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A COMPARISON OF BIPHENYL 4-HYDROXYLATION AND 4-METHOXYBIPHENYL *O*-DEMETHYLATION IN RAT LIVER MICROSOMES

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Abstract—The conversion of biphenyl and 4-methoxybiphenyl to 4-hydroxybiphenyl in rat liver microsomes has been compared. Both reactions are mediated by the microsomal monooxygenase system and both substrates bind to cytochrome P-450 to give a Type I binding spectrum, although the maximal and minimal wavelengths differ between the 2 substrates. The differential effect of enzyme inducers, metyrapone inhibition and ethanol inhibition upon biphenyl 4-hydroxylation and 4-MBP *O*-demethylation strongly suggest that these two reactions are mediated by different cytochrome P-450 haemoproteins.

The microsomal monooxygenase (MMO)* system of mammalian liver has a major responsibility for the Phase I metabolism of a wide variety of drugs and other xenobiotics. The MMO system is centred around the haemoprotein cytochrome P-450 and recent evidence has demonstrated that this is in fact a family of haemoproteins, each haemoprotein having differing substrate specificities [1, 2].

In the course of studies into the interrelation of Phase I and Phase II metabolism there arose a need for a pair of substrates which are metabolized by different cytochrome P-450 haemoproteins to yield the same product (i.e. Phase I metabolite). A study of the literature suggested that biphenyl (BP) and 4-methoxybiphenyl (4-MBP) might be a suitable substrate pair since each is metabolized by the MMO system to 4-hydroxybiphenyl (4-OHBP) by hydroxylation and *O*-demethylation respectively. In addition, BP 4-hydroxylation has been reported to be well induced by phenobarbitone (PB) and polycyclic aromatic hydrocarbons [3, 4] whereas 4-MBP *O*-demethylation has been reported to be well induced by PB but is not induced by polycyclic aromatic hydrocarbons [5], this suggesting the involvement of different cytochrome P-450 types in the metabolism of these two substrates to 4-OHBP.

However, no direct comparison of these two enzymes has previously been made and thus a comparison has now been made of the responses of these two enzymes to the effects of various inducers and inhibitors in order to establish if these two reactions are mediated by different cytochrome P-450 types.

* Abbreviations used: ANF, α-naphthoflavone; AP, aminopyrine; ATN, apparent turnover number; BNF, β-naphthoflavone; BP, biphenyl; DMF, *N*,*N*^{1}-dimethylformamide; 2-OHBP, 2-hydroxybiphenyl; 4-OHBP, 4-hydroxybiphenyl; 4-MBP, 4-methoxybiphenyl; MMO, microsomal monooxygenase; PB, phenobarbitone.

MATERIALS AND METHODS

Animals and pretreatment. Male Wistar albino rats (weight range 80–150 g) were obtained from the University of Nottingham Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K. Within 24 hr after birth the animals (together with their mothers until weaning) were bedded on synthetic bedding (Lablit, W. P. Usher, London) so as to minimize any possible enzyme induction due to components of wood-based bedding. The animals were allowed free access to standard laboratory diet (diet 41B, Wherry and Son, Bourne, U.K.) and tap water at all times. For the induction studies the animals were injected intra-peritoneally either with phenobarbitone dissolved in isotonic saline at 80 mg/kg, once daily for 3 days the last dose being approx 24 hr prior to sacrifice, or with one dose of β-naphthoflavone (BNF) dissolved in Arachis oil at 80 mg/kg given 42–44 hr prior to sacrifice.

Chemicals. BP, 2-OHBP, 4-OHBP and sodium PB were obtained from BDH Chemicals (Poole, U.K.). Aminopyrine (AP), dimethylformamide (DMF), bovine serum albumin (Fraction V), menadione, glucose-6-phosphate dehydrogenase (Type XI), glucose-6-phosphate (monosodium salt), NADP (monosodium salt) and NADH (disodium salt) were obtained from Sigma London Chemical Co. (Poole, U.K.). Naphthoflavones were purchased from Aldrich Chemical Co. (Wembley, U.K.) and 4-MBP (*p*-phenylanisole) was purchased from Eastman-Kodak Ltd. (Kirkby, U.K.). Metyrapone was a generous gift of Ciba Laboratories Ltd. (Horsham, U.K.). All other chemicals were of analytical grade.

