

Acute biotoxic effect of styrene on rat liver

Correlation with enzyme-mediated mutagenicity of benzpyrene and acrylonitrile

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ROBERFROID, M., PONCELET, F., LAMBOTTE-VANDEPAER, M., DUVERGER-VAN BOGAERT, M., DE MEESTER, C. and MERCIER, M. Acute biotoxic effect of styrene on rat liver: Correlation with enzyme-mediated mutagenicity of benzpyrene and acrylonitrile. *Scand. j. work environ. & health* 4 (1978): suppl. 2, 163—168. Styrene is commonly used in western Europe for the manufacture of plastics suitable for packaging foodstuffs. This report demonstrates that, injected intraperitoneally at a dose as low as 10 mg/kg, styrene modifies the catalytic properties of aryl hydrocarbon hydroxylase by reducing its K_M value. A similar effect is reported for two potent chemical carcinogens, 3-methylcholanthrene and benzo(a)pyrene. Ethylbenzene and benzo(e)pyrene and phenobarbital do not produce the same effect. Pretreatments of the rats with chemicals which modify aryl hydrocarbon hydroxylase also increase the capacity of the liver enzymes to activate benzopyrene to a mutagenic intermediate in vitro, as measured by the Ames test for mutagenicity. Exposure to both styrene and the other modifiers of the xenobiotic-metabolizing enzymes could thus influence the carcinogenic and toxic effects of chemicals which are activated by these enzymes. This hypothesis needs further investigation.

Key words: microsomal enzyme modification, mutagenesis, styrene.

Among the various vinyl monomers used for the manufacture of plastics suitable for packaging foodstuffs, styrene is commonly utilized, at least in western Europe. Like many other unsaturated hydrocarbons, styrene is metabolized, in vivo, by the liver microsomal enzymes to styrene oxide and styrene glycol.

Thus far there has been no convincing evidence that styrene or styrene oxide is carcinogenic in animals or man even if it has been demonstrated that both styrene and styrene oxide are mutagenic in vitro towards *Salmonella typhimurium* TA 1535

(5) and if it has been recently reported that occupational exposure to styrene increased the frequency of chromosomal aberrations in workers (6). It has recently been demonstrated by our laboratory (2, 8) that indirect chemical carcinogens and mutagens, which require enzymatic activation in order to exert their deleterious effects, have a common property. When injected into animals, they selectively modify the catalytic properties of those microsomal enzymes which specifically catalyze their activation. The biochemical modification of these enzymes correlates well with the variation of their ability to activate promutagens into active intermediates (7). The present study demonstrates that styrene in vivo also exerts a

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modifying effect on some rat liver microsomal enzymes and that this biochemical modification is correlated with an enhanced capacity of those enzymes to activate various chemical promutagens.

MATERIALS AND METHODS

Reagents

Benzo(a)pyrene, styrene, ethylbenzene and acrylonitrile were obtained from Aldrich; reduced nicotinamide adenine dinucleotide, glucose-6-phosphate and benzo(e)pyrene, from Sigma; oxidized nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate dehydrogenase from Boehringer; 3-methylcholanthrene from Fluka. The purest grades available of all the other chemicals were obtained from Merck.

Instrument

Fluorimetric determinations were carried out on a Perkin Elmer spectrofluorometer type 204, equipped with a universal digital readout Perkin Elmer UDR-1.

Animals and treatments

Adult male Wistar rats (220–260 g), fed ad libitum and fasted 24 h before sacrifice, were used.

Styrene and ethylbenzene in paraffin oil were injected intraperitoneally in a constant volume of 0.5 ml per animal and at doses of 10 and 500 mg/kg, respectively. Control animals received paraffin oil only.

3-Methylcholanthrene, benzo(a)pyrene, and benzo(e)pyrene in corn oil were administered intraperitoneally at a dose of 40 mg/kg. Control animals received corn oil only.

The animals were killed by decapitation 24 h after the treatment.

Preparation of subcellular fraction

The livers were immediately removed after the sacrifice. They were weighed,

rinsed free of blood, and chilled. The chilled livers were minced and homogenized at 4°C in a Potter Elvehjem homogenizer in 0.25 M sucrose 3 mM imidazole (pH 7.4) to make a 33 % (w/v) homogenate. The microsomal fraction was prepared according to the procedure described by de Duve, in which the liver homogenate is subjected to differential centrifugation in a Heraeus Christ ultracentrifuge.

