem to have been undertaken based on this assumption. The initial studies by Brooks *et al.*[6] on the action of insect epoxide hydrases on cyclodiene insecticides were extended as the juvenile hormones of insects and their synthetic mimics or juvenoids showed promise as insecticides. Epoxide hydration was soon shown to be an important route of insect metabolism of these epoxidized terpenes both in the natural juvenile hormone[31] and in its mimics.[32] Studies of subcellular distribution indicated that all insect epoxide hydrase activity on the substrates examined was membrane-bound[33,34] The insect metabolism of juvenile hormone and juvenoids has been reviewed[35] and such work continues to be important in the development of new insect control agents and in the circumvention of insect resistance to existing agents.

In the process of developing any insect control chemical, the environmental chemistry and metabolism of the chemical in nontarget organisms must be investigated. It was no surprise when the diol of an epoxide containing juvenoid (R 20458, Figure 1) under consideration for commercial development was found to be an important *in vivo* and *in vitro* mammalian metabolite.[22,32,36-38] It was surprising, however, that an enzyme(s) in the 100,000-g soluble fraction made a major contribution to this hydration.[32,36] Although there is a plethora of reports on microsomal epoxide hydrases, only one group of workers has investigated soluble mammalian epoxide hydrases. The purpose of this review is to examine these preliminary but provocative investigations. Information on the soluble epoxide hydrase is considered in the light of knowledge on the microsomal epoxide hydrase. Reference to work on epoxide hydration occurring in the microsomal fraction is not exhaustive, but an attempt has been made to provide some primary sources and reviews.

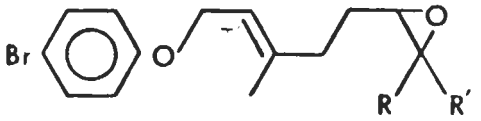
RESULTS AND DISCUSSION

Identification of the Diol as a Soluble Fraction Metabolite of R 20458

The interest of this laboratory in the soluble epoxide hydrases grew from an initial interest in the metabolism of a single xenobiotic.



R20458



Bromine Analogs

Figure 1. Radiolabeled and brominated phenylgeranyl ether epoxides assayed in this study. R 20458 is a juvenoid considered for commercial development by Stauffer Chemical Company. R and/or R' were varied from H through nC_3H_7 on the *p*-bromophenyl ether series with and without the 2,3-olefin.

Thus, this *p*-ethylphenylgeranyl ether (R 20458, Figure 1) was used for early studies, just as styrene oxide (Figure 11) was used as a model substrate for the investigation of the microsomal hydrases. Unless otherwise noted, R 20458 was used as the substrate and epoxide hydrase activity refers to that found in the 100,000-g soluble fraction. The compound was radiolabeled with tritium at C-1 of the terpene side chain and on the aromatic ring[39] and with ^{14}C in the aromatic ring.[40]

In retrospect, an overly intense effort led to the confirmation of the diol as the principle metabolite in the 100,000-g soluble fraction. However, at the time the studies were done, a mounting body of evidence from many other laboratories appeared to indicate that all epoxide hydrase

activity was membrane-bound. The radioactive diol was isolated following incubation of the substrate with rat and mouse liver soluble fraction which had been passed through Sephadex G-25. The metabolite, whether radiolabeled in the aromatic ring or in the terpene chain was found to co-chromatograph in several one- and two-dimensional, thin-layer chromatography systems with diols synthesized by acid hydrolysis or base hydrolysis of the epoxide or by osmium tetroxide oxidation of its olefinic precursor (Figure 2). The majority of the radioactivity continued to co-chromatograph with authentic diol derivatives after acetylation with acetic anhydride; oxidation with lead tetraacetate to the corresponding aldehyde, oxidation with Tollen's reagent to the acid, and esterification with diazomethane; derivatization with *n*-butylboronic acid (BBA); and failure to react with several reagents indicative of different functional groups[32,36,38] (Gill, Hammock and Casida, unpublished) (Figure 2). In each case the derivative's structure was verified by co-chromatography with a standard synthesized on a larger scale and identified at least by nuclear magnetic resonance (NMR), infrared (IR) and mass spectrophotometry (MS). Subsequently, the metabolite was found to co-chromatgraph with the authentic

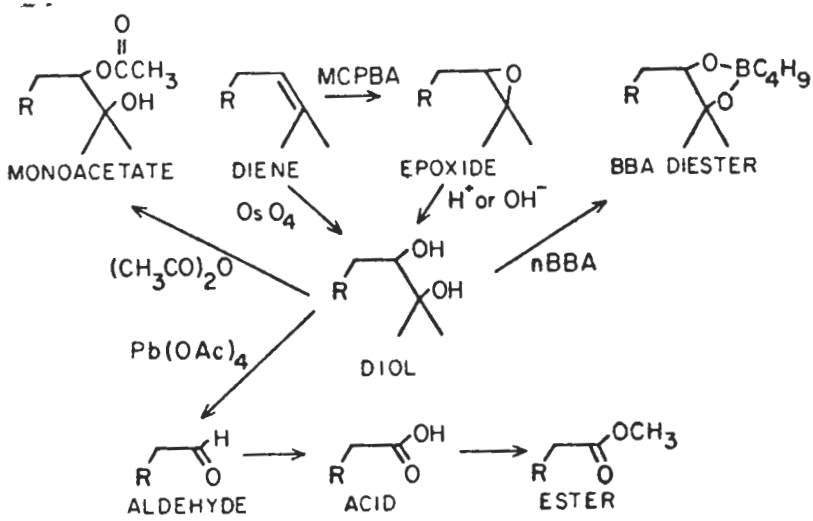


Figure 2. Synthetic steps leading to an authentic standard of the diol of R 20458, and microchemical procedures used to confirm that the radiolabeled metabolite formed in the 100,000-g soluble fraction was the 6,7-diol of R 20458. Lead tetraacetate was also used to convert the diol of the *p*-bromophenyl series into the aldehyde used for the Wittig reactions shown in Figure 6.

diol before and after derivatization on gas-liquid chromatography (GLC) and on normal and reversed phase high-resolution liquid chromatography (HRLC). Enough of the metabolite was isolated to allow its identification by NMR (Gill, Hammock and Casida, unpublished) and later by MS and IR.

An equally grave question concerned contamination of the soluble fraction by membrane-bound hydrase or generation of the diol metabolite by glutathione-S-epoxide transferases. The possibility that the soluble epoxide hydrase is loosely membrane-bound and is dissociated during preparation has not been excluded. However, similar levels of soluble epoxide hydrase activity are obtained when tissues are homogenized in various buffer systems including phosphate, tris, KCl, sucrose and the exact systems reported by previous workers.[27,41-45] Proteolytic enzymes present in the liver homogenate may release the epoxide hydrase from a membrane, but addition of diisopropyl phosphorofluoridate to the homogenate slightly increases rather than decreases epoxide hydrase activity in the 100,000-g supernatant.

The later question regarding glutathione involvement was resolved by showing that (1) no hydrase activity was lost after Sephadex G-25^{R} gel filtration, (2) no enhanced activity was found after the addition of cysteine or glutathione to the soluble fraction from gel filtration and (3) the substrate did not react chemically or enzymatically with glutathione or other thiol reagents.[32,36,46-51] The enzymatic nature of the hydrolysis of the epoxide was demonstrated by precipitating the activity with ammonium sulfate or destroying it by the addition of proteases.[51] Marker enzymes for mitochondrial and microsomal fractions were followed during differential centrifugation, and contamination of the soluble fraction by membrane-bound enzymes was found to be negligible[36] (Gill, Hammock and Casida, unpublished). Centrifugation of the 100,000-g soluble fraction for extended periods at high speed or passage through Amicon filters failed to reduce enzyme activity, although either procedure should remove membrane contaminants.

Molecular weight estimates discussed below[46-51] indicate that the soluble epoxide hydrase is different from glutathione-S-epoxide transferases[16-20] and the soluble form of the microsomal hydrase studied in other laboratories.[8,52-56] The microsomal enzyme hydrating styrene oxide is inhibited by trichloropropene oxide and cyclohexene oxide, while these compounds have minimal effect on hydration of R 20458 by the soluble enzyme (discussed below).

