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Induction and repression of the major phenobarbital-induced cytochrome *P*-450 measured by radioimmunoassay

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Two independent radioimmunoassay techniques for the major phenobarbital-inducible cytochrome *P*-450 (PB *P*-450) of rat liver microsomal membranes are described. The first technique employs as the source of radiolabelled antigen the products of translation *in vitro* labelled with [^{35}S]methionine. The second technique employs purified antigen labelled with ^{125}I and is quicker, less expensive and more precise. Both assays are highly specific for PB *P*-450 and can detect quantities of this variant as small as 1 ng. This is several orders of magnitude more sensitive than any method described previously for the quantification of cytochromes *P*-450, and consequently the technique is particularly well suited for the quantification of so-called constitutive cytochrome *P*-450 variants that are present in very low amounts. The results of the radioimmunoassays demonstrate that the apparent 2.6-fold induction of total cytochromes *P*-450 after phenobarbital treatment is due to a 43-fold increase in PB *P*-450. Although β-naphthoflavone increases the total content of cytochrome *P*-450 of microsomal membranes 1.4-fold, it actually causes a 55% decrease in the amount of PB *P*-450. Thus different xenobiotics can have differential effects on the expression of the genes for specific cytochrome *P*-450 variants.

The cytochrome-*P*-450-mediated mixed-function mono-oxygenase system is one of the major enzyme systems involved in the metabolism of steroid hormones and xenobiotics, including many drugs and carcinogens (Conney, 1967; Gelboin, 1967; Estabrook & Lindenlaub, 1979; Guengerich, 1979; Coon *et al.*, 1980; Lu & West, 1980). The enzyme system is present in many tissues in a wide range of organisms (Kato, 1979). Any one organism usually possesses a multiplicity of cytochromes *P*-450, which differ from each other in molecular weight, amino acid composition, substrate and reaction specificities and immunochemical properties (Guengerich, 1979; Johnson, 1979; Botelho *et al.*, 1979, 1982; Lu & West, 1980). The remarkable diversity of this protein is illustrated by the fact that, to date, 13 individual cytochrome *P*-450 fractions have been purified from just one tissue, rabbit liver (Sato *et al.*, 1982). The cytochromes *P*-450 are inducible by a variety of foreign compounds, such as phenobarbital and β-naphthoflavone. Different variants of cytochrome *P*-450 can be induced by different xenobiotics, and thus an absolute requirement for studying the molecular biology of the induction process is an assay that is sensitive, accurate and specific for a single cytochrome *P*-450 variant. Enzyme assays using specific substrates have limited use because of overlap in the substrate specificities of individual variants (Johnson, 1979). Fortunately there is a much higher degree of individual specificity in the immunochemical characteristics of the different cytochrome *P*-450 variants, and our approach has been to develop radioimmunoassays specific for individual cytochromes *P*-450.

We previously developed a radioimmunoassay for the major cytochrome *P*-450 variant induced by phenobarbital in rat liver microsomal membranes (PB *P*-450). This assay was based on the use of ^{35}S-labelled products of translation of polyribosomes *in vitro* as the source of radiolabelled antigen (Phillips *et al.*, 1981). The results obtained showed that the small increase observed in the specific content of total cytochromes *P*-450 after treatment with phenobarbital was due to a large induction of a specific cytochrome *P*-450 variant. The studies reported in the present paper demonstrate the

Abbreviations used: PB *P*-450, the major phenobarbital-inducible cytochrome *P*-450, of mol.wt. 52000; NF *P*-450, the major β-naphthoflavone-inducible cytochrome *P*-450, of mol.wt. 54000; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

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specificity of the radioimmunoassay and extend the results to include the effect of β-naphthoflavone (a class II inducer) on PB *P*-450. We also report the development of a quicker, less expensive and more accurate radioimmunoassay technique for PB *P*-450 based on the use as the radiolabelled antigen of purified PB *P*-450 labelled *in vitro* with ^{125}I. The results from both radioimmunoassays demonstrate that the amount of PB *P*-450 in rat liver microsomal membranes is greatly increased by phenobarbital, but is decreased by β-naphthoflavone.

Materials and methods

Animals

Male Sprague–Dawley rats (180–200g) bred at the University College animal facility were used in these experiments. The rats were fed (diet GR3 EK; Dixons and Sons, Ware, Herts., U.K.) and watered *ad libitum* under controlled lighting conditions of 12h light/12h dark. Rats treated with sodium phenobarbital were fed with a 0.1% (w/v) solution for 4 days and injected intraperitoneally on day 5 with a 4% (w/v) solution of sodium phenobarbital in 0.9% (w/v) NaCl (saline) at a dose of 40mg/kg. Rats treated with β-naphthoflavone were injected daily for 3 days with a 0.3ml injection of β-naphthoflavone in corn oil at a dose of 40mg/kg. Control animals received injections of 0.9% NaCl or corn oil. No difference in the amount of total cytochromes *P*-450 or of PB *P*-450 was observed when animals were treated with saline or corn oil. Animals were starved overnight before use and killed by cervical dislocation.

Isolation and solubilization of microsomal membrane vesicles

The following procedures were performed at 4°C. Total microsomal membrane vesicles were isolated from liver as described by van der Hoeven & Coon (1974). The microsomal membrane pellet was resuspended (at a final protein concentration of about 20mg/ml) in 10mM-potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol/1mM-EDTA, then flushed with nitrogen and stored at −78°C.

