

Interaction of styrene and acetone with drug biotransformation enzymes in rat liver

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VAINIO, H. and ZITTING, A. Interaction of styrene and acetone with drug biotransformation enzymes in rat liver. *Scand. j. work environ. & health* 4 (1978): suppl. 2, 47—52. In the presence of hepatic microsomes, styrene produced a type I difference spectrum, which demonstrates that styrene binds to the catalytic site of ferri-cytochrome P-450. A comparison of the binding parameters for the interaction of styrene with noninduced, phenobarbital-induced, and 3-methylcholanthrene-induced microsomes indicated that styrene is predominantly bound by cytochrome P-450 and not by cytochrome P-448. Inhalation exposure to a mixture of acetone (1,000 ppm, 6 h/d) and styrene (300 ppm, 6 h/d) for 5 d caused a distinct decrease in hepatic free nonprotein sulfhydryl groups. This decrease could be observed both with and without phenobarbital treatment. Acetone inhalation alone also enhanced ethoxycoumarin O-deethylase activity in rats without pretreatments. Acetone inhalation also increased the cytochrome P-450 content of liver microsomes, but it had no effect on NADPH cytochrome c reductase or epoxide hydratase activity. Combined exposure to styrene and acetone enhanced NADPH cytochrome c reductase activity in non-phenobarbital-treated rats, but no effect was seen in the phenobarbital-treated animals. Phenobarbital treatment of animals can greatly modify the biotransformation and toxicity of styrene, phenobarbital inducible P-450 hemoprotein playing a predominant role in its metabolism. Simultaneous inhalation exposure to acetone also interacts with the metabolism of styrene.

Key words: acetone, cytochrome P-450, microsomal enzymes, styrene.

A multitude of information has been accumulated concerning the biotransformation and toxicity of styrene, an important source of plastics. The metabolism of styrene takes place in the liver and in a number of extrahepatic tissues (4). The biotransformation is stimulated by styrene itself (15), and by phenobarbital, and it is suppressed by coadministered toluene (7). When laminating, workers are also exposed to acetone, which is used to clean the tools.

Both styrene and acetone are volatile liquids, and industrial workers are mainly exposed via inhalation. During the commonly used hand lay-up, glass fiber/polyester resin process, the air concentrations (time-weighted average) of styrene and acetone were 66 ppm (range 20—299) and 90 ppm (range 33—165), respectively, in a recent Finnish investigation (8).

The present study was undertaken to examine the interaction of styrene and acetone in vitro and in vivo with cytochrome P-450 and the related enzyme system of the hepatic endoplasmic reticulum, which is proposed to function in the activation of styrene to a dangerous toxin.

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MATERIAL AND METHODS

Adult male Wistar rats were used in all the experiments. A group of rats was exposed to 1,000 ppm (2.4 mg/l) of acetone, 5 h/d, for 5 d. Another group of rats was simultaneously exposed to 300 ppm (1.3 mg/l) of styrene for the same period. In addition, two similar exposure groups received phenobarbital (0.1 %, w/v) in their drinking water. During the exposure water was given *ad libitum*, but food (mice pellet, obtained from Hankkija Oy, Finland) was not.

Exposure took place in 1-m³ chambers, and the exposure levels were monitored continuously with infrared analysis (Miran 1, Wilks Scientific Corp., U.S.A.). The control animals were sham-exposed under identical conditions.