Analysis of Epoxide Hydrase Activity

The procedures used to measure soluble epoxide hydrase activity have varied with the substrate used. The most sensitive (and also the most

laborious) procedures involve the use of a radiolabeled substrate and chromatography. Following incubation, the substrate and product are extracted, mixed with synthetic, unlabeled standards, and spotted on thin-layer chromatograms. After development the plates are qualitatively analyzed by exposure to X-ray plates and/or scanning with a proportional flow counter, then they are quantitatively analyzed by scraping and liquid scintillation counting (LSC).[32,33,35,36,38,51,57]

Gas-liquid chromatography (GLC) with a flame ionization detector provides a relatively universal method of analysis for volatile epoxides; however, it is also laborious and of mediocre sensitivity. The incorporation of a halogen into the substrate allows the use of the highly sensitive electron capture detector (EC) on GLC.[46,58,59] For analysis by GLC the relative areas of epoxide and diol peaks can be compared (phenylgeranyl ethers, epoxy methyl fatty acids, glycidyl ethers, stilbene oxides) or internal standards can be used. The mono- and dialkoxides (*e.g.*, OCH_3 and OC_2H_5) of squalene were found to be useful internal standards for squalene oxide and dioxide while the diols of styrene oxide and allylbenzene oxide were useful standards for allylbenzene oxide and styrene oxide, respectively, and hexadecane is a useful internal standard for *trans*-β-methylstyrene oxide hydration.[46,49] Figure 3 illustrates the use of internal normalization on GLC for the analysis of a halogen-labeled epoxide and the use of an internal standard for the analysis of squalene oxide. The latter procedure led to the discovery that traces of peroxide in the ethyl ether used for extraction could epoxidize the 23,24 olefin of squalene 2,3 oxide or its diol[49] In all cases, derivatization with *n*-butylboronic acid was found to yield sharper, more reproducible diol peaks, although this derivatization procedure can lead to anomalous results if underivatized diols are subsequently assayed on the same GLC column.

A colorimetric procedure was developed for the soluble epoxide hydrase based upon the reactivity of 4-(*p*-nitrobenzyl)-pyridine with the epoxide containing substrate but not the product diol. The assay procedure is feasible and relatively universal and is a nice screening procedure, but it is also tedious and of poor sensitivity.[60] The addition of a partially purified fraction of the hydrase to a compound known to be mutagenic via its epoxide in a microbial assay such as the Ames assay[61,62] allows a qualitative assessment of the enzyme's role in hydrating that compound.[49,51]

The use of a partition method to separate epoxides and diols of radiolabeled substrates greatly reduces analysis time. After the examination of several solvent systems, it was found that partitioning between isooctane and aqueous methanol offered many advantages: (1) both phases can be counted with minimal quench; (2) emulsions are easily broken; (3) the hyperphase is relatively nonvolatile; (4) the methanol concentration can

be varied to gain a wide range of polarities; and (5) the diols and epoxides of several substrates were found to be largely in separate phases. Statistical treatment indicated that for two substrates, TLC and partition methods yielded similar results.[57]

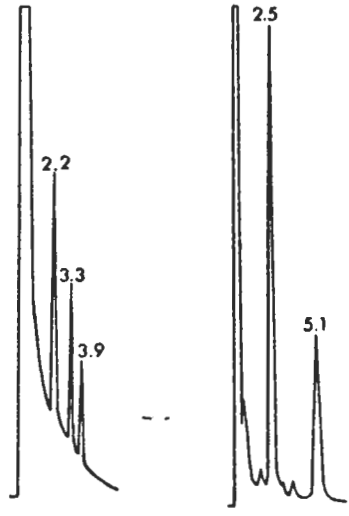


Figure 3. Typical GLC assays of epoxide hydrase activity. A. Shows a tracing of the conversion of squalene 2,3-oxide (retention time 2.2 min) to its 2,3-diol (assayed as the boronic diester, retention time 3.9 min). The reaction is normalized on an internal standard of 2-0-butyl squalene (retention time 3.3 min, 310°C column, N_2 = 35 ml/min). B. Shows a tracing of the conversion of a brominated phenylgeranyl ether (retention time 2.5 min) to its 6,7-diol (assayed as the boronic diester, retention time 5.1 min, 250°C column, argon:methane (95:5) = 14 ml/min; both squalene oxide and phenylgeranyl ethers were assayed on a 2-mm x 6-ft. glass column packed with 2% OV101 on Gas Chrom Q with flame ionization or electron capture detector, respectively).

Before any analysis is performed based on a partition method or a GLC procedure using internal normalization, one must be certain that epoxide hydration is the only reaction occurring. No competing reactions were found with any substrate when the soluble fraction was used except for some epoxy-ester compounds. In these cases, epoxide hydrase and esterase activity were either separated by gel filtration or the esterase activity was

inhibited by treatment with diisopropyl phosphofluoridate.[46,47,49] Reduced glutathione levels in the soluble fraction drop rapidly at neutral pH. Once it is established that competing reactions do not occur with the substrate, then numerous assay procedures described for analyzing epoxide hydrase activity in other subcellular fractions can be applied to the soluble fraction.[6,8,27,29,34,63-71] For instance, the use of cellulose prelayer silica gel plates[67] has proven very useful in the analysis of the action of the soluble epoxide hydrase on terpenoid, steroid and fatty acid epoxides if the reaction is quenched by the addition of tetrahydrofuran. Similarly, some of the assay procedures developed for the soluble epoxide hydrase may offer significant advantages over existing assays for epoxide hydrase activity in the microsomal or other subcellular fractions. A method generally applicable to all epoxides which allows the continuous monitoring of a hydration reaction rather than point monitoring (all literature assays) is needed to investigate epoxide hydrases, regardless of fraction.[72]

Distribution of Epoxide Hydrase Activity

Species Distribution

Epoxide hydrase activity has been found in the soluble fraction of all mammals examined. In the case of the hepatic soluble epoxide hydrase activity in the rat, rabbit and mouse, the experiments have been repeated enough under carefully controlled conditions to have confidence in the distribution pattern shown in Figure 4.[46,47] The rat has very low soluble epoxide hydrase activity when R 20458 is used as the substrate. These results are quite different from those reported by Oesch[8,73] for hepatic microsomal activity using styrene oxide as a substrate. Relative microsomal epoxide hydrase levels were monkey > human > guina pig > rabbit ≥ rat > mouse. Surprisingly, rabbits are reported to hydrate HEOM much faster than rats.[74] It should be cautioned that strain, age, sex and possibly other factors may influence the apparent epoxide hydrase activity in different species, although available information indicates that these factors influence the soluble hydrase levels more than the microsomal levels. Microsomal epoxide hydrase activity has also been found in the livers of every mammalian species examined.[6,8,27,73-78]

Distribution with Strain, Age and Sex

A survey of 4 strains of 7-week-old male mice indicated that C57B1 had the highest hepatic-epoxide-hydrase-specific activity of ~1700 pmoles/min mg^{-1} and Swiss-Webster the lowest, with ~1200 pmoles/min mg^{-1} in the unpurified soluble fraction. The levels of hepatic epoxide hydrase activity

increase with the age of both male and female Swiss-Webster mice. The hydrase levels in males, however, increase much more dramatically than in females and significant differences in hydrase levels are found by 8 weeks of age (Figure 4). Such large sex-linked differences suggest that the hydrase levels may be regulated by the sex hormones. Such involvement may be indicated with the microsomal epoxide hydrase, since Salmona *et al.*[79] reported a decrease in enzyme activity following administration of lynesterol and mestranol. In contrast, Oesch and others[8 27 73] report that no significant differences were noted between sexes in the microsomal epoxide hydrase levels in five mammalian species, although microsomal epoxide hydrase activity was noted to increase as male rats matured as does the monooxygenase system,[80] and Oesch[81] later reported significantly higher levels of hepatic microsomal eposide hydrase in male than in female adults but not newborn rats.

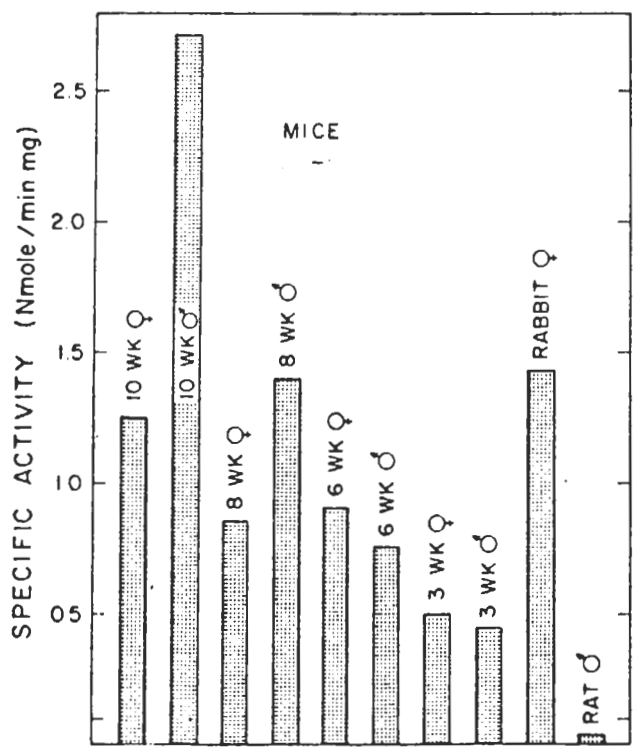


Figure 4. Specific activity of the epoxide hydrase metabolizing R 20458 in the hepatic soluble fraction of male and female mice of various ages and rats and rabbits.