Microsomal membrane vesicles were thawed, flushed with nitrogen, homogenized briefly and solubilized by a modification of the procedure of Imai (1976). Membrane vesicles were diluted to a final protein concentration of 1.9mg/ml in 100mM-potassium phosphate buffer (pH 7.25)/20% glycerol/1mM-dithiothreitol/1mM-EDTA/2μM-FMN/23μM-butylated hydroxytoluene/0.4mM-phenylmethanesulphonyl fluoride. A 20% (w/v) solution of recrystallized sodium cholate (Guengerich, 1978) was added dropwise, with stirring at 0°C to a final concentration of 0.7% (w/v) (Guengerich & Martin, 1980). The sample was stirred for a further 15min and centrifuged at 100000*g* for 1h at 4°C. The supernatant was stored in batches at −78°C.

Purification of PB P-450, NF P-450 and NADPH–cytochrome P-450 reductase

PB *P*-450 (mol.wt. 52000) and NADPH–cytochrome *P*-450 reductase (mol.wt. 76000) were isolated from phenobarbital-treated rats, and NF *P*-450 (mol.wt. 54000) was isolated from β-naphthoflavone-treated rats by the method of Guengerich & Martin (1980).

Preparation of antibodies

Antibodies were raised in male New Zealand White or Red rabbits. Pure antigen was always mixed 1:1 (v/v) with Freund's complete adjuvant before injection. Rabbits were initially injected in the subscapular space with 50μg of antigen, and 3 weeks later they were re-injected with half this dose. After a further 5 weeks animals were given a second booster injection of 12.5μg of antigen. At 7 days after each booster injection, blood was withdrawn from the marginal ear vein and allowed to clot at room temperature for 30min. The clot was broken, left at 4°C overnight and then centrifuged at 10000*g* for 15min at 4°C. Antiserum was decanted and stored in batches at −20°C. Antibodies to injected antigens were always present in relatively high titre after the first booster injection, but sometimes increased in titre after the second booster injection.

Preparation of 'variant-specific' anti-(PB P-450) IgG

An enriched NF *P*-450 fraction (specific activity 12.6nmol/mg of protein) was coupled to CNBr-activated Sepharose-4B (Pharmacia) by the method of Thomas *et al.* (1979). Anti-(PB *P*-450) IgG [isolated as described by Phillips *et al.* (1981)] was chromatographed on the NF *P*-450 affinity column. The first IgG to be eluted from the column was termed 'variant-specific' anti-(PB *P*-450) IgG.

Iodination of PB P-450

To purified PB *P*-450 in 10mM-Tris/acetate (pH 7.4)/1mM-EDTA/20% (v/v) glycerol were added (final concns.) 0.4% (w/v) recrystallized sodium cholate and 0.2% (w/v) Lubrol PX (Sigma). The sample was dialysed overnight at 4°C against 500vol. of 0.1M-potassium phosphate (pH 7.5)/0.4% recrystallized sodium cholate/0.2% Lubrol PX (buffer A) with one change of dialysis buffer. Approx. 5μg of the protein in 20μl of buffer A was added to 0.5mCi of dried Bolton & Hunter (1973) reagent {*N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]-iodophenyl)propionate} (approx. 2000Ci/mmol; Amersham International). The sample was gently

agitated and left for 1h at room temperature with occasional shaking. The reaction was terminated by adding 0.5ml of 0.2M-glycine in buffer A and incubating for a further 2min. ^{125}I-labelled protein was separated from unincorporated Bolton & Hunter reagent by Sephadex G-75 column chromatography. The column (0.9cm × 30cm) had been previously equilibrated with buffer A at room temperature, washed with 100mg of bovine serum albumin (Sigma, fraction V, RIA grade) and re-equilibrated with buffer A. The iodinated protein was eluted with buffer A containing 0.25% (w/v) gelatin; 1ml fractions were collected into tubes containing 100μl of bovine serum albumin at a concentration of 60mg/ml. The fractions containing iodinated PB *P*-450 were made 1% with respect to both Triton X-100 and sodium deoxycholate and stored in batches at −78°C.

Radioimmunoassay of PB P-450 with ^{125}I-labelled PB P-450

All radioimmunoassays were performed in a final volume of 300μl in 50mM-Tris/HCl (pH 7.4) containing 1% (w/v) Triton X-100/1% (w/v) sodium deoxycholate/150mM-NaCl/5mM-EDTA/0.02% (w/v) NaN_3/0.25% (w/v) bovine serum albumin/0.6mM-phenylmethanesulphonyl fluoride. Samples containing 2×10^4c.p.m. were mixed with various quantities of the detergent-solubilized sample being analysed. An amount of anti-(PB *P*-450) serum sufficient to precipitate 50% of the precipitable iodinated protein in the absence of any competitor was added to each sample. The samples were incubated at 4°C overnight. Fixed *Staphylococcus aureus* cells [10μl of a 10% (w/v) suspension] were added and the samples incubated at room temperature for 1h. The cells were pelleted by centrifugation at 1700*g* for 30min at 4°C. The supernatants were removed by aspiration and the radioactivity in the pellets was determined by using a LKB–Wallac 1270 Rack-gamma II counter. Radioiodinated PB *P*-450 was very stable and was usable in the radioimmunoassay for up to 3–4 months.

Staphylococcus aureus cells

Fixed *Staph. aureus* cells were obtained from Michelle Ginsberg of the Imperial Cancer Research Fund Laboratories (Lincoln's Inn Fields, London) and were prepared for use as described previously (Phillips *et al.*, 1981), except that sodium deoxycholate was present in the first wash buffer at a final concentration of 1% and methionine was omitted from both wash buffers.

