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MEMBRANE-BOUND AND SOLUBLE-FRACTION EPOXIDE HYDROLASES

Methodological Aspects

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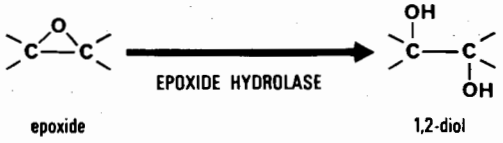
1. INTRODUCTION

Epoxides are highly strained three-membered cyclic ethers which vary considerably in reactivity (Figure 1). The toxicity of these compounds often is related directly to their reactivity, and many of the electrophilically reactive epoxides are mutagenic, carcinogenic, or cytotoxic.[1]

The occurrence of epoxides is widespread. They are formed *in vivo* in several important biosynthetic pathways and as metabolites of xenobiotics. Leukotriene A_4 (**2**)[2] and squalene 2,3-oxide (**1j**)[3,4] are two examples of epoxidized biosynthetic intermediates. Numerous olefinic and aromatic xenobiotics, including many drugs used clinically, are metabolized to epoxides by microsomal cytochrome P-450-dependent monooxygenases,[5] and epoxides may also be formed by peroxide rearrangement. Two well-known examples of xenobiotics metabolized to epoxides are aflatoxin B_1 (**3**)[1] and benzo (a)pyrene (see **1h**). Many natural products contain epoxides, including mycotoxins such as the 12,13-epoxytrichothecenes which are important contaminants of animal and human foodstuffs.[6] Epoxides are also used extensively in industry as intermediates and stabilizers. Manson has provided an extensive review of the vast number of epoxides to which humans are exposed.[7]



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Epoxide hydrolases (EC 3.3.2.3)—formerly named epoxide hydrases or epoxide hydratases (EC 4.2.1.63)—catalyze the conversion of epoxides to 1,2 diols. The diols resulting from such hydration are more polar, more readily excretable, and generally represent a detoxification process. However, the diols of certain polycyclic aromatic hydrocarbons can be metabolized further by

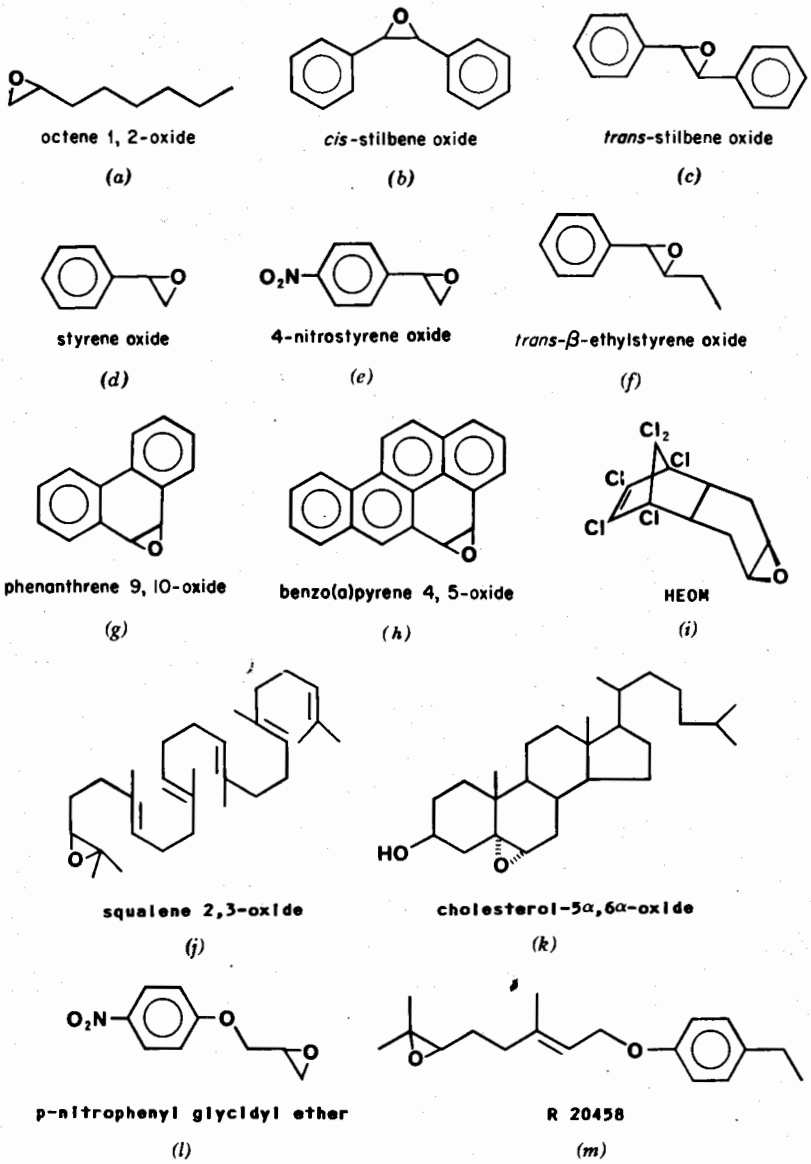
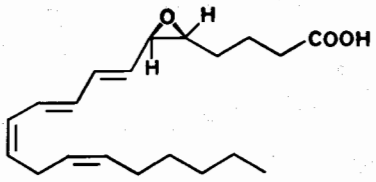


Figure 1. Commonly used substrates of epoxide hydrolases. HEOM = 1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-methanonapthalene. R 20458 = 1-(4′-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-2*E*-octene. *Note*: Epoxides may be named as either the oxide of an alkane (e.g., octane 1,2-oxide) or trivially as the oxide of an alkene (e.g., octene oxide). Both methods of nomenclature have been used extensively in the literature.

cytochrome P-450 monooxygenases to generate more reactive diol epoxides, in which case the epoxide hydrolase serves an activation role.[8]

A number of reviews on different aspects of epoxide hydrolases have been published.[9–15] Thus the major emphasis of this chapter is to review the methodology used to investigate epoxide hydrolases in vertebrates.



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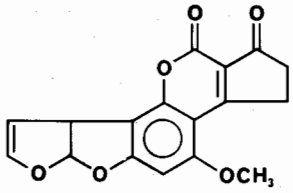

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2. SUBCELLULAR DISTRIBUTION OF EPOXIDE HYDROLASES

2.1. Distribution in Normal Tissue

Initial studies utilizing arene oxides as substrates indicated that epoxide hydrolase activity was localized solely in the microsomal fraction of cells.[9] However, later studies using different substrates demonstrated the presence of epoxide hydrolase activity in the cytosolic fraction as well.[11,16] The enzyme activities in the two subcellular fractions are due to two immunologically distinct forms of epoxide hydrolase,[17] which also differ in pH optima, substrate specificity, molecular weight, and response to inhibitors (see Table 1 and Section 3).

The "microsomal" form is present primarily in the microsomal and nuclear subcellular fractions.[9,18–21] The highest levels are found in the smooth endoplasmic reticulum, whereas lower levels are observed in rough endoplasmic reticulum, nuclear membrane, Golgi apparatus, and plasma membranes.[22] Okada and coworkers[23] showed that the microsomal epoxide hydrolase is syn-

thesized in membrane-bound polysomes and inserted cotranslationally into the endoplasmic reticulum membranes.

The "cytosolic" form is found primarily in the cytosolic subcellular fraction.[16,24–27] Lower levels of epoxide hydrolase activity with similar substrate specificity and pH optima have also been described in mitochondrial matrices,[26,27] peroxisomes,[28] and microsomes.[29]

In human and rhesus monkey liver, the microsomal form of epoxide hydrolase, as assayed by *cis*-stilbene oxide (**1b**), *p*-nitrostyrene oxide (**1e**), or styrene oxide (**1d**), is also present in significant amounts in the cytosol.[30–32] Apparently the microsomal form is detached fairly easily from human liver microsomes. In some cases the detached microsomal epoxide hydrolase is referred to as the preneoplastic antigen (see Section 2.2).

This raises some problems in regard to nomenclature. The microsomal form is also present in the cytosol, and the cytosolic form is also present in the microsomes. Some workers have suggested differentiating the enzymes on the basis of pH optima; however, pH optima vary with substrates, and some substrates specific for the microsomal enzyme are hydrolyzed with a pH optimum near that normally associated with the cytosolic enzyme. In this chapter we refer to the form present predominantly in the microsomal and nuclear fractions as the microsomal epoxide hydrolase and the form present primarily in the soluble and mitochondrial fractions as the cytosolic epoxide hydrolase. These definitions rest largely on studies of subcellular distribution and substrate selectivity of the enzymes in rat and mouse liver. As these studies are extended to other experimental animals,[33,34] and especially to primates,[30–32,35–39] the definitions may become even more ambiguous. Even at this point, it appears that there are major differences between primate and rodent systems.

An additional form of epoxide hydrolase present in rat liver microsomes, which catalyzes the hydration of cholesterol epoxides, has been described recently.[40–42] This "cholesterol epoxide hydrolase" differs from the two forms discussed above in substrate specificity and in response to inducers and inhibitors. Possibly still other forms of the enzyme will be discovered as the hydration of a wider variety of substrates is examined in more species.

TABLE 1. A Comparison of the Major Forms of Epoxide Hydrolase

Property	Microsomal Expoxide Hydrolase	Cytosolic Epoxide Hydrolase	Cholesterol Epoxide Hydrolase
Subcellular distribution	Predominantly microsomal and nuclear	Predominantly cytosolic	Predominantly microsomal
Substrate specificity	Rapidly hydrolyzes epoxides on cyclic systems as well as mono, 1,1-di, and *cis*-1,2-disubstituted epoxides	Hydrates epoxides on cyclic systems very slowly, but hydrates a wide variety of aliphatic epoxides rapidly. Trisubstituted terpenoid epoxides show low K_m's, whereas fatty acid and ester epoxides are hydrated very quickly	Cholesterol epoxides
Diagnostic substrates			
Benzo(a)pyrene 4,5-oxide	+	−	−
Phenanthrene 9,10-oxide	+	−	?

HEOM	+	−	?
cis-Stilbene oxide	+	±	?
Styrene oxide	+	±	−
Squalene oxide	−	+	?
trans-β-Ethyl styrene oxide	−	+	?
trans-9,10-Epoxystearate	±	+	?
trans-Stilbene oxide	−	+	?
1,2-Epoxyoctane	+	+	−
α and β Cholesterol epoxides	−?	−	+
Induction			
trans-Stilbene oxide	+	−	−
Butylated hydroxyanisole (BHA)	+	−	?
2-Acetylaminofluorene	+	−	−
Phenobarbital	+	−	−
Arochlor 1254	+	−	−
Clofibrate	+	+	−
Di(2-ethylhexyl) phthalate	+	+	?
Cholesterol epoxides	−	?	+

TABLE 1. (*Continued*)

Property	Microsomal Expoxide Hydrolase	Cytosolic Epoxide Hydrolase	Cholesterol Epoxide Hydrolase
Inhibition			
Cyclohexane 1,2-oxide	+	−	?
1,1,1-Trichloropropane 2,3-oxide	+	±	−
2-Bromo-4′-nitroacetophenone	+	+	?
Styrene oxide	−	+	?
Substituted chalcone oxides	−	+	?
p-Hydroxymercuri-phenylsulfonate	−	+	?
Cu^{2+}	?	+	?
Iminocholestan-3-β-ols	−	?	+
Stimulation			
Chalcone oxide	+	−	?
Metyrapone	+	−	?
1,1,1-Trichloropropane 2,3-oxide	−	−	+
pH Optima	Usually above 8	ca. 7.4	ca. 7.4
Molecular weight	49,000 (Forms aggregates)	120,000 (59,000 dimer)	?

pI	?	Acidic	?
Immunoreactivity			
Cross reacts with antibody to microsomal	+	−	−
Cross reacts with antibody to cytosolic	−	+	?
Tissue distribution			
Liver	High	High	?
Kidney	High	High	?
Testis	High	Low	?
Species distribution			
Rat	Intermediate	Very low	?
Mouse	Low	High	?
Rabbit	Intermediate	High	?
Monkey	High	Intermediate	?
Human	Intermediate	Intermediate	?
Sexual dimorphism	Similar in male and female	Higher in mature male	?

The need to examine the subcellular distribution of activity whenever one is measuring epoxide hydrolase activity in a new tissue or with a new substrate is essential. The failure to perform such experiments resulted in cytosolic activity being overlooked for a number of years.

Further careful studies are needed to resolve ambiguities regarding the possibility of multiple forms of epoxide hydrolase in each subcellular fraction. Evidence from several laboratories suggests that there may be multiple forms of both the microsomal and cytosolic epoxide hydrolases. Lyman and Poland[43] reported that 26 strains of mice can be separated into two phenotypic classes based upon differences in the pH optima and heat sensitivity of the enzymes that hydrated styrene oxide in the microsomes. Analogous experiments suggested by Poland failed to illustrate differences in the cytosolic epoxide hydrolase activity (K. Ota, unpublished results). These differences could be explained, in part, by varying proportions of the microsomal form of the cytosolic epoxide hydrolase being present. The best evidence to date for multiple forms comes from the work of Guengerich et al.[44,45] These workers reported that they separated several forms of the enzyme using chromatography on ion-exchange and hydroxylapatite media. However, the substrate specificities, amino acid compositions, and peptide maps were similar among the forms. The enzymes also appeared very similar based upon molecular weight and immunochemical criteria. However, evidence is mounting in this laboratory for the existence of several forms of epoxide hydrolase, based upon ratios of the rate of hydration of different substrates in various subcellular fractions (D. N. Loury, unpublished observations). By using either specific antibodies (Section 6.6) or affinity chromatography (Section 3.5.2) it should be possible to remove classical forms of epoxide hydrolase from homogenates and thus detect new forms whose activity has been masked.

2.2. The Preneoplastic Antigen

The preneoplastic antigen (PNA) is of current interest as a tool in the study of the progression of hepatocellular carcinoma and possibly as an aid in its diagnosis as well. To appreciate its relationship

to the epoxide hydrolase, one must examine the history of its discovery. Evidence is good that in a variety of tissues, and notably in the liver, cancer develops by proliferation and evolution of cells that have somehow changed from the parent cell type. In liver tissue this change has been suggested to take the form of progression from hyperplastic foci to hyperplastic nodules (presumptive preneoplastic nodules) to neoplastic nodules. Critical to the study of such hyperplastic nodules in the liver are positive markers which allow the nodules to be identified biochemically and immunohistochemically. To this end Farber and co-workers[46] raised antiserum in rabbits against postmitochondrial supernatants from rat hepatic preneoplastic lesions. Following exhaustive absorption of this antiserum with soluble proteins from normal rat liver, there remained antibodies to a soluble protein termed the PNA. This protein was found in every early and late hyperplastic nodule and in every hepatocellular carcinoma induced in three rat strains (CFN, F344, BUF) by five different chemical carcinogens. It was also found in two transplanted hepatomas that showed limited dedifferentiation (Sidransky and Morris, 9618 hepatomas) but not in several others. It was not found in tumors from three extrahepatic tissues. The antigen was not found in any normal rat livers by Ouchterlony double diffusion, immunohistochemistry, or microcomplement fixation. Since most cancerous liver cells express fetal genes, the lack of detectable PNA in fetal or regenerating liver and amniotic fluid was also promising.[46,47] The fact that PNA was shown to be antigenically distinct from α-fetoprotein (AFP) is very important. AFP is probably the best serum marker for a variety of tumors. However, there are obvious false positives,[48,49] and only a minority of the nodules that stain positive for PNA are also positive for AFP.

During the purification of PNA from hyperplastic nodules Griffin and Kizer[50] noticed that it was present in microsomal fractions from both normal and hyperplastic liver. Although PNA readily dissociated from microsomes from hyperplastic liver, detergents were required to remove it from microsomes isolated from normal liver.[51] Based upon the molecular weight, Griffin hypothesized that rat PNA might be related to the microsomal epoxide hydrolase, and this hypothesis was unequivocally proven by Levin et al.[52] Although the PNA may show epoxide hydrolase activity and is

present in the cytosol, it should not be confused with the cytosolic epoxide hydrolase. Since the PNA is defined by its reaction with an antibody, the number of proteins that constitute the PNA remains controversial. However, it appears certain that a protein antigenically similar to the microsomal epoxide hydrolase comprises a major portion of the PNA. The microsomal epoxide hydrolase has been "induced" following exposure to some carcinogens and has been measured at very high levels in neoplastic nodules.[53] The epoxide hydrolase is not attached covalently to the microsomes, but rather is dissolved in the membrane because of its lipophilic "tail." Possibly the proliferation of the endoplasmic reticulum in the preneoplastic nodule[54] and the marked change in the lipid composition of the microsomes[55,56] leads to a decreased affinity of the epoxide hydrolase for the membrane. The reduction of superoxide dismutase (and subsequent increase in radical production) in many cancer cells[57] could lead to the release of some microsomal enzymes from the endoplasmic reticulum.

Several avenues of research on the PNA are being pursued. A number of groups are investigating the immunochemical localization of the epoxide hydrolase during the progression of liver cancer and following treatment with inducing agents.[53,58–60] Still other teams are investigating the comparative biochemistry of the microsomal epoxide hydrolase and the PNA.[31,61–63] Another important unanswered question is the mechanism by which the microsomal enzyme is released from the membrane.

Several workers have reported their inability to detect PNA in the serum of animals with hepatocellular carcinoma. Research in this laboratory has concentrated on the development of analytical methods for measuring PNA in the serum. The enzyme-linked immunosorbent assay (ELISA) developed for the microsomal epoxide hydrolase[64] is of adequate sensitivity to detect high levels of PNA in serum. This assay is only useful in those species whose epoxide hydrolase cross reacts with an available antibody.

A radiochemical assay has been developed to monitor the ability of serum to hydrate *cis*-stilbene oxide (CSO) (**1b**). This assay has the advantage of being generally applicable to a variety of species. As discussed further in Section 6.4.6 the assay is based upon the enzymatic hydration of CSO radiolabeled at about 15 Ci/mmol. A

detailed procedure for this assay is presented in Section 6. Using this assay a positive correlation has been observed between the ability of the serum to hydrate CSO and exposure to aflatoxin B_1 (3) in rats. Serum from a variety of apparently healthy human patients hydrates CSO very slowly. However, there is an increase in the ability of the serum from patients with hepatocellular carcinoma to hydrate CSO (D. N. Loury, unpublished results).