Organ Distribution

The soluble fraction was found to contain some epoxide hydrase activity in every organ examined in female New Zealand white rabbits, male Swiss-Webster mice, and Sprague-Dawley rats; however, the relative levels varied dramatically. Figure 5 shows the relative levels in several tissues. Hepatic and renal activity is very high while significant activity is present in the duodenum, skeletal muscle, colon and lung. The spleen had the lowest level[36,46,47,51] In contrast, microsomal epoxide hydrase activity is high in the liver, but low in the kidney.[8] As assay procedures have become more sensitive, microsomal epoxide hydrase activity appears ubiquitous in mammalian tissues, although nervous tissue has very low levels and tissue activities vary dramatically.[8,79,82,83] Possibly the same trend will hold true for the soluble hydrase activity.

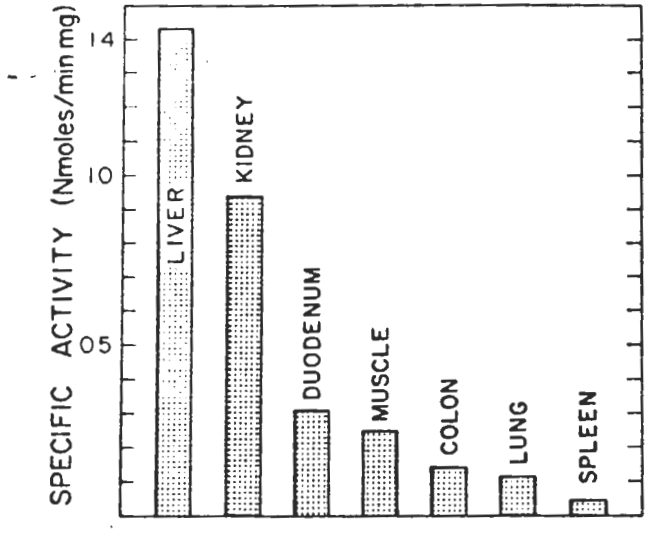


Figure 5. Specific activity of the epoxide hydrase metabolizing R 20458 in the soluble fraction of female rabbit tissues. Tissues from male mice demonstrate a similar distribution of specific activity.

Subcellular Distribution

Differential centrifugation indicates that when R 20458 or several other substrates such as fatty acid epoxides are used, high epoxide hydrase activity

appears largely in the soluble fraction. This laboratory has not yet elucidated the relative contribution that intrinsic activity, soluble contamination and microsomal contamination make to the high hydrolytic activity observed in the mitochondrial fraction.[32,36,46,51,84] However, twice-washed rat liver mitochondria with minimal microsomal contamination still rapidly hydrate epoxy methyl oleate. Much of the epoxide hydrase activity in the microsomal fraction observed with some substrates is due to contamination by the soluble fraction.

In spite of the tremendous body of literature related to microsomal epoxide hydrases, there is a paucity of data concerning subcellular distribution of epoxide hydrase activity using various substrates. Oesch *et al.*[8,27] demonstrated that styrene oxide (Figure 11) was hydrated only in subcellular membrane fractions and that the activity was largely microsomal if isotonic sucrose was used as the homogenization medium. This study was extended to several other species, including man.[73] This laboratory has recently repeated the work of Oesch *et al.*[27] using styrene oxide. We also find that the soluble fraction is unable to hydrate styrene oxide while rapidly hydrating most other epoxide substrates. This study provides additional evidence that the 100,000-g fraction is not contaminated with microsomal hydrases.[50]

Substrate Selectivity

When model substrates were first chosen to study the properties of the soluble epoxide hydrase, it was assumed that almost all epoxide hydrase activity was microsomal and that the soluble enzyme was selective for trisubstituted epoxides of a structure similar to R 20458 (Figure 1). Thus, a series of *p*-bromophenylgeranyl ethers analogous to R 20458 were synthesized. Surprisingly, the soluble hydrase metabolizes a broad range of substrates, and, in retrospect, much simpler series of substrates could have been utilized.

Synthesis of Substrates

The synthesis of the phenylgeranyl ether compounds has been described in detail in several publications.[32,38,39,58,59,85] Briefly, *trans*-pure geranyl bromide was reacted under basic conditions with *p*-bromophenol and then the 6,7-olefin selectively epoxidized to give the dimethyl member of the series. As shown in Figure 2, the epoxide was converted via its diol to the hexenal derivative which was combined with phosphonium salts to yield a large number of mono-, di-, and trisubstituted olefins which could be oxidized to the corresponding epoxides (Figure 6). This prodecure yielded a *cis*-rich mixture of geometrical isomers about the epoxide. Alternatively, the epoxide was converted by anhydrous acid to its allylic alcohol and reacted with

various dialkyl cuprates to yield pure *trans*-dienes. The 1,2-disubstituted epoxides were separated into pure *cis*- and *trans*-isomers by preparative HRLC. In each case none of the opposite isomer could be detected by HRLC or GLC. The isomers were identified based on (1) synthetic method; (2) chromatographic behavior of the diene, epoxide and diol; (3) ^{1}H and ^{13}C nuclear magnetic resonance; and (4) microchemical procedures (Figure 7).[58,59]

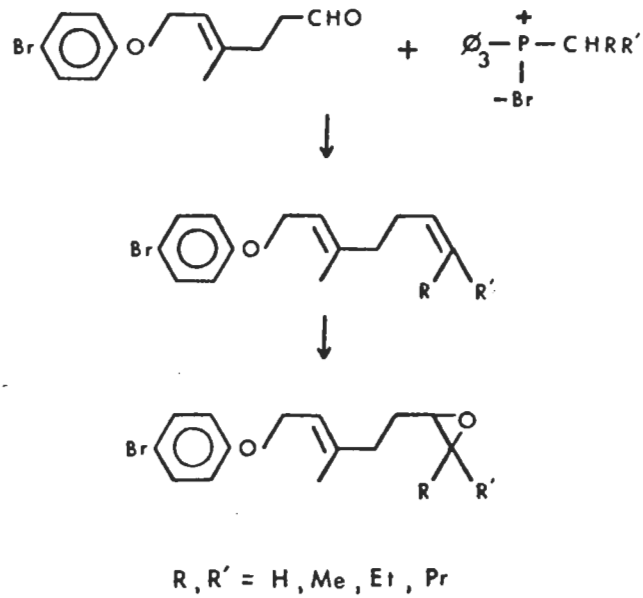


Figure 6. Wittig and subsequent reactions leading to mono-, di-, and trisubstituted epoxides used in these studies. The aldehyde was synthesized from the corresponding diol as shown in Figure 2. The 2,3-saturated compounds were synthesized by an identical pathway starting from citronellol rather than geraniol.

Initial Rates of Hydration of Phenylgeranyl Ethers and Related Compounds

The term substrate specificity, when rigidly defined, refers to k_{cat}/K_m.[87] Substrate specificity is thus a function of the enzyme's affinity for the substrate (Apparent K_m) and its ability to act on it (k_{cat}). There are not sufficient data to discuss the true substrate specificity of the soluble epoxide hydrase; in fact, a careful review of the literature on the microsomal

epoxide hydrases indicates that very little is actually known about the substrate specificity of these enzymes (see below).

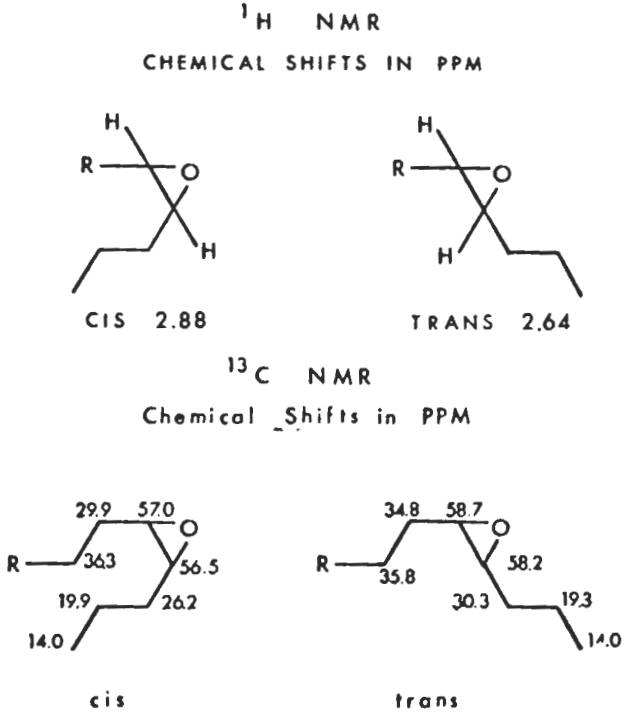


Figure 7. Chemical shifts from ^{1}H and ^{13}C nuclear magnetic resonance spectra used to distinguish *cis*- and *trans*-proton propyl substituted *p*-bromophenyl ether epoxides (Figure 1).

