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ACCUMULATION OF AN EPOXY INTERMEDIATE DURING THE HEPATIC MICROSOMAL METABOLISM OF *cis*-STILBENE TO *threo*-STILBENE GLYCOL DUE TO THE INHIBITION OF EPOXIDE HYDROLASE BY *trans*-STILBENIMINE

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Abstract—Hepatic microsomal epoxide hydrolase catalyzing the conversion of a wide variety of epoxides, including *cis*-stilbene oxide (the model substrate used in the present study), to glycols was inhibited by *trans*-stilbenimine. *cis*-Stilbenimine was less active, and polar aziridines such as mitomycin C, ethylenimine, and iminocyclohexane had little effect. The inhibition by *trans*-stilbenimine was competitive, suggesting the identity of epoxide hydrolase with aziridine hydrolase which has previously been shown to catalyze the conversion of aziridines to the corresponding amino-alcohols in the same stereochemical manner as that in the enzymatic hydrolysis of epoxides. Addition of *trans*-stilbenimine to a microsomal reaction system, containing NADPH and *cis*-stilbene as the substrate, led to the accumulation of the epoxy intermediate, *cis*-stilbene oxide, with concomitant formation of *threo*-stilbene glycol. The aziridine also inhibited epoxidase and reduced the rates of formation of the *threo*-glycol from *cis*-stilbene and of heptachlor epoxide from heptachlor.

THE ALIPHATIC three-membered heterocyclics, epoxides and aziridines, many of which are known to be carcinogenic or toxic,[1] undergo hydrolytic ring cleavage by hepatic microsomal enzymes to yield the corresponding glycols[2–5] and amino-alcohols,[6,7] respectively. A similar reaction occurs in a variety of arene oxides.[8,9] The enzymes, epoxide hydrolase[2,3] (epoxide hydrase[4,8]; epoxide hydratase: EN, 1972, 4.2.1.63) and aziridine hydrolase,[6] therefore, play a key role *in vivo* in the detoxication and excretion of their substrates as polar metabolites.

Hydrolysis of the epoxide, 2β,3β-epoxy-5α-cholestane, by hepatic microsomes has recently been shown to be inhibited by the aziridine, 2β,3β-imino-5α-cholestane,[6] suggesting that aziridines prolong the half-life of the epoxy intermediate formed from an olefin by microsomal epoxidase. In the microsomal metabolism of olefins to glycols via epoxides, epoxidation of the olefin is the rate-limiting step,[3,11] and in most cases the existence of epoxide hydrolase makes it very difficult to detect the intermediate without using epoxide hydrolase inhibitors such as epoxides of other olefins although recently the epoxy intermediates from olefins such as styrene,[5] cyclohexene,[5] indene,[5] and stilbene[12] have been detected without the use of inhibitors.

The use of other olefin oxides to the epoxy intermediate was first reported by Watabe and Maynert to detect epoxides formed from 1- and 4-octenes[3,10] and has recently been applied by other workers to detect K-region epoxides as labile intermediates in the microsomal metabolism of carcinogenic polyaromatic hydrocarbons to dihydrodiols, e.g. cyclohexene oxide for the preliminary detection of benzpyrene

oxide[13] and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene for the detection of pyrene and benzpyrene oxides.[14] In the previous paper,[12] we demonstrated by using *cis*-stilbene as the substrate that the level of epoxy intermediate in the microsomal incubation system is largely affected by lipid peroxidation of microsomes.

In the present paper, the mechanism of the inhibition of enzymatic hydrolysis of *cis*-stilbene oxide by the aziridine, *trans*-stilbenimine an enzymatically unhydrolyzable inhibitor,[15] and the accumulation of the epoxy intermediate, *cis*-stilbene oxide, formed after incubation of *cis*-stilbene with hepatic microsomes in the presence of NADPH and the aziridine are reported. Evidence shows that the aziridine is also a weak inhibitor of epoxidase.

MATERIALS AND METHODS

Materials. cis-Stilbene oxide,[16] *threo*-stilbene glycol,[17] *meso*-stilbene glycol,[18] *cis*- and *trans*-stilbenimines,[19] iminocyclohexane,[20] and heptachlor epoxide[21] were prepared by established methods. Ethylenimine was purchased from Wako Pure Chemicals Co., Tokyo, and NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase from Boehringer Mannheim Co.

Assay of enzymatic reactions. Microsomes were prepared from the liver of five male albino rabbits (2·5–2·8 kg) as previously described,[22] washed twice with 30 volumes of 0·1 M phosphate buffer, pH 7·4, and suspended in the same buffer.

