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STYRENE INDUCED MODIFICATIONS OF SOME RAT LIVER ENZYMES INVOLVED IN THE ACTIVATION AND INACTIVATION OF XENOBIOTICS*

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Abstract—Rats were injected intraperitoneally with single doses of styrene. Its effects on the kinetic parameters of liver microsomal monooxygenases and epoxide hydratase were investigated. The results were compared with those produced either by ethylbenzene, the vinyl-saturated analog of styrene or by phenobarbital and 3-methylcholanthrene, the classical inducers of those enzymes. The biochemical modifications were correlated with the altered ability of homogenates obtained from similarly pretreated rats to activate benzo(a)pyrene into intermediates mutagenic towards *Salmonella typhimurium*. Administration of styrene or 3-methylcholanthrene decreased the K_m of benzo(a)pyrene hydroxylase and aldrin epoxidase; styrene, but not 3-methylcholanthrene, decreased the K_m of styrene oxide hydratase; none of the two compounds modified the K_m of styrene epoxidase.

Pretreatment of the rats by styrene or 3-methylcholanthrene enhanced the S_9 mediated mutagenicity of benzo(a)pyrene several-fold, when compared to the mutagenic response mediated by liver preparations from control rats. Phenobarbital and ethylbenzene did not modify either the K_m of the investigated enzymes or the liver-mediated mutagenicity of benzo(a)pyrene.

The recent demonstration of the carcinogenicity of vinyl chloride in man [1] and animals [2–3] has focused attention on the possible health hazards associated with several other vinylic monomers, such as vinylidene chloride, acrylonitrile and styrene. Styrene (vinylbenzene) is, among several uses, widely utilized as a basic monomer for the manufacture of plastics, including polystyrene and several copolymers; an appreciable proportion of the total amount of those polymeric materials is commonly used as packaging materials for different foodstuffs.

Human exposures to styrene may therefore occur either in industrial environment or as a result of the leaching of residual styrene monomer from polystyrene food containers.

The first step in the metabolism of styrene is the formation of styrene oxide, catalysed by the cytochrome P-450 mixed-function oxidase system [4–6]; styrene oxide is subsequently hydrated to styrene glycol, or conjugated with glutathione [7].

It has been demonstrated [8–9] that styrene is mutagenic towards *Salmonella typhimurium* TA1535 strain after its metabolic activation by the Arochlor 1254-induced rat liver "S9 mix", used in the Ames test. Styrene oxide is mutagenic by itself in the same test system and it binds covalently to rat liver macromolecules both *in vivo* and *in vitro* [10]. More recently, Meretoja *et al.* [11] have reported an increase in the rate of chromosomal aberrations among workers occupationally exposed to styrene, there is up to now no convincing evidence that styrene or styrene oxide are carcinogenic in animals or in man [12].

It has been demonstrated in our laboratory [13–15] that some chemical carcinogens and mutagens could selectively modify (by decreasing the K_m), the microsomal enzymes which catalyse their own metabolic activation. It was therefore of interest to analyze the effect of i.p. administered styrene on the catalytic properties of typical microsomal enzymatic activities.

Our attention has been mainly focused on four characteristic enzymes: two of them (styrene epoxidase and styrene oxide hydratase) are implicated in the metabolism of styrene itself; the two others (benzo-[a]-pyrene hydroxylase and aldrin epoxidase) are typical cytochrome P-450 dependent enzymes which play an important role in the activation of exogenous compounds. The Ames test was used as a biological reagent to correlate the biochemical modifications of the enzymes with their ability to activate chemicals into mutagenic intermediates.

The effect of styrene was compared with the effect of its saturated analog, ethylbenzene, which is not a substrate for epoxidase, and which is not mutagenic in the Ames test‡. The pretreatments with phenobarbital and with 3-methylcholanthrene, inducers of cytochrome P-450 and cytochrome P_1-450, were used as references.

