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HIGH AFFINITY PHENACETIN *O*-DEETHYLASE IS CATALYSED SPECIFICALLY BY CYTOCHROME P450d (P450IA2) IN THE LIVER OF THE RAT

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Abstract—Phenacetin is metabolized primarily by O-deethylation to paracetamol (POD activity), a reaction catalysed by cytochrome P450. The high affinity component of POD activity is inducible in rat liver by treatment of the animals with polycyclic aromatic hydrocarbons. Following treatment with hydrocarbons such as 3-methylcholanthrene (MC) and isosafrole (ISF) both cytochromes P450c (P450IA1) and P450d (P450IA2) are also induced in rat liver. Studies with the reconstituted enzymes have shown that both forms of P450 catalyse phenacetin O-deethylation at rates that exceeded that of the high affinity component of activity of hepatic microsomal preparations from 3-methylcholanthrene-treated rats (at 4 μM phenacetin: P450c, 440 ± 40 pmol/nmol/min; P450d, 1030 ± 10 pmol/nmol/min; microsomal fraction, 163 pmol/mg/min). Specific inhibitory antibodies (both monoclonal and monospecific polyclonal) were used to define the specificity of microsomal POD activity. These studies have shown that hepatic high affinity POD activity is exclusively catalysed by cytochrome P450d in both untreated rats and in rats pretreated with MC.

Polycyclic aromatic hydrocarbon (PAH‖)-inducible forms of cytochrome P450 are products of the cytochrome P450IA gene family, of which two members have been detected in the rat: cytochrome P450IA1 (P450c) and P450IA2 (P450d) [1]. These isoenzymes have been the subject of intensive research, not only because they are inducible by environmental pollutants but also because they are capable of activating otherwise non-toxic compounds into reactive intermediates which can cause toxicity or carcinogenicity [2–4]. There is now good evidence that cytochrome P450d is constitutively expressed, comprising between 4 and 6% of total hepatic cytochrome P450 content, whereas the constitutive levels of P450c are much lower, less than 0.1–1% [2–6]. Both of these isoenzymes are highly and coincidently inducible in the liver of rats by treatment with many toxic xenobiotics such as 3-methylcholanthrene (MC) and polyhalogenated aromatic hydrocarbons [7]. Although no compound has been found to date which exclusively induces only one of these two isoenzymes, MC preferentially induces P450c and isosafrole preferentially induces P450d [3, 8].

From studies of the induction, kinetics and inhibition of cytochromes P450c and P450d, it is apparent that these isoenzymes show different substrate preferences. Whereas in PAH-induced rats cytochrome P450c is predominantly responsible for benzo[*a*]-pyrene hydroxylase (AHH) activity [9, 10], cytochrome P450d is much more active than P450c at converting a number of aromatic amines to mutagenic and carcinogenic intermediates, primarily by N-hydroxylation [11, 12].

The specificity of the isoenzymes of cytochrome P450 can be studied using a number of different approaches, the most common of which involves purification and reconstitution of the individual isoenzymes. However, such studies are limited due to the overlap in substrate specificity that exists amongst the isoenzymes. As a consequence, the rate of metabolism of a compound will be determined by the relative amounts of the different isoenzymes present [8]; for example P450c does not contribute towards AHH activity of untreated rats [10]. In an attempt to overcome this problem, it has been suggested that the overall rate of metabolism of a compound could be predicted from a knowledge of the amount and kinetics of each isoenzyme present [13]. However, an additional difficulty is that the catalytic activity of some forms of P450 is altered during purification and/or reconstitution [14, 15].

As an alternative, the contribution of a given isoenzyme to metabolism could be determined by selectively inhibiting its activity. Antibodies offer the prospect of the greatest specificity, in particular monoclonal antibodies, which react with a single epitope, usually comprising 4–7 amino acid residues [16]. Inhibitory monoclonal antibodies to individual forms of cytochrome P450 should enable the con-

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‖ Abbreviations used: AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase; BSA, bovine serum albumin; DA, dark agouty; EROD, ethoxyresorufin *O*-deethylase; IGSS, immunogold silver stain; ISF, isosafrole; MAb, monoclonal antibody; MC, 3-methylcholanthrene; P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; PB, phenobarbitone; POD, phenacetin *O*-deethylase; PBS, phosphate buffered saline.

tribution of an isoenzyme to any particular monooxygenase activity to be determined. Although the epitope with which a monoclonal antibody reacts will not always be involved in the catalytic activity of the enzyme, such antibodies are often of use in the accurate quantification of a particular isoenzyme in a given tissue.

Phenacetin *O*-deethylase (POD) activity is highly inducible in the liver of rats by treatment of the animals with MC [17]. POD activity in the rat comprises a low affinity component and a high affinity component [18]. Only the high affinity component is induced by PAH treatment of the animals [17]. Cigarette smoking is also an effective inducer of phenacetin O-deethylation, in man as well as in animals [19, 20] and it is high affinity POD activity that is increased in the liver of cigarette smokers [21, 22]. We have now investigated which form(s) of cytochrome P450 catalyses this reaction in rat liver using specific inhibitory antibodies.

