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The Epoxide Hydratase Inducer Trans-Stilbene Oxide Shifts the Metabolic Epoxidation of Benzo(a)pyrene from the Bay- to the K-Region and Reduces Its Mutagenicity

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SUMMARY

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Trans-stilbene oxide is a potent inducer of epoxide hydratase in rat liver which does not significantly alter cytochrome P-450 content or the specific activities of aminopyrine N-demethylase and benzo(a)pyrene monooxygenase measured by tritium release or fluorescence of phenols. Although three of these monooxygenase parameters are directly related to the metabolism of benzo(a)pyrene, we now report the surprising observation that the pattern of benzo(a)pyrene metabolites is substantially altered by trans-stilbene oxide treatment. The formation of metabolites oxidized at the 7,8,9,10-benzo ring was decreased by a factor of 3 whereas the formation of 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene was increased 6-fold. The ratio between 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (dihydrodiol bay region epoxide precursor) and 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene was decreased more than 20-fold. We also found that ethoxycoumarin O-deethylase activity was increased 2.5-fold and preferentially inhibited by metyrapone. These marked effects on monooxygenase activities severely limit the selectivity of trans-stilbene oxide as epoxide hydratase inducer. The mutagenicity with *his*$^-$ *Salmonella typhimurium* was up to 10 times lower when benzo(a)pyrene was activated by microsomes or postmitochondrial fraction from trans-stilbene oxide-treated as compared to control animals. Two factors contribute to this protection: (1) A decreased formation of mutagenic dihydrodiol epoxides at the 7,8,9,10-benzo ring due to the shift of the metabolism to the 4,5-position, (2) a more efficient inactivation of the mutagenic benzo(a)pyrene 4,5-oxide due to the induction of epoxide hydratase. Thus trans-stilbene oxide causes two effects in the rat liver, which can synergistically reduce the harmful effects of benzo(a)pyrene. This study shows that benzo(a)pyrene metabolite patterns and consequent biological effects can be substantially altered without any significant change in the fluorescence intensity of the alkali extractable metabolites, the so called AHH (aryl hydrocarbon hydroxylase) activity.

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[1] This study is part of the Ph.D. theses of M. Bücker (mutagenicity) and M. Golan (metabolism).

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INTRODUCTION

Epoxide hydratase (epoxide hydrase) is a key enzyme in the inactivation of many monofunctional epoxides (1, 2). However, with some angular polycyclic aromatic hydrocarbons it catalyses the production of the precursors of highly mutagenic dihydrodiol epoxides (3–8). Moreover, it has been identified as the neoplastic antigen present in large amounts in preneoplastic liver nodules of acetylaminofluorene-treated rats (9). To establish the *in vivo* role of this enzyme, animals different in epoxide hydratase activity and equal in other respects would be ideal. We therefore set out to develop selective epoxide hydratase inducers which do not affect functionally related enzymes. Monooxygenases especially, which produce the substrates for epoxide hydratase, should not be affected. A problem is that monooxygenases are easily induced by many different chemicals (10, 11). In spite of this difficulty, we found that TSO[3] induced epoxide hydratase in rat liver up to 3-fold without significantly altering any of the following monooxygenase parameters (12): cytochrome P-450 content and CO-difference spectrum, benzo(a)pyrene monooxygenase determined by tritium release or fluorescence of the phenolic fraction and aminopyrine *N*-demethylase. These monooxygenase activities were chosen since they are often induced differently and therefore are under different control. Since, in addition, monooxygenases possess broad and overlapping substrate specificities it could be expected from these results that many, if not all, monooxygenase activities are unaffected by the treatment with TSO. However, several different cytochrome P-450 forms are known. Nebert even postulates the existence of a potentially infinite number of different cytochrome P-450's (summarized in reference 13). Thus, unchanged monooxygenase activity with only a few substrates does not prove unchanged activity with others.

Indeed, in this study, we have to report that at least two monooxygenase parameters are affected by TSO: (a) O-Deethylation of ethoxycoumarin is increased, and (b) most interestingly, we observed a strong shift of the site at which benzo(a)pyrene is oxidized, although the total production of metabolites was not significantly changed. We show in this study that this qualitative alteration in the metabolism of benzo(a)pyrene has strong biological consequences. This is demonstrated by the activation of benzo(a)pyrene *in vitro* to mutagens for *Salmonella typhimurium*.

