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# A form of cytochrome P450 in man, orthologous to form d in the rat, catalyses the *O*-deethylation of phenacetin and is inducible by cigarette smoking

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**1** In previous studies (Boobis *et al.*, 1985b) it was shown that a monoclonal antibody (MAb 3/4/2), raised against rat cytochrome P450 form c, reacts with an isoenyzme(s) of cytochrome P450 in human liver. It was predicted that the epitope with which this antibody reacts should be present on both isoenzymes of the P450IA gene sub-family (the orthologues of forms c and d) in man (Edwards *et al.*, 1987).

**2** This antibody was used to probe 45 different samples of human liver, by the technique of Western blotting. With one exception, all of the samples contained immunoreactive protein, a single band at $M_r$ 54,000 (orthologous to rat form d), which ranged in content from $< 0.5$ to 33.5 pmol $mg^{-1}$ microsomal protein. The content of the human orthologue of form c was below 0.5 pmol $mg^{-1}$, the limit of detection of the assay.

**3** Thirteen of the samples were from patients of known smoking status. Immunoreactive P450 content was 3.5-fold higher, and phenacetin *O*-deethylase activity was four-fold higher, in the smokers than in the non-smokers.

**4** There was a highly significant correlation between the amount of immunoreactive cytochrome P450 and the high affinity component of phenacetin *O*-deethylase activity in both smokers and non-smokers.

**5** It is concluded that the high affinity component of phenacetin *O*-deethylase activity in man is catalysed by the orthologue of rat cytochrome P450d, and that this isoenzyme is inducible by cigarette smoking.

**6** In a number of previous publications it has been suggested that there is an association between the poor metaboliser (PM) phenotype for debrisoquine and impaired phenacetin *O*-deethylation. In the present study it was shown that not all subjects PM for debrisoquine are poor metabolisers of phenacetin.

**Keywords** phenacetin *O*-deethylase cigarette smoking enzyme induction cytochrome P450d human cytochrome P450 monoclonal antibodies

## Introduction

There are two polycyclic aromatic hydrocarbon (PAH)-inducible forms of cytochrome P450 in most, if not all, mammalian species (Reik *et al.*, 1982; Thomas *et al.*, 1984). These are the products of the P450IA gene sub-family: forms c and d in the rat, forms $P_1$ and $P_3$ in the mouse and forms 6 and 4 in the rabbit, respectively (Nebert *et al.*, 1987). There is now good evidence that form d in the rat, and the corresponding forms in mouse and rabbit, are constitutively expressed, comprising between 4–6% of total hepatic cytochrome P450 content, whereas the constitutive levels of form c are very low (Guengerich *et al.*, 1982; Thomas *et al.*, 1983; Goldstein & Linko, 1983).

In liver, both isoenzymes are highly inducible by many toxic xenobiotics e.g. 3-methylcholanthrene (MC) and polyhalogenated aromatic hydrocarbons (Parkinson *et al.*, 1983). To date, no compound has been found that exclusively induces only one of these two isoenzymes in the liver (Kelley *et al.*, 1987), although, in the rat MC preferentially induces form c and isosafrole preferentially induces form d. Thus, these isoenzymes appear to be differentially regulated.

The phenomenon of induction has been recognised in man for some time, although it has been difficult to study its specificity and toxicological implications. The genes for forms of cytochrome P450 orthologous to most of the inducible forms in the rat, i.e. b, c, d, j (Thomas *et al.*, 1987) and p (Wrighton *et al.*, 1985), have now been identified in man. Ethanol consumption induces the human orthologue of rat form j (Wrighton *et al.*, 1986b) and treatment with dexamethasone or phenobarbitone will induce the orthologue of form p in man (Watkins *et al.*, 1985). However there is, as yet, no conclusive evidence for the induction of either form c or d in human liver, although several groups have reported the presence of one or both of these isoenzymes in man (Adams *et al.*, 1985; Wrighton *et al.*, 1986a; Jaiswal *et al.*, 1987). Wong *et al.* (1986) have reported that the orthologue of form c was induced in the placentae of women in Taiwan exposed to polychlorinated biphenyls.