The formation of *threo*-stilbene glycol during the incubation of *cis*-stilbene oxide with the microsomes was determined as follows. The oxide dissolved in benzene (2 ml), was suspended by vigorous agitation in 0·1 M phosphate buffer, pH 7·4 (96 ml), containing 0·1% Tween 80, and the benzene was removed *in vacuo*. After adjusting the volume of the aqueous residue to 96 ml with the buffer so that the final substrate concentration was 7·7 mM, aliquots of the suspension were diluted with the phosphate buffer containing 0·1% Tween 80 to give various substrate concentrations. The substrate suspensions (4·8 ml each) were incubated at 37° with the microsomal suspension (5 ml) after the addition of acetone (0·2 ml). When aziridines were added to the reaction mixtures, they were included in the acetone. The reaction was terminated by the addition of 5 N NaOH (2 ml) at various times so that enzymatic glycol formation corresponded to 5–10 per cent of the substrate used. *threo*-Stilbene glycol formed was quantitatively extracted with ether following saturation of the mixture with sodium chloride. It was isolated from ether-extractable microsomal components by preparative t.l.c. (silica gel containing an inorganic phosphor agent (Wakogel B-5 UA), benzene-acetone (8:1)); *meso*- stilbene glycol was added to the extract as an internal reference for subsequent g.l.c. analysis. The isomeric *threo*- and *meso*-glycols, visualized under ultraviolet light (225 nm), both had an R_f value of 0·28. The glycols were eluted from the chromatogram with ethanol and then trimethylsilylated in the standard manner. Trimethylsilyl ethers of *threo*- and *meso*-stilbene glycols appeared at 25·3 and 28·6 min, respectively, in gas-chromatograms obtained by using a 1.5% succinate polyester column as described previously.[12] Incubation of *cis*-stilbene and heptachlor with hepatic microsomes in the presence of NADPH, generated from NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase, identification of enzymatically formed *cis*-stilbene oxide, *threo*- and *meso*-stilbene glycols, and heptachlor epoxide by g.l.c. and g.l.c.-mass spectrometry, and quantitative determina-

tion of reaction products in these reactions by g.l.c. were carried out as described previously.[12]

RESULTS

Inhibition of enzymatic hydrolysis of cis-*stilbene oxide by aziridines.* The previously demonstrated stereospecific conversion of *cis*-stilbene oxide to *threo*-stilbene glycol by hepatic microsomal epoxide hydrolase[23,24] was inhibited by *trans*- and *cis*-stilbenimines but not by polar aziridines such as mitomycin C, ethylenimine, and iminocyclohexane (Table 1).

TABLE 1. INHIBITION BY AZIRIDINES OF *threo*-STILBENE GLYCOL FORMATION FROM *cis*-STILBENE OXIDE BY HEPATIC MICROSOMAL EPOXIDE HYDROLASE

% Inhibition by aziridines				
trans SI	*cis*-SI	Mit C	EI	IC
39·0	26·8	ns	ns	ns

The reaction mixture consisted of *cis*-stilbene oxide (1 mM), rabbit liver microsomes (0·04 mg protein/ml), Tween 80 (0·048%, w/v), acetone (2%, v/v), and 0·1 M phosphate buffer, pH 7·4. Concentration of each aziridine in the mixture was 1·85 mM. The rate of *threo*-stilbene glycol formation from *cis*-stilbene oxide in the control reaction mixture was $3{\cdot}58 \times 10^{-7}$ M/mg protein/min. Under these conditions some of the *cis*-stilbenimine was separated as crystals from the reaction medium, and consequently its concentration was lower than 1·85 mM. Abbreviations used for aziridines are *trans*-SI, *trans*-stilbenimine; *cis*-SI, *cis*-stilbenimine; Mit C, mitomycin C; EI, ethylenimine, and IC, iminocyclohexane. ns: not significant.

The mechanism of the inhibition of *cis*-stilbene oxide hydrolysis by aziridines was investigated by using *trans*-stilbenimine, the most potent inhibitor that we tested. Double-reciprocal plots of data indicated that the inhibition was competitive. The Michaelis constant (K_m) for the substrate was 0·99 mM, the maximum velocity (V_{max}) was $4{\cdot}07 \times 10^{-7}$ moles/mg protein/min, and the inhibitor constants (K_i) were 2·04 mM and 4·0 mM at 1·85 mM and 3·7 mM concentrations of the inhibitor, respectively (Fig. 1).

