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CHARACTERIZATION OF THE MICROSOMAL CYTOCHROME P-450 SPECIES INDUCED IN RAT LIVER BY *TRANS*-STILBENE OXIDE

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Abstract—*trans*-Stilbene oxide differs from the classical inducers of drug-metabolizing enzymes, phenobarbital and 3-methylcholanthrene, in that it induces the so-called phase II activities, epoxide hydrolase and glutathione *S*-transferase, to a much larger extent than it induces cytochrome P-450. Nonetheless, the level of cytochrome P-450 in liver microsomes from rats treated with *trans*-stilbene oxide is increased significantly to twice the control value.

The existence of a number of different isozymes of cytochrome P-450 has now been clearly demonstrated and in the present study we have posed the question: What form(s) of cytochrome P-450 is induced by *trans*-stilbene oxide? A number of criteria including substrate specificity, pattern of benzo(a)pyrene metabolism, sensitivity to inhibitors, substrate binding spectra, ethylisocyanide binding spectra, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and crossed immunoelectrophoresis were used to answer this question. It seems clear that *trans*-stilbene oxide induces the same form(s) of cytochrome P-450 as phenobarbital.

A variety of compounds including drugs, carcinogens, pesticides, steroids and fatty acids can be metabolized by the hepatic microsomal monooxygenase system [1–3]. This system consists of the enzymes NADPH-cytochrome P-450 reductase and cytochrome P-450§ and requires phospholipids for catalytic activity [4, 5]. The system is also present in the nuclear envelope [6] and, in a modified form, the inner mitochondrial membrane [7].

The cytochrome P-450 system catalyses a number of different reactions, including dealkylation, dehalogenation, amine oxidation, and sulphoxidation [1, 8]. Among the most commonly studied of these reactions are the epoxidation of unsaturated bonds in aromatic hydrocarbons or aliphatic chains and the apparently direct hydroxylation of saturated bonds in compounds such as steroids and fatty acids. Epoxides may rearrange in an aqueous environment to alcohols or may be hydrated by epoxide hydrolase to yield the corresponding *trans*-dihydrodiols. Epoxides and other reactive intermediates may also be conjugated with glutathione, either non-enzymatically or via the glutathione *S*-transferases, or these intermediates may react with cellular macromolecules (DNA, RNA, and protein), thereby giving rise to toxic effects, mutations, and, perhaps ultimately, cancer [2, 9].

The existence of several different isozymes of cytochrome P-450 differing in, among other things, substrate specificity, amino acid composition, electrophoretic mobility, amino acid sequence and immunological properties is now well-established. Multiple forms of cytochrome P-450 have now been isolated from rabbit liver and lung, rat liver, mouse liver and human liver. It has been clearly shown that the form(s) of cytochrome P-450 induced by phenobarbital is not identical with that induced by 3-methylcholanthrene [10, 11].

In the present investigation we have compared the form(s) of cytochrome P-450 induced by *trans*-stilbene oxide to those induced by phenobarbital and 3-methylcholanthrene on the basis of substrate specificity, mol. wt, sensitivity to different inhibitors and immunological properties. Two questions were of primary interest. (1) Is the cytochrome P-450 species induced by *trans*-stilbene oxide different from the forms which have already been identified?; and (2) does *trans*-stilbene oxide induce a form of cytochrome P-450 which is specialized for a certain type of reaction, as the forms of cytochrome P-450 induced by 3-methylcholanthrene are effective in the metabolism of polycyclic hydrocarbons?

trans-Stilbene oxide is an especially interesting inducer of drug-metabolizing enzymes, since it induces the amount of the phase II enzymes microsomal epoxide hydrolase and cytosolic glutathione *S*-transferase(s) to a much greater extent than the total content of the phase I cytochrome P-450 system.

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§ The term 'cytochrome P-450' is used here to designate all those cytochromes which demonstrate an absorption maximum at or near 450 nm in the difference spectrum between the reduced form and the complex formed between carbon monoxide and the reduced form.

Abbreviations: ANF, α-naphthoflavone; BP, benzo(a)pyrene; 9OHBP, 9-hydroxybenzo(a)pyrene; 3OHBP, 3 hydroxybenzo(a)pyrene; MC, 3-methylcholanthrene.

The classical inducers phenobarbital and 3-methylcholanthrene are exactly the opposite in this respect. It seems quite likely that these different patterns of induction will have different effects on the toxicity and carcinogenicity of xenobiotics [12].

MATERIALS AND METHODS

Chemicals. Benzo(a)pyrene, hydroxyresorufin and 3-methylcholanthrene (Eastman Kodak, Rochester, New York, U.S.A.); ethylmorphine, hexobarbital, and phenobarbital (Apoteksbolaget, Stockholm, Sweden); aniline and hydroxycoumarin (E. Merck Aq., Darmstadt, West Germany); *trans*-stilbene oxide and metyrapone (1,2-di-(3-pyridyl)-2-methyl-1-propanone) (EGA-Chemie, Steinheim/Albuch, West Germany); aminopyrine (4-dimethylaminoantipyrin) and α-naphthoflavone (7,8-benzoflavone) (Fluka AB, Buchs SG, Switzerland); isocitric acid dehydrogenase (type IV form porcine heart), $NADP^+$, NADPH (type I), and sodium dodecyl sulphate (Sigma, St. Louis, MO, U.S.A.); and acrylamide and *N,N'*-methylene bisacrylamide (BDH, Poole, U.K.) were all obtained from commercial sources. SKF-525-A (2-diethylaminoethyl-2,2'-diphenylvalerate–HCl) was a generous gift from Smith, Kline and French Laboratories (PA, U.S.A.). [G-^{3}H]Benzo(a)pyrene (19 Ci/mmole) and [7,10-^{14}C]benzo(a)pyrene (21.7 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, U.K.