3. PROPERTIES OF THE MAJOR FORMS OF EPOXIDE HYDROLASE

In discussing the properties of the two major forms of epoxide hydrolase, the cytosolic form will receive some emphasis, since most previous reviews have concentrated on the microsomal form.[9,10,12,13,15] Where sufficient data exist, the microsomal enzyme that hydrates the cholesterol epoxides will also be discussed. There are clear differences among the three different forms of epoxide hydrolase so far described (see Table 1). However, predictions from these data to the situation in other species and with other substrates should be made cautiously.

3.1. Substrate Specificity

3.1.1. General Information. The term substrate specificity can have many interpretations, and very few studies in this area have adhered to a strict definition. For instance, in many cases initial rates have been used as an indication of specificity. Though not all of these studies were carried out under conditions in which the rates approached V_{max}, they probably do give a reasonable indication of the relative rates of hydrolysis of different substrates. However, one must be very careful when extrapolating such information to mixtures of substrates or different enzyme concentrations.

Alternatively, one can measure the ability of a variety of epoxides to inhibit the hydration of a single substrate. The relative potency of various inhibitors may give some indication of their ability to bind to the enzyme which, with some reservations, can be used

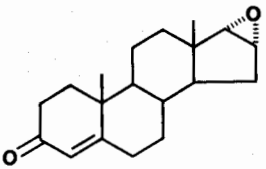
as an approximation of relative K_m. However, such data yield no information on turnover. For instance, a compound could have a very high affinity for the enzyme, yet be turned over very slowly. In fact, most inhibitors of the microsomal and cytosolic epoxide hydrolases probably act as alternate substrates with a low K_m.

More reasonable estimates of specificity are given by the expressions k_{cat}/K_m or V_{max}/K_m.[65] These values take into account both turnover and affinity and are less dependent on assay conditions than the above mentioned values. One can also estimate the specificity between two substrates by examining their rates of hydration as mixtures. Very few studies provide sufficient data to evaluate true specificity; however, the differences in substrate preference among the established forms of epoxide hydrolase are so marked that the limitations of experimental technique are not essential for distinguishing the various forms (Table 1). Still if a detailed knowledge of substrate specificity is to be applied to the evaluation of the hydrolytic mechanism or to predicting the fate of xenobiotics *in vivo*, more detailed information on substrate specificity is needed.

The studies performed to date are very limited in regard to the number of species examined (especially higher primates), the number and variety of substrates evaluated, and the detail of the kinetic analysis. Owing to its lipophilicity, kinetic experiments with the microsomal enzyme are especially difficult.[67] Careful work on substrate specificity may not be attractive, since it involves both chemical and biochemical expertise; however, such research is very important. Based on the limited work available, several diagnostic substrates for the different forms are suggested in Table 1 and Section 6. It must be cautioned that development of these diagnostic substrates—and in fact our total appreciation of the substrate specificity of the various epoxide hydrolases—is based upon data from only a few substrates examined in a paucity of species.

3.1.2. Specificity of the Microsomal Epoxide Hydrolase. In an early study with the microsomal epoxide hydrolase, Maynert et al.[67] demonstrated that octane, 1,2-oxide (**1a**) was hydrated much faster than di- and trisubstituted isomers. Shortly thereafter Brooks et al.[68,69] showed that although the microsomal epoxide hydrolase

rapidly hydrated isomers of cyclodiene epoxides (e.g., **1i**) that were open to a back-side attack, sterically hindered isomers were very resistant to hydration. The work of Leibman and Ortiz[70] as well as the studies from the National Institutes of Health (NIH) also demonstrated that epoxides on cyclic systems were susceptible to hydration.[13,71,72] In reviewing a prodigious amount of earlier work, including these key studies, Oesch[9] outlined the substrate specificity of the microsomal epoxide hydrolase. Although most of this information was based on estimates of initial rate and/or inhibitory potency, his conclusions still define the substrate specificity for the microsomal enzyme from rats[10,12] and seem to apply to other species, including man.[12,73,74] Phenanthrene 9,10-oxide (**1g**), 16α,17α-epoxyandrosten-3-one (**4**[75] and benzo(a)pyrene 4,5-oxide (**1h**) are illustrative of good substrates; dibenz(a,h)anthracene 5,6-oxide (**5**) is illustrative of a poor substrate. An interesting toxicological aspect is that some of the very carcinogenic diol epoxides are very poor substrates for this enzyme.[76]



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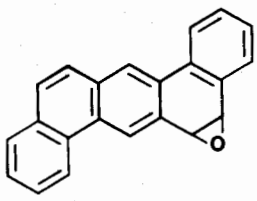

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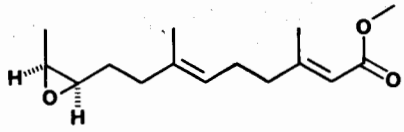
The microsomal epoxide hydrolase readily hydrates several mono-1,1-disubstituted, and *cis*-1,2-disubstituted epoxides. *trans*-1,2-Disubstituted epoxides trisubstituted, and tetrasubstituted epoxides are neither good substrates nor good inhibitors.[9,77,78] However, a trisubstituted epoxide on a cyclic system was recently reported to be hydrolyzed rapidly by microsomes,[79] although a methyl substituent on the HEOM epoxide (**1i**) greatly slowed its rate of hydration.[80] The rate of hydration as well as the avidity of binding seem to increase with the lipophilicity of the epoxide, especially with low molecular weight epoxides.[9,12,73,81] Lu and Miwa[10] reviewed the work of several investigators who made a more detailed evaluation of the substrate specificity of the micro-

somal epoxide hydrolase toward arene oxides,[82–85] steroid oxides,[86,87] stilbene oxides,[88] and styrene oxides.[88,89]

3.1.3. Specificity of the Cytosolic Epoxide Hydrolase. The substrate preference of the cytosolic epoxide hydrolase appears to be the antithesis of the microsomal enzyme in that it poorly hydrates epoxides on cyclic systems, but hydrates a wide variety of aliphatic epoxides.[11] In an early study Hammock et al.[90] reported that trisubstituted terpenoid epoxides (e.g., Juvenile Hormone III, **6**) were hydrated faster than



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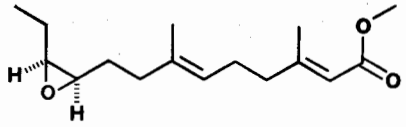

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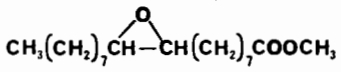
homoterpenes (e.g., **6** Juvenile Hormone II, **7**) which were hydrated much faster than similar tetrasubstituted epoxides. The most complete study of initial rates of hydration was reported by Mumby and Hammock[91] using homologs of (2E)-1-(4′-bromophenoxy)-3-methyl-6,7-epoxy-2-octene (e.g., R 20458, **1m**). They found that mono- and *cis*-1,2-disubstituted epoxides were hydrated faster than *trans*-disubstituted epoxides. Tri- and especially tetrasubstituted epoxides were hydrated much more slowly. It followed from this study that lipid epoxides such as the epoxysterates (**8**), squalene oxides (**1j**), and



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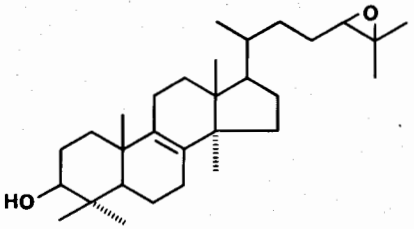

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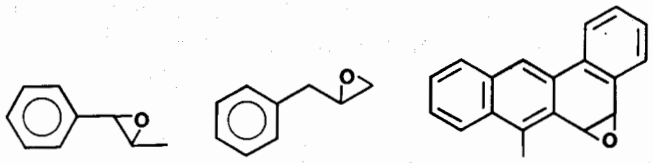
lanosterol epoxide (**9**) might be intrinsic substrates.[11,25,92] This work also led to an examination of homologs of styrene oxide (**1d**)

which indicated in part, how the cytosolic epoxide hydrolase was overlooked.[16] At the high substrate concentrations of styrene oxide routinely used, the cytosolic epoxide hydrolase is inhibited and thus styrene oxide is only a substrate for the microsomal enzyme. In contrast, *trans*-β-methylstyrene oxide (**10**) is hydrated largely by the cytosolic form, and allylbenzene oxide (**11**) by both forms.

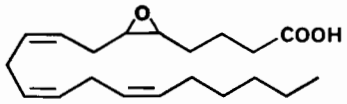


10 **11** **12**

Oesch and Golan[93] report that in mouse liver cytosol, under conditions where *trans*-stilbene oxide (TSO) (**1c**) is rapidly hydrated, a variety of arene oxides are only very slowly hydrated. 7-Methylbenz(a)anthracene 5,6-oxide (**12**) was the only arene oxide reported to be hydrated rapidly by the cytosolic fraction, and it has not been demonstrated that the same enzyme hydrates both this substrate and TSO. Hammock and Hasagawa[79] compared the hydration of a variety of substrates by enzymes in the cytosol and microsomes of mouse liver. Cyclodiene epoxides were not metabolized in the cytosol, and the rate of benzo(a)pyrene 4,5-oxide hydration was so low that it could be accounted for by contamination with the solubilized microsomal enzyme. Subsequent studies with homogenous cytosolic epoxide hydrolase from mouse indicate that no hydration of benzo(a)pyrene 4,5-oxide can be detected (D. N. Loury, unpublished results). Hydration of epoxides on aliphatic rings by enzymes in the cytosol could not be detected, and a 1,1-disubstituted epoxide was hydrated much faster by the microsomal than the cytosolic fraction. With epoxides alpha to aromatic systems, *trans* epoxides are among the best substrates tested for the cytosolic enzyme, with the initial rate of hydration increasing with hydrophobicity. The analogous *cis* epoxides, however, are hydrated very slowly. Glycidyl ethers (e.g., *p*-nitrophenyl glycidyl ether, **11**) are hydrated by both the microsomal and cytosolic forms of the enzyme, but a systemic study in this area is needed.[79,94,95]

Wang et al.[30] report a somewhat different substrate selectivity with the cytosolic epoxide hydrolase purified from human liver, as do Glatt et al.[33] for the rabbit enzyme, suggesting that the trends observed in mice must be applied cautiously to other species.

Among the lipid epoxides, the epoxides of arachidonic acid (with the exception of the 5,6-epoxide **13**) are hydrated fastest followed by *cis*-and *trans*-9,10-epoxysterates (**8**)[25,96] (R. N. Wixtrom, unpublished results). It is of interest that neither leukotriene A_4 methyl ester (see **2**) nor the 5,6-epoxide of arachidonic acid appear to be hydrated rapidly by the hepatic enzyme.



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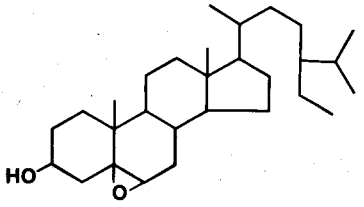
trans-β-Ethylstyrene oxide (TESO) (**1f**) is hydrated with an initial rate 30 times higher than a terpenoid epoxide such as R 20458 (**1m**). The difference in their K_m values is of a similar magnitude with the terpenoid epoxide showing the higher apparent affinity for the enzyme. Thus when one expresses the specificity of the enzyme in terms of V_{max}/K_m there is surprisingly little difference in the specificity of the cytosolic epoxide hydrolase from mice with these vastly different substrates. These data illustrate how expressing substrate specificity as either rate of hydration or affinity for the enzyme can be very misleading, for in this case each set of data support the opposite conclusion regarding specificity.[97]

3.1.4. Specificity of the Cholesterol Epoxide Hydrolase There is little information available on the hydrolase that appears to hydrate the cholesterol epoxides. Aringer and Eneroth[40] noted that the cholesterol epoxides were hydrated much faster when compared with the corresponding β-sitosterol epoxides (**14**). Bindel et al.[86] demonstrated that the 16,17-α-epoxide of estratrien-3-ol (**15**) was hydrated by an enzyme that was similar immunochemically to the more commonly studied microsomal epoxide hydrolase and thus probably different from the cholesterol epoxide hydrolase. Indirect evidence indicates that the cholesterol epoxide hydrolase fails to

hydrate rapidly the common substrates used to monitor the microsomal epoxide hydrolase.[41,42]



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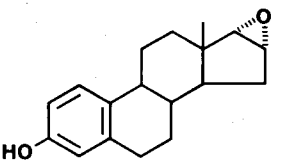

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3.1.5. Diagnostic Substrates. Based on data available at this time, several good diagnostic substrates are listed in Table 1. Our knowledge of the cholesterol epoxide hydrolase is so limited that no model substrate can be recommended. Arene oxides such as benzo(a)pyrene 4,5-oxide (**1h**), phenanthrene 9,10-oxide (**1g**), and HEOM (**1i**) appear to be excellent diagnostic substrates for the microsomal form. *cis*-Stilbene oxide (**1b**) is also useful in this respect if a few simple controls are run, but one must be very cautious in using styrene oxide (**1d**). *trans*-β-Alkylstyrene oxides (e.g., **1f** or **10**) and TSO (**1c**) appear to be useful diagnostic substrates for the cytosolic form. Application of these diagnostic substrates to other species should be done with caution, since new forms of the enzymes may be discovered, and the substrate specificity of the epoxide hydrolases may differ dramatically among species.

3.2. Induction

3.2.1. Induction of the Microsomal Epoxide Hydrolase. Numerous inducers have been reported from several laboratories for the microsomal epoxide hydrolase (Table 2). In most studies inducers are defined as simply compounds that increase the total and/or specific activity of the epoxide hydrolase in microsomal preparations. However, in one study styrene and 3-methylcholanthrene were reported to decrease the apparent K_m of the hydrolytic activity on styrene oxide.[123] Investigators have been interested in determining if the regulation of epoxide hydrolase is coupled to that of the various forms of cytochrome P-450. Although there are

TABLE 2. Some Inducers of the Microsomal Epoxide Hydrolase

	Reference
2-Acetylaminofluorene	52
α-1-Acetylmethadol	98,99
Butylated hydroxyanisole (BHA)	100–102
Butylated hydroxytoluene (BHT)	102
Chalcone	103
γ-Chlordane	59
1,2-Dibromo-3-chloropropane (DBCP)	104
Ethoxyquin	102,105
Isosafrole	42
3-Methylcholanthrene	106,107
α-Naphthoflavone	103
Phenobarbital	59,99,106–112
Polychlorinated and brominated biphenyls	59,113–120
Pregnenolone-16α-carbonitrile	106
trans-Stilbene oxide	59,99,121,122

many common inducers of the two enzyme systems, Oesch[106] and Schmassmann and Oesch[121] concluded that there is differential control.

As discussed by Lu and Miwa,[10] one can also obtain evidence for the existence of multiple forms of epoxide hydrolase by monitoring the activity of a variety of substrates following treatment with several inducers. It is critical that such comparisons of enzymatic activity are kinetically valid if this approach is to yield useful data, and it is always possible that the substrates selected are all metabolized by the same form of the enzyme or that the several forms are regulated similarly. With the exception of some anomalous data on 3-methylcholanthrene, the information to date supports the concept that a single form of microsomal epoxide hydrolase metabolizes the arene oxides.[107,109,110] Further studies need to be performed on the apparent microsomal form of the cytosolic epoxide hydrolase and the microsomal enzyme that hydrates cholesterol epoxide, but the available information indicates that they are regulated differently from the classical microsomal epoxide

hydrolase[29,42] (D. E. Moody et al., unpublished). For example, Levin et al.[42] report that no correlation was observed between the induction by 12 xenobiotics of the epoxide hydrolases acting on 1,2-epoxyoctane (**1a**) and the 5,6-α-epoxide of cholesterol (**1k**). The same technique can be used to examine the regulation of the same enzyme in different tissues or organelles. For instance, TSO was shown to induce the epoxide hydrolase of the nuclear envelope, whereas phenobarbital only induced the microsomal form.[124]

Compounds that can selectively induce one enzyme system involved in xenobiotic metabolism can be useful for determining the importance of competing pathways *in vivo*. A wide variety of xenobiotics induce the microsomal form of the epoxide hydrolase (Table 2), but most also induce a variety of other enzymes involved in xenobiotic metabolism. 3-Methylcholanthrene is interesting in that it causes only low induction of the microsomal epoxide hydrolase while significantly inducing other xenobiotic metabolizing enzymes.[109] *trans*-Stilbene oxide and several related compounds have received attention as selective, but certainly not specific, inducers of the microsomal epoxide hydrolase.[59,99,103,121,122,125–129] A variety of carcinogens, and notably 2-acetylaminofluorene,[52] are potent inducers of the microsomal epoxide hydrolase. Hypocholesterolemic agents that are also peroxisome proliferating agents have been shown to induce microsomal epoxide hydrolase activity.[42,103] The antioxidants, butylated hydroxyanisole and -toluene (BHA and BHT) potently induce both glutathione S-transferases and the microsomal epoxide hydrolase.[43,100–102,130–132] Rouet et al.[133] recently demonstrated fluctuations in microsomal epoxide hydrolase activity during gestation in mice. This observation may indicate a direct or indirect effect of hormones on epoxide hydrolase activities.

