The Effects of Benzopyrene and Safrole on Biphenyl 2-Hydroxylase and other Drug-Metabolizing Enzymes

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A study was made of the nature and specificity of the increase in biphenyl 2-hydroxylase activity after preincubation of liver microsomal preparations with various carcinogens *in vitro*. This enhancement of enzyme activity *in vitro* was investigated in mouse, hamster and rat, and, although the rat appears to be atypical in the variation of the pattern of 2- and 4-hydroxylation with age, similar enhancements were detectable in each species examined. An increase in biphenyl 2-hydroxylase activity was apparent 2h after intraperitoneal administration of safrole or benzopyrene to mature Wistar albino rats and appeared to be similar in nature to that observed after preincubation of liver microsomal preparations with the same chemical *in vitro*. Investigation of other drug-metabolizing enzyme systems suggests that the enhancing effects of carcinogens *in vitro* are specific for biphenyl 2-hydroxylase. No correlation between the enhancement of biphenyl 2-hydroxylase and inhibition of biphenyl 4-hydroxylase was apparent.

The hepatic mixed-function oxidases that catalyse the hydroxylation of drugs and other xenobiotics generally exhibit very low activity in neonatal animals, which develops rapidly after birth (Dickerson & Basu, 1975). At all ages, but especially in the young, these enzymes tend to exhibit marked substrate-mediated induction (Parke, 1975). The activities of the hepatic enzymes that catalyse the 2- and 4-hydroxylation of biphenyl have been shown to vary markedly with age in the rat. The 4-hydroxylation increases from birth to maturity, characteristic of most hepatic microsomal mixed-function oxidases, whereas biphenyl 2-hydroxylation shows decreased activity with age after birth (Basu *et al.*, 1971).

Pretreatment of rats with various foreign compounds produce different effects on the stimulation of the two modes of hydroxylation of biphenyl (Creaven & Parke, 1966; Parke & Rahman, 1970). Phenobarbitone and other non-carcinogenic compounds administered to the rat either selectively stimulate biphenyl 4-hydroxylation or have no enhancing effect, whereas carcinogenic compounds such as 3-methylcholanthrene, 3,4-benzopyrene and safrole preferentially stimulate the 2-hydroxylation of biphenyl with a smaller or no effect on biphenyl 4-hydroxylation. We have shown that the preincubation of various carcinogens with normal hepatic microsomal preparations in the presence of an NADPH-regenerating system produces enhancement of biphenyl 2-hydroxylation (Bridges *et al.*, 1973; McPherson *et al.*,

1974a), but not biphenyl 4-hydroxylation. Preincubation with non-carcinogens, however, produced no significant increase in the hydroxylation of biphenyl in either the 2- or 4-positions. This system has potential as a preliminary screening test *in vitro* for chemical carcinogens (McPherson *et al.*, 1974b).

Activation of microsomal mixed-function oxidases *in vitro* is not unique; it has been observed with the *p*-hydroxylation of aniline by ethyl isocyanide (Imai & Sato, 1966), acetone, pentan-2-one (Anders, 1968), 2,2'-bipyridyl (Buhler & Rasmussen, 1968; Anders, 1971), volatile anaesthetics (Korten & Van Dyke, 1973) and metyrapone (Netter *et al.*, 1969). Further, Gigon *et al.* (1969) have reported that compounds that exhibit a spectrally characterized type I binding to cytochrome *P*-450 produce an activation of part of the electron-transport system, namely cytochrome *P*-450 reductase, which together with cytochrome *P*-450 is involved in the oxidation of many drug substrates.

We now present evidence to show that the enhancement of biphenyl 2-hydroxylation *in vitro* by carcinogens such as safrole or benzopyrene is highly specific for biphenyl 2-hydroxylase and involves a mechanism dissimilar to the other known activations of drug-metabolizing enzyme activity.

Materials and Methods

Materials

2-Hydroxybiphenyl and 4-hydroxybiphenyl and 2,2'- and 4,4'-hydroxybiphenyl were recrystallized before use as described previously (Burke, 1973).

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3,4-Benzopyrene and glucose 6-phosphate dehydrogenase [Sigma (London) Chemical Co., London S.W.6, U.K.], sodium pentobarbitone (BDH Chemicals, Poole, Dorset, U.K.), NADP⁺ and glucose 6-phosphate (P-L Biochemicals, Chicago, IL, U.S.A.) were used as purchased. All other reagents and compounds used were of the highest commercially available purity.

Animals

Male Wistar rats (approx. 150g) bred by the University of Surrey Animal Unit, male Syrian hamsters (pure line, golden strain; approx. 100g) and mature white mice (strain A12; approx. 50g) from commercial breeders were used. Animals were maintained as litter-mate cage groups with a maximum of six animals per group. Water and food (Spratt's Laboratory Diet no. 1) were provided *ad libitum*. All animals were under constant environmental conditions of temperature, humidity and lighting regime. Except for the time-course experiments, animals were killed between 9 and 10 a.m.