[3] The abbreviations used are TSO, *trans*-stilbene α,β-oxide; HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Chemicals. 7-Hydroxycoumarin (Merck-Schuchardt, Hohenbrunn, Germany) as sodium salt was alkylated with ethyl iodide in absolute ethanol at 80° for 8 hours. The ethanol was removed by distillation and the unreacted starting material by extraction with 5% sodium carbonate solution. After two recrystallizations, 7-ethoxycoumarin was obtained as white crystals with a melting point of 91°.

Trans-stilbene oxide was obtained from EGA-Chemie, Steinheim, Germany. It was purified by charcoal treatment and recrystallization from ether until it was pure according to NMR-spectroscopy, melting point and thin layer chromatography on silica gel with ether or chloroform-petroleum ether (1:1).

[7-^{3}H]styrene oxide was prepared as described previously (14). [^{14}C]-Benzo(a)pyrene was purchased from Radiochemical Centre, Amersham, England, Rotiszint 22 from Karl Roth AG, Karlsruhe, Germany.

Animals. Adult male Sprague Dawley rats, weighing 210–230 g, were obtained from Versuchstier-Zuchtanstalt WIGA Sulzfeld, Germany. Trans-stilbene oxide-treated animals received intraperitoneal injections of 2 mmole per kg body weight trans-stilbene oxide (dissolved in 0.5 ml sunflower oil) on three consecutive days. Control animals received three times 0.5 ml sunflower oil at 24 hr intervals. Animals were sacrificed by cervical dislocation 24 hr after the last injection.

Tissue preparations. 9000 *g* supernatant and microsomes were prepared as described previously (15). Protein concentrations were determined by the method of Lowry

et al. (16) using bovine serum albumin as standard. The following parameters were all determined using aliquots of the same preparations: monooxygenase and epoxide hydratase activities, benzo(a)pyrene metabolite profile and mutagenicity. Subcellular fractions from six trans-stilbene oxide-treated and from four control animals were prepared, preparations of two animals being pooled. To avoid any influence of storage they were used on the day of preparation.

Enzyme assays. Monooxygenase activity with 7-ethoxycoumarin as substrate was determined by fluorescence spectrophotometry according to Ullrich and Weber (17) using three diagnostic inhibitors as a crude measure for different monooxygenase forms (18). Epoxide hydratase activity was determined with styrene oxide as substrate using the radiometric extraction assay (14) but without Tween 80.

Study of the benzo(a)pyrene metabolite pattern by HPLC. Benzo(a)pyrene and microsomes were incubated under the following conditions: the incubation mixture (2 ml) consisted of 50 mM K phosphate buffer pH 7.4, 5 mM $MgCl_2$, 0.62 mM NADPH, 0.36 mM NADH, 8 mM glucose 6-phosphate, 0.5 Kornberg units of glucose 6-phosphate dehydrogenase, 1 mg bovine serum albumin and 1.8–2.2 mg microsomal protein. The incubation was started by addition of 0.19 μmole [^{14}C]-labelled benzo(a)pyrene (3.75 mCi/mmole) dissolved in 50 μl ethanol. After a 20 min incubation at 37° in the dark, the reaction was stopped by addition of 6 ml chilled acetone/ethylacetate (1:2 v/v) and extraction for 10 min, which was followed by a second extraction. The organic layers of five parallel incubations were pooled, dried with anhydrous $MgSO_4$ and evaporated to dryness under an argon stream. The products were dissolved in 100 μl ethanol and stored at −70° prior to HPLC analysis. This analysis was carried out on a Spectra Physics SP 3500 B high performance liquid chromatograph fitted with a Spherisorb ODS 10 μ column. The column was maintained at 30°. The column was eluted with a linear gradient of acetonitrile and water with 20 to 60% acetonitrile and a constant flow of 0.8 ml/min. A 10 μl sample was injected into the chromatograph. The elution pattern was monitored by absorption at 280 nm. The eluate was collected in 20 sec fractions and its radioactivity was determined by scintillation spectrophotometry with Rotiszint 22 as scintillation liquid.

Mutagenicity tests. The mutagenicity experiments were performed as described by Ames *et al.* (19) with minor modifications. The histidine-dependent *Salmonella typhimurium* strains TA 1537, TA 100 and TA 98 were grown overnight in nutrient broth (8 g Bacto Nutrient Broth (Difco) and 5 g NaCl per liter medium).