MATERIALS AND METHODS

Chemicals. Phenacetin and paracetamol were obtained from BDH Chemicals Ltd (Poole, U.K.) and deuterated [$^2H_3$]paracetamol was synthesized as described by Murray and Boobis [23]. NADPH, *n*-undecane, phenobarbitone (PB), 3-methylcholanthrene (MC) and prestained molecular weight markers were from Sigma Chemical Co. (Poole, U.K.). Isosafrole (ISF) (*cis* and *trans*) was purchased from Eastman-Kodak (Liverpool, U.K.). 3,5-Bistrifluoromethylbenzoyl chloride was supplied by Fluorochem Ltd (Glossop, U.K.). Phosphatidylcholine was from Calbiochem (Cambridge Bio-Science, Cambridge, U.K.). All gel filtration and ion-exchange resins were from Pharmacia (Milton Keynes, U.K.). Hydroxylapatite and all SDS–polyacrylamide gel electrophoresis and Western blotting reagents were from BioRad Laboratories (Watford, U.K.) with the exception of nitrocellulose membrane (Hybond-C) which was obtained from Amersham International plc (Amersham, U.K.) and anti-mouse, horseradish peroxidase conjugated Ig which was obtained from ICN Biomedicals Ltd (High Wycombe, U.K.). All other chemicals were obtained from either Sigma or BDH and were of AnalaR grade or the best alternative.

Treatment of animals and preparation of microsomal fractions. Male Wistar rats (Olac, Bicester, U.K.), weighing between 180–250 g, were maintained under constant heating and lighting cycles. Animals were treated with inducing compounds as follows: MC, 80 mg/kg in corn oil by i.p. injection 48 hr before they were killed; ISF, 160 mg/kg in corn oil by i.p. injection daily for 4 days; PB, 80 mg/kg in saline for 4 days by i.p. injection. Control animals received no treatment. Animals were permitted free access to food (PRD diet, Labsure Animal Products, Poole, U.K.) until 18 hr before death and tap water *ad lib.* Rats were killed by stunning and exsanguination. Livers were removed and the microsomal fraction prepared from individual animals by differential ultracentrifugation as previously described [24]. Samples were stored at −80° in 0.25 M potassium phosphate buffer, pH 7.25 containing 30% (v/v) glycerol and at protein concentrations of above 10 mg/mL. Under these conditions monooxygenase activity of the microsomal fractions was stable for at least 6 months. All samples were used within 3 months of preparation.

Purification of enzymes. Cytochrome P450c was purified from the livers of rats pretreated with MC as previously described [25]. The preparation was apparently homogeneous on SDS–polyacrylamide gel electrophoresis, with specific content of 14 nmol/mg. Cytochrome P450d was purified, from the livers of rats treated with MC, as previously described [26]. The specific content of this preparation was 10 nmol/mg. Purification was monitored by immunoblotting with MAb 12/2/3/2, which reacts only with cytochromes P450c and P450d in the rat. The final preparation of P450d, although not completely homogeneous on SDS–polyacrylamide gel electrophoresis, contained no detectable P450c. Microsomal NADPH-cytochrome *c* (P450) reductase was prepared by affinity chromatography on 2′,5′-ADP-Sepharose 4B by established procedures [27]. The specific activity of this preparation was 9.35 units/mg protein, where 1 unit catalyses the reduction of 1 μmol of cytochrome *c* per min at 25°.

*Preparation of antibodies to cytochromes P*450. All of the antibodies used in the present study have been fully characterized previously. Monoclonal antibodies specific to rat cytochrome P450c were 3/4/2 [28], C8 and C9 [10]. Only C8 was inhibitory. Monoclonal antibody 12/2/3/2 reacted with and was inhibitory to only cytochromes P450c and P450d in the rat [25]. A monospecific, inhibitory polyclonal antibody to rat cytochrome P450d, designated anti-P450d(-c) [2, 3, 29], was also used. Monoclonal antibody 107, specific and inhibitory to rabbit cytochrome P450 form 4 [30, 31], was used as a negative control.

Monoclonal antibodies were prepared in quantity by inoculating female pristane-pretreated Balb/c or Balb/c × CBA F_1 (as appropriate for the MAb) mice (Tuck and Son Ltd, Battlesbridge, U.K.), 15–20 g, with actively growing hybridoma cells and collecting the ascites fluid that ensued. The immunoglobulin (Ig) fraction of the ascites fluid was isolated by precipitation with 50% saturated ammonium sulphate and ion-exchange chromatography by fast protein liquid chromatography using a MonoQ column (Pharmacia, Milton Keynes, U.K.). Antibody was applied in 20 mM triethanolamine–hydrochloride buffer pH 7.7, and eluted with a linear gradient of sodium chloride (0–0.35 M) in the same buffer, at a flow rate of 1 mL/min, over a period of 20 min [26]. The retention times of the antibodies were 8.2, 9.0 and 16.0 for 12/2/3/2, 3/4/2 and 107, respectively. The antibody preparations were more than 95% pure, as determined by SDS–polyacrylamide gel electrophoresis. Pooled antibody fractions were dialysed against phosphate buffered saline (PBS: 10 mM sodium–potassium phosphate buffer pH 7.4, containing 137 mM sodium chloride and 2.6 mM potassium chloride) prior to storage at −80°. The preparation and purification of the MAbs C8 and C9 and the monospecific polyclonal antibody, anti-P450d(-c) were as previously described [2, 3, 10].

Western blotting. Western blotting was performed essentially as described previously [25]. Proteins and prestained molecular weight standards were subjected to SDS–polyacrylamide gel electrophoresis, using 10% polyacrylamide [32] and then transferred to nitrocellulose membrane. The probe monoclonal antibody was used at a concentration of 0.5–1 $\mu g/mL/cm^2$ nitrocellulose membrane in PBS containing 0.1% (w/v) bovine serum albumin (BSA). The detecting antibody, anti-mouse IgG conjugated to horseradish peroxidase, was used at a dilution of 1:2000 in PBS containing 0.1% (w/v) BSA. Peroxidase activity was detected with 4-chloro-1-naphthol and hydrogen peroxide [33].

