

Review Article

Mammalian Epoxide Hydrases: Inducible Enzymes Catalysing the Inactivation of Carcinogenic and Cytotoxic Metabolites Derived from Aromatic and Olefinic Compounds

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1. Several aromatic and olefinic compounds are converted to intermediate arene and alkene oxides by mammalian mono-oxygenases. Intermediate arene oxides rearrange non-enzymically to phenols. Arene and alkene oxides are converted by epoxide hydrases to vicinal diols and by glutathione *S*-epoxide conjugases to glutathione conjugates. Due to their high electrophilic reactivity, such oxiranes also bind to proteins, RNA and DNA. Mutagenic, carcinogenic and cytotoxic effects of several aromatic and olefinic compounds appear to be due to the formation of intermediate epoxides and their reaction with tissue constituents. Whether a given aromatic or olefinic compound produces such an effect would thus depend on a variety of factors, such as the relative rate of formation and degradation of the intermediate oxirane, on its stability with respect to spontaneous isomerization to the corresponding phenol and on its chemical electrophilic reactivity.

2. Epoxide hydrases, which convert such intermediate oxiranes to much less reactive vicinal diols, have been studied in greater detail. Epoxide hydrase activity is found in mouse, rat, guinea-pig, rabbit, pig, Rhesus monkey and human liver. Activity is high in liver, low in kidney, very low in intestine and lung and not detectable in muscle, spleen, heart and brain. The enzyme is located exclusively in microsomal membranes. Epoxide hydrase activity is markedly increased after pretreatment of rats with phenobarbital or 3-methylcholanthrene and during maturation of rats. These increases are reminiscent of similar increases in microsomal mono-oxygenases. However, the extents of induction of *total* levels of these two enzymes or enzyme families are not comparable and are under separate genetic control.

3. Several stereochemical properties of the reaction catalysed by epoxide hydrases have been studied with microsomal preparations. With styrene oxide and naphthalene oxide as substrates, attack by $H_2^{18}O$ occurs virtually exclusively at the 2-position. Product glycols which are stereochemically fixed by a ring structure invariably have the *trans*-configuration. Hydration of some acyclic alkene oxides has also been found to proceed via a *trans*-opening of the oxirane-ring. Cyclohexene oxide, benzene oxide and naphthalene 1,2-oxide are converted predominantly to 1*R*,2*R*-*trans*-diols, while in the case of phenanthrene 9,10-oxide the 1*S*,2*S*-*trans*-diol predominates.

4. Epoxide hydrase from guinea-pig liver microsomes was solubilized and purified, based on an assay with styrene oxide as substrate. The specific activity after the last purification step is about 40 times higher than in the crude homogenate. This increase is not due to the removal of an inhibitor. About 30% of the activity of the purified preparation is lost within 1-2 days. However, the remaining activity is remarkably stable. Gel electrophoresis of the final (stable) preparation

shows one major band corresponding to a mol. wt. of approx. 50 000. However, several minor bands are also present.

5. Several properties of epoxide hydase were investigated with this purified preparation. While no clearcut pH optimum could be observed with microsomal preparations (broad 'optimum' between 7 and 9) a sharp pH profile was obtained with the purified preparation with its optimum at pH 9. Non-enzymic hydration was significant (>5%) only below pH 6.5. The K_M with respect to styrene oxide as substrate is $2.8 \times 10^{-4} M$ and the apparent V_{max} 2-4 μmol product/mg N per 5 min. No metal ions or other low mol. wt. co-factors are necessary for maximal activity. High concentrations of substrate inhibit the enzyme, whereas product diols have no effect. Several inhibitors of drug-metabolizing enzymes (SKF 525-A), piperonyl butoxide, α -naphthoflavone) do not influence epoxide hydase activity, while sulphhydryl reagents slightly, but significantly, inhibit the enzyme. Several alcohols, ketones and imidazoles stimulate the enzyme. Kinetic analysis of the activation by the potent stimulator, metyrapone, indicates negative co-operativity with the substrate.

6. The active site of the enzyme readily accommodates, as substrates or competitive inhibitors, monosubstituted oxiranes with a lipophilic substituent larger than an ethyl group, suggesting hydrophobic binding sites near the active site. With oxiranes having such a lipophilic substituent, the enzyme interacts with mono-, 1,1-di- and *cis*-1,2-disubstituted oxiranes, but not with *trans*-1,2-disubstituted oxiranes or tri- or tetra-substituted oxiranes, suggesting that increasing bulk around the oxirane ring prevents the approach of the oxirane to the active site. Several oxiranes fused to alicyclic rings (cyclohexene oxide, 1,2,3,4-tetrahydronaphthalene 1,2-epoxide) are potent inhibitors but very poor substrates. Kinetic analysis revealed non-competitive inhibition with respect to the substrate, styrene oxide. Styrene sulphide (an analogue of the competitive inhibitor, styrene oxide) but not cyclohexene sulphide (an analogue of the non-competitive inhibitor, cyclohexene oxide) has inhibitory activity, suggesting differing structural requirements for the sites involved in competitive and non-competitive inhibition. The most potent inhibitor discovered so far is 1,1,1-trichloropropene 2,3-oxide, which is on the other hand a poor substrate. Inhibition by this compound is of the un-competitive type. Structure-activity relationship for substrates and inhibitors of purified human epoxide hydase are qualitatively identical to the ones discussed above for the purified guinea-pig enzyme.

7. Evidence suggesting the presence of more than one liver enzyme capable of hydrating epoxides include differential stabilities, different ratios of hydase activity towards various epoxides in preparations from different species, a different purification factor (activity of the purified preparation compared to liver homogenates) towards benzene oxide as compared to several other epoxides, inability to inhibit hydration of styrene oxide (in purified or particulate preparations) with much higher concentrations of benzene oxide.

8. Evidence indicating the presence of a coupled mono-oxygenase-epoxide hydase multienzyme complex include the following observations. Substantial amounts of dihydrodiols in the urine of animals treated with aromatic hydrocarbons, despite the high instability of intermediate arene oxides, lack of equilibration between pools of naphthalene oxide formed *in situ* and of exogenous naphthalene oxide, differential inhibition of 'free epoxide hydases' at concentrations of 1,1,1-trichloropropene 2,3-oxide which do not affect the 'coupled mono-oxygenase-epoxide hydase system', selective induction of the coupled system by 3-methylcholanthrene, and high epoxide hydase activities in solubilized and purified cytochrome P-450 and P-448 fractions, where other microsomal enzymes (glucose-6-phosphatase, cytochrome c reductase) were absent. Such a coupled mono-oxygenase-epoxide hydase system may be of great relevance to problems such as carcinogenic properties of arene oxides derived from several polycyclic hydrocarbons, and hepatotoxicity of intermediate arene oxides derived from halobenzenes, by circumventing these adverse effects by their rapid conversion

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to dihydrodiols. Indeed, pretreatment of rats with 3-methylcholanthrene, which selectively induces this coupled system, provides protection from chlorobenzene-evoked hepatotoxicity, whereas pre-treatment with phenobarbital increases the toxic effect, although it induces the *total* level of epoxide hydrase to a much greater extent than 3-methylcholanthrene.

Introduction

For many years the mechanism(s) whereby chemically unreactive substances, e.g. aromatic hydrocarbons, exerted their carcinogenic or cytotoxic action has puzzled researchers. However, in recent years evidence has accumulated which shows that many of these compounds are converted enzymically to chemically highly reactive derivatives which act as the ultimate carcinogens or cytotoxins (for references see Shuster, 1964; Miller & Miller, 1966; Uehleke, 1971). Their high reactivity makes such ultimate agents often so unstable that proof of their formation is a difficult task. Boyland proposed the formation of arene oxides* from aromatic hydrocarbons and suggested their importance in carcinogenicity as early as 1950, but the first proof of the biological formation of an arene oxide was not realized until many years later (Jerina, *et al.*, 1968 b; 1970 b). Strong evidence has recently been put forward that such arene oxides are the causative agents for the carcinogenicity of (at least some) polycyclic aromatic hydrocarbons (Marquardt, *et al.*, 1972), and for the liver necrosis evoked by several halobenzenes (Brodie, *et al.*, 1971). These intermediate oxides rearrange non-enzymically to phenols, are metabolized to glutathione conjugates by glutathione S-epoxide conjugases and to vicinal diols by epoxide hydrases. The glutathione S-epoxide transferases are localized in the cytoplasm (Boyland & Chasseaud, 1969), are not inducible (E. Boyland, personal communication) and the glutathione S-epoxide conjugation reaction also occurs non-enzymically to a high extent (Boyland & Chasseaud, 1969; Jerina, *et al.*, 1970 b). The epoxide hydrases are localized in the endoplasmic reticulum, i.e. possibly near the site of formation of the arene oxides (Oesch, Jerina & Daly, 1971 a), they are inducible (Oesch, *et al.*, 1971 a) and non-enzymic hydration of arene oxides has not been observed (Vogel & Klärner, 1968; Jerina, *et al.*, 1968 b, 1970 a; Sims, 1971). These epoxide hydrases appear therefore to be especially worthy of detailed investigation.

Biological formation of oxiranes from aromatic and olefinic substrates

Arene oxides would be expected to be chemically very unstable because of the great gain of resonance energy obtained upon isomerization to the corresponding phenol. It is therefore not surprising that the first arene oxides became synthetically available only recently, when Newman & Blum (1964) prepared K-region oxides of phenanthrene and benz(a)anthracene, closing the oxirane ring from *bis*-aldehyde precursors with the aid of Mark's reagent, *tris*-dimethylaminophosphine. Soon afterwards arene oxides which were not of the K-region oxide type became synthetically available *via* dehydrohalogenation of cyclic haloepoxides (Vogel, Böll & Günther, 1965; Vogel & Günther, 1967). Jerina,

* Compounds which formalistically result from the epoxidation of one of the double bonds of an aromatic nucleus are called arene oxides. For those resulting from the epoxidation of an olefinic double bond, the term alkene oxide is suggested.

Boyd & Daly (1970 a) showed arene oxide formation by direct transfer of oxygen from photo-excited pyridine-*N*-oxide to naphthalene. This constituted the first example of chemical epoxidation of an aromatic double bond.

With the aid of synthetically available arene oxides it became possible to demonstrate their formation upon incubation of aromatic hydrocarbons with liver microsomes fortified with NADPH. Jerina, *et al.*, (1968 b, 1970 b) demonstrated the formation of ¹⁴C-naphthalene 1,2-oxide from ¹⁴C-naphthalene by trapping the radioactive oxide in a large pool of unlabelled synthetic naphthalene 1,2-oxide. Using short incubation times and low temperatures, they also succeeded, without using trapping techniques, in isolating by counter-current distribution a sufficiently large quantity of enzymically formed naphthalene 1,2-oxide to allow identification of this metabolite by chemical and chromatographic characteristics. Subsequently arene oxides were also reported as microsomal metabolites of several polycyclic hydrocarbons, namely dibenz-(a,h)anthracene (Selkirk, Huberman & Heidelberger, 1971), phenanthrene and benz(a)anthracene (Grover, Hewer & Sims, 1971 b), pyrene and benzo(a)pyrene (Grover, Hewer & Sims, 1972), and 7,12-dimethylbenz(a)anthracene (Keysell, *et al.*, 1972). Finally, formation of naphthalene oxide was demonstrated in reconstituted enzyme systems, consisting of solubilized and purified cytochrome P-450 or P-448, NADPH, cytochrome c reductase and lipid fraction (Oesch, *et al.*, 1972). Although no other examples are available to date, it may be concluded that in all likelihood the formation of arene oxides from aromatic hydrocarbons by the action of mono-oxygenases is a general phenomenon. However, attempts to trap radioactive benzene oxide formed from radioactive benzene with rabbit liver microsomes, in the presence of exogenous carrier benzene oxide have not been successful, possibly due to relatively great instability of benzene oxide caused by the gain of resonance energy through isomerization to a monocyclic aromatic compound. Thus, the recovery of added benzene oxide was very poor. Moreover, benzene is a poor substrate for the microsomal mono-oxygenases (Jerina, *et al.*, 1970 b). Isolation of alkylarene oxides after incubation of the corresponding aromatic hydrocarbons is also unfeasible, since arene oxides with alkyl substituents at the oxirane ring are not formed by hepatic microsomal mono-oxygenases (with the possible exception of mesitylene oxide) as demonstrated by comparing isomerization products of synthetic alkylarene oxides to microsomal metabolites of the parent hydrocarbons, whereas arene oxides with alkyl substituents at other ring positions are very unstable, rearranging spontaneously even at 0° (Kaubisch, Daly & Jerina, 1972).