The activating system for the mutagenicity experiment was prepared by mixing one volume 9000 *g* supernatant (30 mg protein/ml) or microsomal fraction (10 mg protein/ml) of the liver homogenate with one volume 24 mM $MgCl_2$–100 mM KCl and one volume NADPH generating system (12 mM NADP, 15 mM glucose 6-phosphate, 6 units glucose 6-phosphate dehydrogenase per ml, 150 mM Na phosphate pH 7.4). Benzo(a)pyrene (dissolved in 10 μl dimethylsulfoxide), 500 μl activating system, 100 μl bacterial overnight culture (1–2 × 10^9 bacteria/ml) and 2000 μl histidine-poor soft agar (0.55% agar, 0.55% NaCl, 50 μM histidine, 50 μM biotin, 25 mM Na phosphate, pH 7.4, 45°) were mixed in a test tube and poured onto a petri dish with minimal agar (1.5% agar, Vogel-Bonner E medium (20) with 2% glucose). After incubation for two days in the dark at 37°, colonies (his$^+$ revertants) were counted.

RESULTS

Effect of TSO treatment on epoxide hydratase and monooxygenase activities. TSO treatment of the rats increased epoxide hydratase activity in liver microsomes threefold (Table 1), which corresponds to previously reported values (12). Although in a previous study (12) it was found that TSO did not significantly alter four monooxygenase parameters, we now observed that another monooxygenase activity, O-deethylation of ethoxycoumarin is markedly increased (Table 1). In liver microsomes of TSO-treated animals this activity

TABLE 1

Effect of trans-stilbene oxide administration on epoxide hydratase and ethoxycoumarin O-deethylase activity in rat liver microsomes

Male Sprague Dawley rats (210–230 g) received intraperitoneal injections of trans-stilbene oxide (2 mmoles per kg body weight, dissolved in 0.5 ml sunflower oil) or sunflower oil only 72, 48 and 24 hr before sacrifice. Epoxide hydratase activity was determined with styrene oxide as substrate (14) without Tween 80 and ethoxycoumarin O-deethylase according to Ullrich and Weber (17). The values represent means ± SD from 3 experiments with trans-stilbene oxide-treated and from 2 experiments with control animals. For each experiment organs from two animals were pooled.

Treatment	Epoxide hydratase		Ethoxycoumarin O-Deethylase	
	nmoles styrene glycol/min/mg protein	*% of control*	*pmoles 7-OH-coumarin/min/mg protein*	*% of control*
Sunflower oil only	8.9 ± 0.8	100	790 ± 120	100
Trans-stilbene oxide	27.4 ± 2.1[a]	309	1980 ± 240[a]	250

[a] Data differ significantly from controls ($p < 0.005$).

was 2.5-fold higher than in microsomes of control animals.

It is known that ethoxycoumarin can be deethylated by several forms of monooxygenase. The phenobarbital-induced enzyme activity is preferentially inhibited by metyrapone, the 3-methylcholanthrene-induced activity by α-naphthoflavone and the ethanol-induced activity by tetrahydrofuran (18). We therefore used these diagnostic inhibitors for characterization of the TSO-induced ethoxycoumarin O-deethylase activity. Figure 1 shows that the induced enzyme is not significantly inhibited by α-naphthoflavone. If the activity of control microsomes is subtracted from the activity of TSO-induced microsomes the inhibition by 10^{-3} M tetrahydrofuran was only 7% whereas the control enzyme activity was inhibited by 44%. The converse was found with metyrapone which inhibited the control enzyme only slightly. A concentration of 10^{-5} M inhibited it by 24%. At this concentration, microsomes from TSO-treated animals showed exactly the same specific enzyme activity as control microsomes. This indicates that the newly formed enzyme was completely inhibited. Thus, the induced monooxygenase resembles more that inducible by phenobarbital than by 3-methylcholanthrene or ethanol. However it should be noted that phenobarbital, in contrast to TSO, also markedly induces cytochrome P-450 and cytochrome c reductase, aminopyrine *N*-demethylase and benzo(a)pyrene monooxygenase activity determined by tritium release or fluorescence of the phenolic fraction.