MATERIALS AND METHODS

Reagents. All chemicals were of the purest grade available and used without further purification:

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‡ F. Poncelet, personal communication.

benzo(a)pyrene, styrene, styrene oxide and ethylbenzene were obtained from Aldrich; phenobarbital, from Rhône-Poulenc; 3-methylcholanthrene, from Eastman Organic Chemicals; aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-endo-1,4,exo-5,8-dimethanonaphthalene) dieldrin (1,2,3,4,10,10-hexachloro-exo-6,7-epoxy-1,4,4a,5,6,7,8,8a, octahydro-1,4-endo-exo-5,8,-dimethanonaphthalene) and endrin (1,2,3,4,-10,10-hexachloro-exo-6,7-epoxy-1,4,4a,5,6,7,8,8a octahydro-1,4,endo-endo-5,8-dimethanonaphthalene) were gifts from the Shell Co.

Pentafluorobenzoyl chloride was purchased from Macherey-Nagel; NADH and glucose-6-phosphate were obtained from Sigma; NADP and glucose-6-phosphate dehydrogenase, from Boehringer. All other chemicals, of the purest grade available, were obtained from Merck.

3-Hydroxybenzo(a)pyrene was kindly donated by Dr. C. Malaveille, IARC, Lyon, France.

Instruments. A Hewlett–Packard model 5750 G gas chromatograph, equipped with a ^{63}Ni-electron capture detector was used. A glass column (2 m × 4 mm I.D.) packed with 3% OV-1 on supelcoport (80–100 mesh), was employed. The column temperatures were isothermal at 195° and 210° for the measurement of dieldrin and the quantitative estimation of the volatile derivative of styrene glycol, respectively.

The temperature of both injector and detector was 250°; argon–methane (95:5) was used as the carrier gas at a flow rate of 50 ml/min.

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

Animals and treatments. Adult male Wistar rats (220–260 g) were fed with ordinary laboratory food and water *ad lib.* Food was withdrawn 24 hr before preparation of the microsomes. The rats were killed by decapitation.

The animals were injected i.p. with styrene or ethylbenzene in paraffin oil.

Styrene was administered at doses of 10, 100 and 500 mg/kg, and ethylbenzene at a single dose of 100 mg/kg. Control animals received paraffin oil only.

The animals were killed by decapitation, 6, 12, 24, 48 and 72 hr after styrene treatments and 24 hr after ethylbenzene injection.

3-Methylcholanthrene (2 × 40 mg/kg) and phenobarbital (2 × 80 mg/kg) were given i.p. 48 hr and 24 hr prior to death.

Preparation of subcellular fractions. The livers were removed, weighed, and rinsed free of blood. The chilled livers were minced and homogenized at 4° in a Potter–Elvejhem homogenizer in 0.25 M sucrose 3 mM imidazole (pH 7.4) to make a 33% (w/v) homogenate. The mitochondrial–lysosomal (ML) and microsomal (P) fractions were prepared according to the procedure described by de Duve [16]. Protein concentrations were determined by the method of Lowry *et al.* [17] with crystalline bovine serum albumin as a standard.

Enzyme assays. Cytochrome P-450 was measured using the procedure of Raj and Estabrook [18] on an Unicam SP 800 spectrophotometer; NADPH:cytochrome *c* reductase was assayed by monitoring cytochrome *c* reduction at 550 nm according to the method of Hogeboon and Schneider [19].

Glucose-6-phosphatase was measured using the procedure described by de Duve *et al.* [16].

N-Acetyl-β-glucosaminidase activity was determined according to Sellinger *et al.* [20].

Sulfite:cytochrome *c* reductase was assayed using the method of Wattiaux-De Coninck and Wattiaux [21].

The activity of aryl hydrocarbon hydroxylase was measured using benzo(a)pyrene as substrate, by the method of Dehnen [22] as previously described [15]. However, all incubations were performed at 37° instead of 40°, which was the temperature utilized in the original method.

The incubation mixture contained 0.180 mM $NADP^+$, 0.200 mM NADH, 5 mM $MgCl_2$, 25 μM $MnCl_2$, 8 mM glucose-6-phosphate, 350 U/L glucose-6-phosphate dehydrogenase, 0.05 M histidine buffer pH 7.6, in a final volume of 3.3 ml of bidistilled water. After 30 min preincubation at 37°, in a Grant shaking incubator, 15 to 30 μg of microsomal protein were then added in 0.25 ml of water, to reach an appropriate dilution of the microsomal fraction. The reaction was initiated by the addition of 0.2 ml of benzo(a)pyrene solution in acetone (final concentration in the incubation medium, from 0.5 to 10 μM for microsomes from control, styrene or phenobarbital-treated rats, and from 0.1 to 5 μM for microsomes from 3-methylcholanthrene-treated rats).