Experiments using an epoxide substrate as a competitive inhibitor proved the naphthalene oxide to be the obligatory intermediate in the formation of dihydrodiol and glutathione conjugates (Jerina, *et al.*, 1970 b). The absence of deuterium in the 4-chlorocatechol and its *O*-methylated products produced by *in vivo* metabolism of chloro[4-³H]benzene demonstrated that catechols arise by dehydrogenation of dihydrodiols rather than by further hydroxylation of a phenol (Jerina, Daly & Witkop, 1967). Observation of dihydrodiols, catechols or glutathione conjugates (the latter with the exception of those formed by a direct replacement of a labile halo- or nitro-group: Booth, Boyland & Sims, 1961; Al-Kassab, Boyland & Williams, 1963; Clark, Darby & Smith, 1967) as metabolites of foreign aromatic compounds is therefore a good indication for the formation

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of an intermediate arene oxide. Such metabolites have been shown to be formed from several compounds of common concern, namely from carcinogenic aromatic hydrocarbons (e.g. dibenz(a)anthracene, Boyland & Sims, 1965 a; benzo(a)pyrene, Sims, 1967 c; 3-methylcholanthrene and 7,12-dimethylbenz(a)anthracene, Sims, 1970), from halobenzenes which are widely used as solvents and which can produce liver necrosis (e.g. chlorobenzene, Smith, Spencer & Williams, 1950; fluoro-, bromo- and iodobenzene, Azouz, Parke & Williams, 1953; Jollow, *et al.*, 1972; dichlorobenzenes, Parke & Williams, 1953), from pesticides (e.g. 2,6-dichlorobenzonitrile, Wit & van Genderen, 1966; Carbaryl, Sullivan, *et al.*, 1972), and from clinically used pharmaceuticals (e.g. diphenylhydantoin, Horning, *et al.*, 1971; Lorazepam, Schillings, Schrader & Ruelius, 1971; phenobarbital and mephobarbital, Harvey, *et al.*, 1972 a). Hydroxylation of deuterated zoxazolamine is the only reported case (Tanabe, *et al.*, 1970) which is not compatible with a hydroxylation mechanism proceeding via an intermediate arene oxide, since the rate of phenol formation appeared to be different whether hydrogen or deuterium was present at the position of hydroxylation. This suggests insertion of oxygen rather than addition. A preliminary report of isotope effects during hydroxylation of nitrobenzene has appeared (Daly, Jerina & Witkop, 1972). However, in all other investigated cases no such primary isotope effects could be observed during hydroxylation of substrates which were deuterated or tritiated at the appropriate position (Guroff & Daly, 1967; Tanabe, *et al.*, 1967; Perel, *et al.*, 1967; Daly & Jerina, 1969).

Several olefinic compounds are metabolized to alkene oxides* which are resistant enough to spontaneous and enzymic degradation so that they can be isolated as such without recourse to special techniques such as enzyme inhibition and/or radiotracer trapping. Examples are heptachlor oxide (Davidow & Radomski, 1953), dieldrin (Winteringham & Barnes, 1955; Bann, *et al.*, 1956), 16 α ,17 α -epoxy-estratriene-3-ol (Breuer & Knuppen, 1961), 1,4:2,3-diepoxy-1,2,3,4-tetrahydronaphthalene (Sims, 1965), endrin (Wong & Terrierre, 1965) and chlordane epoxide (Brooks, 1966). 1- Δ^8 -Tetrahydrocannabinol, the compound believed responsible for most of the psychopharmacological effects of marijuana (Gaoni & Mechoulam, 1964), allyl-substituted barbiturates, such as secobarbital, allobarbital and alphenal, sedatives of short, intermediate and long duration respectively, and carbamazepine, widely used as an antiepileptic agent or in the treatment of trigeminal neuralgia, are interesting examples of drugs which are converted to such alkene oxides, as demonstrated very recently (Gurny, *et al.*, 1972; Harvey, *et al.*, 1972 b; Frigerio, *et al.*, 1972). However, frequently glycols (and no epoxides) are observed as products arising from olefinic compounds by oxidative metabolism (Brooks & Young, 1956; El Masri, Smith & Williams, 1958; Boyland & Sims, 1960; Booth, *et al.*, 1960; Douglas, 1965; Waddell, 1965; Fukami, Yamamoto & Casida, 1967; Leibman & Ortiz, 1968, 1969, 1971; Fukami, *et al.*, 1969; Sisenwine, *et al.*, 1970). Until the late sixties the role of epoxides in the biological formation of glycols was not established. Glycols could conceivably arise by enzymic or spontaneous hydrolysis of intermediate epoxides or by direct dihydroxylation of an ethylenic moiety. Maynert, Foreman & Watabe (1970) showed that addition of a potential competitive inhibitor (4,5-epoxy-*n*-octane) of an enzymic hydration of an

* See footnote p. 3.

intermediate epoxide to a microsomal incubation mixture containing *n*-1-octene as substrate allowed isolation of a considerable quantity of 1,2-epoxy-*n*-octane. In the absence of the potential inhibitor only the corresponding glycol was formed. *n*-4-Octene was also converted to the corresponding glycol with no trace of epoxide in the absence of an excess of another epoxide, 1,2-epoxy-*n*-octane, but only to epoxide in its presence. This indicates that the epoxide is an obligatory intermediate in the conversion of *n*-4-octene to the glycol and that in many cases where glycols are observed as metabolites from olefinic compounds epoxides may be intermediates. Leibman & Ortiz (1970) also reported observation of intermediate alkene oxides from olefinic compounds (styrene, cyclohexene and indene) which hitherto had been known only to produce glycols.

Hence it appears that oxiranes, although in many instances not stable enough to survive isolation, are very widespread primary oxidation products in the metabolism of aromatic and olefinic foreign compounds. Firm establishment of the generality of the formation of (intermediate) oxiranes must await further investigation.

Fate of oxiranes

The fate of arene oxides is summarized in Fig. 1. Reaction 1 shows the valence bond tautomerism to oxepins. Both extremes of the oxide-oxepin equilibrium have been observed: Naphthalene 1,2-oxide exists exclusively as the oxide (Vogel & Klärner, 1968; Boyd, Jerina & Daly, 1970) presumably because tautomerization to the corresponding oxepin would cause loss of aromaticity in the benzene ring. Attempts to prepare naphthalene 2,3-oxide, on the other hand,

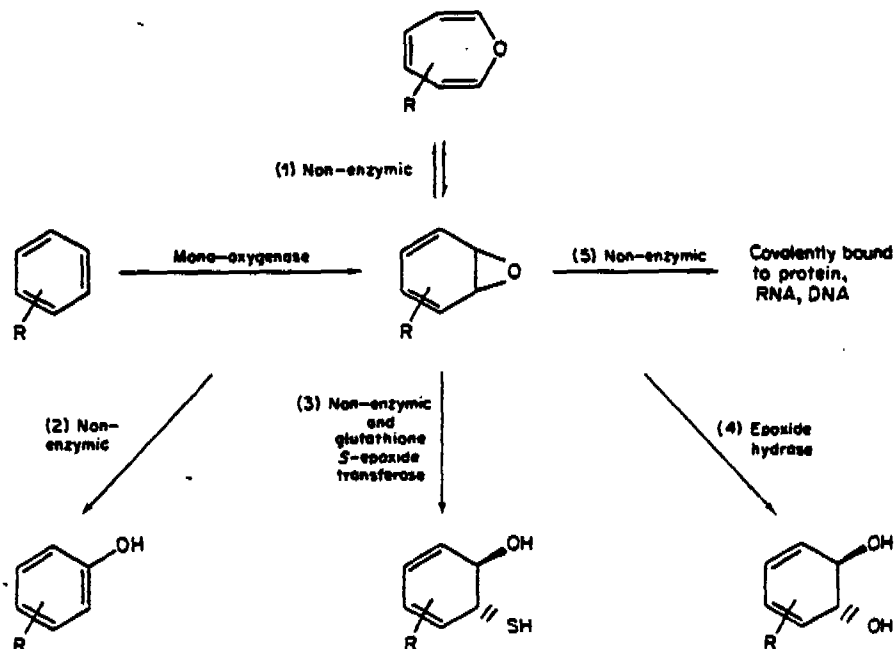


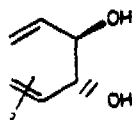
Fig. 1. Biological formation and degradation of arene oxides.

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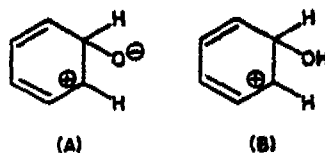
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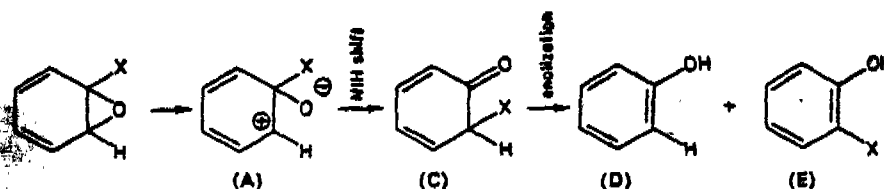


have produced only the oxepin (Jeffrey & Jerina, 1972). Oxepins which are not in equilibrium with the corresponding arene oxide are stable and do not isomerize to phenol. As yet, only arene oxides which exist at least partly in the oxide form have been established as metabolic intermediates.

Reaction 2 (Fig. 1) shows the isomerization of arene oxides to phenols. This reaction occurs non-enzymically as a spontaneous rearrangement in a neutral or basic environment and as an acid-catalysed isomerization at pH < 6 as indicated by recent kinetic studies (Kasperek & Bruice, 1972; Kasperek, *et al.*, 1972; Yagi, *et al.*, 1972). The rate determining step in the spontaneous rearrangement is the heterolytic opening of the carbon-oxygen bond to the zwitter ion (A). In the acid-catalysed isomerization the protonated form (B) predominates (Kasperek, *et al.*, 1972). The isomerization of arene oxides to phenols is accompanied by an



intramolecular displacement of a substituent X (e.g. isotopic hydrogen, halogen, methyl) from the place of the final location of the phenolic group to a neighbouring carbon atom. This phenomenon, the 'NIH Shift', has now been observed concomitant with the rearrangement of many arene oxides to phenols (Jerina, Daly & Witkop, 1968 a; Jerina, Kaubisch & Daly, 1971; Boyd, Daly & Jerina, 1972; Kaubisch, *et al.*, 1972). A complete mechanism for the isomerization of arene oxides to phenols must therefore be compatible with the observation of the NIH shift. Formation of a keto tautomer (C) prior to enolization to the phenol (D, E) satisfies the requirement. The mechanism is shown for the spontaneous reaction (A) which predominates at physiological pH. A more detailed description of this mechanism is given by Boyd, *et al.* (1972).