Methods

Treatment of animals and preparation of liver homogenates. Compounds studied for their effects *in vivo* were normally administered as a single intraperitoneal dose. 3,4-Benzopyrene and safrole (75 mg/kg body wt.) were administered in ground-nut oil (1.0 ml/animal), and phenobarbitone (75 mg/kg body wt.) was administered in 1.15% (w/v) KCl (1.0 ml/animal), similar volumes of vehicle being given to control animals. In one series of experiments a second intraperitoneal dose of benzopyrene was administered 6 h after the first. Actinomycin D (1 mg/kg body wt.) was administered intraperitoneally immediately after a single dose of benzopyrene where appropriate.

Animals were killed by cervical dislocation at various times after drug administration, the livers were rapidly removed into chilled 1.15% (w/v) KCl buffered to pH 7.6 with 0.3 M-NaH₂PO₄/K₂HPO₄, blotted with filter paper, weighed and transferred to fresh-chilled 1.15% (w/v) KCl. The weighed livers were homogenized in a Potter-Elvehjem homogenizer (size C; A. H. Thomas and Co., Philadelphia, PA, U.S.A.) by using two strokes (each of 9 s) of the pestle, power-driven at 2400 rev./min. Homogenate volumes were diluted to 250 mg of tissue/ml of homogenate, and centrifuged in 50 ml polycarbonate centrifuge tubes in an MSE High Speed 18 with 8 × 50 angle rotor (*r*_{av}, 7.4 cm) for 10 min at 15000g. The 15000g supernatant was decanted into 25 ml polycarbonate tubes and further centrifuged at 104000g for 1 h in an MSE Super Speed 50 with 8 × 25 rotor. The 104000g_{av} supernatant was decanted, and the microsomal pellet washed with buffered KCl (see above) and resuspended in the same volume

of buffered KCl. The homogenate was again centrifuged at 104000g for 1 h and the washed pellet was resuspended in the same volume of buffered KCl.

Effect of some chemicals on biphenyl hydroxylation. Hepatic microsomal preparations (to give a final protein concentration of 2 mg/ml) were added to a NADPH-regenerating system containing glucose 6-phosphate (25 mM), NADP⁺ (5.0 mM), glucose 6-phosphate dehydrogenase (2 units/ml) and MgSO₄ (0.5 mM) in a total volume of 2.0 ml. The test compounds, benzopyrene (1 mM), safrole (0.1 μM–1 mM) in 0.5 ml of groundnut-oil, or phenobarbitone (1 mM) in 1.15% (w/v) KCl, were added and the system preincubated (10 min) at 37°C in a shaking water bath (100 cycles/min). After this preincubation period, biphenyl [13 mM in 0.3 ml of 1.15% (w/v) KCl containing 1.5% (w/v) Tween 80] was added and the incubation continued for a further 5 min. The reaction was then terminated by the addition of 1 ml of 4 M-HCl, and the 2- and 4-hydroxybiphenyl metabolites were extracted and determined fluorimetrically by the method of Creaven *et al.* (1965). Separate controls were run in which either the test substance or biphenyl compound was omitted from the system, or the test compound was added after termination of the enzymic reaction.

Similar procedures were used when investigating the effects of preincubation on substrates other than biphenyl. With biphenyl as substrate, optimal homogenization, cofactor concentration and incubation conditions were determined for each animal species used, at various ages.

4-Hydroxylation of aniline. The method of Nakanishi *et al.* (1971), modified for optimum conditions in the Syrian hamster, was used. Incubation mixtures (2 ml) were composed of: NADPH-regenerating system (glucose 6-phosphate, 25 mM; NADP⁺, 5.0 mM; glucose 6-phosphate dehydrogenase, 2 units/ml); MgSO₄, 0.05 mM; microsomal protein, 2 mg/ml; aniline hydrochloride, 0.01 mmol; 1.15% KCl buffered to pH 7.4 with 0.3 M-NaH₂PO₄/K₂HPO₄. The reaction was initiated by addition of aniline and continued for 10 min at 37°C under oxygen in a shaking water bath at 100 cycles/min until the reaction was terminated by the addition of 5 ml of diethyl ether [containing 1.5% (v/v) petan-2-ol]. The incubation mixture was then saturated with NaCl and extracted (rotatory extractor) for 30 min at room temperature with 10 ml of diethyl ether. The phases were separated by centrifugation and the ether layer (7 ml) was extracted for 30 min with 4 ml of freshly prepared 0.5 M-K₃PO₄ containing 0.5% phenol. Phases were again separated by centrifugation, the ether layer was aspirated off and *E*₆₃₀ of the lower layer determined. Standards consisted of a heat-inactivated microsomal incubation mixture containing 0.1 ml of 0.5 mM-4-amino-phenol.