Effect of TSO treatment on the pattern of benzo(a)pyrene metabolites. To investigate the effect of the induction of epoxide hydratase and alteration of the monooxygenases by TSO pretreatment on the pattern of benzo(a)pyrene metabolites, we incubated [^{14}C]-benzo(a)pyrene with control and with TSO-induced rat liver microsomes and separated the metabolites by HPLC (Fig. 2). Standard compounds and two different elution systems were used to characterize the metabolites. The results presented in Fig. 2 and in Table 2 are derived from a separation using an acetonitrile-water gradient. Quantitatively similar results were obtained using a methanol-water gradient with the exception that the 4,5-epoxide peak was separated from the 3,6-quinone peak when the acetonitrile-water but not when the methanol-water gradient was used. Furthermore it was confirmed that metabolites which were (from their mobilities) tentatively identified as dihydrodiols disappeared or were markedly reduced in parallel incubations where the epoxide hydratase inhibitor 1,1,1-trichloropropene 2,3-oxide (21) was present (cf. Fig. 2). By these means all of the major metabolite peaks could be characterized. Using [^{14}C]-labelled benzo(a)pyrene as substrate these peaks were quantitated. As seen from Table 2, the total of the metabolites was not significantly increased after TSO-treatment of the rats. However a most remarkable shift of the metabolism was observed. The quantity of metabolites which were oxidized at the benzo ring (7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, 9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene, 9-hydroxybenzo(a)pyrene) was greatly decreased, and far more K-region metabolites (4,5-dihydroxy-4,5-

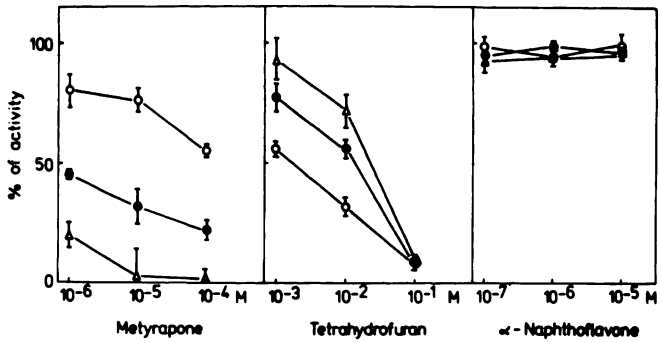

FIG. 1. *Effect of metyrapone, tetrahydrofuran and α-naphthoflavone on ethoxycoumarin O-deethylase activity in liver microsomes of control and trans-stilbene oxide-treated rats*

Male Sprague Dawley rats (210–230 g) received intraperitoneal injections of trans-stilbene oxide (2 mmoles per kg body weight, dissolved in 0.5 ml sunflower oil) or sunflower oil only 72, 48 and 24 hr before sacrifice. Ethoxycoumarin O-deethylase activity was determined according to Ullrich and Weber (17) using inhibitors to characterize the monooxygenase forms present (18). The values represent means ± SD from 3 experiments with trans-stilbene oxide and 2 experiments with control animals. For each experiment organs from two animals were pooled. It was always confirmed, in the absence as well as in the presence of the inhibitors, that the enzymic reaction proceeded linearly with respect to time and protein. —○—○— Microsomes from control animals (100% = 790 pmoles 7-OH-coumarin/min/mg protein). —●—●— Microsomes from trans-stilbene oxide-treated animals (100% = 1980 pmoles 7-OH-coumarin/min/mg protein). —△—△— Difference between microsomes from trans-stilbene oxide-treated and control animals (100% = 1190 pmoles 7-OH-coumarin/min/mg protein).

dihydrobenzo(a)pyrene, benzo(a)pyrene 4,5-oxide) were produced. The ratio between 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene and 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene was more than 20 times lower with TSO-induced than with control microsomes (calculated from Table 2). Only insignificant changes were observed in the peaks containing the quinones and 3-hydroxybenzo(a)pyrene. As expected from the induction of epoxide hydratase, a much higher percentage of the metabolically produced benzo(a)pyrene 4,5-oxide was converted to the corresponding dihydrodiol with TSO-induced microsomes in comparison with control microsomes.

Effect of induction by trans-stilbene oxide on the formation of mutagenic metabolites from benzo(a)pyrene. Benzo(a)pyrene can be activated both at the benzo ring and at the 4,5-K-region to highly mutagenic metabolites; at the benzo ring mainly to dihydrodiol epoxides, at the K-region to the 4,5-oxide (3, 4, 7, 22–27). Therefore it was of interest to investigate how the shift of metabolism which is caused by TSO would affect the mutagenicity of benzo(a)pyrene. For this purpose, we activated various doses of benzo(a)pyrene by microsomal or postmitochondrial fractions to mutagens which were detected by the reversion of various *his*$^-$ *Salmonella typhimurium* strains. In general, induction by TSO markedly decreased the mutagenicity of benzo(a)pyrene. Some differences were observed between the two tissue fractions and the different bacterial strains. The greatest reduction of the mutagenic effect (by more than 90%) was obtained when postmitochondrial fraction and the strain TA 100 were used (Fig. 3A). Using microsomes, TSO treatment reduced the mutagenicity with TA 100 by 65% (Fig. 3B) and with TA 98 by 40% (Fig. 3C, round symbols). However, TSO treatment only slightly reduced the mutagenicity with TA 1537 (Fig. 3D, round symbols). The differences of the effect of TSO induction on the various *Salmonella typhimurium* strains can be easily explained. The Salmonella assay is a backward mutation assay which requires specific mutations to reconstruct a functional *his* gene. Different *his*$^-$ strains vary in their susceptibility to reversion by different benzo(a)pyrene metabolites. TA 100 and TA 98 are easily reverted by benzo(a)-