Under careful conditions of analysis an apparent K_m and V_{max} have been determined for R 20458. The values vary using the crude soluble fraction from mouse liver from preparation to preparation over a relatively narrow range (K_m ~6×10^{-6}M, V_{max} ~2nmol/min/mg) and they compare favorably with values obtained for the microsomal epoxide hydrase by several other workers (Table I). The soluble epoxide hydrase has a substrate affinity high enough to facilitate the hydration of xenobiotics present in very low concentrations.[46-48]

The soluble epoxide hydrase seems to hydrate only the monomeric form of the substrate (Figure 13)[46-48] as may have also been found for

Table I. Apparent K_m and V_{max} for the Microsomal Epoxide Hydration of Several Substrates

Substrate	K_m	V_{max}	Enzyme State	Reference
Styrene Oxide	$2\text{-}8 \times 10^{-4}$ *M*	200-400 nmoles/min/mg *N*	Purified from guinea pig liver microsomes	8
Styrene Oxide	6.7×10^{-4} *M*	800 nmoles/min/mg	Purified from rat liver microsomes	52
Styrene Oxide	–	11 nmoles/min/mg	Crude microsomal from human liver	75
Styrene Oxide	1.8×10^{-4} *M*	6.2 nmoles/min/mg	Crude microsomal from rat liver	116
Styrene Oxide	–	6.3 nmoles/min/mg	Crude microsomal from rat liver	67
Styrene Oxide	2.5×10^{-4} *M*	11 nmoles/min/mg	Crude microsomal from rat liver	66,79
Octene 1,2-Oxide	1.4×10^{-5} *M*	49 nmoles/min/mg	Crude microsomal from induced rat liver	88
HEOM	8.3×10^{-6} *M*	12 nmoles/min/mg	Crude microsomal from rat liver	111
Octene 1,2-Oxide	–	13 nmoles/min/mg	Crude microsomal from rat liver	67
Phenanthrene 9,10-Oxide	–	39 nmoles/min/mg	Crude microsomal from rat liver	67
Phenanthrene 9,10-Oxide	4.6×10^{-6} m	32 nmoles/min/mg	Crude microsomal from rat liver	65, 96
Phenanthrene 9,10-Oxide	5.4×10^{-6} m	87 nmoles/min/mg	Crude microsomal from induced rat liver	88
Benzo(a)pyrene 4,5-Oxide	–	7.2 nmoles/min/mg	Crude microsomal from rat liver	67

Benzo(a)pyrene 4,5-Oxide	$5.9 \times 10^{-6} M$	7.7 nmoles/min/mg	Crude microsomal from rat liver	65,96
Benzo(a)pyrene 4, 5-Oxide	$2.5 \times 10^{-7} M$	17.5 nmoles/min/mg	Crude microsomal from induced rat liver	88
3-Methylcholanthrene 11,12-Oxide		1.2 nmoles/min/mg	Crude microsomal from rat liver	67
3-Methylcholanthrene 11,12-Oxide	$\sim 2 \times 10^{-6} M$	1.4 nmoles/min/mg	Crude microsomal from rat liver	65, 96
3-Methylcholanthrene 11,12-Oxide	$7.9 \times 10^{-6} M$	2.4 nmoles/min/mg	Crude microsomal from induced rat liver	88

the microsomal hydrase,[88] and the initial rates of hydration of the brominated compounds could only be determined below the critical micelle concentrations (CMCs) of the substrates (about 3×10^{-5} *M* determined by surface tension measurements). The conflicting problems of assay sensitivity and CMC precluded valid kinetic studies of the epoxide hydration of the *p*-bromo analogs, and the substrates were thus compared via their initial rates of hydration by the soluble fraction. For most members of phenylgeranyl ether series the initial rates are *cis*-1,2-disubstituted ≳ monosubstituted > *trans*-1,2-disubstituted > trisubstituted.[46-48] It was earlier reported that trisubstituted epoxides were hydrated more rapidly than tetrasubstituted epoxides.[51] For the disubstituted series, the rate of hydration generally increases as the length of the terminal alkyl substituent increases, ultimately obtaining a higher rate than that observed for the monosubstituted compound. The rate of hydration decreases after $R=C_3H_7$, but the decreased CMC of the more lipophilic members of the series may have an effect.[46-48]

The opposite trend occurs with trisubstituted epoxides (Figure 8).[46-48] As earlier pointed out[89] increasing the number of alkyl substituents about an epoxide increases the ease of acid catalyzed epoxide hydration, presumably via stabilization of a carbonium ion-like intermediate. This trend has also been found with acid-catalyzed hydration of the phenylgeranyl ether epoxides,[58,59] but the initial rates of hydration by the soluble fraction are clearly not consistent with the formation of a carbonium ion-like intermediate as the rate-limiting step (Figure 12). As the length of the alkyl substituent increases on trisubstituted epoxides, the rate of enzymatic hydration decreases.

Competition Between Substrates

The initial rate of hydrolysis indicates, but does not equal, the relative k_{cat} for the different substrates. Similarly, the relative ability of compounds to inhibit the hydration of another epoxide may give an indication of their relative affinities for the enzyme active site. Surprisingly, the apparent affinity for the enzyme was highest with tri- and lowest with monosubstituted epoxides. *Trans*-1,2-disubstituted epoxides seem to bind more tightly than the analogous *cis*-epoxides, and, in fact, they are more rapidly metabolized than the *cis* in a 1:1 mixture, although the opposite phenomenon occurs when the epoxides are incubated as pure isomers.[46-48] Interpretation of the competition data is complicated by the fact that some substituted epoxides clearly showed noncompetitive rather than competitive inhibition of the hydration of R 20458. Definitive kinetic studies on this enzyme await substrates which can be synthesized by fewer steps, which are more water soluble, and which can be analyzed by less laborious procedures. The studies of initial rates and apparent affinities indicate that

the soluble epoxide hydrase is capable of metabolizing a wide range of substrates, but elucidation of the true substrate specificity of the enzyme awaits enzyme purification and valid kinetic analysis.

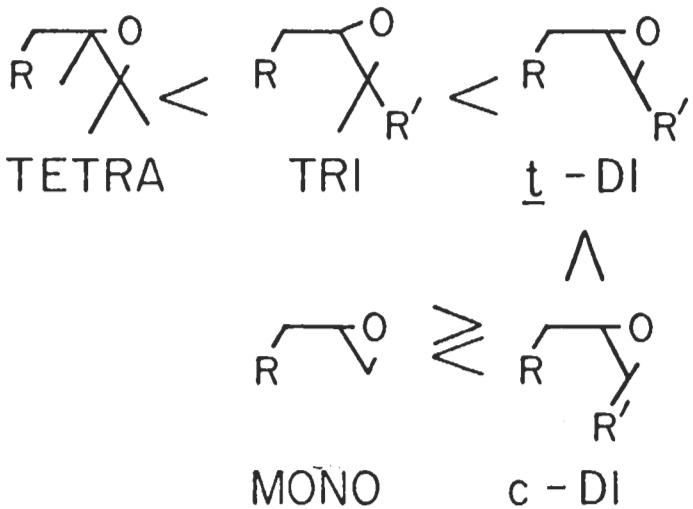


Figure 8. Relative initial rates of hydration as R' is varied. R' = H, CH_3,C_2H_5 or nC_3H_7. Trisubstituted epoxides were assayed as *cis*-rich mixtures while disubstituted epoxides were assayed as mixtures and as pure *cis*- and *trans*-isomers.

Hydration of Natural Product Epoxides

The soluble epoxide hydrase is capable of slowly hydrating squalene oxide and more rapidly, squalene dioxide and apparently lanosterol epoxide (Figure 9).[46,49] Since squalene oxide and dioxide have very low CMCs and the analytical procedures for lanosterol epoxide and diol are very difficult, initial rates of hydration have not been precisely determined. Studies to date, however, indicate that the soluble epoxide hydrase may compete for squalene oxide and its homologs when postmitochondrial rather than microsomal preparations of squalene oxidocyclase are used. Some investigations on the substrate specificity of squalene oxidocyclase using postmitchondrial preparations may reflect the substrate specificity of the competing soluble epoxide hydrase as well as that of squalene oxidocyclase. In fact, the first indication of a soluble epoxide hydrase probably came form the work of Clayton *et al.*[90] and Corey *et al.*[91] when these and other workers were using a postmitochondrial supernatant as well as

a microsomal preparation to study squalene oxidocyclase. Corey *et al.*[91] actually predicted that there might be a scavenger enzyme which hydrated squalene oxide and especially mono- and disubstituted squalene oxide homologs. Brooks *et al.*[6] found no squalene diol produced in rabbit microsomes, while Oesch *et al.*[92,93] indicate that squalene 2,3-oxide is not hydrated and bis-norsqualene-1,2-oxide is only slowly hydrated by the crude homogenate or a partially purified microsomal preparation from guinea pig liver. The substrate concentration used by the later workers is clearly above the CMC of squalene oxide, and the relative quantity of monomeric substrate was probably quite low.

