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EPOXIDE HYDRATASE AND BENZO(A)PYRENE MONOOXYGENASE ACTIVITIES IN LIVER, KIDNEY AND LUNG AFTER TREATMENT OF RATS WITH EPOXIDES OF WIDELY VARYING STRUCTURES

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Abstract—The effect of treatment with various epoxides on epoxide hydratase and benzo(a)pyrene monooxygenase activities in rat liver, kidney and lung was tested with the aim possibly to find a selective inducer of epoxide hydratase. In a first series of epoxides, good substrates of epoxide hydratase and relatively small molecules did not lead to an increase in epoxide hydratase activity in any organ tested. Treatment with the poor substrates dieldrin and *trans*-stilbene oxide(TSO), however, increased epoxide hydratase activity in the liver and with TSO also in the kidney (3-fold). In contrast to all other epoxide hydratase inducers so far discovered, TSO did not affect the benzo(a)pyrene monooxygenase activities, even after doses leading to maximal induction of epoxide hydratase (~ 350 per cent of controls). In an attempt to find an even more potent selective inducer of epoxide hydratase several *trans*-stilbene oxide derivatives were synthesized. All modifications of the TSO molecule led to compounds which were less effective inducers of liver epoxide hydratase than the parent compound and also either increased or drastically decreased the benzo(a)pyrene monooxygenase activities. With TSO, 4-methoxy-TSO, 4-chloro-TSO and 4-nitro-TSO the tests were extended to three other parameters of the monooxygenase system, the cytochrome P450 content, the NADPH-cytochrome *c* reductase and the aminopyrine *N*-demethylase activities. All these derivatives but not TSO itself affected part of the monooxygenase system. Thus, with respect to five measured monooxygenase parameters TSO was found to be a selective inducer of epoxide hydratase in rat liver. An influence of TSO on the pattern of the various cytochrome P450 forms not leading to observable changes in monooxygenase activity towards two substrates known to be preferential substrates of different cytochrome P450 forms (aminopyrine and benzo(a)pyrene) is unlikely but cannot be excluded.

Microsomal monooxygenase and epoxide hydratase have attracted much attention in the endeavour to elucidate the mechanism of chemical carcinogenesis caused by polycyclic aromatic hydrocarbons [1–6].

The microsomal monooxygenase system catalyses the oxidation of a wide variety of lipophilic exogenous and endogenous compounds (insecticides, many drugs, environmental carcinogens, hormones, fatty acids, etc.) [7, 8] and is responsible for the formation of electrophilically reactive epoxide intermediates [9, 10] (for reviews see [1–6]), which can bind to DNA, RNA and protein [11–13] and thereby may exert mutagenic, carcinogenic or cytotoxic effects [14, 15]. Epoxides metabolically formed from carcinogenic polycyclic hydrocarbons were found to be highly mutagenic and are suspected to be ultimate carcinogens [16, 17]. The inactivation of such epoxides is catalyzed by the cytoplasmic glutathione transferases (EC 4.4.1.7) [18, 19] and by the membrane bound epoxide hydratase [2–4, 20]. Epoxide hydratase is of special interest, since it is localized in the same cell compartment as the epoxide producing monooxygenase system and since metabolically formed epoxides from polycyclic hydrocarbons will—due to their lipophilic character—preferentially stay in the membrane and may reach the nucleus by lateral diffusion [21]. The existence of a coupled monooxygenase-epoxide hydratase system [22], would strengthen the importance of this enzyme compared to the cytoplasmic glutathione transferases.

However, the role of epoxide hydratase in the overall metabolism *in vivo* of carcinogenic polycyclic hydrocarbons to potent mutagens and ultimate carcinogens is not as yet clear. Figure 1 shows the interactions of the monooxygenase system and epoxide hydratase in two known pathways of the benzo(a)pyrene metabolism. Epoxide hydratase may act as an inactivating enzyme, as shown for benzo(a)pyrene 4,5-oxide [24]. In the case of benzo(a)pyrene 7,8-oxide which is formed in proportionally higher amounts after 3-methylcholanthrene induction [25], epoxide hydratase may provide the precursor for the highly mutagenic 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene 9,10-oxide [26–29] and thereby may act as a "coactivating" enzyme [29]. The mode of action therefore depends upon the region in which the molecule is oxidized, which is again dependent on the cytochrome P450 species and monooxygenase activity involved [23].