Phenacetin O-deethylase assay and immunoinhibition. POD activity of hepatic microsomal fractions from rat was determined as previously published [23]. Microsomal protein, between 20–200 μg, was incubated at 37°, in a shaking water-bath, for 10 min in a final volume of 1 mL containing Tris–HCl buffer, pH 7.4 (75 mM), magnesium chloride (3 mM), NADPH (1.0 mM) and crystalline bovine serum albumin, fraction V (0.7 mg). The reaction was started by the addition of recrystallized phenacetin in methanol. Deuterated paracetamol was used as internal standard. The product of the reaction, paracetamol, together with its internal standard, were extracted and derivatized with 3,5-bistrifluoromethylbenzoyl chloride as reported previously [23]. Samples in *n*-undecane were analysed by combined gas chromatography/negative ion chemical ionization mass spectrometry using a Finnigan-MAT 4500 system (Finnigan-MAT, San Jose, CA, U.S.A.) on a fused silica capillary GC column (DB 5, 30 m × 0.27 mm, J&W Scientific, Jones Chromatography Ltd, Llanbradach, U.K.). The limit of detection for the assay was 0.2 ng paracetamol per microsomal incubation. The assay was performed under conditions that were linear with respect to protein concentration and time of incubation.

In reconstitution experiments [34], metabolic activity was determined using saturating amounts of purified NADPH-cytochrome P450 reductase and phosphatidylcholine. Each incubation contained purified cytochrome P450 (20 pmol), NADPH-cytochrome P450 reductase (0.03–0.05 units), phosphatidylcholine (150 μg), sodium deoxycholate (120 μM), magnesium chloride (6 mM), NADPH (1.2 mM), crystalline bovine serum albumin, fraction V (0.7 mg), phenacetin (4–20 μM) and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer (50 mM), pH 7.4, in a final volume of 1 mL. The cytochrome P450, reductase and lipid were preincubated for 2 min at 37° prior to addition of the other reactants in buffer. The reaction was started by the addition of substrate. Samples were incubated at 37° for 10 min in a shaking water-bath.

The effect of antibodies on metabolic activity was determined by incubating purified antibody with microsomal protein for 30 min at room temperature in a total volume of 200 μL of PBS, prior to addition of cofactors in reaction buffer and substrate. In reconstituted systems, purified cytochrome P450 was first incubated with antibody for 5 min at room temperature (22°). Reductase and lipid were then added on ice. This was followed by preincubation of all of the components at 37° for 2 min, after which cofactors were added. Metabolic activity was then assayed as described above, the reaction being started by the addition of substrate. The monoclonal antibodies were used at concentrations in the range 0.1 to 1.0 mg Ig/nmol P450. The polyclonal antibody was studied at 5–20 mg Ig/nmol P450.

Other assays. Protein concentrations were assayed by the method of Lowry *et al.* [35], with crystalline BSA (fraction V) as standard. Total cytochrome P450 content was measured by the method of Omura and Sato [36] assuming an extinction coefficient of 91/mM/cm, between 450 and 480 nm, using a model 555 split-beam scanning spectrophotometer with microprocessor controlled background correction (Perkin-Elmer Ltd, Beaconsfield, U.K.).

Difference spectra were recorded with hepatic microsomal fractions from rats pretreated with isosafrole. Microsomal fractions were diluted to 0.25 mg/ml microsomal protein with 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol. Difference spectra were obtained following reduction of the contents of the sample cuvette with sodium dithionite. The effect of cyclohexane on the difference spectrum of microsomes from ISF-treated rats was determined by incubating diluted microsomal fraction with 10 mM cyclohexane, for 30 min at room temperature. Cyclohexane was removed from the samples by ultracentrifugation at 105,000 *g* for 45 min.

Analysis of the results. Results have been expressed as the mean ± SE, where appropriate. Statistical analysis was performed by Student's *t*-test, for unpaired samples. The null hypothesis was rejected at $P < 0.05$. Kinetic data were analysed by iterative, least squares, non-linear regression analysis, as previously described [18]. Goodness of fit was assessed on the basis of the residual sum of squares and the distribution of residuals.

RESULTS

Microsomal phenacetin O-deethylase activity

Treatment of rats with MC induced hepatic POD activity, determined with 4 μM phenacetin, by some 30-fold, from 41.4 ± 11.0 pmol/mg/min to 1270 ± 241 pmol/mg/min (Table 1) ($P < 0.01$). Treatment of rats with either PB or ISF had no significant effect on POD activity. Whereas the cytochrome P450 content of microsomes from PB-treated rats was significantly ($P < 0.01$) increased, from 0.71 ± 0.04 nmol/mg to 1.15 ± 0.08 nmol/mg, that of ISF-treated rats, at 0.67 ± 0.04 nmol/mg, was not significantly different from the P450 content of control rats (Table 1).

When ISF is administered to rats *in vivo* it binds to cytochrome P450, resulting in a type III spectrum [29], characterized by absorption maxima at 428 and 458 nm (Fig. 1, solid line). Thus, hepatic microsomal fractions from rats pretreated with ISF were preincubated *in vitro* with 10 mM cyclohexane (ISF^+ microsomes) prior to their use in metabolic assays. Such treatment with cyclohexane *in vitro* dissociates, at least partially, the spectral complex of ISF with cytochrome P450. The success of this pretreatment

Table 1. Hepatic microsomal phenacetin *O*-deethylase activity in rats following their treatment with inducing agents

Treatment	N	Cytochrome P450 content (nmol/mg)	Phenacetin *O*-deethylase activity (pmol/mg/min)
Untreated	4	0.71 ± 0.040	41.4 ± 11.0
MC	6	1.25 ± 0.125*	1270 ± 241*
ISF	3	0.67 ± 0.043	55.1 ± 5.7
ISF$^+$	3	1.03 ± 0.041*	113 ± 10.3*
PB	3	1.15 ± 0.083*	33.7 ± 3.4

Phenacetin *O*-deethylase activity was determined at a substrate concentration of 4 μM. Values are means ± SE. N indicates the number of different animals used for each determination.