Ethoxycoumarin and ethoxyresorufin were synthesized from the hydroxy compounds by ethylation with ethyl iodide [13]. Ethoxycoumarin was purified by several recrystallizations and ethoxyresorufin by multiple runs on thin layer chromatography. The fluorescence spectra, melting points (86° and 229°, respectively) and NMR spectra were in agreement with those reported in the literature. Ethylisocyanide was synthesized according to Jackson and McKusick [14]. Benzphetamine was synthesized by Dr. Åke Pilotti of the Department of Organic Chemistry, University of Stockholm.

All other chemicals and solvents used were of reagent grade and obtained from common commercial sources.

Treatment of animals. Male Sprague–Dawley rats (obtained from Anticimex AB, Sollentuna, Sweden) weighing 170–200 g were used throughout this study and given free access to food pellets (Astra-Ewos, Södertälje, Sweden) and water. The rats, in groups of three or four animals, were injected intraperitoneally with phenobarbital (80 mg/kg body wt in isotonic saline), 3-methylcholanthrene (20 mg/kg in corn oil), or *trans*-stilbene oxide (400 mg/kg in corn oil) once daily for five days. Control animals received the vehicle alone. These doses of inducer were employed to assure maximal induction of the enzymes investigated here. No toxic effects were observed in any of the animals, although the liver–somatic index was lowered in those rats which received *trans*-stilbene oxide.

Preparation of microsomes. The rats were starved overnight in order to reduce hepatic levels of glycogen before decapitation. The livers were removed immediately after killing and placed in ice-cold 0.25 M sucrose. Liver microsomes were prepared according to Ernster *et al.* [15] and washed once in 0.15 M Tris–HCl, pH 8.0. Finally, the microsomal pellet was resuspended in a vol. of 0.25 M sucrose equal to the original liver wt.

Enzyme assays. All measurements were carried out on freshly prepared microsomes. *Cytochrome P-450* content was measured on an Aminco DW-2 spectrophotometer according to Omura and Sato [16]. *NADPH-cytochrome c reductase* was measured according to Dallner [17] in 50 mM Tris–HCl, pH 7.5.

The measurement of different activities catalysed by the cytochrome P-450 system was performed after the cytochrome P-450 content had been determined. Microsomes containing 0.2–1.0 nmole cytochrome were then used in the different measurements in order to assure linearity with time and protein. Blank incubations were carried out using boiled microsomes.

Aminopyrine N-demethylation and *ethylmorphine N-demethylation* were assayed by measuring the amount of formaldehyde liberated by the method of Nash [18]. The reaction mixture consisted of microsomes containing 1.0 nmole cytochrome P-450, an NADPH-regenerating system (1 mM $NADP^+$, 5 mM isocitric acid, 0.5 i.v. isocitric dehydrogenase), 5 mM $MgCl_2$, 5 μM $MnCl_2$, and 50 mM Tris–HCl, pH 7.5, in a total vol. of 1.0 ml. The tubes were preincubated for 1 min at 37° before addition of the substrate, 10 μmoles aminopyrine or 10 μmoles ethylmorphine dissolved in water. The reaction was terminated after 10 min with 0.25 ml cold 20% trichloroacetic acid, vortexed, chilled on ice and centrifuged. Formaldehyde was determined in 1 ml of the resulting supernatant.

Benzphetamine N-demethylation was assayed at 30° by following the oxidation of NADPH at 340 nm [19]. The reaction mixture had a vol. of 1 ml and contained microsomes. 0.2 M phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 6 mM semicarbazide, 0.15 mM NADPH, and 0.9 mM benzphetamine.

Benzo(a)pyrene monooxygenase was assayed radiometrically by the method of DePierre *et al.* [20]. Microsomes containing 0.25–0.5 nmoles cytochrome P-450 were used. In order to minimize the autoxidation of benzo(a)pyrene the incubation was carried out under dim light. Inhibitors were added in 10 μl acetone and control experiments demonstrated that this vol. of acetone was without effect in the control incubations and that the inhibitors had no effect on the background.

Ethoxycoumarin O-deethylation and *ethoxyresorufin O-deethylation* were monitored continuously at 37° by measuring the formation of the corresponding hydroxy products with an Aminco Bowman spectrofluorimeter [13].

SDS-polyacrylamide gel electrophoresis was performed with slab gels as described by Laemmli [21]. Microsomes were solubilized in 2% SDS and 5% 2-mercaptoethanol by heating for 3 min at 100°. Bovine serum albumin (67,000 daltons), catalase (58,000), glutamate dehydrogenase (53,000) and ovalbumin (43,000) were used as references and the resolution was sufficient to distinguish differences of 1000 daltons in the apparent mol. wts of different polypeptides.

