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MICROSOMAL EPOXIDATION OF *CIS*-STILBENE: DECREASE IN EPOXIDASE ACTIVITY RELATED TO LIPID PEROXIDATION

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Abstract—Incubation of *cis*-stilbene with rabbit liver microsomes in the presence of NADPH yielded *threo*-stilbene glycol and a trace of *meso*-stilbene glycol. The ratio of epoxidase activity to epoxide hydrolase activity decreases markedly so that the epoxy intermediate, *cis*-stilbene oxide, can be demonstrated only during brief incubations. These data indicate that lipid peroxidation of microsomes plays an important role in regulating not only the rate of epoxide formation from the olefin, but also the half life of the epoxide formed *in vitro*.

EPOXIDES, as obligatory intermediates in the metabolism of olefins to glycols by hepatic microsomes of mammals, are rapidly hydrolyzed by microsomal epoxide hydrolase and this makes their recognition difficult without using epoxide hydrolase inhibitors.[1–6] Similar evidence has also been obtained in the microsomal metabolism of aromatic hydrocarbons to the corresponding dihydrodiols.[7–12] However, in the case of some polychlorinated cyclodiene insecticides, heptachlor and related toxicants aldrin and isodrin, epoxides that are rather stable in a number of living organisms are produced by biological oxidation *in vitro*.[13,14]

Recent studies on the metabolic pathway involving epoxide formation from olefins and arenes gave a clue to solving problems concerning proximal active forms of olefinic toxicants and chemical carcinogens such as polyaromatic hydrocarbons, for it is well known that a variety of olefin oxides are toxic or carcinogenic[15] and it has recently been demonstrated that K-region epoxides of a number of carcinogenic aromatic hydrocarbons form covalent bondings with nucleic acids and proteins.[9,16–19] Therefore, it is assumed that factors affecting the half life of the epoxide formed play a key role in the toxicity or carcinogenicity of the olefin or arene, of which the most important and ultimate one could be the difference in activities between epoxidase and epoxide hydrolase, i.e. an increase in the ratio of epoxidase activity to epoxide hydrolase activity will lead to an increase in epoxide level in the animal body.

We wish to report that in rabbit liver microsomes the ratio of epoxidase activity to epoxide hydrolase activity decreases markedly during incubation so that the epoxy intermediate, *cis*-stilbene oxide, in the microsomal metabolism of *cis*-stilbene to *threo*-stilbene glycol can be demonstrated only during brief incubations and also that such phenomena originate at least in considerable part from NADPH-dependent lipid peroxidation of the microsomes.

MATERIALS AND METHODS

Materials. *cis*-Stilbene oxide,[20] *threo*-stilbene glycol,[21] *meso*-stilbene glycol,[22] and heptachlor epoxide[23] were prepared by the previous methods. $NADP^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Boehringer Mannheim Co.

Enzyme studies. Microsomes were obtained from the liver of five male albino rabbits (2·5–2·8 kg) by the previously reported method,[24] washed twice with 30 times the volume of 0·1 M phosphate buffer, pH 7·4, and homogeneously suspended in the same buffer.

When *cis*-stilbene was used as the substrate, it was dissolved in acetone, mixed with the microsomal suspension including an NADPH-generating system, and incubated at 37° in air with shaking. Final concentrations of constituents of the mixtures in 0·1 M phosphate buffer, pH 7·4, were *cis*-stilbene: 2 mM, $NADP^+$: 0·5 mM, glucose 6-phosphate dehydrogenase: 1 IU/ml, glucose 6-phosphate, nicotinamide, magnesium chloride: 5 mM each, microsomal protein: 1·73 mg/ml, and acetone: 2% (v/v).