The mechanism by which the microsomal epoxide hydrolase activity is increased following administration of putative inducers is not fully understood. Kawabata et al.[134] found that TSO and phenobarbital yielded different patterns of induction when the microsomal epoxide hydrolase was monitored immunohistochemically. Goujon et al.[135] showed induction of epoxide hydrolase in primary hepatocyte cultures, and Raphael et al.[136] demonstrated that several compounds capable of inducing monooxygenase in hepatoma

cells *in vitro* failed to induce epoxide hydrolase or glutathione S-transferase activity. The amount of microsomal epoxide hydrolase activity has been shown to be correlated with the protein present (as microsomal epoxide hydrolase), as determined by an immunoassay following induction with TSO (unpublished results). Several studies indicate that induction of microsomal epoxide hydrolase activity can be explained at least partially by *de novo* synthesis of the enzyme and an increase in the mRNA coding for the enzyme.[105,111,112, 137–142]

3.2.2. Induction of the Cytosolic Epoxide Hydrolase. Comparatively little is known about the induction of the cytosolic epoxide hydrolase. Hammock and Ota[103] demonstrated that the cytosolic enzyme appears to be regulated differently from microsomal epoxide hydrolase and glutathione S-transferase activities by examining the relative induction of these enzymes by 14 xenobiotics. For instance, under conditions where BHA gives over a six-fold induction of the microsomal epoxide hydrolase, the cytosolic enzyme activity significantly decreased. However, chemicals known to be peroxisome proliferating, such as the hypolipidemic agents clofibrate and di(2-ethylhexyl) phthalate, proved to be good inducers of the cytosolic epoxide hydrolase. A recently reported nafenopin binding protein could be involved in this process.[143] The increased activity is due to an enzyme with the same isoelectric point and molecular weight on SDS gels as that present in the uninduced control mice. This induction is time and dose dependent and occurs in the liver and kidneys of both rats and mice. These compounds also induce the microsomal form of the cytosolic epoxide hydrolase (D. N. Loury et al. and D. E. Moody et al., unpublished). High micromolar concentrations of clofibrate and diethylhexylphthalate neither stimulate nor inhibit cytosolic epoxide hydrolase activity *in vitro*. Since there is a large sexual dimorphism in the hepatic epoxide hydrolase activity in mice,[92] it is possible that hormones influence the enzyme activity. As with the microsomal enzyme, primary hepatocyte cultures—as well as hepatoma and other cell lines—may prove valuable for investigating the mechanism of induction.[36,144]

3.2.3. Induction of the Cholesterol Epoxide Hydrolase. Research on the induction of the microsomal enzyme that hydrates the epoxides of cholesterol is quite limited. Chan and Black[145,146] reported that the epoxides induced their own detoxification. This observation was not confirmed in hepatic tissue from rats, where administration of cholesterol-5α,6α-oxide (**1k**) resulted in only a very small increase in the cholesterol epoxide hydrolase, but a decrease in the rate of hydration of 1,2-epoxyoctane (**1a**).[42] When several inducers of the classical microsomal epoxide hydrolase were given to rats, no significant increase in the rate of hydration of cholesterol epoxide was observed.

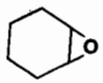
Since potent inducers are known for epoxide metabolizing enzymes, there is the possibility of xenobiotic interaction based on this phenomenon, and this should be considered when potential inducers are used therapeutically. The ability to induce selectively a single enzyme involved in xenobiotic metabolism can prove very valuable in elucidating the routes of metabolism. However, no compound has yet been reported that can specifically induce an epoxide hydrolase activity. Thus experiments based on selective induction of epoxide metabolism must be controlled carefully.

3.3. Inhibition

3.3.1. General Information. Inhibitors capable of blocking a pathway of epoxide metabolism *in vivo* and *in vitro* would be very useful for dissecting the pathways of epoxide detoxification as well as for increasing the sensitivity to epoxides of various short-term assays. If better inhibitors can be developed, they will prove invaluable in elucidating both the role of epoxide hydrolases and the toxicity of epoxide-containing compounds *in vivo*. In addition, such inhibitors may be useful for clarifying the mechanism of enzymatic hydration, for labeling the active site of the enzyme, for determining enzyme multiplicity, or even for affinity purification. Some good inhibitors are known for epoxide hydrolases, yet none are the highly potent, specific, and irreversible inhibitors that allow the above experiments to be performed with a minimum of controls. As discussed in Section 3.1 with regard to substrate specificity, there is a need to perform careful studies on the kinetics of inhi-

bition of epoxide hydrolases if more than a rough idea of potency is desired from the data. A rough comparison of potency can be made by plotting the percent of inhibition versus the log of the inhibitor concentration. Both the slope of the resulting line and the concentration of the inhibitor yielding 50% inhibition (I_{50}) can be used in such a comparison. As illustrated below, one must insure that the experiments are performed under steady-state conditions[147] or that nonsteady-state approaches are taken.[148] Since it appears that epoxides are involved as intermediates or byproducts in several biosynthesis pathways, potent inhibitors of epoxide hydration may be of pharmacological and toxicological interest.

3.3.2. Inhibition of the Microsomal Epoxide Hydrolase. Oesch and co-workers[9,78] reported that 1,2,3,4-tetrahydronaphthalene 1,2-oxide, cyclohexane 1,2-oxide (**16**), and 1,1,1-trichloropropane 2,3-oxide (**17**)



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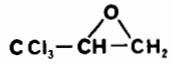

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were inhibitors of the microsomal epoxide hydrolase from several species. The third compound is the most widely used inhibitor of the microsomal epoxide hydrolase in spite of its lack of specificity, weak mutagenicity, rapid turnover, and cellular toxicity. However, with appropriate controls for cellular toxicity, mutagenicity, and inhibition of other pathways of metabolism, cyclohexane oxide and trichloropropane oxide can be used to demonstrate very effectively the involvement of epoxide hydration in xenobiotic metabolism *in vitro*.[149,150] Data on the inhibition of epoxide hydrolases have been largely generated using enzyme preparations from rodents, and therefore one can anticipate differences as other species are examined. For instance, Pacifici and Rane[39] report that 1,1,1-trichloropropane 2,3-oxide is a better inhibitor of the epoxide hydrolase activity from human fetal liver than from rat liver. The inhibition of the microsomal epoxide hydrolase by drugs and drug me-

tabolites such as cyproheptadine epoxide and norethisterone epoxide raises the possibility of drug–drug and drug–carcinogen interactions resulting from the hindered epoxide detoxification.[151,152] Craven et al.[153] report that some glycidyl ethers are potent inhibitors of the microsomal epoxide hydrolase. Since related compounds are important industrial chemicals,[7] their effect on the epoxide hydrolases of workers should be noted.

Many of the epoxides that are good substrates are competitive inhibitors of the hydration of other substrates. In fact, data from two laboratories support the theory that trichloropropane oxide acts as an alternate substrate with a low K_m.[84,89] Based on this hypothesis, Hanzlik and Walsh[89] evaluated a variety of α-substituted styrene oxides as inhibitors and found some that approached the activity of trichloropropane oxide. Other compounds including *trans*-1,2-di-, tri- and tetrasubstituted epoxides are poor inhibitors of the microsomal epoxide hydrolase.[73,77]

The kinetic behavior of inhibitors of the microsomal epoxide hydrolase is rather complex since the enzyme has a lipophilic tail and active site, is partially imbedded in a membrane, and often is evaluated in an impure form and/or in the presence of detergents. Since many inhibitors are also lipophilic, one must consider the interaction of lipophilic substrates and inhibitors with sites other than the active site of the enzyme. The kinetics observed with an inhibitor often differ depending upon the substrate, the enzyme concentration, and other conditions of the assay.[77,84,85,89,154] Because of these problems there is a tendency to overinterpret the results of kinetic experiments. If kinetic experiments are undertaken, it is important to demonstrate that the enzyme obeys Michaelis–Menton kinetics over the range of substrate concentrations used and that the inhibitor is being evaluated under steady-state conditions.

One approach to determining the mechanism of enzymatic epoxide hydration involves the judicious use of amino acid modifiers. For example, DuBois et al.[155] report an extensive survey of potential inhibitors of the microsomal epoxide hydrolase. The enzyme was insensitive to the vast majority of these compounds, providing some evidence that tyrosine, tryptophan, and arginine are not catalytically important. Some amino group modifiers were also active.

The enzyme was very sensitive to inactivation by oxidation, but several sulfhydryl reagents caused only minor inhibition. Titration indicated that there were two cysteine residues. By far the most potent inhibitors found were the phenacyl bromides. These investigators succeeded in isolating 1-(*p*-nitrophenacyl)-5-histidine from the inhibited enzyme, indicating that the histidine may be involved in general base catalysis. That covalent binding of 2-bromo-4′-nitroacetophenone to the histidine residue of serine esterases is a slow reaction following the reversible attack of the serine on the carbonyl to yield a tetrahedral transition state intermediate was not discussed. The potency of phenacyl bromides as inhibitors of both the microsomal and cytosolic epoxide hydrolases may indicate that the polarized carbonyl mimics some of the characteristics of an epoxidized substrate in addition to binding to an essential histidine or cysteine. If epoxide hydrolases employ a general acid catalyst, the high electron density of the carbonyl may interact with and partially abstract a proton. It is also cautioned that such reagents can be expected to be much more potent on pure rather than crude enzymes.

Cadmium and to a lesser extent mercury and zinc inhibit epoxide hydrolase activity as well as glutathione conjugation and P-450-catalyzed oxidation.[156–158] When cyclohexane 1,2-oxide gave competitive inhibition, cadmium inhibited the hydration of styrene oxide noncompetitively, but benzo(a)pyrene 4,5-oxide hydration competitively. This observation may indicate that cadmium does not bind at the catalytic site, but close enough to disrupt the binding of large substrates.

3.3.3. Inhibition of the Cytosolic Epoxide Hydrolase. There has been comparatively less work on inhibition of the cytosolic epoxide hydrolase, and as with the microsomal enzyme, interpretation of inhibition data is complicated by several factors. As discussed earlier, certain substrates appear to bind tightly (have a low K_m), yet are turned over slowly; other substrates are turned over rapidly, but have a high K_m. With the former compounds high concentrations of enzyme must be used to obtain detectable rates of metabolism, thus reducing the apparent potency of stoichiometric inhibitors or nonspecific alkylating agents which will react with a variety

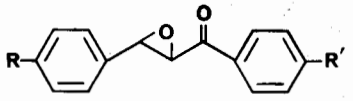
of proteins in the cytosol. Similarly, an inhibitor that must compete with a substrate for binding will appear less potent in assays with a substrate of high affinity for the enzyme rather than with one of low affinity. In this regard, hydration of the terpenoid epoxide R 20458 ($K_m = 2.0 \times 10^{-6}\ M$, $V_{max} = 2.4$ nmol/min·mg) would be difficult to inhibit, hydration of TSO (**1c**, $K_m = 1 \times 10^{-5}\ M$, $V_{max} = 6.9$ *nmol/min·mg*) less difficult, and hydration of TESO (**1f**, $K_m = 7.2 \times 10^{-5}\ M$, $V_{max} = 65$ nmol/min·mg) comparatively easy to inhibit.

Using the juvenoid R 20458 (**1m**) it was found that a variety of compounds, including oxetanes, diols, thiaranes, and notably trichloropropane oxide and cyclohexane 1,2-oxide, failed to inhibit hydration significantly.[24] *O*-Alkyl compounds have been used successfully to mimic the epoxide function of insecticidal juvenile hormone mimics.[159,160] These compounds proved to be weak but very valuable inhibitors of the cytosolic epoxide hydrolase (see Section 3.5.2). When 20 homologous terpenoid-like epoxides were compared as inhibitors of the hydration of R 20458 (**1m**), it was found that the best inhibitors were trisubstituted epoxides, followed by *trans*-1,2-disubstituted, *cis*-1,2-disubstituted, and monosubstituted epoxides. This order was generally the inverse of the initial rate of their hydration (see Section 3.1.3).[91] Trisubstituted epoxides were better inhibitors of monosubstituted epoxides than 1,2-disubstituted epoxides and poor inhibitors of other trisubstituted epoxides. For the same reasons *trans*-1,2-disubstituted epoxides were good inhibitors of the corresponding *cis* compounds, but the converse was not true.[91]

A similar approach was taken using TESO as a substrate. In this case TSO could inhibit the hydration of TESO, but the converse was not true. Interestingly, styrene oxide was found to be a time-dependent inhibitor of the cytosolic epoxide hydrolase, as would be expected for a sulfhydryl enzyme.[161] In fact styrene oxide can be considered a site-directed alkylating agent for the cytosolic epoxide hydrolase. This explains why it is a poor substrate for the cytosolic epoxide hydrolase at high concentrations,[16] yet a good substrate at low concentrations.[79]

The best inhibitors to date came from a survey of over 150 compounds in which it was found that substituted chalcone oxides (**18**)

were potent inhibitors of the cytosolic epoxide hydrolase. Based on a series of these inhibitors it appears that hydrophobicity is a predominant factor in determining the potency imparted by a *para* substituent. Thus 4 and 4′-phenylchalcone oxides proved to be potent, selective inhibitors of the cytosolic epoxide hydrolase.[97] In a separate study, the corresponding chalcones were found to be moderately potent inhibitors of glutathione sulfotransferase activity.[162] The chalcone oxides are rather selective in their inhibition of this enzyme. The mechanism of inhibition is not known; it seems reasonable to assume that the compounds bind to the catalytic site, but have a low turnover. Preliminary kinetic data indicate that the chalcone oxides may act as tight-binding inhibitors,[148] and under such conditions the assumptions made for steady-state analysis may not be valid. Many such tight-binding inhibitors act as "transition-state" mimics.



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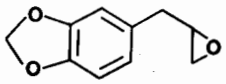
The application of amino acid modifiers to the study of the cytosolic epoxide hydrolase has not been as extensive as with the microsomal enzyme. When a variety of compounds were screened as inhibitors of the cytosolic epoxide hydrolase, the best inhibitors proved to be the organomercurial sulfhydryl reagents. The action of most of the inhibitors could be explained based on their binding to an essential sulfhydryl. For instance, the inhibitory activity of chalcones is explained by their propensity to form Michael adducts. *p*-Nitro-2-bromoacetophenone was used to derivatize histidine on the microsomal epoxide hydrolase, but it is likely that this compound also blocks a sulfur on the cytosolic enzyme.[79] The activity of these compounds is dependent upon the purity of the cytosolic epoxide hydrolase, as would be expected. The concept of an essential cysteine was further advanced by noting that a variety of cations are good inhibitors of the cytosolic epoxide hydrolase and that their potency correlates with the solubility product constants of their sulfides.[92,97] The failure of diethylmaleate to cause any

inhibition of the cytosolic epoxide hydrolase at millimolar concentrations provides a simple way of depleting glutathione in a crude assay system. Obviously one should not use this reagent in buffers containing dithiothreitol or mercaptoethanol (see Section 3.5).

3.3.4. Inhibition of the Cholesterol Epoxide Hydrolase. Watabe et al.[41] found that using trichloropropane oxide led to stimulation rather than inhibition of the hydration of cholesterol epoxide under conditions where the hydration of safrole oxide (**19**) and styrene oxide (**1d**) were inhibited. The 5,6-α- and -β-aziridines corresponding to cholesterol epoxide inhibited the hydration of cholesterol epoxide, but not that of safrole and styrene oxides. Although it is difficult to prove the multiplicity of enzyme activities by inhibition studies, these data provide very convincing evidence for multiple forms, especially in light of subsequent induction studies.[42]



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3.4. Stimulation

A number of effective *in vitro* stimulators of microsomal epoxide hydrolase have been identified (see Table 3); an important point to emphasize is that their activation of catalytic activity is substrate dependent. For example, Levin et al.[85] showed that metyrapone enhances the rate of hydration of styrene oxide and octene oxide, but inhibits the hydration of benzo(a)pyrene 11,12-oxide and dibenz(a,h)anthracene 5,6-oxide. As Guengerich[12] and others[163,166] have noted, several stimulators of microsomal epoxide hydrolase activity are also effective inhibitors of microsomal monooxygenase enzymes.

Little is known about the stimulation of the cytosolic form of epoxide hydrolase. Hammock et al.[11] report that under certain conditions, some alcohols and other co-solvents stimulate cytosolic epoxide hydrolase activity. This effect, which is primarily seen at high substrate concentrations above the critical micelle concentra-

TABLE 3. Stimulators of Microsomal Epoxide Hydrolase

	Reference		Reference
Metyrapone	73,78,85	Flavone	164
1-(2-Isopropylphenyl)-imidazole	73	5,6-Benzoflavone	164
1-(2-Cyanophenyl)-imidazole	73	7,8-Benzoflavone	164
Ethanol	73	Benzil	165
Cyclobenzaprine	151	Ellipticine	166
Cyclobenzaprine epoxide	151	Harman	166
Benzophenone	163	Norharman	166
Propyl phenyl ketone	163	Isoquinoline	166
Benzyl phenyl ketone	163	Quinoline	166
9-Fluorenone	163	4-Azafluorene	166
Diphenylcyclopropenone	163	4-Phenylpyridine	166
Chalcone	163	Pyridine	166
Chalcone oxide	163		

tion (CMC), has been attributed to increased solvation of the substrate from micelles, since the cytosolic epoxide hydrolase does not appear to hydrate substrates in micellular form. An interesting but unexplained observation is that Fe^{3+} salts (but not Fe^{2+} salts) stimulate cytosolic epoxide hydrolase activity.[167]

3.5. Purification

3.5.1. Purification of the Microsomal Epoxide Hydrolase. Numerous procedures have been reported for purifying the microsomal epoxide hydrolase. The methods are based on the early studies by Watabe and Kanehira[168] demonstrating solubilization of the microsomal epoxide hydrolase by deoxycholate and partial purification by ammonium sulfate and acetone precipitation. Several workers reported partial purification, and in 1975 the enzyme was purified from rat liver.[169–171] The three-step procedure of Lu et al.[171] was unique in using a detergent gradient to elute a DE52 column followed by hydroxyapatite chromatography to remove the detergent. A similar procedure yielded pure epoxide hydrolase from human liver,[74] but the hydroxyapatite step gave poor recovery of liver activity with human as with rat. Bentley and Oesch[169] de-

scribed a five-step procedure in which chromatography on phosphocellulose and butyl Sepharose replaced the hydroxyapatite step and detergent contamination was minimized. Slight modifications of these procedures have appeared in *Methods in Enzymology*.[172,173]

Knowles and Burchell[174] describe a simple, two-step method for the purification of the rat microsomal epoxide hydrolase following Lubrol solubilization. Lubrol (an alkyl polyethoxylate) does not absorb ultraviolet light and simplifies the monitoring of column effluents. This procedure has been adopted by several laboratories and was used by Guengerich et al.[44] to provide evidence for multiple forms of the microsomal epoxide hydrolase. These procedures were further modified to facilitate the purification of P-450, reductase and epoxide hydrolase from the same preparation using an *n*-octylamine Sepharose column as the first step.[175] Recently a single-step purification of Lubrol-solubilized epoxide hydrolase using immobilized monoclonal antibodies was described.[176]

*3.5.2. **Purification of the Cytosolic Epoxide Hydrolase.*** Recent reports from three laboratories describe the purification of epoxide hydrolase from the cytosolic fractions of human, rabbit, and mouse liver.[30,34,177] The reports are complicated because very little is known regarding the substrate specificity or multiplicity of epoxide hydrolases in the cytosol of mammals other than mice. In two purification schemes ammonium sulfate precipitation was used as a first step.[30,34] In one case there was a substantial loss of total activity with no increase in specific activity.[34] Experience in this laboratory indicates that with mice and rabbit preparations, ammonium sulfate precipitation is counterproductive and higher yields can be obtained by using a high-capacity chromatography step (such as DEAE) or polyethylene glycol precipitation in place of salt precipitation (Table 4).[178] As expected for an enzyme with an essential sulfhydryl, zinc acetate precipitation caused a total loss of activity, but could be a good way of removing the cytosolic epoxide hydrolase from other enzymes. This enzyme is remarkably stable to organic solvents, but differential precipitation with ethanol gave poor recovery. Gel permeation chromatography in a variety of matrices gives reasonable purification in good yield and

TABLE 4. Application of Polyethylene Glycol (PEG) Precipitation to the Purification of Murine Cytosolic Epoxide Hydrolase[a]

Percentage PEG (MW = 6000)	Fraction	Total Protein (mg)	Total Activity Recovered (%)	Purification Factor
0	Control	21	100	1.0
8	Supernatant	12	80	1.4
8	Pellet	12	15	0.3
15	Supernatant	8	7	0.2
15	Pellet	4	46	2.4

[a] A 50% (w/w) solution of PEG in I = 0.2 pH 7.4 phosphate buffer is slowly added to a stirred crude cytosol solution held at 4°C. Following stirring for 1 hr (the reaction is not very time dependent), the mixture is centrifuged at 4°C and the process is repeated. PEG 400 at 50% (w/w) gives only 40% precipitation of epoxide hydrolase activity with substantial loss of enzyme activity. All samples should be dialyzed before analysis. Percentages of PEG giving the optimum separation must be determined for each lot of PEG (D. Schridetzki, unpublished results).

is a rapid, low resolution technique for detecting possible multiple forms of the enzyme. This method clearly shows that both the monomer and dimer forms of the cytosolic enzyme differ from the microsomal enzyme.

