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Trans-stilbene Oxide: A Selective Inducer of Rat Liver Epoxide Hydratase

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SUMMARY

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Trans-stilbene oxide (TSO) was shown to be a potent rat liver epoxide hydratase inducer which up to doses leading to maximal epoxide hydratase induction did not increase monooxygenase activities. The selectivity of rat liver epoxide hydratase induction by TSO was established using the cytochrome P-450 content, the NADPH-cytochrome c reductase, the aminopyrine N-demethylase and two benzo(a)pyrene monooxygenase activities as indicators of the monooxygenase system. TSO treatment caused a dose-dependent increase of epoxide hydratase activity of up to 300% the control value, whereas apart from a slight increase of the NADPH-cytochrome c reductase no significant changes in the five monooxygenase parameters were observed. Due to the broad and overlapping specificities of the various monooxygenases it is unlikely that an increase in any of them would not be reflected by an increase of activity towards aminopyrine or benzo(a)pyrene. Since this still cannot fully be excluded, we do not term this induction specific but selective. The limits of the selectivity of epoxide hydratase induction by TSO were investigated by using excessive doses of TSO, which produced slightly less induction of the epoxide hydratase than optimal doses. At such doses of TSO the monooxygenase activities were also induced. Studies on the kinetics of the epoxide hydratase induction showed that two days after a single injection of 2.5 mmoles TSO per kg body weight, maximal induction of epoxide hydratase was reached and thereafter the enzyme level declined slowly reaching control values after 12 days. Over the whole induction period no significant increase of the five monooxygenase parameters was found with the exception of a slight (30%) shortlived increase in NADPH-cytochrome c reductase activity. Twelve hours after administration of the inducer the aminopyrine N-demethylase and the two benzo(a)pyrene monooxygenase activities were reduced to about 60% of controls, probably because of the presence of relatively high concentrations of TSO in the microsomes at this early time point. Addition of TSO *in vitro* (10–1000 μM) did not influence the determination of the cytochrome P-450 content and had no effect on the epoxide hydratase activity, but inhibited the aminopyrine N-demethylase and the benzo(a)pyrene monooxygenase activities. However, no inhibition of the monooxygenase activities was seen when control liver microsomes were incubated together with various amounts of

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liver microsomes from TSO treated rats (under conditions of maximal epoxide hydratase induction), indicating that no induction of monooxygenase activities occurred which was overshadowed by the presence of TSO or its metabolites in the microsomes. The TSO-induced increase in epoxide hydratase activity toward four structurally very different substrates was virtually identical indicating that the induced enzyme or at least its active site is very similar if not identical to the basal enzyme. Concomitant treatment with actinomycin D or cycloheximide and TSO showed that the epoxide hydratase induction was dependent on RNA and protein synthesis. Moreover, SDS-gel electrophoresis performed with liver microsomes from TSO-treated rats clearly showed an increased intensity of the band, which co-migrated with a standard of epoxide hydratase purified to homogeneity. Thus, the increased epoxide hydratase activity after TSO treatment is due to the presence of higher amounts of enzyme protein and not to an activation of the enzyme by TSO or its metabolites.

INTRODUCTION

Electrophilically reactive epoxides of carcinogenic polycyclic hydrocarbons, which are metabolically produced by the microsomal monooxygenase (mixed function oxidase) system (for reviews see 1–6) present in most of the mammalian tissues, are known to be mutagenic (7–9, for reviews see 4, 5) and represent prime candidates for the ultimately carcinogenic species of these compounds (10). The adverse effects of epoxides of polycyclic hydrocarbons may be a consequence of their ability to bind irreversibly to cellular macromolecules such as DNA, RNA and protein (4, 5, 11, 12). Epoxide intermediates may rearrange nonenzymically to phenols or may be inactivated by the microsomal epoxide hydratase (epoxide hydrase) (2, 14) or by the cytoplasmic glutathione-S-transferases (15, 16). Epoxide hydratase is probably most critical (17), since it is localized in the same subcellular fraction as the epoxide-producing monooxygenase system. However, since the finding that 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene 9,10-oxide is an extremely potent mutagen (18–21) and 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene a mouse skin carcinogen (22), epoxide hydratase has to be considered not only as an inactivating enzyme (23) but also as the supplier of the precursor for the highly mutagenic 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene 9,10-oxide which is not inactivated by epoxide hydratase (24).

In order to elucidate the role of epoxide hydratase in the mechanism of tumorigenesis caused by polycyclic hydrocarbons, we attempted to find an epoxide hydratase inducer which does not affect the monooxygenase system. This task was very difficult since the monooxygenase system is very easily induced by many compounds of widely differing structures and functions (25–27). Indeed all epoxide hydratase inducers so far discovered also induce microsomal monooxygenase (28, 29). Moreover, striking similarities between the ontogenetic development of epoxide hydratase and microsomal monooxygenases have been observed (29) indicating that endogenous inducers may also affect both systems. However, the discovery of a selective transplacental induction of benzo(a)pyrene monooxygenase activity in fetal rat liver (29) suggested that a selective induction of epoxide hydratase should not be intrinsically impossible. In this study we wish to report on a selective induction of rat liver epoxide hydratase activity by TSO.[3] The dose and time-dependent effect of TSO treatment on epoxide hydratase and on several parameters of the monooxygenase system such as the cytochrome P-450 content, the NADPH-cytochrome c reductase, the aminopyrine N-demethylase and the benzo(a)pyrene monooxygenase activities are shown. By cumulative treatment with TSO the limits of this selective epoxide hydratase induction are demonstrated. Furthermore, it will be shown that TSO had no stimulatory effect on epoxide hydratase ac-

[3] The abbreviations used are: TSO, trans-stilbene oxide; CH, cycloheximide; AD, actinomycin D; EH, epoxide hydratase; SDS, sodium dodecyl sulfate; HEOM, 1,2,3,4,9,9 hexachloro-6,7 epoxy 1,4,4a,5,6,7,-8,8a-octahydro-1,4 methanonaphthalene; i.p., intraperitoneal; wt., weight.

tivity when added to the *in vitro* incubation medium but did increase the amount of enzyme of the treated animals, as determined by the intensity of the bands obtained by SDS-gel electrophoresis.

