Comp. Biochem. Physiol. Vol. 96C, No. 1, pp. 163–176, 1990
Printed in Great Britain

0306-4492/90 $3.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF CYTOCHROME P-450 ISOZYMES FROM PHENOBARBITAL-INDUCED ADULT HEN LIVER*

RAM P. GUPTA, DANIEL M. LAPADULA and MOHAMED B. ABOU-DONIA†
Laboratory of Neurotoxicology, Department of Pharmacology, Duke University Medical Center, Durham, North Carolina, U.S.A. Telephone: (919) 684-2221

(*Received* 12 *December* 1989)

Abstract—1. Two cytochrome P-450 isozymes (P-450 PB-A, PB-B) and cytochrome b_5 were purified from livers of phenobarbital-treated adult hens.

2. Both the enzymes exhibited the same apparent molecular weight (54,000).

3. They could be distinguished on the basis of immunochemical properties, spectral properties, peptide pattern after partial proteolysis, tryptic peptide pattern, and N-terminal sequence.

4. The antibodies raised against P-450 PB-A and PB-B did not cross-react with microsomal P-450s of rat, mice, cat, or catfish species by immunoblotting.

INTRODUCTION

A wide variety of drugs, endogenous substrates (e.g. fatty acids and steroids), and environmental pollutants are oxidized by a microsomal mixed-function oxidase system that consists of cytochrome P-450 and NADPH cytochrome P-450 reductase (Wislocki *et al.*, 1980). The oxidation product may be further degraded by epoxide hydrolase and by glutathione alkyl and aryl transferases, esterases, and hydrolases. The intermediate product may act as a carcinogenic, mutagenic, or toxic agent (Wislocki *et al.*, 1980). The broad substrate specificity of the mono-oxygenase system is partially ascribed to the presence, induction, or suppression of distinct forms of cytochrome P-450 in the host liver (Haugen and Coon, 1976; Guengerich *et al.*, 1982; Wang *et al.*, 1983; Duignan *et al.*, 1987; Funae and Imaoka, 1987). There is overlapping in the substrate specificity of individual P-450 isozymes, even though some substrates are more efficiently metabolized than others by distinct P-450 isozymes (Guengerich *et al.*, 1982; Funae and Imaka, 1987). The metabolism of xenobiotics by the monooxygenase system may lead to either bioactivation or detoxification depending on the level and properties of cytochrome P-450 isozymes modulating various pathways of degradation (Hansen, 1984).

The purification of a large number of cytochrome P-450 isozymes from several animal species demonstrates the existence of multiple forms of this enzyme. They have been generally purified from mammalian liver and lung microsomes, e.g. rat, rabbit, dog, mouse, and human (Nebert *et al.*, 1989). There are few reports of purification of P-450s from chick embryos (Brooker *et al.*, 1983; Sinclair *et al.*, 1989), and probably only one report on the purification of this hemoprotein from adult rooster liver (Oron and Bar-Nun, 1984). Cytochrome P-450 isozymes are usually differentiated from each other on the basis of molecular weights, spectral properties, immunochemical characteristics, rate of metabolism, inhibition by inhibitors, substrate specificity, and chromatographic behaviour. Phenobarbital and 3-methylcholanthrene are traditional inducers of two types of P-450, and have been invariably used to induce these hemoproteins in animal species.

The adult hen is currently specified as a test animal by regulating agencies for evaluating delayed neurotoxic potential of organophosphorous esters (Abou-Donia, 1981). It is also being used in laboratories for evaluating neurotoxicity of other compounds (Abou-Donia, 1981; Abou-Donia *et al.*, 1985). Despite increasing recognition of the hen in neurotoxic studies, the P-450-dependent metabolism of xenobiotics has not been well explored in this non-mammalian species. In this paper, we report the purification and characterization of at least two cytochrome P-450 isozymes (P-450 PB-A and PB-B) from phenobarbital-treated hens. The cytochrome P-450 PB-B, however, seemed to appear at three places in our purification procedure, since fractions PB-B_1, PB-B_2, and PB-B_3 were very similar and could not be unambiguously distinguished from each other.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: sodium cholate, sodium deoxycholate, DL-dithiothreitol, phenylmethylsulfonyl fluoride, Sepharose 4B, Lubrol PX, α-chymotrypsin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, glycerol, citric acid, ethylenediaminetetraacetic acid, aminopyrine, and benzo(α)pyrene (Sigma Chemical Co., St. Louis, MO); 1,8-diamino-octane, 1,4-dioxane, cyanogen bromide, sodium dithionite, 7-ethoxycoumarin, 7-hydroxycoumarin (Aldrich Chemical Co., Inc., Milwaukee, WI); hydroxylapatite (Bio-Gel HT); electrophoretic reagents (Bio-Rad Laboratories, Richmond, CA);

*This work was supported by grant No. OHO 0823 from the National Institute of Occupational and Safety Health of the Centers for Disease Control.
†To whom requests for reprints should be addressed.