The so-called S-9 mix used in the tests for mutagenicity was prepared according to the procedure of Ames et al. (1).

Enzyme assay

The activity of the microsomal aryl hydrocarbon hydroxylase was measured by the method of Dehnen et al. (3) as described by Cumps et al. (2), with benzo(a)pyrene as the substrate.

Testing for mutagenicity

Benzo(a)pyrene. The tests with benzo(a)pyrene were performed in duplicate with *S. typhimurium* strain TA 1538 and according to the plate incorporation method described by Ames et al. (1). Mixtures of bacteria from an overnight culture in nutrient broth (Difco) ($2-8 \times 10^7$ /plate), benzo(a)pyrene (10 µg/plate) and S-9 mix (0.5 ml/plate) in top agar supplemented with histidine-biotin were layered on a minimal glucose agar in petri dishes. After incubation of the plates for 48 h at 37°C, the numbers of macroscopic colonies were calculated.

Table 1. K_M values of liver microsomal benzo(a)pyrene hydroxylase 24 h after pretreatment of rats. (Means $\pm$ standard errors)

Pretreatment	K_M (10^{-6} M)
Control	2.48 ± 0.07
3-Methylcholanthrene	0.23 ± 0.03
Benzo(a)pyrene	0.40 ± 0.04
Benzo(e)pyrene	3.19 ± 0.87
Styrene (10 mg/kg)	1.09 ± 0.23
Styrene (500 mg/kg)	0.82 ± 0.26
Ethylbenzene (100 mg/kg)	2.79 ± 0.70

Acrylonitrile. The tests with acrylonitrile were performed in duplicate with *S. typhimurium* strain TA 1530. The petri plates containing S-9 mix and bacteria were prepared as described by Ames et al. (1) and incubated in the presence of gaseous acrylonitrile as described previously (4). The numbers of revertants his⁺ were calculated.

RESULTS

The reported experiments were carried out in order to reveal specific effects of styrene on liver microsomal enzymes. In order to rule out the possibility of an effect due to severe necrosis of the hepatic tissue, we measured and analyzed the activities and the distribution pattern of several so-called marker enzymes and calculated their recoveries.

We tested the integrity of the sub-cellular structures (mainly mitochondria and lysosomes) by controlling the latency of some of their consecutive enzymes. At the doses injected, neither styrene nor the various inducers significantly modify these biochemical parameters (personal communication from Lambotte-Vandepaer).

Effects of the various pretreatments on the K_M of benzo(a)pyrene hydroxylase

As previously reported, when the activity of liver microsomal benzo(a)pyrene hydroxylase is measured under strictly defined Michaelis-Menten conditions, pretreatment with 3-methylcholanthrene 24 h prior to sacrifice reduces the K_M of this enzyme by a factor of 10 (2). Benzo(a)pyrene itself, another polycyclic aromatic hydrocarbon carcinogen, also reduces the K_M of the same enzyme by a factor of 6 (table 1). On the contrary, benzo(e)pyrene, a noncarcinogenic isomer of benzo(a)pyrene, has been shown not to cause the same qualitative modification of benzo(a)pyrene hydroxylase.

Table 2. Effects of the various pretreatments of the enzyme-mediated mutagenicity of benzo(a)pyrene toward *Salmonella typhimurium* TA 1538. (Means $\pm$ standard errors)

Pretreatment	Number of reversions/plate ^a
Control	5 $\pm$ 5
3-Methylcholanthrene	200 $\pm$ 30
Benzo(a)pyrene	175 $\pm$ 28
Benzo(e)pyrene	16 $\pm$ 5
Styrene (500 mg/kg)	50 $\pm$ 13

^a Spontaneous reversion rate = 17 $\pm$ 7.

Table 3. Effects of styrene pretreatment on the enzyme-mediated mutagenicity of acrylonitrile toward *Salmonella typhimurium* TA 1530. (Means $\pm$ standard errors)

Pretreatment	Number of reversions/plate ^a
Control	30 $\pm$ 10
Styrene	72 $\pm$ 12

^a Spontaneous reversion rate = 15 $\pm$ 6.