Aldrin epoxidase activity was evaluated by the measurement of the amount of dieldrin, formed after incubation of the microsomal preparation with aldrin, according to the method described by Dubois-Krack *et al.* [23]. Both the mixture and the conditions for the incubation were essentially the same as for benzo(a)pyrene hydroxylase. The final concentration of aldrin in the incubation medium ranged from 0.5 to 25 μM for microsomes from control, styrene- or phenobarbital-treated rats and from 0.2 to 25 μM for 3-methylcholanthrene-induced microsomal preparation.

Styrene epoxidase and styrene oxide hydratase activities were measured, using an electron capture gas chromatographic method recently developed by Duverger-van Bogaert [24].

Measurement of both activities is based on the evaluation of the quantity of styrene glycol formed, either from styrene or from styrene oxide. For the epoxidase, the incubation medium contained NADH, $MgCl_2$ and $MnCl_2$, plus a NADPH-generating system, in a 0.1 M Tris buffer, pH 7.2.

After 30 min preincubation at 37°, 0.75 ml of the microsomal suspension (800 μg protein) was then added and the reaction was initiated by the addition of an ethanolic solution of styrene (final concentration: 50–2000 μM). For the epoxide hydratase, a 0.1 M phosphate buffer, pH 7.5 (0.2 ml), was maintained for 2 min at 37°.

After addition of 0.05 ml of the microsomal suspension (70 μg protein), the reaction was initiated by the addition of 10 μl of a solution of styrene oxide in acetonitrile in order to obtain final substrate concentrations of 10–1000 μM.

Mutagenicity testing. The tests were performed in duplicate with the *Salmonella typhimurium* strain TA1538, using the plate incorporation method as described by Ames *et al.* [25].

The liver postmitochondrial fractions (S-9) were

Table 1. Characterization of the microsomal fractions in liver of styrene treated rats*

Enzyme	Control	Styrene 500 mg/kg	Styrene 100 mg/kg	Styrene 10 mg/kg
Glucose-6-phosphatase (U/g liver)	21.95 ± 5.1	16.03 ± 2.90	17.70 ± 7.70	23.03 ± 6.60
NADPH:cytochrome *c* reductase (U/g liver)	3.82 ± 0.80	3.26 ± 0.06	2.76 ± 0.20	2.69 ± 0.60
Cytochrome P-450 (nmoles/mg protein)	0.79 ± 0.04	0.89 ± 0.19	0.93 ± 0.25	0.94 ± 0.13
Protein (mg/g liver)	26.26 ± 4.94	22.75 ± 1.88	19.73 ± 2.42	21.38 ± 2.42

* Microsomal fractions were obtained from rats treated with styrene at doses of 500, 100 or 10 mg/kg and killed 12 hr after injection. Entries are mean values ± S.D. from four (treated rats) to ten experiments (control rats).

Glucose-6-phosphatase, NADPH-cytochrome *c* reductase, cytochrome P-450 and protein contents were determined as described in Materials and Methods.

obtained from rats which have been pretreated as described above. The liver homogenates were centrifuged and added together with a NADPH-generating system to prepare the S-9 mix according to Ames *et al.* [25], by adding $MgCl_2$ (8 μmoles/ml mix), KCl (33 μmoles/ml mix), sodium phosphate (100 μmoles/ml mix), glucose-6-phosphate (5 μmoles/ml mix, NADP (4 μmoles/ml mix) and 100 μl (25 mg wet liver/ml mix) of the S-9.

Assays were carried out by mixing 5–8 × 10^7 viable bacteria cells from an overnight culture in nutrient broth (Difco)/plate, dilutions of benzo(a)pyrene in dimethylsulfoxide (0.1 ml/plate) and S-9 mix (0.5 ml/plate), in histidine–biotin-supplemented (0.05 mM) top agar (2 ml/plate) which was layered on minimal glucose agar (Vogel Bonner E medium) in Petri dishes. The number of his^+ revertants/plate were counted after incubation of the plates for 48 hr at 37° in the dark.