High-pressure liquid chromatography was used to analyse the individual metabolites of benzo(a)pyrene as described previously [22]. *Spectral analysis* was performed with an Aminco DW-2 spectrophotometer at 30°. For determination of substrate binding spectra each cuvette contained microsomes with 2 nmoles cytochrome P-450 in 1 ml 50 mM Tris–HCl, pH 7.5. Aniline or hexobarbital was added to the sample cuvette to give a final concn of 2.5 or 7.5 mM, respectively, while an equivalent vol. (25 μl) of the solvent (dimethylsulfoxide) was added to the reference cuvette. The difference spectrum was recorded between 350 and 450 nm.

Ethylisocyanide difference spectra were obtained by a modification of a published procedure [23]. Each cuvette contained microsomes containing 1 nmole cytochrome P-450 in 1 ml 50 mM Tris–HCl, pH 7.5. Dithionite was added to both cuvettes and the baseline recorded between 400 and 500 nm. Ethylisocyanide in 15 μl water was added to the sample cuvette to give a final concn of 16 mM, water was added to the reference, and the difference spectrum was recorded.

Immunological analysis. Rabbit antibodies raised towards the phenobarbital-inducible cytochrome P-450 and the cytochrome P-448 induced by β-naphthoflavone ('B'-fractions) were prepared as described earlier [24]. Microsomal proteins were separated using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and the resolved proteins were electrophoretically transferred to sheets of nitrocellulose. The nitrocellulose sheets were treated sequentially (with intermediate washes) with: (1) rabbit antisera raised to purified

Table 1. The effects of different inducers on cytochrome P-450 levels in rat liver microsomes

Treatment	*n**	nmoles cytochrome P-450/ mg microsomal protein	% of control
Control†	11	0.365 ± 0.071	100
Phenobarbital	11	1.29 ± 0.26	353
3-Methylcholanthrene	11	1.24 ± 0.27	340
trans-Stilbene oxide	11	0.772 ± 0.111	212

Induction was performed and cytochrome P-450 assayed as described in Materials and Methods.

* Number of animals.

† Microsomes from untreated animals or animals receiving vehicle only (0.9% NaCl or corn oil) demonstrated the same levels of cytochrome P-450 and these values were therefore pooled.

Table 2. Rat liver microsomal cytochrome P-450-catalysed activities known to be preferentially induced by phenobarbital*

	Activities (nmoles metabolized/min/nmole cytochrome P-450)		
Treatment	Aminopyrine-*N*-demethylase	Ethylmorphine-*N*-demethylase	Benzphetamine-*N*-demethylase
Control	7.06 ± 1.15 (14)	8.23 ± 2.21 (12)	4.16 ± 2.00 (13)
Phenobarbital	12.5 ± 2.31 (12)	14.5 ± 1.06 (12)	10.7 ± 2.58 (9)
3-Methylcholanthrene	2.96 ± 0.98 (14)	2.67 ± 1.75 (11)	0.54 ± 0.31 (9)
trans-Stilbene oxide	13.0 ± 2.10 (15)	12.5 ± 2.36 (12)	13.1 ± 2.88 (8)

*Rats were induced and microsomes prepared as described in Materials and Methods. Values represent means ± S.D.; the number of animals used is given in parentheses.

Table 3. Rat liver microsomal cytochrome P-450-catalysed activities known to be preferentially induced by 3-methylcholanthrene*

	Activities (nmole metabolized/min/nmole cytochrome P-450)		
Treatment	Benzo(a)pyrene monooxygenase	Ethoxycoumarin-*O*-deethylase	Ethoxyresorufin-*O*-deethylase
Control	0.51 ± 0.17 (15)	0.83 ± 0.19 (10)	0.42 ± 0.11 (13)
Phenobarbital	1.42 ± 0.14 (12)	0.91 ± 0.20 (10)	0.17 ± 0.03 (15)
3-Methylcholanthrene	3.68 ± 0.47 (15)	2.35 ± 0.38 (7)	24.4 ± 6.37 (13)
trans-Stilbene oxide	1.68 ± 0.38 (18)	1.42 ± 0.28 (9)	0.21 ± 0.04 (9)

* Rats were induced and microsomes prepared as described in Materials and Methods. Values represent means ± S.D.; the number of animals used is given in parentheses.

Table 4. Pattern of benzo(a)pyrene metabolites obtained with liver microsomes from induced and control rats

Treatment	Percentage distribution of metabolites							
	9,10-Diol	4,5-Diol	7,8-Diol	1,6-Quinone	3,6-Quinone	6,12-Quinone	9-Hydroxy	3-Hydroxy
Control	5.05 ± 0.07	3.08 ± 0.48	3.70 ± 1.53	7.08 ± 0.91	5.08 ± 1.40	4.11 ± 0.55	5.00 ± 0.28	67.0 ± 2.7
Phenobarbital	3.59 ± 0.20	20.1 ± 0.9	2.09 ± 0.28	9.93 ± 0.60	12.9 ± 0.7	6.04 ± 0.62	1.44 ± 0.42	43.0 ± 2.3
3-Methylcholanthrene	12.2 ± 2.6	8.31 ± 0.35	10.6 ± 0.5	7.97 ± 0.20	7.52 ± 0.57	2.58 ± 0.14	13.1 ± 1.5	37.9 ± 1.6
trans-Stilbene oxide	1.78 ± 0.54	27.3 ± 2.4	1.03 ± 0.30	7.11 ± 0.76	9.06 ± 1.49	4.42 ± 0.07	0.78 ± 0.13	47.9 ± 0.8