Protein determination. For determination of the protein content of the microsomal preparations, the method of Lowry *et al.* (1951) was used, with bovine serum albumin as standard.

Cytochromes b_5 and P-450. The microsomal concentrations of cytochromes b_5 and P-450 were determined by the method of Ullrich (1969) in a Pye Unicam spectrophotometer SP.1800. Cytochrome concentrations were expressed as nmol/mg of microsomal protein, by using extinction coefficients of $170\text{ mm}^{-1}\cdot\text{cm}^{-1}$ for cytochrome b_5 ($E_{409}-E_{424}$) and $91\text{ mm}^{-1}\cdot\text{cm}^{-1}$ for cytochrome P-450 ($E_{480}-E_{500}$) (Ullrich, 1969).

Other microsomal enzymes. These were determined by the following procedures: *p*-nitrobenzoate reductase (Gingell, 1970); cytochrome P-450 reductase (Gigon *et al.*, 1969); *p*-nitroanisole *N*-demethylation (Netter & Seidel, 1964); benzopyrene hydroxylase (Kuntzman *et al.*, 1966); 4-chloromethylaniline demethylase (Kupfer & Bruggeman, 1966); 7-ethoxycoumarin de-ethylase (Ullrich & Weber, 1972); ethoxyresorufin de-ethylase (Burke & Mayer, 1974).

NADPH- and NADH-cytochrome *c* reductase. The activities of NADPH- and NADH-cytochrome *c* reductase were determined by the method of Masters *et al.* (1967). The reaction mixture contained 1.5 mg

of microsomal protein, 150 μmol of phosphate buffer, pH 7.4, and 150 μmol of ferricytochrome *c* in 2.8 ml. The reaction was started by the addition of 100 μmol of NADPH or 100 μmol of NADH. To examine the effects of test compounds on this reaction *in vitro*, microsomal preparations were preincubated with the above compounds for 15 min at 37°C.

Results and Discussion

Preincubation of biphenyl 2-hydroxylase with safrole and 3,4-benzopyrene in various species

Significant differences in the ability to hydroxylate biphenyl in the 2- and 4-positions have been established in mature animals of a number of species (Creaven *et al.*, 1965). The adult hamster and mouse were reported to show a greater ability than the rat to metabolize biphenyl in the 2-position. However, in the neonatal rat the activity of biphenyl 2-hydroxylase is relatively high and decreases with increasing age (Basu *et al.*, 1971). However, this unusual age-dependence of this enzyme seen in rat is not exhibited by the hamster or mouse, which show the expected pattern of increased enzyme activity with increased age (see Table 1). These differences are not associated with age-related changes in cofactor requirements or

Table 1. *Effects of age on biphenyl 2- and 4-hydroxylase activity in rats, hamsters and mice*

Results are given as the means $\pm$ S.D. for six animals. For details of experimental procedure see the Materials and Methods section.

Species	Biphenyl 2-hydroxylase (nmol/min per mg of microsomal protein)		Biphenyl 4-hydroxylase (nmol/min per mg of microsomal protein)	
	17 days old	60 days old	17 days old	60 days old
Rat (Wistar albino)	0.51 ± 0.08	0.31 ± 0.07	0.8 ± 0.14	2.4 ± 0.41
Hamster (Syrian)	0.42 ± 0.06	0.61 ± 0.04	2.1 ± 0.2	2.7 ± 0.2
Mouse (strain A/2, white)	0.64 ± 0.09	0.92 ± 0.10	1.0 ± 0.1	1.9 ± 0.1

Table 2. *Effects of preincubation of hepatic microsomal preparations of different species with carcinogenic and non-carcinogenic compounds on biphenyl 2-hydroxylase activity*

Results for mature Wistar albino rats, strain A/2 white mice and Syrian hamsters are expressed as nmol/min per mg of protein. The means $\pm$ S.D. for six animals of each species, with percentage changes relative to control values in parentheses, are given. For details of the experimental procedure see the Materials and Methods section.

Treatment	Rat	Mouse	Hamster
Control	0.31 ± 0.02	0.62 ± 0.03	0.65 ± 0.02
3,4-Benzopyrene	1.03 ± 0.04 (+232)*	2.35 ± 0.13 (+280)*	2.40 ± 0.17 (+270)*
3-Methylcholanthrene	0.70 ± 0.03 (+125)*	1.36 ± 0.11 (+120)*	1.33 ± 0.11 (+105)*
Safrole	0.56 ± 0.03 (+82)*	0.99 ± 0.09 (+61)*	0.94 ± 0.13 (+52)*
Isosafrole	0.50 ± 0.04 (+61)*	0.97 ± 0.10 (+59)*	1.06 ± 0.09 (+64)*
Phenobarbitone	0.33 ± 0.02 (+6)	0.54 ± 0.08 (-12)	0.69 ± 0.03 (+11)
Nikethamide	0.27 ± 0.03 (-12)	0.60 ± 0.06 (-3)	0.56 ± 0.06 (-10)
1,2,3,4-Dibenzopyrene	0.35 ± 0.03 (+12)	0.64 ± 0.09 (+4)	0.67 ± 0.05 (+8)

* Values significantly different from controls; $P < 0.05$.