MATERIALS AND METHODS

Chemicals. [^{3}H]benzo(a)pyrene 4,5-oxide was prepared according to the method of Dansette and Jerina (30) under conditions previously described (31) and [7-^{3}H]styrene oxide as described previously (32). [G-^{3}H]-benzo(a)pyrene (5 Ci/mmole), [dimethylamine-^{14}C]aminopyrine (11.6 mCi/mmole) and [7-^{3}H]styrene were obtained from the Radiochemical Centre, Amersham, England. Trans-stilbene oxide was purchased from EGA Chemie, Steinheim, West Germany and its purity was established by NMR-spectroscopy, melting point and thin layer chromatography in ether and chloroform/petroleum ether (1:1). Cycloheximide, actinomycin D, NADPH, NADH and glucose-6-phosphate were from Serva, Heidelberg, West Germany, glucose-6-phosphate dehydrogenase from Boehringer Mannheim, West Germany, cytochrome c from Sigma Chemicals Company, St. Louis, Mo., USA, activated coconut charcoal from Fisher Scientific Company, Fair Lawn, N.J., USA, 2-(4-biphenyl)-5-(p-tert-butylphenyl)-1,3,4-oxadiazol (butyl PBD) and Unisolve 1 from Zinsser, Frankfurt/Main, West Germany. Other chemicals were of the purest grade commercially available.

Animal treatment. Adult male Sprague-Dawley rats (180–280 g) were obtained from Versuchstier-Zuchtanstalt WIGA Sulzfeld, West Germany and were kept in plastic cages with hardwood bedding under standardized conditions of light (light-dark cycle: 07:00–19:00) and temperature (21–24°) for at least four days before treatment. They had free access to Altromin pellets (Samen-Schmitt-Jakobi, Frankfurt a. M., West Germany) and tap water. Care was taken to avoid any environmental influence such as cigarette smoke, insecticides, noise, etc. Animals were always treated between 8 and 10 a.m. (and additionally between 8 and 9 p.m. when 12 hour treatment intervals were chosen). At all dosages, trans-stilbene oxide was injected i.p. dissolved in 0.5 ml of sunflower oil except for the experiment where rats were treated daily for 12 days. In this experiment TSO was given dissolved in 0.2 ml of oil. Doses and duration of treatment are indicated in legends to figures and tables. Cycloheximide and actinomycin D were dissolved or suspended in 0.9% NaCl (1 mg/ml). Rats received at 12 hour intervals an intraperitoneal injection of 1 mg/kg body wt. of either cycloheximide or actinomycin D. In all experiments control animals received the appropriate volume of vehicle alone.

Preparation of microsomes. The animals were always killed between 8 and 10 a.m. by a blow on the head and cervical dislocation. Liver microsomes washed and resuspended in 1.15% KCl containing 10 mM K phosphate buffer pH 7.4 were prepared as described (33). Protein concentrations were determined by the method of Lowry et al. (34) using bovine serum albumin as standard.

Enzyme Assays

General. Cytochrome P-450 content and NADPH-cytochrome c reductase activity were always determined immediately after preparation of the microsomes and aminopyrine N-demethylase activity after storing the freshly prepared microsomes in an ice bath for <24 hours. Losses in aminopyrine N-demethylase activity after such a storage for 24 hours were always <20%. The benzo(a)pyrene monooxygenase and the epoxide hydratase assays were performed after storage of <36 hours under the same conditions. No measurable losses of the benzo(a)pyrene monooxygenase and the epoxide hydratase activities from control or TSO pretreated rats could be observed when liver microsomes were kept in ice for 48 hours. Deviations of enzyme activities from theoretical linearity with respect to protein concentrations and time were less than 6% for epoxide hydratase, less than 10% for aminopyrine N-demethylase and less than 20% for benzo(a)pyrene monooxygenase.

Epoxide hydratase. Epoxide hydratase activities were determined using the radiometric extraction assays with [^{3}H]-benzo(a)pyrene 4,5-oxide (31), [7-^{3}H]sty-

rene oxide (32) and in one experiment with [^{3}H]3-methylcholanthrene 11,12-oxide (35) as substrates. The styrene oxide assay was performed (without Tween 80) under conditions previously described (36). Enzyme activities are corrected for 80% recovery of benzo(a)pyrene-4,5-dihydrodiol and 3-methylcholanthrene-11,12-dihydrodiol, and 86% recovery of styrene glycol. In one case epoxide hydratase activity was also determined using HEOM as substrate (37).

Benzo(a)pyrene monooxygenase. The benzo(a)pyrene monooxygenase activity (also called "aryl hydrocarbon hydroxylase") was measured by fluorimetric determination of the phenolic benzo(a)pyrene products using 3-hydroxybenzo(a)pyrene and quinine sulfate as standards, essentially as described by Nebert and Gelboin (38) (in this study labeled "3-OH-BP"). This assay was always used in combination with the radiometric assay originally described by Hayakawa and Udenfriend (39), since these two methods may provide quite different results with respect to monooxygenase induction (29). Results obtained by the latter assay are labeled "overall" (for a thorough discussion of these two assays see ref. 29). Assay conditions were as described (33). The tritiated water was counted after addition of Unisolve 1 in a Packard Tri-Carb liquid scintillation spectrometer, Model 3380, with a counting efficiency of 23–28%.

Aminopyrine N-demethylase. Aminopyrine N-demethylase activity was determined with the radiometric assay exactly as described by Poland and Nebert (40), except for the NADPH generating system, where the one as described for the benzo(a)pyrene monooxygenase assay (33) was used. The radioactive aminopyrine was always purified by thin-layer chromatography immediately before use.

NADPH-cytochrome c reductase. NADPH-cytochrome c reductase activity was measured by the method of Dallner et al. (41) using an incubation temperature of 37°C.