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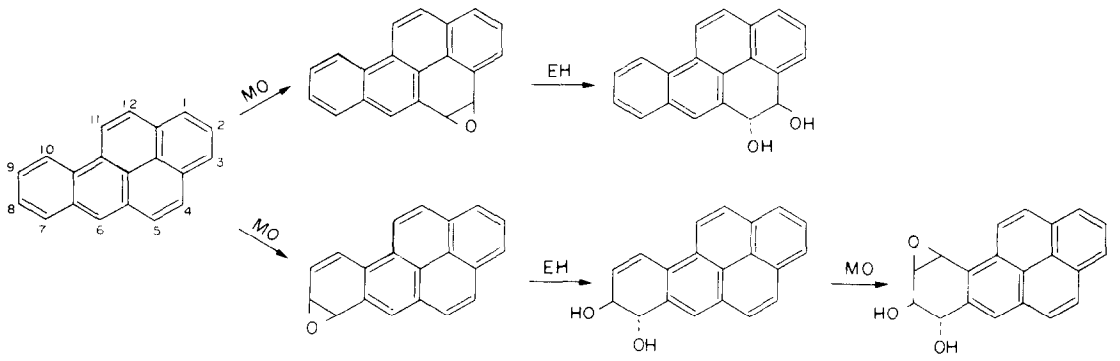

Fig. 1. Interactions of the monooxygenase system (MO) and epoxide hydratase (EH) in two known pathways of the benzo(a)pyrene metabolism.

To elucidate the role of epoxide hydratase *in vivo* in the mechanism of tumor formation caused by polycyclic hydrocarbons, we have tried to develop a selective epoxide hydratase inducer. This task appeared to be very difficult if not impossible because of the ease by which the monooxygenase system is induced by such different compounds as barbiturates, hormones, insecticides or carcinogens [30, 31] and by a great number of environmental factors [32]. Moreover, all epoxide hydratase inducers so far discovered also induce the monooxygenase system suggesting that epoxide hydratase and a rate limiting entity of the monooxygenase system might be under common biosynthetic control. However, in a recent study on transplacental induction of epoxide hydratase and benzo(a)pyrene monooxygenase activities, the latter could be selectively induced in the fetal liver by treatment of pregnant rats with benzo(a)pyrene or 3-methylcholanthrene [33]. This finding gave rise to the hope that a selective induction of epoxide hydratase should also be possible. Since epoxides were thought to be the most likely class of compounds possessing, as monooxygenase products or product mimics, only a low or no affinity to the monooxygenase system, and the receptor(s) being responsible for its induction, but possibly having a high affinity to the receptor(s) responsible for epoxide hydratase induction, a series of structurally widely varying epoxides was tested in order to find a selective epoxide hydratase inducer.

MATERIALS AND METHODS

Chemicals. Substituted *trans*-stilbene oxides were prepared by two methods: in the case of di- and tetra-substituted compounds, the procedure of Mark [34] was used, and for the mono-substituted oxides that of Trippett and Walker [35]. Identity and purity were established by n.m.r. spectroscopy, melting point (m.p.) and thin layer chromatography (t.l.c.). [^{3}H]benzo(a)pyrene 4,5-oxide was prepared according to the method of Dansette and Jerina [36] under conditions as described in [37]. Diethylstilbestrol α,β-oxide and the corresponding diacetate were a generous gift of Dr. H. G. Neumann, Würzburg, West Germany. 4-Vinylcyclohexene dioxide, exo-2,3-epoxynorbornane and *N*-(3,4-epoxypropyl)-phthalimide were purchased from Aldrich-Europe, Belgium, 16α,17α-epoxypregnenolone was obtained from Fluka, Switzerland, dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy - 1,4,5,6,7,8,9,10-octahydro-exo-(1,4)-endo-(5,8)-dimethanonaphthalene) from Ferak, Berlin, *trans*-stilbene (TS) and TSO from EGA-Chemie, Steinheim, West Germany. *Trans*-stilbene oxide was purified by treatment with charcoal in ether and recrystallization from benzene–methanol. The purity of TSO was established by m.p. and t.l.c. in ether and chloroform–petroleum ether (1:1). [G-^{3}H]benzo(a)pyrene (5 Ci/m-mole) and [dimethylamine-^{14}C]aminopyrine (11.6 Ci/mole) were obtained from the Radiochemical Centre, Amersham, U.K., and [7-^{3}H]styrene oxide from NEN, Dreieichenhain, West Germany. NADPH, NADH and glucose-6-phosphate dehydrogenase from Boehringer Mannheim, West Germany, cytochrome *c* from Sigma Chemicals Co., St. Louis, MO, U.S.A., activated cocoanut charcoal from Fisher Scientific Co., Fair Lawn, NJ, U.S.A., 2-(4-biphenyl)-5-(*p*-tert-butyl-phenyl)-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 hard wood bedding under standardized conditions of light (light–dark cycle; 07.00–19.00) and temperature 21–24° for at least 4 days before treatment.