Preparation of microsomes. Animals were killed by cervical dislocation between 9.00–10.00 a.m. so as to minimize any diurnal variation in enzyme activity. The livers were rapidly removed and blotted dry, and a 1 in 4 homogenate prepared by homogenization at 4° in 1.15 per cent (w/v) KCl using a

motor-driven Potter-Elvejhem homogenizer operating at 1500 rpm. The homogenate was centrifuged at 10,000 g_{av} for 20 min and a microsomal fraction was obtained from the 10,000 g supernatant by isoelectric precipitation (for enzyme analysis) or differential centrifugation (for binding studies) as outlined previously [6, 7].

Enzyme assays. The basic enzyme incubation mixture contained, in a total volume of 1.0 ml, microsomal suspension (0.1 ml, containing 1–2 mg protein), 100 mM phosphate buffer pH 7.4, 500 μg albumin, 1 U glucose-6-phosphate dehydrogenase, 0.5 mM NADP, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$ and substrate (5 mM AP, 1 mM BP, 1 mM 4-MBP). The AP was added as an aqueous solution whilst the BP and 4-MBP were added in 5 μl DMF. The incubation mixture (-substrate) was pre-incubated at 37° for 2 min prior to addition of the substrate and incubation for a further 10 min.

For AP *N*-demethylation the reaction was terminated by the addition of 10 per cent TCA (1 ml) and the reaction product, formaldehyde, was measured on a sample of the TCA-supernatant by use of the Nash reagent [8].

For BP hydroxylation and 4-MBP *O*-demethylation, the reaction was terminated by the addition of 4 N HCl (0.25 ml). The acidified incubation mixture was extracted with 4 ml *n*-heptane containing 1.5 per cent (v/v) *i*-amyl alcohol (30 min) and, after centrifugation, a sample (3 ml) of the heptane layer was back extracted with 0.1 N NaOH (4 ml). Fluorescence of the 4-OH BP present in the alkali layer was measured at pH 13 for 4-MBP *O*-demethylation (λ_{ex} 295 nm, λ_{fl} 390 nm), or at pH 6 for BP hydroxylation as outlined by Atlas and Nebert [9].

Inhibitor studies. Metyrapone and ANF were added to the incubation mixture (substrate) in 2 μl DMF and the rest of the assay was performed as outlined above; control incubations received 2 μl DMF. Ethanol was added directly to the incubation mixture (substrate).

Other assays. Cytochrome P-450 was measured by the method of Omura and Sato [10] using an extinction coefficient of 91 $mM^{-1} cm^{-1}$ for $A_{450-490}$ and microsomal protein by the method of Lowry *et al.* [11] using bovine serum albumin as the standard. Substrate-induced binding spectra were recorded by the method of Remmer *et al.* [12] using a PE 550S scanning spectrophotometer. Thin layer chromatography of ether extracts of the incubation mixtures was performed as outlined previously [13].

Statistical evaluation was performed by means of an unpaired Student's *t*-test [14].

RESULTS AND DISCUSSION

Preliminary studies, utilizing fluorescence spectrum and t.l.c. analyses, confirmed that 4-OH BP was the major metabolite of both BP and 4-MBP metabolism mediated by rat liver microsomes (data not shown).

MMO characteristics of BP 4-hydroxylation and 4-MBP O-demethylation in liver microsomes

Omission of NADP (and hence NADPH) in the incubation mixture effectively abolished both enzyme activities as did the presence of menadione, an artificial electron acceptor (Table 1). The presence of carbon monoxide, which avidly binds to the reduced form of cytochrome P-450, greatly inhibited both reactions. The reactions were not mediated by metal ions as evidenced by the lack of significant inhibition by EDTA, but Mg^{2+} was required for optimal activity of both enzymes. These points, taken together, strongly suggested that both BP 4-hydroxylation and 4-MBP *O*-demethylation reactions are mediated by the NADPH-dependent MMO system. Product formation proceeded linearly during the 10 min incubation period for both enzymes and both enzyme activities increased linearly with protein concentration up to 5 mg protein/ml (data not shown).