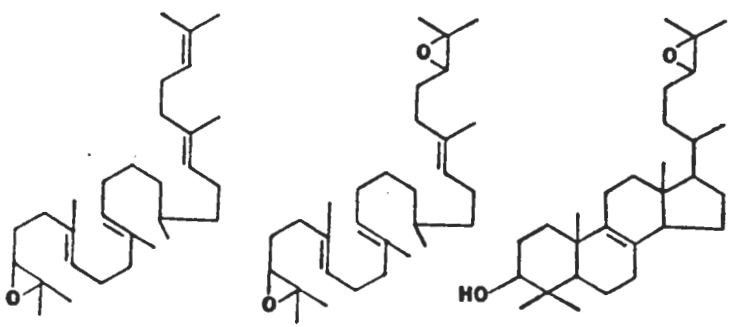
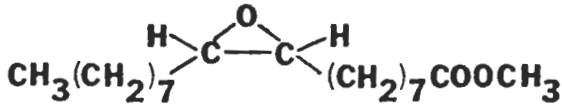


Figure 9. Structure of squalene 2,3-oxide, squalene 2,3-23,24-dioxide and lanosterol epoxide which were hydrated to their respective diols, tetraols or triols by the mouse liver soluble epoxide hydrase.

The cholesterol-5α,6α-epoxide has been shown to be a carcinogen, and it is metabolized by epoxide hydrases in mouse skin[94] and rat lung.[95] It has not been established which subcellular fraction(s) is responsible for this hydration, but preliminary studies indicate that cholesterol-5α,6α-epoxide is a very poor substrate for the mouse soluble hepatic epoxide hydrase (Sevanian and Hammock, unpublished). Watabe *et al.*[42] indicate that 2α, 3α are slowly hydrated and 2β,3β-epoxy-5α cholestanes are rapidly hydrated by rabbit liver microsomes, but hydration by a soluble enzyme has not been ruled out.

Two of the best substrates for the soluble epoxide hydrase are the epoxides of the fatty esters methyl oleate (*cis*) and methyl elaidate (*trans*)

(Figure 10). The two substrates are metabolized at similar rates and, in contrast to the phenylgeranyl series, the *trans*-epoxide appears to be metabolized at a slightly higher rate.[46,84] Both the free acids and the methyl esters are rapidly hydrated, although the relative rates of hydration have not yet been determined. The hydration of these fatty acids was earlier studied in rabbit liver microsomes.[43,45]



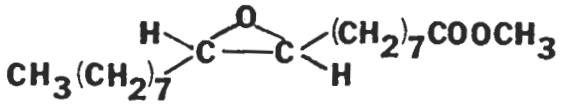


Figure 10. Structure of the methyl esters of epoxy oleic and epoxy elaidate acids (*cis*- and *trans*-epoxymethylstearate, respectively). Both compounds are rapidly hydrated by the soluble fraction from rabbit and mouse liver, kidney and lung homogenates.

Relative Hydration of Styrene Oxide and Allylbenzene Epoxide

Since styrene has been used for so many studies on the microsomal epoxide hydrases, its metabolism in the soluble fraction of mouse liver was investigated. In several soluble preparations which were highly active on other substrates, levels of styrene diol significantly above background were not found, even when varying pH's (6.8-9.0), substrate concentrations (10^{-3} to $^{-6}$ *M*), enzyme concentrations (0.01%-10% fresh weight equivalent), and incubation times (5-60 min) were used.[50] However, if the epoxide is β- to the aromatic ring as in allylbenzene epoxide (Figure 11), the epoxide is very rapidly hydrated by the soluble fraction. Also β-methyl styrene (Figure 11), which was reported by Oesch *et al.*[93] not to be a substrate for the guinea pig microsomal enzyme, is very rapidly metabolized by the soluble fraction. These three substrates could be very useful in monitoring the activities of microsomal and soluble epoxide hydrases. When the livers from three mammalian species were used, it was found that

styrene oxide is hydrated only by the microsomal fraction, *trans*-β-methyl styrene oxide is hydrated exclusively by the soluble fraction, and allylbenzene oxide is hydrated by both fractions.[50] The *cis*- and *trans*-isomers of stilbene oxide could similarly be used to distinguish soluble and microsomal activity in a postmitochondrial supernatant (see below).

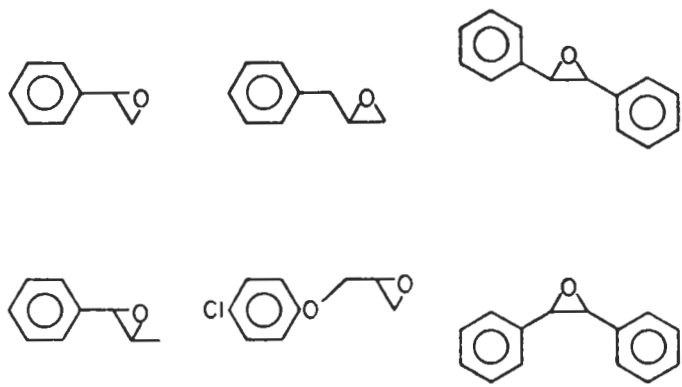


Figure 11. Structures (left to right top line first) of styrene oxide, allylbenzene oxide, *trans*-stilbene oxide, *trans*-β-methylstyrene oxide, *p*-chlorophenylglycidyl ether and *cis*-stilbene oxide. Styrene oxide and *cis*-stilbene oxide are not hydrated by the mouse liver soluble fraction, while *trans*-β-methylstyrene oxide and *trans*-stilbene oxide are hydrated exclusively by the soluble fraction.

Comparison of Substrate Selectivity of Microsomal and Soluble Hydrases

When defined in the strict sense, substrate specificity data for the microsomal epoxide hydrase is also very limited. Recently, comparative kinetic studies have been performed with several arene oxides in two laboratories (Table 1).[65,88,96] Several studies indicate that the arene oxides have a high apparent affinity for the microsomal enzyme and are rapidly hydrated by it. No studies on the hydration of arene oxides by the soluble hydrase have been reported, but the lack of catalytic effect on styrene oxide and *cis*-stilbene oxide by the mouse liver soluble fraction[50] and the lack of metabolism of 3-methylcholanthrene-11,12-oxide by the rat liver soluble fraction[28] may indicate that the soluble epoxide hydrase may not possess a broad spectrum of activity against epoxides alpha to aromatic nuclei. The rapid hydration of β-methylstyrene and *trans*-stilbene oxide is provocative information to the contrary especially in the light of the high mutagenicity of some arene diol epoxides (see below).[12] It is hoped that more

definitive studies on arene oxide and especially arene diol epoxide hydration by the soluble epoxide hydrase will be undertaken in the near future.

There is a plethora of reports indicating comparative initial rates of hydration of a variety of epoxide-containing substrates, but there is little information on substrate specificity or selectivity which lends itself to a comparison with currently available information on the soluble epoxide hydrase. Jerina *et al.*[14] concluded that an olefin alpha to the epoxide increased the rate of its hydration by the hepatic microsomal fraction. Maynert *et al.*[5] indicated that for both microsomal and soluble fractions from rat liver, disubstituted epoxides were hydrated faster than trisubstituted but slower than monosubstituted epoxides. The most comprehensive study was that by Oesch and co-workers,[92,93] yet these workers shared our problem of a lack of a rapid, sensitive, general assay for epoxide hydrase activity. Thus, Lineweaver-Burke plots were reported only for styrene oxide and other substrates were compared based on their initial rates of hydration and as inhibitors for the hydration of styrene oxide. From these studies it was concluded that the microsomal epoxide hydrase would hydrate lipophilic epoxides which were mono-, 1,1-di- and *cis*-1,2 disubstituted, but not *trans*- 1, 2- , tri- or tetrasubstituted epoxides.[8,92,93] Most of the data apparently were generated using unwashed guinea pig liver microsomes, but some information was derived from incubations with the 600-g supernatant. Some of the activity reported for some substrates such as octene 1,2-oxide or *p*-chlorophenyl 2,3-epoxypropyl ether may have been due to soluble epoxide hydrases. The soluble epoxide hydrase shows a similar substrate selectivity when initial rates of hydration are compared; however, it seems to have a lipophilic binding site on either side of the enzyme catalytic site as evidenced by the rapid hydration of 1,2-disubstituted epoxides. The soluble epoxide hydrases seem to have a greater ability to hydrolyze tri- and tetrasubstituted epoxides than do those of the microsomal fraction.[51] There was partial correlation between inhibition of hydration of three alkene oxides[5] and styrene oxide by compounds and their efficacy as a substrate for the microsomal hydrase[8,92,93] and this correlation has been extended to investigations with human liver microsomes.[73,75] In contrast, based on the data now available with the soluble enzyme, there generally appears to be an inverse correlation between initial rate of hydration by and competitive substrate binding with the soluble hydrase.[46-49]