Cytochrome P450 content was determined from the CO-complex of dithionite reduced microsomes.

Rats were pretreated with 3-methylcholanthrene (MC), isosafrole (ISF) or phenobarbitone (PB) as described in Materials and Methods.

$^+$ Isosafrole microsomes were pretreated with 10 mM cyclohexane as described in the text (Fig. 1) before determination of the parameters.

* $P < 0.01$ compared with corresponding untreated group by Student's *t*-test.

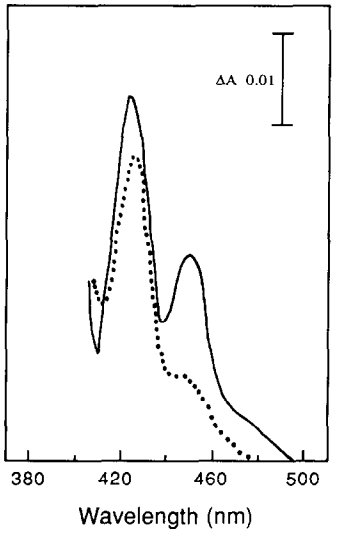

Fig. 1. Isosafrole induced difference spectra of hepatic microsomal fraction from male Wistar rats. Microsomal fraction from the liver of rats pretreated with isosafrole (160 mg/kg) was diluted to a protein concentration of 0.25 mg/mL and the difference spectrum recorded after chemical reduction of the sample in the sample cuvette with sodium dithionite (solid line). The spectrum of an identical microsomal sample was recorded after incubation with 10 mM cyclohexane for 30 min at room temperature (dotted line).

was apparent from the considerable reduction in absorption at 458 nm in the type III spectrum (Fig. 1, dotted line) and from the significant ($P < 0.01$) increase in spectrophotometrically determined cytochrome P450 content of these microsomes, to 1.03 ± 0.04 nmol/mg (Table 1).

POD activity of microsomes from ISF treated rats, preincubated with cyclohexane (ISF$^+$ microsomes), was significantly ($P < 0.01$) greater, at 113 ± 10.3 pmol/mg/min, than that of microsomes from untreated rats, an increase of 2.7-fold. When microsomes from control animals were treated with cyclohexane there was no increase in POD activity (data not shown).

The kinetics of POD activity of microsomal fractions were determined over a substrate concentration range from 0.4 to 1000 μM. Hepatic microsomal POD activity of untreated, MC- and ISF$^+$-treated rats was best described by a 4-parameter model, i.e. POD activity was biphasic [18]. However, with microsomes from rats pretreated with PB the data were fitted best by a 2-parameter model, i.e. monophasic kinetics. The Michaelis–Menten parameters for the different microsomal preparations are shown in Table 2. High affinity POD activity of control animals was characterized by K_m1 of 4.4 ± 1.9 μM and $V_{max}1$ of 76.2 ± 23.2 pmol/mg/min. Treatment of animals with MC resulted in an increase in $V_{max}1$ to 678 ± 139 pmol/mg/min, without any marked change in K_m1. ISF-treatment had no effect on the high affinity component of POD activity. Following cyclohexane treatment of these microsomes, $V_{max}1$ showed a very modest increase. Michaelis–Menten constants for low affinity POD activity of control rats were K_m2: 569 μM and $V_{max}2$: 1760 pmol/mg/min. Following treatment of rats with MC there was a small increase in $V_{max}2$, to 3800 pmol/mg/min. In microsomes from PB-treated rats, no high affinity activity could be detected. V_{max} of the single component of activity was higher, at 3440 pmol/mg/min, than $V_{max}2$ of control microsomes, and K_m of this component, at 207 μM, was lower than K_m2 of untreated animals.

Western blotting and immunoquantification

Treatment of rats with MC, ISF or PB increased the intensity of staining of protein bands in the region of M_r 52,000–58,000, as determined by SDS–polyacrylamide gel electrophoresis of the hepatic microsomal fraction (Fig. 2A). These samples were subjected to Western blotting with MAb 12/2/3/2, which reacts with only cytochromes P450c and P450d

Table 2. Michaelis–Menten parameters for hepatic microsomal phenacetin *O*-deethylase activity of rats following induction

Treatment	Michaelis–Menten parameter			
	$V_{max}1$ (pmol/mg/min)	K_m1 (μM)	$V_{max}2$ (pmol/mg/min)	K_m2 (μM)
Untreated	76.2 ± 23.2	4.44 ± 1.85	1760 ± 490	569 ± 220
MC	678 ± 139	12.6 ± 3.28	3800 ± 100	442 ± 179
ISF	85.5 ± 4.64	4.08 ± 0.84	1800 ± 170	310 ± 45
ISF$^+$	105 ± 35	3.30 ± 1.70	1470 ± 350	125 ± 43
PB	—	—	3440 ± 110	207 ± 42

$^+$ Isosafrole microsomes were incubated with 10 mM cyclohexane *in vitro* as described in the text (Fig. 1).