Fig. 1. Effects of a single intraperitoneal dose of safrole administered to male adult Wistar albino rats

Results are shown for biphenyl 2-hydroxylase (Δ), biphenyl 4-hydroxylase ($\blacktriangle$), aniline hydroxylase ($\circ$), cytochrome P-450 ($\blacksquare$) and cytochrome b_5 ($\bullet$) after intraperitoneal administration of safrole (75 mg/kg body wt.). Mean percentage increases in enzyme activity for six animals are shown. Significant increases in enzyme activity ($P < 0.05$) were detectable after 4h only for biphenyl 2-hydroxylase.

enzyme stability, since an investigation of the required optimal concentrations of NADP^+ , Mg^{2+} , glucose 6-phosphate, glucose 6-phosphate dehydrogenase and substrate, the rate of product formation for different periods of incubation and the enzyme stability after storage at 2°C , showed no significant change with age in any of these species.

These changes of biphenyl 2-hydroxylation with age in the rat might be interpreted as a conversion of active biphenyl 2-hydroxylase into a latent form within the endoplasmic reticulum. On this basis its enhancement *in vitro* would be explained by re-activation. However, since the hamster and mouse, which do not exhibit this unusual age-dependency, are similar to the rat in that their hepatic microsomal preparations show comparable enhancement of 2-hydroxylase activity after addition of benzopyrene, 3-methylcholanthrene, safrole or isosafrole *in vitro* (see Table 2), it is unlikely that this explanation is valid.

Correlation of the enhancement of biphenyl 2-hydroxylase *in vitro* and *in vivo*

The enhancement of biphenyl 2-hydroxylase has

been shown to occur *in vitro* after 10 min preincubation with benzopyrene or 3-methylcholanthrene (Bridges *et al.*, 1973), but after intraperitoneal administration of similar aromatic-hydrocarbon carcinogens induction of biphenyl 2-hydroxylase activity has been shown to occur only after 24 h (Creaven & Parke, 1966). In an attempt to correlate these two observations, benzopyrene, safrole or phenobarbitone was administered intraperitoneally to rats, the animals were killed at intervals between 15 min and 48 h after administration and the activities of biphenyl 2- and 4-hydroxylase and other related enzymes determined.

Fig. 1 shows that after the intraperitoneal administration of safrole, increases in biphenyl 2-hydroxylation were detectable after 2 h, whereas biphenyl 4-hydroxylation was even inhibited. The activity of biphenyl 2-hydroxylase increased to reach a maximum at about 4–6 h after administration, falling again to basal values after 10 h. A second phase of increased biphenyl 2-hydroxylase activity occurred after 24 h, at which time increases in cytochrome P-450 and other parameters of drug metabolism were also seen. The initial inhibitions observed with biphenyl 4-hydroxylation and aniline hydroxylation are probably due to inhibition by safrole of general cytochrome P-450-dependent enzymic activity (Elcombe *et al.*, 1975).

Administration of 3-methylcholanthrene and 3,4-benzpyrene produced similar effects to safrole; the effects of 3,4-benzopyrene are shown in Table 3. Animals killed 2 h after administration of the carcinogen

Fig. 2. Effects of a single intraperitoneal dose of phenobarbitone administered to male Wistar albino rats

Results shown are for biphenyl 2-hydroxylase (Δ), biphenyl 4-hydroxylase ($\blacktriangle$), aniline hydroxylase ($\circ$), cytochrome P-450 ($\blacksquare$) and cytochrome b_5 ($\bullet$). Mean percentage increases in enzyme activities for six animals are shown. No significant increases in enzyme activity were detectable after 4 h. The dose of phenobarbitone was 100 mg/kg body wt.

Table 3. *Effects of benzopyrene administration to male Wistar albino rats on the activity of hepatic microsomal biphenyl 2-hydroxylase in vitro*

A single dose of 3,4-benzopyrene (75 mg/kg body wt.) or of 3,4-benzopyrene (20 mg/kg body wt.) plus actinomycin D (1 mg/kg body wt.) or two doses of 3,4-benzopyrene (75 mg/kg body wt.), the second administered 6 h after the first, were administered intraperitoneally in ground-nut oil. Results shown are the means $\pm$ S.D. for six animals, expressed as nmol of 2-hydroxybiphenyl/min per mg of microsomal protein; percentage changes relative to control values are in parentheses.