Radioimmunoassay of PB P-450 with ^{35}S-labelled PB P-450

[^{35}S]Methionine-labelled products of translation *in vitro* were used as a source for labelled PB *P*-450 in a radioimmunoassay as described previously (Phillips *et al.*, 1981).

SDS/polyacrylamide-gel electrophoresis

Purified proteins and microsomal membrane samples were analysed on 10%-acrylamide slab gels containing SDS as described previously (Phillips *et al.*, 1981).

Ouchterlony double-immunodiffusion analysis

This was done as described by Thomas *et al.* (1981). Antiserum was concentrated to 200mg/ml in an Amicon Minicon B clinical sample concentrator.

Determination of total cytochromes P-450

Cytochrome *P*-450 was assayed by the CO-reduced difference-spectral method of Omura & Sato (1964). Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. Samples containing non-ionic detergents were assayed for protein content by the method of Wang & Smith (1975).

Results

Purification of PB P-450 and NF P-450

The major cytochrome *P*-450 variants induced by phenobarbital (PB *P*-450) and β-naphthoflavone (NF *P*-450) were purified by chromatography on n-octylamino-Sepharose 4B and DEAE-cellulose columns to specific activities of 18.4 and 17.4nmol/mg of protein respectively (E. A. Shephard, I. R. Phillips, U. Gaul, S. F. Pike & R. M. Bayney, unpublished work). Analysis by electrophoresis on SDS/polyacrylamide gels (Fig. 1) demonstrated that both these protein preparations were homogeneous and that each cytochrome *P*-450 variant was apparently free of contamination by the other. On these gels the molecular weight of PB *P*-450 is 52000 and that of NF *P*-450 is 54000.

Specificity of antibodies to PB P-450

The apparent homogeneity of the PB *P*-450 does not rule out the possibility of contamination by small amounts of other microsomal proteins, which could also produce antibodies on injection into rabbits. This risk was lessened by the use of a protocol specifically designed to favour strongly the selective production of antibodies against only the major antigen. This procedure has been demonstrated to give this selectivity even in the presence of amounts of contaminating proteins that are visible on SDS/polyacrylamide gels (Harboe & Inglid, 1973; E. A. Shephard, unpublished work). Antiserum raised by this method to purified PB *P*-450 gave a single immunoprecipitation band on reaction with purified

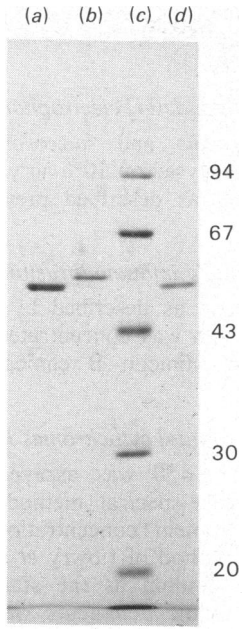

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of purified PB P-450 and NF P-450*

SDS, 2-mercaptoethanol and glycerol were added to the purified proteins to give final concentrations of 2, 1 and 15% respectively. Samples were heated at 100°C for 3 min, then electrophoresed on a 10%-polyacrylamide gel containing SDS. The gels were stained with Kenacid Blue. Gels: (*a*) 1.0 μg and (*d*) 0.5 μg of purified PB P-450; (*b*) 0.5 μg of purified NF *P*-450; (*c*) molecular-weight markers. Numbers give the molecular weights of marker proteins ($\times 10^{-3}$).

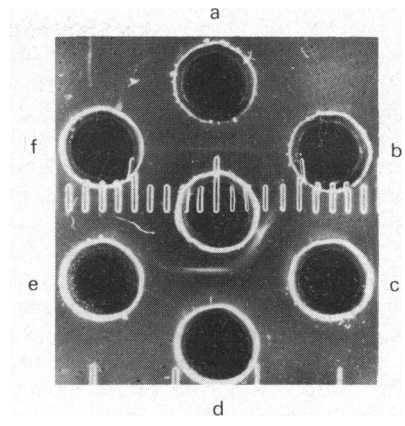

Fig. 2. *Ouchterlony double-immunodiffusion analysis using an antibody raised to PB P-450*

Antiserum (200 mg/ml) was placed in the centre well. Solubilized microsomal membranes isolated from control (4.5 mg/ml), β-naphthoflavone-treated (4.5 mg/ml) or phenobarbital-treated (3.8 mg/ml) rats were placed in wells a, b and c respectively. Purified PB *P*-450 (0.2 mg/ml), purified NF *P*-450 (0.2 mg/ml) and purified NADPH–cytochrome *P*-450 reductase (0.2 mg/ml) were placed in wells d, e and f respectively. All samples were added in a volume of 12 μl. Plates were incubated in a moist chamber at room temperature. Precipitin bands formed between the antiserum and either PB *P*-450 or membranes isolated from phenobarbital-treated animals were visible within 2 h, and those formed between the antiserum and the other solubilized microsomal membranes were visible after 24 h.