Reaction 3 (Fig. 1) is a conjugation of the arene oxide with glutathione (Boyland & Sims, 1965 a, b; Jerina, *et al.*, 1968 c, 1970 b). Such glutathione conjugates lead after loss of glutamate and glycine and *N*-acetylation of the remaining cysteine residue, to the mercapturic acids which in acidic environment easily dehydrate to the mercapturic acids (Boyland, Ramsay & Sims, 1961). This conjugation of arene oxides with glutathione proceeds spontaneously but there is also a cytoplasmic liver enzyme, glutathione *S*-epoxide transferase, which catalyses this reaction (Boyland & Sims, 1965 a, b; Jerina, *et al.*, 1970 b).

Reaction 4 (Fig. 1) is the hydration of arene oxides to the corresponding *trans*-dihydrodiols. This reaction is catalysed by epoxide hydrases which are

localized in the hepatic endoplasmic reticulum (Oesch, *et al.*, 1971 a). These enzymes will be dealt with in greater detail in the Bio-inactivation section of this review. The dihydrodiols are then conjugated with glucuronic acid or are dehydrogenated to catechols by a soluble hepatic dehydrogenase (Ayengar, *et al.*, 1959; Jerina, Ziffer & Daly, 1970 c). Catechols may then be *meta* or *para* O-methylated by a non-particulate (Creveling, *et al.*, 1970, 1972; Meyer & Scheline, 1972) or microsomal (Inscoc, Daly & Axelrod, 1965; McCormick, Flanagan & Lloyd, 1972) hepatic catechol O-methyltransferase.

Reaction 5 of Fig. 1 shows covalent binding of arene oxides to tissue macromolecules. Arene oxides form covalent bonds with many nucleophiles such as methanol, azide and sulphhydryl compounds (Jerina, *et al.*, 1968 c, 1970 b; De Marines & Berchtold, 1969; Yagi, *et al.*, 1972). They would therefore be expected to react with nucleophilic moieties in proteins, DNA and RNA. In fact, aromatic hydrocarbons bind to DNA, RNA and proteins *in vitro* if a metabolically active oxidative system (liver microsomes) is present but not if such a system is absent or relatively inactive (no NADPH added) (Grover & Sims, 1968; Gelboin, 1969). Moreover, the amount of the hydrocarbon which is bound to DNA is considerably increased if microsomes are used from animals pre-treated with 3-methylcholanthrene (Gelboin, 1969). Such treatment is known to induce the microsomal enzyme system which oxidatively transforms aromatic hydrocarbons (Conney, 1967). Thus, the oxidative enzyme system in the endoplasmic reticulum appears to transform aromatic hydrocarbons to a reactive intermediate which then binds to tissue constituents. In the light of the studies which are discussed in this section and which show that arene oxides are formed by liver microsomes it appears likely that the active metabolites which bind to DNA, RNA and proteins are arene oxides. Gelboin (1969) showed furthermore that the binding was probably not an adsorption but rather of a covalent nature since the ratio of aromatic hydrocarbon : DNA remained constant during repeated precipitation, extractions with organic solvents and fractionation in a CsCl gradient. Grover & Sims (1970) showed that in the absence of a microsomal enzyme system arene oxides did firmly bind *in vitro* to DNA, RNA and histone whereas the corresponding aromatic hydrocarbons and dihydrodiols did not. In cell cultures possessing some oxidative metabolizing potential, aromatic hydrocarbons, arene oxides, dihydrodiols and phenols did firmly bind to DNA, RNA and proteins, with arene oxides clearly binding to a much greater extent than the other compounds (Grover, Forrester & Sims, 1971 a; Kuroki, *et al.*, 1972). Kuroki & Heidelberger (1972) report similar results in mouse embryo cells with respect to binding to the 'h' protein, which they believe plays a role in chemical oncogenesis. Arene oxides react *in vitro* readily with poly(G), slower with poly(A), but not with poly(U) or poly(C) (Grover & Sims, 1972 a). Arene oxides also behave as active alkylating agents towards *p*-nitrobenzylpyridine (Sims, 1972 b), a widely used model acceptor for alkylating agents. Physical affinity of arene oxides to nucleic acids (i.e. 'solubility' in nucleic acid solution as compared to buffer solution) may be an essential prerequisite for the formation of covalent bonds since for aromatic hydrocarbons positive correlations appear to exist between this physical affinity and the chemical reactivity with nucleic acids *in vivo* (Brookes & Lawley, 1964; Goshman & Heidelberger, 1967) and *in vitro* (Grover & Sims, 1968). The lifetime of arene oxides, which under physiological conditions would be expected to be

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Alkene oxides are also substrates of glutathione S-epoxide-transferases (Boyland & Williams, 1965) and epoxide hydrases (Oesch, *et al.*, 1971 c and references therein) and bind covalently with DNA or related compounds (see below). Thus, they undergo metabolic reactions which are analogous to reactions 3, 4 and 5 of the arene oxides (Fig. 1). Lawley & Wallick (1957) showed that ethylene oxide and propylene oxide react with guanosine at the N-7 position. Windmueller & Kaplan (1961) noted that ethylene oxide reacts with adenine nucleotides predominantly at the N-1 position. Recently, propylene oxide was reported to bind covalently with DNA (Lawley & Jarman, 1972). At neutral pH values at 37° 7-(2-hydroxypropyl)guanine and 3-(2-hydroxypropyl)adenine hydrolysed out of the alkylated DNA. Quite a number of alkene oxides have been assessed to have strong alkylating properties using *p*-nitrobenzylpyridine as a model acceptor (Preussmann, Schneider & Eppe, 1969).

In studies on transformation and disposition of aromatic or olefinic drugs a measurable amount is often unaccounted for after adding together what is excreted from the body by various means. This missing amount may represent that which has bound covalently as a consequence of formation of electrophilically reactive intermediate oxiranes. This suspicion becomes even stronger if measurable levels can be observed in tissues and if these levels are persistent, although drugs, especially those with a high lipid solubility, can also be retained in tissue fat without being covalently bound. Covalent binding of low molecular material to substances which play a key role in the normal functioning of a cell such as proteins, RNA and DNA, would be expected to lead to disturbances. Some adverse effects of arene oxides and alkene oxides will be discussed in the following chapter.

Carcinogenicity and cytotoxicity of oxiranes

The oxides have been synthetically available for a much longer time than the arene oxides, due to the considerably greater stability of the former, such alkene oxides have for a longer time been known to cause malignant tumours. Several di-epoxides having the two oxirane rings separated from each other by a simple aliphatic hydrocarbon chain (Hendry, *et al.*, 1951; Van Duuren, *et al.*, 1963, 1967 a) as well as some mono-epoxides with additional functional groups, for example glycidaldehyde, (Van Duuren, *et al.*, 1967 a, b) have been reported to be carcinogenic. Propylene oxide and ethylene oxide are mutagenic for *Drosophila* (Loveless, 1966). Recent indirect evidence suggests that the destruction of cytochrome P-450 which is observed *in vivo* and *in vitro* after administration of several allyl-containing compounds (secobarbital, aprobarbital, allobarbital, 2-allyl-2-isopropylacetamide) is due to the formation of an alkene oxide in the allyl moiety of these compounds (De Matteis, 1971; Levin, *et al.*, 1972 b; Levin, Jacobson & Kunzman, 1972 a).

Early investigations on the carcinogenicity of arene oxides in whole animals were inconclusive (Boyland & Sims, 1967; Miller & Miller, 1967; Sims, 1967 a; Van Duuren, *et al.*, 1967 a) which is not surprising considering their great instability. In order to produce adverse effects they may have to be generated

Table 1. Malignant transformation of cells derived from mouse prostate by epoxides and other derivatives of aromatic hydrocarbons

The G 23 clone of cells derived from C3H mouse ventral prostate was used. 10^3 cells were plated in 60 mm dishes. Test compounds were dissolved in dimethyl sulphoxide or acetone and the cells were treated with them for a period of 24 h beginning 24 h after plating. The compounds were then removed by media changes which were twice weekly. After 56 days the dishes were fixed, stained and scored for piled-up foci.

Data from Marquardt, *et al.*, (1972).

Derivatives of:	Number of transformed foci/number of dishes* produced by					
	Control	Parent hydrocarbon	K-region epoxide	K-region <i>cis</i> -dihydrodiol	K-region <i>trans</i> -dihydrodiol	K-region phenol
Benz(a)anthracene	0/30	0/13 (1-0)	9/19 (0-5)	0/10 (1-0)	0/10 (5-0)	0/12 (1-0)
	(DMSO, 0-5%)	0/18 (5-0)	23/25 (1-0)	0/10 (10-0)	0/8 (10-0)	0/18 (3-0)
Dibenz(a,h)anthracene	0/30	0/15 (1-0)	4/20 (0-5)	0/12 (1-0)	0/10 (5-0)	0/6 (1-0)
	(DMSO, 0-5%)	0/18 (10-0)	9/17 (1-0)	0/15 (10-0)	0/12 (10-0)	0/10 (5-0)
			12/15 (10-0)			
3-Methylcholanthrene	0/30	1/13 (1-5)	38/27 (0-75)	0/10 (5-0)	0/10 (5-0)	0/8 (1-0)
	(DMSO, 0-5%)	10/33 (10-0)	58/26 (1-5)	0/18 (10-0)	0/12 (10-0)	8/16 (2-5) 0/10 (5-0)
Phenanthrene	0/20	0/12 (1-0)	0/12 (1-0)	—	—	—
	(Acetone, 0-5%)	0/12 (5-0)	0/15 (5-0)			

* Numbers in parenthesis represent concn. of test compounds in $\mu\text{g/ml}$.

in situ. Recent studies in cell cultures showed more clearcut effects. Hamster embryo cells (Grover, *et al.*, 1971 c) and cells derived from mouse prostate (Marquardt, *et al.*, 1972) underwent more malignant transformation in the presence of oxides of benz(a)anthracene, dibenz(a,h)anthracene, and 3-methylcholanthrene than in the presence of their parent hydrocarbons, dihydrodiols or phenols. The 9,10-oxide of the non-carcinogenic aromatic hydrocarbon anthracene was inactive (Table 1). The latter study also showed K-region oxides to be more active at producing malignant transformation than non-K-region oxides of the same hydrocarbon. The piled-up foci of these cell cultures were injected into mice and found to produce malignant tumours. Cells isolated from non-piled-up areas of carcinogen-treated dishes and cells from control dishes did not give rise to tumours. This cell culture system therefore appears to be a valid model for carcinogenesis. Although free radicals (Nagata, Kodama & Tagashira, 1967) and carbonium ions (Dipple, Lawley & Brookes, 1968) have been invoked as reactive intermediates involved in the induction of cancer by polycyclic hydrocarbons, it now appears from the facts discussed in this and in the preceding chapter that arene oxides are the causative agents. This seems to be true at least for polycyclic hydrocarbons without substituents.

Since cancer is inherited from mother to daughter cells it may be caused by mutation of the genome. Experiments with chinese hamster cells (Huberman, *et al.*, 1971) showed that aromatic hydrocarbons require metabolic activation in order to become mutagenic. In the benz(a)anthracene and 3-methylcholanthrene series the K-region oxides were clearly more mutagenic than parent hydrocarbons, dihydrodiols or phenols. In the dibenz(a,h)anthracene series, however, the phenol appeared more mutagenic than the epoxide. K-region oxides of dibenz(a,h)anthracene, benz(a)anthracene and 7-methylbenz(a)anthracene produce frame shift mutations in *Salmonella typhimurium* whereas parent hydrocarbons, K-region dihydrodiols and phenols are inactive (Ames, Sims & Grover, 1972). K-region oxides are also effective mutagens in bacteriophage (Cookson, Sims & Grover, 1971).