Gill[177] presents a classical method for purifying the cytosolic epoxide hydrolase from mice. The enzyme is reasonably stable to a pH of 5, and does not readily bind to CM cellulose or Sephadex.[90] Thus, simply passing the cytosol through a cation exchange column at pH 6 or below effects 1.3 to 2-fold purification and allows the removal of glutathione sulfotransferase activity for subsequent purification. The cytosolic epoxide hydrolase binds readily to DEAE matrices at neutral pH and can be removed in high yield by a salt gradient. Approximately 10-fold purification can be obtained by binding the cytosolic epoxide hydrolase to phenyl Sepharose and eluting it with a 0–0.3% gradient of Lubrol. Gill[177] reports the use of a hydroxyapatite column eluted with a potassium phosphate gradient to give a high yield of apparently homogeneous epoxide hydrolase. Meijer et al.[179] recently reported other approaches to purification of the cytosolic epoxide hydrolase of mice.

Recently, Prestwich (unpublished) developed an affinity column for the purification of the cytosolic epoxide hydrolase. The column is based upon the low K_m values observed with trisubstituted epoxides and the ability of *O*-alkyl groups to mimic the epoxide moiety. Thus the epoxide hydrolase activity in crude mouse cytosol will bind specifically to a 7-methoxy citronellol derivative on Sepharose CL6B and can be eluted with geraniol 6,7-oxide, citronellol epoxide, or a chalcone oxide. This procedure gives a quantitative yield of apparently homogeneous cytosolic epoxide hydrolase in a single step when the cytosol from livers of induced mice are passed through the affinity column. This procedure has now been applied successfully to liver homogenates of rhesus monkeys and humans as well (M. H. Silva, unpublished).

The cytosolic epoxide hydrolase from mice is stable in crude form at a wide range of pH and buffer concentrations. Thus differential centrifugation can be performed under a variety of conditions selected for other reasons. Since the enzyme has an essential thiol, it is prudent to include ethylenediaminetetraacetic acid (EDTA) as well as mercaptoethanol and/or dithiothreitol in buffers made in high-quality distilled water, although in crude form the enzyme is quite stable without these precautions. One can safely predict that the enzyme will also be more stable at lower pH's, which result in protonation of the essential sulfhydryl.

4. TISSUE DISTRIBUTION

Epoxide hydrolases appear to be ubiquitous in mammalian tissues. Oesch and co-workers identified microsomal epoxide hydrolase activity in some 26 different rat organs and tissues.[113] These observations have been extended to other species as well.[35,180–184] Gill and Hammock[11,92] observed cytosolic epoxide hydrolase activity in all rabbit, mice, and rat organs examined. The highest levels of both major forms of the enzyme (i.e., microsomal and cytosolic) are generally found in the liver. High levels of the microsomal enzyme are also found in kidneys, testes,[113,183,184] and adrenal glands.[35] There is some evidence for a new form of epoxide hydrolase in testes (D. N. Loury, unpublished). Cytosolic enzyme

levels are similarly high in kidneys, but very low in testes[92] (B. D. Hammock, unpublished observations). Several comparative studies of epoxide hydrolase levels in different tissues of a number of species have been published.[75,127,183,184] The relative ranking of tissues according to epoxide hydrolase activity varies with the strain and species of animal being examined. Information on the tissue distribution of epoxide metabolizing enzymes such as epoxide hydrolase may help to clarify tissue-specific carcinogenicity among different species. Of further note are reports of low but measurable levels of epoxide hydrolase activity in human placenta.[38] This may be a very valuable observation since placenta is a readily available tissue source.

5. VARIATION AMONG SPECIES, AGE, SEX, AND STRAIN

Epoxide hydrolase activity has been identified in all mammalian species examined, as well as in birds,[185,186] fish,[186] amphibians,[182] insects,[69,160] protozoans,[187] plants,[188] and bacteria.[189] Significant variations in the levels of both microsomal and cytosolic epoxide hydrolase activity among vertebrate animals of different species, age, and sex have been noted.[11,92,182–184,186,190] The highest levels of hepatic microsomal epoxide hydrolase activity are found in baboons and monkeys, whereas mice possess among the lowest levels.[37] In contrast, high levels of liver cytosolic epoxide hydrolase activity are found in rabbits and mice, and very low levels are present in rats.[11,92] Levels of both major forms of the enzyme in human liver appear to be intermediate.[31] Knight and Walker[185] report a lower level of microsomal epoxide hydrolase in a variety of avian species than that observed in most mammals. In comparing data on the levels of epoxide hydrolase activity in different species, it is important to realize that the relative ranking of species according to enzyme levels will differ depending on the substrate used to measure activity, the tissue being examined, and a variety of factors related to the physiological state of the organism. Significant structural similarities and differences among the liver microsomal epoxide hydrolases from different species have been dem-

onstrated by the use of both monoclonal and polyclonal antibodies produced against the purified enzymes.[74,190,191]

An increase in epoxide hydrolase activity with age has been observed for both the microsomal and cytosolic enzymes. Stoming and Bresnick[192] report very low rates of hydration in fetuses and neonates of Sprague–Dawley rats with a rapid increase in activity to 25 days. Oesch and co-workers noted a four-fold increase in microsomal epoxide hydrolase activity between 28- and 40-day-old male rats.[18] Similarly, Gill and Hammock observed a 10-fold difference in cytosolic epoxide hydrolase activity between 2- and 10-week-old male Swiss Webster mice, but only a two-fold difference between female mice of the same ages.[92] Birnbaum and Baird noted that even very old rats retain their ability to metabolize epoxides.[193] Very few studies have extended these observations of age effects to extrahepatic tissues.[39,181]

In the species examined so far, only small differences between the sexes in the levels of microsomal epoxide hydrolase activity present have been noted.[127,183,185,194] However, fairly large differences between the sexes in the levels of cytosolic epoxide hydrolase activity have been observed in adults of several of these species.[11,92]

Interstrain differences in the levels of enzyme activity for both major forms are quite small in mice and rats.[92,182,195] However, as previously mentioned (Section 2.1), two different phenotypic classes of microsomal epoxide hydrolase have been identified in 26 inbred strains of mice.[43]

6. ANALYTICAL METHODS

6.1. General Information

6.1.1. Selecting the Appropriate Assay. As with any enzyme assay, one must not expect more information from the assay than it can provide. For instance, if one is interested in the metabolism of cholesterol epoxide in lymphocytes, one ultimately must examine the substrate of interest in the actual tissue of interest. Our knowledge of epoxide hydrolases is too fragmentary to extrapolate

with confidence from information obtained using model substrates, tissues, or species. Therefore, this section not only covers the use of model substrates, but also discusses how to develop assays for epoxides of future interest. Particular attention is given to the development of partition assays (see Section 6.4).

With any published assay there is a tendency to follow it in cookbook fashion. Such a course of action has advantages in that it minimizes start-up time and facilitates comparison of data generated in different laboratories. For this reason some detailed procedures are given in Table 5. However, the experimentalist should adapt any published assay to a particular problem. If a point assay (in contrast to a continuous assay) is being used, extra care is needed to assure that the reaction is linear with regard to time and protein concentration under all experimental conditions. It is particularly important to insure that both of these plots yield lines that pass through the origin at zero time. If one is comparing tissue homogenates with very different activities (e.g., induced vs. uninduced), several controls must be run to assure that the values are, in fact, comparable. When homogenates are being compared on the basis of single assays, the assays must be performed under conditions that closely approach V_{max} for the enzyme.

Obviously, with catalytic assays one is measuring enzyme activity not enzyme, and the data should be interpreted accordingly. Alternatively, with immunochemical assays one is measuring antigen(s) and not necessarily enzyme. Furthermore, there is often a need to know the substrate specificity of an enzyme, and as discussed in Sections 3.1 and 3.3, initial rate studies (estimates of V_{max}) or comparative inhibition data (estimates of K_m) cannot provide such information when used independently.

The analytical method selected should be based on the problem at hand, as illustrated by the following four examples. First, if numerous samples will be generated, as in a comparison of species or studies of induction, the analytical method should be capable of handling them. Even with autosamplers, most chromatographic methods are tedious. Thus few chromatographic methods are well suited for such tasks. Second, enzyme kinetics can be done using any assay method, yet a single experiment based on a continuous assay becomes a prodigious task when one uses a point assay,

because numerous samples must be assayed. Hence continuous assays are especially useful for detailed study of the kinetics of epoxide hydration, since a single experiment can yield a complete time course. Third, if one is comparing the relative rates of hydration of a variety of substrates, the time required to develop a spectrophotometric or partition assay is probably not warranted for each substrate, and chromatographic assays become advantageous. Chromatographic assays always have the advantage over other assay methods in that they give information on the identity and multiplicity of metabolites. Thus, as described in Section 6.1.2, they should always be used to check the integrity of other assay methods when a new system is being evaluated. Finally, budget and equipment limitations may dictate the assay used. For instance, radiochemical assays are extraordinarily expensive. The studies by Oesch et al.[77] and Mullin and Wilkinson[196] are excellent examples of workers employing a variety of analytical techniques, each appropriately chosen for the substrate used.

6.1.2. Verification of the Identity of Products. When the existence of epoxide hydrolases was not so well established, a major effort was sometimes expended in identifying as a diol the product formed during the exposure of an epoxide to a tissue homogenate. Such major efforts are no longer warranted in most cases. However, it is inexcusable not to provide reasonable verification of a metabolic product when a new substrate and/or system is examined. In general, an epoxide will be converted rapidly to a diol, a glutathione conjugate, or in some cases an olefin. Further metabolism of diols requires cofactors and usually is slow relative to diol formation. Thus, if an enzyme system converts a simple epoxide to a product of moderate polarity, the product will most likely be a diol.

The comparison of the resulting metabolite with a standard synthesized via an unambiguous pathway is of course optimum. Most epoxides can be converted into diols by treatment with aqueous organic or mineral acids. Trifluoroacetic and perchloric acid are poor nucleophiles and thus good catalysts, although acetic and sulfuric acid are often used successfully as well. The acids normally are used as dilute aqueous solutions in a nonreactive cosolvent,

such as tetrahydrofuran, which enhances solubility (e.g., 0.05 *N* perchloric acid in 40% aqueous tetrahydrofuran). In those few cases where the tetrahydrofuran ring opens and can react with the epoxide, the higher boiling solvent dioxane can be substituted. These reactions to generate diols are not universal, however. Pinacol rearrangements can occur with some epoxides, arene oxides tend to eliminate, and many terpenes rearrange or cyclize. Thus spectral verification of the product is important.

Gas-liquid chromatography–mass spectrometry (GLC-MS) provides a very useful, sensitive method for comparing a standard with a metabolite.[197] One needs to be aware, though, that many diols and some epoxides do not give parent peaks on electron impact and sometimes decompose either in the gas jet leading to the spectrometer or on some packing materials. Chemical ionization can be used to enhance parent peaks, and capillary columns and/or nonpolar supports reduce decomposition problems. Also, such decomposition does not preclude comparison of the metabolite and standard and is becoming less of a problem with modern spectrometers. Infrared spectrophotometry is also a sensitive technique which should not be overlooked for structure confirmation. With GLC-FTIR interfaces such confirmation should become more commonplace.

Gill et al.[24] invested a major amount of time in isolating enough diol metabolite to confirm its identity by nuclear magnetic resonance (NMR). Although such time is probably no longer warranted in most cases, new instrumentation allows very small sample sizes, and NMR spectra are usually quite diagnostic. Hammock et al.[198] used the large differences in chemical shifts of adjacent protons to follow a chemical reaction involving an olefin, an epoxide, and a diol in the same molecule. Similarly, the large differences in ^{13}C chemical shifts of the carbon atoms alpha to the epoxide functionality were useful in determining the geometry of the ring opening catalyzed by the cytosolic epoxide hydrolase.[91,199] Risley et al.[200] took advantage of the ^{18}O isotope effect in ^{13}C NMR spectroscopy to simultaneously determine the position of bond cleavage and the rate of hydrolysis of isobutylene oxide in dilute acid using mouse liver microsomes and purified rat liver microsomal epoxide hydrolase. As pulse and Fourier-transform methods become more so-

phisticated and magnets larger and more homogeneous, one can predict even greater application of NMR technology for the structural elucidation of metabolites both in pure form and in the presence of large amounts of biomass. These techniques will certainly have application in future detailed studies of the mechanism of enzyme-catalyzed epoxide hydration.

Obviously, cochromatography in several thin-layer chromatography (TLC) systems or high-performance liquid chromatography (HPLC) systems is very useful. If there is still uncertainty, these techniques can be extended further by derivatizing both the diol standard and the metabolite, followed by rechromatography. A number of micro methods for derivatizing 1,2-diols have been discussed.[11,198] These methods include formation of diesters with boronic acids, formation of esters with acetic anhydride, and formation of aldehyde with lead tetraacetate. A generally applicable procedure for confirming the identity of metabolites involves isolating a reasonable amount of radiolabeled metabolite, mixing it with an unlabeled diol standard, and dividing the mixture into several aliquots. Each aliquot can be derivatized by using a separate reagent, and the resulting materials are examined by cochromatography. Prelayer TLC plates (see Section 6.3.1) are convenient for such studies because they can handle large amounts of inorganic reagent.

6.1.3. Potential Problems. There are several potential problems related specifically to working with epoxides. Obviously some of these materials can present a risk to health, as can many of the solvents and reagents used in their analysis. Many epoxides are volatile and may be lost either during enzyme incubation or work-up or while spotting on a TLC plate. This problem is especially severe with prelayer plates (see Section 6.3.1). Some epoxides are also quite reactive. Thus it is important to control for the spontaneous hydrolysis and decomposition that occurs during enzyme incubation, work-up, and analysis. Epoxides also can react with nitrogen and especially with sulfur nucleophiles present in an enzyme reaction. Such alkylation may even inhibit the enzyme under investigation. For example, as discussed in Section 3.3, epoxides such as styrene oxide and some glycidyl ethers can inhibit the cy-

tosolic epoxide hydrolase. With especially reactive epoxides traces of acid present in some solvents may lead to decomposition. This problem can be reduced by using high-quality solvents, distilling the solvent from base, or adding a trace of pyridine or triethylamine. Some epoxides are lipophilic enough that binding to glass presents a problem at low substrate concentrations. This problem can be reduced by coating glassware with a dilute solution of inexpensive polyethylene glycol followed by heat treatment.[90] Certainly one should avoid the use of plastics with lipophilic compounds.

Many of the substrates used for the cytosolic and microsomal epoxide hydrolases are relatively lipophilic and tend to form micelles in aqueous solution. Since the cytosolic epoxide hydrolase fails to recognize substrates in micellular form,[11,91] one should be careful to work below the CMC of the substrate or to correct for the substrate in the micellular form, as suggested by Armstrong et al.[82]

One seldom faces the problem of volatility with diols; rather, the high polarity conferred by the diol moiety may be disconcerting. If the diol is present on a small molecule, one may have to use very polar solvents to extract the diol efficiently from aqueous solution. In some cases it is necessary to add large amounts of salt to the aqueous phase in order to improve extraction efficiency (see also Section 6.3.1). The high polarity may also lead to problems in eluting the diol from chromatography columns or from TLC.

6.2. Colorimetric Methods

Ideally a simple, universal colorimetric procedure could be used to monitor the disappearance of epoxide and/or appearance of diol in an enzyme system. Unfortunately, such a universal system has yet to be devised. A methyl red-sodium iodide spray has been used to detect epoxides on TLC.[18] Many epoxides react with 4-(*p*-nitrobenzyl)pyridine to give an unstable blue color on TLC plates or in solution.[203–205] An improved procedure for analysis in solution employs an analogous alkylation of epoxides by nicotinamide, which yields a fluorescent product.[206,207] Both of these approaches suffer from high background and are less sensitive than other methods. A different technique uses the greater nucleophilicity of sulfur

to attack the epoxide.[208] It is likely that a useful colorimetric procedure can be developed based on such reactions, but the reagents so far developed do not lend themselves to enzyme assays. A text by Dobinson et al.[209] covers numerous classical methods for the analysis of epoxides. Possibly some of these assays as well as some of the general reactions of epoxides can be used for the development of sensitive methods.[210]

Selective methods for colorimetric detection of diols would be advantageous since they could be used to evaluate appearance of product rather than disappearance of substrate. Leibman and Ortiz[211] describe the detection of diols by oxidation with periodic acid followed by reaction with 4-nitrophenyl hydrazine. Oesch et al.[18] found that detection of styrene glycol by oxidation with potassium triacetylosmate lacked sensitivity, but this method has been used for detecting diols on TLC.[196] In addition, several other oxidizing reagents such as lead tetraacetate have been used to detect diols.[203,212] However, these methods have limited use for enzyme assays, but have proven very useful for qualitative detection of diols on TLC and for spot tests to support structural assignments of synthetic standards. Possibly some of the reactions of bifunctional compounds reviewed by Poole and Zlatkis[213] could be modified to develop fluorescent or UV dense materials suitable for detecting diols on TLC or HPLC.