PB *P*-450 by Ouchterlony double immunodiffusion (Fig. 2). No cross-reactivity was observed between the anti-(PB *P*-450) serum and purified NF *P*-450 or NADPH–cytochrome *P*-450 reductase. A single immunoprecipitation band was also formed between the antiserum and solubilized total liver microsomal membrane proteins isolated from phenobarbital- or β-naphthoflavone-treated or control rats (Fig. 2). The band formed between the antiserum and purified PB *P*-450 gave a fusion pattern of identity with that formed between the antiserum and the microsomal membrane proteins. The band formed between the antiserum and microsomal membrane proteins isolated from phenobarbital-treated rats is more intense than that formed between the antiserum and microsomal membrane proteins from either β-naphthoflavone-treated or control animals (Fig. 2). These data suggest that the antiserum is specific for a single microsomal membrane protein (namely PB *P*-450) that is present in greater amounts in microsomal fractions of phenobarbital-treated rats. Further evidence for the specificity of anti-(PB *P*-450) antibodies is provided by our previous finding that the antibodies precipitate a single polypeptide band of the same molecular weight as purified PB *P*-450 from the products of liver RNA translation *in vitro* (Phillips *et al.*, 1981), but not from the translation products of soleus-muscle poly(A)-containing RNA (Shiels *et al.*, 1982) or pancreas total RNA (I. R. Phillips, E. A. Shephard & M. White, unpublished work).

^{35}S *radioimmunoassay of PB P-450*

A far more sensitive test for cross-reactivity of anti-(PB *P*-450) antibodies with other microsomal membrane proteins is provided by radioimmunoassay. Competition curves of purified PB *P*-450 and NF *P*-450 (Fig. 3) were obtained by using ^{35}S-labelled products of translation *in vitro* as a source of labelled PB *P*-450 in the radioimmunoassay system described previously (Phillips *et al.*, 1981). The radioimmunoassay detects PB *P*-450 from 2 to 200 ng, but does not detect NF *P*-450 in amounts as

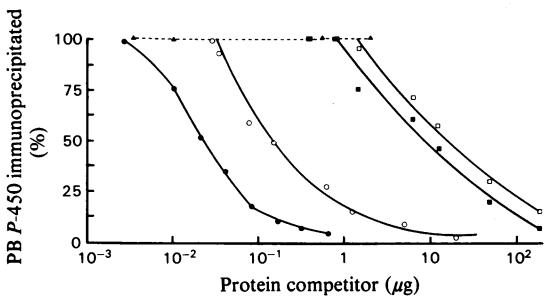

Fig. 3. *Radioimmunoassay of PB P-450 by ^{35}S technique*
^{35}S-labelled products of translation *in vitro* of polyribosomes isolated from the livers of phenobarbital-treated rats (1×10^6 acid-precipitable c.p.m.) were mixed with indicated quantities of unlabelled purified PB *P*-450 (●), unlabelled purified NF *P*-450 (▲), solubilized microsomal membrane proteins isolated from the livers of phenobarbital-treated (○), β-naphthoflavone-treated (□) or untreated (■) rats. Protein mixtures were incubated in a final volume of 1 ml with an amount of PB *P*-450 antiserum sufficient to precipitate 50% of the labelled protein in the absence of competitor. Antibody–antigen complexes were precipitated with *Staph. aureus* cells and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Radioactivity in the 52000-mol.wt. band was determined as described previously (Phillips *et al.*, 1981) and expressed as the percentage of the ^{35}S-labelled PB *P*-450 precipitated in the absence of competitor.

high as 2μg (i.e. 1000 times the minimum amount of PB *P*-450 detectable). The cross-reactivity between NF *P*-450 and anti-(PB *P*-450) serum is less than 0.1%, and the antiserum appears to be 'variant specific', thus establishing the specificity of the radioimmunoassay, which was used to quantify the amount of PB *P*-450 in liver microsomal membrane vesicles isolated from phenobarbital-, β-naphthoflavone- or untreated rats (Fig. 3). Phenobarbital treatment caused a 2.6-fold induction in the specific content of total cytochromes *P*-450 in microsomal membranes (Table 1). However, radioimmunoassay showed that PB *P*-450 was increased from 3.6 to 156μg/mg of microsomal membrane protein (Table 1), a 43-fold increase. The change in terms of the percentage of total cytochromes *P*-450 is from 5.1 to 86% on induction by phenobarbital. Treatment with β-naphthoflavone resulted in a 1.4-fold induction in the total content of cytochromes *P*-450 in microsomal membranes, but with an actual decrease of PB *P*-450 from 3.6 to 1.6μg/mg of microsomal membrane protein (Table 1). This represents a decrease of 56% in the amount of this cytochrome *P*-450 variant, which falls from 5.1 to 1.6% of the total cytochromes *P*-450. The very small amount of PB *P*-450 detected by the radioimmunoassay in control microsomal membranes indicates that there can be very little cross-reactivity between the many 'constitutive' cytochrome *P*-450 variants and anti-(PB *P*-450) serum. Although the results demonstrate that radioimmunoassay can be successfully used to

Table 1. *Quantification of PB P-450 in rat liver microsomal membrane vesicles*
Each determination was made in duplicate on microsomal membranes isolated from pools of 4 to 15 rat livers. Duplicates varied by less than 0.5%. (*a*), Total cytochromes *P*-450 were determined by CO difference spectral analysis as described in the Materials and methods section. (*b*) and (*c*), Data derived from Figs. 3 and 6. The amount of PB *P*-450 per mg of membrane protein was obtained from the point on the curve corresponding to 50% competition. (*d*), Calculated by the formula: $\% = 100 \times c/a$. (*e*), All cytochrome *P*-450 species that are not PB *P*-450; calculated by the formula: $e = a - c$.