Cytochrome P-450 content. The total content of cytochrome P-450 was determined according to the method of Omura and Sato (42), with a Perkin Elmer 365 Dual-Wavelength Double Beam Spectrophotometer.

Electrophoresis. SDS-gel electrophoresis was performed in 2 mm thick slab gels with a 10% acrylamide concentration. Gels were stained in Coomassie blue (43).

Statistics

Student's *t*-test was used to establish the significance of differences between means. If not otherwise indicated $p < 0.025$ was chosen as the level of significance.

RESULTS AND DISCUSSION

Effect of trans-stilbene oxide in vitro on the assays used in this study. Trans-stilbene oxide (TSO) bears an epoxide group and is a highly lipophilic compound. Thus an interaction with both the epoxide hydratase and the monooxygenase system had to be considered. Therefore, the *in vitro* effect of various concentrations of TSO on the determination of all the parameters measured in this study was investigated in order to allow estimation of possible inhibition or activation of the liver microsomal enzyme activities by any TSO remaining in the microsomal membranes.

As can be seen from Fig. 1 the determination of the cytochrome P-450 content and epoxide hydratase activity using benzo(a)pyrene 4,5-oxide as substrate was not affected by TSO at the concentrations used. The NADPH-cytochrome c reductase activity was reduced to 68% of control, but only at the highest concentration of TSO (1000 μM), the concentration at which TSO started to precipitate, which might have influenced the spectrophotometric determination. TSO at one tenth of the substrate concentration in the benzo(a)pyrene monooxygenase assay had no inhibitory effect, but at a concentration equal to the substrate concentration the specific enzyme activities were reduced to 83% (3-OH-BP) and 57% (overall) of control. A further decrease to 41% and 21%, respectively, was observed when ten-fold more TSO (1000 μM) (solubility of TSO exceeded) than benzo(a)pyrene was added to the incubation mixture. With respect to the substrate concentration TSO was a more potent inhibitor of the aminopyrine N-demethylation, in that one tenth of the substrate concentration was enough to inhibit this enzyme activity to 64% of control. However,

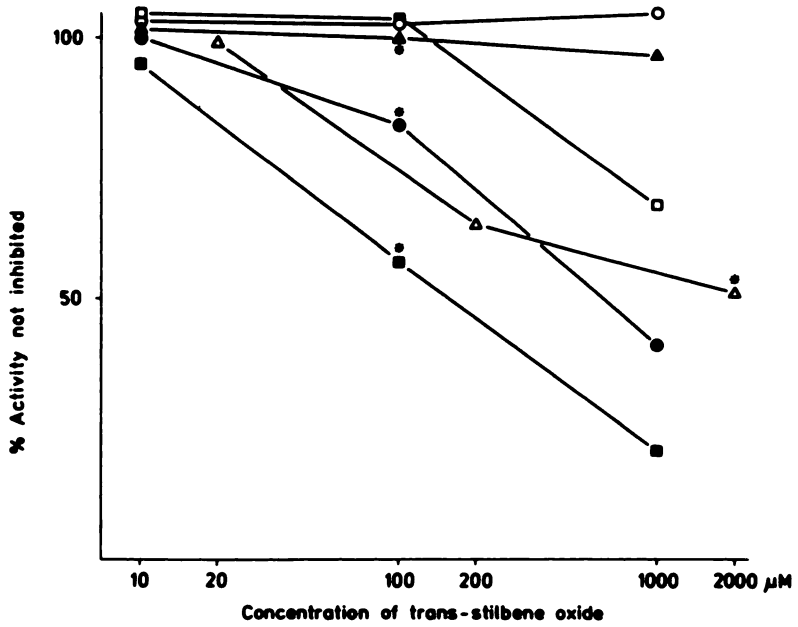

FIG. 1. *In vitro effect of trans-stilbene oxide (TSO) on the assays used in this study*

Liver microsomes from untreated rats were used. TSO was added dissolved in DMSO except for the benzo(a)pyrene monooxygenase assays, where ethanol was used. The final solvent concentration in the assays was always 1% (v/v). For the aminopyrine N-demethylase, the benzo(a)pyrene monooxygenase and the epoxide hydratase assays the substrates were added after 2 minutes of preincubation with TSO at 37°C. Values represent the means of 3–4 determinations performed at 2 protein concentrations, except for the determination of the cytochrome P-450 content where duplicate determinations were done. Results are expressed as percent of solvent controls. * indicates equimolar concentrations of TSO and substrate. Symbols for the assays are: ○——○ cytochrome P-450 content, ▲——▲ epoxide hydratase using benzo(a)pyrene 4,5-oxide as substrate, □——□ NADPH-cytochrome c reductase, △——△ aminopyrine N-demethylase, ●——● benzo(a)pyrene monooxygenase (3-OH-BP) and ■——■ benzo(a)pyrene monooxygenase (overall). The two highest concentrations of TSO used (1000 and 2000 μM) refer to the amount TSO added and not to the amount dissolved (TSO started to precipitate).

equimolar concentrations of TSO (solubility of TSO exceeded) and aminopyrine resulted in an inhibition to 51% of control, which is comparable to that observed in the radiometric benzo(a)pyrene monooxygenase assay with 100 μM TSO.

Effect of treatment with various amounts of trans-stilbene oxide on liver epoxide hydratase activity and the monooxygenase system. To establish the dose dependency of the TSO-induced increase of epoxide hydratase activity, rats were treated on three successive days with the doses indicated in Fig. 2 and were killed 24 hours after the last intraperitoneal injection. At the same time the selectivity of the epoxide hydratase induction was checked by determining the cytochrome P-450 content and the NADPH-cytochrome c reductase activity (two important components of the monooxygenase system) as well as by measuring the preferentially cytochrome P-450 dependent and phenobarbital inducible aminopyrine N-demethylase activity, and the predominantly cytochrome P-448 (P_1-450) dependent and polycyclic hydrocarbon inducible benzo(a)pyrene monooxygenase activities.