For estimating the effect of lipid peroxidation of microsomes on epoxidase activity using heptachlor as the substrate, the microsomal suspension was preincubated at 37° in air in the absence and in the presence of the NADPH-generating system. The cofactor system, consisting of NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and magnesium chloride in 0·1 M phosphate buffer, pH 7·4, to make a final volume of 1 ml was incubated at 37° for 10 min prior to the addition to the preincubation flask. The final concentration of heptachlor was 1 mM. Other constituents of the reaction mixture, their final concentrations, and incubation conditions were the same as described in *cis*-stilbene. Ferrous sulfate (0·2 mM) and EDTA (1 mM) were also preincubated with microsomes in the presence of the cofactor system when their effects on epoxidase and epoxide hydrolase activities were examined. For estimating the effect of lipid peroxidation of microsomes on epoxide hydrolase activity using *cis*-stilbene oxide (1 mM) as the substrate, the above mentioned preincubated microsomal systems were diluted 40 times with 0·1 M phosphate buffer, pH 7·4, mixed with the epoxy substrate dissolved in acetone, and incubated under the same conditions as described above.

All the enzymatic reactions were terminated by the addition of 5 N NaOH to make a final concentration of 1 N. Following saturation of the mixtures with sodium chloride, unchanged *cis*-stilbene, heptachlor and their epoxides were extracted with three volumes of light petroleum (b.p. 30–40°) and stilbene glycols with three volumes of peroxide-free ether. An aliquot of each organic layer separated by centrifugation was transfered into a test tube and then the solvent evaporated under very mild conditions. Residues obtained from light petroleum extracts were dissolved in hexane and the ones from ether extracts in isopropyl ether for subsequent chromatographic analyses.

Chromatography. Thin-layer chromatography (t.l.c.) was carried out using plates coated with silica gel containing an inorganic phosphor agent (Wakogel B-5 UA, Wako Pure Chemicals Co., Tokyo). Chromatograms of biological extracts containing *cis*-stilbene and its oxide were developed once more in the same solvent system for the complete separation of the latter from the former and from microsomal components extracted. Elution of biologically formed *cis*-stilbene oxide from chromatograms was carried out with ethanol using the usual preparative t.l.c. technique.

Trimethylsilylation of the ethanolic eluates containing stilbene glycols was carried out in the standard manner[25] after evaporation of the solvent under a nitrogen stream.

A Shimadzu Model GC-1C gas-chromatograph equipped with a flame ionization detector was used for g.l.c., and conditions used were: column—0·75% SE-30 on Chromosorb W (60–80 mesh, 4 mm × 180 cm); column temperature—130°; nitrogen as carrier gas—35 ml/min for *cis*-stilbene oxide; column—15% succinate polyester on Shimalite (60–80 mesh, 4 mm × 180 cm); column temperature—150°; nitrogen—35 ml/min for trimethylsilyl ethers of stilbene glycols; and column—the same as that used for *cis*-stilbene oxide; column temperature—160°; nitrogen—40 ml/min for heptachlor and its epoxide.

Determination of lipid peroxidation. Lipid peroxidation of microsomes was determined by the standard method using thiobarbituric acid.[26]

RESULTS AND DISCUSSION

Conversion of cis-*stilbene to* threo-*stilbene glycol via* cis-*stilbene oxide.* For detecting and determining the epoxy intermediate, *cis*-stilbene oxide, in an incubation mixture, including *cis*-stilbene as the substrate, rabbit liver microsomes, and NADPH, the reaction was terminated at a brief interval (15 min), and it was extracted with light petroleum. The crude extract obtained was subjected to t.l.c. for elimination of light petroleum-extractable microsomal components that interfere with subsequent g.l.c. and g.l.c.–mass spectrometry. In thin-layer chromatograms obtained in hexane–benzene (3:1), authentic *cis*-stilbene, *cis*-stilbene oxide, and *threo*-stilbene glycol had R_f values of 0·85, 0·53 and 0·0, respectively. Gas–liquid chromatography–mass spectrum of an ethanolic eluate obtained from the thin-layer chromatogram, corresponding to the area of adsorbent layer for the intermediate, indicated the extract to contain *cis*-stilbene oxide (Fig. 1A). The retention time (8·0 min on a 0·75% SE-30 at 110°) and mass spectrum of the biologically formed *cis*-oxide were identical with those of the authentic one. That no epoxide peak was observed in gas-chromatograms with samples similarly obtained from extracts of control reaction mixtures using boiled microsomes indicated the epoxide formation to be enzymatic.