Cigarette smoking has long been known to increase the oxidation of a number of substrates, both in animals and in man (Pantuck *et al.*, 1972, 1974; Kuntzman *et al.*, 1977) presumably due to induction of drug metabolising enzymes. Cigarette smoke is a complex mixture of organic and inorganic compounds, and in animals it has inducing properties similar to PAH (Uotila *et al.*, 1977; Conney, 1982, 1986). Whereas treatment of animals with PAH results in considerable elevation of hepatic aryl hydrocarbon hydroxylase activity, such a change has never been observed in man following cigarette smoking (Boobis *et al.*, 1980; Pelkonen *et al.*, 1986). However, cigarette smoking does cause a profound induction of phenacetin oxidation in man determined both *in vivo* and *in vitro* (Pantuck *et al.*, 1972, 1974; Boobis *et al.*, 1981; Kahn *et al.*, 1985).

Using specific inhibitory monoclonal antibodies, we have previously shown that, in the rat, the hepatic *O*-deethylation of phenacetin is highly selectively catalysed by cytochrome P450d (Boobis *et al.*, 1987). We have now used both immunological and metabolic methods to identify a major hepatic form of cytochrome P450 induced by cigarette smoking in man, and determined its role in the oxidation of phenacetin.

## Methods

### *Chemicals*

Phenacetin and paracetamol were obtained from BDH Chemicals Ltd (Poole, UK). Phenacetin was recrystallised from benzene and purified by washing with aqueous sodium hydroxide solution prior to use. [$^2H_3$]-Paracetamol was synthesised as previously described (Murray & Boobis, 1986). NADPH, n-undecane and prestained molecular weight markers for use on Western blots were obtained from Sigma Chemical Co. Ltd (Poole, UK). 3,5-Bistrifluoromethylbenzoyl chloride was supplied by Fluorochem Ltd (Glossop, UK). All gel filtration and ion-exchange resins were from Pharmacia (Milton Keynes, UK), and all sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis (PAGE) reagents were from BioRad Laboratories (Watford, UK). Nitrocellulose membrane (Hybond-C) was obtained from Amersham International plc (Amersham, UK) and anti-mouse horseradish peroxidase conjugated Ig was from ICN Biomedicals Ltd (High Wycombe, UK). All other chemicals were of AnalaR grade or the best available.

### *Human liver samples and preparation of microsomal fraction*

Biopsy samples (15) of human liver were obtained at laparotomy from patients undergoing abdominal surgery, where biopsy was indicated for diagnostic reasons. Only those samples with preserved hepatic architecture were used in this study. The use of human tissue in this way had local Research Ethics Committee permission. Details of social and therapeutic drug use in these patients was obtained by interview and from the case records. Full clinical chemical and biochemical tests were performed 3 days prior to biopsy (Boobis *et al.*, 1980).

Human liver samples (28) from organ transplant donors (Kremers *et al.*, 1981; Boobis *et al.*, 1985a) were also used in this study. Two human liver samples, HL3 and HL4 were made available by Dr M. S. Lennard, Department of Therapeutics, Royal Hallamshire Hospital, Sheffield.

Hepatic microsomal fractions were isolated from all samples by differential ultracentrifugation as previously described (Boobis *et al.*, 1980), and resuspended in 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol. Samples were stored in small aliquots, with a protein concentration of above 10 mg ml$^{-1}$, at −80° C until required.

*Preparation of monoclonal antibody*

Monoclonal antibody (MAb) 3/4/2, the production and characterisation of which have previously been described (Boobis *et al.*, 1985b; Sesardic *et al.*, 1986), was used. The MAb was prepared in quantity by inoculating female, pristane-pretreated, Balb/c mice with actively growing hybridoma cells and then collecting the resultant ascites fluid. The Ig fraction of the ascites fluid was isolated by ammonium sulphate precipitation (50% saturation), gel filtration on Sephadex G-25 and ion-exchange chromatography by fast protein liquid chromatography (f.p.l.c.) on a MonoQ column (Pharmacia, Milton Keynes, UK). Antibody was applied to the column in 20 mM triethanolamine-HCl 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$^{-1}$, over a period of 20 min. Fractions containing antibody were pooled and 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° C. The final antibody preparation was greater than 95% pure, as determined by SDS/polyacrylamide gel electrophoresis.