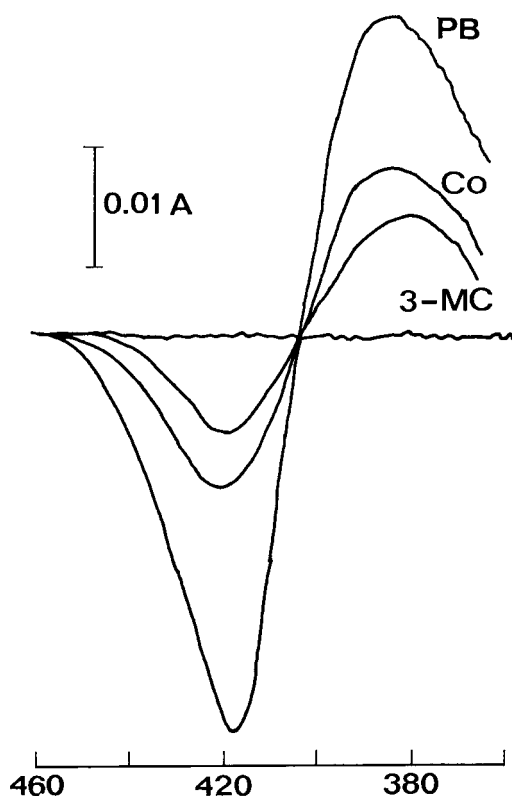


Fig. 1. Difference spectra of styrene in microsomes from 3-methylcholanthrene-induced (MC), noninduced (Co) and phenobarbital-induced (PB) male rats. (Microsomal concentration 1.6 mg/ml, 0.15 M KCl; styrene concentration, 1.4 mM; 25°C).

The animals were sacrificed by decapitation on the last day of exposure; they had received no food the night before. Their livers were removed, weighed, immediately frozen, and stored at -70°C until further preparation.

For the *in vitro* spectral studies the rats were induced by the provision of phenobarbital in their drinking water (0.1 %, w/v) for 5 d or by an intraperitoneal injection of 3-methylcholanthrene (40 mg/kg) (obtained from Koch-Light Ltd., England) in olive oil. The 3-methylcholanthrene-treated rats were decapitated 4 d after the injection.

Ca⁺⁺-aggregated liver microsomes were prepared according to the method of Aitio and Vainio (2). Protein was measured with the technique of Lowry et al. (9).

The binding spectra were recorded with a Varian Cary 219 spectrometer. Styrene (obtained from Fluka AG, Switzerland) was dissolved in acetone and added, with efficient mixing, to 3 ml of the microsomal suspension in 0.15 M potassium chloride in a 1-cm sample cuvette. The volume of acetone never exceeded 30 μ l. An equal volume of acetone was added to the suspension in the reference cuvette.

The content of cytochrome P-450 was determined from the carbon monoxide difference spectrum of dithionite-reduced microsomes recorded according to the method of Omura and Sato (12). 7-Ethoxycoumarin O-deethylase was measured with a Perkin Elmer 512 Fluorometer by the method of Ullrich and Weber (17) as modified by Aitio (1). Epoxide hydratase activity was determined with (7-³H) styrene oxide (Radiochemical Centre, Amersham, England) as the substrate, as described by Vainio and Parkki (21). UDP-glucuronosyltransferase activity was assayed with p-nitrophenol (E. Merck AG, Darmstadt, Federal Republic of Germany) as the aglycon (18).

Determination of the free glutathione in the tissue was performed by the method of Cohn and Lyle (5) with slight modifications. The proteins of 20 % liver homogenate were sedimented with 5 % trichloroacetic acid (final concentration). After centrifugation at 3,000 g for 10 min, 100 μ l aliquots of the supernatants were taken for the free reduced glutathione determi-

Table 1. Effects of inducing agents on the interaction of styrene and microsomal cytochrome P-450 in vitro.

Treatment of rats	Cytochrome P-450 (P-448) (nmol/mg microsomal protein)	K_s^a (mM)	$\Delta A_{\max}/\text{mg}$ microsomal protein ^b
None	0.85	0.75	0.0165
3-Methylcholanthrene	1.12	0.57	0.0053
Phenobarbital	1.97	0.05	0.0207

^a Values calculated from plots of $1/\Delta A$ versus $1/(s)$.

^b $\Delta A = A_{\text{peak}} - A_{\text{through}}$ of difference spectrum.

Table 2. Effects of inhaling acetone (1,000 ppm, 6 h/d) or acetone (1,000 ppm, 6 h/d) and styrene (300 ppm, 6 h/d) in combination for 5 d on liver reduced glutathione (GSH) levels and microsomal drug-metabolizing enzymes in rats without (—Pb) and with (+ Pb: 0.1 % w/v in drinking water for two weeks) phenobarbital treatment.