Time after administration (h)	3,4-Benzopyrene (one dose)	3,4-Benzopyrene (two doses)	3,4-Benzopyrene + actinomycin D
0	0.23 $\pm$ 0.02 (100)	—	0.24 $\pm$ 0.03 (100)
0.25	0.37 $\pm$ 0.01 (161)*	—	0.36 $\pm$ 0.02 (150)*
2	0.51 $\pm$ 0.03 (221)*	—	0.52 $\pm$ 0.04 (216)*
4	0.80 $\pm$ 0.04 (347)*	—	0.82 $\pm$ 0.05 (341)*
6	0.44 $\pm$ 0.03 (191)*	0.42 $\pm$ 0.02 (182)*	0.43 $\pm$ 0.04 (179)*
8	0.28 $\pm$ 0.04 (121)†	0.29 $\pm$ 0.01 (126)*	0.28 $\pm$ 0.02 (116)
24	0.45 $\pm$ 0.03 (196)*	0.49 $\pm$ 0.02 (213)*	0.32 $\pm$ 0.04 (133)*
40	0.51 $\pm$ 0.04 (221)*	0.57 $\pm$ 0.04 (247)*	0.37 $\pm$ 0.03 (154)*
48	0.65 $\pm$ 0.02 (282)*	—	0.39 $\pm$ 0.04 (162)*

* Values significantly different from controls; $P < 0.05$.

† Addition of 1 mM-3,4-benzopyrene *in vitro* did not further enhance the biphenyl 2-hydroxylase activity.

Table 4. *Changes in rat hepatic microsomal biphenyl 2- and 4-hydroxylase activity after preincubation and after intraperitoneal administration of 3,4-benzopyrene, 3-methylcholanthrene, safrole or phenobarbitone*

Results are expressed as percentage increase of enzyme activity $\pm$ S.D. for six animals, observed after 10 min preincubation or 2 h after intraperitoneal administration. For details of the experimental procedure see the text.

Treatment	Biphenyl 2-hydroxylase activity		Biphenyl 4-hydroxylase activity	
	10 min preincubation <i>in vitro</i>	2 h after intraperitoneal administration	10 min preincubation <i>in vitro</i>	2 h after intraperitoneal administration
3,4-Benzopyrene	+253 $\pm$ 23*	+240 $\pm$ 19*	-9 $\pm$ 6	-17 $\pm$ 12
3-Methylcholanthrene	+125 $\pm$ 11*	+137 $\pm$ 21*	-4 $\pm$ 7	-11 $\pm$ 7
Safrole	+82 $\pm$ 7*	+80 $\pm$ 14*	-34 $\pm$ 6*	-37 $\pm$ 11*
Phenobarbitone	-7 $\pm$ 9	-6 $\pm$ 7	-8 $\pm$ 4	+3 $\pm$ 6

* Values significantly different from controls; $P < 0.05$.

showed even greater increases in biphenyl 2-hydroxylase than occurred after treatment with safrole. These findings are in contrast with the effects on drug-metabolizing-enzyme activity observed after treatment with phenobarbitone (see Fig. 2), which produced no significant increases in activity of biphenyl 2-hydroxylase or any other drug-metabolizing enzymes during the first 8 h after administration.

This very rapid initial increase of biphenyl 2-hydroxylase activity after pretreatment with safrole, 3,4-benzopyrene or 3-methylcholanthrene *in vivo* would tend to preclude protein synthesis *de novo* as the cause of enhancement of this enzyme activity. However, the secondary phase of increases in enzyme activities, after 24 h, is probably due to enzyme synthesis *de novo*, since both 2- and 4-hydroxylation are increased. Further, the time-profile of induction is similar to that observed with other enzymes after 3-methylcholanthrene treatment, which has been

unambiguously attributed to protein synthesis *de novo*. In support of this interpretation, the second phase of biphenyl 2-hydroxylase stimulation, but not the initial phase, was largely prevented by administration of actinomycin D (see Table 3).

The extent of enhancement of biphenyl 2-hydroxylase in the early phase of stimulation by safrole and polycyclic carcinogens *in vivo* shows good correlation with that found when these compounds are added to hepatic microsomal preparations containing an NADPH-regenerating system (see Table 4). In contrast, addition of phenobarbitone *in vitro* produced no significant changes (see Tables 2 and 4).

An investigation of the changes in K_m and V_{max} , that take place during this initial increase of biphenyl 2-hydroxylase activity mediated by 3,4-benzopyrene indicates that this initial increase in enzyme activity is due both to a lowering of the K_m and to an increase in V_{max} . (normal values, K_m 0.51 mM, V_{max} 2.2 nmol/

min per mg of microsomal protein; 4h values, K_m 21 μM , V_{max} 7.6 nmol/min per mg of microsomal protein), whereas after 8h the K_m and V_{max} values have almost returned to basal values (see Table 5). When a second administration of benzopyrene was given 6h after the first dose no further stimulation of biphenyl 2-hydroxylation occurred, indicating that the gradual loss of activity of this enzyme 6h after the initial dose is an irreversible effect.