The best illustration of the use of a colorimetric test for diols in an enzyme assay was provided by Mullin and Wilkinson.[196] The principle of this method involves oxidation of the diol product by periodate to yield formaldehyde,[214] which is then determined colorimetrically by the Nash reagent.[215,216] Mullin and Wilkinson[196] used 1,2-epoxypropane (**1a**) as a substrate, but the method could, in theory, be applied to a variety of other monosubstituted or 1,1-disubstituted epoxides. It should be cautioned that this method is a point assay; a continuous assay would be more advantageous (see Section 6.1.1).

6.3. Chromatographic Detection

6.3.1. Thin-Layer Chromatography. The very simple, inexpensive TLC method is often overlooked when one is developing ana-

TABLE 5. General Procedures for Using *trans*-Stilbene Oxide (TSO) and *cis*-Stilbene Oxide (CSO) for Monitoring Epoxide Hydrolase Activity[a]

Analysis of Cytosolic Epoxide Hydrolase

1. Prepare solution of 5×10^{-3} *M* TSO in ethanol with approximately 20,000 cpm/μl. TSO is available from Aldrich Chemical Co.
2. Dilute cytosol in 76 m*M* phosphate buffer (pH 7.4) to give a protein concentration that will yield a rate in the linear range (100–250 μg/ml for mouse). Pipet 100 μl of diluted cytosol into either 6×50 or 10×75 mm round bottom tubes (conical tubes may yield poor mixing at a later step).
3. Preincubate diluted cytosol at 37°C for 1 min. Start the reaction by adding 1 μl of the ethanol solution from step 1 to give a final substrate concentration of 5×10^{-5} *M*. We routinely use a 50-μl Hamilton syringe (#705N) with a Hamilton repeating dispenser to rapidly add substrate.
4. Sample one tube before dodecane extraction to yield maximum counts and use thermally denatured enzyme as a control.
5. Stop the reaction by adding 200 μl of a hydrocarbon solvent such as isooctane or dodecane. Vigorous shaking on a Vortex is needed to obtain complete extraction. A table top centrifuge is used to break the emulsion.
6. With a Hamilton syringe penetrate the organic layer while extruding a small bubble. Remove 50 μl of the aqueous phase for liquid scintillation counting. A 50-μl Hamilton syringe with a #3 point is useful, but a variety of sampling devices are adequate. We use 3 ml of Aqueous Counting Scintillant (ACS) (Amersham) in 4 ml Wheaton Omni-Vials, which yields about 40% efficiency.
7. One method for calculating results is shown below:

$$\frac{\frac{101}{50}(\text{CPM sample} - \text{CPM boiled})}{\text{CPM/assay}} \times \frac{5 \text{ nmol}}{t\,(0.92)} \Big/ \text{mg protein/assay} = \text{nmol hydrated per mg min}$$

$\frac{101}{50}$ —Adjusts for volume of incubation and volume sampled. Can be varied to suit assay.

CPM/assay—Adjusts for total counts used in assay.

TABLE 5. (*Continued*)

CPM/boiled—Adjusts for background, epoxide not extracted, and other factors. See Section 6.4.7. These components can be treated separately for precise studies.

5 nmol—Mass of substrate used in study.

t—Time of assay in minutes. Since TSO is very stable and relatively nonvolatile, long incubation times can be used.

0.92—Proportion of diol in aqueous phase. This will vary depending upon the volume, solvent quality, and number of extractions with a hydrocarbon solvent.

Analysis of Microsomal Epoxide Hydrolase

1. Prepare solutions of 5×10^{-3} *M* CSO in ethanol with approximately 20,000 cpm/μl. CSO can be prepared from the corresponding *cis*-stilbene from Aldrich Chemical Company by a slight modification of the procedure described in *Organic Synthesis*[20] in which a 25% excess of *m*-chloroperbenzoic acid is substituted for peracetic acid.
2. Dilute microsomes in 100 m*M* tris-HCl buffer (pH 9.0) to give a protein concentration that will yield a rate in the linear range (10–75 μg/ml for mouse). Pipet 100 μl of diluted microsomes into either 6 × 50 or 10 × 75 mm round bottom tubes.
3. Proceed as in the case of the assay described above.

Analysis of Glutathione Transferase

1. Use either the TSO or CSO solutions prepared above.
2. Dilute cytosol with 76 m*M* phosphate buffer (pH 7.4) (this is a compromise between maximal enzyme activity and minimal background), to give a protein concentration that will yield a rate in the linear range (75–150 μl/ml). Pipet 50 μl of diluted cytosol into either 6 × 50 or 10 × 75 mm round bottom tubes. Immediately prior to assay add 50 μl of 10 m*M* reduced glutathione solution to yield a final concentration of 5 m*M*.
3. Identical to above.
4. Identical to above.
5. Stop the reaction by adding 200 μl of hexyl alcohol. Shake tube and break the emulsion using a table top centrifuge.
6. Same as above. The isooctane and hexanol extractions can be used in sequence to assay both epoxide hydration and glutathione conjugation.
7. The same procedure is used except that 0.99 is substituted as the percent conjugate in the aqueous phase.

TABLE 5. (*Continued*)

Preneoplastic Antigen in Serum

1. Prepare a solution of CSO in absolute ethanol containing ca 100,000 cpm/μl. This assumes a specific activity of 15Ci mmol.
2. Dilute unhemolyzed serum 1:1 with 100 m*M* tris-HCl buffer (pH 9.0) containing 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF) and place 50 μl of this in a 6 × 50 mm tube. Then add 1 μl of the substrate as described in step 1. Cover the tube with parafilm and incubate for varying times (we use 3 hr as a standard time) at 37°C.
3. Terminate the reaction by adding 25 μl of methanol containing 10 mg/ml CSO and *meso*-diol. Vortex, centrifuge, and spot 50 μl of the supernatant (25 μl at a time) on the cellulose prelayer of Whatman LK5DF silica gel plates. Be careful that the aqueous material neither touches the silica layer nor extends below the level of the developing solvent.
4. Let the plate air dry for a minimum of 20 min. For very tight spots develop the plate in methanol up to the cellulose-silica gel boundary. Let dry and develop 20 cm with toluene:*n*-propanol (20:1) as a solvent system. When the toluene has evaporated visualize the epoxide (upper) and diol (lower) bands under 254 nm UV light and mark with a pencil. Spray the plate lightly with water and scrape the diol spot into a scintillation vial. The water reduces chemiluminescence and reduces the hazard of radioactive dust. Either add ACS scintillation solution (4 ml) or methanol (50 μl) to the silica gel followed by a xylene- or toluene-based solution. Note that CSO evaporates from the prelayer so one cannot take a ratio of epoxide to diol counts on the plate, but rather one must express diol produced as a function of epoxide added.
5. Chemiluminescence is a serious problem with the fluoroscent indicator used in LK5DF plates. Let the vials stand at least 12 hr in the dark and always run background vials with silica gel present.

Calculations:

$$\frac{76}{60}(\text{dpm as diol} - \text{dpm boiled}) \times \frac{1}{t \times 0.025\ \text{ml}} = 33.3 \times 10^3\ \text{dpm/pmol per ml serum/hr}$$

$\frac{76}{50}$—Adjusts for volume sampled.

TABLE 5. (*Continued*)

33.3×10^3 dpm/pmol	—Converts dpm to pmol substrate metabolized. Will vary depending upon specific activity of substrate.
t	—Time in hours, usually 3 hr for routine studies.
0.025	—Since 25 μl of serum are used, this factor converts data to a milliliter of serum basis.

[a] The methods described represent slight modifications to those suggested by Mullin and Hammock,[202] Gill et al.,[162] and unpublished results from Loury of this laboratory.

lytical methods for monitoring the hydration of epoxides. The chromatographic separation of epoxides and diols is trivial, and a multilane TLC plate allows numerous separations to be run in parallel. If the substrate is hydrolyzed rapidly and a tissue rich in epoxide hydrolase is used, the TLC plate can be run with minimal background. Cautions regarding volatility and stability of the epoxides are discussed below (Section 6.4.2). For substrates that fail to absorb UV light, the chromagenic reagents described above can give a qualitative assessment of hydration. If the substrate and diol are either fluorescent or UV dense, the plate can be scanned or the appropriate bands scraped off the plate and their fluorescence or absorbance read in a solvent such as methanol. This procedure can be very quantitative since one has an internal standard (the epoxide), and devices to scan TLC plates for both absorbance and emission are becoming increasingly sensitive. As it evolves, this technology may replace, in part, the heavy reliance now placed on the use of radioisotopes. The work of Oesch et al.[77] provides an excellent example of this simple, but powerful, technique. Certainly, the potential of TLC should not be overlooked, especially when one is screening a variety of substrates.[78]

A worker who has not experienced the tedium of performing multiple extractions on an enzyme reaction, carefully drying and concentrating the solvent, spotting the extract in a tight band on TLC, developing the plate, detecting the radioactive bands, and finally scraping and counting will not appreciate the improvements that partition assays and prelayer TLC plates have made in the

analysis of epoxide hydrolases (Section 6.4). However, the longer technique has its place in an initial metabolism study. There is no excuse for failing to perform a careful balance study to insure that recovery is high and that the diol is the sole product. One should monitor both organic and aqueous phases as well as the TLC plate for levels of radioactivity. Such careful extraction followed by a well-designed two-dimensional TLC system provides a cost-effective way of supporting metabolite identification and insuring that minor metabolites are not overlooked. Extraction of polar diols from an aqueous phase may not be easy. Ethyl ether is a convenient solvent, but even traces of peroxides may lead to the epoxidation of reactive olefins. Ether:ethanol mixtures or the more toxic ethyl acetate may be needed to improve extraction efficiencies. The addition of sodium chloride increases extraction efficiencies, breaks emulsions, and leads to drier organic phases, while allowing one to use more polar organic solvents. However, high salt concentration makes it difficult to count radioactivity in the aqueous phase. The method of Randerath[217] still provides a useful way to qualitatively evaluate radioactive metabolites. Several TLC based assays discussed below using radioactive substrates illustrate some new approaches.

Black and Lenger[218] employed TLC on silica gel to separate the corresponding diol from cholestan-3β,5α,6α-epoxide (**1k**) following extraction. It is important to avoid spotting water when using silica gel plates such as these. The procedure described is rather laborious[218], but TLC procedures do result in a lower background than partition methods. These workers avoided liquid scintillation counting by using a TLC scanner. This is a rather slow, insensitive procedure when older proportional flow counters are used and one must be very careful to control for counting efficiency. However, newer (but very expensive) scanners promise to make this method very time efficient.[219] Several researchers have used modifications of the TLC method described below to monitor the hydration of the cholesterol epoxides. This assay is interesting in that apparently minor modifications in technique can lead to major variations in the rate of hydration detected.[40–42,107,220]

Jerina et al.[107] used Quantum Industries LQDF TLC plates. These prescored plates have a nonabsorbant layer at the origin

which allows one to spot aqueous samples. Following evaporation of the water the plate is developed as a normal TLC plate. A further advantage is that most compounds are concentrated in a thin band at the silica–cellulose interface. These workers used the method with 11 substrates and concluded that the combined attributes of benzo(a)pyrene 4,5-oxide (**1h**) made it the substrate of choice. Benzo(a)pyrene 4,5-oxide is turned over rapidly by the microsomal epoxide hydrolase, but very slowly by the cytosolic form. It is much more stable than many arene oxides, is nonvolatile, and the epoxide and diol are easily separated by TLC. This general procedure can be applied, with some caution, to a wide variety of substrates (see below). In this and other comparative studies octane 1,2-oxide (**1a**) proved to be rapidly hydrated by the microsomal epoxide hydrolase. Since it should be a good substrate as well and have a favorable octanol/water partition coefficient, it is a prime candidate for the development of a partition assay, as discussed in Section 6.4.

This TLC method has been used in this laboratory to verify results from partition assays. The TLC method is slower than partition methods for epoxides such as the stilbene (**1b,c**) or alkylstyrene oxides (**1f** and **10**); however, it gives lower background values. Thus, to detect the epoxide hydrolase activity of PNA in serum, high specific activity substrate is used with the TLC method (Table 5). As pointed out by Mullin and Hammock,[202] one can use high concentrations of *trans*-β-alkylstyrene oxides or stilbene oxide in enzyme preparations with glutathione and monitor both the diol and glutathione conjugate in the same TLC track.

It has been noted in this laboratory that the enzymatic hydration of an epoxide does not necessarily stop when the aqueous enzyme solution is applied to the TLC plate. The reaction is usually arrested by adding 25 μl of a solvent such as tetrahydrofuran (containing nonradioactive standards) to an equal volume of assay mixture prior to spotting on a TLC plate. Also it is important to evaporate most of the water from the prelayer before the plate is developed to avoid streaking. If bands are not sharp, predevelopment in a polar solvent such as methanol or a methanol:toluene mixture to the level of the cellulose–silica interface often improves the situation. The regions of the TLC plate containing standards are lo-

cated colorimetrically or by UV quenching and then analyzed by liquid scintillation counting. The fluorescent indicator used in these plates may cause serious difficulties with liquid scintillation counting, owing to its phosphorescent properties. This problem can be reduced by (1) lightly spraying the plate with water before scraping (which also reduces health hazard), (2) scraping the material into a scintillation vial with a small amount of methanol, (3) letting the plate and/or vials stand in the dark for several hours to days before counting, (4) always running control regions of the plate to insure low background, (5) correcting for chemiluminescence, and (6) counting representative samples several times to insure that a constant number of events are being detected. Most epoxides are stable on TLC; however, some will break down on silica or alumina plates. Predevelopment of the plate in a 5% solution of triethylamine in ether helps reduce this problem.

Even epoxides that demonstrate no detectable volatility from a silica gel-TLC plate over a period of days may evaporate in minutes from the prelayer of LQDF plates. For example, stilbene oxides rapidly volatilize, apparently at two rapid rates, the first presumably due in part to codistillation with water. We have experienced no similar loss with diols or glutathione conjugates. Thus, with nonvolatile substrates such as benzo(a)pyrene 4,5-oxide, one can take a ratio of metabolite to substrate; with more volatile compounds, one must spot a known amount of radioactivity on the plate and express the diol as a proportion of total counts applied, since the volatile epoxide cannot be used as an internal standard. Approximately equal counting efficiency has been observed for all epoxides and diols tested in a number of scintillation solutions. Thus, after an initial study, one is probably justified in using counts per minute rather than disintegrations per minute in calculations for routine assays. In contrast, even some very polar liquid scintillation solutions gave poor counting efficiency with glutathione conjugates of small epoxides. In such cases it is critical to calculate the efficiency of detection of the epoxide, diol, and conjugate. Apparently, the scintillation solution is unable to extract the conjugate from the silica gel. This hypothesis can be tested by adding a small amount of methanol to the silica gel and cellulose containing the conjugate before adding the scintillation solution.

6.3.2. High-Pressure Liquid Chromatography. High-pressure liquid chromatography (HPLC) offers all of the advantages of TLC with the added benefits of increased sensitivity, higher resolution, and the ability to detect and integrate the reaction products on-line. The separation of epoxide and diol from interfering substances and injection solvent can be more difficult to achieve than the usually trivial separation of substrate and product. Thus one often can use small and/or inexpensive columns for such simple separations. When dealing with biological materials, one obviously should use a column that can withstand some build-up of biomass, so reversed phase methods are preferred. The disadvantages of using HPLC to monitor epoxide hydration include the required dedication of expensive instrumentation to such projects and the relative slowness of the method. Until better detectors or universal derivatizing reagents become available for diols, one is restricted to the analysis of either compounds with a good chromaphore or radioactive materials. If a refractive index detector is to be used, one is essentially limited to the use of isocratic solvent systems. In attempting to derivatize diols to enhance sensitivity on HPLC, our experience has been that the derivatizing reagent caused such interference on HPLC that sensitive analysis of the diol was prevented.

Two excellent studies illustrate some of the factors that must be considered when developing an HPLC-based assay. Westkaemper and Hanzlik[221] describe an assay based on the hydration of 4-nitrostyrene oxide. They give detailed synthetic procedures and these simple procedures could be adapted readily to the radiosynthesis of the epoxide as well. Giuliano et al.[222] describe an assay based on 3-(4-nitrophenoxy)-1,2-epoxypropane (**11**), 4-nitrophenyl glycidyl ether). This material is easy to synthesize from epichlorohydrin and thus can be radiolabeled from commercially available starting materials. Both groups used an internal standard (4-nitrobenzyl alcohol and 4-nitroanisole, respectively). The importance of the internal standard is discussed in more detail in Section 6.3.3. Both methods have the advantage of using a substrate of lower volatility and greater stability than styrene oxide,[221,222] while being hydrated at a similar rate (in many species).[88] The method of Giuliano et al.[222] offers some advantage in that the higher extinction coefficient and wavelength of maximum absorbance of 3-(4-nitro-

phenoxy)-1,2-propanediol leads to greater sensitivity, although a more expensive UV monitor must be used (for further discussion see Section 6.5). However, Dansette et al.[84] report that 4-nitrophenyl glycidyl ether is unstable. Both 4-nitrostyrene oxide and 4-nitrophenyl glycidyl ether are potentially useful probes for the cytosolic epoxide hydrolase and have been used for monitoring glutathione sulfotransferase as well. In fact, a recent study reports a system that separates the diol and glutathione conjugate of phenyl glycidyl ether during a single run.[223]

Like other chromatographic techniques, HPLC is useful for comparing the kinetics of hydration of a variety of substrates, as illustrated by the work of Dansette et al.[88] The power of HPLC is best seen when one needs to separate a variety of products and by-products. This point was illustrated first by the Nesnow and Heidelberger[224] epoxide hydrolase assay using 3-methylcholanthrene 11,12-oxide as substrate followed by analysis with normal phase HPLC. An even more complex separation is encountered if one is dealing with both activation (by P-450) at multiple sites and several deactivation processes. Methodologies for the separation of such mixtures by HPLC have been developed for polycyclic aromatic hydrocarbons in numerous laboratories.[225–227] A similar approach has been applied to the investigation of the metabolism of arachidonic acid to its corresponding epoxides and diols.[96,228–230] The HPLC technique is especially appropriate for analyzing the hydration of LTA_4 (**2**), since a variety of positional isomers are produced by solvolysis, but only a single isomer seems to be produced enzymatically.