They had free access to Altromin pellets (Samen–Schmitt–Jakobi, Frankfurt/Main, West Germany) and tap water. Care was taken to avoid possible environmental influences on the enzyme activities under study, such as from cigarette smoke, insecticides, noise, etc. Animals were always treated between 8 and 10 a.m. on three successive days by an i.p. injection of a solution or suspension of the stated compound in sunflower oil (usually 0.5 ml per injection), if not otherwise indicated. 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 24 hr after the last treatment. The organs from groups of three to six animals were excized and pooled in ice-cold 1.15% KCl containing 10 mM K phosphate buffer pH 7.4.

The organs were minced and homogenized in 3 vols of 1.15% KCl containing 10 mM K phosphate buffer pH 7.4 in a Potter–Elvehjem homogenizer. The homogenates were centrifuged at 10,000 *g* for 15 min and the resulting supernatant fractions were centrifuged at 100,000 *g* for 1 hr. When the cytochrome P450 content was determined the microsomal pellets were resuspended in the same medium and recentrifuged at 100,000 *g* for 1 hr. In the other cases the surface of the microsomal pellets was washed with cold 1.15% KCl containing 10 mM K phosphate buffer pH 7.4. The 100,000 *g* pellets were resuspended in the same medium to give protein concentrations of 5–10 mg/ml for liver and kidney and 3–6 mg/ml for lung. Protein concentrations were determined by the method of Lowry *et al.* [38] using bovine serum albumin as standard.

Enzyme assays

General. Determinations of epoxide hydratase and benzo(a)pyrene monooxygenase activities were performed with freshly prepared microsomes, which were kept in an ice bath and were used within 36 hr after killing the animals. No measurable losses of these enzyme activities were found when microsomes were kept on ice for 48 hr. The other monooxygenase parameters were determined immediately after preparation of the microsomes in the following sequence: cytochrome P450 content, NADPH-cytochrome *c* reductase, aminopyrine *N*-demethylase. The enzyme assays proceeded linearly with respect to the protein concentrations and incubation times used.

Epoxide hydratase. Epoxide hydratase activities were determined using the radiometric extraction assays with [^{3}H]benzo(a)pyrene 4,5-oxide [37] and [7-^{3}H]styrene oxide [39] as substrates. The latter assay was performed using the modification described [40] (in the absence of Tween 80). Enzyme activities are corrected for 80 per cent recovery of 4,5-dihydroxy-4,5-dihydrobenzo(a)pyrene and 86 per cent recovery of styrene glycol. Although previous studies indicated that the same enzyme is responsible for the hydration of benzo(a)pyrene 4,5-oxide and styrene oxide in liver, kidney and lung of untreated rats [41], in this study epoxide hydratase activity was usually determined with both substrates, since it could not be excluded that pretreatment with various epoxides might have caused the formation of an enzyme possessing different substrate specificities and therefore might have led to misinterpretations when only one substrate was used.

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 [42]. Results obtained by this assay are labeled "3-OH-BP". Since the pattern of hydroxylated benzo(a)pyrene metabolites possessing different fluorimetric properties [43] is dependent on the composition of the cytochrome P450 species present in the microsomes [23], which in turn may be changed upon treatment with various compounds [44], determination of the benzo(a)pyrene monooxygenase activity by the fluorimetric method only may in certain cases be misleading. Therefore a combination of this assay [33], with the radiometric assay described by Hayakawa and Udenfriend [45] was used. The latter assay is based on the tritium release when epoxide intermediates rearrange to phenols (NIH shift, cf. [3]) or when hydroxylation occurs by direct insertion. Benzo(a)pyrene monooxygenase activities determined by the latter method in which the tritiated water is measured, are labeled "overall". Since increases in monooxygenase activities as determined by these procedures are not always parallel [33], the two assays must differentially reflect monooxygenase activities catalyzed by some different cytochrome P450 forms (for a thorough discussion of these two assays, see [33]).

The assays were performed in dim light as follows. An aliquot of a [^{3}H]benzo(a)pyrene (150 μCi/μmole) stock solution in benzene (stored protected from light at $-10°$) was always freshly purified before use according to the method of DePierre *et al.* [46]. The purified [^{3}H]benzo(a)pyrene was dissolved in absolute ethanol to give a concentration of 4 μmoles/ml. The incubation, which was carried out at 37° usually for 10 min, was started by addition of 25 μl of this solution to 1 ml of an incubation mixture containing 50 μmoles of K_3PO_4 buffer pH 7.4, 0.8 mg bovine serum albumin, 5 μmoles of $MgCl_2$, 0.31 μmole NADPH, 0.18 μmole NADH, 3.95 μmoles glucose-6-phosphate, 0.2 unit of glucose-6-phosphate dehydrogenase and 0.07–0.35 mg microsomal protein. The reaction was stopped by addition of 1 ml of cold (-10–$-20°$) acetone. After removal of the precipitated protein, substrate and products were extracted into 6 ml of petroleum ether (b.p. 40–60°) by rotating the tubes for 10 min on a Rotoshake (Kühner AG, Basel). From 3 ml of the organic phase the phenolic products were extracted into 3 ml of 1 N NaOH and determined fluorimetrically as described by Nebert and Gelboin [42]. For quantitation of the tritium release a modification of the method of Hayakawa and Udenfriend [45] was used. The aqueous phase was extracted two more times with 6 ml of petroleum ether as described above. 0.5 ml of the remaining water phase was then put on a small charcoal column (Pasteur pipette loaded with about 0.5 g of activated cocoanut charcoal, which was washed with distilled water prior to use) to remove the remaining substrate and products and run directly into a scintillation vial. After the tritiated water had been washed out with 0.5 ml of distilled water, 10 ml of Unisolve 1 was added and the sample was counted with a counting efficiency of 23–28 per cent in a Packard Tri-Carb liquid scintillation spectrometer, Model 3380.