For detecting and determining the corresponding glycol in the incubation mixture, the reaction was terminated at 15 min and it was extracted with ether. The crude ethereal extract obtained was also pretreated by t.l.c., carried out in benzene–acetone (8:1) by the same reason as mentioned in the case of the epoxy intermediate. An ethanolic eluate obtained from the area of the adsorbent layer, corresponding to stilbene glycol (R_f 0·28), was trimethylsilylated and gas-chromatographed. In gas-chromatograms obtained on 15% succinate polyester at 150° appeared two peaks with the same retention times as those of di-trimethylsilyl ethers of *threo*- and *meso*-stilbene glycols (28·6 and 25·3 min, respectively). Both isomeric glycols were inseparable under the above mentioned t.l.c. conditions. Gas-chromatograms indicated the ratio of the *meso*- to *threo*-glycols to be 2·1:97·9. Further identification of the major glycol formed was carried out by g.l.c.–mass spectrometry (Fig. 1B). The glycols formed by the incubation for 15 min was 3·8 per cent of the substrate used and the epoxide 0·79 per cent (Table 1).

It has been demonstrated in the previous paper that hepatic microsomal epoxide

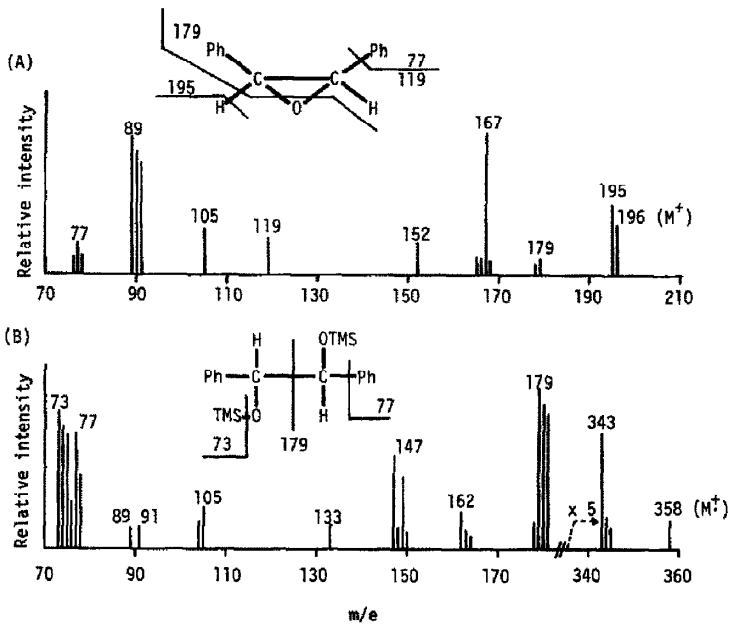
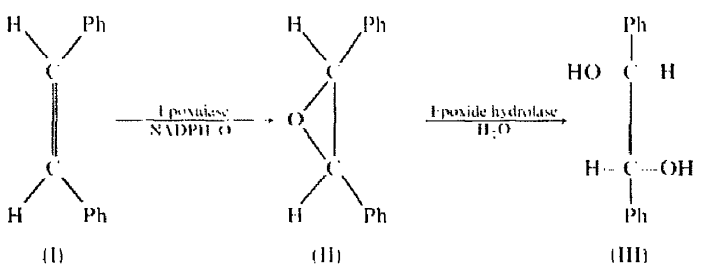


FIG. 1. Gas-liquid chromatography mass spectra of the epoxy intermediate (A) and the trimethylsilyl ether of the *threo*-glycol (B) formed from *cis*-stilbene by rabbit liver microsomes in the presence of NADPH. A Hitachi g.l.c.-mass spectrometer Model K-53-RM-7L was used. The base peak with *m/e* 167 in spectrum (A) was assigned as Ph_2CH^+ arising from rearrangement of the phenyl group in an epoxide ion and subsequent fragmentation with loss of a hydrogen (yielding a fragment ion peak with *m/e* 195) and carbon monoxide.