Data were analysed assuming a four parameter, two component system as previously described [17].

Values are computer estimates, obtained using iterative non-linear regression analysis, for pooled microsomal fractions from the livers of three animals for each experimental group ± the radex errors, an indication of the goodness of fit of the predicted parameters to the observed data [17].

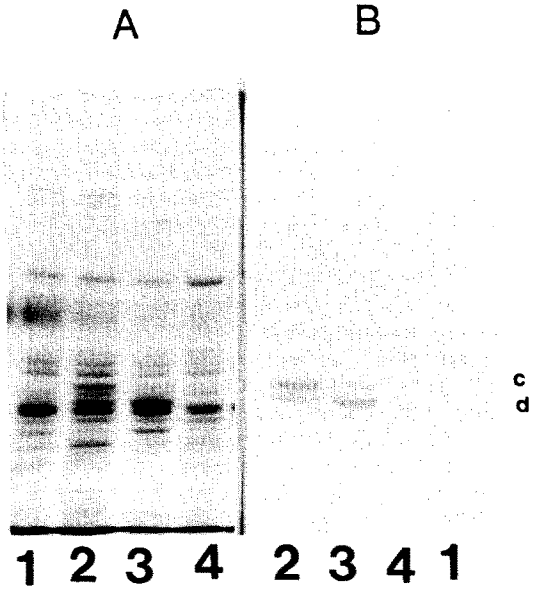

Fig. 2. SDS–polyacrylamide gel electrophoresis (A) and Western blot with MAb 12/2/3/2 (B) of hepatic microsomal fractions from rats treated with various inducers. Microsomal proteins (20 μg) from livers of rats pretreated with phenobarbitone (lane 1); 3-methylcholanthrene (lane 2); isosafrole (lane 3) and from untreated rats (lane 4) were separated on 7.5% SDS–polyacrylamide gels and stained with Coomassie blue [32] (A) or further subjected to Western blotting [25] (B). **c** and **d** stand for cytochrome P450c and P450d, at M_r 57,000 and M_r 54,000, respectively.

in the rat (Fig. 2B). In microsomes from untreated rats, no P450c (M_r 54,000) or P450d (M_r 57,000) could be detected. However, when the detecting antibody was replaced with one conjugated to gold, and silver enhancement was used (IGSS), P450d could be detected in these microsomes, although P450c was still not detectable (data not shown) (specific content of P450c: <0.5 pmol/mg, the limit of detection of the immunoquantification technique, and of P450d: 9.0 ± 1.0 pmol/mg). Treatment of rats with either MC or ISF induced both P450c and P450d (P450c: 356 ± 60 and 106 ± 32 pmol/mg, P450d: 124 ± 22 and 183 ± 42 pmol/mg, following MC and ISF, respectively). Thus, with MC, P450c was induced 3-fold more than P450d, whereas with ISF, P450d was induced 2–3-fold more than P450c. Following treatment with PB, P450d content was decreased to <2 pmol/mg, P450c levels remaining below 0.5 pmol/mg.

Phenacetin O-deethylase activity of reconstituted cytochromes P450

Purified preparations of cytochromes P450c and P450d were reconstituted with NADPH-cytochrome *c* (P450) reductase and lipid and their POD activity determined with 4–20 μM phenacetin. Both PAH-inducible forms of cytochrome P450 were very active in catalysing the O-deethylation of phenacetin, the activity of P450d exceeding that of P450c (P450c: 440 ± 40, 560 ± 37 and 778 ± 43 pmol/nmol/min; P450d: 1030 ± 10, 1160 ± 70 and 1160 ± 60 pmol/nmol/min with 4, 10 and 20 μM phenacetin, respectively. Results are means ± SE of the average of duplicate determinations from at least three different experiments).

Effect of antibodies on reconstituted phenacetin O-deethylase activity

A number of antibodies against cytochrome P450c and P450d were screened for their ability to inhibit POD activity of reconstituted preparations of cytochromes P450c and P450d (Fig. 3). The results have been expressed as a percentage of control activity, where samples were incubated with an equivalent volume of PBS instead of an antibody, prior to the assay. Under these conditions, control activity of reconstituted P450c was 568 pmol/nmol/min and of P450d it was 1290 pmol/nmol/min. MAb 12/2/3/2 was equally effective at inhibiting cytochrome P450c and P450d (Fig. 3). Activity was inhibited maximally, by 87 and 83%, respectively, at an antibody con-

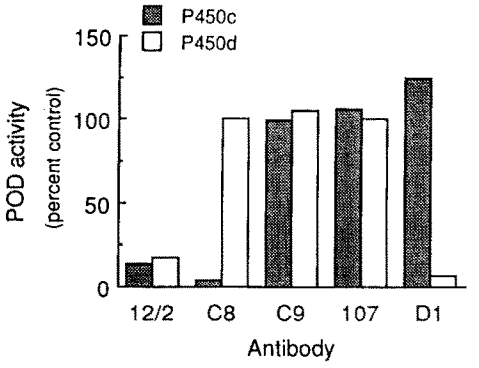

Fig. 3. Effect of antibodies on phenacetin *O*-deethylase activity of reconstituted rat cytochromes P450c and P450d. Cytochromes P450 (20 pmol) were incubated with monoclonal (1 mg Ig/nmol P450) or mono-specific polyclonal anti-P450d(-c) (15 mg Ig/nmol P450) antibodies for 5 min at 22° prior to addition of NADPH-cytochrome *c* (P450) reductase and lipid on ice. After addition of the other components the samples were incubated for 10 min at 37°. Phenacetin concentration was 10 μM. Basal activities, in the absence of any antibody, were: cytochrome P450c, 568 pmol/nmol/min and P450d, 1289 pmol/nmol/min.