Statistical analysis. The program of Cleland as modified and completed by Cumps [26] has been applied for the quantitative estimation of the enzymic kinetic parameters.

The program includes:

(i) The determination of the kinetic parameters (V_{max} and K_m) and their standard deviation by an iterative process of non-linear [27] regression "steepest descent", further corrected by using a Taylor series linearization.

(ii) A decision test (are the parameters different from zero?).

(iii) A comparison test (are the calculated numeric parameters different from those of the normal population?).

RESULTS

Characterization of the microsomal preparation

The quality of the microsomal preparation, obtained from rats previously treated with various doses of styrene, was controlled by means of several enzymatic assays.

The microsomal fractions can be contaminated by membranes of damaged lysosomes or mitochondria (damage caused either by chemicals or by the fractionation procedure). The integrity of both organelles were therefore carefully assessed, by measuring the percentage of latency of two marker enzymes, i.e. by verifying that in isotonic conditions, those enzymes are inactive because of their inaccessibility to their respective substrates; for both sulfite:cytochrome *c* reductase (marker enzyme of the intermembranar space of mitochondria) and *N*-acetyl-β-glucosaminidase (marker enzyme of the lysosomes) the ratio of free to total activity was not significantly different from values from untreated animals (not shown).

The direct characterization of the microsomal preparations was carried out by determining the activities of several microsomal enzymes. Table 1 shows that neither the activities of glucose-6-phosphatase and NADPH:cytochrome *c* reductase, nor the amount of cytochrome P-450 and protein were significantly affected by the treatment, in the animals sacrificed 12 hr after the administration of styrene.

Effect of styrene administration on the kinetic parameters of metabolizing enzymes

A complete Michaëlis–Menten kinetic analysis of four microsomal enzymatic activities involved either in the activation (benzo(a)pyrene hydroxylase, aldrin and styrene epoxidase) or in the detoxification (styrene oxide hydratase) of xenobiotics, has been undertaken.

In the optimal conditions (time, protein, pH) for the Michaëlis–Menten type analysis of those enzymes, the effect of substrate concentration on the reaction velocities was investigated. The results were then submitted to

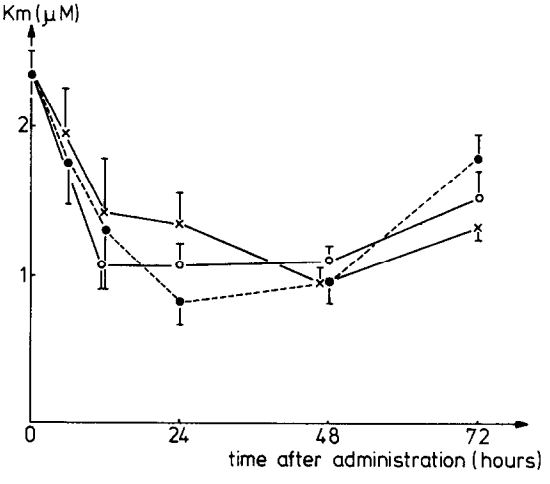

Fig. 1. Evolution of the K_m of benzo(a)pyrene hydroxylase, determined in liver microsomes from rats treated with a single dose of styrene (500 mg/kg ●------; 100 mg/kg ×———; 10 mg/kg ○———) and killed 6 hr, 12 hr, 24 hr, 48 hr or 72 hr after injection. The value at zero time corresponds to the control value. Assay of enzymatic activity is described in Materials and Methods. The values are given with their standard deviation and with at least 22 degrees of freedom.