The activity of some, but not all, enzymes of the MMO system can be increased by the additional presence of NADH in an amount equimolar to that of NADPH [15, 16], and it is generally believed that this indicates the involvement of an NADH-cytochrome b_5 reductase pathway in the supply of the second electron necessary for completion of the reaction sequence [17, 18]. In this context, it is interesting to note that the presence of 0.5 mM NADH in addition to 0.5 mM NADPH produced a large increase in BP 4-hydroxylase activity relative to 0.5 mM NADPH alone (in agreement with other

Table 1. MMO characteristics of BP 4-hydroxylation and 4-MBP *O*-demethylation

Assay conditions	Activity as per cent of control	
	BP 4-hydroxylase	4-MBP *O*-demethylase
Complete system	100	100
−NADP	0	0
+NADH (0.5 mM)	180	107
+Menadione (0.5 mM)	0	0
+EDTA (0.1 mM)	138	82
+CO (bubbled for 1 min)	16	6
$-Mg^{2+}$	83	63

Enzyme incubation and analyses were performed as outlined in the text. All additions or gassing to the basic incubation mixture were performed prior to addition of substrate. Each value is the mean of 2 experiments.

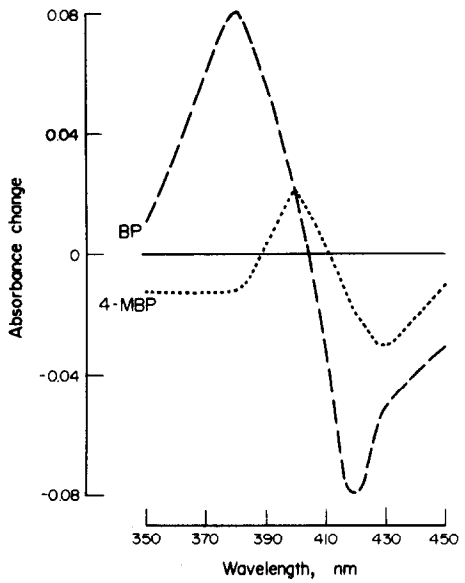

Fig. 1. Spectral changes caused by the addition of BP or 4-MBP to rat liver microsomes. Microsomes were isolated from the livers of PB-treated rats as outlined in the text. Suspensions of microsomes containing 1 mg of protein/ml of buffer were placed in reference and sample cuvettes. Substrate, dissolved in ethanol, was added to the sample cuvette; the final concentrations of the substrates were 80 μM. An equivalent amount of ethanol (10 μl) was added to the reference cuvette. The difference spectrum was then recorded. Graphs are baseline corrected.

studies [4]) but produced no increase in 4-MBP *O*-demethylase activity (Table 1), this possibly indicating the presence of different reaction sequences for the two enzymes. This point was not studied further.

BP and 4-MBP both bound to cytochrome P-450 to give a Type I binding spectrum (Fig. 1) and this is further evidence that BP and 4-MBP are metabolized in rat liver microsomes by MMO-linked systems. However, the appearance of the binding spectra differed in respect of the wavelength maxima and minima, thus suggesting that differences may exist in the binding of these substrates to the cytochrome P-450 haemoprotein(s).

Effect of pretreatment with enzyme inducers on BP 4-hydroxylase and 4-MBP O-demethylase activities in rat liver microsomes

It is established that PB and polycyclic aromatic hydrocarbons (e.g. BNF) are capable of inducing different cytochrome P-450 types each of differing substrate specificity [2, 19]. It was, therefore, of interest to compare the responses of BP 4-hydroxylase and 4-MBP *O*-demethylase to pretreatment with PB or BNF. In addition, AP *N*-demethylase and BP 2-hydroxylase activities were also measured after such pretreatments since these are known to be selectively induced by PB and BNF respectively [4, 19]. The results of this study are shown in Table 2. As expected, cytochrome P-450 content was increased by both inducers whereas AP *N*-demethylase activity was increased only by PB, and BP 2-hydroxylase activity was moderately induced by PB and massively induced by BNF. BP 4-hydroxylase activity was well induced by both PB and BNF, in agreement with previous studies [3, 4]. 4-MBP *O*-demethylase activity, on the other hand, was well induced by PB but only modestly induced by BNF.