Rats were induced, microsomes prepared and the metabolite pattern determined as described in Materials and Methods. The values represent the means ± S.D. for three rats in each group.

rat liver cytochromes P-450; (2) goat anti-rabbit immunoglobulin G; (3) a complex of horseradish peroxidase and rabbit antiperoxidase; and (4) a mixture of 3,3′-diaminobenzidine and H_2O_2. The procedure is described in detail elsewhere [24]. Brown bands on a white background correspond to proteins in the gel which react with the primary antibody.

Protein. Assayed using a slight modification of the method of Lowry *et al.* with bovine serum albumin as standard [25].

RESULTS

Substrate specificity of the microsomal cytochrome P-450 induced by trans*-stilbene oxide*

Table 1 demonstrates the effects of interperitoneal administration of different inducers on the levels of cytochrome P-450 in microsomes from rat liver. It can be seen that *trans*-stilbene oxide induces cytochrome P-450 to a highly significant extent, even though the extent of induction with this xenobiotic is considerably less than that observed after treatment with phenobarbital or 3-methylcholanthrene. *trans*-Stilbene oxide induction does not result in a blue shift in the absorption maximum of the carbon monoxide-reduced cytochrome P-450 complex, indicating that *trans*-stilbene oxide does not induce the same isozyme of cytochrome P-450 as does 3-methylcholanthrene.

Since different isozymes of cytochrome P-450 demonstrate different substrate specificities, the activities catalysed by cytochrome P-450 in control and induced animals were compared. Table 2 illustrates the activities per nmole cytochrome P-450 obtained with substrates known to be preferentially metabolized by microsomes from phenobarbital-treated rats. As can be seen, the activities with all three substrates are increased approx. 2-fold with microsomes from phenobarbital-treated rats; while the corresponding activities from methylcholanthrene-induced microsomes are decreased 2–8-fold compared with the control activities. The activity pattern obtained with microsomes from rats treated with *trans*-stilbene oxide closely resembles that seen with phenobarbital as inducer.

The activities with three substrates whose metabolism is known to be preferentially increased by 3-methylcholanthrene treatment were also assayed (Table 3). The increase in specific activity in the case of 3-methylcholanthrene induction was 7-, 3- and 60-fold for benzo(a)pyrene mono-oxygenase, ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase, respectively. Thus, the latter activity is a good marker for 3-methylcholanthrene induction. The corresponding changes in the case of phenobarbital induction were +178%, +10% and −60%; while the values for *trans*-stilbene oxide-induced microsomes were +229%, +71% and −50%, respectively.

Products of benzo(a)pyrene metabolism after injunction

Table 4 reveals the metabolite patterns obtained with benzpyrene using microsomes from control and induced rats. Such patterns obtained with different

Table 5. Effects of some inhibitors on benzo(a)pyrene monooxygenase activity in liver microsomes from induced and control rats*

Treatment	Inhibitor (percentage of corresponding activity in the absence of inhibitor)			
	SKF 525-A (5×10^{-4} M)†	Metyrapone (10^{-4} M)†	α-Naphthoflavone (10^{-4} M)†	Progesterone (10^{-4} M)†
Control	37.9 ± 5.4	68.6 ± 2.5	102.5 ± 12.4	56.9 ± 5.2
Phenobarbital	23.3 ± 2.6	46.7 ± 5.8	84.0 ± 2.6	56.6 ± 3.8
3-Methylcholanthrene	65.6 ± 7.0	98.8 ± 8.4	19.9 ± 2.8	81.3 ± 7.6
trans-Stilbene oxide	27.6 ± 6.6	52.2 ± 3.3	68.5 ± 1.6	67.6 ± 4.2

* Rats were induced and microsomes prepared as described in Materials and Methods. The values represent means ± S.D. for 3–6 rats.

† The final concns of the inhibitors are shown in parentheses.

isozymes of cytochrome P-450 are known to differ clearly. As also reported previously, induction with 3-methylcholanthrene can be seen to cause increases in the relative amounts of 9-hydroxybenzo(a)pyrene and the dihydrodiols, among them the benzo(a)pyrene 7,8-dihydrodiol which can be recycled through the cytochrome P-450 system and give rise to a 9,10–oxide which may be the major ultimate carcinogen of benzo(a)pyrene. Treatment with phenobarbital resulted in a large increase in the relative formation of benzo(a)pyrene 4,5-dihydrodiol and benzo(a)pyrene 3,6-quinone, but a decrease in the formation of the phenols and benzo(a)pyrene 9,10- and 7,8-dihydrodiols. In the case of *trans*-stilbene oxide the metabolite pattern obtained closely resembled that seen after phenobarbital induction; the increased formation of benzo(a)pyrene 4,5-dihydrodiol at the expense of the 9,10-and 7,8-dihydrodiols was even somewhat greater with *trans*-stilbene oxide.