Radioimmunoassay method	Pretreatment of rats	Total cytochromes *P*-450 (nmol/mg of microsomal membrane protein) (*a*)	PB *P*-450 (μg/mg of microsomal membrane protein) (*b*)	PB *P*-450 (nmol/mg of microsomal membrane protein) (*c*)	PB *P*-450 (% of total cytochromes *P*-450) (*d*)	Non-PB *P*-450 (nmol/mg of microsomal membrane protein) (*e*)
^{35}S	Untreated	1.37	3.6	0.070	5.1	1.30
	Phenobarbital	3.54	156	3.03	86	0.51
	β-Naphthoflavone	1.94	1.6	0.031	1.6	1.91
^{125}I	Untreated	1.37	2.9	0.057	4.2	1.31
	Phenobarbital	3.54	126	2.44	69	1.10
	β-Naphthoflavone	1.94	1.3	0.026	1.3	1.91

quantify a specific cytochrome *P*-450 variant in microsomal membranes, this particular radioimmunoassay technique is too expensive and time-consuming for the routine assay of relatively large numbers of samples. Therefore we developed a less complicated, more rapid and less expensive radioimmunoassay technique based on purified PB *P*-450 labelled *in vitro* with ^{125}I as the radiolabelled antigen.

Radioiodination of PB P-450

When purified PB *P*-450 was radioiodinated with the Bolton & Hunter reagent as described in the Materials and methods section, 18% of the radioactivity was incorporated into the protein, giving a specific radioactivity of 18 μCi/μg.

Specificity of the interaction between ^{125}I-labelled PB P-450 and anti-(PB P-450) serum

A maximum of about 60% of the ^{125}I-labelled PB *P*-450 was precipitable by anti-(PB *P*-450) serum (Fig. 4*a*), indicating that the antiserum is still able to interact with PB *P*-450 after the latter had been radioiodinated. The inability of the antiserum to precipitate all the iodinated antigen is not unusual (see, e.g., Heath *et al.*, 1982) and is probably due to either the blocking of some of the antigenic sites by the incorporated Bolton & Hunter reagent or radiation damage to the protein. The specificity of the immunoprecipitation reaction was demonstrated by determining the effect of substituting different antigens or antibodies. Anti-(PB *P*-450) serum was unable to precipitate ^{125}I-labelled NADPH–cytochrome *P*-450 reductase (Fig. 4*b*), and ^{125}I-labelled PB *P*-450 was precipitable only by anti-(PB *P*-450) serum and not by non-immune or anti-(NF *P*-450) serum (Fig. 4*a*).

Specificity of radioimmunoassay for PB P-450

It was first necessary to establish that unlabelled purified PB *P*-450 could compete with the radioiodinated antigen for binding sites on the antibody under the conditions used for the immunoprecipitation reaction. Radioiodinated PB *P*-450 was mixed with various amounts of unlabelled PB *P*-450, and a quantity of antiserum, sufficient to precipitate 50% of the total precipitable radioactivity in the absence of any competitor protein, was added. Samples were processed as described in the Materials and methods section. Fig. 5 shows that PB *P*-450 competes for essentially all the antibody-binding sites that recognize the radioiodinated antigen. The assay was capable of detecting PB *P*-450 in amounts as low as 1 ng (~20 fmol) and could be used to measure quantities up to 100 ng (2 pmol). We routinely use up to 100 μg of competitor protein in the assay. Thus it is possible to detect PB *P*-450 in amounts as low as 10 ng/mg of microsomal membrane protein, equivalent to concentrations of 0.2 nM. By increasing the total volume to 1 ml, the assay can accommodate up to 10 mg of competitor protein. The only major factor limiting

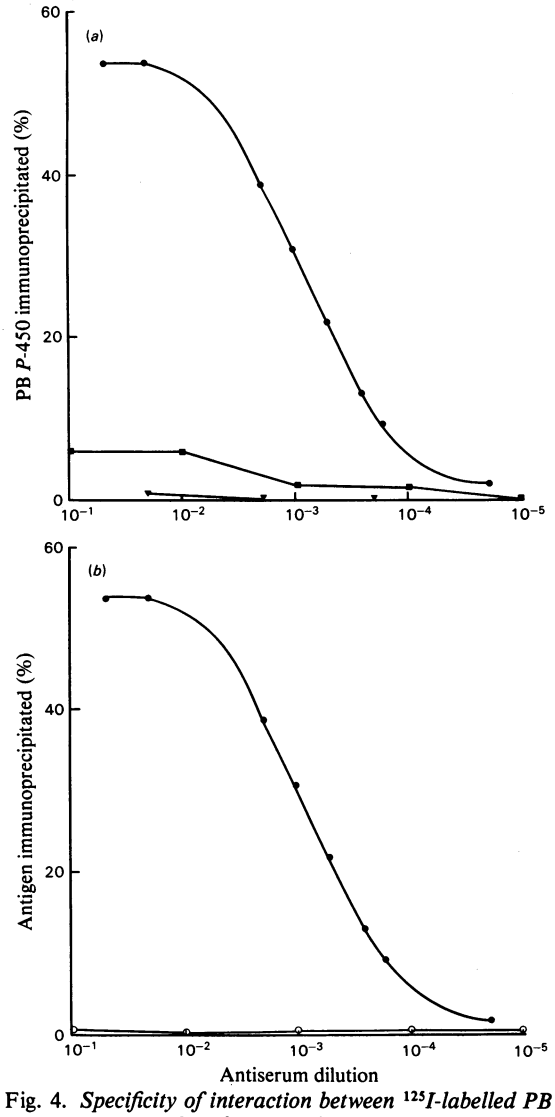

Fig. 4. *Specificity of interaction between ^{125}I-labelled PB P-450 and PB P-450 antiserum*