As can be seen from Fig. 2, the epoxide hydratase activity determined with styrene oxide as substrate was increased in a dose-dependent fashion up to about 300% of control activity after daily treatment with 2.0 mmoles (about 400 mg) TSO per kg body wt. for three days. The same dose dependency was seen when epoxide hydratase activity was measured with benzo(a)pyrene 4,5-oxide as substrate (data

not shown in figure). No activation or inhibition of epoxide hydratase activity was observed when control and liver microsomes from rats treated with the highest dose of TSO were incubated together. Over the whole range of doses the cytochrome P-450 content, the aminopyrine N-demethylase and the benzo(a)pyrene monooxygenase activities determined fluorimetrically (3-OH-BP) and radiometrically (overall) were never significantly different from control. Only the NADPH-cytochrome c reductase activity was slightly but significantly increased to 140% and 130% of controls after doses of 1.0 and 2.0 mmoles TSO, respectively. An attempt to provoke a higher induction of epoxide hydratase by treatment with daily doses of 4.0 mmoles TSO per kg body wt. failed in that the hepatic epoxide hydratase activity was only induced to 225% of control. This dose was already highly toxic (some of the treated animals did not survive the treatment period) while at the dose leading to maximal epoxide hydratase induction no signs of overt toxicity were observed (for pathological examination see below). Moreover, all measured monooxygenase parameters were considerably reduced. The cytochrome P-450 content was decreased to 57% of control possibly by a repressing effect of such acute toxic doses of TSO on the cytochrome P-

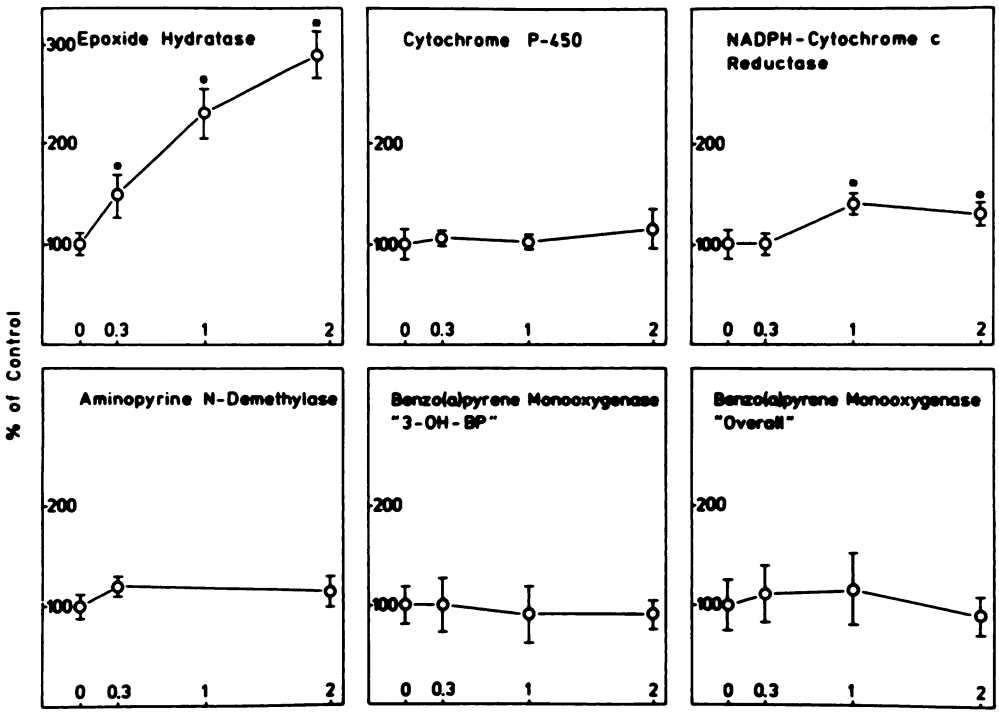

FIG. 2. *Dose-response curves of liver epoxide hydratase activity and monooxygenase parameters after daily intraperitoneal injections of trans-stilbene oxide for three days.*

The microsomal enzyme activities in rat livers were determined in duplicate at each of 2 protein concentrations. The cytochrome P-450 content was determined in duplicate. Activities or contents are expressed as percent of controls and represent the means ± SD of determinations performed on liver microsomes of at least three individuals. Control activities expressed as nmoles product per mg microsomal protein per min ± SD were: 6.97 ± 0.88 styrene glycol, 6.96 ± 1.13 benzo(a)pyrene-4,5-dihydrodiol, 324 ± 49 cytochrome c reduced, 4.09 ± 0.52 formaldehyde and 0.292 ± 0.058 3-hydroxybenzo(a)pyrene. Control benzo(a)pyrene monooxygenase activity determined by the radiometric assay was 6280 ± 1560 CPM (1 pmole benzo(a)pyrene = 92 ± 7 CPM) per mg microsomal protein per min. Cytochrome P-450 content in controls was 0.852 ± 0.128 nmoles per mg microsomal protein. $^{*}p < 0.025$ compared to control.

450 biosynthesis. The aminopyrine N-demethylase activity was decreased to 69% of controls. The benzo(a)pyrene monooxygenase activity was no longer linear with protein concentration and showed a decrease to 51% at the lower concentration of microsomal protein (0.13 mg per assay) and a reduction to 30% at the higher protein concentration (0.26 mg per assay). Incubations of mixtures of control microsomes with equal amounts (0.13 mg) of microsomes derived from the rat which was treated with daily doses of 4.0 mmoles (about 800 mg) TSO per kg body wt. for 3 days resulted (after preincubation for 3 min) in an inhibition of the control benzo(a)pyrene monooxygenase activity to about 70% (3-OH-BP and overall). This indicates that the inhibition of monooxygenase activity was at least in part caused by TSO remaining in the microsomes after treatment with such massive doses.