Sensitivity to inhibitors

Sensitivity to inhibitors was also employed as a criterion for comparison of the form of cytochrome P-450 induced by *trans*-stilbene oxide with those induced by phenobarbital and 3-methylcholanthrene. The effect of SKF 525-A, metyrapone, α-naphthoflavone, and the potentially competing substrate progesterone on benzo(a)pyrene monoxygenase activity were tested (Table 5). SKF 525-A was an effective inhibitor with microsomes from control and phenobarbital- and *trans*-stilbene oxide-treated animals; but this inhibitor was less effective in the case of 3-methylcholanthrene induction. Metyrapone inhibited benzo(a)pyrene monooxygenase activity after phenobarbital and *trans*-stilbene oxide induction about 50%; in control microsomes about 30%; but this inhibitor had no effect in the case of 3-methylcholanthrene induction. α-Naphthoflavone had no inhibitory effect on the control activity and at higher concentrations even stimulated it. The corresponding activities after treatment with phenobarbital, *trans*-stilbene oxide and 3-methylcholanthrene were slightly, somewhat more, and very strongly inhibited by α-naphthoflavone, respectively. Progesterone was found to give nearly 50% inhibition in control microsomes and after phenobarbital administration, less inhibition after *trans*-stilbene oxide treatment, and even less after 3-methylcholanthrene induction.

Substrate binding spectra

The affinity of the different cytochrome P-450s for aniline and hexobarbital was determined by record-

Table 6. Spectral interaction of the ligands aniline, hexobarbital and ethylisocyanide with liver microsomes from induced and control rats*

Treatment	Aniline (2.5 mM)†	Hexobarbital (7.5 mM)†	Ethylisocyanide (16 mM)†	
			A_{450}/A_{430}	Absorbance maxima (nm)
Control	11.1 ± 1.5 (9) (II)	11.8 ± 2.2 (12) (I)	0.28 ± 0.15 (9)	455/426
Phenobarbital	19.1 ±2.2 (12) (II)	23.0 ± 5.3 (12) (I)	0.30 ± 0.12 (9)	452/425
3-Methylcholanthrene	41.2 ± 7.7 (9) (II)	21.8 ± 6.3 (6) (RI)	0.93 ± 0.24 (5)	450/427
trans-Stilbene oxide	21.6 ± 4.7 (16) (II)	18.4 ± 4.7 (17) (I)	0.38 ± 0.13 (14)	453/425

* Rats were induced and microsomes prepared as described in Materials and Methods. The values represent mean absorbance units/nmole cytochrome P-450 ± S.D. for the number of animals given in the first parentheses. The second set of parentheses indicates the type of spectrum obtained: RI, reversed type I spectrum; I, type I spectrum; II, type II spectrum.

† Final concn of the ligands.

ing so-called type spectra (Table 6). Aniline gave rise in all cases to a type II spectrum whose magnitude was increased about two-fold by treatment with phenobarbital and *trans*-stilbene oxide and about four-fold after 3-methylcholanthrene induction. Hexobarbital binding resulted in a type I spectrum in the case of control and phenobarbital- and *trans*-stilbene oxide-treated rats; and both of these inducers resulted in a two-fold increase in the size of the spectral maximum. On the other hand, microsomes from 3-methylcholanthrene-induced rats gave a reversed type I spectrum with hexobarbital which was about twice as large as the type I spectrum seen with control microsomes.

Ethylisocyanide binding spectrum

The characteristic spectrum obtained when ethylisocyanide interacts with reduced cytochrome P-450 was also recorded using microsomes from control and induced animals (Table 6). The ratios between the absorption peaks at approx. 455 and 430 nm were calculated. This ratio was increased about 230% in the case of 3-methylcholanthrene, increased 36% with *trans*-stilbene oxide and remained unchanged after phenobarbital treatment. The exact peak maximum was lowered from 455 to 450 nm by 3-methylcholanthrene treatment and to 452 and 453 nm in the case of phenobarbital and *trans*-stilbene oxide, respectively. The other absorbance maximum was at 426 nm for control microsomes and was decreased 1 nm in the case of phenobarbital and *trans*-stilbene oxide and increased 1 nm after 3-methylcholanthrene treatment.

SDS-polyacrylamide gel electrophoresis

As can be seen from the electrophoresis profiles (Fig. 1), there is a great similarity in the microsomal protein bands induced by treatment of rats with phenobarbital and *trans*-stilbene oxide. Epoxide hydrolase (with a mol. wt of approx. 49,000) was strongly induced by *trans*-stilbene oxide and induced to a lesser extent by phenobarbital. Above the epoxide hydrolase band is a band corresponding to the major form of cytochrome P-450 induced by phenobarbital, with a mol. wt of approx. 52,000. Treatment of rats with 3-methylcholanthrene gives rise, on the other hand, to two new bands in the region corresponding to mol. wts of 49,000–60,000. Neither of these bands is seen in the other preparations.