6.3.3. Gas-Liquid Chromatography. Gas-liquid chromatography (GLC) offers the most universal method for analyzing the products of epoxide hydrolase activity. Most epoxides of interest are small enough that they pass readily through GLC columns. Even if the epoxide cannot be detected by GLC, this does not preclude analysis of the diol. In addition to being rather universal, most GLCs are rugged, dependable instruments, and they need not be overly expensive. Like other separation methods, cochromatography provides the investigator with some degree of assurance of the identity and purity of both the metabolite and the substrate.

However, as with HPLC, some metabolites and impurities may not emerge from the column.

Several years ago some degree of skill was required to get an epoxide and diol through a GLC column, but improved technology has alleviated this problem. Some epoxides rearrange if they contact metal or acid at high temperatures. As a general rule it is best to avoid metal columns, to use neutral stationary phases, and in some cases to add a trace of a basic liquid phase (such as phenyldiethanolamine succinate) to the main liquid phase. Since the separations involved are usually simple, a liquid phase that has little interaction with the diol and/or epoxide allows analysis at relatively low temperatures. Isothermal conditions provide better quantitative results, especially on older instruments. Conditions should be adjusted so that the products elute as sharp, symmetrical peaks as soon after the solvent peak as possible. On nonpolar phases diols often give sharp peaks without derivatization; however, trimethylsilyl and boronic diesters commonly are used to improve peak shape.[213] One must be careful, however, not to inject underivatized diols onto a column previously used with boronic acids. In many cases no diol peak will emerge from such a column. Furthermore, boronic acids will not react with *trans* diols that are not free to rotate. With unstable epoxides it may be necessary to reduce the epoxide with hydride or convert it to an alkoxyhydrin before analysis so that it does not form additional diol during sample preparation or analysis.

Since both GLC and HPLC assays usually require at least one extraction step, the use of an internal standard greatly simplifies analysis. If an internal standard is not used, one must be exceptionally careful in each extraction step and make quantitative injections onto the GLC. If the internal standard shows a similar solubility to the diol, and in some cases the epoxide, it is possible to attain higher accuracy and precision in the assay with less labor. In some cases the epoxide itself has been used as an internal standard for the diol.[91] This procedure is useful with higher molecular weight materials, because the epoxide and diol will have similar retention times. It is necessary that the detector have a large, linear dynamic range, since small amounts of diol must be compared with large amounts of substrate to determine initial rates. When the

epoxide is used as the internal standard it is critical that hydration of the epoxide is the only reaction, that both the diol and the epoxide are extracted in equal proportions, and that the epoxide is not volatile.

Alkoxides have proven to be very useful internal standards for the analysis of rates of hydration of lanosterol epoxide (**9**) and squalene epoxides (**1j**).[11] Alkoxides can be made readily from the corresponding olefin by oxymercuration–demercuration,[231] and the volatility can be adjusted by shifting from methoxy to ethoxy or propoxy. Reduction products of epoxides as well as alkoxyhydrins formed by the hydrolysis of epoxides in acidic alcohols also provide products of intermediate volatility that can be used as internal standards.

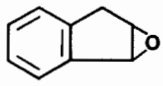
With relatively small molecules, not only are the epoxides too volatile to use as internal standards, but their retention times are very short. Hammock and Hasagawa[79] used easily synthesized diols of similar retention times (and polarities) to the diol of interest as internal standards. Such standards control for extraction efficiency, extent of derivatization, some measurement errors, and detector response. When selecting the amount of standard to be used, it is important that it approaches the amount of product expected from the reaction rather than the amount of substrate used.

Both GLC-MS and GLC-IR provide reliable methods for confirming the structure of metabolites; however, unless the cost drops dramatically, the use of such expensive instrumentation seldom will be warranted for routine analysis. As shown by the trace analysis of insect juvenile hormone, selective ion monitoring can yield exceptionally sensitive analytical methods for epoxide containing compounds.[232] The GLC-MS method also is useful for monitoring the ratio of heavy isotopes for mechanistic studies.[9,233–235]

Electron capture detection (EC) has been used by several laboratories for diols that either capture electrons efficiently themselves or can be made to do so following derivatization. Since increased sensitivity and/or specificity often is associated with numerous other problems, one should question whether the added sensitivity is really needed. Although the following technique has been little used, in drug analysis one sometimes injects a biological sample directly onto a GLC column. With a polyhalogenated sub-

strate this approach could possibly yield a very rapid analytical method.

Probably the most useful application of GLC technology is for examining the hydration of a variety of substrates. Thus the early literature on the microsomal epoxide hydrolase relied heavily on GLC for the analysis of products. For instance, Leibman and Ortiz monitored the hydration of indene epoxide (**20**)



20

to its diol,[70] while Maynert et al.[67] studied several epoxyoctanes. The latter assay was used subsequently by several other workers.[77,168,196,236] Brooks et al.[68,69] examined the hydration of epoxides of cyclodiene insecticides. Most cyclodiene epoxides are remarkably resistant to hydration, but one polyhalogenated compound termed HEOM (**1i**) is hydrated rapidly by epoxide hydrolases of mammals and insects. This model substrate has been used by numerous workers,[14,153,196,236–238] and recently it has been used as the basis of a highly sensitive assay for epoxide hydrolase activity in human liver.[239] With these cyclodienes, the peak shape obtained on GLC is good when either the diol itself or its silylated derivative is injected. The epoxide of HEOM is nonvolatile and exceptionally stable in water, thus one can use long incubation times to enhance the sensitivity of the assays. It is not hydrolyzed detectably by the cytosolic epoxide hydrolase of mouse, so it is also useful for the selective analysis of microsomal epoxide hydrolase without any subcellular distribution experiments.[79]

As a verification for their photometric assay, Watabe and Akamatsu[240] described a GLC method using safrole oxide (**19**) as the substrate. The method illustrates that differential partitioning can be used to clean up a sample before GLC analysis. These workers removed the epoxide from the aqueous assay mixture by hexane extraction prior to extracting the diol with ethyl acetate. The acetonide of the diol was then quantiated by GLC.

Belvedere et al.[241] described an assay for monooxygenase activity using styrene. The product, styrene oxide, was analyzed as

the diol. The assay was also applicable to the analysis of epoxide hydrolase. It was subsequently modified for greater sensitivity by derivatizing the diol with pentafluorobenzoyl chloride or trifluoroacetic anhydride for analysis by EC-GLC.[242]

Stoming and Bresnick[243] developed a method for monitoring the hydration of 3-methylcholanthrene 11,12-oxide based on analysis by GLC of the trimethylsilylated diol. The hydration of several arene oxides has also been monitored by EC-GLC.[197,244] These assays are quite sensitive, owing to the large conjugated system present in the arene oxides.

Analysis of products by GLC was used extensively in early studies on the substrate selectivity of the cytosolic epoxide hydrolase. Mumby and Hammock[91] prepared a series of homologous epoxides, all containing the 4-bromophenoxy moiety so that they could be analyzed by EC-GLC. Because these substrates were so lipophilic, they had to be used at low substrate concentrations in the enzyme assay, which posed a difficult analytical problem. Detection by EC, however, allowed the studies to be conducted below the CMCs of the substrates. Gill and Hammock[25] used GLC analysis to monitor the hydration of boronic diesters of diols formed from fatty acid epoxides, and this technique should be generally applicable to a variety of such compounds. Gas-liquid chromatography also has been used for monitoring the comparative hydration of a variety of epoxides catalyzed by microsomal and cytosolic fractions from mouse liver.[16,26,79,162]

6.4. Solvent Partition Methods

6.4.1. General Considerations. As mentioned in Section 6.1 there is a large increase in polarity as an epoxide is converted to a diol, and a further increase in polarity as an epoxide is converted to a glutathione conjugate. Bush[245] provides a review of partition methods, suggests a systematic approach to evaluating the potential of a partition based method for separating metabolites, and discusses the optimization of partition procedures. Since octanol:water partition coefficients (P) commonly are used for correlating structure with biological activity,[246] a first step in developing a partition assay may be to determine P (usually ex-

pressed as log *P*) for the substrate and its product. If the difference in log *P* between the epoxide and its diol is large, designing a partition-based assay should be easy. In general, the greater the contribution of functionalities other than the epoxide and diol to the polarity or lipophilicity of a molecule, the more tedious will be the partition assay. Thus developing a partition system to separate styrene oxide (**1d**), β-alkylstyrene oxides (**1f,10**), or stilbene oxides (**1b,c**) from their product diols is trivial,[18,162,202] the juvenile hormones **6** and **7** lipid epoxides **8** and **13** present a more difficult problem,[247] and the large arene oxides **1k, 5,** and **12** may require multiple extractions.[83,248]

6.4.2. Assays with Styrene Oxide. Oesch et al.[18] described the first partition assay for epoxide hydrolase using tritiated styrene oxide (**1d**), and this is still the most widely used method for analysis of the microsomal epoxide hydrolase. Two extractions with petroleum ether remove over 99.8% of the epoxide but only 8.8% of the diol from the aqueous phase. The speed of this separation step is enhanced by freezing the aqueous phase before decanting. The diol then is extracted into ethyl acetate, dried, and counted by liquid scintillation spectrometry. This basic assay has been changed slightly by a number of workers,[12,172] but a report by Seidegard et al.[249] illustrates several systematic ways in which the partition assay can be manipulated to improve it for a particular application.

The styrene oxide assay offers a number of advantages. It has been used widely, thus facilitating comparisons with other systems, and styrene oxide is also a good substrate for some glutathione sulfotransferases (obviously this may be a disadvantage if proper controls are not run). At high substrate concentrations styrene oxide can be used as a substrate to detect the microsomal epoxide hydrolase in the presence of the cytosolic form.[16] Styrene oxide is very soluble in water, allowing one to use even millimolar concentrations of the substrate. Both labeled and unlabeled material is available commercially, and the assay is rather simple. There are large differences among the polarities of the epoxide, diol, and glutathione conjugate which lead to straightforward partition-based assays.

When one examines the evolution of substrates used at NIH, one can see the reasoning leading to the conclusion that styrene oxide may be a good model substrate for investigating the metabolism of some arene oxides. This hypothesis was elegantly verified by Oesch and Bentley[250] for the microsomal epoxide hydrolase using benzo(a)pyrene 4,5-oxide (**1h**), thus substantiating earlier studies with a variety of other substrates.[77]

Still, there are several limitations of the assay using styrene oxide which indicate that one should consider alternate substrates before initiating a large study. Styrene oxide is unstable on storage, yielding a variety of products including polymers. However, these impurities can be removed readily by partition immediately before assay.[249] Of greater concern is the instability of styrene oxide in buffer, invariably leading to a high background and limiting its use at lower pH values and with long incubation times. Styrene oxide is a potent alkylating agent which may present a minor health risk.[251] In terms of the assay its reactivity causes problems in that it readily alkylates a variety of nucleophiles. Thus it often gives a high background in the presence of biological material. This binding to protein leads to the extra steps involved in the multiple petroleum ether extractions and the ethyl acetate extraction. The reactivity of styrene oxide limits its use as a substrate for the cytosolic enzyme since this enzyme apparently possesses a thiol group that is important for enzyme activity and is readily inhibited by styrene oxide (as are some other enzymes).[79,161] The compound is volatile, adding to a potential health risk and necessitating the use of capped reaction vessels or a large excess of substrate to maintain V_{max} conditions during the assay. This volatility also precludes monitoring both diol and epoxide in a single partition. Although the substrate currently is available from several reputable suppliers, its apparently simple synthesis is far from trivial for the neophyte[252] (see Table 6 for alternate GLC methods).

If the assay with styrene oxide is used, several minor modifications may reduce the time required for analysis. Certainly, periodic purification of the substrate is critical, since a low background greatly enhances the sensitivity of the assay. Multiple extractions of the assay mixture are not warranted in all cases, since the background resulting from hydrolysis in the buffer and

TABLE 6. Some Substrates Examined for Their Hydration by Epoxide Hydrolases[a]

Substrate	Subcellular Fraction(s) Examined	Method (Reference)
Allylbenzene oxide (**11**)	m,c	GLC (79)
Arachidonic acid epoxides (**13**)	c	HPLC (96,230)
Benzene oxide	m	TLC/UV (77)
	m,c	TLC (71)
Benz(a)anthracene 5,6-oxide	m	EC-GLC (197,244)
	m	GLC-MS (197)
	m	Continuous spectrophotometric (82)
[^{3}H]-Benz(a)anthracene 5,6-oxide	m	LSC (83)
[7-^{3}H]-Benz(a)anthracene 5,6-oxide	m	TLC/LSC (19)
Benzo(a)pyrene 4,5-oxide (Figure 1*h*)	m	EC-GLC (197)
	m	GLC-MS (197)
	m	Fluorometric (254)
	m	Continuous fluorometric (84)
	m	Continuous spectrophotometric (82)
	m	HPLC (227)
[^{3}H]-Benzo(a)pyrene 4,5-oxide	m	LSC (83,162,248)
[6-^{3}H]-Benzo(a)pyrene 4,5-oxide	m	TLC/LSC (19,107)
Benzo(a)pyrene 7,8-oxide	m	HPLC (227)
[6-^{3}H]-Benzo(a)pyrene 7,8-oxide	m	TLC/LSC (19,107)
[6-^{3}H]-Benzo(a)pyrene 9,10-oxide	m	TLC/LSC (19,107)
[11,12-^{3}H]-Benzo(a)pyrene 11,12-oxide	m	TLC/LSC (19,107)

TABLE 6. (*Continued*)

Substrate	Subcellular Fraction(s) Examined	Method (Reference)
Benzo(e)pyrene 4,5-oxide	m	Continuous spectrophotometric (82)
Bisnorsqualene-2-^{3}H-1,2-oxide	m	TLC/LSC (77)
3-Bromophenanthrene 9,10-oxide	m	Continuous spectrophotometric (82)
4-Chlorophenyl-2,3-epoxypropylether	m	TLC/UV (77)
	m,c	GLC (79)
5-Cholestene 5α,6α-oxide	m	LC/GLC-MS (41)
5-Cholestene 5β,6β-oxide	m	LC/GLC-MS (41)
Cholesterol-5α,6α-oxide (Figure 1*k*)	m	LC/GLC-MS (41)
[^{14}C]Cholesterol-5α,6α-oxide	h	LC/TLC/TLC scanner (218)
	m	TLC/LSC (42)
[4-^{14}C]-Cholesterol-5α,6α-oxide	h	TLC/LSC (145)
	m,c	LC,TLC,GLC-MS (40)
Cholesterol-5β,6β-oxide	m	LC/GLC-MS (41)
[4-^{14}C]Cholesterol-5β,6β-oxide	m,c	LC,TLC,GLC-MS (40)
Cyclodiene epoxides	m	GLC (69,196)
Cyclohexene oxide (**16**)	m	GLC (196)
[^{3}H]Cyclohexene oxide	m	LSC (77)
[^{3}H]-Dibenz(a,h) anthracene 5,6-oxide	m	LSC (83)
[5,6-^{3}H]-Dibenz(a,h) anthracene 5,6-oxide	m	TLC/LSC (107)
7,12-Dimethylbenz(a) anthracene 5,6-oxide	m	Continuous fluorometric (84)
16α,17α-Epoxyandrost-4-en-3-one (**4**)	m	LSC (87)
[17-^{3}H]-16α,17α-Epoxy-1,3,5(10)-estratrien-3-ol ("estroxide") (**15**)	m	LSC (86)

TABLE 6. (*Continued*)

Substrate	Subcellular Fraction(s) Examined	Method (Reference)
Epoxymethyl stearates (**8**)	m,c	GLC (25)
cis-Epoxymethyl stearate	m	TLC/GLC (259)
[1-^{14}C]*cis*-Epoxymethyl stearate	m	TLC/LSC (25)
1,2-Epoxypropane	m	Spectrophotometric (196)
cis-β-[2-^{3}H]Ethylstyrene oxide	m,c	LSC (202)
trans-β-[2-^{3}H]Ethylstyrene oxide	m,c	LSC (202)
trans-β-Ethylstyrene oxide (Figure 1*f*)	m,c	GLC (79)
HEOM (Figure 1*i*)	m	EC-GLC (69,153,182,196,239)
Indene oxide (**20**)	m	TLC/UV (77)
	m	GLC (70)
[10-^{3}H]Juvenile hormone I	c	LSC (247)
Limonene 1,2-oxide	m,c	GLC (79)
Limonene 7,8-oxide	m,c	GLC (79)
[^{3}H]-7-Methylbenz(a) anthracene 5,6-oxide	m	LSC (83)
3-Methylcholanthrene 11,12-oxide	m	EC-GLC (197)
	m	GC-MS (197)
	m	HPLC (224)
	m	GLC (243)
[^{3}H]-3-Methylcholanthrene 11,12-oxide	m	LSC (83)
[11,12-^{3}H]-3-Methylcholanthrene 11,12-oxide	m	TLC/LSC (19,107)
2-Methyl-2,3-epoxyheptane	m	GLC (196)
20-Methylpregnenol 5α,6α-oxide	m	LC/GLC-MS (41)
20-Methylpregnenol 5β,6β-oxide	m	LC/GLC-MS (41)
cis-β-Methylstyrene oxide	m,c	GLC (79)

TABLE 6. (*Continued*)