Consequently the question arose, whether the monooxygenase activities were also inhibited after the doses indicated in Fig. 2 and whether an apparent selectivity of induction of epoxide hydratase was only the result of such an inhibition. However, when liver microsomes from control animals were incubated together with various amounts of liver microsomes from rats which had been treated with daily doses of 2.0 mmoles TSO per kg body wt. for 3 days, no inhibition of the aminopyrine N-demethylase or benzo(a)pyrene monooxygenase activities could be observed when determined 24 hours after the last application of TSO. It seems unlikely that different monooxygenase activities would be inhibited to the same extent as they were induced by various doses of TSO, especially because no changes in the cytochrome P-450 content and no spectral shift in the Soret peak of the reduced cytochrome-CO complex were observed. A change in the pattern of the various cytochrome P-450 forms present in the liver microsomes cannot be excluded. However, such a change would have to result in a similar overall ability to oxidize aminopyrine and benzo(a)pyrene as control microsomes. Since these two substrates are preferentially oxidized by different forms of cytochrome P-450, such a change in the pattern of cytochromes is very unlikely.

Effect of a single dose of trans-stilbene oxide (TSO) on liver epoxide hydratase and the monooxygenase system as a function of time. Twelve hours after administration of a single intraperitoneal dose of 2.5 mmoles TSO per kg body wt. the epoxide hydratase activity (only results from the styrene oxide assay are depicted) increased rapidly reaching a maximum after two days (Fig. 3). The enzyme activity then declined reaching the control level twelve days after treatment. Epoxide hydratase activity at each time point was always very similar when determined with benzo(a)pyrene 4,5-oxide and styrene oxide as substrate.

Over the whole time course the cytochrome P-450 content, the aminopyrine N-demethylase and the benzo(a)pyrene monooxygenase activities when determined fluorimetrically (3-OH-BP) and radiometrically (overall) were never significantly increased. Only the NADPH-cytochrome c reductase activity was slightly increased to 130% of control 24 hours after treatment. The aminopyrine N-demethylase and benzo(a)pyrene monooxygenase activities were reduced to about 60% of control 12 hours after application of TSO. This effect is most likely due to the relatively high concentration of TSO in the microsomes at this time point. Thus, with the possible exception of a small increase of the NADPH-cytochrome c reductase activity, the induction of liver epoxide hydratase by TSO was found to be selective with respect to the measured monooxygenase parameters, whether measured at different times following a single dose or at the same time following different doses. Preliminary experiments also revealed no effect of TSO treatment on the cytoplasmic glutathione transferase activity, determined with benzo(a)pyrene 4,5-oxide as substrate (data not shown).

Effect of repeated treatment with trans-stilbene oxide on three, six and twelve successive days. This experiment, where rats were treated with daily doses of 1.0 mmole (about 200 mg) TSO per kg body wt. for three, six and twelve days, was performed to answer the following questions: (1) Is it

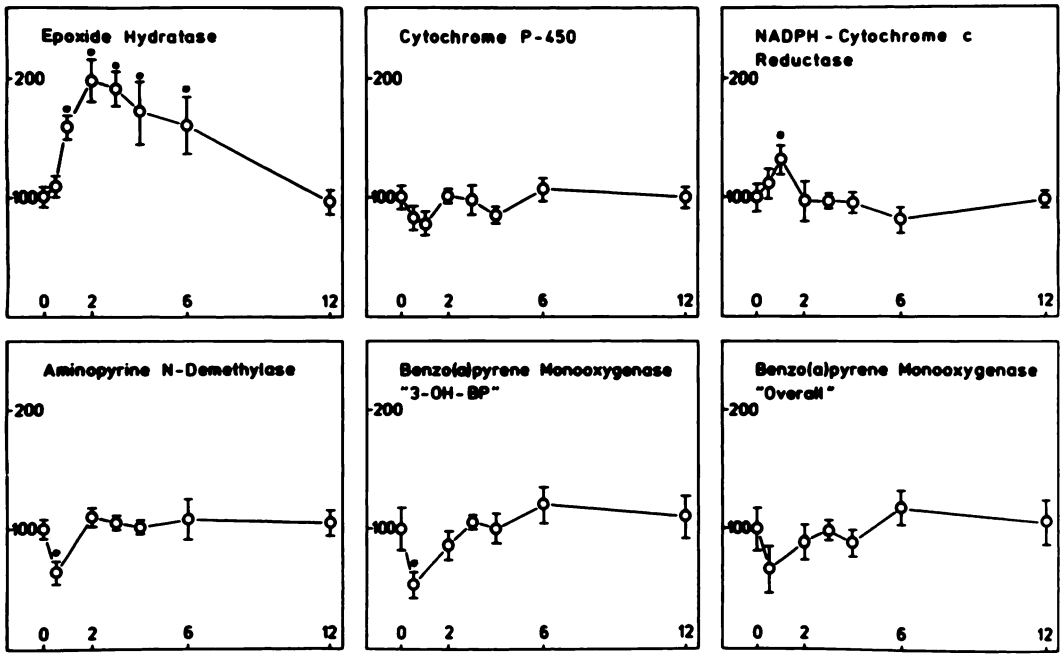

FIG. 3. *Time-response curves of liver epoxide hydratase activity and monooxygenase parameters after a single intraperitoneal treatment with trans-stilbene oxide (TSO)*

Male Sprague-Dawley rats were treated with a single intraperitoneal injection of 2.5 mmoles TSO per kg body wt. as described in MATERIALS AND METHODS. Enzyme activities were determined in duplicate at each of 2 protein concentrations and duplicate determinations were performed for the cytochrome P-450 content. Activities or contents are expressed as percent of controls and represent the mean ± SD of determinations performed on liver microsomes of at least three individuals. Control activities expressed as nmoles product per mg microsomal protein per min. ± SD were: 7.10 ± 0.57 styrene glycol, 6.49 ± 0.81 benzo(a)pyrene-4,5-dihydrodiol, 371 ± 44 cytochrome c reduced, 3.88 ± 0.31 formaldehyde and 0.334 ± 0.059 3-hydroxybenzo(a)pyrene. Control benzo(a)pyrene monooxygenase activity determined by the radiometric assay was 5490 ± 950 CPM per mg microsomal protein per min and cytochrome P-450 content in controls was 0.815 ± 0.081 nmoles per mg microsomal protein. $^{*}p < 0.025$ compared to controls.

possible to achieve a higher induction of epoxide hydratase by repeated administration of TSO or will a plateau be reached after a certain time and dosage? (2) What is the effect of such a long term treatment with relatively high doses of TSO on the monooxygenase system and the body weight gain? (3) Can any gross pathological changes be observed after such an extreme exposure of the rats to TSO?