*Immunoquantification*

Microsomal proteins (30 μg) from human liver samples were separated on 10% SDS/polyacrylamide gels, 1.5 mm × 10 cm × 14 cm, following the method of Laemmli (1970). Western blotting of the separated proteins was performed as previously described (Sesardic *et al.*, 1986), using a modification of the method of Towbin *et al.* (1979). Immunoquantification of human cytochrome P450 was achieved by computerised integration of the peaks obtained by densitometric scanning of Western blots probed with MAb 3/4/2. Stained bands were quantified by reflectance densitometry using a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoeffer Scientific Instruments, San Francisco, USA) and analysed using a Waters model 840 Data and Chromatography control station (Millipore (UK) Limited, Harrow, UK). Standard curves were constructed using highly purified rat cytochrome P450c (specific content 14 nmol mg$^{-1}$) prepared as previously described (Sesardic *et al.*, 1986).

*Phenacetin O-deethylase (POD) assay*

POD activity was determined using the method of Murray & Boobis (1986). Microsomal protein, 200 μg, was incubated in a final volume of 1 ml containing Tris-HCl buffer pH 7.4 (75 mM), magnesium chloride (3 mM) and NADPH (1.0 mM), at 37° C for 10 min. The reaction was started by the addition of recrystallised phenacetin in methanol (20 μl), to give a final concentration of 4 μM. The volume of methanol used in these studies was virtually without effect on phenacetin *O*-deethylase activity (data not shown). The reaction was terminated by the addition of 300 μl of 0.25 M sodium hydroxide. Trideuteroparacetamol (40 ng) was added to the samples as internal standard (Murray & Boobis, 1986). Paracetamol, the product of the *O*-deethylation of phenacetin, together with internal standard, was differentially extracted into dry diethyl ether. The extracts were analysed following derivatisation with 3,5-bistrifluoromethylbenzoyl chloride, by combined gas chromatography/negative ion chemical ionization mass spectrometry on a Finnigan-MAT 4500 system (Finnigan-MAT, San Jose, California, USA) with a fused silica capillary column (SE54, 30 m × 0.25 mm, J & W Scientific Inc, Folson, California, USA). The gas chromatographic and mass spectrometric conditions were essentially as described previously (Murray & Boobis, 1986), with a limit of detection at 0.2 ng paracetamol per microsomal incubation.

*Other assays*

Protein concentration was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin (fraction V) as standard. Cytochrome P450 content was measured by reduced CO-difference spectroscopy (Omura & Sato, 1964) on a model 555 split-beam scanning spectrophotometer with microprocessor controlled background correction (Perkin-Elmer Ltd, Beaconsfield, Bucks, UK). An extinction coefficient of 91 mM$^{-1}$ cm$^{-1}$ was assumed for reduced CO-cytochrome P450 between 450–480 nm.

**Results**

As previously reported (Sesardic *et al.*, 1986), MAb 3/4/2, raised to rat cytochrome P450c, recognises hydrocarbon-inducible forms of cytochrome P450 in other species. On Western blotting, within the limits of detection of the method, MAb 3/4/2 reacted with only a single form of cytochrome P450, $M_r$ 54,000 (very close to the mol wt of rat P450d run in the same system), in microsomal fractions of human liver (Figure 1).

As the binding sites for MAb 3/4/2 on rat P450c and on the forms in man orthologous to P450c and P450d are identical (Edwards *et al.*,

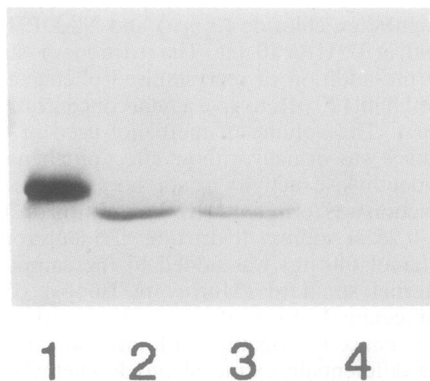


**Figure 1** Western blot with MAb 3/4/2 of microsomal proteins from rat and human liver. Microsomal proteins were first separated by SDS/polyacrylamide gel electrophoresis and then subjected to Western blotting with MAb 3/4/2, as described in **Methods**. Microsomal preparations were as follows: lane 1, 3-methylcholanthrene (MC) treated rat liver (3 μg); lane 2, human liver T5, as a positive control (30 μg); lane 3, human liver HL3 (30 μg); lane 4, human liver HL4 (30 μg).