Table 2. Effects of styrene, phenobarbital and 3-methylcholanthrene on the catalytic properties (K_m and V_{max}) of benzo(a)pyrene hydroxylase and aldrin epoxidase*

Treatment	Benzo(a)pyrene hydroxylase K_m (μM)	Benzo(a)pyrene hydroxylase V_{max} (nmole min^{-1} mg prot^{-1})	Aldrin epoxidase K_m (μM)	Aldrin epoxidase V_{max} (nmole min^{-1} mg prot^{-1})
None	2.36 ± 0.12	3.43 ± 0.46	2.23 ± 0.28	7.74 ± 0.36
Styrene 10 mg/kg	1.09 ± 0.28†	2.61 ± 0.15	1.21 ± 0.12†	5.77 ± 0.12†
100 mg/kg	1.37 ± 0.37†	3.75 ± 0.12	0.90 ± 0.23†	4.64 ± 0.13†
500 mg/kg	0.82 ± 0.26†	2.75 ± 0.08	1.02 ± 0.22†	6.05 ± 0.16†
Phenobarbital	3.51 ± 0.44	3.56 ± 0.23	2.75 ± 1.06	18.69 ± 3.36†
3-Methylcholanthrene	0.27 ± 0.02†	8.22 ± 0.14†	0.75 ± 0.17†	4.26 ± 0.14†

* Styrene (500 mg/kg, 100 mg/kg and 10 mg/kg) was given (i.p.) 24 hr before sacrifice; phenobarbital, (2 × 80 mg/kg, i.p.) and 3-methylcholanthrene (2 × 40 mg/kg, i.p.), were injected 48 hr and 24 hr before sacrifice. The values are given with their standard deviation and with at least 22 degrees of freedom.

† Significantly different from control rats (P = 0.05). Benzo(a)pyrene hydroxylase and aldrin epoxidase activities were determined as described in Materials and Methods.

a statistical analysis in order to determine the Michaëlis–Menten parameters and to compare the effect of the various pretreatments.

Benzo(a)pyrene hydroxylase. As can be seen from Fig. 1, the K_m of benzo(a)pyrene hydroxylase decreases significantly as a function of time after styrene treatment. At a dose of 500 mg/kg, the minimal value of 0.82 μM is reached after 24 hr. At 100 mg/kg, the minimal value (0.93 μM) is obtained after 48 hr and at 10 mg/kg, the minimum (1.10 μM) is observed between 12 and 48 hr after treatment. In the three cases, the K_m of benzo(a)pyrene hydroxylase tends to progressively return to normal values (untreated rats), after 72 hr.

No relevant effect of styrene administration is observed on the V_{max}. As shown in Table 2, the V_{max} of this enzyme remains unchanged after phenobarbital treatment, but is increased by the 3-methylcholanthrene induction as previously reported by Kuntzman *et al.* [28]; the K_m of benzo(a)pyrene hydroxylase is not modified by phenobarbital, but is significantly decreased by 3-methylcholanthrene.

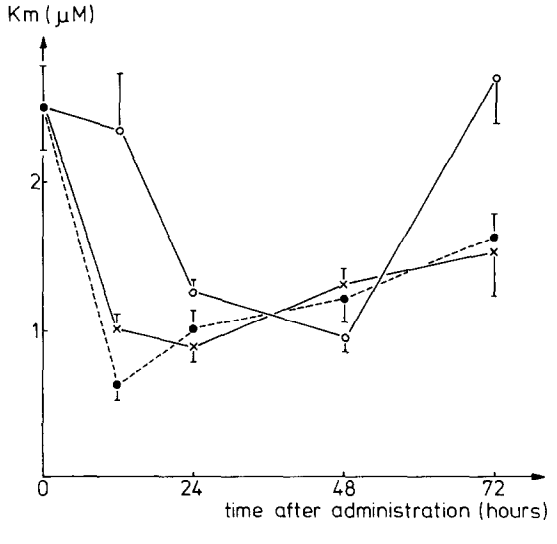

Fig. 2. Evolution of the K_m of aldrin epoxidase measured in liver microsomes from rats treated with a single dose of styrene (500 mg/kg ●------; 100 mg/kg ×———; 10 mg/kg ○———) and killed 12 hr, 24 hr, 48 hr or 72 hr after administration. The value at zero time corresponds to the control value. Assay of enzymatic activity is described in Materials and Methods. The values are given with their standard deviation and with at least 35 degrees of freedom.

Aldrin epoxidase. As observed for benzo(a)pyrene hydroxylase, the K_m values decrease significantly as a function of time after styrene treatment (Fig. 2). At the lowest dose however, the effect is perceptible only from 24 hr, the K_m reaching its minimal value after 48 hr. At the two highest doses, the minimum is observed between 12 and 24 hr. At the three doses, the K_m of aldrin epoxidase tends to return to normal values within 72 hr.