TABLE 1. OXIDATIVE METABOLISM OF *cis*-STILBENE TO *threo*-STILBENE GLYCOL VIA *cis*-STILBENE OXIDE BY RABBIT LIVER MICROSOMES IN THE PRESENCE OF NADPH



Incubation time (min)	*cis*-Stilbene oxide (II) formed (μM)	*threo*-Stilbene glycol (III) formed (μM)
15	15·9	76·7
30	2·7	106·1
60	0·0	124·3

Reaction conditions and methods for product analysis are as described in the text.

hydrolase catalyzes the stereospecific conversion of *cis*-stilbene oxide to *threo*-stilbene glycol and of *trans*-stilbene oxide to *meso*-stilbene glycol.[27] The simultaneous formation of the *threo*-glycol and of the trace amount of the *meso*-glycol appeared

resulted by the catalytic action of enzyme(s) bound to microsomes which seemed to be originally localized in the soluble supernatant fraction; the *meso*-glycol formed from *cis*-stilbene by the 15 min incubation with the 9000 *g* supernatant fraction of the rabbit liver homogenate in the presence of the same cofactor system as used for the microsomal incubation system accounted for 30·1 per cent of the total glycols formed.

Prolonged incubation of *cis*-stilbene up to 60 min led to the complete disappearance of the epoxy intermediate from the reaction mixture in spite of increasing formation of the *threo*-glycol (Table 1). The transient formation of the epoxy intermediate appeared to be due to a facile decrease in epoxidase activity during incubation of the microsomes since it is well known that microsomal epoxide hydrolase is stable enough on various treatments.[28,29] The falling off in monooxygenase activity with time is a general phenomenon in microsomal systems, being remarkably accelerated in the presence of NADPH and oxygen as a result of peroxidation of microsomal lipids.[30] Our attention, therefore, was payed on the problem whether or not lipid peroxidation of hepatic microsomes affected the half life of the epoxy intermediate *in vitro*.

Effect of lipid peroxidation of microsomes on epoxidase and epoxide hydrolase activities. An attempt was made to clarify whether or not relative activities of both enzymes were altered during the incubation of the microsomes with NADPH. In this study heptachlor whose sole metabolite in the microsomal system has been shown to be its epoxide[13,14] was used as the substrate for the assay of epoxidase activity and *cis*-stilbene oxide for epoxide hydrolase activity.

Preincubation of the microsomes at 37 in the presence of NADPH caused a marked decrease in epoxidase activity (Fig. 2A). However, when the microsomes were preincubated in the absence of NADPH, the decrease was much smaller. The curve for the NADPH-dependent decrease in epoxidase activity was almost coincident with a reciprocal pattern of the NADPH-dependent lipid peroxidation of the microsomes which was determined by the thiobarbituric acid method and expressed as malonaldehyde values[20] (Fig. 2B).

Evidence for the participation of the lipid peroxidation in the decrease in epoxidase activity was further supported by the effects of ferrous sulfate and EDTA added to the preincubation flasks. Preincubation of microsomes with NADPH in the presence of ferrous ion, which is known as stimulant for the lipid peroxidation of microsomes[31] and has been shown to decrease microsomal *N*-demethylase activity,[32] caused a larger extent of decrease in epoxidase activity whereas EDTA, an inhibitor for the lipid peroxidation[31] and consequently a potentiator for microsomal aromatic hydroxylase and *N*-demethylase,[32] inhibited the NADPH-dependent decrease in epoxidase activity (Fig. 2A).