Ampholines (LKB-Produkter AB, Bromma, Sweden); DE-51, DE-52, DE-53, CM-52 (Whatman BioSystems Ltd, Maidstone, Kent); Freund's complete and incomplete adjuvant (Difco Laboratories, Detroit, MI); *Staphylococcus aureus* V8 protease (Miles Laboratories, Elkhart, IN), and ^{125}I-Protein A (New England Nuclear, Newark, DE). All other chemicals were of the highest purity commercially available.

Buffers and solutions

(A) 0.1 M KH_2PO_4, pH 7.25, 1 mM EDTA, 20% glycerol. (B) 0.1 M KH_2PO_4, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.35% sodium cholate, 0.15% sodium deoxycholate, 2 μM FMN. (C) 40 mM KH_2PO_4, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.3% Lubrol PX. (D) 5 mM KH_2PO_4, pH 7.7, 0.1 mM EDTA, 20% glycerol, 0.1% Lubrol PX, 0.2% sodium cholate. (E) 5 mM KH_2PO_4, pH 6.5, 0.1 mM EDTA, 20% glycerol, 0.2% Lubrol PX. (F) 5 mM KH_2PO_4, pH 7.7, 0.1 mM EDTA, 20% glycerol, 0.05% Lubrol PX, 0.1 mM DTT. (G) 20 mM Tris-acetate, pH 8.1, 0.1 mM EDTA. (H) 10 mM Tris-acetate, pH 8.1, 0.1 mM EDTA. (I) 10 mM Tris-acetate, pH 7.4, 1 mM EDTA, 20% glycerol. (J) overlay solution, 5% NP-40, 8 M urea, 0.8% Ampholines, pH 5–7, 0.2% Ampholines, pH 3–10. (K) 1.5% SDS, 0.375 M Tris-HCl, pH 6.8. (L) 5 mM KH_2PO_4, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.2 mM DTT. (M) 50 mM KH_2PO_4, pH 7.4, 1.0 mM EDTA, 20% glycerol, 0.1 mM DTT. PMSF was made 0.4 M in ethanol and added to the buffer just before using. All pH measurements were made at the temperature at which they were going to be used (4°C or room temperature).

Purification of cytochrome P-450s

Adult leghorn hens, 14 months old and weighing 1.69 ± 0.25 kg (mean ± SD), were obtained from Featherdown Farm, Raleigh, NC. The hens were specific pathogen- and chemical medication-free. They were intraperitoneally injected once daily for four days with 20 mg/ml of phenobarbital sodium (80 mg/kg), and feed was withheld for 24 hr prior to experimentation. They had free access to water during treatment. The birds were anesthetized with carbon dioxide and killed by decapitation 24 hr after the last dose. The microsomes were prepared and solubilized as described by Guengerich and Martin (1980), with the following exceptions. The livers were homogenized twice with a Polytron (setting 7) for 30 sec, and the microsomes were suspended with dounce homogenizer during washing with pyrophosphate buffer. The microsomal cholate solution was used for *n*-octylamino-Sepharose 4B column chromatography as described by Guengerich *et al.* (1982). The columns were eluted with buffer A containing 0.33% cholate and 0.06% Lubrol PX (1200 ml), buffer A containing 0.33% cholate and 0.25% Lubrol PX (1000 ml), buffer A containing 0.33% cholate and 0.5% Lubrol PX (1000 ml), and buffer B (1500 ml). Throughout column chromatography of the P-450 isozymes, selected fractions were read at 280 and 417 nm and scanned by SDS-PAGE followed by silver staining. The pooled fractions were concentrated in Amicon Ultrafiltration Cell using PM-30 diaflo-membrane.