During the first six days of treatment epoxide hydratase activity increased to about 300% of control, but no further increase was observed after treatment for another six days; a slightly lower induction was found after 12 days (Table 1). In this experiment the epoxide hydratase activity was measured with 3-methylcholanthrene 11,12-oxide as well as styrene oxide and benzo(a)pyrene 4,5-oxide substrates. The same level of induction was also found when epoxide hydratase activity was determined using a structurally very different substrate, the insecticide 1,2,3,4,9,9-hexachloro-6,7 epoxy 1,4,4a,5,6,7,8,8a-octahydro-1,4 methanonaphthalene.[4] The high correlation of the increases (% of control) of epoxide hydratase activities, which were the same for the four substrates in that the deviations between substrates were not greater than the experimental deviation for the individ-

[4] C. H. Walker, P. Bentley and F. Oesch, unpublished results

TABLE 1

Effect of repeated administration of trans-stilbene oxide (TSO) on rat liver epoxide hydratase activities and parameters of the microsomal monooxygenase system

Male Sprague-Dawley rats (200–280 g) were treated by daily intraperitoneal injections of 1.0 mmole TSO per kg body wt. for 3, 6 and 12 days and were killed 24 hours after the last injection. Enzyme activities are expressed as nmoles product (trans-dihydrodiols) per mg microsomal protein per min for epoxide hydratase and as nmoles cytochrome c reduced per mg microsomal protein per min for NADPH-cytochrome c reductase. The benzo(a)pyrene monooxygenase activities are expressed as pmoles 3-hydroxybenzo(a)pyrene per mg microsomal protein per min (3-OH-BP) and as CPM per mg microsomal protein per min (overall). Cytochrome P-450 content is expressed as nmoles per mg microsomal protein. Values represent the means ± SD of determinations performed individually on liver microsomes from three animals.

		Epoxide hydratase		
		Styrene oxide	Benzo(a)pyrene 4,5-oxide	3-Methylcholanthrene 11,12-oxide
Oil	3 days	6.42 ± 0.92	6.74 ± 0.99	1.08 ± 0.09
Oil	6 days	6.43 ± 1.07	6.61 ± 0.78	1.01 ± 0.13
Oil	12 days	6.52 ± 0.17	6.14 ± 0.83	1.15 ± 0.07
TSO	3 days	14.85 ± 1.75[b]	15.78 ± 2.42[b]	2.56 ± 0.17[c]
TSO	6 days	21.43 ± 1.51[c]	20.37 ± 2.28[c]	2.87 ± 0.50[b]
TSO	12 days	18.20 ± 2.30[c]	16.62 ± 0.65[c]	2.57 ± 0.42[b]

		NADPH cyt. c reductase	Cytochrome P-450	Benzo(a)pyrene monooxygenase	
				3-OH-BP	Overall
Oil	3 days	249 ± 49	0.811 ± 0.026	260 ± 45	5141 ± 757
Oil	6 days	280 ± 48	0.835 ± 0.042	298 ± 52	4950 ± 1848
Oil	12 days	308 ± 34	0.807 ± 0.049	330 ± 48	5185 ± 1150
TSO	3 days	348 ± 7[a]	0.825 ± 0.042	231 ± 79	5980 ± 1908
TSO	6 days	368 ± 20[a]	1.045 ± 0.009[b]	323 ± 70	7655 ± 2116
TSO	12 days	391 ± 52	1.095 ± 0.098[a]	434 ± 48	13504 ± 4014[a]

[a] $p < 0.025$.
[b] $p < 0.005$.
[c] $p < 0.0005$.

ual substrates, suggests that the active site of the TSO-induced epoxide hydratase is very similar to that of the enzyme in control animals. This indication becomes even more convincing when considering that liver microsomes from animal species phylogenetically quite far apart from each other had very different substrate preferences toward the individual substrates used in this study (44). Thus these substrates are quite diagnostic to differentiate between different epoxide hydratase forms.

The parameters of the monooxygenase system were, as already seen in the dose response curve, not significantly different from controls after three days of treatment, apart from an increase of the NADPH-cytochrome c reductase activity to 140% of control. This augmentation of NADPH-cytochrome c reductase activity was not dependent on the duration of treatment and remained at about 130% of control after six and twelve days. The cytochrome P-450 content was also slightly enhanced after six and twelve days of treatment. The benzo(a)pyrene monooxygenase activities, however, were not significantly increased after six days. Only treatment for twelve days, which did not cause a further increase of the epoxide hydratase activity, led to a significant induction of the benzo(a)pyrene monooxygenase activity (overall). The increased level of components of the monooxygenase system under these extreme conditions may be explained either by a low affinity of TSO to (a) receptor(s) responsible for the induction of one or more cytochrome P-450 forms, or by a secondary

effect such as the release of an endogenous inducer of cytochrome P-450 after repeated administration of TSO. No acute toxic effects could be noticed and this treatment seemed to be tolerated by the animals. Moreover, no gross lesions were observed on pathological examination and no difference in the body weight gain between control and TSO treated rats was registered.