Both the microsomal and the soluble epoxide hydrases appear to hydrate partially overlapping broad spectra of substrates. This observation is substantiated by recent work from this laboratory on several simple substrates. For instance, when substrates are arranged in order of decreasing rates of hydration by the mouse hepatic soluble fraction, one finds: allylbenzene

oxide > *t*-β-methylstyrene oxide > *t*-stilbene oxide > *p*-chlorophenylglycidyl ether ≫ *c*-stilbene oxide ≫ styrene oxide, while the corresponding order in the microsomal fraction is: *cis*-stilbene oxide > styrene oxide > *p*-chlorophenylglycidyl ether ≫ *t*-stilbene oxide and *t*-β-methylstyrene oxide. An appreciation of substrate specificity for enzymes from both fractions is clearly important in assessing the potential risk of some xenobiotics. Studies on the substrate specificity of the epoxide hydrases is hampered by the lack of a good general assay procedure and by the lipophilic nature of many of the substrates. Valid kinetic data are thus difficult to obtain and are sometimes confusing.[8,48,49,51,65,88] The availability of high-specific-activity substrates and sensitive assay techniques which allow substrates to be assayed below their CMC may simplify interpretation of kinetic data. One approach to the investigation of substrate specificity is to study those compounds which are known to pose immediate environmental risks. An alternate course which yields less dramatic short-term results, but will ultimately be useful in the prediction of hydrolytic lability, is the synthesis of specific substrates which will allow a QSAR (quantitative structure-activity relationship)[97] approach to the understanding of the substrate specificities of the epoxide hydrases as well as the chemical and biological properties of the epoxides themselves.[12,29,58,59,89] Such information will be useful in drug design and in the prediction of environmental risks of pesticides, industrial by-products and other potential xenobiotics.

Mechanism of Epoxide Hydration

The hydration of twenty 1,2-disubstituted epoxides including epoxymethyloleate and epoxymethylelaidate by the epoxide hydrases in the soluble fraction has been shown to proceed in a stereospecific *trans*-manner ner.[46-49] In other words, the pure *cis*-epoxides are hydrolyzed only to the corresponding *threo*-diols and the *trans*-epoxides are hydrolyzed only to the *erythro*-diols. This is the same stereochemistry that occurs with acid hydration (Figure 12).[89] However, the initial rates of hydrolysis of di- vs trisubstituted epoxides is not consistent with mechanisms of epoxide hydration in which the formation of a carbonium ion-like intermediate is the rate limiting step.[46-49,58,59,89] Mass spectral analysis of the hydration of the ^{18}O epoxide (1-[4'-ethylphenoxy]-3,7-dimethyl-6,7-epoxyoctane) by the soluble epoxide hydrases demonstrates that the ^{18}OH is almost entirely at C-7. These results indicate that in this molecule nucleophilic attack of water occurs at the least hindered carbon. Simultaneous studies of acid-catalyzed hydration demonstrated ^{18}OH to be almost entirely at C-6 (Ratcliff, Schooley and Hammock, unpublished). If low-molecular-weight cofactors are necessary for hydration by the soluble

epoxide hydrase, they are not easily removed by gel filtration or chelating agents. Epoxide hydration by the microsomal fraction also occurs in a *trans*-manner,[6,8,41-45,92,93,101,102] and recent studies provide information that hydration involves the nucleophilic addition of water[8,69,98-101] and that a histidine may be involved at the active site![102] In contrast to many excellent studies on the absolute stereochemistry of microsomal hydration[8,101] there has been only one superficial experiment on the stereochemistry of hydration using the hepatic soluble fraction from mouse. Using racemic methyl (2*E*, 6*E*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate (juvenile hormone III) as substrate, the enzyme produced a preponderance of the 10R over the 10S isomer (60:40) when the products were analyzed by HRLC of their α-methoxy-α-trifluoromethylphenylacetic acid esters[103] (Schooley and Hammock, unpublished information). Different mechanisms of epoxide hydration have been found in fungi![104-106] bacteria[107] and possibly trypanosomes![108] Clearly, further studies are needed on the mechanism of epoxide hydration by the microsomal and especially other epoxide hydrases.

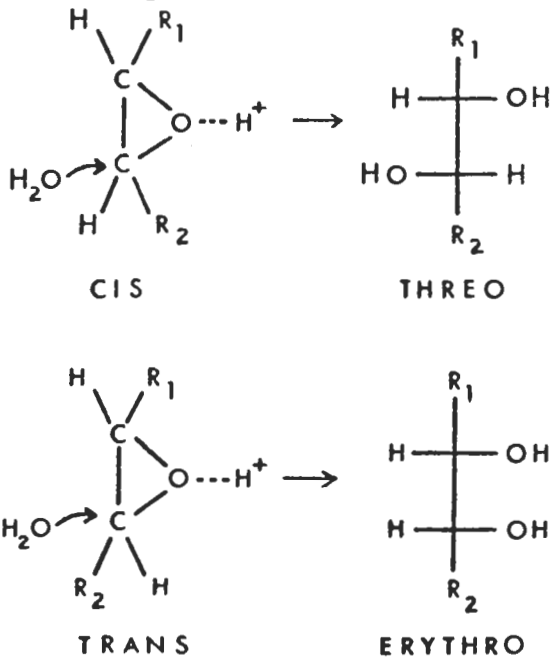


Figure 12. Stereochemistry of acid catalyzed epoxide hydration.

Purification of the Soluble Epoxide Hydrase

Only very preliminary purification studies have been performed on the soluble epoxide hydrase from mouse liver. Enzyme activity in the crude supernatant can be partially stabilized by the use of protease inhibitors and the activity is quite stable when the crude or partially purified fraction is frozen or held just above freezing. Gel filtration indicates an apparent molecular weight of about 130,000 and yields a purification factor of 20-30X and a recovery of 75-80% of the enzyme activity.[46-49] Other purification techniques such as ammonium sulfate precipitation, anion and cation exchange chromatography, electrophoresis and isoelectric focusing have yielded disappointing enzyme recoveries, but they have not been exhaustively explored.[49,51]

In contrast, several laboratories have reported the purification of the epoxide hydrase acting on styrene oxide to apparent homogeneity[52-54,56] in addition to reports of partial purification such as the enzyme hydrating styrene oxide from human liver microsomes.[73,75] The purification factors from the isolated or solubilized microsomes are often around 60X, and they may give the impression that our 40-60X purification (gel filtration and ion exchange) of the soluble enzyme has resulted in a preparation approaching homogenicity. The soluble fraction, however, normally contains about ten times as much protein as the microsomal fraction so our attempts to purify the soluble epoxide hydrase must be considered as preliminary.

The epoxide hydrase activity when R 20458, epoxymethyloleate, or a mono-, di- or trisubstituted epoxide from the *p*-bromophenylgeranyl ether series are used as substrates emerges in a single sharp peak from gel filtration. This information does not indicate that only a single epoxide hydrase is present in the soluble fraction, but at least it indicates that these five substrates are degraded by an enzyme or enzymes of similar Stoke's radii.[46-48]

Inhibition, Stimulation and Induction of the Soluble Epoxide Hydrase

The activity of the soluble epoxide hydrase is strongly inhibited by some ions, notably the cupric ion. At relatively high concentrations the ferric ion stimulates epoxide hydrase activity, possibly suggesting that some transition metals can polarize the epoxide and aid its opening by the hydrase.[46,49] The soluble epoxide hydrase is not strongly inhibited by the organophosphates tested, nor is it inhibited by cyclohexene oxide or trichloropropene oxide with R 20458 as the substrate. It is also not inhibited by the thiirane analog of R 20458 or the corresponding diol, and inhibition by limonene epoxide and a cyclic analog of R 20458 is weak.[36,38,48,49]