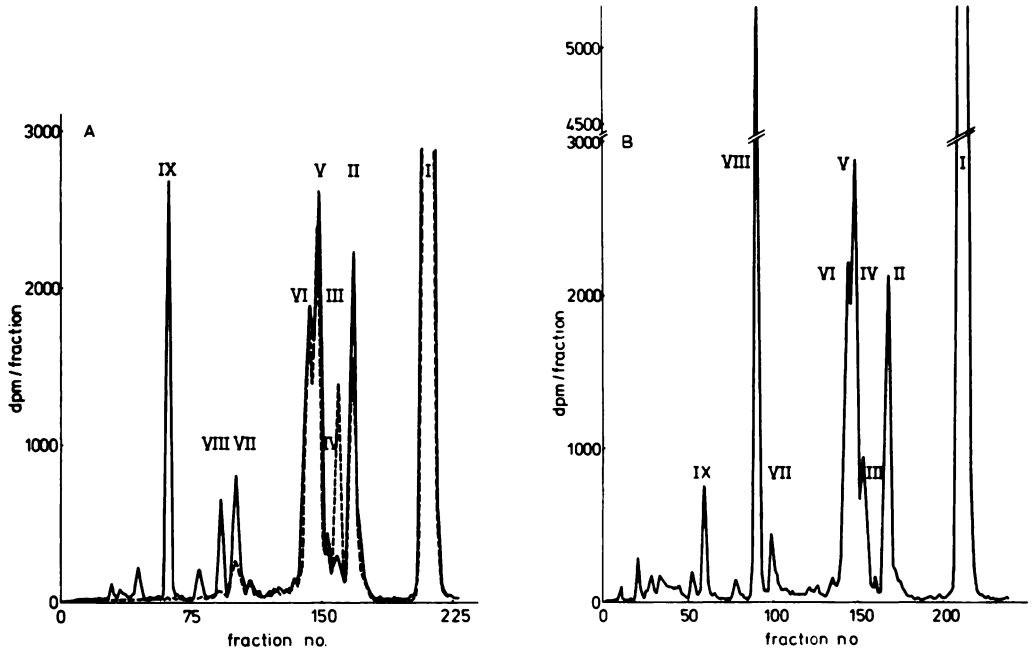

FIG. 2. *Pattern of metabolites produced from benzo(a)pyrene by liver microsomes from control and trans-stilbene oxide-treated rats*

Microsomes were incubated with [^{14}C]-benzo(a)pyrene, NADPH and NADH for 20 min at 37°. Ethyl acetate-acetone (2:1 v/v) extracts were separated by high performance liquid chromatography using a linear acetonitrile-water gradient (for details see MATERIALS AND METHODS). Roman numbers indicate the fractions where reference compounds appeared, benzo(a)pyrene (I), 3-hydroxybenzo(a)pyrene (II), 9-hydroxybenzo(a)pyrene (III), benzo(a)pyrene 4,5-oxide (IV), benzo(a)pyrene 3,6-quinone (V), benzo(a)pyrene 1,6-quinone (VI), trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (VII), trans-4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene (VIII), and trans-9,10-dihydroxy-9,10-dihydrobenzo(a)pyrene (IX).

A) Pattern of metabolites from control rats (treated with sunflower oil only) in the absence (——) and in the presence (- - -) of the epoxide hydratase inhibitor 1,1,1-trichloropropene 2,3-oxide.

