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# THE INTERACTION OF STYRENE OXIDE WITH HEPATIC CYTOCHROME P-450 *IN VITRO* AND EFFECTS OF STYRENE OXIDE INHALATION ON XENOBIOTIC BIOTRANSFORMATION IN MOUSE LIVER AND KIDNEY

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**Abstract**—Female mice (strain CB-20) were killed 0.5 hr, 18 hr and 5 days after inhaling styrene oxide for 6 hr per day for 3 consecutive days at concentrations of 200, 200 and 100 p.p.m., respectively. Acute intoxication was manifest, both clinically and as a depression of non-protein sulfhydryl content in liver and kidney. During the recovery period of 5 days a transient rise in microsomal 7-ethoxycoumarin *O*-deethylase activity in both tissues paralleled changes in cytochrome P-450 content. The activity of microsomal epoxide hydratase (measured with styrene oxide as the substrate) was not affected by the treatment, neither was the UDP-glucuronosyltransferase activity. In the presence of hepatic microsomes from phenobarbital-treated mice, styrene oxide produced a characteristic Type I difference spectrum. A comparison of the binding parameters for the interaction of styrene oxide with uninduced and phenobarbital- and 3-methylcholanthrene-induced microsomes indicates that the binding of styrene oxide is catalyzed by more than one type of P-450 hemoprotein, but predominantly by phenobarbital-induced cytochrome P-450. In addition, in phenobarbital-pretreated microsomes styrene oxide had two spectral dissociation constants ($K_s$), 0.05 mM and 0.4 mM.

Styrene oxide (phenylethylene oxide) has been detected as a volatile component in tobacco concentrate [1] as a by-product in commercial samples of styrene chlorohydrin [2] and in effluent water from various latex manufacturing plants [3]. It is used as a reactive diluent in epoxy resins [4] and as an intermediate in the preparation of agricultural and biological chemicals, cosmetics, coatings and in the treatment of textiles and fibers. In view of its large production, about 2.5 million kg in 1976 [5], and wide use, astonishingly few studies have been done with styrene oxide. In the present study effects of intermittent styrene oxide inhalation on drug-metabolizing enzymes and free glutathione content in liver and kidney were examined. We have also reported the results of an investigation of the interaction of styrene oxide *in vitro* with the cytochrome P-450 enzyme system of the hepatic endoplasmic reticulum.

## MATERIALS AND METHODS

In a dynamic exposure chamber 20 dm$^3$ in size nine female CB-20 mice (8–12 weeks old) were exposed for 6 hr per day for 3 consecutive days to 200, 200 and 100 p.p.m. (490 mg/m$^3$) of styrene oxide, respectively. Control mice of the same age were sham exposed to air in a similar chamber.

*Course of the exposure.* Styrene oxide (phenylethylene oxide, purum grade, obtained from Fluka AG, Buchs, Switzerland) was fed by an infusion pump at a constant rate into a heated injection device for volatilization. Thereafter the styrene oxide, mixed with air to the desired concentration with the help of purgemeters, was fed into the exposure chamber at a pump rate of 7 l. per min. The styrene oxide concentration in the chamber air was monitored continuously with a recording infrared Miran 1 A spectrophotometer (Wilks Scientific Corp., U.S.A.). The ranges of the given air concentrations varied within ± 10 per cent.

Three exposed and three control animals were killed and bled by decapitation 0.5 hr, 18 hr and 5 days after the last treatment. Livers and kidneys were removed, washed in ice-cold 0.9% saline and stored at − 70° until the biochemical analysis.

*Enzyme assays.* With a Potter–Elvehjem tube and a Teflon pestle, liver and kidney tissues were homogenized in 0.25 M sucrose–0.15 M KCl–20 mM HEPES buffer (pH 7.4) to a 20% (w/v) homogenate. $Ca^{2+}$-aggregated microsomes were obtained in a previously described manner [6].

The concentration of non-protein sulfhydryl groups was measured directly from tissue homogenates with a colorimetric method for thiols [7]. Cytochrome P-450 was determined from the recording made of the difference spectrum of the dithionite-reduced carbon monoxide complex in a Pye Unicam SP-1800 spectrophotometer [8]. The molar extinction coefficient of the reduced P-450–CO complex was taken as 91 cm$^{-1}$mM$^{-1}$.

Microsomal 7-ethoxycoumarin *O*-deethylase activity was measured by a modified fluorometric method of Ullrich and Weber [9], as described previously [10]. The fluorescence of 7-hydroxycoumarin was determined at a wavelength of 390 nm for excitation and 460 nm for emission in a Perkin Elmer Double Beam 512 spectrofluorometer.