(*a*) Specificity of ^{125}I-labelled PB *P*-450. ^{125}I-labelled PB *P*-450 (2×10^4 c.p.m.) was incubated with the indicated amounts of anti-(PB *P*-450) serum (●), anti-(NF *P*-450) serum (■) or non-immune serum (▼). (*b*) Specificity of anti-(PB *P*-450) serum. The indicated amounts of this antiserum were incubated with 2×10^4 c.p.m. of ^{125}I-labelled PB *P*-450 (●) or ^{125}I-labelled NADPH-dependent cytochrome *P*-450 reductase (○). After subtraction of the small amount of radioactivity (approx. 2.5% of the total) that was precipitated in the absence of serum, radioactivity in the pellets was expressed as a percentage of total radioactivity.

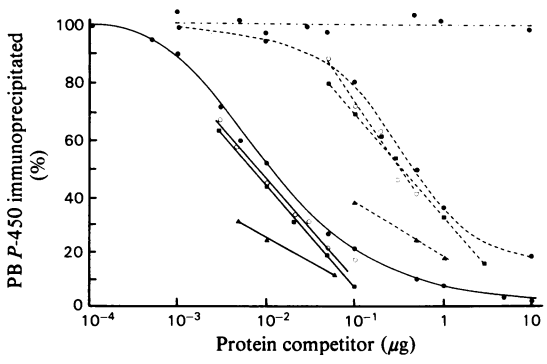

Fig. 5. *Specificity of radioimmunoassay of PB P-450 by the ^{125}I technique*

^{125}I-labelled PB *P*-450 (2×10^4 c.p.m.) was mixed with the indicated amounts of unlabelled purified PB *P*-450 (——), unlabelled purified NF *P*-450 (– – – –) or unlabelled purified NADPH-dependent cytochrome *P*-450 reductase (–·–·). Protein mixtures were incubated with the anti-(PB *P*-450) serum used in Figs. 2, 3, 4 and 6 (●), with anti-(PB *P*-450) serum isolated from a different rabbit (○), with antiserum to PB *P*-450 purified by a different method (described in the text) (■), or with the anti-(PB *P*-450) serum used in curve ○ after having been made 'variant-specific' (▲). The amount of each antiserum used was sufficient to precipitate 50% of the total immunoprecipitable radioactivity in the absence of competitor. Antibody–antigen complexes were precipitated with *Staph. aureus* cells. After subtraction of the amount immunoprecipitated by non-immune serum (approx. 2.5% of total), radioactivity in the pellet was expressed as the percentage of that immunoprecipitated in the absence of competitor.

the sensitivity of the assay is the upper concentration at which it is possible to solubilize microsomal membrane proteins. Thus it is possible with this assay to detect amounts of PB *P*-450 as low as 0.1 ng/mg of protein and concentrations as low as 2 pM. As a control for interference by microsomal membrane proteins other than cytochrome *P*-450, NADPH–cytochrome *P*-450 reductase was used as a competitor protein. This had no effect on the assay when added at 10 μg (i.e. <0.01% cross-reactivity) (Fig. 5). A more exacting test of the specificity of the radioimmunoassay is the degree of cross-reactivity observed with other cytochrome *P*-450 variants. A very small (~1.5%) degree of cross-reactivity was observed between purified NF *P*-450 and anti-(PB *P*-450) serum (Fig. 5). This was significantly more than was found for the [^{35}S]-methionine-based radioimmunoassay (Fig. 3). Since the purified NF *P*-450 and the anti-(PB *P*-450) serum were the same in both radioimmunoassay systems, the differences in cross-reactivity must be due to some difference in the labelled PB *P*-450. Perhaps modification during radioiodination produces some labelled PB *P*-450 molecules with affinity for the antibody decreased sufficiently to permit NF *P*-450 to compete with them for antibody binding sites.

Before using the ^{125}I-based radioimmunoassay to quantify PB *P*-450, we attempted to decrease the cross-reactivity. Anti-(PB *P*-450) sera of different titres, raised in different rabbits, and antisera raised to PB *P*-450 purified by an entirely different method [precipitation with $(NH_4)_2SO_4$, followed by column chromatography on DEAE-cellulose, CM-cellulose and hydroxyapatite], all gave essentially the same degree of cross-reactivity (Fig. 5). It should be possible to remove, by affinity chromatography, antibodies that recognize antigenic sites common to both PB *P*-450 and NF *P*-450. By the technique of Thomas *et al.* (1979), anti-(PB *P*-450) serum was passed through a column of partially purified NF *P*-450 (specific activity 12.6 nmol/mg of protein) bound to CNBr-activated Sepharose 4B. Antibodies that did not bind to the column were collected in small fractions and tested for cross-reactivity with purified NF *P*-450. However, these antibodies still exhibited the same degree of cross-reactivity with NF *P*-450 (Fig. 5). This was probably not due to a failure of the column to bind antibodies to NF *P*-450, because we have affinity-purified IgG species for NADPH–cytochrome *P*-450 reductase and serum albumin under similar conditions (E. A. Shephard & S. F. Pike, unpublished work). Fig. 5 shows that the radioimmunoassay for PB *P*-450 does not detect amounts of NF *P*-450 less than 100 ng. Thus the cross-reactivity could cause potential difficulties only when assaying small amounts of PB *P*-450 in the presence of large amounts of NF *P*-450, for example when assaying membranes isolated from β-naphthoflavone- or 3-methylcholanthrene-treated animals. However, even for these maximally unfavourable situations the errors are negligible, since every 10 ng of NF *P*-450 in excess of 100 ng is assayed as only 0.15 ng of PB *P*-450.