As shown in table 1, styrene, administered intraperitoneally at doses of 10 and 500 mg/kg, significantly modifies the catalytic properties of liver microsomal benzo(a)pyrene hydroxylase. Ethylbenzene, the saturated analog of styrene, is without effect.

Effects of the various pretreatments on the capacity of the rat liver enzymes to activate promutagens

The biochemical analysis of the liver microsomes prepared from pretreated rats demonstrated that administration of 3-methylcholanthrene, benzo(a)pyrene and styrene specifically modifies the catalytic properties of aryl hydrocarbon hydroxylase; this enzyme is known to play a role in the metabolic activation of polycyclic aromatic hydrocarbons. This modification corresponds to an increase in the affinity of the enzyme for its substrate.

The results presented in table 2 demonstrate that this biochemical modification

correlates with an increase in the capacity of the modified enzymatic preparation to activate benzo(a)pyrene to a mutagenic intermediate. Likewise, treatments which do not modify the enzymatic system, do not enhance their capacity to produce the active mutagenic intermediate.

We have previously shown that acrylonitrile is mutagenic towards *S. typhimurium* in the presence of a rat liver enzyme preparation; therefore, it was of particular interest to test the effect of a pretreatment of the rat with styrene (500 mg/kg) on the capacity of its enzymes to activate this other vinyl monomer, to which it is often associated in copolymeric materials. As shown in table 3, the mutagenicity of acrylonitrile is significantly increased when a liver preparation from styrene (500 mg/kg) pretreated rat is used, as compared to a liver homogenate from a control rat.

DISCUSSION

When injected intraperitoneally into rat at a dose as low as 10 mg/kg, styrene modifies the catalytic properties of aryl hydrocarbon hydroxylase by reducing its K_M value. This effect has been confirmed in an analysis of another microsomal enzymatic activity, i.e., that of aldrin epoxide synthetase (M. Lambotte-Vandepaer, personal communication).

A similar effect has been observed with potent chemical carcinogens such as 3-methylcholanthrene or benzo(a)pyrene; however, styrene reduces the K_M of the analyzed enzyme only by a factor of 2 to 3, while both polycyclic aromatic hydrocarbons decrease it 8- to 10-fold.

The absence of effect of the chemical analog ethylbenzene seems to indicate that the modifying effect of styrene is related to the presence of a vinyl group, and it is most probably metabolically mediated.

The fact that benzo(e)pyrene does not act as an enzyme modifier could indicate that this property is restricted to specific

chemical structures which are also the most reactive biologically.

These data thus clearly demonstrate that styrene is far from being an inert chemical in vivo. Its metabolism produces an active intermediate which reacts with subcellular structures or macromolecules which are consequently qualitatively modified. According to our previously proposed hypothesis that such a modifying effect, which corresponds to an increase in enzyme affinity, could "at the cellular levels, when the concentration of the carcinogenic agents is rather low, have much more dramatic or beneficent consequences than a change in the maximum velocity by increasing the enzyme concentration" (2), we have tried to correlate this biochemical information with more direct biological parameters.

This report demonstrates that pretreatments of the rats with several chemicals, including styrene, which have been demonstrated to modify aryl hydrocarbon hydroxylase, also increase the capacity of the liver enzyme preparations (S-9 mix) to activate benzo(a)pyrene to a mutagenic intermediate in vitro towards *S. typhimurium*.

Moreover, the effect of styrene is not limited to benzo(a)pyrene since, administered in vivo, it enhances the recently reported (5) enzyme-mediated mutagenicity of acrylonitrile in vitro towards the same microorganism.

It is evidently extremely difficult to draw conclusions from these preliminary experiments. They however allow the proposal of a working hypothesis which will require further experiments, i.e., since styrene is acting, in vivo, as a modifier of enzymes such as aryl hydrocarbon hydroxylase or aldrin epoxide synthetase, which are known to play a role in the metabolism of various chemicals, and since this biochemical effect correlates with an enhancement of the capacity of enzyme preparations to activate promutagen, it could be possible that the exposure of an animal or even man to styrene could influence its capacity to activate those chemicals and thus could increase the risk for a carcinogenic or a toxic effect resulting from exposure to these compounds.