In contrast, the microsomal hydrase acting on styrene oxide appears to be strongly inhibited by trichloropropene oxide and several other epoxide-containing substrates, although thiiranes except for styrene sulfide also give poor inhibition.[8,79,109] If Corey *et al.*[91] were actually looking at the soluble epoxide hydrase in their studies, then hydration of squalene homo logs, at least, is not inhibited by 2,3-iminosqualene. The organophosphates tested are poor inhibitors of the mammalian soluble or microsomal epoxide hydrases. Some organophosphates have been found to inhibit some insect and mammalian microsomal epoxide hydrases acting on the cyclodiene epoxide HEOM,[34,110,111] but this laboratory has failed to detect potent inhibition of juvenile hormone epoxide hydrase,[33,35] so their inhibitory activity does not appear ubiquitous. Glycidyl ethers have also been found to be potent inhibitors of mammalian epoxide hydrase activity on HEOM.[111] DuBois *et al.*[102] have recently surveyed numerous microsomal epoxide hydrase inhibitors, and 2-bromo-4'-nitroacetophenone proved to be especially interesting as it bound to a histidine, possibly at the active site, and several ketones have been found to inhibit the soluble epoxide hydrase.[49] Although potent inhibitors of epoxide hydrases have not yet been reported among the clinically used drugs, several studies have cautioned that weak inhibition may increase the risk from epoxide-containing mutagens or carcinogens by potentiation.[112-114]

Several alcohols, ketones and imidazoles, including metyrapone, stimulate microsomal epoxide hydrase activity.[8,75,115,116] Some alcohols and other co-solvents have been found to stimulate the soluble epoxide hydrase activity at high substrate concentrations. This effect appears to be due, in part, to increasing the solubility and CMC of the substrate which would give an apparent increase in reaction rate because the soluble epoxide hydrase does not seem to hydrate the micellular form of substrates (Figure 13). With R 20458 as substrate the reaction rate increases with substrate concentration until the CMC of the substrate is reached. At concentrations above the CMC the reaction rate does not increase with additional substrate, although the production of diol is linear for longer periods. Addition of varying levels of methanol as co-solvent increases the reaction rate at substrate concentrations above the CMC but has little effect on substrate concentrations below the CMC.[49] Since CMCs have not been reported for many of the apparently lipophilic substrates of the liver microsomal epoxide hydrases and relatively high substrate concentrations are normally used, some of the data presented on the kinetics of microsomal epoxide hydrases may may be explained in terms of micelle formation of the substrate as well as the substrate's solubility in microsomes and added lipid micelles.[65,88,116]

There have been numerous investigations into the induction of microsomal epoxide hydrase activity, and inducers have been found among drugs, pesticides, food additives and other possible environmental

contaminants.[8,67,71,79,81,109,117-121] In comparison, only very preliminary studies have been performed on the soluble epoxide hydrase, but phenobarbital was found to induce the levels of soluble epoxide hydrase activity in the rat.[36]

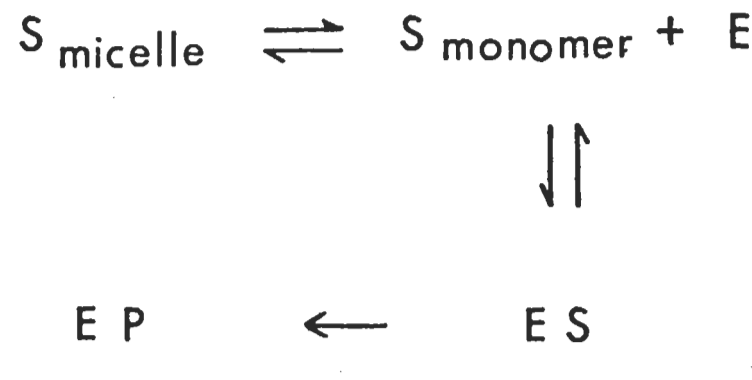


Figure 13. Hydration of lipophilic substrates (S) by the soluble epoxide hydrase in the monomeric but not the micellular form.

Environmental Implications of the Soluble Epoxide Hydrase

Hydrolysis of Mutagenic Epoxides

Several workers have shown that the microsomal epoxide hydrase in either its free or its membrane-bound form is capable of reducing the mutagenicity of epoxide-containing compounds.[55,71,76,112,113,122-125] Recently, this laboratory has found that when crude or partially purified epoxide hydrase from the soluble fraction of mouse liver is added to the Ames *Salmonella typhimurium* assay[61,62] the revertant colonies caused by vinyl cyclohexene dioxide and several other alkene oxides are reduced (El-Tantawy, unpublished).[49,50] This blockage of mutagenic activity seems to occur in a dose-dependent manner with the soluble hydrase, and the same effect occurs with partially purified enzyme from gel filtration. These results indicate that the soluble epoxide hydrase could be a useful microchemical tool for derivatizing epoxides[51] or testing if epoxides are involved in the mutagenic action of some compounds.[55]

The continuing debate rages concerning the reproducibility and predictability of various *in vitro* mutagenicity assays.[62,126] This debate

was recently summarized with some new data which may concern the soluble epoxide hydrase.[127,128] These workers pointed out the variability of the S9 fraction used in the assays, and they were particularly critical of enzymes present in the S9 fraction, which both activate (mixed-function oxidases) and deactivate (microsomal epoxide hydrases and glutathione-S-epoxide transferases) potential mutagens such as benzo(a)pyrene. This "weakness" of the Ames' assay is also its strength. Once components of the S9 fraction have been identified and characterized from different species, they can be used to define the nature of the activation and inactivation processes involved with a specific mutagen to help or explain differences in susceptibility of strains or species to mutagens or carcinogens as recently illustrated by work on acetylaminofluorene.[129] Such assays can then be more precisely used to assess the role of metabolism in the action of mutagens, especially when active metabolites are unstable.

The soluble epoxide hydrase appears to metabolize somewhat different substrates and to be inhibited by different compounds than the microsomal enzyme. It may thus be an important component of the activation-deactivation system whose role has not been considered in either *in vitro* or *in vivo* systems. For instance, the soluble epoxide hydrase does occur in guinea pig liver[49] and may be involved as one of the epoxide-inactivating factors in the soluble component of guinea pig S9 fraction[127] or other species.[125] The properties of the soluble epoxide hydrase should also be considered when attempts are made to correlate *in vivo* toxicity or carcinogenicity of compounds which may act as epoxides with deactivating enzymes.[119,127,128,130-133]

Organisms are faced with metabolizing and eliminating natural and man-made xenobiotics in their diet as well as potentially toxic by-products of metabolism. The exact roles of the soluble, and also the microsomal, epoxide hydrases in these processes are not clear, and such overviews can come only from an enhanced body of knowledge on xenobiotic pharmacodynamics in general and epoxide hydrases in particular. Several lines of evidence support the close association of a monooxygenase and epoxide hydrase system.[36,81,130,134] However, the mutagenicity and carcinogenicity of numerous alkenes and arenes probably indicates that epoxides do move from the endoplasmic reticulum to the cytosol where they may be hydrated by the soluble enzyme.

Our preliminary studies on the hydration of the epoxides of oleic and elaidic acid may have environmental implications. Epoxymethylstearate is known to occur in pulp mill effluents and to be toxic to fish. This and other epoxidized fats are used as plasticizers and stabilizers in some plastics.[135] It is conceivable that they could find their way into man's diet or water supply, so the *in vivo* metabolism of epoxymethylstearate has been

recently studied in rats.[136] Epoxidized fats may occur naturally in man's diet. Holloway and Deas,[137] in discussing the occurrence of epoxyoctadecanoic acid in plant cutins and suberins, pointed out that earlier techniques of qualitative lipid analysis could have destroyed any epoxides present and that natural epoxidized fats may be more widespread than earlier thought. Several studies have shown reasonable levels of epoxidized fats in natural lipid fractions.[138-141] There is also indication that lipid epoxides can form via peroxidation[142] or oxidation[143] pathways. Such lipid epoxides present in our diet, or possibly formed *in vivo*, may be metabolized, in part, by the epoxide hydrases present in the cytosol. An increase has been reported in the levels of linoleic and oleic acid epoxides as well as cholesterol epoxides in rat lung parenchymal tissue after exposure to NO_2.[95] We have found that epoxymethyloleate and elaidate are rapidly hydrated in the soluble fraction of rabbit lung homogenate, and a further investigation of the epoxide hydrase activity in the lung in relation to atmospheric components or smoking may thus be appropriate.

A cursory review of the literature reveals many potential substrates for the soluble epoxide hydrase. Many of the di- or *bis*-epoxide monomers used in the plastics industry have long been known as potential toxins and mutagens,[144] and preliminary evidence indicates that some are metabolized by the soluble hydrase. The epoxide of allylbenzene may be a good model for the epoxide of safrole[2,145] and other allyl-containing natural products, and possibly some allyl-containing drugs such as allobarbital, 2-allyl-2-isopropylacetamide, alphenal and secobarbital,[8] some of which are known to destroy cytochrome P-450. Other possibly substrates include still other drugs,[113] glycidyl ethers and esters used in epoxy resins, and a plethora of natural products ranging from the aflatoxins to the trichothecenes.[2,146-149] The relative levels of the enzymes involved in metabolizing a xenobiotic should be considered when assessing the potential risk which this xenobiotic poses to man.