A little decrease of the V_{max} of this enzyme, is observed, without dependence on either the dose or the time after injection. Pretreatment of the rats with phenobarbital induces the activity of aldrin epoxidase by a factor of 2.5 but does not modify the K_m value. The effect of 3-methylcholanthrene is clearly a reduction of both the K_m and the V_{max}.

Styrene epoxidase. This enzymatic activity was of peculiar interest in this study, because of its implication in the activation of styrene. Because of the technical difficulties of the Michaëlis–Menten analysis of this enzyme, the determinations were carried out solely for a constant time of 24 hr after styrene administration, when its effects on benzo(a)pyrene hydroxylase and aldrin epoxidase are generally maximum. Table 3 shows that styrene treatment does not significantly modify the K_m and the V_{max} of styrene epoxidase. Similarly, except for an increase of V_{max}, induced by phenobarbital, no modification is observed after treatment with phenobarbital or 3-methylcholanthrene (already reported by Salmona [29]).

Styrene oxide hydratase. A complete kinetic analysis of styrene oxide hydratase was performed using rat liver microsomal preparations obtained at different times after injection of 500 mg/kg styrene. A preliminary study had shown that 24 hr after the administration of styrene at doses of 500 mg/kg and 100 mg/kg, the K_m values were different from control; at 10 mg/kg, no effect was observed. Figure 3 shows that, at the highest dose, the K_m of styrene oxide hydratase is decreased as soon as 6 hr after administration, remains at this low value up to 48 hr and goes back thereafter to a value which is higher than the control value. No

Table 3. Effects of styrene, phenobarbital and 3-methylcholanthrene on the catalytic properties (K_m and V_{max}) of styrene epoxidase and styrene oxide hydratase*

Treatment	Styrene epoxidase K_m (μM)	Styrene epoxidase V_{max} (nmole min^{-1} mg $prot^{-1}$)	Styrene oxide hydratase K_m (μM)	Styrene oxide hydratase V_{max} (nmole min^{-1} mg $prot^{-1}$)
None	463 ± 36	5.29 ± 0.15	28.9 ± 5.1	4.8 ± 0.39
Styrene 10 mg/kg	610 ± 105	6.14 ± 0.43	31.7 ± 8.5	3.05 ± 0.19
100 mg/kg	611 ± 132	4.14 ± 0.36	8.32 ± 1.42	3.39 ± 0.93
500 mg/kg	391 ± 42	5.77 ± 0.22	12.8 ± 1.9†	5.50 ± 0.16
Phenobarbital	378 ± 37	15.48 ± 0.43†	33.77 ± 7.2	7.99 ± 0.27†
3-Methylcholanthrene	598 ± 77	7.64 ± 0.40	35.18 ± 10.1	4.41 ± 0.21

*Styrene (500 mg/kg, 100 mg/kg or 10 mg/kg) was administered (i.p.) 24 hr prior to sacrifice; phenobarbital (2 × 80 mg/kg i.p.) and 3-methylcholanthrene (2 × 40 mg/kg i.p.) were injected 48 hr and 24 hr before preparation of the microsomes. The values are given with their standard deviation and with at least 22 degrees of freedom.

† Significantly different from control rats ($P = 0.05$). Styrene epoxidase and styrene oxide hydratase activities have been evaluated as described in Materials and Methods.

relevant effect of the styrene administration on the V_{max} is observed. If one excepts the enhancement of V_{max} produced by phenobarbital (reported by Oesch *et al.* [30]), the kinetic parameters of styrene oxide hydratase remain unchanged after phenobarbital or 3-methylcholanthrene pretreatment (Table 3).

Effects of ethylbenzene. Table 4 shows that a single dose of 100 mg/kg ethylbenzene has no significant effect, 24 hr after injection, on the K_m's of benzo(a)pyrene hydroxylase, aldrin epoxidase, styrene epoxidase and styrene oxide hydratase. The V_{max} of those enzymes remain unchanged, after ethylbenzene treatment (not shown).