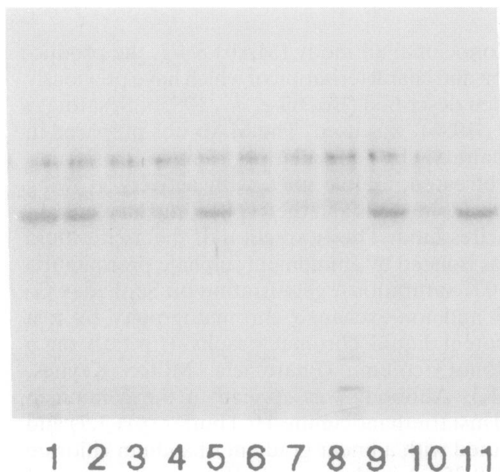


**Figure 2** Western blot with MAb 3/4/2 of microsomal proteins from biopsy samples of human liver from smokers and non-smokers. Microsomal proteins (30 μg) from the following samples: smokers – lanes: 1 (011), 2 (021), 5 (022), 9 (026) and 11 (031); non-smokers – lanes: 3 (046), 4 (047), 6 (064), 7 (056), 8 (066) and 10 (043), were separated by SDS/polyacrylamide gel electrophoresis and probed with MAb 3/4/2. Details of the patients (patient number in parentheses) from whom the samples were obtained are shown in Table 1.

1987), immunoquantification of cytochrome P450 in different samples of human liver was achieved by reference to a standard curve constructed using purified rat P450c. As little as 30 fmol P450c could be detected by this method. There was a linear relationship between the amount of P450c and the intensity of staining on a Western blot, to 1 pmol. Probit transformation extended the linear relationship to 4 pmol P450c. A limited number of standards were included on each Western blot to compensate for any between day variation in the staining reaction.

Biopsy samples from smokers ($n$ = 7) and non-smokers ($n$ = 8) were subjected to Western blotting with MAb 3/4/2 in order to quantify their levels of immunoreactive cytochrome P450 (Figure 2). In all of these samples only a single immunoreactive band, at $M_r$ 54,000, was detected. Details of the individual patients from whom the biopsies were obtained, together with data on the immunoreactive P450 content and POD activity, are shown in Table 1. The amount of this immunoreactive cytochrome P450 (Figure 3) in the liver samples from cigarette smokers was significantly (3.5-fold) higher ($P < 0.01$) than in those from the non-smokers: 16.3 ± 2.7 compared with 4.7 ± 1.9 pmol $mg^{-1}$ (mean ± s.d.).

As reported previously (Boobis *et al.*, 1981), the *O*-deethylation of phenacetin by microsomal fractions of human liver is biphasic, enzyme velocity being best described as the sum of two components, each characterized by their own values for $K_m$ and $V_{max}$. High affinity POD activity was determined in hepatic microsomal samples of biopsies from smokers ($n$ = 5) and non-smokers ($n$ = 8). This component of POD activity was significantly higher (4.3-fold) in smokers ($P < 0.01$) than in non-smokers: 230 ± 70 compared with 54 ± 11 pmol $mg^{-1}$ $min^{-1}$ (mean ± s.d.) (Table 1).

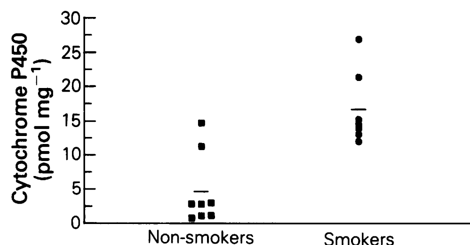


**Figure 3** Immunoquantification of the human orthologue of cytochrome P450 form d in hepatic biopsies from smokers and non-smokers. The levels of immunoreactive P450, determined by Western blotting with MAb 3/4/2, in liver samples from seven smokers were compared with those in eight non-smokers.