Measured parameter	Treatment of animals					
	Control		Acetone		Acetone + styrene	
	—Pb	+Pb	—Pb	+Pb	—Pb	+Pb
GSH ($\mu\text{mol/g}$ liver w.wt.)	2.27 ± 0.13	1.58 ± 0.08	1.49 ± 0.19	1.56 ± 0.37	1.12 ± 0.23	1.31 ± 0.11
NADPH cytochrome c reductase ($\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$)	83.15 ± 11.96	154.2 ± 11.65	84.55 ± 7.89	155.3 ± 11.93	110.3 ± 16.56	158.2 ± 8.31
Cytochrome P-450 (nmol/mg)	0.80 ± 0.08	2.42 ± 0.19	0.92 ± 0.05	2.37 ± 0.04	1.05 ± 0.09	2.49 ± 0.17
Ethoxycoumarin O-deethylase ($\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$)	0.400 ± 0.03	1.32 ± 0.08	0.890 ± 0.05	1.55 ± 0.09	0.780 ± 0.06	1.56 ± 0.14
Epoxide hydratase (nmol styrene glycol formed $\times \text{min}^{-1} \times \text{mg}^{-1}$)	13.50 ± 3.02	55.70 ± 3.14	11.65 ± 0.50	57.92 ± 6.85	11.80 ± 1.92	56.05 ± 4.28
UDPGlucuronosyl- transferase (nmol p-nitrophenol bound $\times \text{min}^{-1} \times \text{mg}^{-1}$)	1.04 ± 0.23	1.83 ± 0.11	0.69 ± 0.04	2.77 ± 0.41	1.09 ± 0.18	2.18 ± 0.09

nation, as described by Hissin and Hilf (6).

The data were statistically treated with Student's t-test.

RESULTS

The binding of styrene to cytochrome P-450 in vitro was investigated spectrally in microsomes from noninduced, 3-methylcholanthrene-induced and phenobarbital-induced rats. Styrene was bound to the hepatic microsomal cytochrome P-450 in all types of microsomes investigated, and

it produced a type I difference spectrum (fig. 1). The K_s and $\Delta A_{\max}$ values are presented in table 1.

The coadministration of acetone and styrene caused a depression in the free glutathione level in the liver of animals which did not receive phenobarbital. Phenobarbital induction seemed to cause a small reduction in the free glutathione level, and there was no significant further decrease when acetone was inhaled alone or in conjunction with styrene (table 2).

Phenobarbital in the drinking water for 5 d increased the cytochrome P-450 content in the liver microsomes by threefold,

as it also did the ethoxycoumarin O-deethylase and epoxide hydratase activities. The NADPH² cytochrome c reductase activity was doubled, and the measurable UDPglucuronosyltransferase activity increased 1.8-fold. Acetone singly or in combination with styrene was not able to modify the response caused by phenobarbital to any great extent (table 2).

Acetone inhalation caused a reduction in the liver free glutathione content. Acetone treatment also doubled the ethoxycoumarin O-deethylase activity in liver microsomes, whereas UDPglucuronosyltransferase activity was depressed by acetone exposure. The cytochrome P-450 level was slightly increased ($P < 0.05$), but no effect on either NADPH cytochrome c reductase or epoxide hydratase activities could be seen. In animals pretreated with phenobarbital the enhancement in ethoxycoumarin O-deethylase was smaller but still detectable. When styrene was administered together with acetone, the NADPH cytochrome c reductase activity and the cytochrome P-450 level were slightly increased, together with the doubled ethoxycoumarin O-deethylase activity. UDPglucuronosyltransferase in the liver microsomes remained however at the same level as in the untreated control rats.

DISCUSSION

We have attempted to evaluate the interactions of styrene with hepatic microsomal cytochrome P-450 *in vitro* and the interactions of styrene and acetone *in vivo*. The appearance of type I difference spectra following the addition of styrene to hepatic microsomes indicates that styrene binds to hepatic microsomal cytochrome P-450 in a manner characteristic of the binding of substrates to this hemoprotein. The results attained with the various inducing agents used in the present study suggest that styrene does not bind to hepatic microsomal cytochrome P-448. This conclusion was drawn on the basis of

the following points: phenobarbital-induced microsomes, which have elevated cytochrome P-450 levels, exhibit markedly enhanced difference spectra with styrene in comparison to noninduced microsomes, and 3-methylcholanthrene-induced microsomes, which have markedly enhanced cytochrome P-448 levels, have no enhanced difference spectra in comparison to non-induced controls (fig. 1., table 1).