Substrate	Subcellular Fraction(s) Examined	Method (Reference)
trans-β-Methylstyrene oxide (**10**)	m,c	GLC (79)
Naphthalene 1,2-oxide	m	TLC/UV (77)
[2-^{3}H]Naphthalene 1,2-oxide	m	TLC/LSC (107)
3-(*p*-Nitrophenoxy)-1,2-propene oxide (Figure 1*l*)	m	HPLC (222)
m-Nitrostyrene oxide	m	EC-GLC (196)
p-Nitrostyrene oxide (Figure 1*e*)	m	HPLC (221)
	m	Continuous spectrophotometric (264)
Octene 1,2-oxide (Figure 1*a*)	m	GLC (77,168)
	m	Continuous spectrophotometric (81)
[7,8-^{3}H]Octene 1,2-oxide	m	TLC/LSC (19,107)
Phenanthrene 9,10-oxide (Figure 1*g*)	m	TLC/UV (77)
	m	Continuous fluorometric (84)
	m	Continuous spectrophotometric (82)
[^{3}H]-Phenanthrene 9,10-oxide	m	LSC (83)
[3-^{3}H]-Phenanthrene 9,10-oxide)	m	TLC/LSC (19,107,155)
Pregnenolone 5α,6α-oxide	m	LC/GLC-MS (41)
Pregnenolone 5β,6β-oxide	m	LC/GLC-MS (41)
trans-β-Propylstyrene oxide	m,c	GLC (79)
Pyrene 4,5-oxide	m	Continuous fluorometric (84)
	m	Continuous spectrophotometric (82)
[^{14}C] R 20458	c	LSC (247)
Safrole oxide (**19**)	m	Spectrophotometric (240)

TABLE 6. (*Continued*)

Substrate	Subcellular Fraction(s) Examined	Method (Reference)
β-Sitosterol-4-^{14}C-5α,6α-oxide	m,c	LC,TLC,GLC-MS (40)
β-Sitosterol-4-^{14}C-5β,6β-oxide (**14**)	m,c	LC,TLC,GLC-MS (40)
Squalene-3-^{3}H-2,3-oxide (Figure 1*j*)	m	TLC/LSC (77)
cis-Stilbene oxide (Figure 1*b*)	m,c	GLC (79)
	m	GLC (259)
	m	TLC/GLC (256)
[^{3}H]*cis*-Stilbene oxide	m,c	LSC (162)
trans-Stilbene oxide (Figure 1*c*)	c	Continuous spectrophotometric (260)
	m,c	GLC (79)
	m	GLC (259)
	m	TLC/GLC (256)
[^{3}H]*trans*-Stilbene oxide	m	LSC (93)
	m,c	LSC (162)
Styrene oxide (Figure 1*d*)	m	GLC (151,241,259)
	m,c	GLC (79)
	m	EC-GLC (242,275)
	m	Continuous spectrophotometric (81)
[^{3}H]Styrene oxide	m	LSC (173,249)
	m	TLC/LSC (149,194)
[7-^{3}H]Styrene oxide	m	LSC (18,77,172)
	m	TLC/LSC (19,107,155)
[8-^{14}C]Styrene oxide	m	LSC (252)
1,2-Vinylcyclohexene oxide	m,c	GLC (79)
7,8-Vinylcyclohexene oxide	m,c	GLC (79)

[a] Abbreviations: m, microsomal (includes nuclear subcellular fraction); c, cytosolic; h, liver homogenate; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; UV, ultra-violet spectroscopy; EC-GLC, electron capture detection–gas-liquid chromatography; GLC-MS, gas-liquid chromatography–mass spectrometry; LSC, liquid scintillation counting; LC, liquid chromatography.

the reaction of styrene oxide with proteins is high relative to the epoxide not extracted by a single partition. If reaction of the substrate with glutathione and proteins can be kept to a minimum, one can use a single partition, as described below for the stilbene oxides. When the diol must be extracted to minimize background, it is best to avoid ethyl acetate even though it often seems to possess the optimum polarity for extraction of diols. Ethyl acetate is somewhat toxic and is a quenching agent, which necessitates evaporation before liquid scintillation counting. Long-chain alcohols such as hexanol or pentanol do not lead to serious quenching problems and can be added directly to scintillation solutions, as discussed below. Of greater complexity would be a systematic study of the specificity of both cytosolic and microsomal epoxide hydrolases as well as glutathione transferases for substituted sytrene oxides. Undoubtedly, better substrates that are less volatile and reactive could be found.[88] A major advantage of styrene oxide or less volatile styrene oxide homologs may be that they are hydrated by a variety of epoxide hydrolases, and thus can be used to monitor total epoxide hydration.

6.4.3. Assays with Arene Oxides, Steroid Oxides, and Related Compounds. The work by Schmassmann et al.[248] illustrates the application of the partition assay to a particularly difficult separation when benzo(a)pyrene 4,5-oxide (**1h**) was used as substrate. These workers decreased the polarity of the aqueous solvent by adding 50% dimethylsulfoxide so that the majority of the diol remained in the aqueous phase following multiple extractions with petroleum ether. In the case of arene oxides, the problem of extraction is further complicated by the rearrangement of the epoxides to yield phenols of intermediate polarity. The diol was then extracted with ethyl acetate, as in the case of styrene glycol. This method gives a very low background and is a good illustration that maximum sensitivity is obtained when the majority of the substrate is extracted from the aqueous phase, even if it means a substantial loss of diol.

This basic method of decreasing the polarity of the aqueous phase with a co-solvent has been applied to a number of different epoxides including arene oxides[83] and steroid epoxides.[86,87] This

work illustrates that a partition method can be applied even when there is only a minor difference between the partition coefficient of the substrate and the product, and the method can also be modified slightly to monitor glutathione conjugates.[253] If a large number of samples are to be processed, even a procedure involving multiple extractions can be time efficient.

As with the styrene oxide assay, some minor modifications can lead to improvement. For example, petroleum ethers are often somewhat more polar than normal alkanes, and thus pentane, hexane, or a higher alkane may give slightly better differential extraction of the diol and epoxide. With substrates that readily alkylate proteins, extraction of the diol from the aqueous phase may be necessary, but for those that do not alkylate proteins readily, addition of methanol as a cosolvent and direct counting of the aqueous phase speeds the analysis. One obviously should use assay conditions that minimize the rearrangement of arene oxides and maximize enzyme activity.

Most workers think of partition assays as applicable only to the use of radiolabeled substrates, but Hukkelhoven et al.[254] describe a sensitive partition assay based on the fluorescence of the 4,5-*trans*-dihydrodiol of benzo(a)pyrene. These workers use a slight modification of the partition method developed for the radiochemical assay and then monitor the diol in the aqueous phase by its fluorescence. The assay is sensitive enough to measure hydrolase activity in hair follicles, and it nicely illustrates that the partition method can be applied to any material that has a large extinction coefficient or is fluorescent, especially if there is a difference in the spectrum of the substrate and product. It also shows that a partition can serve as a quick cleanup step before a chromatographic or spectrophotometric assay. It is important that volatile solvents are completely removed prior to reading a change in absorbance.

6.4.4. Assays with Juvenile Hormone. Unless one is interested in insect hormones, there is little need to assay epoxide hydrolase using insect juvenile hormone (**6,7**) or its synthetic mimics (juvenoids). However, three radiolabeled juvenile hormones are available commercially, the compounds are nice models for trisubstituted

epoxides, and the assay illustrates a slightly different approach to partition assays which is discussed further in subsequent sections. In this and subsequent assays a hydrocarbon solvent such as isooctane or dodecane is used to extract the epoxide from the incubation mixture while methanol is added to increase the amount of the diol remaining in the aqueous phase by decreasing its polarity. The work by Mumby and Hammock[247] is valuable in that it illustrates the power of a cosolvent to dramatically alter partition characteristics of substrates and products. For example, a hydrocarbon solvent will extract quantitatively juvenile hormone I diol from aqueous solution. If 20% methanol is added this level drops to about 95%, and at 80% methanol it is under 5%. This work demonstrates how one can optimize a partition assay for a particular substrate and analytical problem. In terms of studies with vertebrate systems, juvenile hormones are interesting in that they are hydrated almost exclusively by the cytosolic form of the epoxide hydrolase. Although they are turned over slowly, these trisubstituted epoxides demonstrate very low apparent K_m values.

6.4.5. Assays with β-Alkylstyrene Oxides. It was noted previously that *trans*-β-methylstyrene oxide (**10**) appeared to be a poor substrate for the microsomal epoxide hydrolase;[9] however, it is a good substrate for the cytosolic epoxide hydrolase.[16,79] As the alkyl chain of 1,2-disubstituted epoxides is increased, the speed of hydration catalyzed by the cytosolic epoxide hydrolase increases.[79,91] Thus, *trans*-β-ethyl (**1f**) and propylstyrene oxides give high rates of hydration with the cytosolic epoxide hydrolase and minimal hydration with the microsomal epoxide hydrolase. Mullin and Hammock[202] developed a simple procedure for radiolabeling these materials by reducing the corresponding haloketone with tritiated sodium borohydride. The assay consists of adding substrate to the enzyme mixture, then terminating the reaction by vigorous mixing with a hydrocarbon solvent which extracts the epoxide.

Detailed procedures for performing the assay are given in reference 202, and the assay offers some particular advantages. Although some substrates appear to be hydrolyzed by the cytosolic epoxide hydrolase slightly faster than the *trans*-β-alkylstyrene oxides, the alkylstyrene oxides demonstrate very favorable parti-

tioning characteristics, are easy to radiolabel, and are turned over very quickly by the enzyme (but do not show exceptionally low K_m values). They have proven exceptionally valuable in this laboratory for studying the inhibition of the cytosolic epoxide hydrolase and for a variety of experiments in which very sensitive assays are needed. The assay also has been used for the purification of the cytosolic epoxide hydrolase[30] and for the characterization of epoxide hydrolases from insects and mites.[255]

In spite of its many attributes, several limitations have led us to make stilbene oxide based assays available to other laboratories for routine use rather than the alkylstyrene oxide assays. Neither the radiolabeled nor the unlabeled standards are commercially available. Although neither synthesis is particularly difficult, and detailed procedures are published,[202] the synthetic procedures require several steps and some skill in organic synthesis. The synthetic methods used yield either pure *cis* epoxides or a mixture of cis and trans isomers which must be separated by a chromatographic system of moderate efficiency. For someone not familiar with radiosynthesis this separation is tedious and could lead to the contamination of valuable equipment. The reduction of haloketones with commercially available tritiated sodium borohydride does provide a general, high-yield method for the synthesis of a variety of epoxides, as illustrated by alkylstyrene oxides, the stilbene oxides discussed below, the three commercially available juvenile hormones, and the radiosynthesis of 4-nitrostyrene oxide.[30] Since the bond between tritium and carbon is not broken during hydration, secondary isotope effects should be minor, and even can be used to investigate the mechanism of epoxide hydration. The *trans* epoxides are excellent substrates for the mammalian cytosolic epoxide hydrolase and glutathione S-transferases. However, the *cis* epoxides are very poor substrates for all three major epoxide metabolizing systems in vertebrate species that have been studied in some detail. Thus approximately half of the radiolabel prepared is of little use to the average investigator. When compared with styrene oxide, the alkylstyrene oxides present minimal difficulties with regard to volatility (and mutagenicity); however, the compounds are sufficiently volatile to necessitate the use of covered reaction vessels for long incubations and extreme care (and

vigreux condensers) for concentrating the epoxide. Although very stable when compared to styrene oxide, *trans*-β-ethylstyrene oxide is not as stable in aqueous solution as the stilbene oxides.

6.4.6. Assays with Stilbene Oxides. The stilbene oxides were among the first substrates used to monitor the hydration of epoxides.[240,256] They have been used as model compounds by chemists to study the chemistry of epoxidation as well as the reactions of epoxides. Thus there are many synthetic methods published for these compounds, including syntheses of optically active materials.[257] Based upon separation of the products by GLC, it was noted that CSO (**1b**) and TSO (**1c**) were selective substrates for the microsomal and cytosolic epoxide hydrolases, respectively.[11,167] Mullin and Hammock[202] proposed that the stilbene oxides could be radiolabeled readily from a commercially available starting material (2-chloro-2-phenylacetophenone, desyl chloride) by the same pathway that was used for the β-alkylstyrene oxides. Based on this work, two partition assays have been developed. Oesch and Golan[93] report an assay analogous to those previously described for styrene oxide and other substrates in Section 6.4.3., based on a 2,3-tritiated substrate synthesized from *N*-tosylimidazole.[258] Gill et al.[162] describe a partition assay analogous to the procedures described in Section 6.4.5 and similar to those in Table 5.

No assay is optimal for all applications, and new and better substrates are continually evolving. However, for those applications in which measurement of the amount of product formed at a single time point is desirable, the CSO/TSO based radiochemical assays appear generally applicable. One major advantage is that CSO may be used to monitor the microsomal epoxide hydrolase selectively, and TSO for the cytosolic epoxide hydrolase. A further advantage is that both substrates can be used to monitor the glutathione S-transferase catalyzed conjugation of epoxides. However, one must be careful to control for such conjugation as a competing pathway. It is convenient that compounds having similar partition characteristics and other chemical properties can be used to monitor the major epoxide metabolizing systems now known in vertebrates. By taking advantage of the selective hydration of CSO and TSO and the different pH optima of the major epoxide me-

tabolizing enzymes, it is possible to monitor both the microsomal and cytosolic enzymes in crude homogenates, such as the "S9" systems used in some mutagenicity assays and even in whole cells. By using similar compounds of lower volatility, one could extend this concept to the examination of epoxide metabolism *in vivo*.

trans-Stilbene oxide is hydrated at roughly 6% of the rate of *trans*-β-ethylstyrene oxide (**1f**) in the cytosol from mouse liver.[79] Nevertheless, TSO is adequate for monitoring epoxide hydrolase activity even in species or tissues that have very low activity. The sensitivity of the assay depends in part on the stability of the substrate over a wide range of pH's and its lack of volatility in aqueous systems. The apparent K_m is lower as compared with alkylstyrene oxides, thus some inhibitors are less potent when a TSO assay is used.

cis-Stilbene oxide is hydrated at almost three times the rate of styrene oxide (**1d**) in microsomes from mouse liver. Depending upon the species, it is hydrated at 50–100% of the rate of benzo(a)pyrene 4,5-oxide (**1h**) when each substrate is assayed at the same concentration. *cis*-Stilbene oxide is superior to styrene oxide for selective monitoring of the microsomal epoxide hydrolase in the presence of the cytosolic form, but somewhat inferior to benzo(a)pyrene 4,5-oxide. However, by using selective pH's for the assays, CSO is adequate in this regard for most applications. Though one must be cautious whenever using model substrates, the epoxide of CSO is alpha to aromatic rings, as in arene oxides and styrene oxide, and the hydrolase activity active on CSO is precipitated by an antibody raised against the microsomal epoxide hydrolase that metabolizes styrene oxide and benzo(a)pyrene 4,5-oxide. It appears that CSO may be hydrated by enzymes present in certain tissues that are different from the classical microsomal epoxide hydrolase. Thus, by comparing ratios of benzo(a)pyrene 4,5-oxide hydration to CSO hydration, one can generate evidence for new isozymes (D. N. Loury, unpublished results).

Most of the advantages of CSO and TSO are common to both compounds. They are stable when stored, even when labeled at very high specific activity. Both are also stable in a variety of solvents, including water. When impurities form, they can be removed by partition, simple chromatography, or even recrystallization. The

compounds are poor alkylating agents which, it is hoped, means minimal health risk. Poor capacity for alkylation of proteins means that one seldom must extract the diol product of CSO and TSO from the aqueous phase to remove it from radioactive protein adducts. Since the compounds are relatively nonvolatile, long assay periods can be used. Although the partition characteristics are not as good as those of styrene oxide or the small alkylstyrene oxides, they are far superior to larger compounds such as benzo(a)pyrene 4,5-oxide and the juvenile hormones. Thus a single extraction is usually sufficient to remove unreacted epoxide and reduce the background to acceptable levels. Since there are alternate ways to monitor the hydration of CSO and TSO based upon TLC and GLC,[79,93,256,259] as well as a spectrophotometric assay for TSO,[260] it is possible to verify the partition assay by independent methods.

From a practical standpoint, both unlabeled substrates are available commercially and can be purified easily by crystallization. The *meso* diol also is available, and both the *meso* and *threo* diols can be made readily from the epoxides and separated by liquid chromatography.[162] Furthermore, there are numerous published synthetic pathways to the stilbene oxides which can be adapted for the fascile synthesis of carbon or tritium labels in a variety of locations.[261] Reduction of desyl chloride with tritiated sodium borohydride gives quantitative incorporation of tritium in a synthesis that requires no specialized equipment and can be carried out in a single sealed vessel. Using this procedure and commercially available borotitride one can label CSO and TSO at over 15 Ci/mmol. Since CSO and TSO can be separated readily by TLC, there is no need to contaminate expensive glassware or equipment in the synthesis and purification. Thus, if radioactive CSO and TSO are not commercially available, they can be synthesized readily with a reasonable specific activity. Although the synthesis is trivial, it should be done in a laboratory suited for the safe handling of moderate levels of tritium.[162]

As discussed in Section 2.2, CSO can be used to monitor the low levels of epoxide hydrolase activity in serum. It is thought that this epoxide hydrolase activity is contributed by PNA. Since there is some interest in correlating PNA levels in the serum with the

progression of hepatocellular carcinoma (and possibly other disorders), a procedure for this assay is provided in Table 5.

Several attributes of CSO make it particularly suitable for such an assay. Of prime importance is that it can be radiolabeled readily at very high specific activity. High specific activity allows one to use small amounts of serum, relatively low substrate concentrations to increase the percentage conversion to diol, and high levels of radioactivity so that even small levels of hydration can be detected. Its hydrolytic stability and lack of volatility allow one to increase the sensitivity of assays still further by incubating for long periods of time.

The utility of the PNA assay for the possible early detection of cancer in both man and experimental animals needs to be evaluated; the CSO assay is promising in this regard. However, the assay only detects enzymatically active PNA, whereas an immunoassay[64] may detect denatured enzyme as well. Thus this radiochemical assay is best used in conjunction with an immunoassay.