Cytochromes P-450 PB-B_1 and PB-B_2. Fractions eluted with buffer A containing 0.33% sodium cholate and 0.06% Lubrol PX from *n*-octylamino-Sepharose 4B columns were concentrated, diluted five-fold with 20% glycerol, and loaded on an HA column (4.4 × 15 cm) equilibrated with buffer C. The flow was about 90 ml/hr. The column was washed with equilibrium buffer and eluted with buffer C containing 90 mM KH_2PO_4 (1500 ml) and 180 mM KH_2PO_4 (1500 ml). Fractions eluted with buffer C containing 90 mM KH_2PO_4 were used for isolation of cytochromes P-450 PB-B_1 and PB-B_2. The selected fractions were pooled, concentrated, dialyzed twice against 20 volumes of buffer D, and applied on a series of three columns of DE-51 (1.5 × 10 cm), DE-52 (1.5 × 15 cm), and DE-53 (1.5 × 25 cm) (Guengerich *et al.*, 1982). This column chromatography was performed at room temperature (23°C). The majority of cytochrome P-450 passed through the column with buffer D. This was concentrated, dialyzed against 20 volumes (two times) of buffer E, and loaded onto a CM-52 column (1.5 × 15 cm) equilibrated with the same buffer. The flow rate was 20 ml/hr. The column was washed with buffer E containing 20 mM NaCl and then eluted with buffer E containing either 30 or 40 mM NaCl to get fractions for further purification of cytochrome P-450 PB-B_1 or PB-B_2, respectively.

Cytochrome P-450 PB-B_1. Fractions eluted with buffer E containing 30 mM NaCl were concentrated, dialyzed (two times) against 20 volumes of buffer C containing 20 mM KH_2PO_4, and applied to an HA column (1.5 × 15 cm) equilibrated with the same buffer. The flow rate was 20 ml/hr. The column was washed with the dialysis buffer, and then the fractions were eluted with buffer C containing 80 mM KH_2PO_4 to obtain pure cytochrome P-450 PB-B_1.

Cytochrome P-450 PB-B_2. Fractions eluted above from the CM-52 column with buffer E containing 40 mM NaCl were concentrated, dialyzed against buffer C containing 20 mM KH_2PO_4, and loaded onto an HA column (1.5 × 15 cm) at a flow rate of 20 ml/hr. The column was washed with the buffer C containing 30 mM KH_2PO_4 and eluted with buffer C containing 70 mM KH_2PO_4 to obtain pure cytochrome P-450 PB-B_2.

Cytochrome P-450 PB-B_3. Fractions eluted with buffer C containing 180 mM KH_2PO_4 from HA-column (4.4 × 15 cm) were subjected to DE-51, DE-52 and DE-53 column chromatography as described above. This enzyme passed through these columns during washing with the equilibration buffer D. The fractions were concentrated, dialyzed two times against 20 volumes of buffer E, and loaded onto a CM-52 column (1.5 × 15 cm) at a flow rate of 20 ml/hr. The column was washed with buffer E containing 10 mM NaCl and then eluted with buffer E containing 40 mM NaCl to obtain pure P-450 PB-B_3.

Cytochrome P-450 PB-A. Fractions eluted with buffer A containing 0.33% sodium cholate and 0.25% Lubrol PX from *n*-octylamino-Sepharose 4B columns were concentrated, diluted five-fold with buffer C containing 5 mM KH_2PO_4 and 0.5 mM DTT, and applied onto an HA column (1.5 × 25 cm) equilibrated with the same buffer. The column was washed with buffer C containing 20 mM KH_2PO_4 and 0.5 mM DTT at a flow rate of 20 ml/hr. The washed material was concentrated, dialyzed against buffer F, and loaded onto a DE-52 column (1.5 × 25 cm) equilibrated with buffer F. The column was washed with buffer F, and pure P-450 PB-A was eluted with buffer F containing 25 mM KCl.

Cytochrome b_5 and NADPH-cytochrome P-450 reductase

Cytochrome b_5 was purified from hen livers treated for four days with phenobarbital sodium. The isolation of microsomes, extraction with cold acetone, solubilization with 2% Triton X-100 and loading onto a DE-52 column (1.5 × 50 cm) was performed as described by Strittmatter *et al.* (1978). The flow rate was 45 ml/hr. The DE-52 column was washed with buffer G containing 0.3% sodium deoxycholate until there was no appreciable absorption by the effluent at 280 nm. Further purification of cytochrome b_5 was accomplished by modification of the method of Waxman and Walsh (1982). The DE-52 containing cytochrome b_5 was suspended in buffer G containing 0.3% DOC and loaded on another DE-52 column (1.5 × 50 cm) equilibrated with the same buffer. The enzyme was eluted with a 0–0.2 M NaSCN gradient (1 liter each) in buffer G containing 0.3% DOC. The cytochrome b_5-containing fractions were pooled and dialyzed (2 times) against 4 l of buffer G containing 0.3% DOC. The dialysate was passed through a 2′,5′-ADP-agarose column (10 ml) equilibrated with the