^{125}I-based radioimmunoassay of PB P-450 in microsomal membranes

The technique was used to determine the amount of PB *P*-450 in solubilized liver microsomal membrane vesicles isolated from phenobarbital- or β-naphthoflavone-treated or untreated rats (Fig. 6). The amount of PB *P*-450 in control animals was 2.9 μg/mg of microsomal membrane protein, and 4.2% of the total cytochromes *P*-450 (Table 1). Phenobarbital treatment increased the amount of this cytochrome *P*-450 variant to 126 μg/mg of microsomal membrane protein and 69% of total cytochromes *P*-450, representing a 43-fold induc-

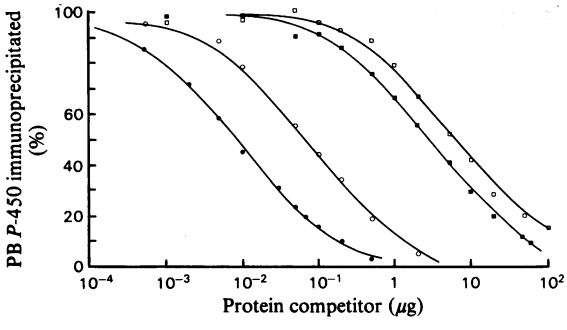

Fig. 6. *Radioimmunoassay by the ^{125}I method of PB P-450 in liver microsomal membrane vesicles isolated from phenobarbital-treated, β-naphthoflavone-treated and control rats*

^{125}I-labelled PB *P*-450 (2×10^4 c.p.m.) was mixed with the indicated amounts of unlabelled purified PB *P*-450 (●), or with solubilized microsomal membranes isolated from the livers of phenobarbital-treated (○), β-naphthoflavone-treated (□) or untreated (■) rats. Mixtures were incubated with an amount of anti-(PB *P*-450) serum sufficient to precipitate 50% of the total immunoprecipitable radioactivity. Antibody–antigen complexes were precipitated with *Staph. aureus* cells. After subtraction of the amount immunoprecipitated by non-immune serum, radioactivity in the pellets was expressed as the percentage of that immunoprecipitated in the absence of competitor.

tion of PB *P*-450. The very low amount of PB *P*-450 detected by this radioimmunoassay in control microsomal membranes demonstrated the lack of cross-reactivity between the many constitutive cytochrome *P*-450 variants and anti-(PB *P*-450) serum. A more exacting test of the assay is provided by the experiment using microsomal membranes from animals treated with β-naphthoflavone. This caused a 1.4-fold induction of the total cytochromes *P*-450 content of rat liver microsomal membranes. However, the amount of PB *P*-450 was found to be decreased on treatment with this xenobiotic from 2.9 to 1.3 μg/mg of microsomal membrane proteins, representing a net decrease in amount of 55%. The relative proportion of this cytochrome *P*-450 variant as a percentage of the total cytochromes *P*-450 decreased from 4.2 to 1.3. The results obtained by the ^{125}I radioimmunoassay (Fig. 6; Table 1) were very similar to those obtained with the ^{35}S radioimmunoassay (Fig. 3; Table 1), demonstrating that any alteration or damage sustained by the PB *P*-450 during radioiodination *in vitro* did not affect the results obtained by radioimmunoassay.

Further evidence for the decrease in certain cytochromes *P*-450 after xenobiotic treatment is provided by the finding (Table 1) that phenobarbital treatment, although greatly increasing PB *P*-450, causes an overall decrease in the amount of total non-phenobarbital-inducible cytochromes *P*-450.

Discussion

We have developed two independent radioimmunoassay techniques for the precise quantification of a specific cytochrome *P*-450 variant. The first involved the use of [^{35}S]methionine-labelled products of translation *in vitro* as a source of radiolabelled antigen, and the second used ^{125}I-labelled purified PB *P*-450. The latter method is operationally simpler and very much cheaper, and is the method of choice. It involves no manual measurement, is several orders of magnitude more sensitive and is more precise than the other techniques used immunochemically to quantify specific cytochrome *P*-450 variants, such as radial immunodiffusion (Thomas *et al.*, 1979, 1981), rocket immunoelectrophoresis (Pickett *et al.*, 1981), or gel electrophoresis followed by immunochemical staining (Guengerich *et al.*, 1982). An additional advantage of the radioimmunoassay over other techniques is that it is possible to check for very low extents of cross-reactivity. The specificity of our antibodies compared favourably with that reported by Thomas *et al.* (1979) and is greater than that found by Dus *et al.* (1980) or Guengerich *et al.* (1981). The ^{125}I radioimmunoassay is particularly well suited to the quantification of small amounts of specific cytochrome *P*-450 variants present in 'control' samples, in addition to samples from animals treated with a variety of inducers. The technique should be extremely useful for the analysis of so-called 'constitutive' forms of cytochrome *P*-450.