B) Pattern of metabolites of TSO-induced rats (treated with 2 mmoles TSO per kg body weight, dissolved in 0.5 ml sunflower oil, 72, 48 and 24 hr before sacrifice).

pyrene 7,8-dihydrodiol 9,10-oxides and by benzo(a)pyrene 4,5-oxide. The strain TA 1537 is less sensitive toward the 7,8-dihydrodiol 9,10-oxides but is highly sensitive to the 4,5-oxide[4] (27). The greater decrease of the mutagenicity by TSO induction with the strains which are sensitive to 7,8-dihydrodiol 9,10-oxides (TA 100, TA 98) when compared with a strain which is relatively insensitive toward these dihydrodiol epoxides (TA 1537) suggests that the reduction of the mutagenicity is caused to a significant extent by the decreased oxidation of benzo(a)pyrene at the benzo ring. The decreased benzo ring metabolism is accompanied by increased K-region metabolism, leading to the mutagenic benzo(a)pyrene 4,5-oxide, but epoxide hydratase, which is induced by TSO, can inactivate this mutagen (7, 26, 27). The presence of the potent epoxide hydratase inhibitor 1,1,1-trichloropropene oxide (21) in the mutagenicity assay potentiated the mutagenic effects both with control and with TSO-induced microsomes (Figs. 3C, 3D). This experiment could be performed only with TA 98 and TA 1537 since 1,1,1-trichloropropene oxide is too mutagenic with TA 100 (22). Using TA 98, inhibition of epoxide hydratase increased the mutagenicity of benzo(a)pyrene activated with control microsomes about 3-fold, and with TSO-induced microsomes about 5-fold (cf. Fig. 3C). Also, with TA 1537, inhibition of epoxide hydratase potentiated the mutagenicity more strongly when

[4] Glatt, H. R., unpublished results.

TABLE 2

Effect of trans-stilbene oxide administration on the pattern of benzo(a)pyrene metabolism by rat liver microsomes

Male Sprague Dawley rats (210–230 g) received intraperitoneal injection of trans-stilbene oxide (2 mmoles per kg body weight dissolved in 0.5 ml sunflower oil) or sunflower oil only 72, 48 and 24 h before sacrifice. [^{14}C]-Benzo(a)pyrene, microsomes, NADPH and NADH were incubated for 20 min at 37°. Ethyl acetate/acetone (2:1 v/v)-extractable metabolites were separated by high performance liquid chromatography with a linear acetonitrile/water gradient (for details see MATERIALS AND METHODS). The peaks were characterized by reference compounds (roman numbers are equivalent to Fig. 2) with the same elution volume and expressed (1) as the total amount present after the incubation time, corrected for small (range of less than ± 10% of the mean) variations in protein content and (2) as percentage of the radioactivity of all but the benzo(a)pyrene peak. Values represent means ± SD from 3 (trans-stilbene oxide-treated) or 2 (control animals) independent experiments. For each experiment organs from two animals were pooled.

Radioactivity with mobility of the following reference compounds		Control rats		Trans-stilbene oxide-treated rats		Ratio[a] TSO-treated/control
		pmoles/mg protein × 20 min	*% of metabolites*	*pmoles/mg protein × 20 min*	*% of metabolites*	
9,10-Dihydroxy-9,10-dihydrobenzo(a)pyrene	IX	1240 ± 90	13.5	460 ± 10	4.3	0.37
4,5-Dihydroxy-4,5-dihydrobenzo(a)pyrene	VIII	430 ± 10	4.7	2670 ± 250	25.0	6.21
7,8-Dihydroxy-7,8-dihydrobenzo(a)pyrene	VII	330 ± 10	3.6	90 ± 30	0.8	0.27
Benzo(a)pyrene 1,6-quinone	VI					
and 3,6-quinone	V	3690 ± 20	40.2	3750 ± 480	35.0	1.01
Benzo(a)pyrene 4,5-oxide	IV	430 ± 90	4.7	760 ± 70	7.1	1.76
9-Hydroxybenzo(a)pyrene	III	320 ± 10	3.5	120 ± 10	1.1	0.37
3-Hydroxybenzo(a)pyrene	II	2190 ± 170	23.9	1840 ± 320	17.2	0.84
Others		550 ± 40	5.9	1030 ± 80	9.5	1.87
Total		9180 ± 390	100	10700 ± 1250	100	1.16

[a] Ratio of metabolites present after 20 min incubation with microsomes from trans-stilbene oxide-treated and control rats.

TSO-induced microsomes were used for the activation (Fig. 3D). This illustrates the increased importance of epoxide hydratase for the inactivation of mutagenic benzo(a)pyrene metabolites after a shift of the metabolism to the K-region.