**Table 1** Details of human liver biopsy samples from smokers and non-smokers

| *Patient* | *Sex* | *Age (years)* | *Cytochrome P450* ($pmol\ mg^{-1}$)* | *POD activity** ($pmol\ mg^{-1}\ min^{-1}$)* | *Smoking status* |
|---|---|---|---|---|---|
| 011 | F | 42 | 11.9 | 119 | Smoker |
| 021 | M | 52 | 15.2 | 189 | Smoker |
| 022 | M | 61 | 13.8 | ND | Smoker |
| 026 | M | 74 | 26.8 | 467 | Smoker |
| 031 | F | 27 | 21.3 | ND | Smoker |
| 040 | M | 63 | 13.0 | 64.6 | Smoker |
| 044 | M | 49 | 14.4 | 309 | Smoker |
| 037 | M | 70 | 11.2 | 61.2 | Non-smoker |
| 043 | F | 57 | 2.95 | 25.4 | Non-smoker |
| 046 | F | 84 | 2.79 | 79.7 | Non-smoker |
| 047 | M | 68 | 2.76 | 72.0 | Non-smoker |
| 054 | F | 42 | 14.7 | 107 | Non-smoker |
| 056 | M | 69 | 0.72 | 23.6 | Non-smoker |
| 064 | M | 59 | 1.11 | 25.2 | Non-smoker |
| 066 | F | 63 | 1.05 | 39.3 | Non-smoker |

Smokers, on the basis of self-reporting, smoked more than 10 cigarettes per day. Non-smokers claimed not to smoke at all.
ND = not determined.
* Cytochrome P450 (orthologous to form d) was immunoquantified using MAb 3/4/2 as described in **Methods**.
** POD activity, high affinity component, was determined at a phenacetin concentration of 4 μM, as described in **Methods**.
None of the drugs given prior to biopsy would affect the activity of the enzymes of drug metabolism.

There was a good correlation between high affinity POD activity and the level of immunoreactive cytochrome P450. In 13 human liver biopsies (five from smokers and eight from non-smokers), the correlation between these two parameters was highly significant ($r_s = 0.846$, $P < 0.001$). To confirm this correlation, a further 28 human liver samples, obtained from kidney transplant donors, were analysed. Total cytochrome P450 content, and several monooxygenase activities, of these samples have previously been published (Kremers *et al.*, 1981; Boobis *et al.*, 1985a, c). The smoking status of these patients was not known. High affinity POD activity was determined at a substrate concentration of 4 μM. The range of activities was from < 0.66 to 119 $pmol\ mg^{-1}\ min^{-1}$ (33.2 ± 24.3; mean ± s.d.), the activity of one sample (P60) being below the detection limit of the assay.

The cumulative frequency distribution of POD activity of these samples and the probit transformed data were plotted to test normality of distribution (Figure 4). Activity was not normally distributed. A small number of samples had higher activity than would be predicted from a normal distribution of the data. The distribution of POD activity, amongst these 28 samples, was significantly skewed (1.873, $P < 0.01$) and also showed significant positive kurtosis (5.285, $P < 0.01$). The range for POD activity was more than 180-fold, and the coefficient of variation was relatively high, at 73%. POD activity in all of the smokers, with one exception, was greater than 110 $pmol\ mg^{-1}\ min^{-1}$, placing these samples at the upper extreme of the range of all of the samples analysed.

The 28 liver samples from transplant donors were subjected to Western blotting with antibody 3/4/2, to enable immunoquantification of the human cytochrome P450 at $M_r$ 54,000. A Western blot for 16 of these samples (selected at random) is shown in Figure 5. The amount of immunoreactive protein varied by more than 60-fold amongst these samples, ranging from < 0.5 to 33.5 $pmol\ mg^{-1}$ (6.03 ± 5.92 $pmol\ mg^{-1}$; mean ± s.d.). The samples at the extremes of this range are amongst those shown in Figure 5, in lanes 15 and 2, respectively.