In all the cases examined, little alteration was observed in microsomal epoxide hydrolase activity (Fig. 2A). It is of interest that epoxide hydrolase activity is not affected by lipid peroxidation of microsomes, for the recent studies have demonstrated that so far as examined with naphthalene and naphthalene oxide, epoxide hydrolase appears to be very closely associated with the epoxidase system in microsomes.[33,34] The present evidence for the selective decrease in epoxidase activity seems to be attributable to the suggested catabolic effect of lipid peroxidation on microsomal hemoprotein, including cytochrome P-450.[35]

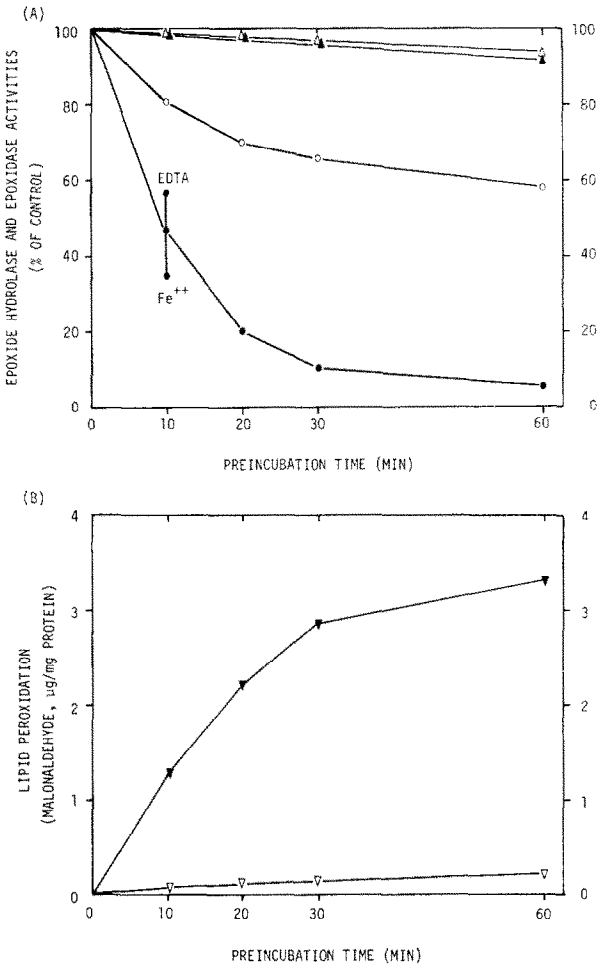


FIG. 2. Effect of NADPH-dependent lipid peroxidation of hepatic microsomes on epoxidase and epoxide hydrolase activities. (A) Epoxidase activity was assayed using heptachlor as the substrate. Rabbit liver microsomes were preincubated at 37° in air in the absence and in the presence of NADPH. Fe^{2+} (0·2 mM) and EDTA (1 mM) were added only to the 10 min incubation flasks, respectively. Epoxide hydrolase activity was assayed using *cis*-stilbene oxide as the substrate and the preincubated microsomal systems which were diluted 40 times with 0·1 M phosphate buffer, pH 7·4, at the end of preincubation in order to obtain adequate reaction velocities. Control values for the heptachlor epoxide formation from heptachlor and the *threo*-stilbene glycol formation from *cis*-stilbene oxide were $4{\cdot}72 \times 10^{-9}$ M and $2{\cdot}34 \times 10^{-7}$ M/mg microsomal protein/min, respectively. The incubation time of the control and preincubated preparations was 20 min. (B) Lipid peroxide formed during preincubations of the microsomes was determined by the thiobarbituric acid method and expressed as malonaldehyde values. Data are arithmetic mean values of at least three experiments. Deviations of data from the mean were 3, 2, and 5 per cent for the heptachlor epoxide, *threo*-stilbene glycol, and lipid peroxide formations, respectively. Epoxide hydrolase activities: (△) and (▲) and epoxidase activities: (○) and (●) in the absence and in the presence of NADPH during preincubations, respectively. NADPH-independent, (▽) and dependent, (▼) lipid peroxide formations.

The above results indicate the participation of microsomal lipid peroxidation in determining the production rate and the half life of the epoxy intermediate not only *in vitro* but also presumably *in vivo* since lipid peroxidation of hepatic endoplasmic reticulum has been observed *in vivo*.[36]

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