DISCUSSION

Previously we reported (12) that TSO induced epoxide hydratase in rat liver microsomes without significantly affecting four monooxygenase parameters (cytochrome P-450 content and CO-difference spectrum, aminopyrine *N*-demethylase and benzo(a)pyrene monooxygenase activity determined by tritium release from [^{3}H]-benzo(a)pyrene or by the fluorescence intensity of the phenolic fraction. In the present study we have found that two further monooxygenase parameters were markedly changed: (a) O-Deethylation of ethoxycoumarin was increased 2.5 fold; (b) The pattern of oxidation products of benzo(a)-pyrene was substantially altered. With respect to some of the investigated parameters, the effects observed after TSO treatment are similar to the effects of phenobarbital treatment: (a) The phenobarbital-induced (18) as well as the TSO-induced (this study) ethoxycoumarin O-deethylase activities are preferentially inhibited by metyrapone, but not by tetrahydrofuran or by α-naphthoflavone which preferentially inhibit the ethoxycoumarin O-deethylase activities induced by ethanol and 3-methylcholanthrene, respectively. (b) Phenobarbital (23, 24) as well as TSO (this study) increase the metabolism of benzo(a)pyrene mainly at the K-region. (c) Furthermore,

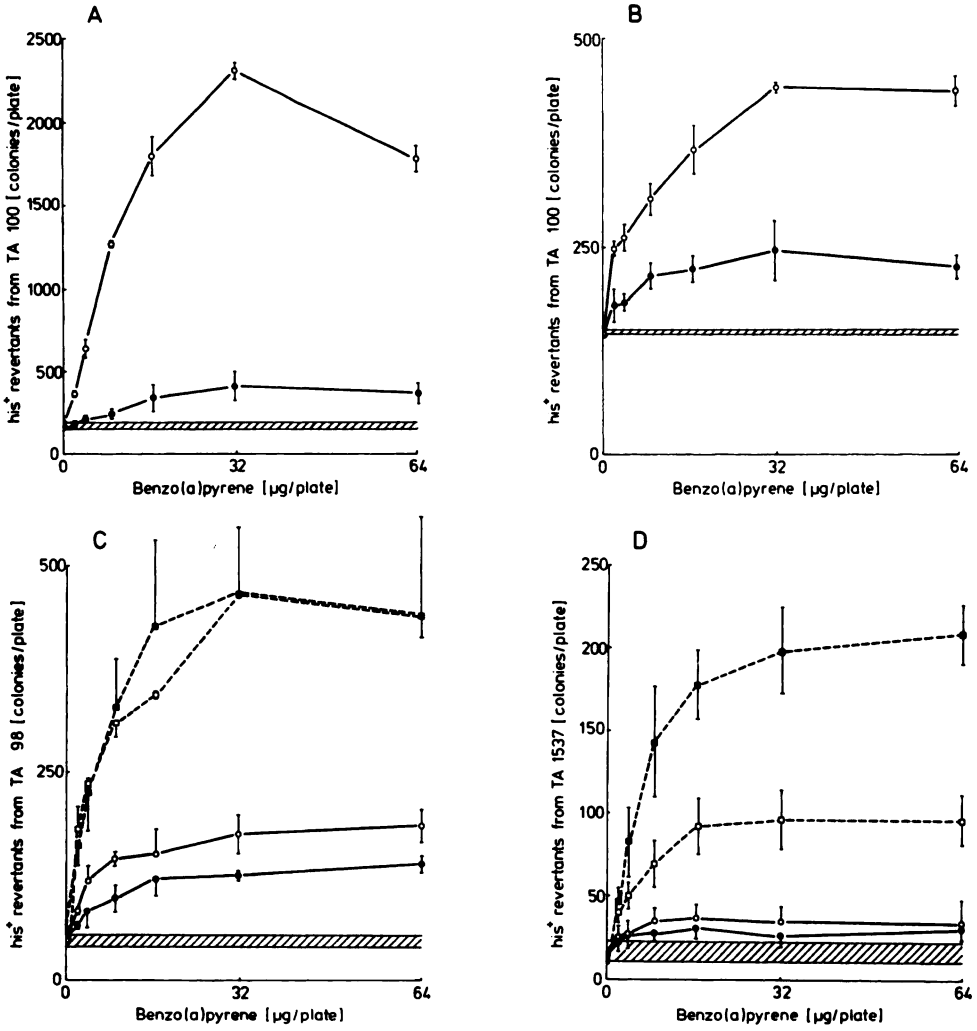

FIG. 3. *Effect of trans-stilbene oxide treatment on the activation of benzo(a)pyrene to mutagens*