centration of 1.0 mg Ig/nmol P450 (data not shown). Maximal inhibition by MAb C8 was achieved at concentrations of Ig lower than this (data not shown), but data using 1.0 mg Ig/nmol P450 are shown in Fig. 3. Only cytochrome P450c activity was inhibited by this antibody, by 96%. Higher concentrations of polyclonal antibody anti-P450d(-c) were necessary to produce inhibition. Thus, at a concentration of 15 mg Ig/nmol P450, this antibody inhibited POD activity of only P450d, by a maximum of 95% (Fig. 3). MAbs 107 and C9, at concentrations at which C8 and 12/2/3/2 produced maximum inhibition, had no effect on POD activity catalysed by either cytochrome P450c or P450d. Like C8, C9 is specific to cytochrome P450c, but this MAb has no effect on the catalytic activity of the protein [10]. In contrast, MAb 107 is inhibitory only to rabbit P450 4, and does not cross-react with the rat isoenzymes [31].

Effect of antibodies on rat hepatic microsomal phenacetin O-deethylase activity

The contribution of PAH-inducible forms of cytochrome P450 to microsomal POD activity was determined using specific inhibitory antibodies. In initial studies, POD activity was assayed at 4 μM phenacetin, at which concentration the majority of POD activity would be from the high affinity component. The overall contribution of PAH-inducible forms of P450 was first determined with MAb 12/2/3/2. Hepatic microsomal fractions from untreated (Fig. 4A), MC- (Fig. 4B), ISF- (Fig. 4C) and PB- (Fig. 4D) treated rats were incubated with a range of concentrations of this MAb and of MAb 107, as a negative control. MAb 12/2/3/2 inhibited POD activity of microsomal fractions from untreated, MC- and ISF-treated rats, by a maximum of 75–80% in each case. Whereas maximum inhibition with control microsomes was achieved at an antibody concentration of 0.2 mg Ig/nmol P450, with microsomes from MC- or ISF-treated rats, antibody concentrations of 1 mg Ig/nmol P450 were necessary to produce maximum inhibition. MAb 12/2/3/2 had no effect on POD activity of microsomes from PB-treated rats, at MAb concentrations of up to 2 mg Ig/nmol P450. The control antibody, MAb 107, was without effect on POD activity of any of the microsomal fractions.

Inhibition of POD activity by MAb 12/2/3/2 was determined at several concentrations of phenacetin, at which the contribution of the two components of activity would vary (Table 3). Pooled microsomal fractions from the livers of three animals were incubated with 1.0 mg Ig/nmol P450. The contribution of high affinity POD activity, expressed as a per cent of total activity, was calculated from the Michaelis–Menten parameters given in Table 2. The percentage of total POD activity inhibited by MAb 12/2/3/2 was very similar to the predicted contribution of the high affinity component. At higher concentrations of phenacetin the degree of inhibition with microsomes from MC- and ISF-treated rats was higher than expected from the kinetic data. The individual contribution of cytochromes P450c and P450d to POD activity was determined with inhibitory antibodies to each of the isoenzymes. MAb C9 was included as negative control. MAb C8, although capable of inhibiting POD activity of P450c when reconstituted (Fig. 3) was without effect on POD activity of hepatic microsomal fractions from either untreated (Fig. 5A) or MC-treated (Fig. 5B) rats. However, this antibody did inhibit AHH activity of microsomal fractions from rats pretreated with MC, catalysed by cytochrome P450c (Ref. 10, and confirmed in these studies, data not shown). Polyclonal anti-P450d(-c) inhibited hepatic microsomal POD activity of both untreated (Fig. 5A) and MC-treated (Fig. 5B) rats. The extent of inhibition with this antibody was very similar to that found with MAb 12/2/3/2, although the concentration of the polyclonal antibody necessary to produce maximum inhibition was ten times higher than with the MAb. Both the control MAb, C9 (Fig. 5A and B) and preimmune rabbit serum (data not shown) were without effect on POD activity.

DISCUSSION

The O-deethylation of phenacetin by hepatic microsomal fractions is biphasic [18]. The Michaelis–Menten constants for POD activity obtained in the present study, both in untreated and MC-treated Wistar rats, are in good agreement with the values obtained previously with Fischer and DA rats [17]. Thus, amongst these three strains of rat, there is very little variation in POD activity. In previous studies, and confirmed here, it was shown that the high affinity component of POD activity is increased by treatment of rats with MC [17]. This provided evidence that one or more PAH-inducible form(s) of cytochrome P450 is involved in the O-deethylation of phenacetin.

Although treatment of rats with ISF results in the induction of P450 [29], there is no apparent increase in either total cytochrome P450 content or metabolic activity. This is because the induced P450 is complexed with ISF, which is apparent from difference