It was observed that the extent of PB induction of cytochrome P-450 and BP 2-hydroxylation paralleled each other as did BNF induction of cytochrome P-450 and 4-MBP *O*-demethylation, this suggesting that the increase of these two enzyme activities was due not to altered affinity of the haemoproteins for the substrates but rather to increases in the amount of cytochrome P-450 present. To examine this further the apparent turnover numbers (ATN) for the four enzymes were calculated (Table 2). The ATN for AP *N*-demethylase was not altered by PB treatment but was significantly decreased by BNF treatment. The ATN for BP 2-hydroxylase was also not altered by PB treatment but was massively increased by BNF treatment. These findings are in agreement with those of Alvares and Kappas [19]

Table 2. Effect of pretreatment with PB or BNF on various parameters of the rat liver MMO system

Parameter	Control rats	PB-induced rats	BNF-induced rats
(a) Measured relative to protein content of microsomes			
Cytochrome P-450 (nmole/mg protein)	0.109 ± 0.013	0.349*** ± 0.045 (220 per cent)	0.221*** ± 0.001 (103 per cent)
AP *N*-demethylase†	3.47 ± 0.61	9.97*** ± 0.61 (187 per cent)	3.74 ± 0.26 N.S.
4-MBP *O*-demethylase†	1.40 ± 0.23	6.28*** ± 0.46 (348 per cent)	2.68*** ± 0.12 (91 per cent)
BP 4-hydroxylase†	0.39 ± 0.04	2.78*** ± 0.33 (613 per cent)	2.01*** ± 0.07 (415 per cent)
BP 2-hydroxylase†	0.039 ± 0.004	0.125*** ± 0.011 (220 per cent)	0.416*** ± 0.022 (967 per cent)
(b) Measured relative to cytochrome P-450 content of microsomes (ATN)			
AP N-demethylase‡	26.53 ± 2.77	27.62 ± 1.74 N.S.	17.05** ± 1.12
4-MBP *O*-demethylase‡	12.97 ± 1.22	17.45* ± 0.96	12.43 ± 0.93 N.S.
BP 4-hydroxylase‡	3.64 ± 0.14	7.67*** ± 0.41	9.26*** ± 0.48
BP 2-hydroxylase‡	0.380 ± 0.053	0.344 ± 0.023 N.S.	1.922*** ± 0.136

Enzyme activities are expressed as: †nmole product/mg protein/min; ‡ nmole product/nmole cytochrome P-450/min. Values are mean ±SEM of 8 rats. Figures in brackets indicate per cent increase above control values.

N.S. = not significantly different from controls ($P > 0.05$). Significantly different from controls *$P < 0.02$; **$P < 0.01$; ***$P < 0.001$.

which demonstrate that the ATN for ethylmorphine *N*-demethylase (an enzyme selectively induced by PB treatment) is affected in a like manner to the ATN for AP *N*-demethylase, whilst the ATN for benzpyrene 3-hydroxylase (an enzyme selectively induced by polycyclic aromatic hydrocarbon treatment) is affected in a like manner to the ATN for BP 2-hydroxylase. The ATN for BP 4-hydroxylase was greatly increased by PB and BNF treatment whereas the ATN for 4-MBP *O*-demethylase was significantly increased by PB treatment but was unaffected by BNF treatment. These differences in the effect of inducers on the ATNs for BP 4-hydroxylase and 4-MBP *O*-demethylase support the contention that the increase in 4-MBP *O*-demethylase activity obtained after induction with BNF was due simply to the increase in amount of cytochrome P-450 present whereas the inductions of 4-MBP *O*-demethylase by PB and of BP 4-hydroxylase by PB and BNF were due to induction of cytochrome P-450 species of increased substrate affinity.

These results, taken together, strongly indicate that 4-MBP *O*-demethylase and BP 4-hydroxylase activities are differentially affected by treatment with PB and BNF and, since PB and BNF are known to induce different cytochrome P-450 haemoproteins, it seems reasonable to assume that 4-MBP *O*-demethylation and BP 4-hydroxylation are catalyzed by different cytochrome P-450 haemoproteins.