There is good, although indirect evidence that the causative agents for liver necrosis after treatment of animals with halobenzenes are also arene oxides. Bromobenzene is converted by microsomes *in vitro* in an NADPH and O₂ dependent reaction to an active intermediate which forms a covalent complex with glutathione. Pre-treatment of rats with phenobarbital, which stimulates the synthesis of microsomal enzymes involved in the oxidative metabolism of many foreign compounds (Remmer, 1959), potentiates both covalent binding of bromobenzene and necrosis elicited by this halobenzene, while blockage of this metabolism by β -diethylaminoethyl diphenylpropylacetate (SKF 525-A) (Axelrod, Reichenthal & Brodie, 1954) prevents both binding and necrosis. Moreover, radioautography of paraffin liver sections after administration of [¹⁴C]bromobenzene showed that the label was firmly bound at the sites of necrosis (centrolobular zones), indicating a close relationship between covalent binding and necrosis (Brodie, *et al.*, 1971; Mitchell, *et al.*, 1971). Duncan & Brookes (1971) also report a positive correlation between the extent of binding of dibenz(a,c)anthracene and dibenz(a,h)anthracene and their carcinogenic potency. With mouse embryo cells in culture the binding index (i.e. the extent of binding of the hydrocarbon to the macromolecule in question resulting from metabolism of 1 nmol of hydrocarbon per ml of medium) to DNA and RNA was at least

10 times higher for the carcinogenic dibenz(a,h)anthracene than for the non-carcinogenic dibenz(a,c)anthracene.

Bio-inactivation of oxiranes by epoxide hydrases

Several intermediate oxiranes bind firmly to proteins, RNA and DNA, cell constituents which are of prime importance for the normal functioning of the cell (see section on fate of oxiranes) and several, as would be expected, appear to be ultimate mutagens, carcinogens or cytotoxins (see section on carcinogenicity and cytotoxicity). Whether a given olefinic or aromatic compound does produce such adverse effects may depend on the chemical reactivity of the intermediate alkene oxide or arene oxide and on the relative rate of its formation and degradation. The level and activity of epoxide hydrases, which transform such reactive oxiranes to much less reactive vicinal diols (see section on carcinogenicity and cytotoxicity) may therefore be of critical significance. The importance of epoxide hydrases for the degradation of arene oxides is illustrated by the recent observation of Grover, *et al.* (1972) that arene oxides can be isolated after incubation of pyrene and benzo(a)pyrene with liver microsomes in the presence but not in the absence of a potent *non*-competitive epoxide hydrase inhibitor, 1,2,3,4-tetrahydronaphthalene 1,2-epoxide (Oesch, *et al.*, 1971 c). Moreover, Sims (1970) reported that rat liver preparations transform benz(a)-anthracene and dibenz(a,c)anthracene predominantly to dihydrodiols but convert dibenz(a,h)anthracene to about equal amounts of dihydrodiols and phenols. Thus, the arene oxide(s) formed from the former two hydrocarbons appear to be much better substrates for epoxide hydrase(s) compared with those formed from dibenz(a,h)anthracene. This may be causally related to the much weaker carcinogenic activity of the former hydrocarbons compared with the latter. Although correlating these two facts largely oversimplifies the very complex events which eventually lead to cancer after exposure to a chemical carcinogen, it nevertheless shows the potential importance of this enzyme. Moreover, a great variety of arene and alkene oxides, widely differing from each other with respect to the chemical environment of the oxirane ring, are converted to the corresponding diols by liver preparations (Table 2). It therefore seems that the enzyme(s) which catalyse this reaction deserve detailed investigation.

Assay

Arene oxides are impractical as substrates for a routine assay of epoxide hydrase activity because of their generally great instability. A sensitive and rapid radiometric assay has therefore been developed using the relatively stable [³H]styrene oxide as a substrate (Oesch, *et al.*, 1971 a). Only after a whole complex of questions had been solved with this convenient assay was it then checked whether the information also held true for arene oxides and other alkene oxides (Oesch, Jerina & Daly, 1971 b; Oesch & Daly, 1972).

The substrate [7-³H] styrene oxide, was synthesized from commercially available [7-³H]styrene. After incubation the unreacted substrate is extracted quantitatively into petroleum ether, while the enzymically formed styrene glycol is polar enough to be almost completely unaffected by this extraction. The low freezing point of the petroleum ether allows freezing of the aqueous phase in acetone-dry ice followed by simple decantation of the petroleum ether. After

thawing, the styrene glycol is extracted into ethyl acetate and an aliquot assayed by scintillation spectrometry. Radiochromatography proved the radioactivity in this extract to be solely associated with carrier styrene glycol (Oesch, *et al.*, 1971 a). This assay is very quick. However, for organs where epoxide hydase is very low, this assay is not sensitive enough, the blank being the limiting factor. (With liver preparations the blank can easily be kept below 3%). The same problem was encountered when it was attempted to determine epoxide hydase activity in needle biopsy specimens of human liver. For such problems an assay was developed with increased sensitivity at the expense of simplicity and rapidity (Oesch, Thoenen & Fahrlander, 1973 c). (For epoxide hydase assays with other substrates see Jerina, *et al.*, 1968 c; Leibman & Ortiz, 1968; Brooks, Harrison & Lewis, 1970; Maynert, *et al.*, 1970; Oesch, *et al.*, 1971 b; Oesch, *et al.*, 1971 c; Watabe & Akamatsu, 1972.)

Distribution of epoxide hydase

Diol formation from arene or alkene oxides (Table 2) has been demonstrated almost invariably with liver homogenates or microsomes from rats, rabbits or pigs. Using the above assay with [^3H]styrene oxide as substrate, quantitative data on the levels of hepatic epoxide hydase in various species were obtained (Oesch, *et al.*, 1971 a; Oesch, *et al.*, 1973 c). Figure 2 shows that levels of the human enzyme are more comparable to rodents than to Rhesus monkeys. No significant ($0.3 > P > 0.2$) differences in epoxide hydase activity were noted between sexes in humans, Rhesus monkeys, rabbits, rats or guinea-pigs. El Zorgani, Walker & Hassall (1970) had also reported that liver microsomes from rabbits were more active in hydrating an epoxide than those from rats. They used HEOM (1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene) as substrate.

Using the assay with [^3H]styrene oxide as substrate, epoxide hydase was determined in several organs of rat and guinea-pig. Activity was high in liver, low in kidney, very low (i.e. only detectable with the more sensitive assay) in

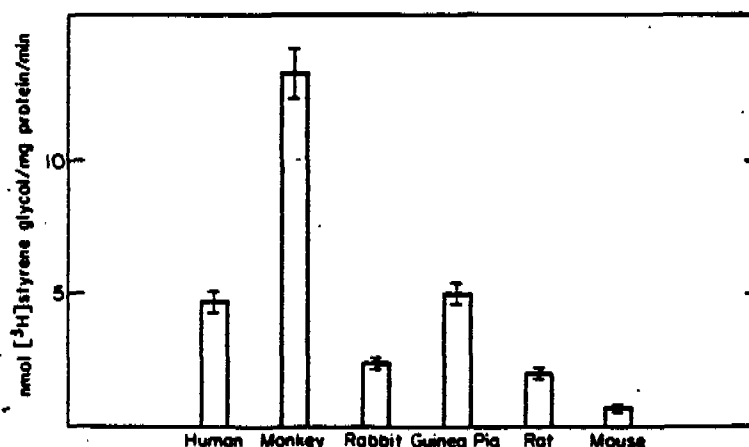
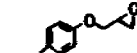
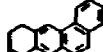
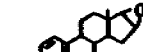
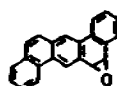


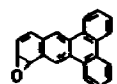
Fig. 2. Epoxide hydase activities in liver homogenates from various species. Data from Oesch, *et al.* (1973 b). Assay with [^3H]styrene oxide as substrate.

Table 2. Oxiranes converted to Diols by Liver Preparations

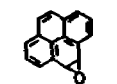
Arene oxides		Cycloalkene oxides		Open-chain alkene oxides			
				Monosubstituted oxiranes		Polysubstituted oxiranes	
Structure	Reference	Structure	Reference	Structure	Reference	Structure	Reference
	Jerina, <i>et al.</i> , 1968 c		Jerina, <i>et al.</i> , 1968 c		Jerina, <i>et al.</i> , 1968 c		Oesch, <i>et al.</i> , 1971 c
	Jerina, <i>et al.</i> , 1970 b		Oesch, <i>et al.</i> , 1971 c		Oesch, <i>et al.</i> , 1971 c		Oesch, <i>et al.</i> , 1971 c
	Daly, <i>et al.</i> , 1970		Oesch, <i>et al.</i> , 1971 c		Oesch, <i>et al.</i> , 1971 b		Oesch, <i>et al.</i> , 1971 c
	Boyland and Sims, 1965 a		Leibman and Ortiz, 1968		Oesch, <i>et al.</i> , 1971 c		Watabe, Akamatsu and Kiyonaga 1971 a
	Boyland and Sims, 1965 b		Sims, 1971		Oesch, <i>et al.</i> , 1971 c		Maynert, <i>et al.</i> , 1970
	Sims, 1971		Sims, 1972 a		Maynert, <i>et al.</i> , 1970		Oesch, <i>et al.</i> , 1971 c
	Sims, 1967 b		Breuer and Knuppen, 1961		Oesch, <i>et al.</i> , 1971 c		Watabe, Veno and Imazumi 1971 c
							Watabe, <i>et al.</i> , 1971 c



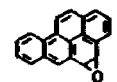
Boylard and
Sims, 1965 b



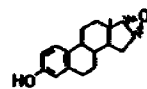
Sims, 1972 a



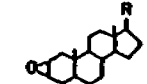
Grover, *et al.*,
1972



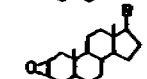
Grover, *et al.*,
1972



Breuer and
Knuppen,
1961

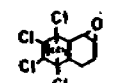


Watabe, *et al.*,
1971 b

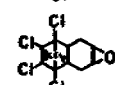


Watabe, *et al.*
1971 b

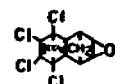
$R = \text{CH}(\text{CH}_3)-$
 $(\text{CH}_2)_3-\text{CH}(\text{CH}_3)_2$



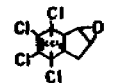
Brooks, Lewis
and Harrison
1968



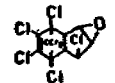
Brooks,
1969



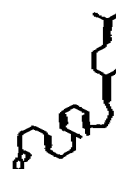
Brooks, *et al.*,
1970



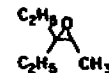
Brooks, *et al.*,
1968



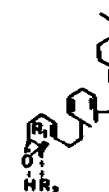
Brooks, *et al.*,
1970



Corey, *et al.*,
1968



Maynert, *et al.*,
1970



$R_1 = R_2 = \text{CH}_3$

Oesch, *et al.*,
1971 b

$R_1 = \text{CH}_3, R_2 = \text{H}$

Clayton, *et al.*,
1968

$R_1 = \text{H}, R_2 = \text{CH}_3$

Clayton, *et al.*,
1968

$R_1 = \text{C}_2\text{H}_5, R_2 = \text{H}^*$

Corey, *et al.*,
1968

Epoxyde Hydrases

22

* Stereochemistry not indicated

SL 066150

319

intestine and lung and not detectable in brain, heart, spleen and muscle. Adult mouse skin also exhibited very low epoxide hydrase activity, whereas in foetal mouse skin, the enzyme could not be detected (Oesch, *et al.*, 1971 a; 1973 a).

After differential centrifugation of rat and rabbit liver homogenates prepared with isotonic KCl, epoxide hydrase activity was associated with mitochondrial and microsomal fractions to a similar extent on a per protein basis. However, the same was also true for a microsomal marker enzyme, glucose-6-phosphatase, suggesting heavy microsomal contamination of the mitochondrial fraction. In contrast, when isotonic sucrose was used, both epoxide hydrase and glucose-6-phosphatase activities were found virtually exclusively in the microsomal fraction (Oesch, *et al.*, 1971 a). This was also true for human epoxide hydrase (Oesch, *et al.*, 1973). Because of the simplicity of the above assay, epoxide hydrase might be used as a marker enzyme for microsomal membranes.