Preincubation of hepatic microsomal preparations with various xenobiotics and their effects on parameters of drug metabolism

Anders (1968) has demonstrated that acetone and various other compounds markedly enhance aniline hydroxylase when added directly to incubation mixtures. To determine whether these compounds enhance biphenyl 2-hydroxylase, acetone (0.25–0.5 mM)

or metyrapone (0.1–5.0 mM) was added to rat hepatic microsomal preparations *in vitro*. In contrast with benzopyrene or safrole, acetone or metyrapone produced inhibition of biphenyl 2-hydroxylase and biphenyl 4-hydroxylase at all concentrations, the latter being inhibited to a greater extent (see Table 6). In agreement with the work cited above, enhancement of aniline hydroxylase was apparent only with high concentrations of acetone or metyrapone added *in vitro*. These findings indicate that the enhancement of biphenyl 2-hydroxylation by addition of benzopyrene or safrole *in vitro* is a different phenomenon from that observed for the *p*-hydroxylation of aniline after addition of acetone or metyrapone (Table 6). Addition of benzopyrene to rat hepatic microsomal preparations *in vitro* had no effect on a number of other drug-metabolizing enzyme systems, e.g. ethylmorphine *N*-demethylase, 4-chloromethylaniline demethylase

Table 5. Comparison of the effects of benzopyrene, *in vitro* and *in vivo*, on rat hepatic microsomal biphenyl 2-hydroxylation

The effects of addition of 1 mM-3,4-benzopyrene to control male Wistar rat hepatic microsomes and the administration of 3,4-benzopyrene intraperitoneally (75 mg/kg body wt.) to rats on K_m and V_{max} values for biphenyl 2-hydroxylase. The results are expressed as mean values $\pm$ s.d. for six animals.

Time after administration (h)	Increase in biphenyl 2-hydroxylase activity (%)	$10^4 \times K_m$ (M)	V_{max} (nmol/min per mg of microsomal protein)
0	0	5.1 ± 2.1	2.2 ± 1.4
4	212	0.21 ± 0.6	7.6 ± 2.1
8	33	3.9 ± 1.2	3.2 ± 2.3
24	63	1.4 ± 0.9	0.8 ± 0.7
Benzopyrene addition <i>in vitro</i>	250	0.14 ± 0.2	6.7 ± 0.6
Solvent addition <i>in vitro</i>	0	4.6 ± 1.4	2.2 ± 0.3

Table 6. Effect of addition of metyrapone and acetone *in vitro* on rat hepatic microsomal biphenyl 2- and 4-hydroxylase and aniline hydroxylase

Results are expressed as mean values $\pm$ s.d. (nmol/min per mg of microsomal protein) for six animals, with percentage changes relative to the control values in parentheses.

Test compound	Hydroxylation (nmol/min per mg of microsomal protein)		
	Biphenyl 2-hydroxylation	Biphenyl 4-hydroxylation	Aniline hydroxylation
Acetone			
Control	0.23 ± 0.03	2.14 ± 0.12	1.8 ± 0.05
0.25 mM	0.20 ± 0.01 (–13)	1.64 ± 0.11 (–25)*	1.6 ± 0.04 (–12)
0.20 M	0.17 ± 0.02 (–27)*	1.01 ± 0.09 (–53)*	2.1 ± 0.05 (+16)*
0.50 M	0.15 ± 0.04 (–35)*	0.82 ± 0.04 (–62)*	2.8 ± 0.05 (+55)*
Metyrapone			
Control	0.24 ± 0.02	2.27 ± 0.14	1.8 ± 0.07
0.1 mM	0.19 ± 0.01 (–21)*	1.44 ± 0.14 (–37)*	1.30 ± 0.09 (–28)*
2.0 mM	0.12 ± 0.01 (–50)*	0.71 ± 0.09 (–69)*	0.80 ± 0.08 (–66)*
5.0 mM†	0.08 ± 0.03 (–66)*	0.38 ± 0.07 (–84)*	2.6 ± 0.09 (+44)*

* Values significantly different from controls; $P < 0.05$.

† Results for only three animals.