Immunological analysis

In line with the other results presented here, preliminary double-diffusion immunoprecipitation and enzyme activity inhibition studies indicated that the major cytochrome P-450 induced by *trans*-stilbene oxide was much more similar to the major phenobarbital-induced form of cytochrome P-450 than to the form induced by 3-methylcholanthrene or by β-naphthoflavone (data not shown). The most definitive immunological evidence in support of this view is presented in Fig. 2. A crossed immunoelectrophoretic technique was used in which proteins resolved by polyacrylamide gel electrophoresis are stained with specific antibodies raised to the major forms of cytochrome P-450 induced by phenobarbital and β-naphthoflavone [24]. Other evidence indicates that the major forms of rat liver cytochrome P-450 induced by 3-methylcholanthrene and by β-naphthoflavone are indistinguishable as judged by catalytic activity [26, 27], quantitative micro-complement fixation analysis [28] and crossed immunoelectrophoresis [24].

In Fig. 2(A), the antibody raised against the phenobarbital-induced cytochrome P-450 was found to react with a protein of the same apparent mol. wt as the antigen in microsomes prepared from phenobarbital- or *trans*-stilbene oxide-treated rats but not control or 3-methylcholanthrene-treated rats. The data presented in Fig. 2(B) indicates that the antibody raised against the β-naphthoflavone-induced cytochrome P-450 recognized a protein of the same mol. wt as the antigen in microsomes prepared from 3-methylcholanthrene-treated rats but not control or phenobarbital- or *trans*-stilbene oxide-treated rats. Other experiments have shown that the two forms of cytochrome P-450 considered here can be distinguished readily by apparent mol. wt with this technique when electrophoresed in the same gel [24]. In the present experiments some aggregation appeared to occur as the result of lyophilization of samples for shipping, thus giving rise to high mol. wt stains; but this phenomenon does not affect the validity of the conclusions drawn.

DISCUSSION

trans-Stilbene oxide is a potent inducer of rat liver microsomal epoxide hydrolase and cytosolic glutathione *S*-transferase, while the induction of cytochrome P-450 is only two fold ([29, 30] Table 1). In recent years it has become apparent that multiple forms of cytochrome P-450 exist in rat liver microsomes, some of which can be preferentially induced by different xenobiotics. To date, the induction of cytochrome P.450 has been classified as phenobarbital-, 3-methylcholanthrene-, or mixed type-induction, mainly on the basis of substrate specificity and the electrophoretic polypeptide pattern. In the present study we have posed the question, 'What form(s) of cytochrome P-450 is induced by *trans*-stilbene oxide?'

An earlier report by Bend and coworkers [31] showed a 50–100% increase in certain cytochrome P-450-linked activities (recalculated as activity per nmole cytochrome P-450) with no significant increase in the content of cytochrome P-450 in liver microsomes isolated from immature male rats (100–120 g) treated with different doses of *trans*-stilbene oxide. An early report form Oesch's laboratory [32] also indicated no induction of cytochrome P-450 or of benzo(a)pyrene monooxygenase activity by this xenobiotic.

However, a later report from the same group [33] showed a 2.5-fold increase in ethoxycoumarin *O*-deethylase activity in liver microsomes from *trans*-stilbene oxide-treated rats. Furthermore, this activity was found to be inhibited by metyrapone, but not affected by α-naphthoflavone in the concns used.

The following criteria from the present study all indicate that *trans*-stilbene oxide induces the same form(s) of cytochrome P-450 as induced by