The amounts of this immunoreactive cytochrome P450, in the 28 liver samples, was significantly skewed (3.944, $P < 0.001$) and also showed highly significant kurtosis (18.33, $P < 0.001$). The coefficient of variation for the levels of immunoreactive cytochrome P450, like that for POD activity, was high, at 98%. Again, as was found for POD activity, the amount of immunoreactive P450 in several of the samples was higher than would be predicted from a normal

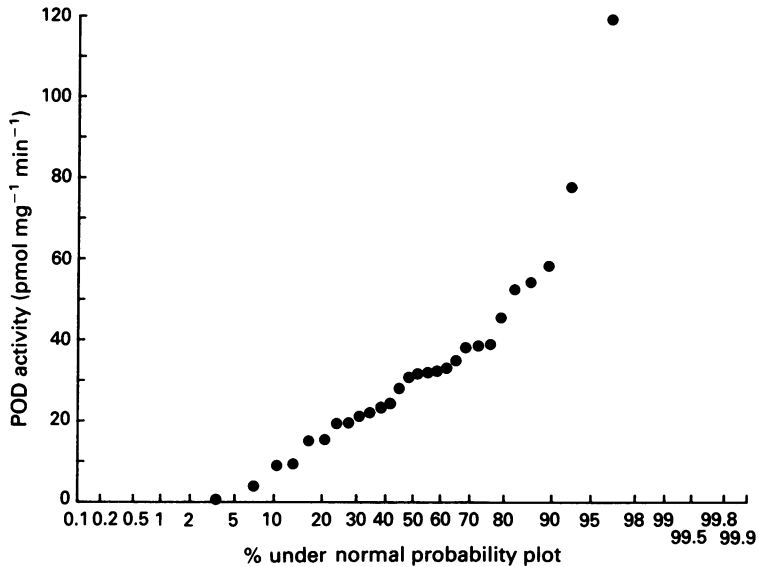


**Figure 4** Cumulative percent relative frequency distribution (probit plot) of phenacetin *O*-deethylase activity amongst 28 human liver samples from organ transplant donors.

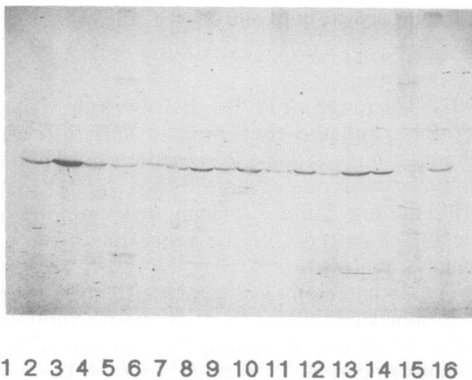


**Figure 5** Western blot with MAb 3/4/2 of microsomal proteins from samples of human liver from organ transplant donors. Microsomal proteins (30 μg) were separated by SDS/polyacrylamide gel electrophoresis and then subjected to Western blotting with MAb 3/4/2. Lanes 1–16 correspond to the following samples: 1 (P51), 2 (P41), 3 (P54), 4 (P49), 5 (P34), 6 (P63), 7 (P70), 8 (P55), 9 (P44), 10 (P66), 11 (T7), 12 (P50), 13 (P47), 14 (P70), 15 (P60) and 16 (P61).

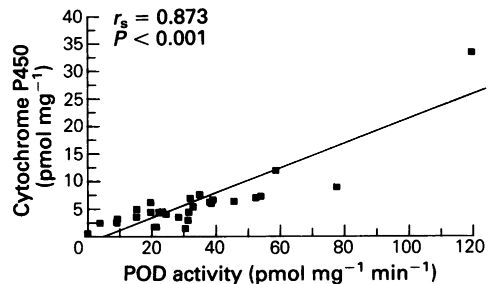


**Figure 6** Relationship between phenacetin *O*-deethylase activity and the content of immunoreactive cytochrome P450 (orthologous to form d) in 28 human liver samples. The *symbols* show values determined experimentally and the *solid line* shows the computed regression line. $r_s$ is the Spearman rank correlation coefficient.

distribution of the data (data not shown). Those samples containing most immunoreactive cytochrome P450 also had the highest POD activity. Further, the only sample with no detectable POD activity (P60), was also the only one negative on Western blotting with MAb 3/4/2 (Figure 5, lane 15). Again, comparison with the data obtained with the biopsy samples, revealed that the samples from all known smokers had levels of immunoreactive cytochrome P450 greater than 12 pmol mg$^{-1}$, placing them at the upper extreme of the population of samples investigated.