Properties of epoxide hydrase determined in microsomal preparations

Epoxide hydrase activity in liver microsomes as determined with styrene oxide as substrate amounted to 150% of controls after pre-treatment of rats with 3-methylcholanthrene and to 300% of controls after pre-treatment with phenobarbital (Oesch, *et al.*, 1971 a). The relative increases after these pretreatments were similar with respect to styrene oxide, naphthalene 1,2-oxide or benzene oxide as substrates, while benzene had no effect on activity towards any of these substrates (Oesch, *et al.*, 1973 a). During maturation of male rats an increase of hepatic epoxide hydrase by a factor of 4 took place between 28 and 40 days of age (Oesch, *et al.*, 1971 a). These increases in specific activity of epoxide hydrolases are reminiscent of similar increases in other microsomal drug metabolizing enzymes, i.e., mono-oxygenase(s) (Conney, 1967; Nebert & Gelboin, 1969). However, the extents of induction of total levels of these two enzymes or enzyme families are not comparable (cf. Conney & Burns, 1963, and Oesch, *et al.*, 1971 a) and are under separate genetic control (Nebert, *et al.*, 1972; Oesch, *et al.*, 1973 c). These latter observations strictly refer to total levels of epoxide hydrolases, whereas certain epoxide hydrase(s) and mono-oxygenase(s) appear to exist as fairly stable multienzyme complexes as will be discussed below.

The stereochemistry of the enzymic hydration of several oxiranes has also been studied in microsomal preparations. With naphthalene 1,2-oxide (Jerina, *et al.*, 1970 b), and styrene oxide (Jerina, *et al.*, 1970 c) the stereochemical course of the entering oxygen was investigated. With both substrates, attack by $H_2^{18}O$ occurred almost exclusively at the 2-position. Formation of dihydrodiol from naphthalene by microsomes involves incorporation of oxygen from air into the 1-position and hence from water into the 2-position (Holtzman, Gillette & Milne, 1967). Thus, dihydrodiol formation from naphthalene and from naphthalene 1,2-oxide is identical with regard to the source of oxygen at the 2-position providing an additional argument for the intermediacy of oxiranes in the formation of diols from aromatic and olefinic compounds.

Product glycols which are stereochemically fixed by a ring structure invariably have the *trans*-configuration (Breuer & Knuppen, 1961; Leibman & Ortiz, 1968; Jerina, *et al.*, 1968 c; 1970 b; Daly, *et al.*, 1970; Pandov & Sims, 1970; Brooks, *et al.*, 1970; Watabe, *et al.*, 1971 b; Sims, 1971; 1972 a). Hydration

of some acyclic alkene oxides has also been shown to proceed via a *trans*-opening of the oxirane ring. Thus, *threo*-9,10-dihydroxy-stearic acid was produced from *cis*-9,10-epoxy-stearic acid, *threo*-1,2-diphenyl-1,2-ethanediol from *cis*-stilbene oxide but *meso*-1,2-diphenyl-1,2-ethanediol from the *trans*-isomer (Watabe & Akamatsu, 1972). It is noteworthy, that urinary diol metabolites of arenes and cycloalkenes also possess *trans*-configuration. The only exceptions known to-date are the metabolites of indene (Brooks & Young, 1956) and acenaphthylene (Hopkins, Brooks & Young, 1962) representing a mixture of *cis*- and *trans*-dihydrodiols. However, even there, in all likelihood, the primary metabolites are the *trans*-isomers since *cis*- and *trans*-mixtures were found in rat urine after administration of the carefully purified *trans*-dihydrodiols (Hopkins, Lewis & Young, 1964). With *trans*-indene-1,2-dihydrodiol the interconversion appears to occur through an intermediate ketol, 2-hydroxy-indan-1-one (Lewis, 1970).

The absolute stereochemistry of several *trans*-diols produced by the action of microsomal epoxide hydrase(s) has also been investigated. Cyclohexene oxide, benzene oxide and naphthalene 1,2-oxide are converted predominantly to 1*R*, 2*R*-*trans*-diols whereas in the case of phenanthrene 9,10-oxide the 1*S*,2*S*-*trans*-diol predominates. Optical purity varied between 30–70% with these product diols. Incubation of either naphthalene or naphthalene 1,2-oxide with liver microsomes from several species lead to diols with the same absolute stereochemistry and similar (~30–50%) optical purity. Benzene dihydrodiol produced as an *in vivo* metabolite from benzene also had the same absolute stereochemistry as the diol produced from benzene oxide by incubation with liver microsomes (Jerina, *et al.*, 1970 c). This implies that the enantiomers of the intermediate arene oxide are either in equilibrium with the oxepin (Vogel, *et al.*, 1965) leading to a racemic mixture, or that the arene oxide is enzymically formed with very low stereoselectivity. The latter appears to be true for naphthalene 1,2-oxide which is not in equilibrium with the corresponding oxepin. No spontaneous racemization of optically active naphthalene oxide takes place at neutral pH. However, the optical activity of naphthalene dihydrodiol is ~10% lower when racemic naphthalene oxide is employed as substrate for microsomal epoxide hydase in place of naphthalene oxide enzymically generated *in situ* from naphthalene. This suggests an asymmetric synthesis of naphthalene oxide by microsomal mono-oxygenases of a low stereoselectivity (Boyd, *et al.*, 1970). Due to molecular symmetry, the ease of opening of the oxirane ring of *cis*-stilbene oxide would be expected to be equal for either C–O bond. However, enzymic hydration stereo-selectivity produces 1*R*,2*R*-1,2-diphenyl-1,2-ethanediol, probably due to the structure of the enzyme-substrate complex. Similarly, racemic *cis*-9,10-epoxystearic acid is enzymically hydrated in an optically stereoselective manner to yield the *threo*-glycol with dextrorotatory properties (Watabe & Akamatsu, 1972). Enzymic hydration of 2 α , 3 α - and 2 β ,3 β -epoxy-5 α -cholestane occurs with a high stereospecificity. Only one of the four possible stereoisomeric diol products was observed, namely the 2,3-*trans*-diaxial dihydroxysteroid 2 β ,3 α -dihydroxy-5 α -cholestane. The same product was formed from either of the two isomeric substrates although at very different rates (Watabe *et al.*, 1971 b). Acid-catalysed hydration of these two isomeric epoxysteroids also leads to the same stereoisomer (Schoppee, Jones & Summers, 1957). This may be an indication that hepatic epoxide

hydrase(s) exert their catalytic effect by means of protonation of the oxirane substrates as a consequence of the presence of an easily dissociable proton in the active site of the enzyme. On the other hand, in an extensive study on structure-activity relationship with a purified epoxide hydrase preparation, the relative activity of various oxiranes as substrates or inhibitors of epoxide hydrase did not correlate with that expected from a mechanism involving an initial acid-catalysed opening of the oxirane ring (Oesch, *et al.*, 1971a). The enzymic hydration of indan-8,9-oxide is unique in that it occurs homoallylically by 4,9- and not by 8,9-addition of water (Daly, *et al.*, 1970). Steric factors possibly prevent 'normal' hydration and lead to stereospecific entry of water at the homoallylic position.



The arene dihydrodiol products are in turn substrates for a dihydrodiol dehydrogenase which converts them to catechols. Surprisingly, the more readily dehydrogenated isomer is the minor enantiomer formed by the action of the epoxide hydrase. This inverse correlation between the two enzymes holds true with all the substrates studied, i.e. even with phenanthrene 9,10-dihydrodiol in which the absolute stereochemistry of the major isomer formed by the hepatic epoxide hydrase(s) is *S,S* and not *R,R* as in all other dihydrodiols which were investigated (Jerina, *et al.*, 1970 c).

Solubilization and purification of epoxide hydrase

Hepatic epoxide hydrase is localized exclusively in the microsomal fraction (Oesch, *et al.*, 1971 a). Solubilization of epoxide hydrase activity from guinea-pig liver microsomes met with difficulties. Use of hypotonic buffer or high salt concentrations (e.g. 6M guanidine hydrochloride) preserved the activity, but failed to release the enzyme from the microsomes. Attempts to prepare acetone powders were unsuccessful and use of several detergents destroyed the enzymic activity. Finally, solubilization without loss of activity was effected under optimal conditions with a neutral detergent, Cutacum. The solubilized preparation contained more than 200% of the epoxide hydrase activity originally present in the liver homogenate (Oesch & Daly, 1971). Epoxide hydrase activity in the homogenate was linear with respect to protein concentrations. Repeated dialysis of the liver homogenate or Sephadex G-25 chromatography of the solubilized preparation did not alter the enzymic activity and combinations of the homogenate and the solubilized preparation in various proportions always resulted in an additive activity, i.e. an activity corresponding to the sum of the two components (Oesch, *et al.*, 1973 c). Therefore, this increase in activity after solubilization cannot be due to the removal of an inhibitor but rather may reflect exposure of active sites by the action of the detergent. No activity was observed in the sediment after centrifugation at $100\,000 \times g$ for 1 h. This is the criterion by which the preparation was called 'solubilized'. No electron microscopic investigations have been performed with the preparation.

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Table 3. Purification of an epoxide hydrase from guinea pig liver

Data from Oesch & Daly (1971).

Fraction	Protein recovery (%)	Specific activity*	Activity recovery (%)	Purification factor
Liver homogenate	100	102	100	1
Microsomes	20.4	334	66.7	3.3
Supernatant after Cutscum treatment and centrifugation at 100 000 x g for 1 h	16.9	1242	206.2	12.2
Supernatant after removal of top layer and filtration	10.0	1918	188.0	18.8
(NH ₄) ₂ SO ₄ ppt. after Sephadex G-25 chromatography	2.5	2837	69.4	27.8
Calcium phosphate gel adsorption and desorption	0.5	4210	20.6	41.3

* Specific activity expressed as nmol styrene glycol/mg N/5 min.

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Table 3 summarizes the purification procedure. An overall purification of 40-fold with a recovery of 20% of the epoxide hydrazase activity originally present in liver homogenates was achieved (Oesch & Daly, 1971). The final preparation lost about 30% of its activity within 1-2 days at -15° or at $0-5^{\circ}$. However, the remaining epoxide hydrazase activity was very stable and lost virtually no ($<10\%$) activity during storage at -15° for at least 6 months. Polyacrylamide disc gel electrophoresis showed under several conditions a single protein band which moved into the separating gel, in addition to protein which did not migrate from the stacking gel. No hydrazase activity could be detected in slices of unstained gel. Sodium dodecyl sulphate gel electrophoresis allowed all protein to move into the separating gel. One major band corresponding to a molecular weight of approximately 50 000 dominated the picture. However, several minor bands were also present (unpublished results).

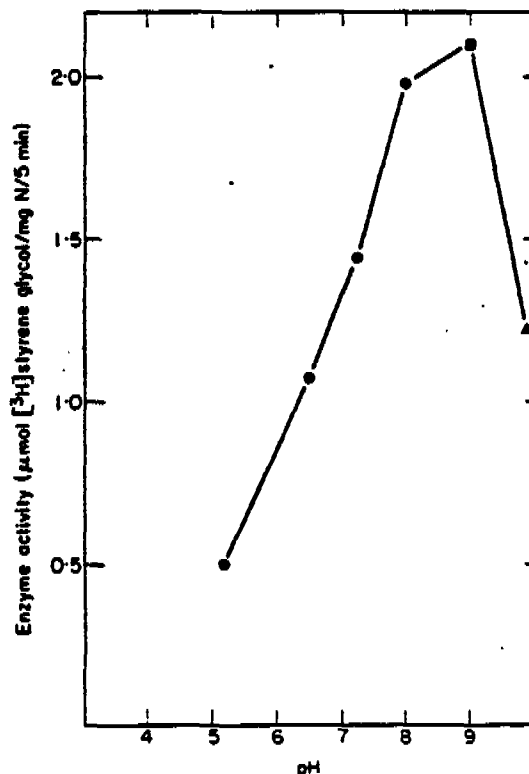


Fig. 3. Epoxide hydrazase activity in purified epoxide hydrazase preparation from guinea pig liver as a function of pH.