Aminopyrine N-demethylase. Aminopyrine *N*-demethylase activity was determined using the radiometric assay as described by Poland and Nebert [47]. The radioactive aminopyrine was always purified by t.l.c. immediately before use. The NADPH generating system used was the same as described above for the benzo(a)pyrene monooxygenase assays.

NADPH-cytochrome c reductase. NADPH-cytochrome c reductase activity was measured as described by Dallner *et al.* [48] using an incubation temperature of 37°.

Cytochrome P450 content. The cytochrome P450 content was determined by the method of Omura and Sato [49] using a Perkin–Elmer 356 Dual-Wavelength Double Beam Spectrophotometer.

RESULTS AND DISCUSSION

Effect of treatment with epoxides of widely varying structures on epoxide hydratase and benzo(a)pyrene monooxygenase activities in male rat liver, kidney and lung

In the search for a selective epoxide hydratase inducer, i.e. a compound which does not affect the benzo(a)pyrene monooxygenase activity, rats were treated with different epoxides using dosage schedules as described in Methods and legends to the Tables. As can be seen from Tables 1 and 2, *N*-(2,3-epoxypropyl)-phthalimide, a good substrate of epoxide hydratase, did not affect the epoxide hydratase activities in liver, kidney or lung, but at the higher dose slightly inhibited the benzo(a)pyrene monooxygenase activity in the liver. The lack of epoxide hydratase induction* after pretreatment with this compound as well as the styrene oxide [50], another good substrate, may be due to their fast hydration by epoxide hydratase.

A receptor responsible for epoxide hydratase induction may indeed share a specificity for epoxides with the enzyme active site, but the affinity and intrinsic activity of an epoxide towards such a receptor may depend on structural features different to those required for a good substrate of the enzyme. Therefore not only good substrates, but also epoxides similar to the non-competitive epoxide hydratase inhibitor cyclohexene oxide [51] (4-vinylcyclohexene dioxide and exo-2,3-epoxynorbornane) as well as the poor substrates 16α,17α-epoxypregnenolone, dieldrin and TSO were tested. When rats were treated with the two cyclohexene oxide derivatives 4-vinylcyclohexene dioxide and exo-2,3-epoxynorbornane as well as after treatment with 16α,17α-epoxypregnenolone the liver epoxide hydratase activities were never significantly different from controls.† On the other hand, after treatment with these compounds the benzo(a)pyrene monooxygenase activity was decreased to 40–70 per cent of the control values. Whether these decreases were due to a reduction of the cytochrome P450 content or to an inhibition *in vitro* of the benzo(a)pyrene monooxygenase by the presence of these compounds in the microsomes was not further investigated.