We have shown, using both radioimmunoassay techniques, that phenobarbital causes an approximately 43-fold induction of PB *P*-450, whereas β-naphthoflavone depresses the amount of this cytochrome *P*-450 below the low values present in controls. Our results confirm and extend those of Thomas *et al.* (1979, 1981) by radial immunodiffusion, Pickett *et al.* (1981) by rocket immunoelectrophoresis and Guengerich *et al.* (1982) by electrophoresis followed by immunochemical staining. They support our earlier conclusion (Phillips *et al.*, 1981) that the relatively small (2–3-fold) induction of the specific content of cytochromes *P*-450 in rat liver microsomal membranes by phenobarbital is due mainly to a large increase in the amount of a specific cytochrome *P*-450 variant (PB *P*-450). The larger phenobarbital induction of PB *P*-450 found in the present study compared with that reported previously (Phillips *et al.*, 1981) is probably due to a difference in the regime of phenobarbital treatment, which results in a greater increase

in the specific content of total cytochromes *P*-450. β-Naphthoflavone treatment, although inducing a different cytochrome *P*-450 variant (namely NF *P*-450) does not induce PB *P*-450, indicating that the induction of specific cytochrome *P*-450 variants occurs via a very selective mechanism.

The results in Table 1 and those found previously by Phillips *et al.* (1981) give indirect evidence that long-term phenobarbital treatment causes a decrease in the total amount of non-phenobarbital-inducible cytochromes *P*-450. This suggests that, in addition to causing an increase in one or a few specific cytochrome *P*-450 variants, a xenobiotic can decrease the amounts of other cytochrome *P*-450 variants. Several other groups have drawn attention to this possibility from results obtained by one- and two-dimensional gel electrophoresis (McIntosh *et al.*, 1980; Vlasuk & Walz, 1982; Vlasuk *et al.*, 1982), and from the observed decrease in testosterone 16α-hydroxylase activity in microsomal membranes after treatment by 3-methylcholanthrene (Kuntzman *et al.*, 1968; Kremers *et al.*, 1978).

The radioimmunoassay data reported in the present study provide direct evidence that β-naphthoflavone treatment decreases the quantity of PB *P*-450. Thus the process of induction of cytochromes *P*-450 by xenobiotics could be more complicated than originally thought, involving both a large increase of one or a few specific cytochrome *P*-450 variants from a low content in control membranes, together with a decrease in the amounts of other cytochrome *P*-450 variants. Analysis by a radioimmunoassay specific for NADPH-dependent cytochrome *P*-450 reductase of the same solubilized microsomal membrane preparations from control and β-naphthoflavone-treated animals showed that β-naphthoflavone treatment had no effect on the amount of this enzyme (Shephard *et al.*, 1983). This indicates that the repressive effect of this xenobiotic on components of the mixed-function mono-oxygenase system is at least partially specific to PB *P*-450. The decrease in PB *P*-450 on treatment by β-naphthoflavone could be due to an induced proliferation of microsomal membranes that has been reported (Ernster & Orrenius, 1965; Orrenius *et al.*, 1965; Fouts & Rogers, 1965; Stäubli *et al.*, 1969; Phillips *et al.*, 1981), with no compensating increase in the expression of PB *P*-450 genes. However, we have found that β-naphthoflavone treatment results in a 53% decrease in translatable mRNA coding for PB *P*-450 (Shephard *et al.*, 1982), indicating that the observed decrease of the protein by this xenobiotic could be due to a decrease in PB *P*-450 gene expression.

During the course of this investigation, Vlasuk *et al.* (1982) have reported the existence of as many as four phenobarbital-inducible cytochrome *P*-450 variants that have >95% fingerprint homology and are immunochemically identical. It should be noted that these cytochrome *P*-450 variants were identified in Long–Evans and Holtzmann rat strains, and they vary between strain and breeding colony. In any one breeding colony only two or three of the phenobarbital-induced variants were present. Although it is not clear whether a similar microheterogeneity exists in the phenobarbital-inducible cytochrome *P*-450 of mature Sprague–Dawley rats of the University College breeding colony, this possibility must be considered. The existence of multiple, immunochemically indistinguishable, phenobarbital-inducible cytochrome *P*-450 variants would mean that the immunochemical quantification of a 'specific' cytochrome *P*-450 variant would, in fact, represent the sum of all the immunochemically indistinguishable cytochrome *P*-450 variants. Owing to the similarity of the molecular weights of these proteins, even the technique of electrophoresis in one dimension followed by immunochemical staining (Guengerich *et al.*, 1982) could not distinguish them adequately. If we assume that our radioimmunoassay detects multiple related cytochrome *P*-450 variants, this will not affect our main conclusions that the induction process involves the enhanced expression of genes for specific cytochrome *P*-450 variants.

The observed 43-fold induction by phenobarbital of PB *P*-450 would then represent the average induction of all the proteins immunoprecipitated, with the possibility that there is even greater enhancement of the expression of particular genes. Thus the small increase in total cytochrome *P*-450 content after phenobarbital treatment is due to a relatively very much larger induction of one or a few specific cytochrome *P*-450 variants, which represent only a small proportion of the cytochromes *P*-450 in microsomal membranes from control rats. β-Naphthoflavone treatment decreases the amount of at least one cytochrome *P*-450 variant that is induced by phenobarbital.

The molecular mechanisms responsible for the changes in particular cytochromes *P*-450 are not yet understood. It is, however, apparent that increases in the amounts of specific cytochrome *P*-450 variants are accompanied by increases in their mRNA (Dubois & Waterman, 1979; Colbert *et al.*, 1979; Kumar & Padmanaban, 1980; Phillips *et al.*, 1981; Bresnick *et al.*, 1981; Adesnick *et al.*, 1981; Negishi & Nebert, 1981).

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