Epoxide hydratase (EC 4.2.1.63) activity was determined by the radiometric assay of Oesch *et al.* [11], but

with the following modifications. To start the reaction, [7-$^3$H]styrene oxide (code No. TRQ1107, Radiochemical Centre, Amersham, England) in 25 $\mu$l of acetone was pipetted into 1.0 ml of the incubation mixture at a specific activity of 140 $\mu$Ci/m-mole and a radioactive concentration of 12 $\mu$Ci/ml. Radioactivity was measured from an aliquot of the aqueous phase in a LKB Wallac 81000 liquid scintillation counter with the external standardization method for quench correlations.

UDP-glucuronosyltransferase (EC 2.4.1.17) was assayed with 0.35 mM *p*-nitrophenol as the aglycone and 4.5 mM UDP-glucuronate (ammonium salt, 98%, Sigma Chemical Co, U.S.A.) as described by Hänninen [12]. Protein was analyzed by the method of Lowry *et al.* [13].

*Styrene oxide binding studies* in vitro. Nine littermate female mice (strain CB-20, 9 weeks old) were divided into 3 groups for intraperitoneal treatment with sodium phenobarbital (80 mg/kg per day in 0.9% saline for 3 consecutive days), 3-methylcholanthrene (25 mg/kg per day in olive oil as a single injection four days before decapitation, obtained from Koch Light Laboratories, Ltd) or olive oil.

Liver microsomes were isolated immediately after the decapitation of the animals as described above, but this time they were gathered by centrifugation at 105,000 *g* for 60 min. The microsome preparations from 9 livers were kept in an ice bath until the spectral measurements were made, during the same day. The protein content of each liver preparation was determined so that the concentration could be adjusted in

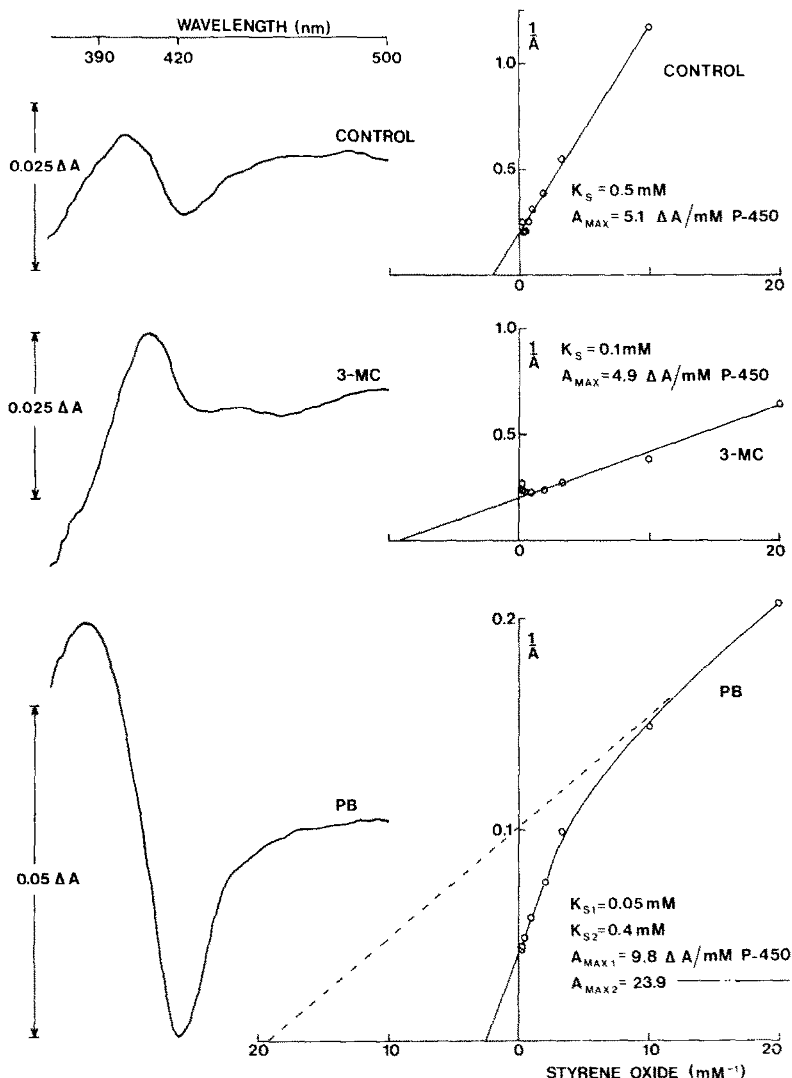


Fig. 1. Left: typical difference binding spectra of styrene oxide to hepatic microsomes from female mice treated with olive oil (Control), 3-methylcholanthrene (3-MC) and phenobarbital (PB). Right: double-reciprocal plot demonstrations of respective styrene oxide binding properties based on spectrophotometric titrations from three animals for each plot. The spectra shown were recorded at a 1 mM concentration of styrene oxide and at 2 mg/ml of microsomal protein in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 MKCl. Cytochrome P-450 contents ($\pm$ S.D.) were 1.10 $\pm$ 0.03 (Control), 1.25 $\pm$ 0.03 (3-MC) and 2.08 $\pm$ 0.07 (PB) nmoles per milligram of protein. All spectra were recorded with a Cary 219 spectrophotometer at room temperature after the addition of styrene oxide dispersed in water, in a cuvette with a light path length 1 cm.