Finally dieldrin and TSO were tested. Treatment with a low dose of the insecticide dieldrin caused an induction of liver epoxide hydratase to about 170 per cent of control activity, but showed no significant increase of the benzo(a)pyrene monooxygenase activity. A single dose of 0.1 m-mole dieldrin/kg body wt, however, induced also the benzo(a)pyrene monooxygenase activity to 170 per cent of control activity after 3 days. These results, together with another study on the effect of dieldrin on epoxide hydratase and benzo(a)pyrene monooxygenase activities [52], which appeared in press while these experiments were in progress, indicate that epoxide hydratase activity can be preferentially induced by dieldrin compared to benzo(a)pyrene monooxygenase activity but only in a narrow range of low doses. However, the cytochrome P450 content and other monooxygenase activities are also increased after low doses of dieldrin [52]. Therefore this compound was also excluded from further investigations. After treatment with TSO, the epoxide hydratase activities in liver microsomes increased dose dependently up to 350 per cent of controls, whereas none of the given doses had a significant effect on the benzo(a)pyrene monooxygenase activity. Treatment with TSO up to 2 m-moles kg body wt/day for 3 days had no harmful effects on the rats, but after a single i.p. injection of 5 m-moles TSO/kg body wt the animals showed signs of convulsions. Therefore, this treatment could not be continued on the second day, and on the third day the animals received only 2.5 m-moles TSO/kg body wt. It is noteworthy that, even after this massive treatment with almost lethal amounts of TSO,* no induction of the benzo(a)pyrene monooxygenase activity could be observed. Moreover, benzo(a)pyrene monooxygenase activity was not increased at earlier and later time points up to 12 days after application of TSO (Schmassmann and Oesch, unpublished results). Therefore a simulation of a selective induction of epoxide hydratase by differences in the half-lifes or in the onset of epoxide hydratase and benzo(a)pyrene monooxygenase induction can be excluded. Mixtures of liver microsomes from control rats with various amounts of liver microsomes from TSO treated rats (2 m-moles TSO/kg body wt/day for 3 days) always resulted in benzo(a)pyrene monooxygenase activities corresponding to the sum of the two components. This indicated that the lack of increases in *in vitro* benzo(a)pyrene monooxygenase activity was not due to a possible combination of an enzyme induction balanced by an inhibition of similar magnitude due to residual traces of inducer or metabolites derived therefrom. This indication is corroborated by the observation of similarly unchanged benzo(a)pyrene monooxygenase activity at the widely varying dosages of TSO which all led to epoxide hydratase induction† (Table 1).

* The term induction is used in this study in its broader sense to denote an increase in enzyme activity regardless of the underlying mechanism. More recently, we have obtained evidence that the TSO induced increase of epoxide hydratase activity is accompanied by an increase in epoxide hydratase enzyme protein (Schmassmann and Oesch, article in preparation).

† Enzyme activities ± 15 per cent of controls were considered as not significantly different from control activities, since this corresponds to the S.D. usually observed in controls.

* LD_{50} for male Sprague–Dawley rats was estimated to be about 1200 mg TSO per kg body wt, i.p.

† The term induction is used in this study in its broader sense to denote an increase in enzyme activity regardless of the underlying mechanism. More recently, we have obtained evidence that the TSO induced increase of epoxide hydratase activity is accompanied by an increase in epoxide hydratase enzyme protein (Schmassmann and Oesch, article in preparation).

Table 1. Effect of various epoxides on male rat liver epoxide hydratase activity and benzo(a)pyrene monooxygenase activity

Compound tested	Dose* m-moles/kg body wt	Activity as per cent of controls†			
		Epoxide hydratase		BP Monooxygenase	
		BP 4,5-oxide	Styrene oxide	'3-OH-BP'	'Overall'
		(a) Various epoxides			
N-(2,3-Epoxypropyl)phthalimide	0.2	97	95	113	ND
	0.7	109	111	78	ND
4-Vinylcyclohexene dioxide	0.7	96	96	63	ND
	2.3	100	102	47	ND
exo-2,3-Epoxynorbornane	0.5	95	105	68	ND
	1.8	84	96	50	ND
16α,17α-Epoxypregnenolone	0.3	100	92	44	ND
Dieldrin	0.05	164	171	114	ND
	0.10§	141	152	170	ND
Trans-stilbene oxide (TSO)	0.2	147 ± 21	153 ± 27	111 ± 28	114 ± 30
	0.5	170 ± 18	173 ± 25	90 ± 15	97 ± 23
	2.0	295 ± 33	321 ± 40	100 ± 33	112 ± 27
	'5.0'	326	350	100	ND
	(b) *Trans*-stilbene oxide derivatives				
4-Methoxy-TSO	0.5	128	145	132	185
4-Nitro-TSO	1.5	129	133	128	150
4-Chloro-TSO	0.5	150	171	146	195
4,4'-Dichloro-TSO	0.5	122	ND	140	220
2,2',4,4'-Tetrachloro-TSO	0.5	25	ND	340	370
Trans-stilbene	0.2	110	120	90	100
	2.0	144	154	31	57
Diethylstilbestrol α,β-oxide	0.3	133	153	34	32
Diethylstilbestrol diacetate α,β-oxide	0.3	141	150	33	27
		nmoles · mg^{-1} protein · min^{-1}			CPM · mg^{-1} protein · min^{-1}
Control activities‡		6.65 ± 0.76	7.21 ± 1.02	0.287 ± 0.04	5260 ± 1230

* Male Sprague–Dawley rats (180–280 g) were treated by a daily i.p. injection of the indicated dose for 3 days (1.000 mg TSO/kg body wt was toxic and therefore this treatment could not be continued, see text) and were killed 24 hr after the last injection. The compounds were dissolved in sunflower oil (usually 0.5 ml per injection), except for 16α,17α-epoxypregnenolone which was given in propylene glycol. Control animals received the appropriate vehicle alone.