Assay with $[^3\text{H}]$ styrene oxide as substrate (Oesch, Jerina & Daly, 1971 a); $\circ$, phosphate; $\blacksquare$, Tris; $\blacktriangle$, glycine. Data from Oesch & Daly, 1971.

Subsequent to our preliminary report (Oesch, *et al.*, 1970), a procedure was described for solubilizing and purifying epoxide hydrazase from rabbit liver microsomes (Watabe & Kanehira, 1970). The method appears less satisfactory,

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resulting in a final purification factor from liver microsomes of 3.8 with only 5% recovery of enzyme activity.

Properties of the purified epoxide hydrase

Full enzymic activity is not dependent on the presence of metal ions or other low molecular weight co-factors, since chelating agents such as EDTA or α, α -dipyridyl, or repeated dialysis or Sephadex G-25 or G-200 chromatography had no effect on epoxide hydase activity. Moreover, if the purified preparation was combined with the original homogenate in various proportions, the resulting activity was always additive. Carbonyl reagents such as hydroxylamine, did not influence the enzymic activity. On the other hand, sulphhydryl reagents, such as mersalyl or *p*-chloromercuribenzoate, slightly but significantly inhibited epoxide hydase activity, possibly indicating that sulphhydryl group(s) are important for the most favourable conformation of the enzyme protein but are probably not involved in the catalytic mechanism at the active site. Product diols, such as styrene glycol and several dihydrodiols derived from arene oxides did not inhibit the enzyme even at twice (4 mM) substrate concentration whereas high substrate concentrations (8-16 mM styrene oxide) inhibited enzyme activity by 20-50%.

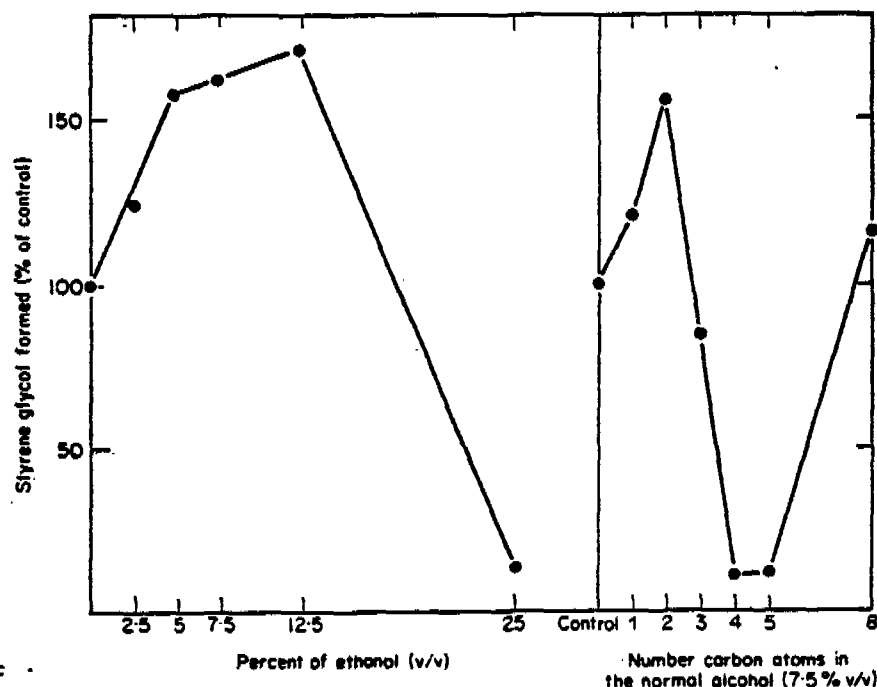


Fig. 4. Effect of concentration and chain length of 1-alkanols upon epoxide hydase activity with $[^3\text{H}]$ styrene oxide as substrate.

Alcohols were added in 20 μ l acetonitrile at zero time with no pre-incubation. Purified epoxide hydase preparation from guinea pig liver (Oesch & Daly, 1971) was used. Assay with $[^3\text{H}]$ styrene oxide (2 mM) as substrate (Oesch, *et al.*, 1971 a). Data from Oesch, *et al.* (1971 c).

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β -Diethylaminoethyl diphenylpropylacetate (SKF 525-A), piperonyl butoxide and α -naphthoflavone, potent inhibitors of several reactions catalysed by microsomal drug-metabolizing enzymes (Axelrod, *et al.*, 1954; Anders, 1968; Wiebel, *et al.*, 1971), had no effect on epoxide hydrase (Oesch, *et al.*, 1971 a, c, 1973 a, c).

In contrast to crude homogenates or microsomal preparations, where a very broad pH profile with its optimum for enzymic hydration of styrene oxide between 7 and 9 was observed, the purified preparation displayed a sharp pH profile with its optimum at pH 9 (Fig. 3). Non-enzymic hydration of styrene oxide was significant (>5%) only below pH 6.5 (Oesch & Daly, 1971; Oesch, *et al.*, 1973 c).

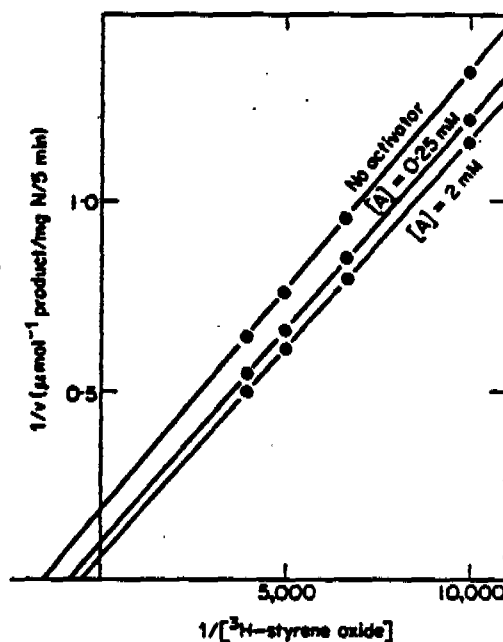


Fig. 5. Activation of epoxide hydrase by metyrapone.

Double-reciprocal plot of velocity (μmol styrene glycol/mg of N/5 min) versus concn. of substrate styrene oxide (M) (Oesch, *et al.*, 1971 c). Assay as described (Oesch, *et al.*, 1971 a). Metyrapone (A) was added in 20 μl acetonitrile at zero time. Purified epoxide hydrase preparation from guinea-pig liver was used (Oesch & Daly, 1971).

Compounds which stimulate epoxide hydrase activity could serve as tools for the study of the role of this enzyme in drug metabolism and they might even prove useful in alleviating or preventing mutagenic, carcinogenic or cytotoxic effects of aromatic or olefinic compounds which are caused by their epoxide derivatives. A number of simple alcohols such as cyclohexanol, 2-cyclohexen-1-ol and glycidol were found to activate epoxide hydrase *in vitro*. The activation by 1-alkanols was maximal at very high (1-2 M) concentrations and was a function of chain length (Fig. 4). Certain imidazoles, such as 1-(4-propylphenyl)-imidazole and certain ketones, such as α -tetralone, also stimulated the enzyme.

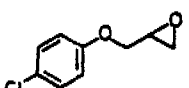
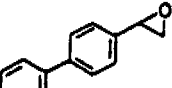
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A marked activation was obtained with the ketonic *bis*-pyridine, metyrapone, whereas pyridine or nicotinamide were inactive. The stimulating effect was also completely lost on reduction of the ketonic group of metyrapone to the alcohol. Kinetic analysis of the activation of epoxide hydrase by metyrapone (Fig. 5) indicated negative co-operativity with the substrate (Oesch, *et al.*, 1971 c, 1973 a). Attempts to stimulate epoxide hydrase *in vivo* so far have been unsuccessful.

Table 4. Monosubstituted oxiranes as inhibitors towards hydration of [³H]styrene oxide

Data from Oesch (1973 b). Substrate ([³H]styrene oxide) and inhibitor concn. 2 mM. The inhibitor was added in 20 μ l acetonitrile at zero time with no preincubation. This amount of solvent had no significant effect on enzyme activity. Incubation mixture, conditions and assay as described (Oesch, *et al.*, 1971 a).

Inhibitor	% Inhibition* of epoxide hydrase purified from liver of:		
	Man	Guinea pig	Rat
	ns	ns	ns
	ns	ns	ns
	ns	ns	ns
	12 $\pm$ 1.2	9 $\pm$ 2.3	17 $\pm$ 2.1
	37 $\pm$ 5.2	39 $\pm$ 2.1	43 $\pm$ 6.0
	59 $\pm$ 4.4	50 $\pm$ 3.1	82 $\pm$ 3.2
	56 $\pm$ 2.3	28 $\pm$ 1.3	40 $\pm$ 2.1
	37 $\pm$ 5.4	38 $\pm$ 6.2	28 $\pm$ 2.7
	51 $\pm$ 2.1	49 $\pm$ 1.5	52 $\pm$ 2.2
	68 $\pm$ 4.3	74 $\pm$ 9.2	79 $\pm$ 2.3

*Significant effect with $P < 0.001$ (ns = not significant).

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The rapidity of the radioassay (Oesch, *et al.*, 1971 a) allowed indirect delineation of structural requirements necessary for effective interaction with epoxide hydrazase by measuring the inhibitory effect of more than 100 compounds towards hydration of [^3H]styrene oxide. Assays for determination of product formation were then developed for several key-compounds and their relative ability to inhibit hydration of styrene oxide compared to their relative ability to serve as substrates of epoxide hydrazase. Moreover, kinetic analysis of the type

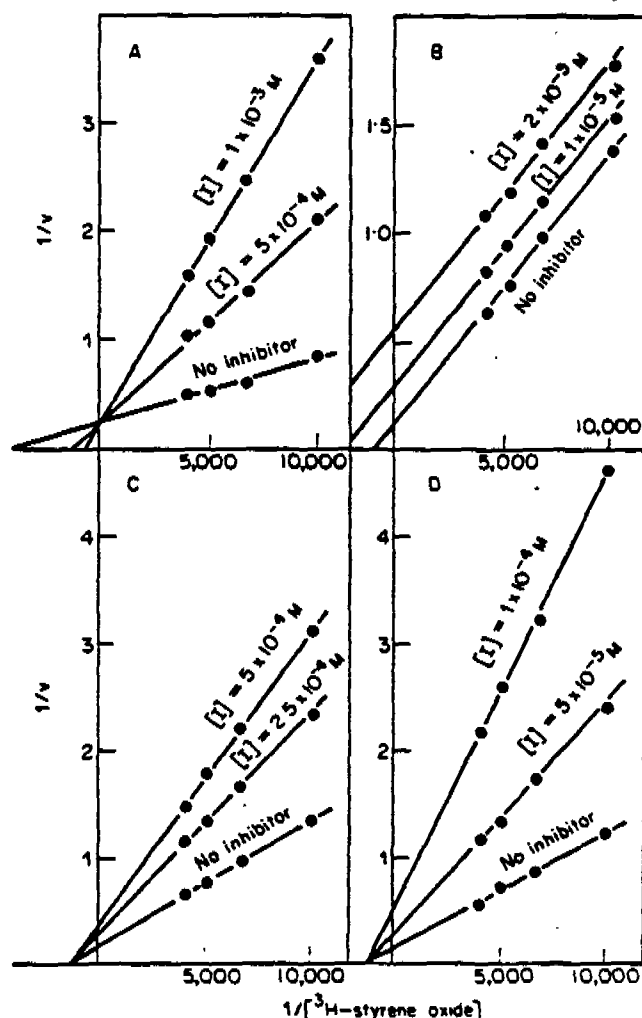


Fig. 6. Inhibition of epoxide hydrazase with [^3H] styrene oxide as substrate. Inhibitor: (A) 4-chlorophenyl 2,3-epoxypropyl ether, (B) 1,1,1-trichloropropene 2,3-oxide, (C) cyclohexene oxide, and (D) 1,2-epoxy-1,2,3,4-tetrahydronaphthalene. Double-reciprocal plots of velocity (μmol styrene glycol formed/mg N^5 min) versus concn. of substrate (M). Assay as described (Oesch, *et al.*, 1971 a). Inhibitor was added in 20 μl of acetonitrile at zero time with no pre-incubation (Oesch, *et al.*, 1971 c). Either 10 μl (A) or 20 μl (B-D) of purified epoxide hydrazase preparation from guinea-pig liver (Oesch & Daly, 1971) was used per incubation.