Table 7. Effects of preincubation with carcinogenic and non-carcinogenic compounds on parameters of microsomal hepatic drug metabolism in adult male Syrian hamsters and Wistar albino rats

Enzyme	Syrian hamster			Rat		
	Control	3,4-Benzopyrene	Phenobarbitone	Control	3,4-Benzopyrene	Phenobarbitone
Biphenyl 2-hydroxylase	0.39 ± 0.03	1.14 ± 0.16 (+192)	0.38 ± 0.02 (-3)	0.23 ± 0.02	0.64 ± 0.03 (+178)	0.20 ± 0.02 (-14)
Biphenyl 4-hydroxylase	4.2 ± 0.18	3.9 ± 0.15 (-8)	4.3 ± 0.06 (+2)	2.02 ± 0.11	2.00 ± 0.10 (-1)	2.03 ± 0.10 (0)
Aniline hydroxylase	1.9 ± 0.05	1.8 ± 0.07 (-5)	1.7 ± 0.05 (-11)	1.6 ± 0.06	1.4 ± 0.01 (-12)	1.7 ± 0.09 (+6)
4-Chloromethylaniline demethylase	3.7 ± 0.21	3.8 ± 0.22 (+3)	3.7 ± 0.24 (0)	4.6 ± 0.16	4.1 ± 0.22 (-11)	4.1 ± 0.24 (-11)
Ethylmorphine N-demethylase*	2.8 ± 0.11	2.8 ± 0.10 (0)	2.9 ± 0.17 (+3)	3.1 ± 0.12	3.2 ± 0.09 (+3)	2.9 ± 0.18 (-6)
Nitroreductase	4.1 ± 0.09	3.4 ± 0.08 (-18)	4.0 ± 0.09 (-3)	3.2 ± 0.07	3.1 ± 0.11 (-3)	3.2 ± 0.09 (0)
Benzopyrene hydroxylase	0.012 ± 0.002	—	0.014 ± 0.003 (+16)	0.09 ± 0.003	—	0.08 ± 0.001 (-12)
Ethoxycoumarin de-ethylase	0.44 ± 0.02	0.41 ± 0.02 (-5)	0.42 ± 0.01 (-4)	—	—	—
Ethoxyresorufin de-ethylase	0.02 ± 0.01	0.02 ± 0.01 (0)	0.02 ± 0.01 (0)	—	—	—
Cytochrome P-450†	1.71 ± 0.04	1.70 ± 0.04 (-1)	1.72 ± 0.09 (+1)	1.34 ± 0.03	1.32 ± 0.19 (-1)	1.37 ± 0.07 (+2)
Cytochrome b ₅ †	0.61 ± 0.03	0.62 ± 0.04 (+2)	0.62 ± 0.07 (+2)	0.82 ± 0.06	0.80 ± 0.06 (-3)	0.81 ± 0.09 (-1)
Cytochrome P-450 reductase	7.6 ± 0.42	6.1 ± 0.32 (-20)	9.0 ± 0.62 (+18)	380 ± 22	372 ± 14 (-2)	396 ± 11 (+5)
NADH-cytochrome c reductase	371 ± 19	368 ± 24 (-1)	379 ± 32 (+2)	5.1 ± 0.23	4.8 ± 0.19 (-6)	6.2 ± 0.21 (+22)
NADPH-cytochrome c reductase	84 ± 9	87 ± 11 (+3)	86 ± 7 (+2)	—	—	—

Results (means ± S.D. for six animals) are expressed as nmol of product/min per mg of microsomal protein, nmol of formaldehyde/min per mg of microsomal protein* or nmol of enzyme/mg of microsomal protein,† and comparisons with control values are shown in parentheses.

and nitro-reductase, and indicated that the observed enhancement of biphenyl 2-hydroxylation *in vitro* is a specific phenomenon for this mixed-function oxidase (see Table 7).

It is particularly worthy of note that cytochrome P-450, NADPH-cytochrome P-450 reductase and NADPH-cytochrome c reductase were not significantly affected by preincubation with benzopyrene or phenobarbitone *in vitro*. There was not any enhancement of the metabolism of substrates such as aniline or ethoxyresorufin, which are known to be induced by pretreatment with benzopyrene *in vivo*.

From these results it seems likely that the enhancement of biphenyl 2-hydroxylase after preincubation with chemical carcinogens, but not non-carcinogens, is not caused by a modification or replacement of the normal cytochrome P-450 by cytochrome P-448, which is a well-known phenomenon (Masters *et al.*, 1973; Waterman *et al.*, 1973). A possible reason for the enhancement of biphenyl 2-hydroxylase *in vitro* might be the isoenzymic conversion of biphenyl 4-hydroxylase into biphenyl 2-hydroxylase (Jaffe *et al.*, 1969). However, our results indicate that such a mechanism is most unlikely, because enhancement of biphenyl 2-hydroxylase by many carcinogens has been shown to occur without a concomitant decrease in 4-hydroxylation (McPherson *et al.*, 1974a). With methylenedioxyphenyl compounds a significant inhibition of biphenyl 4-hydroxylase occurs, but this is due to the binding of a metabolite to cytochrome P-450, with concomitant loss of its enzymic activity (Elcombe *et al.*, 1975).