Male Sprague Dawley rats (210–230 g) received intraperitoneal injections of trans-stilbene oxide (2 mmoles per kg body weight, dissolved in 0.5 ml sunflower oil) or sunflower oil only 72, 48 and 24 hr before sacrifice. Various amounts of benzo(a)pyrene, 9000 *g* supernatant fraction (A) or microsomes (B–D) of liver homogenate, a NADPH generating system and his⁻ *S. typhimurium* (A, B: TA 100; C: TA 98; D: TA 1537) were mixed with histidine-poor soft agar and poured on a minimal agar plate as described by Ames *et al.* (19). In part of the experiments 1 mM of the epoxide hydratase inhibitor 1,1,1-trichloropropene 2,3-oxide (21) was added. Colonies (his⁺ revertants) were counted after an incubation for 2 days at 37°. The values represent means ± SD of 3 experiments with trans-stilbene oxide-treated and of 2 experiments with control animals. Livers from two animals were pooled and two plates were run for each experimental condition. The hatched zones show means ± SD of the number of revertant colonies in the absence of benzo(a)pyrene. ○ = control rats, ● = trans-stilbene oxide-treated rats, □ = control rats with epoxide hydratase inhibitor, ■ = trans-stilbene oxide-treated rats, with epoxide hydratase inhibitor.

A) *S. typhimurium* TA 100, 9000 *g* supernatant

B) *S. typhimurium* TA 100, microsomes

C) *S. typhimurium* TA 98, microsomes

D) *S. typhimurium* TA 1537, microsomes

phenobarbital (28) and TSO (12 and this study) are good inducers of epoxide hydratase. However, the effects of phenobarbital are much less selective. It increases benzo(a)pyrene metabolism not only at the 4,5- but also at the 7,8-position, although more at the former than at the latter (23) and it causes many additional effects which are not caused by TSO, e.g., it induces all four above described monooxygenase parameters which are not affected by TSO (12). Most strikingly, TSO potently induces ethoxycoumarin O-deethylase activity (this study) without a significant increase in total cytochrome p-450 content (12). At least two explanations are possible for this observation: (a) induction of a very small amount of a cytochrome P-450 form which possesses a high ethoxycoumarin O-deethylase activity or (b) alteration of a form of cytochrome P-450 into a form which possesses an increased ethoxycoumarin O-deethylase activity. The second hypothesis could also easily explain the large (about 3-fold) decrease of metabolism at the 7,8,9,10-benzo ring of benzo(a)pyrene which was observed after TSO treatment in this study. Further investigations are required to explain the detailed mechanisms underlying these subtle changes in the monooxygenase system.

Not only the causes but also the biological effects of such changes are of great interest. This is illustrated in this study by the metabolic activation of benzo(a)pyrene to mutagens. Pretreatment of the rats with TSO strongly decreased mutagenicity of benzo(a)pyrene activated by liver microsomes. A decrease is caused by a reduced metabolism at the 7,8,9,10-benzo ring where highly mutagenic dihydrodiol epoxides (3–8) can be formed. As compensation for the decreased metabolism at the 7,8,9,10-benzo ring, a higher proportion of benzo(a)pyrene is metabolized via the benzo(a)pyrene 4,5-oxide. This is also highly mutagenic (3, 7, 26). The dihydrodiol epoxides require epoxide hydratase for their formation and are not inactivated by this enzyme (7, 32), whereas epoxide hydratase does inactivate benzo(a)pyrene 4,5-oxide. The strong induction of epoxide hydratase compensates for the increased formation of this highly mutagenic metabolite. Therefore, the mutagenicity in strain TA 1537, which is mainly mutated by the 4,5-oxide (27), changes little, while the mutagenicity in strains TA 98 and TA 100, which are efficiently mutated by both the dihydrodiol-bay region epoxides (31) and the 4,5-epoxide (27), is substantially reduced. Thus, the two effects of TSO, shift of the site of metabolic oxidation and induction of epoxide hydratase, synergistically provide protection against the mutagenic effects of benzo(a)pyrene.

It should be emphasised that these remarkable changes in the benzo(a)pyrene metabolites and the consequent changes in mutagenic effects occur without any significant alteration of the benzo(a)pyrene monooxygenase activity determined by the widely used assays which measure the fluorescence intensity of the phenolic benzo(a)pyrene metabolites extracted into alkali (29) (the so-called "aryl hydrocarbon hydroxylase activity") or tritium release from [^{3}H]benzo(a)pyrene (30). The frequent attempts to correlate susceptibility to polycyclic hydrocarbon-mediated toxic effects with "aryl hydrocarbon hydroxylase activity" are based on the premise that the enzyme activity measured by these assays gives a sufficiently complete indication of the biotransformation to toxic metabolites. As shown here, this is not always so, and misleading results may be expected when it is not true.

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