Effect of prior treatment on benzo(a)pyrene rat liver mediated mutagenesis

As shown in Table 5, pretreatment of the rats with phenobarbital does not significantly enhance the S-9-mediated mutagenicity of benzo(a)pyrene towards strain TA 1535. However, the addition of a liver fraction obtained from styrene or 3-methylcholanthrene pretreated animals produce an increase in mutagenicity. This enhancing effect is evident at the three concentrations of benzo(a)pyrene. With 3-methylcholanthrene it is more pronounced at the lowest concentration of the promutagen whereas it is relatively constant with styrene.

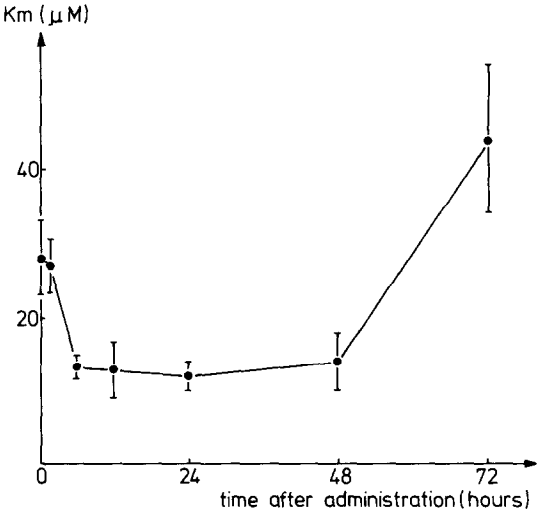

Fig. 3. Evolution of the K_m of styrene hydratase determined in liver microsomes from rats treated with a single dose of styrene (500 mg/kg) and killed 2 hr, 6 hr, 12 hr, 24 hr, 48 hr and 72 hr after injection. Assay of enzymatic activity is described in Materials and Methods. The values are given with their standard deviation and with at least 22 degrees of freedom.

DISCUSSION

The current study deals with the *in vivo* effects of styrene on the kinetic parameters of liver microsomal enzymes involved in activation or inactivation processes of chemicals. At doses ranging from 10 to 500 mg/kg, styrene does not produce severe toxic effects in the liver cells, as indicated by the absence of modification of the integrity of intracellular organelles. The activity of two microsomal enzymatic activities (glucose-6-phosphatase and NADPH:cytochrome *c* reductase), and the amount of cytochrome P-450 remain unchanged, as previously reported by Parkki *et al.* [31].

A Michaëlis–Menten kinetic analysis of some of the microsomal enzymes demonstrates however that styrene or its metabolite(s) may interact with some components of the liver cell. When injected i.p. to rats, it modifies the catalytic properties of several microsomal mixed function oxidases: benzo(a)pyrene hydroxylase (a typical aryl hydrocarbon hydroxylase) and aldrin epoxidase (a proposed model for the quantification of epoxide formation [23]), by significantly reducing their K_m. It does not modify styrene epoxidase, the enzyme which catalyzes its own metabolic activation, but it increases the affinity of the oxide hydratase for styrene oxide. Those effects are not dose-dependent but are time-related.

As previously reported by Parkki *et al.* [31], for a dose of 500 mg/kg, styrene (10, 100 or 500 mg/kg, i.p.) does not significantly induce the activity of benzo(a)pyrene hydroxylase and styrene oxide hydratase. It has no effect on the activity of styrene epoxidase and reduces slightly but significantly the V_{max} of aldrin epoxidase.

According to our previous proposal [13–15], the

Table 4. Effect of ethylbenzene on the K_m (μM) of benzo(a)pyrene hydroxylase, aldrin epoxidase, styrene epoxidase and styrene oxide hydratase*

Enzyme	Control rats	Treated rats
Benzo(a)pyrene hydroxylase	2.36 ± 0.12	2.79 ± 0.70
Aldrin epoxidase	2.23 ± 0.28	1.61 ± 0.30
Styrene epoxidase	463 ± 36	336 ± 31
Styrene oxide hydratase	28.9 ± 5.1	29.9 ± 5.7

* The complete kinetic analysis of those four enzymes was realized on liver microsomes from rats treated with ethylbenzene (100 mg/kg i.p.) and killed 24 hr after injection.