† Enzyme activities are expressed as per cent of the control activity in the corresponding experiment (in the case of TSO the means ± S.D. of four individual experiments are given). The assays were performed on microsomes from pooled livers of three to five animals per group and duplicate determinations for each of at least two protein concentrations were done. Standard deviations of the means of the single determinations were less than 7 per cent for the epoxide hydratase assays and less than 10 per cent for the fluorimetric ('3-OH-BP') and less than 15 per cent for the radiometric ('overall') benzo(a)pyrene monooxygenase assay.

‡ Control activities represent the means ± S.D. of seven to ten experiments each of them performed on microsomes from pooled organs of three to five rats.

§ Forty mg dieldrin was given as a single i.p. injection 72 hr before the animals were killed.

ND = not determined.

The effect of treatment with all these epoxy compounds was also followed in rat kidney and lung (Table 2), because the biosynthetic control of epoxide hydratase in these organs may be different from that in the liver, and therefore an induction of epoxide hydratase activity in an important target organ for carcinogenic polycyclic hydrocarbons such as the lung might have been overlooked. However, as in the liver, most of the epoxides tested had no effect on epoxide hydratase activities in kidney and lung. The exceptions were 4-vinylcyclohexene dioxide, which caused a 30 per cent decrease of epoxide hydratase activities in both organs, and TSO, which was most striking in that it induced epoxide hydratase activities in kidney up to 300 per cent of controls whereas in the lung the activity was slightly decreased (70–90 per cent of controls).

Although no structure-activity relationship could be laid down from this series of epoxides tested, it appeared that good substrates of epoxide hydratase, which may be rapidly hydrated by the enzyme, as well as relatively small molecules, such as the cyclohexene oxide derivatives, are not suitable epoxide hydratase inducers. On the other hand poor substrates of epoxide hydratase such as dieldrin and TSO, which may persist in the body and thereby may exert their inducing effect over a longer period of time, were most promising as epoxide hydratase inducers.

Table 2. Effect of various epoxides on kidney and lung epoxide hydratase activity of male rats

Compound tested	Dose* m-moles/kg body wt	Epoxide hydratase activity as per cent of controls†			
		Kidney		Lung	
		BP 4,5-oxide	Styrene oxide	BP 4,5-oxide	Styrene oxide
(a) Various epoxides					
N-(2,3-Epoxypropyl)phthalimide	0.2	ND	ND	93	104
	0.7	93	96	113	112
4-Vinylcyclohexene dioxide	0.7	71	73	68	70
	2.3	ND	ND	69	70
exo-2,3-Epoxynorbornane	0.5	ND	ND	97	84
	1.8	97	102	106	85
16α,17α-Epoxypregnenolone	0.3	100	110	100	ND
Dieldrin	0.05	84	ND	115	90
Trans-stilbene oxide (TSO)	0.2	108	92	ND	ND
	2.0	161	147	82	71
	5.0	304	264	91	84
(b) *Trans*-stilbene oxide derivatives					
4,4'-Dichloro-TSO	0.5	176	ND	80	ND
2,2',4,4'-Tetrachloro-TSO	0.5	126	ND	51	ND
Trans-stilbene	0.2	111	127	ND	ND
	2.0	230	249	ND	ND
Diethylstilbestrol α,β-oxide	0.3	81	ND	85	ND
Diethylstilbestrol diacetate α,β-oxide	0.3	95	ND	86	ND
Control activities‡ (nmoles · mg^{-1} protein · min^{-1} ± S.D.)		0.75 ± 0.10	0.79 ± 0.12	0.33 ± 0.05	0.43 ± 0.07

* See Table 1.

† Enzyme activities are expressed as per cent of the control activity in the corresponding experiment. Epoxide hydratase assays were performed on microsomes from the pooled organs of three to five animals (same animals from which the livers were taken) and duplicate determinations for each of at least two protein concentrations were done. Standard deviations of the means of single determinations were usually less than 8 per cent.

‡ See Table 1.

ND = not determined.

Effect of TSO derivatives on epoxide hydratase and benzo(a)pyrene monooxygenase activities in male rat liver, kidney and lung

Various TSO derivatives were synthesized in order to possibly find an even more potent selective epoxide hydratase inducer, but all derivatives were less effective than TSO itself (Table 1). Moreover, most of them induced also the benzo(a)pyrene monooxygenase activity when determined either fluorimetrically ("3-OH-BP") or radiometrically ("Overall"). The chlorinated derivatives were most potent with respect to this effect. The more chlorine atoms the TSO derivatives contained, the lower were the epoxide hydratase activities and the higher the benzo(a)pyrene monooxygenase activities compared to the enzyme activities after treatment with TSO.