cuvettes to 2 mg protein/ml microsomal suspension. Styrene oxide, dispersed in water by ultrasonication, was added step-wise into the sample cuvette with a Hamilton syringe to record the difference spectra at final concentrations varying from 0.05 up to 5 mM. Dilution corrections were omitted in the reference cuvette as the total volume added did not exceed 2 per cent of the original sample volume (2.5 ml). Spectral dissociation constants ($K_s$) were obtained from double-reciprocal plots for which the magnitude of spectral changes given per concentration of cytochrome P-450 was calculated from the difference between the peak and the trough absorption values. For further descriptions see the legend of Fig. 1.

## RESULTS

*Binding of styrene oxide to hepatic microsomal cytochromes P-450 in vitro.* Styrene oxide was found to bind to hepatic microsomal cytochrome P-450 with the production of a Type I difference spectrum. However, only in phenobarbital-treated mice did the binding result in the appearance of a characteristic Type I difference spectrum with a peak at 385 nm and a trough at 418. The spectra from control and 3-methylcholanthrene-treated mice resembled Type I spectra but were somewhat shifted to the right; they showed an absorption peak at about 400 nm (control) and 408 nm (3-MC) and a trough at about 422 nm and 426 nm, respectively (Fig. 1).

The cytochrome P-450 content of the liver microsomal fraction showed a 1.9-fold increase after phenobarbital treatment and a 1.1-fold increase after 3-methylcholanthrene treatment. Double reciprocal plots of the titration data were used for the estimation of the apparent spectral dissociation constant $K_s$ (Fig. 1). With microsomes from the phenobarbital-treated animals a biphasic titration curve was illustrated (Fig. 1). At low substrate concentrations these microsomes also showed the highest binding affinity to styrene oxide, with a $K_{s(1)}$ value of 0.05 mM, whereas at higher concentrations the $K_{s(2)}$ value (0.4 mM) was about the same as that of the controls ($K_s = 0.5$ mM). Moreover, approx. 2- and 5-fold increases in the maximal magnitude of the Type I spectral changes (per cytochrome P-450 concentration) were recorded in comparison to the $\Delta A_{max}$ value of the controls (Fig. 1). An intermediary $K_s$ value of 0.1 mM was calculated for the interaction of styrene oxide with 3-methylcholanthere microsomes. No concomitant changes in maximal absorptivity was observed, however.

*Effects of styrene oxide inhalation on drug metabolism in liver and kidney.* Styrene oxide inhalation resulted in signs of intoxication in mice as early as the first hours of the experiment. One mouse succumbed 3 days after the last exposure. Styrene oxide caused observable irritation of the eyes, decreased mobility and abnormally crooked posture of the back that was still prominent 5 days after the last exposure.

The level of non-protein sulfhydryl groups was found to be strongly reduced in the liver and kidney immediately after the last 6 hr exposure to styrene oxide. The hepatic thiol content was restored to half of control within 18 hr and was up to 87 per cent in 5 days, whereas the control level was resumed more rapidly in kidneys (Table 1). Microsomal ethoxycoumarin *O*-deethylase activity revealed its maximal enhancement 18 hr after the last exposure both in liver (1.5-fold) and in kidneys (6-fold). Slightly elevated levels of *O*-deethylase activities were still detectable 5 days after the last exposure in both tissues (Table 1). Changes in hepatic cytochrome P-450 contents followed a pattern similar to the specific ethoxycoumarin *O*-deethylase activities (Table 1).

Hepatic epoxide hydratase activity, responsible for the enzymic conversion of styrene oxide to corresponding dihydrodiol, was practically unaffected by the exposure (data not shown). Only a slight decrease (20 per cent) in the measurable UDP-glucuronosyltransferase activity in liver microsomes was observed 18 hr after the last exposure (data not shown). Although several measured enzyme activities were substantially affected by the styrene oxide exposures, the microsomal protein contents of liver or kidney did not differ from appropriate control groups (data not shown).