Effect of cycloheximide and actinomycin D on the induction of epoxide hydratase activity by trans-stilbene oxide in rat liver. The relatively rapid increase of epoxide hydratase activity after a single dose of TSO enabled us to examine whether this increase was dependent on RNA and protein synthesis. A rapid increase of enzyme activity after application of the inducer was needed, since the rats would not have survived longer than 24 hours or 48 hours with the dosage schedule used for actinomycin D and cycloheximide, respectively. As can be seen from Fig. 4, treatment with 1 mg cycloheximide or actinomycin D per kg body wt. half an hour before application of a single dose of 2.5 mmoles TSO per kg body wt. and repeated treatment with the RNA and protein synthesis inhibitors at intervals of twelve hours clearly prevented the induction of liver epoxide hydratase activity by TSO. The lower induction in the experiment in which actinomycin D was used is because the high toxicity of this compound allowed a treatment for only 24 hours, whereas in the experiment with cycloheximide the rats were killed 48 hours after treatment with TSO.

The consistent slight increase of the NADPH-cytochrome c reductase activity caused by TSO treatment was also reduced after treatment with either protein synthesis inhibitor.

SDS-gel electrophoresis with liver microsomes from trans-stilbene oxide pre-

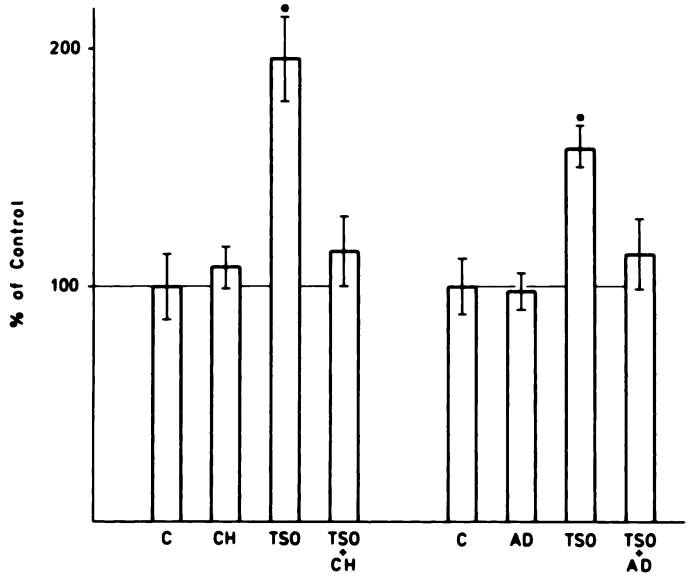

FIG. 4. *Effect of cycloheximide (CH) and actinomycin D (AD) on the induction of epoxide hydratase activity by trans-stilbene oxide*

Where indicated by TSO, animals were treated by a single intraperitoneal injection of 2.5 mmoles TSO per kg body wt. In a first experiment one group received 1 mg CH per kg body wt. alone and another group was treated with the same dose half an hour before TSO was given. CH was injected at intervals of 12 hours. The animals were killed 48 hours after treatment with TSO and 12 hours after the last injection of CH. In a second experiment animals received actinomycin D instead of CH. The same dose schedule was followed but the animals were killed 24 hours after TSO treatment. Bars represent the means of enzyme activities as percent of controls ± SD of determinations performed on liver microsomes from 3 animals per group. Control activities were 7.82 ± 1.12 and 6.33 ± 0.76 nmoles styrene glycol per mg microsomal protein per min, respectively. $^{*}p < 0.01$ compared to controls and TSO + AD.

treated rats. Since epoxide hydratase purified to apparent homogeneity (45) was available as standard, SDS-gel electrophoresis provided a method to test whether the increase of epoxide hydratase activity after treatment with TSO was due to the presence of higher amounts of enzyme protein. Figure 5 is a photograph of an electrophoresis-gel, which shows (alternating with the epoxide hydratase standard) the gel electrophoretic pattern of liver microsomal proteins from a control rat (pos. 3) and a TSO-treated rat (pos. 5). In sample number 5, which shows the pattern of liver microsomal proteins from a TSO-pretreated rat, the band which co-migrated with the epoxide hydratase standard appeared to be much stronger than the same band in control microsomes (pos. 3). The intensity of this band was increased when pure epoxide hydratase was added to control microsomes (pos. 1). These observations are in good agreement with the measured enzyme activities.

In conclusion, TSO was demonstrated to be a potent inducer of rat liver epoxide hydratase activity. This induction was selective for epoxide hydratase when compared with five monooxygenase parameters over a broad range of doses and throughout the whole induction time course of epoxide hydratase after a single dose of TSO. Although an *in vitro* inhibition of the aminopyrine N-demethylase and the benzo(a)pyrene monooxygenase activities by TSO was found, no inhibition of these enzyme activities could be observed when control and liver microsomes of rats, which were treated with doses of TSO leading to maximal induction of epoxide hydratase, were incubated together, indicating that the selective induction of epoxide hydratase was not the result of an inhibition of the monooxygenase activities by TSO present in the microsomes. Moreover, the total cytochrome P-450 content and the difference spectrum of the reduced cytochrome P-450 CO-complex were not changed. It seems rather unlikely, but cannot be excluded, that upon treatment with TSO the composition of the different cytochrome P-450 forms might be changed in a manner which does not lead to differences in the gel electrophoretic pattern or in the substrate specificity for benzo(a)pyrene and aminopyrine (which are oxidized by different cytochrome P-450 forms) but would lead to different properties with respect to other substrates of the monooxygenase system. Because of the complexity of the monooxygenase system, the several cytochrome P-450 forms being quite similar to each other and the uncertainty of their number, it seems impossible to ascertain that this induction is entirely specific for epoxide hydratase with no effect on any monooxygenase activity. We therefore did not term this induction specific, but selective.

By repeated administration of relatively high doses of TSO for 3, 6 and 12 days the limits of the extent of the epoxide hydratase induction as well as the limits of the selectivity of this induction were demonstrated.

The TSO-induced increase of rat liver epoxide hydratase activity was shown to be dependent on RNA and protein synthesis since it could be prevented by pretreatment of the rats with actinomycin D or cycloheximide. The gel electrophoretic observation of an increased intensity of the band which co-migrated with the epoxide hydratase standard indicates that the augmentation of the epoxide hydratase activity in rat liver after treatment with TSO is due to the presence of more enzyme protein and not to an activation of the enzyme by TSO or metabolites derived from it.