Interestingly, treatment with 2,2',4,4'-tetrachloro-TSO caused a decrease of epoxide hydratase activity in the liver to 25 per cent of control activity. This decrease was most probably not due to the presence of 2,2',4,4'-tetrachloro-TSO or its metabolites in the microsomes, since mixtures of liver microsomes from 2,2',4,4'-tetrachloro-TSO pretreated animals with liver microsomes from control rats always gave—after preincubation for 10 min, additive enzyme activities. Moreover, addition of 2,2',4,4'-tetrachloro-TSO to the incubation mixture up to 5-fold the substrate concentration, caused no significant inhibition of epoxide hydratase activity, which may be taken to indicate a repression of the enzyme by this compound. On the other hand 2,2',4,4'-tetrachloro-TSO induced the benzo(a)pyrene monooxygenase activities more than 3-fold. Therefore, because of its lack of selectivity, the repressing effect of this compound on epoxide hydratase activity can not be utilized in studies on the mechanism of tumor formation caused by compounds which are activated via epoxides.

To see whether for the induction of epoxide hydratase by TSO the overall TS structure or the epoxide moiety or both were critical, animals were treated with TS. The results in Table 1 show that the epoxide moiety is essential to achieve a high epoxide hydratase induction, since TS was an at least 4-fold less potent inducer than TSO. A marked difference between TSO and TS was also observed with respect to the benzo(a)pyrene monooxygenase activities. At a dose where TSO showed no effect, TS treatment caused a decrease of the benzo(a)-pyrene monooxygenase activities to 31 and 57 per cent of controls respectively. It was also interesting to test the oxide of the clinically used oestrogenic compound diethylstilbestrol. Diethylstilbestrol α,β-oxide (DESO) and DESO diacetate showed about the same potency as epoxide hydratase inducer as TSO. Treatment with relatively low doses (0.3

m-mole of DESO or DESO diacetate/kg body wt/day for 3 days) caused an induction of epoxide hydratase activity in the liver to about 140–150 per cent of controls, but at the same time these compounds decreased the benzo(a)pyrene monooxygenase activities to about 30 per cent of controls. This very marked reduction of the monooxygenase activities is most probably due to an effect *in vitro* of DESO present in the liver microsomes, since this compound was found to be a potent inhibitor of benzo(a)pyrene monooxygenase activities *in vitro* (data not shown).

Thus, all modifications of the TSO molecule led to compounds which firstly were less potent epoxide hydratase inducers than the parent compound and secondly increased or decreased the benzo(a)pyrene monooxygenase activities. As shown in Table 2, TSO induced the epoxide hydratase activities also in the kidney but not in the lung. It was now checked whether the kidney epoxide hydratase activity showed the same differences as the liver epoxide hydratase activities in the response on pretreatment with the various TSO derivatives and whether lung epoxide hydratase activities would possibly be induced by one of these componds. The kidneys and lungs were taken from the same rats as the livers. As can be seen from Table 2, the effect of the TSO derivatives on kidney epoxide hydratase activities was considerably different from that in the liver. TS and 4,4-dichloro-TSO were about 3-fold more potent epoxide hydratase inducers in the kidney than in the liver, whereas TSO was less effective. Pretreatment with 2,2′, 4,4′-tetrachloro-TSO, which decreased liver epoxide hydratase to 25 per cent of control, slightly induced this enzyme in kidney. Both DESO and the corresponding diacetate had no apparent effect on kidney epoxide hydratase activity, which is in contrast to their inducing ability in the liver. In the lung all the TSO derivatives tested showed a similar slightly reducing effect on epoxide hydratase activities. The different effect of treatment with various TSO derivatives on epoxide hydratase activities in rat liver, kidney and lung suggests a different biosynthetic control of the enzyme in these organs, if the exposure of the responsible receptor in these organs to the ultimate inducing species is the same. Moreover, the different ratios of the epoxide hydratase induction in liver to the induction in kidney after treatment with various TSO derivatives can be taken to indicate a direct effect of these compounds or their metabolites in the corresponding organs, rather than an induction mechanism via a central target such as the pituitary gland. However, a combined central and peripheral effect of these TSO derivatives can not be excluded. Furthermore the high correlation of epoxide hydratase activities when determined either with benzo(a)pyrene 4,5-oxide or with styrene oxide as substrate after treatment with widely varying compounds suggests that at least the active site of the induced and the control epoxide hydratase is the same.

* A further reason for choosing the benzo(a)pyrene monooxygenase activity was that benzo(a)pyrene as a model compound in studies on chemical carcinogenesis represents a relevant substrate.