POD activity of the 28 liver samples from transplant donors was compared with their content of immunoreactive cytochrome P450 (Figure 6). The *symbols* show the observed data and the *solid line* shows the computed regression line. There was a highly significant correlation between POD activity and content of immunoreactive cytochrome P450, $r_s = 0.873$ ($P < 0.001$) (Figure 6). Omitting the outlier with very high

POD activity did not alter significantly this correlation ($r_s = 0.800$, $P < 0.001$). There was a highly significant correlation when all of the samples were analysed together ($n = 41$, $r_s = 0.706$, $P < 0.001$).

POD activity and immunoreactive P450 content were compared in liver samples from subjects of known phenotype for debrisoquine 4-hydroxylation (Figure 1). Sample HL3 (lane 3) was from a poor metaboliser (PM) subject and sample HL4 (lane 4) was from an extensive metaboliser (EM) subject, as determined by analysis *in vitro* (Otton *et al.*, 1988 and unpublished observations). From the Western blot it is apparent that sample HL3 contained more of the immunoreactive P450 than did sample HL4. POD activity was also higher in sample HL3 than in HL4 (data not shown). There was no correlation between debrisoquine 4-hydroxylase and POD activities ($n = 27$, $r_s = 0.312$, $P > 0.1$).

## Discussion

The hepatic expression of PAH-inducible forms of cytochrome P450 (products of the P450IA gene sub-family) has been investigated in man, using a monoclonal antibody (MAb 3/4/2) which, as previously reported (Boobis *et al.*, 1985b), was raised to rat cytochrome P450c. This antibody recognises analogous form(s) of cytochrome P450 in many other species, including man (Sesardic *et al.*, 1986).

It is apparent from Western blotting of human liver samples as reported here, and as indicated in the initial studies with this antibody (Boobis *et al.*, 1985b), that MAb 3/4/2 recognises only a single protein, with $M_r$ 54,000, in microsomal fractions from human liver. This was true of 44 different human liver samples analysed to date. In hepatic microsomal fractions from rats treated with MC, the antibody reacts with only cytochrome P450c, at $M_r$ 57,000 (Figure 1; Sesardic *et al.*, 1986). The relative molecular weights of the human isoenzymes corresponding to P450c (HLc) and P450d (HLd) in the rat have recently been published (Wrighton *et al.*, 1986a) together with data on the relative amounts of these two proteins in human liver microsomes. These authors could identify a protein analogous to P450d in all of the 14 samples analysed, but in only one of these were there detectable amounts of the analogue of P450c. Likewise, Jaiswal *et al.* (1987), using an antibody raised against mouse $P_3$-450, found only a single protein at $M_r$ 54,000 in eight human liver samples. Their antibody reacted equally well with rat P450c and P450d. The relative amounts of PAH-inducible forms of cytochrome P450 in the liver of untreated animals have been investigated in a number of different species. In general, the form analogous to P450d predominates over that analogous to P450c, between 6 to 10-fold in the rat (Goldstein & Linko, 1983; Thomas *et al.*, 1983), by four-fold in the mouse (Negishi & Nebert, 1979; Thomas *et al.*, 1984) and by 20-fold in the rabbit (Chiang *et al.*, 1982). From the work presented here, it would appear that a similar situation holds in man, in that the amount of the form orthologous to P450c is much less than the amount of the form orthologous to P450d, either because P450c is not expressed, or because it is below the detection limit of the assay.

Published estimates of the level of P450c in the liver of control rats vary from less than 1 to 5 pmol $mg^{-1}$ protein (Goldstein & Linko, 1983; Thomas *et al.*, 1983; Yeowell *et al.*, 1985) (less than 0.1–1% of total cytochrome P450 content). With 3/4/2, the limit of detection of P450c in the rat on Western blotting is 0.5 pmol $mg^{-1}$. No P450c can be detected in liver microsomes from untreated rats with this antibody (Sesardic *et al.*, 1986). Given that the limit of detection of the orthologue of P450c in human liver is the same as in the rat, the level of this isoenzyme cannot be greater than that in the rat (< 0.5 pmol $mg^{-1}$).