Accumulation of the epoxy intermediate in the microsomal metabolism of cis-*stilbene to* threo-*stilbene glycol in the presence of* trans-*stilbenimine.* Incubation of *cis*-stilbene with hepatic microsomes in the presence of NADPH yielded *cis*-stilbene oxide together with *threo*-stilbene glycol; the epoxy intermediate decreased in yield during the reaction and disappeared from the mixture within 60 min, whereas the *threo*-glycol was still increasing at that time (Table 2). These results are in accordance with previous data suggesting that a selective decrease in epoxidase activity occurs by NADPH-dependent lipid peroxidation of microsomes.[12] Addition of the epoxide hydrolase inhibitor, *trans*-stilbenimine, markedly increased the formation of the epoxy intermediate at each interval checked: the ratio of the epoxide formed in reaction mixtures with and without the inhibitor were 4·7:1 and 8·3:1 at 15 and 30 min, respectively. The epoxide was detected even at 60 min when the inhibitor was present.

Formation of *meso*-stilbene glycol, which was previously been shown to be formed from the *threo*-glycol by the catalytic action of a dehydrogenase bound to the microsomes,[12] was also observed, but it was less than 2 per cent of the *threo*-glycol formed under these reaction conditions.

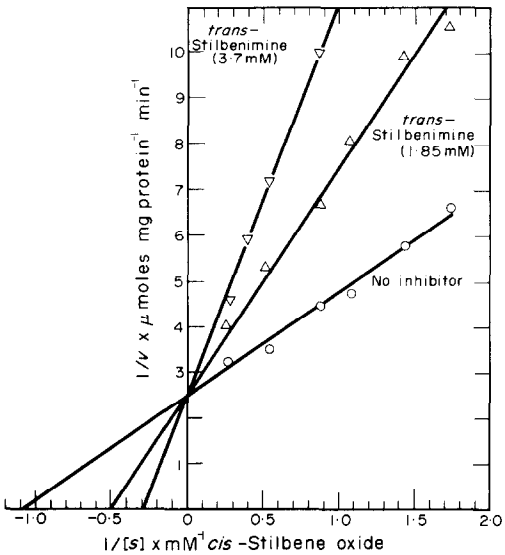
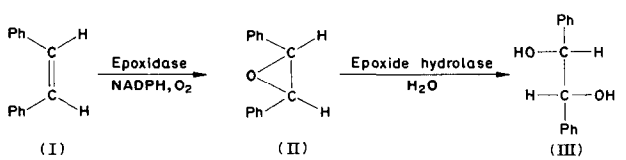


FIG. 1. Double-reciprocal plots of *cis*-stilbene oxide concentration vs rate of *threo*-stilbene glycol formation by rabbit liver microsomal epoxide hydrolase in the presence and in the absence of *trans*-stilbenimine as the inhibitor. Other constituents of the reaction mixture were as described in Table 1.

On the other hand, *trans*-stilbenimine markedly retarded the formation of the *threo*-glycol, suggesting its inhibitory effect on microsomal epoxidase activity: total amounts of the *cis*-oxide and *threo*-glycol formed were 53 and 45 per cent of the control at 15 and 30 min, respectively (Table 2). To determine whether the aziridine inhibits epoxidation of other olefinic substrates, heptachlor whose sole metabolite by hepatic microsomes has been demonstrated to be its epoxide,[25] was used. Data indicated that the aziridine also had an inhibitory effect on this reaction (Table 3).

TABLE 2. ACCUMULATION OF *cis*-STILBENE OXIDE (II) IN THE HEPATIC MICROSOMAL METABOLISM OF *cis*-STILBENE (I) TO *threo*-STILBENE GLYCOL (III) DUE TO THE INHIBITORY EFFECT OF *trans*-STILBENIMINE ON EPOXIDE HYDROLASE



Incubation time (min)	Control II formed (μM)	Control III formed (μM)	*trans*-Stilbenimine added II formed (μM)	*trans*-Stilbenimine added III formed (μM)
15	6·1	84·4	28·9	19·2
30	2·2	116·7	18·2	35·6
60	0·0	136·5	2·6	42·7

The reaction mixture consisted of *cis*-stilbene (2 mM), rabbit liver microsomes (1·73 mg protein/ml), NADP (0·5 mM), glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (1 IU/ml), magnesium chloride (5 mM), nicotinamide (5 mM), acetone (2% v/v), and 0·1 M phosphate buffer, pH 7·4. *trans*-Stilbenimine (10 mM) was added together with the substrate dissolved in the acetone.

TABLE 3. INHIBITION OF MICROSOMAL EPOXIDATION OF HEPTACHLOR BY *trans*-STILBENIMINE

trans-Stilbenimine	Heptachlor epoxide formed (μM)
None	596
Added	72

Heptachlor (2 mM) was incubated with rabbit liver microsomes in the presence of NADPH for 30 min in air under the same conditions as described in Table 2.