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REFERENCES

1. AMES, B. N., MC CANN, J. and YAMASAKI, E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. *Mutat. res.* 31 (1975) 347—364.
2. CUMPS, J., RAZZOUK, C. and ROBERFROID, M. Michaelis-Menten kinetic analysis of the hepatic microsomal benzpyrene hydroxylase from control, phenobarbital and methyl-3-cholanthrene treated rats. *Chem. biol. interact.* 16 (1977) 23—38.
3. DEHNEN, W., TOMINGAS, R. and ROOS, J. A modified method for the assay of benzpyrene hydroxylase. *Anal. biochem.* 53 (1973) 373—380.
4. DE MEESTER, C., PONCELET, F., ROBERFROID, M. and MERCIER, M. Mutagenic activity of acrylonitrile: A preliminary study. *Arch. int. physiol. biochem.* 86 (1978) 16—17.
5. DE MEESTER, C., PONCELET, F., ROBERFROID, M., RONDELET, J. and MERCIER, M. Mutagenicity of styrene and styrene oxide. *Mutat. res.* 56 (1977) 147—152.
6. MERETOJA, T., VAINIO, H., SORSA, M. and HÄRKÖNEN, H. Occupational styrene exposure and chromosomal aberrations. *Mutat. res.* 56 (1977) 193—197.
7. PONCELET, F., DE MEESTER, C., ROBERFROID, M. and MERCIER, M. Correlation between the kinetic parameters of the microsomal benzo(a)pyrene hydroxylase and the mutagenicity of benzo(a)pyrene in vitro. *Arch. int. physiol. biochem.* 86 (1978) 45—46.
8. ROBERFROID, M., RAZZOUK, C. and MERCIER, M. Microsomal N-hydroxylase modification as a first step in carcinogenesis by arylamines. *Arch. int. physiol. biochem.* 85 (1977) 429—431.

QUESTIONS AND ANSWERS

Questions to Prof. ROBERFROID

Dr. HILDEBRAND:

You mentioned very preliminary results of investigations on mice injected with styrene which showed chromosomal aberrations. Since experimental data on the mutagenicity of styrene in mammals are not available, it would be very interesting to learn about some details of the experimental design and the preliminary results.

Prof. ROBERFROID:

The very preliminary experiments I refer to seem to indicate that pretreatment of mice with styrene could increase the chromosomal aberrations in their lymphocytes after treatment with alfatoxin B₁. It does thus not tell of an eventual mutagenic effect of styrene in mammals, but rather it is an indication of a possible effect of styrene pretreatment on the mutagenicity of another chemical, i.e., alfatoxin. It is thus again possible evidence that styrene could act as a comutagen, a hypothesis which needs further proof.

Mr. PARKKI:

Is it possible that after styrene administration the hepatic microsomes contain styrene and cause an artificially wrong value in the K_M determination of styrene epoxides?

Prof. ROBERFROID:

We have never found any residue of styrene in the microsomes we analyzed and which were prepared after the injections of styrene, as similarly in microsomes from 3-methylcholanthrene-treated rats, there is no 3-methylcholanthrene present. The modifying effect is most probably due to a change in the interaction between cytochrome P-450 and PLP.

Questions to Dr. PONCELET

- Dr. SORSA: You were using DMSO as a solvent for styrene-induced mutagenesis. Data of Dr. Ramel's group in Stockholm show that DMSO is a rather potent inhibitor of microsomal activity in *Drosophila*. I wonder, therefore, if it would be worthwhile to use other solvents which could possibly induce stronger mutagenic activity in *Salmonella*.
- Dr. PONCELET: In our experiments with *Salmonella*, we too have used dilutions of styrene in absolute ethanol and then obtained similar mutagenic effects towards TA 1535. (Moreover, dilutions in acetone have given positive results.)
- Dr. VAINIO: What do you think is the basis of the cytotoxicity of styrene?
- Dr. PONCELET: I think there are two possible explanations: (a) styrene might act on the membrane of the bacterial cells and (b) the mutagenic activity might consequently induce lethal effects towards bacteria.