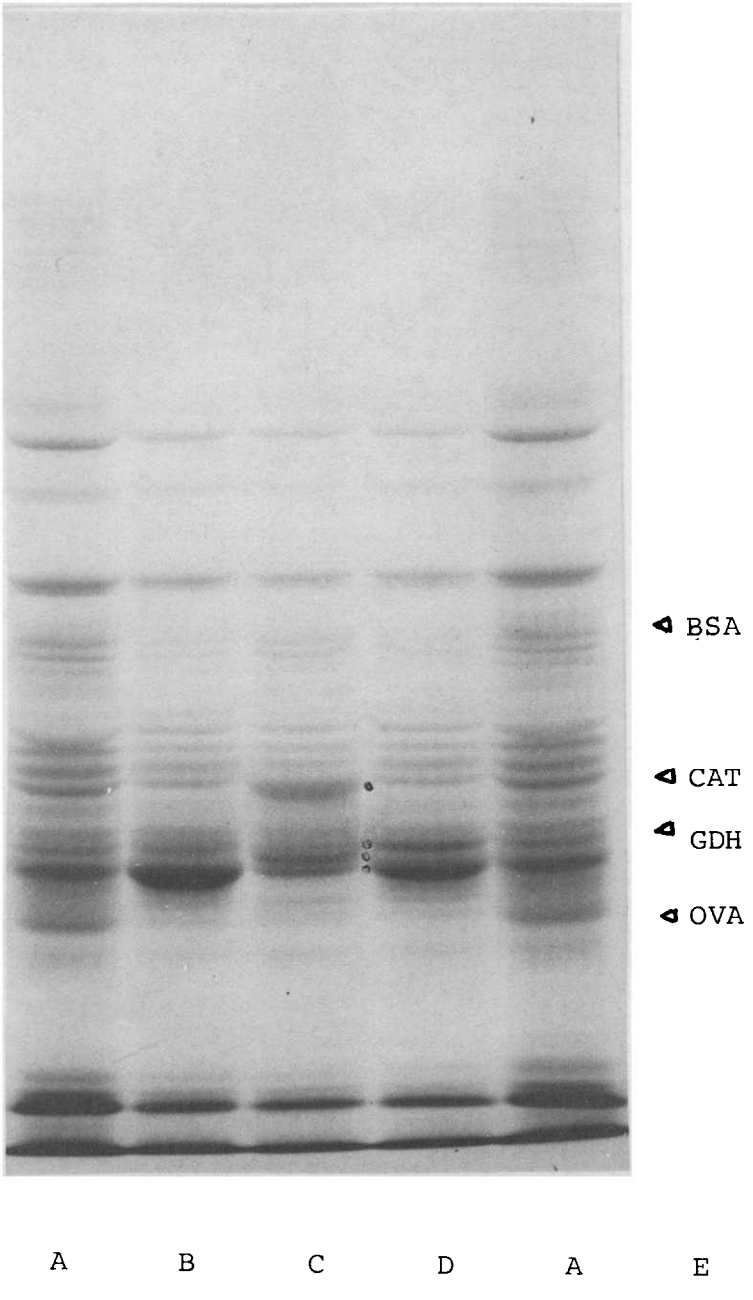

Fig. 1. SDS-polyacrylamide gel electrophoresis of liver microsomes from rats treated with different inducers. Electrophoresis was performed according to Laemmli [21] on slab gels, except that the ionic strength in the running buffer was doubled. This modification improved the resolution somewhat, but changed the relative migration of the polypeptide bands. An amount of microsomes containing 15 pmoles cytochrome P-450 from control rats (A) and rats treated with *trans*-stilbene oxide (B), 3-methylcholanthrene (C), or phenobarbital (D) was applied to each well. The dots between lanes C and D indicate, from bottom to top, epoxide hydrolase, polypeptide induced by 3-methylcholanthrene, polypeptide induced by phenobarbital and polypeptide induced by 3-methylcholanthrene. The migrations of the reference proteins bovine serum albumin (BSA), catalase (CAT), glutamate dehydrogenase (GDH), and ovalbumin (OA) are indicated in lane E.