This failure of styrene to bind to cytochrome P-448 *in vitro* provides an explanation for the failure of 3-methylcholanthrene induction to potentiate the toxicity of styrene *in vivo* (14). Apparently, the levels of total hepatic type P-450 cytochromes in 3-methylcholanthrene-induced rats are insufficient to potentiate styrene toxicity. Since the major component of the cytochromes in this case is cytochrome P-448, which perhaps does not bind styrene at all, no toxic effects are observed.

A monooxygenase with cytochrome P-450 as its core is responsible for the metabolic activation of styrene to the more toxic styrene epoxide (11). Through the covalent binding of epoxide metabolites to nucleic acids and proteins, there exists a possibility of the onset of liver necrosis and/or carcinogenesis (13). Epoxide hydratase can contribute to the inactivation of epoxide via diol formation. Styrene oxide administration has been shown to mediate a destruction of hepatic microsomal cytochrome P-450 *in vivo* (15) and cause a reduction in the nonprotein sulfhydryl groups in liver (10). Styrene inhalation has been found to cause a dose-related depression in free glutathione in rat liver (20).

Acetone is known to have a stimulative effect on aniline p-hydroxylation *in vitro*. But this enhancement requires high concentrations (0.8–1.0 M), and its mechanism is still not clear (3,19). For type I substrates such as ethoxycoumarin, acetone is only inhibitory *in vitro* (1, 19). However, the N-demethylation of dimethylnitrosamine is enhanced by acetone treatment *in vivo*, and even this phenomenon is not attributed to the direct action of acetone in liver microsomes (16). Whatever the mechanism of stimulation by acetone may be, it is noteworthy that it is absent in phenobarbital-treated animals. Pheno-

² NADPH = reduced nicotinamide adenine dinucleotide phosphate.

barbital is a powerful classical enzyme inducer, whose effects are superior to those caused by either acetone or acetone and styrene in combination. Phenobarbital treatment of rats enhanced several-fold both the epoxide forming mixed function oxidase activity (such as ethoxycoumarin O-deethylase) and the activity of enzymes inactivating reactive epoxides (e.g., epoxide hydratase). Phenobarbital treatment can greatly modify the toxicity of styrene by increasing the affinity of styrene (K_s) to cytochrome P-450 and interacting with the toxifying/detoxifying enzymes.

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QUESTIONS AND ANSWERS

Questions to Dr. VAINIO

Prof. WATABE:

1. May I have your opinion on the fact that the biological oxidation of styrene is enhanced both by phenobarbital and by 3-methylcholanthrene, although you showed that styrene binds only to P-450 but not to P-448.
2. Do you have a plan to use purified P-448 or P-450 for confirming the binding specificity?

Dr. VAINIO:

At the moment we can only say that styrene is predominantly bound to cytochrome P-450, and the binding to cytochrome P-448 (induced by 3-methylcholanthrene) takes place only to a small extent if at all. I think we have to do further studies, possibly with purified P-450 hemoproteins to get more knowledge about the binding characteristics. According to in vivo toxicity studies, phenobarbital treatment, although it increases both the cytochrome P-450 level and the epoxide hydratase activity, is able to enhance the toxicity of styrene in hamsters, whereas 3-methylcholanthrene seems to decrease its toxicity. [Ohtsuji and Ikeda, *Toxicol. appl. pharmacol.* 18 (1971) 321—328; Parkki, *Scand. j. work environ. & health* 4 (1978): suppl. 2, 53—59 and Parkki et al., *Proceedings of international conference of biotransformation and toxicity of industrial and environmental xenobiotics*. September 13—15, 1977]

Prof. ROBERFROID:

Have you measured the binding of styrene to micromes from styrene pretreated rats?

Dr. VAINIO:

Not yet.