Effect of TSO derivatives on male rat liver cytochrome P450 content, NADPH-cytochrome c reductase and aminopyrine N-demethylase activities

In the screening experiments for a selective inducer of epoxide hydratase, the benzo(a)pyrene monooxygenase activities were chosen as indicators of induction of the monooxygenase system (Table 1), since it is known that these activities are increased by different types of monooxygenase inducers responsible for induction of different forms of cytochrome P450* [33, 53]. However, since these activities are very drastically increased after treatment with inducers known to augment the cytochrome P448 (P_1450) content and much less by cytochrome P450 inducers, these experiments did not fully exclude the induction of other parameters of the monooxygenase system after treatment with TSO. Therefore, the cytochrome P450 content as well as the NADPH-cytochrome *c* reductase and the aminopyrine *N*-demethylase activities—the latter two parameters being preferentially inducible by the phenobarbital type of inducers—were also determined (Table 3). All these parameters were very similar to the control values after TSO treatment. Moreover, no spectral shift to the blue in the Soret peak of the reduced cytochrome–CO complex could be observed indicating that no cytochrome P448 was formed. The tendency of the 4-methoxy-, 4-chloro- and 4-nitro-TSO to induce the monooxygenase system (Table 1) could be confirmed in these experiments, the induction of the aminopyrine *N*-demethylase activity being most clear (Table 3).

In summary, in an attempt to find a selective epoxide hydratase inducer, the effect of treatment with various epoxides on the epoxide hydratase and the benzo(a)pyrene monooxygenase activities in rat liver, kidney and lung microsomal fractions was investigated. In the first series of epoxides tested only dieldrin and TSO induced epoxide hydratase in the liver. Dieldrin was also found to induce the benzo(a)pyrene monooxygenase activity ("3-OH-BP"), in common with all the other epoxide hydratase inducers discovered so far. However, TSO induced liver epoxide hydratase activities dose dependently up to 350 per cent of controls without having any significant effect on the benzo(a)pyrene monooxygenase activities. Treatment with TSO also caused an increase of epoxide hydratase activities in the kidney but not in the lung. Consequently a series of TSO derivatives were synthesized and tested as potential selective epoxide hydratase inducers. All these TSO derivatives were less effective epoxide hydratase inducers and in the case of 2,2′,4,4′-tetrachloro TSO even depressed the epoxide hydratase activity in the liver. Moreover, all of them affected the benzo(a)pyrene monooxygenase activities. The relative modulating effect of the various TSO derivatives on kidney epoxide hydratase activities was quite different from that in the liver, indicating a direct organ specific biosynthetic control of this enzyme rather than a central control mechanism. Finally, the effect of treatment with TSO and three TSO derivatives on the cytochrome P450 content, the NADPH-cytochrome *c* reductase and the aminopyrine *N*-demethylase activities was

Table 3. Effect of *trans*-stilbene oxide derivatives on male rat liver cytochrome P450 content, NADPH-cytochrome *c* reductase and aminopyrine *N*-demethylase activities

Compound tested	Dose* m-moles/kg body wt	Activity as per cent of control†		
		Cytochrome P450	NADPH-cytochrome *c* reductase	Aminopyrine *N*-demethylase
Trans-stilbene oxide (TSO)	0.5	99 ± 15	97 ± 11	103 ± 11
	2.0	94 ± 18	113 ± 14	108 ± 18
4-Methoxy-TSO	0.5	109	97	127
4-Chloro-TSO	0.5	142	117	158
4-Nitro-TSO	0.5	104	105	110
	1.5	116	115	140
		nmoles mg^{-1} protein	nmoles mg^{-1} protein min^{-1}	
Control activities‡		0.83 ± 0.11	335 ± 45	3.82 ± 0.41

* See Table 1.

† Cytochrome P450 content and enzyme activities are expressed as per cent of control in the corresponding experiment. The activities after TSO treatment are given as the means ± S.D. of three experiments. All determinations were performed on washed microsomes from pooled livers of three animals per group. For measurement of cytochrome P450 content and NADPH-cytochrome *c* reductase activity duplicate or triplicate determinations were performed. Aminopyrine *N*-demethylase assays were done in duplicate determinations for each of two protein concentrations. Standard deviations of the means of the values obtained by the single determinations were usually less than 5 per cent for the cytochrome P450 content and the NADPH-cytochrome *c* reductase activity and less than 8 per cent for the aminopyrine *N*-demethylase activity.

‡ See Table 1.

compared. Again only TSO had no significant effect on all three parameters.

Thus, TSO appears to be a selective inducer of rat liver epoxide hydratase, which has no effect on the total content of liver microsomal cytochrome P450 as well as on the monooxygenase activities towards two substrates (benzo(a)pyrene and aminopyrine) known to be preferentially metabolized by different cytochrome P450 forms. A change in the pattern of the various cytochrome P450 forms present in liver microsomes not leading to a change in the metabolism of benzo(a)pyrene and aminopyrine is rather unlikely but cannot be excluded. More detailed studies on the mode of action of this interesting agent are in progress.

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