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of inhibition was performed with several representative compounds (Oesch, *et al.*, 1971 b, c). Table 4 shows that mono-substituted oxiranes interact with epoxide hydase only if they have a lipophilic substituent larger than an ethyl group such as an isopropyl, *t*-butyl, *n*-hexyl or phenyl group. This may indicate lipophilic binding sites of the enzyme near its active site. Several mono-substituted oxiranes with a large lipophilic substituent, such as *n*-octene 1,2-oxide, styrene oxide, phenylpropene 2,3-oxide and several phenyl 2,3-epoxypropyl ethers were also demonstrated to be excellent substrates for epoxide hydase and kinetic analysis of the inhibition exerted by one of them, 4-chlorophenyl 2,3-epoxypropyl ether, indicated that the inhibition was competitive with respect to substrate, [^3H]styrene oxide, as expected (Fig. 6). The most potent inhibitor of epoxide hydase discovered so far is a monosubstituted oxirane 1,1,1-trichloropropene 2,3-oxide. This oxirane completely inhibited hydration of [^3H]styrene oxide at concentrations of one fifth of substrate. The bulk and strong electron-withdrawing effect of the trichloromethyl group as well as the oxirane ring appeared to be essential, since analogous epoxides with either the bulky *t*-butyl group or the smaller but strongly electron-withdrawing trifluoromethyl group were much less potent and a variety of analogous compounds containing a trichloromethyl group but no oxirane ring had no effect on the hydration of [^3H]styrene oxide. This potent inhibitor was on the other hand a very poor substrate for epoxide hydase, and kinetic analysis proved the inhibition to be uncompetitive with respect to the substrate, styrene oxide (Fig. 6) (Oesch, *et al.*, 1971 c, 1973 a).

In a series of styrene oxides (Table 5) the enzyme readily interacted with mono- and 1,1-disubstituted oxiranes, although less effectively as the bulk of the two substituents was increased. Strikingly, the enzyme interacted with 1,2-disubstituted oxiranes only if they had *cis*-configuration. All the above styrene oxides not only inhibited hydration of [^3H] styrene oxide, but also served as substrates for epoxide hydase. However, quantitatively their relative activity as substrates or inhibitors did not always correlate well. For more complete data and discussion see Oesch, *et al.*, (1971 c). With *trans*-1,2-disubstituted, tri- and tetra-substituted oxiranes of the styrene oxide series (Table 5) no interaction was observed either with respect to inhibition of hydration of [^3H]styrene oxide or with respect to product formation from these compounds. This dependency on the substitution pattern of the oxirane ring was also found with several other classes of compounds (Oesch, *et al.*, 1971 c). Increasing substitution of the oxirane ring may prevent the approach of the oxirane to the active site of epoxide hydase by steric hindrance. Interestingly, increasing substitution of the oxirane ring by electron-releasing substituents including alkyl groups facilitates chemical acid-catalysed conversion of oxiranes to vicinal diols (Prichard & Long, 1956) in distinct contrast to the enzyme-catalysed hydration of oxiranes. Studies on a large series of styrene oxides with various substituents on the aromatic ring, and epoxides with various substituents on the oxirane ring, as well as several other classes of compounds, showed structure-activity relationships which did not correlate well with what would be expected from a catalytic mechanism which initiates hydration of the oxirane ring by protonation of the oxygen as a consequence of an easily dissociable proton of the active site of the enzyme, nor from a mechanism which initiates the reaction by a nucleophilic attack of water (Oesch, *et al.*, 1971 c).

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Liver microsomes exhibit another enzymic activity towards certain oxirane substrates, namely cyclization of endogenous and of several foreign squalene oxides to lanosterol and lanosterol analogs (Clayton, van Tamelen & Nadeau, 1968; Corey, Lin & Jautelat, 1968). The underlying catalytic mechanism might conceivably be the same as the mechanism by which epoxide hydase catalyzes the hydration of arene and alkene oxides and the two reactions might be catalysed by the same enzyme. However, the cyclase activity has the reversed order of substitution requirements for optimal substrate activity (trisubstituted >

Table 5. Effect of the substitution pattern of the oxirane ring on the inhibitory potency of various styrene oxides towards hydration of [^3H]styrene oxide.

Data from Oesch (1973 b). Substrate ([^3H]styrene oxide) and inhibitor concn. 2 mM. The inhibitor was added in 20 μl acetonitrile at zero time with no preincubation. This amount of solvent had no significant effect on enzyme activity. Incubation mixture, conditions and assay as described (Oesch, *et al.*, 1971 a).

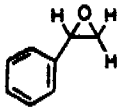
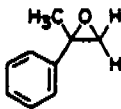
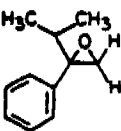
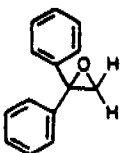
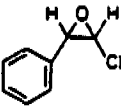
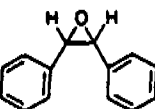
Inhibitor	% inhibition* of epoxide hydase purified from liver of:		
	Man	Guinea pig	Rat
	51 $\pm$ 2.1	49 $\pm$ 1.5	52 $\pm$ 2.2
	32 $\pm$ 4.1	30 $\pm$ 3.1	40 $\pm$ 4.3
	24 $\pm$ 3.8	22 $\pm$ 3.4	29 $\pm$ 5.0
	19 $\pm$ 2.7	18 $\pm$ 2.1	16 $\pm$ 3.2
	46 $\pm$ 3.0	74 $\pm$ 6.1	73 $\pm$ 4.2
	21 $\pm$ 8.1	16 $\pm$ 3.2	14 $\pm$ 2.3

Table 5 (contd.)

Inhibitor	% Inhibition* of epoxide hydase purified from liver of:		
	Man	Guinea pig	Rat
	ns	ns	ns
	ns	ns	ns
	ns	ns	ns
	ns	ns	ns
	ns	ns	ns

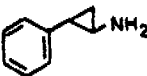
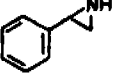
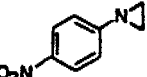
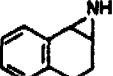
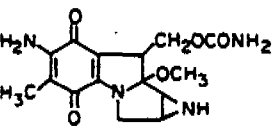
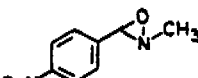
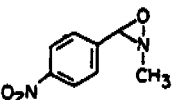
*Significant effect with $P < 0.001$ (ns = not significant).

trans-disubstituted > *cis*-disubstituted > monosubstituted oxiranes) (Clayton, *et al.*, 1968; Corey, *et al.*, 1968) than the ones discussed above for the hydase activity. Moreover, during purification of epoxide hydase, squalene oxidocyclase has been virtually completely removed (Oesch & Daly, 1971) clearly demonstrating that the cyclase is a different enzyme.

Cycloalkene oxides and arene oxides behaved in a similar manner to acyclic alkene oxides in that tri- or tetra-substituted oxiranes did not (or only marginally) interact with epoxide hydase both as substrates or inhibitors. Among a variety of cycloalkene oxides, cyclohexene oxide and 1,2,3,4-tetrahydronaphthalene 1,2-epoxide were outstanding in that they were potent inhibitors while at the same time they were very poor substrates. Kinetic analysis proved the inhibition to be *non*-competitive with respect to the substrate, [^3H]styrene oxide (Fig. 6), thus providing an explanation for the discrepancy between activities as substrate or as inhibitors (Oesch, *et al.*, 1971 c).

Table 6. Epoxide analogs as inhibitors of purified epoxide hydrases with [^3H]-styrene oxide as substrate

Data from Oesch *et al.* (1971 c). Several 5- and 6-membered cyclic ethers had no significant effect on epoxide hydase activity. Substrate ([^3H]styrene oxide) and inhibitor concn. 2 mM. The purified epoxide hydase preparation (Oesch & Daly, 1971) was used (40 μl per incubation). The inhibitor was added in 20 μl of appropriate solvent (in most cases acetonitrile) at zero time with no preincubation. The same amount of solvent was added to the controls. Incubation mixture, conditions and assay as described (Oesch, *et al.*, 1971 a).

Inhibitor	% inhibition*
	ns
	ns
	41
	ns
	ns
	ns
	Stimulates (+45)
	ns
	ns
	43

*Significant effect with $P < 0.001$ (ns = not significant).

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Sulphur, nitrogen or carbon analogues of oxirane compounds proved either inactive or less potent than the parent epoxides (Table 6). Thus, cyclopropanes and aziridines had no inhibitory activity. Styrene sulphide was quite an active inhibitor (comparable to styrene oxide) whereas cyclohexene sulphide was inactive. The former is an analogue to a competitive epoxide inhibitor (styrene oxide) whereas the latter is an analogue to a potent *non*-competitive epoxide inhibitor (cyclohexene oxide), thus suggesting distinctly differing structural requirements for the sites involved in competitive and *non*-competitive inhibition. Interestingly, *cis*-*N*-methyl-*p*-nitrophenyloxaziridine was quite an active inhibitor whereas the *trans*-isomer was completely inactive (Oesch, *et al.*, 1971 c). This behaviour closely parallels the dependency of activity on *cis*- versus *trans*-stereochemistry of 1,2-disubstituted oxiranes which has been discussed above.

Styrene and 1,2-dialin, olefins corresponding to a potent competitive (styrene oxide) and *non*-competitive inhibitor (1,2,3,4-tetrahydro-naphthalene 1,2-epoxide) had weak inhibitory activities (11 and 23%, respectively, at 2×10^{-3} M). Hydration of these olefins to alcohols by the purified epoxide hydase preparation in analogy to a bacterial hydase which adds water both to epoxides and to olefins (Niehaus, *et al.*, 1970) could not be detected (Oesch, *et al.*, 1971 c).

Inhibitors of epoxide hydase *in vivo* could be useful for assessing the role of epoxides in drug toxicity and carcinogenicity and for delineating the role of epoxide hydase in preventing mutagenic, carcinogenic or cytotoxic effects produced by several aromatic and olefinic compounds. The most promising candidates for *in vivo* inhibition of epoxide hydase were the potent oxirane inhibitors which at the same time are very poor substrates for epoxide hydase, 1,1,1-trichloropropene 2,3-oxide and cyclohexene oxide. Besides their potent inhibitory activity towards hydration of [^3H]styrene oxide they are also very potent inhibitors towards hydration of naphthalene 1,2-oxide and benzene oxide but have, on the other hand, no effect on the mono-oxygenase activity of liver microsomes (determined with [4- ^3H]benzenesulphonanilide as substrate) (Oesch, *et al.*, 1973 a). However, all attempts to inhibit epoxide hydase *in vivo* have so far failed, even using very high (toxic) doses of these potent *in vitro* inhibitors.