The binding site for MAb 3/4/2 on rat P450c is a linear sequence of amino acids (Sesardic *et al.*, 1986) and has now been identified (Edwards *et al.*, 1987). From the published primary amino acid sequences (Quattrochi *et al.*, 1986; Jaiswal *et al.*, 1987), it was predicted that the antibody would react with equal affinity in Western blotting with the human forms orthologous to both P450c and P450d. This, together with the molecular weight of the isoenzyme recognised by MAb 3/4/2 in human liver and the data on immunoquantification, make it very likely that the form of P450 recognised by this antibody in human liver is the orthologue of rat form d and not form c.

In previous studies in untreated rat, using specific inhibitory monoclonal antibodies, it was shown that high affinity POD activity in liver is catalysed exclusively by P450d (Boobis *et al.*, 1987). In the present study, a highly significant correlation was found between human hepatic POD activity and content of immunoreactive P450 orthologous to rat form d. Although this is not proof, it does provide evidence that the orthologue of cytochrome P450d in human liver catalyses the *O*-deethylation of phenacetin, as in the rat (but also see below).

It has long been known from studies *in vivo* that cigarette smoking increases the metabolism of a number of compounds in man (Vahakangas *et al.*, 1987) including phenacetin (Pantuck *et al.*,

1972, 1974). However, it has proved more difficult to demonstrate such an effect *in vitro*, with microsomal fractions from human liver. Differences in hepatic aryl hydrocarbon hydroxylaste activity (which is highly inducible in rat liver by PAH) between smokers and non-smokers are low or non-existent (Boobis *et al.*, 1980; Pelkonen *et al.*, 1986). In contrast, the high affinity components of the *O*-deethylation of both phenacetin (Kahn *et al.*, 1985) and 7-ethoxyresorufin (Pelkonen *et al.*, 1986) are two to three-fold greater in liver samples from smokers than in those from non-smokers.

The present study confirms that POD activity (high affinity component) of liver biopsy samples from smokers is greater than that of samples from non-smokers, by 4.2-fold. In the same liver samples, the levels of immunoreactive cytochrome P450, orthologous to rat form d, were 3.4-fold higher in smokers than in non-smokers. Human hepatic POD activity and content of immunoreactive P450 orthologous to rat form d are significantly correlated in samples from smokers and non-smokers. Thus, it appears that cigarette smoking induces the form of cytochrome P450 orthologous to form d in man and that this is responsible for all of the increased POD activity in these subjects. Further, the presence of detectable amounts of this isoenzyme in liver samples from non-smokers, the correlation between these levels and POD activity, and the fact that the only sample of 45 studied with no detectable POD activity was also the only one with no P450d, indicate the involvement of constitutive levels of the orthologue of form d in this activity. However, proof of the sole involvement of the human orthologue of P450d in catalysing high affinity POD activity must await the availability of inhibitory antibodies specific to this isoenzyme.

It is more than 20 years since it was first suggested that there might be a genetic impairment in human POD activity (Shahidi, 1968). Of 43 human liver samples analysed *in vitro* in this study for their ability to *O*-deethylate phenacetin, only one (P60) was found to be deficient in this activity. The same sample was also the only one which was completely negative on Western blotting with MAb 3/4/2 (Figure 5, lane 15). In previous reports (Boobis *et al.*, 1985a, c), it was concluded that sample P60 was from a subject PM for debrisoquine, based on its inability to 4-hydroxylate debrisoquine or to 1′-hydroxylate bufuralol, despite normal levels of aldrin epoxidase and spectrophotometrically determined cytochrome P450 content. Thus, the limited number of subjects characterised as PM for phenacetin to date (two defined *in vitro* and one *in vivo* (Kahn *et al.*, 1985 and unpublished observations)) were also all PM for debrisoquine. However, some subjects who are PM for debrisoquine have normal amounts of the orthologue of P450d and concomitant POD activity.

It has been established that the 4-hydroxylation of debrisoquine and the *O*-deethylation of phenacetin are catalysed by different isoenzymes of cytochrome P450 in man (Kahn *et al.*, 1985; Distlerath *et al.*, 1985), and the genes for these isoenzymes appear to be located on different chromosomes (Jaiswal *et al.*, 1987). Thus, the lack of correlation between the two activities (Kahn *et al.*, 1985 and the present study) is not unexpected. It remains to be determined whether the apparent association between impaired debrisoquine 4-hydroxylation and phenacetin *O*-deethylation in the same subjects is purely coincidental or due to a common regulatory defect.

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