Implications of Inhibition, Stimulation or Induction of the Soluble Epoxide Hydrase

The soluble epoxide hydrase may be significant in the hydration of potentially dangerous xenobiotics as is the epoxide hydrase in the microsomal fraction. Thus, its titer and substrate preference may be important factors in assessing risks posed by xenobiotics. Recent studies have shown that some slowly metabolized epoxides are able to inhibit the hydration of rapidly metabolized epoxides.[46,48] Although the compounds investigated represent likely classes of natural product epoxides from food, cosmetics or environmental contamination, these compounds themselves are

rapidly hydrated and probably present minimal risk of substantial *in vivo* inhibition of the soluble epoxide hydrase.[36] However, these inhibition studies raise the possibility that more stable compounds may significantly inhibit the soluble epoxide hydrase and allow an accumulation of dangerous, epoxidized xenobiotics. Similarly, in the conversion of lipophilic xenobiotics to hydrophilic metabolites, a balance must be maintained between the monooxygenase system which forms electrophilically reactive species and the differentially induce and/or inhibit the monooxygenase system and the microsomal epoxide hydrases resulting in an altered distribution of metabolites.[8,71,109,150] This altered distribution can obviously either increase or decrease the health risks of xenobiotics both activated and inactivated by metabolism. The relative stimulation and induction of activating and deactivating enzymes by xenobiotics or cultural conditions thus become important components of environmental risk assessment, and the epoxide hydrase activity in the soluble fraction should be considered in this assessment.

Activation of Potential Cytotoxins by Epoxide Hydration

Evidence is mounting that benzo(a)pyrene 7,8-diol and benzo(a)anthracene 8,9-diol are further metabolized to "diol-epoxides" which are more mutagenic than the arene oxides.[12,124,151-153] Thus, the microsomal epoxide hydrase can be considered as an activating as well as a deactivating enzyme. Until arene oxides and arene diol epoxides are definitively ruled out as substrates for epoxide hydrase(s) in the soluble fraction, the soluble fraction should be considered in those activation-deactivation reactions. Since some aliphatic diepoxides are known to be mutagens and carcinogens,[144] this laboratory has examined the reactivity and mutagenicity of the corresponding diol-epoxides.[149] The diol-epoxides are highly reactive but apparently they tend to react internally, leading to cyclic products.[38,49] Preliminary data so far do not indicate that epoxide hydration by the soluble fraction is involved in any activation process, but the converse may prove true as more substrates are examined.

Soluble Epoxide Hydrase—An Overlooked Enzyme

A wide variety of epoxides are hydrated by an enzyme or enzymes in the soluble fraction, and in some cases the hydration takes place much faster in the soluble fraction than in any other fraction. This conclusion may superficially seem to be in conflict with a great mass of data concerning the microsomal epoxide hydrase. However, the information which our laboratory has generated on the soluble epoxide hydrase is, in fact, not in opposition with the vast majority of information on the microsomal epoxide hydrases.

In retrospect, it is easy to see how hydrase activity in the soluble fraction was overlooked. The great mass of information on the microsomal enzymes has come from laboratories interested primarily in the arene oxides. Given such an interest, styrene oxide (Figure 11). is a superb model substrate for studying epoxide hydrase activity. The initial prediction that the epoxide alpha to the aromatic ring will be largely metabolized by the same microsomal enzymes hydrating arene oxides has been borne out by subsequent studies, most recently that of Oesch and Bentley.[122] The conclusion from these studies as performed should be applied only to the detergent-solubilized form of microsomal epoxide hydrases. Coincidentally, styrene oxide is one of the few substrates not metabolized by the soluble fraction of mouse liver.[50] Based on our initial interest with the metabolism of terpenoid epoxides, we could have drawn the opposite conclusion that the microsomal fraction from mice made a relatively minor contribution to epoxide hydration compared to the soluble fraction.

Jerina *et al.*[14] reported that benzene oxide was converted to 1,2-diols and other products by both the soluble and the microsomal fractions of rat and rabbit liver. A high pH (8.0) apparently had to be used to stabilize the benzene oxide in these experiments which may have reduced the activity of the soluble enzyme to only 10% of that of the microsomal enzyme. In spite of clearly demonstrating the contribution of a soluble epoxide hydrase to benzene oxide metabolism, many subsequent publications from this laboratory ignored this early 1968 report.

Maynert *et al.*[5] reported that epoxide hydrases (hydrolases) were microsomal. This observation was based on the fact that a rat liver 9000 x g supernatant + NADPH converted *n*-l-octene, *trans-n*-4-octene and 3-ethyl-2-pentene to their respective glycols at the same rate as an equivalent microsomal + NADPH preparation. Such data could also be interpreted to indicate that the epoxidase (monooxygenase) rather than the epoxide hydrase was the rate-limiting step in glycol production from an olefin as recently shown for styrene.[66] These workers also reported that for the substrates 1,2-epoxyoctane, *trans*-4,5-epoxyoctane, and 2,3-epoxy-3-ethylpentane, 85% of the epoxide hydrase could be pelleted at 165,000 g. They concluded that the soluble and microsomal enzymes might be the same. As earlier indicated, the rat has the lowest level of soluble epoxide hydrase activity of any mammal so far examined (Figure 4). Had Maynert *et al.*[5] used the mouse, rabbit or guinea pig as an enzyme source, the existence of the soluble epoxide hydrase might have been recognized a decade earlier. The investigation of the subcellular distribution of epoxide hydrase activity with 3-methylcholanthrene-11,12-oxide as substrate also used rat enzyme at a high pH.[28]

The microsomal enzyme acting on styrene oxide was found to have a

broad pH optimum with an increase in activity toward more basic pH.[52,55,67,73,76] This dependence on a basic pH with some but not all substrates was accentuated when the microsomal enzyme was solubilized and purified. In contrast, the soluble fraction demonstrates its highest epoxide hydrase activity between pH 6.8 and 7.8 and the activity falls off as higher pH ranges are used. Also, the soluble enzyme is exceedingly sensitive to inhibition by some inorganic ions at levels which conceivably could contaminate buffers.[47,49]

Subcellular distribution studies are reported for only a few substrates, and we have reproduced some of these studies in our laboratory.[50] Therefore, even if subcellular distribution studies had been performed with an appropriate substrate, an unfortunate choice of age, sex or species of the test mammal or pH or buffer conditions could have led to the conclusion that there was little or no contribution to epoxide hydration from the soluble fraction.

The early studies on subcellular distribution of epoxide hydrase activity appear to have been carefully done with exhaustive controls.[5,27] The conclusions from these studies indicated that epoxide hydrase activity was microsomal for the substrate(s) used. As these studies were reviewed, the reviews reviewed in their turn, and the reviews ultimately quoted in texts, epoxide hydration became a general microsomal phenomenon with only a very few specific experiments supporting such a conclusion. Faced with such a mass of literature, most workers apparently ignored the requisite studies on subcellular distribution when a new substrate or tissue was used, or have used styrene oxide as the substrate for these studies.[29,30,123] The report of a subcellular distribution experiment by Stoming and Bresnick[28] is a pleasant exception to this trend.

In many cases ignoring the epoxide hydrase activity in the soluble fraction will have little effect on the results or conclusion of the experiment. For instance, in the studies by Watabe and others[43,45] epoxymethyloleate was used as a substrate for studying the microsomal epoxide hydrase. In one early study,[77] the postmitochondrial supernatant was used as the "microsomal preparation" while in other cases carefully "washed microsomes" were utilized.[41-44] The soluble fraction from rabbit liver very rapidly hydrates epoxymethyloleate,[46,84] and it is unfortunate that the chemical studies from Watabe's laboratory were not extended to the soluble fraction. Possibly the low rate of hydration of *trans*-stilbene oxide in rabbit microsomes as reported by Watabe and Akamatsu[45] resulted, in part, from trace contamination by the soluble fraction.

For several years workers have questioned if one or several enzymes were involved in the hydration of epoxides.[10] Recently, information has been mounting on the side of a single enzyme in the solubilized component

of rat liver microsomes which is responsible for the hydration of styrene oxide, benzo(a)pyrene 4,5-oxide, and possibly other epoxide-containing xenobiotics.[67,122] The temptation may exist to conclude from such experiments that epoxide hydrase activity is due to a single enzyme present in the microsomal fraction of mammalian liver. It is hoped that subsequent reviewers of these experiments will limit their conclusions to the substrate and assay condition utilized as cautioned by Bentley *et al.*[65]

Numerous laboratories are currently investigating the correlation of microsomal epoxide hydrase levels with the presence of cancer *in vivo* and the mutagenicity of metabolites using *in vitro* mutagenicity assays. As illustrated earlier, it would be prudent to include the soluble epoxide hydrase in these considerations, at least until the comparative substrate specificities of the soluble and microsomal hydrases are delineated.[71]

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