6.4.7. Designing a Partition Assay. As described in Section 6.4.1, one first must consider what assay method is most suitable for the problem. If the partition method appears suitable, the next step is to insure the chemical and radiochemical purity of the epoxide substrate and diol metabolite. One must then make certain either that the diol is the only metabolite formed in the assay system or that steps are taken to selectively extract the diol. For instance, with esters such as the juvenile hormones or methyl epoxystearates, inhibitors like *O,O*-diisopropylphosphorofluoridate (DFP) must sometimes be used to inhibit esterases. In this laboratory the glutathione present in liver homogenates is largely oxidized during the preparation of subcellular fractions. However, in several species, and when the conditions for enzyme preparations are slightly changed, significant problems are encountered with glutathione conjugation. The glutathione can be removed by either dialysis or gel permeation chromatography, or it can be depleted by compounds such as chalcone and diethyl maleate. Obviously, one must insure that such treatments do not inhibit the enzyme activity to be assayed.

The partition method offers a way to enrich a sample using a relatively nondestructive procedure and to assay rapidly a large number of samples. If the goal is to perform a number of point assays rapidly, one must next consider the difficulty of the separation and the number of samples to be run. If the number is relatively small and the separation is likely to involve several partition steps, a chromatographic system may prove more cost effective. Thus an early step is to determine the partition coefficient P of the epoxide and diol.

The next problem to consider is the sensitivity of the assay. Sensitivity, to a large degree, depends on minimizing background. This background consists largely of unextracted epoxide and reaction products of the epoxide with water and biological nucleophiles. Thus, if one has 10% epoxide left in the aqueous phase following a single partition, but 1% following three partitions, one must decide whether the speed of the assay or its sensitivity is most important. For most applications in this laboratory, a chromatographic method is used if multiple extractions are required for a partition assay to work.

A large number of solvents are potentially suitable for developing partition assays,[245] but we have settled on a very limited quantity. For the organic phase we routinely use a hydrocarbon solvent. A disadvantage of such solvents is that they constitute the hyperphase (upper), while a halogenated solvent would be the hypophase (lower). With a halogenated solvent one does not have to sample through the phase with a large amount of epoxide to detect a small amount of diol. Unfortunately, with a few exceptions,[222] carbon tetrachloride yields poor differential partitioning and does not form biphasic systems with methanol or acetonitrile. Furthermore, halogenated solvents are quenching agents in liquid scintillation counting and necessitate an evaporation step if both phases are to be counted. At early stages in the development of a partition assay it is useful to count both phases. We routinely only sample the phase containing the diol, but sampling both phases reduces the variability of the assay.

If the partition step is to be used for the removal of impurities, a volatile solvent such as pentane is utilized. If multiple extractions are used, petroleum ether represents an inexpensive alternative.

For a single extraction, a nonvolatile solvent such as isooctane or dodecane simplifies quantitative analysis of both phases and is relatively inexpensive. One can use a wide variety of such hydrocarbon solvents and experience only very minor changes in the distribution of the epoxide and diol, unless the solvents are contaminated with other solvents of increased polarity. If the polarity of the hyperphase must be increased, one can add a small amount of a variety of relatively nonvolatile, aromatic solvents such as cymene, diisopropyl or *t*-butylbenzene, mesitylene, pentamethylbenzene, or xylenes.

In metabolism chemistry, the formation of two-phase systems with acetonitrile, dimethylsulfoxide, or methanol in the hypophase and an aliphatic hydrocarbon as the hyperphase is commonly used for the partial purification of a metabolite or reaction product. Even relatively nonpolar materials may be retained in the hypophase by employing a high proportion of methanol. These materials may then be driven back into the hyperphase by increasing the proportion of water and/or salt. In practice we tend to avoid the use of acetonitrile or dimethylsulfoxide. Acetonitrile is not stable on long storage and must be repurified, and it will quench some liquid scintillation solutions. Dimethylsulfoxide may lead to quench and often results in liquid scintillation solutions separating into two phases. High salt concentrations are avoided because they also result in biphasic systems with many cocktails. We routinely use methanol in the hypophase, although propylene or ethylene glycol may be substituted. However, the minor differences in the partition characteristics of a variety of solvents can potentially be exploited to enhance the partition characteristics of the system when a difficult separation is encountered.

Figure 2 shows how increasing the amount of various cosolvents in the aqueous phase changes the partitioning characteristics of diols and epoxides (nonpolar phase = dodecane). One selects the amount of cosolvent that results in an optimal amount of epoxide in the hyperphase and an optimal amount of diol in the hypophase. With the advent of the hand calculator, one can tolerate relatively incomplete separations if high background is acceptable, since factors to correct for these incomplete separations can be included in equations. If the diol needs to be extracted from alkylated materials

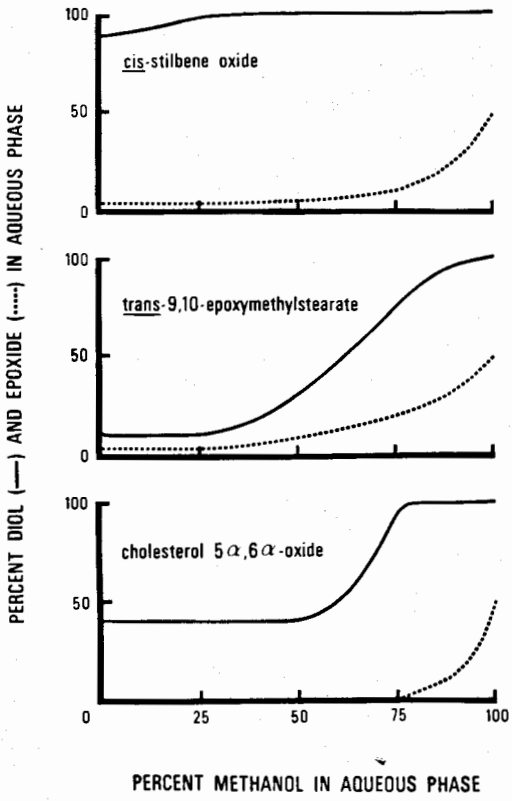


Figure 2. Illustration of the methodology used to optimize a partition assay.

in the hypophase to minimize background or to evaluate the amount of conjugation, aliphatic alcohols are a good choice as a solvent. These solvents extract even the most polar diols, but leave most glutathione conjugates. They demonstrate minimal quench upon liquid scintillation counting. More exotic solvents may prove advantageous, but a pragmatic approach would be to monitor the partition of the epoxide and diol of interest between dodecane and a buffer containing increasing amounts of methanol. The optimum methanol concentration depends, in part, on the analytical question at hand. The ratio of the volumes of hypophase and hyperphase can be optimized according to theory,[245] but for a single partition system, we normally use an excess of hyperphase.

Once conditions are satisfactorily optimized, one must then develop an equation to describe the partitioning behavior. It is important to remember that background for the assay can be divided into time-independent factors such as the amount of epoxide left in the aqueous phase, impurities in the epoxide, and the background of the liquid scintillation system, as well as time-dependent factors including nonenzymatic hydrolysis and alkylation. For routine assays such background values can be determined from one or two controls, but for sensitive assays multiple controls are needed. The assay should then be checked by adding known ratios of epoxide and diol as well as by monitoring the rate of enzyme hydrolysis by partition and an independent method.

Numerous factors can perturb the distribution of epoxide and diol. For assays in which the epoxide and diol have very different solubilities, conditions can be such that minor changes in the polarity of the aqueous phase have little effect on the partition assay. As the polarities of the epoxide and diol become more similar, the conditions chosen for the partition assay are critical. Thus simple partitions such as those for styrene oxide or TSO are remarkably forgiving, whereas complex assays are more dependent on assay conditions. Obvious factors to check include the buffer type and ionic strength, the solvent used for addition of substrate, and lipid and protein concentrations.

When using hydrocarbon solvents it is important to remember that they penetrate aqueous systems poorly so that it is necessary to agitate the reaction mixture vigorously. Narrow conical tubes may retard mixing, and it is quite possible to mix such systems with a vortex without having adequate extraction if the hyperphase simply is spun above the hypophase. Also, if the cosolvent concentration is low, addition of a hydrocarbon may not destroy the enzyme. Thus the reaction does not end until the substrate is extracted from the aqueous system. If low protein concentrations are used, centrifugation is usually not necessary; however, routine centrifugation is often advisable, since even small amounts of hydrocarbon micelles in the aqueous system can increase background.

One usually thinks of partition assays involving a radioligand, but Watabe and Akamatsu[240] demonstrate an excellent photometric method using safrole oxide [1-(3′,4′,-methylenedioxyphenyl)-2,3-

epoxypropane, **19**] as substrate. Detailed synthetic methods are given for the substrate, which appears to be very stable in aqueous solution and much less volatile than styrene oxide, and this work provides an excellent description of the experimental method. One must be careful of increased volatility in the light beam of the spectrophotometer in such assays. The reaction is terminated by the addition of hexane, followed by extraction of the substrate and determination of its absorbance at 288 nm. The major limitation of the method is that it is a substrate disappearance method, which does not distinguish among competing reactions and is inherently less sensitive than a product appearance assay. In cases where the product has a large molar absorption coefficient or an absorbance at wavelengths distinct from interfering materials, it is especially important to monitor the increase in absorbance of the aqueous phase rather than the decrease in absorbance of the hydrocarbon layer. One can also extract the diol from the aqueous phase to remove interfering materials and glutathione conjugates. As Watabe and Akamatsu[240] noted, the solvents that extracted the diol effectively also interfered with UV analysis. Therefore, one would need to evaporate these solvents before analysis or else use long-chain alcohols, as described above, which extract diols efficiently and neither quench in liquid scintillation counting nor absorb UV light. Such a partition method can also be used to greatly increase the sensitivity of some of the continuous assays described below, which are based on the difference in the spectrophotometric properties of the diol and the epoxide.

6.5. Continuous Spectrophotometric and Fluorometric Assays.

As discussed in Section 6.1, continuous assays of enzyme activity offer marked advantages for studying enzyme kinetics, and they are often much less expensive than radiochemical assays. However, improvements in spectrophotometric assays are needed for both the cytosolic and microsomal epoxide hydrolases. The criteria for a good substrate for continuous assays are similar to those discussed in Section 6.4. In addition, it is advantageous to have an epoxide and diol that differ greatly in their absorbance or fluores-

cence spectra. If there is a major difference in the visible region of the spectrum, less expensive instrumentation is needed, the assay can be adapted easily to monitoring epoxide hydrolase activity on native gels (see discussion given below), and there is less chance of interference from biological materials and UV absorbing compounds in the assay. The earliest spectrophotometric assay for epoxide hydrolase activity was described by Watabe and Akamatsu[240] using safrole oxide (**19**). Since the assay was based on differential partitioning and is not a continuous assay, it is discussed in Section 6.4.7.

Fjellstedt et al.[262] describe an assay for glutathione S-transferase based on an increase in absorbance at 360 nm with 1,2-epoxy-3-(4′-nitrophenoxy)propane, **11** (4-nitrophenyl glycidyl ether, see Section 6.3.2) used as a substrate. This assay should be applicable to monitoring epoxide hydrolase activity. Unfortunately, the glycidyl ethers so far examined are not particularly good substrates for either the cytosolic or microsomal epoxide hydrolases,[9,79] and some glycidyl ethers tend to be electrophilically reactive, as evidenced by their use in the transferase assay.[262] This could lead to the inhibition of the cytosolic epoxide hydrolase as well as a high background due to the reaction of sulfhydryls. Dansette et al.[84] examined 4-nitrophenoxy glycidyl ether as a substrate for the microsomal epoxide hydrolase and found it unsuitable because the spontaneous rate of hydrolysis approached that of the enzymatic rate. Glycidyl ethers can be made by the reaction of alcohols or phenols with epichlorohydrin or bromohydrin or by the analogous reaction with an allyl halide followed by epoxidation. Since the synthesis of glycidyl ethers is so simple, perhaps compounds with improved spectral and chemical properties that are good substrates can be found.

With all fluorometric and spectophotometric assays for the microsomal enzyme, one must be careful to minimize or correct for light scatter. Dansette et al.[84] developed an excellent fluorometric assay for the hydration of several arene oxides based on the large difference in both the excitation and emission spectra of the oxides and the corresponding diols. Benzo(a)pyrene 4,5-oxide (**1h**) proved to be a particularly good substrate because of its low rate of spontaneous hydrolysis. This method offers numerous advantages, with

its general application to a variety of substrates of toxicological interest being of prime importance. The assays are very sensitive, and fluorometric assays are less subject to interference than absorbance based assays, since two wavelengths are being used. Fluorometric assays are limited in that most fluorimeters accept only a single sample at one time, and it is best not to expose the substrate continuously to intense UV light. However, with a "slurper" system one could process a large number of samples with great speed. As discussed in Section 6.4, Hukkelhoven et al.[254] described a very sensitive point assay based on the concepts outlined above. This work illustrates that the sensitivity of assays based on difference spectra can be greatly increased by partitioning the substrate and product.

An analogous assay was developed by Armstrong et al.[82] based on the UV difference spectrum of phenanthrene 9,10-oxide (**1g**) and its diol. It is not as sensitive as the fluorimetric assays, but uses less complex equipment. Phenanthrene 9,10-oxide is a good substrate for the microsomal epoxide hydrolase and a very poor substrate for the cytosolic form. It has the advantage of being relatively water soluble for an arene oxide. Armstrong et al.[82] discuss some of the problems involved with the use of a lipophilic substrate in detergent micelles.

The synthesis of substrates suitable for the spectrophotometric analysis of epoxide hydrolase activity has been attempted in several laboratories and the frustration encountered is illustrated by Hanzlik and Hilbert[263] in their synthesis of styrene oxides with potential leaving groups in the alpha position. These compounds are excellent chromophores following hydration, because the diols rearrange to yield conjugated ketones. Unfortunately, with the possible exception of 4-nitro-α-acetoxystyrene oxide, the epoxides proved to be far too unstable in water for convenient use. Possibly this approach can be extended to yield compounds of appropriate stability. Based on this work, Westkaemper and Hanzlik[264] developed a continuous spectrophotometric assay for the microsomal epoxide hydrolase using 4-nitrostyrene oxide. This substrate is nonvolatile and moderately stable in aqueous solution. Using 4-nitrostyrene oxide, these workers employed the integrated form of the Michaelis–Menten equation to obtain kinetic information on a variety

of substrates very rapidly, thus illustrating a further advantage of continuous assay. A nice comparison of the use of this substrate and styrene oxide for the assay of epoxide hydrolase was presented by Hanzlik and Walsh[89], where they demonstrated that 4-nitrostyrene oxide had both a lower K_m and V_{max} with hepatic, microsomal epoxide hydrolase from chickens.

A similar procedure was employed by Hammock and Hasagawa[260] to develop a continuous assay based on the difference in the UV spectra of TSO (**1c**) and the corresponding meso diol and glutathione conjugate. As with the partition assay, this assay is quite selective for the cytosolic form of the enzyme. The assay has proven quite useful, but illustrates some problems that are best avoided in a continuous assay. The substrate is not soluble in the millimolar range, thus a limited range of substrate concentrations are available for kinetic experiments. The assay uses the UV region of the spectrum, which requires an expensive lamp and sometimes results in interference from the absorbance of proteins and inhibitors. Also, the assay is based on substrate disappearance rather than product appearance, which limits the sensitivity of the assay and requires the use of a good spectrophotometer. Thus one must be able to examine 0.1 absorbance units full scale against a background of over 2 absorbance units if the assay is to reach its full potential.

The most general continuous assay for epoxide hydrolase activity was described by Guengerich and Mason.[81] This assay takes advantage of the reduction of NAD^+ by some diols when catalyzed by alcohol dehydrogenase. The assay has been described in some detail as applied to styrene oxide (**1d**) and 1,2-epoxyoctane (**1a**)[12,81] but can be applied to a variety of compounds. Although characterized for the microsomal enzyme, it is applicable to the cytosolic form as well. However, proper use of the assay requires that numerous controls be run.

Another application of colorimetric methods is in the specific detection of epoxide hydrolases on native gels. Such specific stains are valuable in comparing enzymes from various species and strains and also are needed in the search for multiple forms of epoxide hydrolase.

6.6. Immunochemical Methods for Measuring Enzyme Levels

The successful purifications of both the microsomal and cytosolic epoxide hydrolases (see Section 3.6) have led to the development of several useful immunochemical techniques for studying epoxide hydrolases. Immunochemical methods are valuable for distinguishing the causes of observed interindividual or interspecies differences. Whereas routinely used enzymatic activity assays only measure the levels of catalytic activity present, immunochemical procedures allow one to determine the actual levels of enzyme protein that are present. Thus one can differentiate between qualitative and quantitative differences. For instance, Thomas et al.[265] used a radial immunodiffusion assay to demonstrate that the interindividual differences in the rate of hydration of octene oxide in liver microsomal samples from 11 human livers was due to differences in the level of epoxide hydrolase protein present, and was not the result of different levels of endogenous modulators of epoxide hydrolase activity. Similarly, Oesch et al.[195] used an immunoprecipitation technique to show that differences in styrene oxide hydration rates in liver microsomes from 22 strains of rats were correlated with the levels of epoxide hydrolase protein present in the various strains. Gill et al.,[64] using an enzyme linked immunosorbent assay for microsomal epoxide hydrolase from rat liver, found similar levels of enzyme protein in rats and mice, though they observed a significantly higher level of catalytic activity in rats. Immunohistochemical techniques have been employed to examine the distribution of the microsomal form of epoxide hydrolase within the liver lobules of untreated rats;[266–268] 2-acetylaminofluorene-treated rats;[53,269] and phenobarbital-, TSO-, and 3-methylcholanthrene-treated rats.[134] Both unlabeled antibody peroxidase–antiperoxidase[266] and indirect fluorescent antibody-staining techniques[268] have been described. As mentioned in Section 2.2 and illustrated by the work of Griffin and Gengozian,[270] immunoassays have also been used for measuring the levels of PNA.

The immunochemical methods vary considerably in sensitivity and ease of use. Enzyme immunoassays such as the enzyme-linked immunosorbent assay procedure offer 10- to 100-fold greater sen-

sitivity than the radial immunodiffusion, "rocket" immunoelectrophoresis, or immunoprecipitation techniques, and have the added advantages of shorter analysis time and ease of use. Present methods for enzyme immunoassay or radioimmunoassay can be modified by the researcher to further enhance their sensitivity. A number of helpful references in this area have been published.[271–273] Also of note is the recent development in several laboratories of monoclonal antibodies against the microsomal epoxide hydrolase,[176,274] which may be useful in distinguishing closely related forms and in a variety of other procedures.

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