Values are given with their standard deviation and with at least 22 degrees of freedom.

Benzo(a)pyrene hydroxylase, aldrin epoxidase, styrene epoxidase and styrene oxide hydratase activities have been measured as described in Materials and Methods.

decrease of the K_m's of both aryl hydrocarbon hydroxylase and aldrin epoxidase could indicate that styrene pretreatment increases the capacity of those enzymes to activate some chemical carcinogens, such as polycyclic aromatic hydrocarbons, which require the formation of an intermediate epoxide.

Moreover, the fact that styrene modifies the oxide hydratase could indicate that it also stimulates the inactivation of those reactive intermediates. Even if it does not induce the microsomal enzyme in the classical sense, styrene could however, by modifying the affinity of those enzymes, markedly change the equilibrium between the various metabolic pathways.

Some of the effects of styrene can be compared with those of 3-methylcholanthrene; they both modify (decrease K_m) benzo(a)pyrene hydroxylase and aldrin epoxidase and they both do not induce or modify styrene epoxidase.

The other data, however, demonstrate that the two molecules do not act identically. 3-Methylcholanthrene induces the activity of benzo(a)pyrene hydroxylase, while styrene does not; styrene decreases the K_m of the oxide hydratase for styrene oxide but 3-methylcholanthrene does not.

The effect of both styrene and 3-methylcholanthrene on the affinity of benzo(a)pyrene hydroxylase correlates well with a significant increase in the capacity of the modified enzymes to activate benzo(a)pyrene to mutagenic intermediates. The lower effect of the styrene pretreatment could be explained by its specific interaction with oxide hydratase and by the fact that it does not induce aryl hydrocarbon hydroxylase activity.

On the other hand styrene does not behave like phenobarbital, another classical inducer of the microsomal enzymes.

Pretreatment of the rats with ethylbenzene does not affect the kinetic parameters of the investigated microsomal enzymes. Those results are in favour of a metabolite-mediated effect and emphasize the role of the vinylic moiety in the mechanism of styrene activity most probably via styrene oxide which has been demonstrated to be an important reactive intermediate of styrene, able to bind to macromolecules [10] and to cause mutations of *Salmonella typhimurium* [9].

In conclusion, it appears that the intraperitoneal administration of styrene modifies the catalytic properties of some liver microsomal enzymes involved in the metabolism of xenobiotics. This effect is almost exclusively limited to the K_m of those enzymes. This biochemical modification correlates well with an enhancement of the enzyme (S-9) mediated mutagenicity of benzo(a)pyrene. The demonstration that styrene is able to increase the enzyme mediated mutagenicity of benzo(a)pyrene, by modifying the catalytic properties of liver microsomal enzymes, calls for more thorough investigations of the possible effects exerted by styrene

Table 5. Effects of prior treatment on the rat liver activated mutagenicity of benzo(a)pyrene*

Prior treatment	Benzo(a)pyrene added *in vitro* 2.5 μg/plate	5 μg/plate	10 μg/plate
None	$\leqslant 3^{+}$	12 ± 6	10 ± 5
Phenobarbital	$\leqslant 3^{+}$	20 ± 7	20 ± 7
Styrene	15 ± 7	50 ± 13	50 ± 13
3-Methylcholanthrene	320 ± 40	275 ± 35	200 ± 30

* The bacterial assay is described in Materials and Methods. Rats were injected with styrene (500 mg/kg, i.p.) 24 hr before sacrifice, with phenobarbital (2 × 80 mg/kg, i.p.) or 3-methylcholanthrene (2 × 40 mg/kg, i.p.), 48 hr and 24 hr before sacrifice.

All values are histidine positive revertants per plate and are the mean of two separate experiments ± S.E.M.

In the absence of the S-9 fraction, the reversion rate obtained after addition of benzo(a)pyrene, is not different from the spontaneous reversion rate (16 rev./plate) which was subtracted from the given data.

$^{+}$ Not significant.

in the metabolic pattern of other mutagenic and/or carcinogenic compounds. Preliminary results from our laboratory have indeed indicated that styrene pretreatment of rats increases the S-9 mediated mutagenicity of acrylonitrile (de Meester *et al.* [32]).

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