## DISCUSSION

Results of investigations with the various inducing agents used in this study suggest that there is no or very little binding of styrene oxide to hepatic microsomal cytochrome P-448. This conclusion was based on a comparison of phenobarbital-induced microsomes, which have elevated cytochrome P-450 levels, and exhibit spectra with styrene oxide which are markedly enhanced relative to uninduced microsomes or to 3-methylcholanthrene-induced microsomes. The linearity of the double reciprocal plot of the difference spectra data produced by the addition of varying concentrations of styrene oxide to uninduced or 3-methylcholanthrene-induced microsomes indicates that styrene oxide binds to a single site on the enzyme or at least to sites with similar $K_s$ values. However, in phenobarbital-treated microsomes, the double reciprocal plot was biphasic and thus permitted the calculation of two different $K_s$ values. This phenomenon is probably a consequence of the fact that styrene oxide can interact with the P-450 hemoprotein system in more than one way—but the phenobarbital-induced microsomes are by far the most important. The fact that in 3-methylcholanthrene induced microsomes the affinity of styrene oxide to P-450 (P-448) hemoprotein was increased without any change in maximal specific binding suggests that the treatment facilitated the binding, probably due to changes in the microenvironment of the membrane-bound hemoprotein. Phenobarbital pretreatment on the other hand highly increased both the affinity and the total binding capacity of the liver microsomes.

Styrene has also been found to bind predominantly to phenobarbital-induced cytochrome P-450 in rat liver microsomes [14]. Styrene oxide, being the primary metabolite of styrene, effectively depleted thiol content in the liver and kidney. The depletion of tissue non-protein sulfhydryl groups by inhalation is in accordance with earlier data obtained with intraperitoneal administration [15]. Intraperitoneally administered styrene oxide also causes a loss of liver microsomal cytochrome P-450 and decreases the ability of liver microsomes to metabolize drugs [16]. This loss is possibly due to the reaction of nucleophilic sites of P-450 hemoprotein with the epoxide. In the present study, no significant decrease in either cytochrome P-450

Table 1. Effects of styrene oxide inhalation (200, 200 and 100 p.p.m. on 3 consecutive days, 6 hr daily) on non-protein sulfhydryl groups, cytochrome P-450 contents and 7-ethoxycoumarin *O*-deethylase in mouse liver and kidney*

| Time since last exposure (N)† | | Non-protein sulfhydryl groups (μmoles/g wet wt.) | | Cytochrome P-450 (nmoles/mg protein) | 7-Ethoxycoumarin *O*-deethylase (nmoles × min$^{-1}$ × mg$^{-1}$) | |
|---|---|---|---|---|---|---|
| | | Liver | Kidney | Liver | Liver | Kidney |
| Styrene oxide | | | | | | |
| 0.5 hr | (3) | 0.7 ± 0.4¶ | 1.6 ± 0.3¶ | 0.74 ± 0.24 | 1.24 ± 0.20 | 0.061 ± 0.026 |
| 18 hr | (3) | 4.5 ± 1.6¶ | 5.5 ± 0.9 | 1.10 ± 0.12‖ | 1.81 ± 0.19‖ | 0.142 ± 0.023¶ |
| 5 days | (2‡) | 6.8 ± 0.3‖ | 5.5 ± <0.1 | 0.79 ± 0.01 | 1.51 ± 0.43 | 0.076 ± 0.033§ |
| Controls | | | | | | |
| 0.5 hr | (3) | 8.2 ± 0.2 | 6.2 ± 0.3 | 0.92 ± 0.07 | 1.32 ± 0.06 | 0.033 ± 0.004 |
| 18 hr | (3) | 9.7 ± 0.1 | 6.5 ± 0.3 | 0.79 ± 0.06 | 1.05 ± 0.03 | 0.014 ± 0.004 |
| 5 days | (3) | 9.0 ± 0.6 | 6.0 ± 0.8 | 0.85 ± 0.04 | 1.26 ± 0.19 | 0.020 ± <0.001 |

* The results are given as mean values ±S.D.
† Number of animals in each experimental group is given in parentheses.
‡ One of the nine styrene oxide exposed mice died on day 3 and hence this group size was reduced to 2.
Statistical analysis of the data was done with Student's *t* test. Significance levels: §P ⩽ 0.05, ‖P ⩽ 0.01, ¶P ⩽ 0.001.

levels or ethoxycoumarin *O*-deethylase activity could be detected. This obvious discrepancy with the data from intraperitoneal studies may be due to the different dose level and the different animal species used. However, even the dose used, 200 p.p.m. for 6 hr/day, was so toxic that the concentration of styrene oxide had to be lowered on the third day to 100 p.p.m. Thus styrene oxide via inhalation appears to be more toxic than the inhalation of styrene, which is tolerated well at these concentrations, even for longer periods of time [17, 18].

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