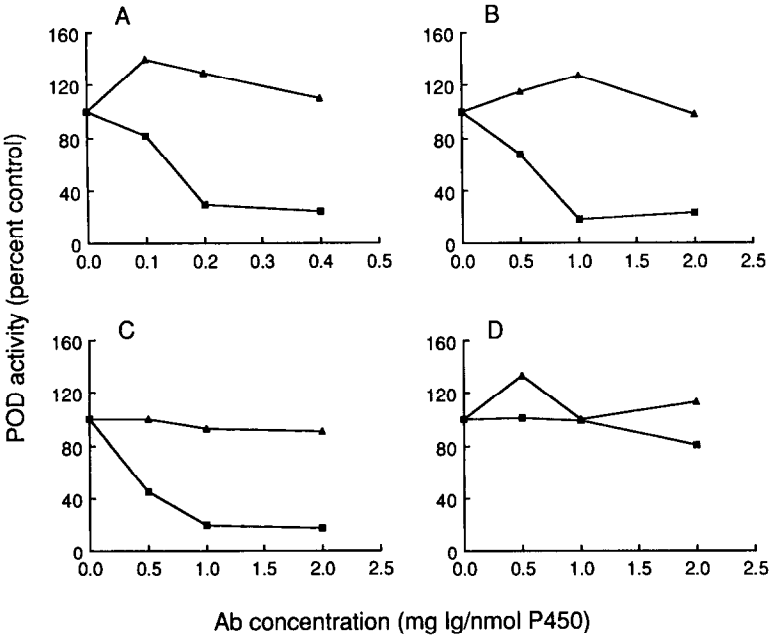

Fig. 4. Effect of monoclonal antibodies on rat hepatic microsomal high affinity phenacetin *O*-deethylase activity. POD activity was determined with hepatic microsomal fractions from untreated (A), 3-methylcholanthrene (B), isosafrole (C) and phenobarbitone (D) treated rats at a substrate concentration of 4 μM in the presence of inhibitory MAbs. Antibodies 12/2/3/2, reacting with cytochromes P450c and P450d in the rat (■) and 107, specific to rabbit cytochrome P450 4 (▲) were incubated with microsomal fractions for 30 min at room temperature before assay. Results have been expressed as per cent of basal activity determined in the absence of MAb. Basal POD activities were as follows; untreated rat, 55.0 pmol/mg/min; 3-methylcholanthrene-treated rat, 497 pmol/mg/min; isosafrole-treated rat, 126 pmol/mg/min; phenobarbitone-treated rat, 30.5 pmol/mg/min.

Table 3. Effect of monoclonal antibody 12/2/3/2, against rat cytochromes P450c and P450d, on rat hepatic phenacetin *O*-deethylase activity

Treatment	Phenacetin concentration (μM)	High affinity activity*	Percent activity inhibited by MAb
Untreated	4	75.0	75.7
Untreated	20	51.0	68.0
Untreated	400	10.4	7.0
MC	4	82.3	81.4
MC	20	68.4	82.8
MC	400	26.3	43.9
ISF$^+$	2	71.0	80.3
ISF$^+$	200	10.7	28.2
PB	2	0	1.3
PB	200	0	0

* High affinity activity was calculated using the Michaelis–Menten parameters given in Table 2.

$^+$ Isosafrole microsomes were preincubated with cyclohexane before assay as described in the text (Fig. 1).

Microsomes were preincubated with a purified preparation of MAb 12/2/3/2 for 30 min at 22° before addition of co-factors and substrate as described in Materials and Methods. Antibody concentration was 1.0 mg Ig/nmol P450 with all preparations of microsomes except those from untreated rats which were incubated with 0.4 mg Ig/nmol P450. Results are for pooled microsomal fraction from the livers of three animals for each group.

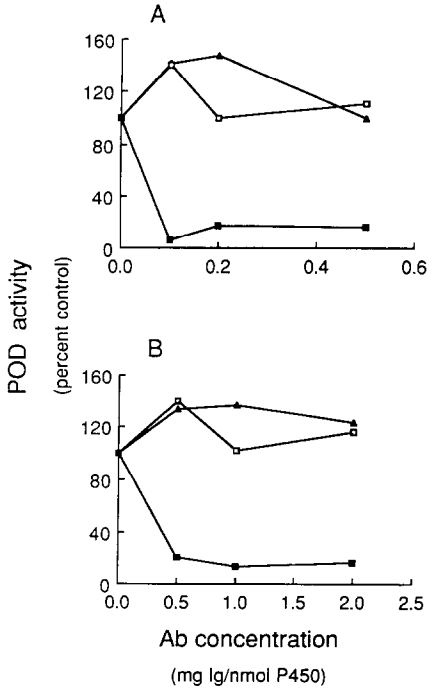

Fig. 5. Effect of antibodies, specific to either rat cytochrome P450c or P450d, on rat hepatic microsomal high affinity phenacetin *O*-deethylase activity. POD activity was determined with hepatic microsomal fractions from untreated rats (A) and from rats pretreated with 3-methylcholanthrene (B) at a substrate concentration of 4 μM. Immunoinhibition was determined in the presence of antibodies specific to either cytochrome P450c, C8 (▲) and C9 (□) or P450d, anti-P450d(-c) (■). Anti-P450d(-c) was used at a concentration 10 times higher than that shown for C8 and C9. Results have been expressed as per cent of basal activity determined in the absence of antibody. Basal POD activity of hepatic microsomes from untreated and MC-treated rats was 20.3 pmol/mg/min and 1252 pmol/mg/min, respectively.

spectroscopy of the reduced microsomes. This spectral complex can be dissociated by incubation with a number of lipophilic compounds such as cyclohexane. Following incubation of microsomes from ISF-treated rats with cyclohexane the P450 from which ISF is displaced is detectable as an increase in total P450 content and in catalytic activity. Following such incubation of microsomes from ISF-treated, but not control, rats POD activity is increased, showing that the form(s) of cytochrome P450 involved in rat hepatic POD activity is (are) inducible by ISF as well as by MC.