Effect of metyrapone and ANF on BP 4-hydroxylase and 4-MBP O-demethylase activities

It has previously been shown that metyrapone selectively inhibits PB-inducible reactions whereas ANF selectively inhibits polycyclic aromatic hydrocarbon-inducible reactions [2, 9]. This being so, the effect of *in vitro* addition of various levels of metyrapone and ANF on BP 4-hydroxylation in control rat liver microsomes was investigated. There was some evidence for a differential response of the two enzymes to ANF (10^{-6}–10^{-4} M) but this was not statistically significant ($P > 0.05$). Metyrapone at 10^{-5} and 10^{-4} M concentrations only marginally inhibited both reactions but 10^{-3} M metyrapone inhibited 4-MBP *O*-demethylation by 41.7 ± 1.8 per cent but only marginally inhibited BP 4-hydroxylation (11.0 ± 5.6 per cent); this differential inhibition at 10^{-3} M metyrapone was statistically significant ($P < 0.01$). Metyrapone is believed to be an inhibitor of MMO-linked enzymes by virtue of its binding to the substrate-binding site of cytochrome P-450 [20, 21] rather than by an effect on microsomal electron transport (as with menadione). Therefore the finding that metyrapone differentially inhibits 4-MBP *O*-demethylase activity and not BP 4-hydroxylase activity argues for the involvement of different binding sites for BP and 4-MBP and, by extrapolation, the involvement of different haemoproteins for each reaction.

Effect of ethanol on BP 4-hydroxylase and 4-MBP O-demethylase activities

Ethanol, added *in vitro* to enzyme incubation mixtures, inhibits a number of MMO-linked enzymes, and it is believed to exert its inhibiting effect by interfering with the binding of substrates to cytochrome P-450 [22–25]. Ethanol does not itself appear to bind to cytochrome P-450 and thus the inhibition is likely to be due to perturbation of the lipid environment surrounding the cytochrome [22]. In the present studies, ethanol added directly to the enzyme incubation mixture inhibited both enzyme activities, BP 4-hydroxylase being the more sensitive (Fig. 2). Calculation of the dose of ethanol required to inhibit the enzyme reaction by 50 per cent (I_{50} value) confirmed this differential sensitivity to ethanol. The I_{50} value for BP 4-hydroxylase was 0.180 ± 0.023 M and that for 4-MBP *O*-demethylase was 0.605 ± 0.125 M. If 4-MBP *O*-demethylation and BP 4-hydroxylation were catalyzed by the same haemoprotein(s) it would be expected that each would respond similarly to the membrane-perturbing effects of ethanol. That they do not provide further evidence for the involvement of different haemoproteins in the two reactions.

A number of different approaches viz. binding spectra, induction profiles, metyrapone inhibition and ethanol inhibition, have been used to ascertain

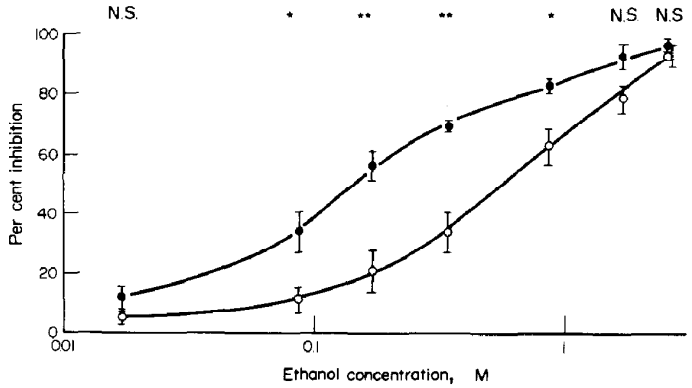

Fig. 2. Ethanol inhibition of BP 4-hydroxylation (●) and 4-MBP *O*-demethylation (○) in control rat liver microsomes. Details of *in vitro* ethanol treatment and enzyme analyses are given in the text. Each point is the mean ± SEM of 4 separate experiments. Symbols, N.S. = mean inhibition values for the 2 enzymes not significantly different at same ethanol concentration ($P > 0.05$); mean inhibition values significantly different from each other at same ethanol concentration *$P < 0.05$; **$P < 0.01$.

if the metabolism of BP and 4-MBP to 4-OHBP by rat liver microsomes are mediated through different P-450 haemoproteins. Whilst conclusive proof of this problem can only be achieved by use of purified cytochrome P-450 fractions the results presented in this study strongly suggest that MMO-linked BP 4-hydroxylation and 4-MBP *O*-demethylation are mediated through different cytochrome P-450 haemoproteins. The availability of a pair of substrates such as BP and 4-MBP which are metabolized by different pathways to yield the same Phase I metabolite should be of value in studies into the interrelation of hepatic Phase I and Phase II metabolism.

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