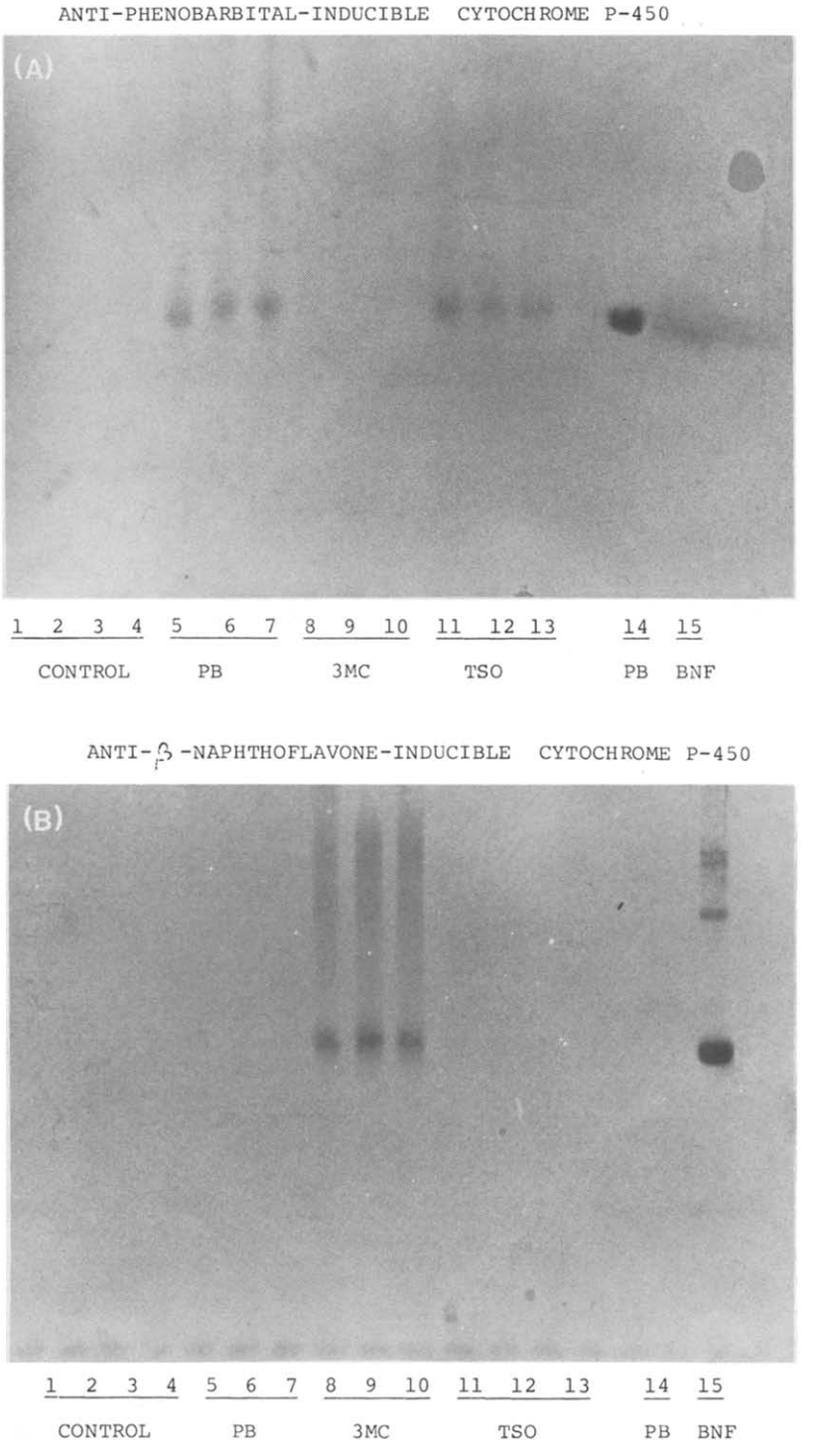

Fig. 2. Immunological analysis of cytochrome P-450 induced by *trans*-stilbene oxide. In each well 10 μg microsomal protein was electrophoresed and the entire procedure was carried out as described in Materials and Methods and in more detail elsewhere [24]. The antisera were diluted 1:100 in both cases. In (A) the antiserum raised against the phenobarbital-induced cytochrome P-450 was used and in (B) the antiserum raised against the β-naphthoflavone-induced cytochrome P-450 was employed. Individual wells in both cases contained microsomes prepared from control rats (1–4), phenobarbital (PB)-treated rats (5–7), 3-methylcholanthrene (3MC)-treated rats (8–10), and *trans*-stilbene oxide (TSO)-treated rats (11–13). In all cases replicate microsomal preparations from individual rats were used in the series of wells. Well 14 contained 20 nmoles purified phenobarbital-induced cytochrome P-450 (PB) and well 15 contained 20 nmoles β-naphthoflavone-induced cytochrome P-450 (β-NF). The anode was at the bottom of the gel in both cases and faint bands corresponding to the tracking dye can be seen.

Table 7. Changes in rat liver microsomal cytochrome P-450 linked activities after induction calculated on the basis of the induced amount of cytochrome P-450*

Inducer	Activity (percent of control)				
	Aninopyrine-*N*-demethylase	Ethylmorphine-*N*-demethylase	Benzphetamine-*N*-demethylase	Benzo(a)pyrene monooxygenase	Ethoxycoumarin-*O*-deethylase
Phenobarbital	174	262	478	415	91
3-Methylcholanthrene	24	8	0.3	1220	224
trans-Stilbene oxide	198	232	870	572	149

* These values are means calculated as described in the Discussion.

phenobarbital.

(1) Substrate specificity. In order to estimate the specific activity of the induced portion of cytochrome P-450 with different substrates we performed the following calculation: the increase in activity per mg microsomal protein was divided by the increase in cytochrome P-450 content per mg microsomal protein and these values were then divided by the activity per nmole cytochrome P-450 in control microsomes to arrive at the percentage change. These calculations assume that the isozymes of cytochrome P-450 present in control microsomes are also present at the same levels in induced microsomes.

The results obtained are shown in Table 7. These findings support those of Tables 2 and 3 and indicate a very close resemblance in the specific activities of the forms of cytochrome P-450 induced by phenobarbital and *trans*-stilbene oxide.

(2) Pattern of benzo(a)pyrene metabolism. This pattern, which may be considered as a sort of 'fingerprint' for the forms of cytochrome P-450 present in microsomes, is very similar after induction with phenobarbital and *trans*-stilbene oxide, while the corresponding pattern in the case of 3-methylcholanthrene differs considerably. These finding agree well with an earlier report from Oesch's laboratory [33].

(3) Sensitivity to inhibitors.

(4) Substrate binding spectra.

(5) Ethylisocyanide binding spectra.

(6) SDS-polyacrylamide gel electrophoresis.

(7) Immunoprecipititation.

Thus, it is clear from the present investigation that *trans*-stilbene oxide induces the same form(s) of cytochrome P-450 as is induced by phenobarbital.

A very recent article by Levin and co-workers [34] arrives at the same conclusion. The level of microsomal cytochrome P-450 was induced nearly twofold in their immature rats by *trans*-stilbene oxide. These investigators quantitated the relative abundance of three different forms of cytochrome P-450 using specific antibodies in a radial immunodiffusion assay. Their results demonstrate a nearly identical distribution of these three isozymes in liver microsomes from phenobarbital- and *trans*-stilbene oxide-treated animals, a distribution pattern which differs markedly from that seen in control rats or after induction with 3-methylcholanthrene.

The report by Levin and coworkers reveals that it is the so-called 'b' form of cytochrome P-450, with an apparent mol. wt of 52,000, which is induced both by phenobarbital and *trans*-stilbene oxide. These basic observations concerning the immunochemical quantitation of the forms of cytochrome P-450 induced by phenobarbital and *trans*-stilbene oxide have been confirmed using the quantitative crossed immunoelectrophoresis assay [24]. However, 47, 34 and 89% of the total cytochrome P-450 measured spectrally in liver microsomes from *trans*-stilbene oxide-treated, phenobarbital-induced and control rats, respectively, did not react with the antibodies used. Thus, the possibility remains that unidentified forms of cytochrome P-450 may also be induced by *trans*-stilbene oxide to a minor extent.

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