Studies on biopsy specimens showed that human liver also possesses the capability to convert epoxides enzymically to vicinal diols. Human epoxide hydase was also localized exclusively in microsomal membranes. Structure-activity relationships for substrates and inhibitors of the purified human enzyme were qualitatively identical with the ones discussed above for the purified guinea-pig enzyme (Tables 4 and 5). The same was true for several other properties such as pH optimum, Michaelis constant and lack of requirement for low molecular weight co-factors (Oesch, 1973 a, b). However several quantitative differences were noted between preparations solubilized and purified simultaneously from liver microsomes of guinea-pig, rat and man (Tables 4 and 5). For a more complete discussion on these aspects see Oesch (1973 b) and Oesch, *et al.*, (1973 c).

Evidence for more than one hepatic epoxide hydase

About 30% of the activity of the epoxide hydase preparation solubilized from guinea-pig liver microsomes was lost within 24–36 h after completion of the last purification step. This occurred whether the preparation was stored

frozen (-15°) or between 0° and 5° . However the remaining activity was remarkably stable. In fact, almost full activity was recovered after heating at 60° for 5 min. Moreover, no decrease in activity was observed after several freeze-thaw cycles, after preincubation for 40 min at 37° or after storage at -15° for more than 6 months (Oesch & Daly, 1971 and unpublished observations). These differential stabilities suggested the presence of more than one liver enzyme capable of hydration of epoxides. The ratio of microsomal hydrazase activity towards naphthalene oxide, benzene oxide and styrene oxide was approximately 5 : 2 : 1 in the guinea-pig (Oesch, *et al.*, 1971 b) but in the rat 6 : 5 : 1 (Oesch, *et al.*, 1973 a), suggesting the possibility that a separate enzyme might be involved in the hydration of benzene oxide. Moreover, cyclohexene oxide, another oxirane fused to a six-membered carbocycle was much more potent an inhibitor towards hydration of benzene oxide than towards hydration of styrene oxide or naphthalene 2,3-oxide. Thus, hydration of benzene oxide was completely prevented by cyclohexene oxide at a concentration one eighth of that of the substrate whereas equimolar concentrations of cyclohexene oxide inhibited hydration of naphthalene oxide or styrene oxide only by about 50% (Oesch, *et al.*, 1973 a). More direct evidence for the existence of a separate benzene oxide hydrazase was obtained from comparing the rate at which hydration of various epoxides proceeded with the crude homogenate (600 g supernatant) and with the purified preparation. The hydration of several structurally unrelated alkene oxides and arene oxides proceeded at an approximately 30-fold enhanced rate with the purified preparation ('purification factors' of 26-30) with the single exception of benzene oxide ('purification factor' of 4) (Oesch, *et al.*, 1971 b). Thus, it appeared that on the one hand either an epoxide hydrazase with a broad substrate 'specificity' was present or several more specific epoxide hydrazases had been purified to the same extent, whereas on the other hand a separate 'benzene oxide hydrazase' had been largely removed by this purification procedure. Accordingly, benzene oxide does not inhibit hydration of styrene oxide even at concentrations four-fold that of styrene oxide while other arene oxides such as naphthalene oxide or phenanthrene oxide and other alkene oxides such as octene 1,2-oxide or phenoxy epoxypropyl ethers effectively inhibit hydration of styrene oxide at concentrations smaller than that of styrene oxide (Oesch, *et al.*, 1971 c). This lack of cross inhibition by benzene oxide was observed not only with the purified epoxide hydrazase preparation but also with microsomes and crude homogenates (from several species) suggesting that the different purification factor with benzene oxide was not due to alterations of the enzyme protein during solubilization but rather to the presence of at least two distinct hepatic epoxide hydrazases, one with a rather broad substrate specificity and another with a rather narrow substrate specificity for benzene oxide and possibly some other closely related epoxides. Attempts to isolate 'benzene oxide hydrazase' are now in progress.

Evidence for a coupled mono-oxygenase-epoxide hydrazase complex

Spectral studies with an olefin, 5 α -androst-16-en-3 α -ol, and a corresponding epoxide, 16 α , 17 α -epoxy-5 α -androstan-3 α -ol, showed that both steroids interacted with cytochrome P-450 with similar affinities ($K_s \sim 45 \mu M$) (Von Bahr, Brandt & Gustafsson, 1972) implying that epoxide hydrazase and cytochrome P-450 might

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However, a variety of other evidence suggests the presence of a relatively stable mono-oxygenase-epoxide hydrase multienzyme complex, besides a 'free' epoxide hydrase in liver microsomes. Despite the property of arene oxides to rearrange quickly to the corresponding phenols substantial amounts of dihydrodiols can be isolated from the urine of animals treated with aromatic hydrocarbons (Jerina, *et al.*, 1970 b, and references therein). This suggests a close physical relationship of mono-oxygenase(s), responsible for the formation of arene oxides, and epoxide hydrase(s), responsible for their conversion to dihydrodiols (cf. section on the fate of oxiranes). If such a coupled mono-oxygenase-epoxide hydrase system catalyzes the overall conversion of naphthalene *via* the obligatory intermediate naphthalene 1,2-oxide (see the section on biological formation of oxiranes) to naphthalene 1,2-dihydrodiol, radioactive naphthalene oxide, generated *in situ* from radioactive naphthalene, should not completely equilibrate with added non-radioactive naphthalene oxide. Indeed, the specific radioactivity of naphthalene dihydrodiol was markedly greater than that of naphthol after such an experiment with guinea-pig liver homogenate*. With the same enzyme preparation, 0.5 mM 1,1,1-trichloropropene 2,3-oxide effectively inhibited dihydrodiol formation from (exogenous) naphthalene oxide (inhibition of 'free epoxide hydrase') but not from naphthalene (no inhibition of 'coupled epoxide hydrase'). Accordingly, when radioactive naphthalene and non-radioactive naphthalene oxide were incubated together, addition of 1,1,1-trichloropropene 2,3-oxide at a concentration which selectively inhibits the 'free epoxide hydrase' (0.5 mM) produced a marked increase in specific radioactivity of the product dihydrodiol. Moreover, dihydrodiol formation from naphthalene oxide was much more enhanced after pre-treatment of rats with phenobarbital than with 3-methylcholanthrene. For the formation of dihydrodiol from naphthalene the converse was true (Oesch & Daly, 1972, and unpublished results). Solubilized and purified cytochrome P-450 and cytochrome P-448 preparations from rat liver contained high levels of both epoxide hydrase and mono-oxygenase activity, whereas other microsomal enzymes, such as glucose-6-phosphatase or cytochrome c reductase, were absent (Oesch, *et al.*, 1972). Thus, it appears that some mono-oxygenase(s) and epoxide hydrase(s) exist as a fairly stable multi-enzyme complex which in contrast to 'free epoxide hydrase' is relatively insensitive to inhibition by 1,1,1-trichloropropene 2,3-oxide and, again in contrast to free epoxide hydrase, is induced selectively by 3-methylcholanthrene. Moreover, this complex can be purified by procedures in which other microsomal enzymes are lost. With these purified cytochrome P-450 and cytochrome

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* Homogenates were used since the coupled mono-oxygenase-epoxide hydrase system did not reproducibly survive the resuspension procedure after isolation of microsomes. Glutathione had been removed from the homogenates by titration with diethyl maleate prior to these experiments.

P-448 preparations it was, after reconstitution with cytochrome c reductase and a lipid fraction, possible to catalyse the following reactions :

1. The mono-oxygenase reaction, namely the molecular oxygen and NADPH dependent transformation of naphthalene to naphthalene oxide and to its isomerization product naphthol. The formation of naphthalene oxide was demonstrated by radiotracer trapping technique, isomerization of the isolated naphthalene oxide and re-isolation as 1-naphthol.
2. The epoxide hydase reaction, namely transformation of synthetic naphthalene oxide to naphthalene dihydrodiol.
3. The mono-oxygenase and epoxide hydase reactions coupled, namely the molecular oxygen and NADPH dependent transformation of naphthalene to naphthalene dihydrodiol (Oesch, *et al.*, 1972).

Mono-oxygenase activity towards naphthalene was similar in the reconstituted P-450 and P-448 systems whereas towards benzo(a)pyrene the mono-oxygenase activity was more than ten-fold higher in the reconstituted P-448 system. Interestingly, the ratio of dihydrodiol to naphthol after incubation of naphthalene was markedly higher with the reconstituted P-448 system (obtained from rats pretreated with 3-methylcholanthrene) as compared to the reconstituted P-450 system (obtained from rats pretreated with phenobarbital), although total levels of epoxide hydase (determined with naphthalene oxide or styrene oxide as substrate) were higher in the cytochrome P-450 preparation (Oesch, *et al.*, 1972). This is in good agreement with earlier observations, that 3-methylcholanthrene selectively induces the coupled mono-oxygenase-epoxide hydase system (Oesch & Daly, 1972), whereas total levels of epoxide hydase are much more induced by phenobarbital than by 3-methylcholanthrene (Oesch, *et al.*, 1971 a, 1973 a. The figure is mislabelled in the first publication but the results are stated correctly in the text).

Such a coupled mono-oxygenase-epoxide hydase enzyme system may be of great relevance to several problems such as the hepatotoxicity of intermediate arene oxides derived from halobenzenes and the carcinogenic properties of intermediate arene oxides derived from several polycyclic aromatic hydrocarbons, by circumventing these adverse effects of intermediate arene oxides by their rapid conversion to dihydrodiols. Thus, modestly decreased glycogen levels and moderate centrilobular necrosis of hepatocytes after treatment of 200-220 g male Sprague-Dawley rats with a moderate dose of chlorobenzene (600 mg/kg in cottonseed oil, injected intraperitoneally 24 h before sacrifice of the animals) was dramatically increased if the animals had been pre-treated with phenobarbital (0.2% in the drinking water for 3 days), a potent mono-oxygenase inducer (Remmer, 1959). However, pre-treatment of the animals with 3-methylcholanthrene (40 mg/kg in cottonseed oil, one intraperitoneal injection/day for 3 days), another potent mono-oxygenase inducer (Conney, Miller & Miller, 1956), did not produce an increase and in more than 90 per cent of the animals completely blocked the necrosis and glycogen depletion after the same dose of chlorobenzene (unpublished results). Similar effects of 3-methylcholanthrene pretreatment of rats on bromobenzene-induced hepatotoxicity led Jollow, Zampaglione & Gillette (1971) to suggest, that this protection from hepatotoxicity by

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3-methylcholanthrene may be afforded by induction of epoxide hydase activity. However, the results presented above show that the total levels of epoxide hydase are increased to a much greater extent by pre-treatment of rats with phenobarbital than with 3-methylcholanthrene, yet phenobarbital does not protect from chloro-benzene-evoked hepatotoxicity but rather dramatically increases necrosis of hepatocytes. The enigma may be solved, by taking into account that, while dihydrodiol formation from naphthalene 1,2-oxide with liver preparations from rats pre-treated with phenobarbital was much more enhanced than after pre-treatment of animals with 3-methylcholanthrene, for dihydrodiol formation from naphthalene the converse was true, i.e. the coupled mono-oxygenase-epoxide hydase system is selectively induced by 3-methylcholanthrene (Oesch & Daly, 1972). Observation of increased levels of dihydrodiol and catechol in the urine of animals pre-treated with 3-methylcholanthrene as compared to controls (Jollow, *et al.*, 1971) is consonant with this view. Thus, it now appears, that this coupled mono-oxygenase-epoxide hydase multienzyme complex is of great importance for the metabolism of foreign aromatic and olefinic compounds, especially with respect to shortening the lifetime of intermediate oxiranes, converting them rapidly to dihydrodiols before they react with nucleophilic moieties of cell constituents and thereby cause cytotoxic, mutagenic or carcinogenic effects. Selective inhibitors of this coupled system may provide insight into the mechanism of these adverse reactions, whereas selective activators or inducers may even prove useful in preventing them.

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