In assessing which isoenzymes might be involved in this increase in POD activity, it is necessary to consider the amounts of the individual forms of cytochrome P450 present in the different microsomal preparations. The level of cytochrome P450c in hepatic microsomes from untreated animals is extremely low [3, 4, 6], <0.5 pmol/mg in the present study. Cytochrome P450d is present at levels of at least 20 times this (Ref. 4, present study). Treatment of rats with MC results in large increases in the microsomal content of both cytochromes P450c and P450d as well as a small increase in cytochrome P450a [3]. In the present investigation cytochrome P450c was induced by at least 100-fold and P450d by 30–40 fold. ISF treatment of rats results in increases in the hepatic levels of cytochromes P450a, P450b, P450c, P450e, P450p and P450d [3, 8]. In this study, P450c was induced by about 40-fold and P450d by 60-fold. The relative induction of cytochromes P450c and P450d by ISF and MC in this investigation is in agreement with that found by others [3, 4].

MC treatment of rats results in a 30-fold increase in hepatic POD activity. In individual MC-treated animals, the change in POD activity parallels the increase of hepatic microsomal cytochrome P450d but not cytochrome P450c content. In contrast, treatment of rats with ISF increases activity by only 3-fold, and there is no correlation with cytochrome P-450d content of these microsomes. However, as ISF inactivates cytochrome P450 by forming a metabolically inactive complex, it is very difficult to relate immunologically determined content of cytochrome P450 with catalytic activity, even after displacement with cyclohexane.

As the kinetics of POD are biphasic, there will be a contribution to activity from the low affinity component, even at low concentrations of substrate. This contribution can be estimated from the Michaelis–Menten constants for POD activity. Thus, the degree of inhibition by an antibody can be compared with predicted high affinity activity. Studies with MAb 12/2/3/2, which is specific for P450c and P450d, showed that all of the high affinity component of hepatic POD activity is catalysed by P450c and/or P450d, not only in microsomes from MC- and ISF-treated rats, but also in those from untreated animals. The low affinity component of activity was either not inhibited or only slighty inhibited by this antibody. In the latter case, this was apparent only at higher substrate concentrations with microsomes from MC- and ISF-treated rats. This might be due to a contribution of PAH-inducible form(s) to this component of activity.

These data show that members of only the P450IA sub-family contribute to high affinity POD activity. Inhibition of activity in microsomes from untreated animals indicates that this reaction is catalysed by the constitutive levels of PAH inducible isoenzyme(s). The concentration of antibody necessary for maximum inhibition of POD activity of MC- and ISF-treated rats is at least five-fold that required with microsomes from untreated animals. This reflects the lower levels of immunoreactive P450 in microsomes from untreated rats, and further supports the conclusion that high affinity POD activity of such microsomes is catalysed by a minor form(s) of cytochrome P450.

When the PAH-inducible forms of cytochrome P450, P450c and P450d, were purified and POD activity determined with the reconstituted isoenzymes, it was found that both forms are very effective at catalysing this reaction. Given the activity of the reconstituted isoenzymes and their specific content within the microsomal fraction, it might be expected that both forms of cytochrome P450 would contribute to POD activity of microsomes from MC-treated rats. However, results of immunoinhibition studies

with isoenzyme-specific antibodies were contrary to this expectation. The anti-P450c and anti-P450d antibodies do inhibit POD activity of the homologous isoenzyme when reconstituted. However, with microsomes from MC- or ISF-treated rats, POD activity is inhibited only by the anti-P450d(-c) antibody. The extent of inhibition by this antibody was almost identical to that obtained with MAb 12/2/3/2, against cytochromes P450c and P450d. This confirms the lack of involvement of cytochrome P450c in high affinity POD activity.

Treatment of rats with PB results in a decrease in hepatic microsomal cytochrome P450d content by 2.3-fold, relative to the content in untreated animals. Similar results have been reported by others [3, 4], where decreases of 1.5-fold and 2.5-fold in P450d content, respectively, were found. Parallel with this loss in hepatic P450d content, high affinity POD activity is lost following treatment of rats with PB, resulting in monophasic kinetics. The loss of high affinity POD activity is due, presumably, to the repression of constitutive levels of cytochrome P450d by PB. In contrast, low affinity POD activity is increased in these animals, confirming previous findings [17]. PB treatment affects both K_m and V_{max} of this component of POD activity. This, together with the lack of any inhibition of activity by MAb 12/2/3/2, suggests that PB treatment alters the profile of cytochrome(s) P450 catalysing O-deethylation.

In microsomes from untreated rats, the levels of cytochrome P450d exceed those of P450c by at least 20-fold. Thus, it might be expected that cytochrome P450d would be responsible for all of high affinity POD activity in such microsomes. This was confirmed with specific antibodies to P450c and P450d. Thus, cytochrome P450d is responsible for high affinity POD activity in microsomes from both untreated and induced (MC or ISF) animals. The contribution of P450d to high affinity POD activity is not affected by the amount of cytochrome P450c present in the liver. This contrasts with the finding of Kelley *et al.* [8] on ethoxyresorufin *O*-deethylase (EROD) activity. These workers have shown that the specificity of PAH-inducible forms of P450 in catalysing EROD activity in the liver is affected by the amount of P450c present. Thus, in untreated rats, as well as in rats treated with inducers which preferentially increase P450d, such as 3-methoxy-4-aminoazobenzene, this activity is catalysed largely by P450d. However, after induction with MC, EROD activity is catalysed primarily by cytochrome P450c.

In conclusion, these studies show that high affinity phenacetin *O*-deethylase activity of rat liver is catalysed exclusively by P450d, not only in PAH-induced rats but also in control animals. Thus, high affinity POD is a specific measure of the activity of hepatic P450d in the rat.

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