A more likely explanation of our findings is that biphenyl 2-hydroxylase normally exists in an active and latent form in the endoplasmic reticulum. The form active in control animals probably involves cytochrome P-450, but the latent form, which is rapidly activated by the carcinogen or its metabolite, may be independent of normal cytochrome P-450, since enhanced activity is not shown for the metabolism of any of the other typical cytochrome P-450 substrates investigated. The results with safrole and 3,4-benzopyrene administered *in vivo* imply that activation of the 2-hydroxylase is irreversible, since although the K_m and V_{max} values return almost to normal at 8 h after pretreatment they cannot be 're-activated' by a second injection of benzopyrene 6 h after the first dose, or by addition of benzopyrene to the microsomal preparation obtained 8 h after benzopyrene administration *in vivo* (see Table 3). These observations would appear to be consistent with a relatively specific change in the physical state of the endoplasmic reticulum, which is dependent on the interaction of carcinogens or their metabolites with this membrane. This may further suggest that carcinogens, or their reactive metabolites, may elicit a change in the endoplasmic reticulum that is fundamental to the phenomenon of carcinogenesis.

References

- Anders, M. W. (1968) *Arch. Biochem. Biophys.* **126**, 269–278
- Anders, M. W. (1971) *Annu. Rev. Pharmacol.* **11**, 37–56
- Basu, T. K., Dickerson, J. W. T. & Parke, D. V. (1971) *Biochem. J.* **124**, 19–24
- Bridges, J. W., McPherson, F. J., King, L. J. & Parke, D. V. (1973) *Proc. Eur. Soc. Study Drug Toxicol.* **15**, 98–104
- Buhler, D. R. & Rasmussen, A. (1968) *Comp. Biochem. Physiol.* **25**, 223–231
- Burke, M. D. (1973) Ph.D. Thesis, University of Surrey
- Burke, M. D. & Mayer, R. (1974) *Drug Metab. Dispos.* **2**, 583–588
- Creaven, P. J. & Parke, D. V. (1966) *Biochem. Pharmacol.* **15**, 7–16
- Creaven, P. J., Parke, D. V. & Williams, R. T. (1965) *Biochem. J.* **96**, 879–885
- Dickerson, J. W. T. & Basu, T. K. (1975) in *Enzyme Induction* (Parke, D. V., ed.), pp. 27–37, Plenum Press, London
- Elcombe, C. R., Bridges, J. W., Gray, T. J. B., Nimmo-Smith, R. H. & Netter, K. J. (1975) *Biochem. Pharmacol.* **24**, 1427–1433
- Gigon, P. L., Gram, T. E. & Gillette, J. R. (1969) *Mol. Pharmacol.* **5**, 109–122
- Gingell, R. (1970) Ph.D. Thesis, University of London
- Imai, Y. & Sato, R. (1966) *Biochem. Biophys. Res. Commun.* **25**, 80–86
- Jaffe, H., Fujii, K., Guerin, H., Sengupta, M. & Epstein, S. S. (1969) *Biochem. Pharmacol.* **18**, 1045–1051
- Korten, K. & Van Dyke, R. A. (1973) *Biochem. Pharmacol.* **22**, 2105–2112
- Kuntzman, R., Mark, L. C., Brand, L., Jacobson, M., Levin, W. & Conney, A. H. (1966) *J. Pharmacol. Exp. Ther.* **152**, 151–156
- Kupfer, D. & Bruggeman, L. L. (1966) *Anal. Biochem.* **17**, 502–512
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Masters, B. S. S., Williams, C. H. & Kamin, H. (1967) *Methods Enzymol.* **10**, 565–573
- Masters, B. S. S., Nelson, E. B., Schacter, B. A., Baron, J. & Isaacson, E. L. (1973) *Drug Metab. Dispos.* **1**, 121–128
- McPherson, F. J., Bridges, J. W. & Parke, D. V. (1974a) *Biochem. Soc. Trans.* **2**, 618–619
- McPherson, F. J., Bridges, J. W. & Parke, D. V. (1974b) *Nature (London)* **252**, 488–489
- Nakanishi, S., Masamura, E., Tsukada, M. & Matsumura, R. (1971) *Jpn. J. Pharmacol.* **21**, 303–309
- Netter, K. J. & Seidel, G. (1964) *J. Pharmacol. Exp. Ther.* **146**, 61–65
- Netter, K. J., Kahl, G. F. & Magnusson, K. (1969) *Naunyn-Schmeideberg's Arch. Pharmacol. Exp. Pathol.* **265**, 205–215
- Parke, D. V. (1975) in *Enzyme Induction* (Parke, D. V., ed.), pp. 207–271, Plenum Press, London
- Parke, D. V. & Rahman, H. (1970) *Biochem. J.* **119**, 53P
- Ullrich, V. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 357–365
- Ullrich, V. & Weber, P. (1972) *Hoppe-Seyler's Physiol. Chem.* **353**, 1171–1177
- Waterman, M. R., Ullrich, V. & Estabrook, R. W. (1973) *Arch. Biochem. Biophys.* **155**, 355–360