However, the effect was much weaker than that of SKF 525-A (β-diethylaminoethyldiphenylpropyl acetate); the ratio of inhibition of enzymatic heptachlor epoxide formation by the aziridine and SKF 525-A was 1:11 at an inhibitor concentration of 0·1 mM.

DISCUSSION

The present study demonstrates that the addition of basic nitrogen analogues, *trans*- and *cis*-stilbenimines, to incubation mixtures containing rabbit liver microsomes inhibits *threo*-stilbene glycol formation from *cis*-stilbene oxide; other more polar aziridines have little inhibitory effect. From these data it appears that the inhibitory effect of aziridines on microsomal epoxide hydrolase may be related to their hydrophobic characters. However, Oesch *et al.*[26] using a soluble epoxide hydrolase preparation obtained from the liver of guinea-pigs demonstrated that some hydrophobic aziridines such as 2-phenyl- and 1-*p*-nitrophenyl-aziridines have little inhibitory effect on the hydrolysis of styrene oxide, whereas a stimulatory effect occurred with the hydrophobic aziridine, iminotetrahydronaphthalene. Our data are in accordance with theirs with respect to mitomycin C. Although it is very difficult to interpret these results, the variance might be due to a difference in the epoxy substrates used in the investigations since the presence of at least two epoxide hydrolases in hepatic microsomes has previously been suggested.[27]

Since only a partially purified hydrolase has been obtained from hepatic microsomes,[28,29] a kinetic method, i.e. whether the inhibition of enzymatic hydrolysis of the epoxide by the aziridine is competitive or not, may resolve the question of identity of epoxide hydrolase with aziridine hydrolase. The present evidence for the competitive inhibition of enzymatic hydrolysis of *cis*-stilbene oxide by *trans*-stilbenimine strongly suggests that hepatic microsomal epoxide hydrolase, or at least one of the hydrolases, if present in multiple forms as has previously been suggested,[27] is identical with aziridine hydrolase and also, as predicted from the results of stereochemical studies of enzymatic hydrolysis of various types of olefin oxides,[5,11,23,24,30,31] that the active site of the enzyme has a dissociating hydrogen with which the oxygen of the epoxide could interact. The nitrogen of the aziridine also could interact more readily with the active site since hydrogen bonding abilities of heteroatoms of three-membered heterocyclics toward a protonic group of an organic molecule are known to be greater in the aziridine than in the epoxide.[32] Thus, the aziridine could compete with the epoxide upon the interaction of the latter with the protonic active site of the enzyme and consequently block the formation of a hydrolase-epoxide complex. The glycol is similarly formed by a push–pull mechanism, which involves the rear side attack of a hydroxyl anion from water and the

simultaneous cleavage of the C—O bond. Moreover, the rate-limiting step in the enzymatic reaction has been shown to be at the stage of the introduction of the hydroxyl anion.[29] The fact that *trans*-stilbenimine is not hydrolyzed by the microsomal hydrolase[15] despite its suggested stronger interaction with the enzyme than *cis*-stilbene oxide, can be attributed to stability of the aziridine ring[7]; the aziridine, in general, has lower ring strain energy than the epoxide.[33]

The rate of formation of the epoxy intermediate, *cis*-stilbene oxide, and its half-life in the microsomal metabolism of *cis*-stilbene to *threo*-stilbene glycol have recently been demonstrated to be affected by lipid peroxidation of microsomes.[12] An increase in the lipid peroxidation which occurs during the incubation of microsomes under aerobic conditions has little influence on epoxide hydrolase activity but it does give rise to a rapid decrease in epoxidase activity. Increasing the relative activity of epoxide hydrolase to that of the epoxidase results in the disappearance of the epoxy intermediate from the reaction mixture.[12] The presence of *trans*-stilbenimine, a competitive inhibitor of epoxide hydrolase markedly increased epoxide concentrations in reaction mixtures and prolonged its half-life. Although the aziridine inhibited both epoxide hydrolase and epoxidase, as estimated from the rate of *threo*-glycol formation or more directly of heptachlor epoxide formation, the present data indicate that it decreases the relative activity of epoxidase to some extent. However, a comparison of the degree of inhibition of both enzymes could not be made since there was a large difference in the amount of microsomal protein used in the epoxide hydrolysis and epoxide formation reactions; an increase in the protein content in the former reaction to that used in the latter reduced the reaction time to an unsuitable degree.

Epoxide accumulation suggests that the well known carcinogenicity or toxicity of aziridines[1] might be attributable in part to their inhibitory effect on epoxide hydrolase which plays a key role in the detoxication and excretion of toxic or carcinogenic epoxides formed as obligatory intermediates in the metabolism of natural or foreign olefins although little is known about the physiological activity of the aziridine used as the model inhibitor in our investigation.

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