The substrate specificity of the induced epoxide hydratase with respect to the four substrates styrene oxide, benzo(a)pyrene 4,5-oxide, 3-methylcholanthrene 11,12-oxide and HEOM was found to be very similar to that of epoxide hydratase from untreated rats suggesting that the active site of the TSO-induced epoxide hydratase is the same as that of the enzyme in control animals.

The fact that selective induction of epoxide hydratase has been found so far only in rat liver obviously limits its range of application. However, since all other epoxide hydratase inducers so far discovered also induced the monooxygenase system, the main task of this study was to determine whether a selective induction of epoxide hydratase is intrinsically possible in any

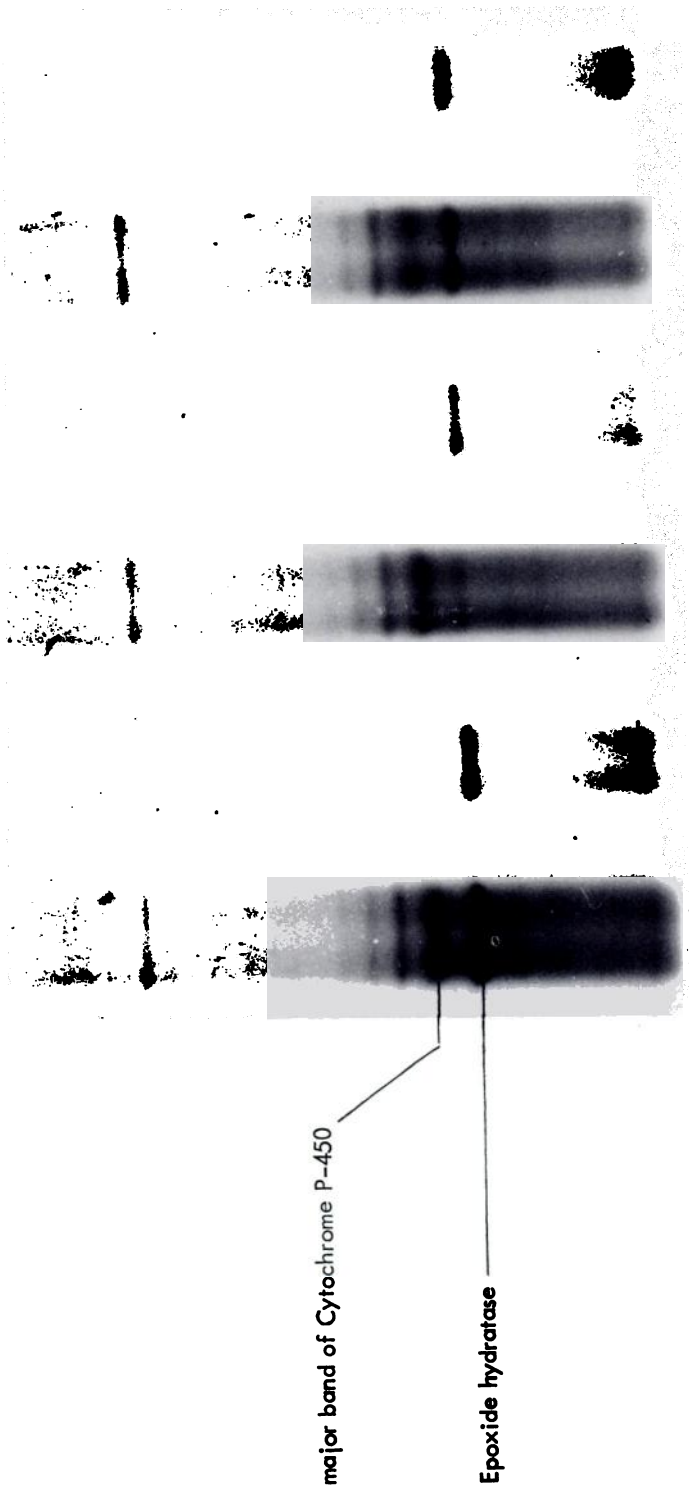

FIG. 5. *SDS-Polyacrylamide slab gel of liver microsomal proteins after treatment with trans-stilbene oxide*

Electrophoretic migration is from top to bottom. Twenty microliter samples of preparations of liver microsomes containing 1 mg protein per ml were applied to 2 mm thick slab gels containing 10% acrylamide and 0.1% SDS. Position 1 contains 20 μg of control liver microsomal protein plus epoxide hydratase standard. Position 3 contains 20 μg control liver microsomal protein alone. To well 5, 20 μg microsomal liver protein from a TSO treated rat was applied. Positions 2, 4 and 6 contain epoxide hydratase standard.

organ. Selective induction of epoxide hydratase in rat liver should prove to be a useful tool to elucidate the role of epoxide hydratase in the overall biotransformation of polycyclic hydrocarbons leading to mutagenic and carcinogenic metabolites in *in vitro* model systems. In contrast to studies of the role of epoxide hydratase in reconstituted systems (24) or by addition of pure epoxide hydratase to liver microsomes (14, 23) we have now the means to investigate the effect of an increased epoxide hydratase activity in its natural environment which may possibly be important for the enzyme's characteristics. Furthermore this study has provided the basis for further investigations to find a selective epoxide hydratase inducer for target organs of polycyclic hydrocarbons, such as skin and lung, which would allow a direct study of the role of epoxide hydratase in the mechanism of tumor formation caused by polycyclic hydrocarbons besides the rather narrow range of experimental conditions where polycyclic aromatic hydrocarbons can cause liver tumors. If studies on the influence of a selective epoxide hydratase induction on tumor formation by olefinic and aromatic compounds activated via epoxides were going to show that epoxide hydratase acts as a predominantly inactivating enzyme, the development of selective epoxide hydratase inducers may even have practical importance in the prevention of tumors caused by such compounds.

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