same buffer. The effluent was diluted with two volumes of buffer H and loaded on an HA-column (1.5 × 15 cm) equilibrated with buffer H. The HA column was washed with buffer H containing 50 mM KCl and 0.1% DOC, buffer H containing 50 mM KCl, 0.1% DOC, and 20 mM KH_2PO_4, and then eluted with buffer H containing 50 mM KCl, 0.2% DOC and 20 mM KH_2PO_4. The effluent was diluted with an equal volume of buffer H and loaded on another HA column (1.5 × 15 cm) equilibrated with buffer H. The column was washed with buffer H containing 0.2% DOC and 10 mM KH_2PO_4, and eluted with the same buffer containing 20 and 30 mM KH_2PO_4 to get two pure cytochrome b_5 fractions.

NADPH-cytochrome P-450 reductase was purified from the fractions eluted with buffer B from *n*-octylamino-Sepharose 4B columns. The procedure described by Guengerich and Martin (1980) was used except that 2 μM FMN was included in all the washing buffers and a column containing 20 ml of 2′,5′-ADP-Agarose was used.

Removal of detergent from cytochrome P-450 preparations

Purified P-450s containing Lubrol PX were concentrated, dialyzed (2 times) against 20 volumes of buffer I, and mildly shaken overnight at 4°C with 1 g of XAD-2 per 100 ml of solution. If lather was still visible, another g of XAD-2 was added to the same tube and mildly mixed again for 4 hr. The absorbent was removed by filtering the solution through glass wool packed in a 5 ml syringe and the glass wool was washed with buffer I. The filtrate was again dialyzed against buffer I and stored in aliquots at −70°C. The cytochrome P-450 PB-A was loaded onto an HA column (1.5 × 10 cm) equilibrated with buffer L containing 0.05% Lubrol PX. The column was washed with 500 ml of buffer L containing 0.2% cholate to remove Lubrol PX, and then eluted with buffer L containing 0.2% cholate and 0.3 M KH_2PO_4. The fractions were concentrated, dialyzed against buffer M and preserved at −70°C.

Immunochemical methods

New Zealand white rabbits were used to raise antibodies against pure proteins. P-450s (0.50 nmol) were subcutaneously injected two times at one month intervals after emulsification with 2 volumes (1 ml) of complete Freund's adjuvant. A booster injection with the same amount of P-450 in incomplete Freund's adjuvant was given 15 days later and the hens were bled 10 days after the last injection. The specificity of these antibodies was assessed by Ouchterlony double diffusion analysis and immunoblotting (Lapadula *et al.*, 1986). Immunodiffusion plates (Cooperbiomedical, Inc., Malvern, U.S.A.) contained agar and sodium azide but no ionic or non-ionic detergent. The antiserum (3–6 μl) was pipetted into the center well, and pure P-450s (6 pmol) were added to the outer wells. The plates were incubated at room temperature for 72 hr under a humid atmosphere, and the immunoprecipitin bands were stained by amido black. The immunoblotting method consisted of SDS-PAGE, electrophoretic transfer onto nitrocellulose sheets, and development of immunoblots with antiserum (1000–2000 times dilution) and ^{125}I-protein A (Lapadula *et al.*, 1986).

Analytical procedures

The protein was estimated by the method of Smith *et al.* (1985) using bovine serum albumin as the protein standard. Cytochrome P-450 was estimated in microsomes by the method of Matsubara *et al.* (1976) with the use of an extinction coefficient of 106 cm^{-1} mM^{-1}, and at other steps of purification by the procedure of Omura and Sato (1964) using 91 cm^{-1} mM^{-1} as the extinction coefficient. NADPH-cytochrome P-450 reductase was determined at room temperature by absorbance difference at 550 nm (Strobel and Dignam, 1978) using cytochrome c as the substrate. Cytochrome b_5 was measured by its reduced minus oxidized spectrum. The photoheme content of P-450 was determined from the difference spectra of dithionite (reduced) versus potassium ferricyanide (oxidized) pyridine hemochromes (Falk, 1964). The SDS-PAGE was performed on 1.5 mm-thick gels at room temperature (Laemmli, 1970). Stacking and resolving gels contained 4 and 7.5% total acrylamide, respectively. Gels were silver stained (Wray *et al.*, 1981). Two-dimensional electrophoresis was carried out according to the method of O'Farrell *et al.* (1977). Samples were

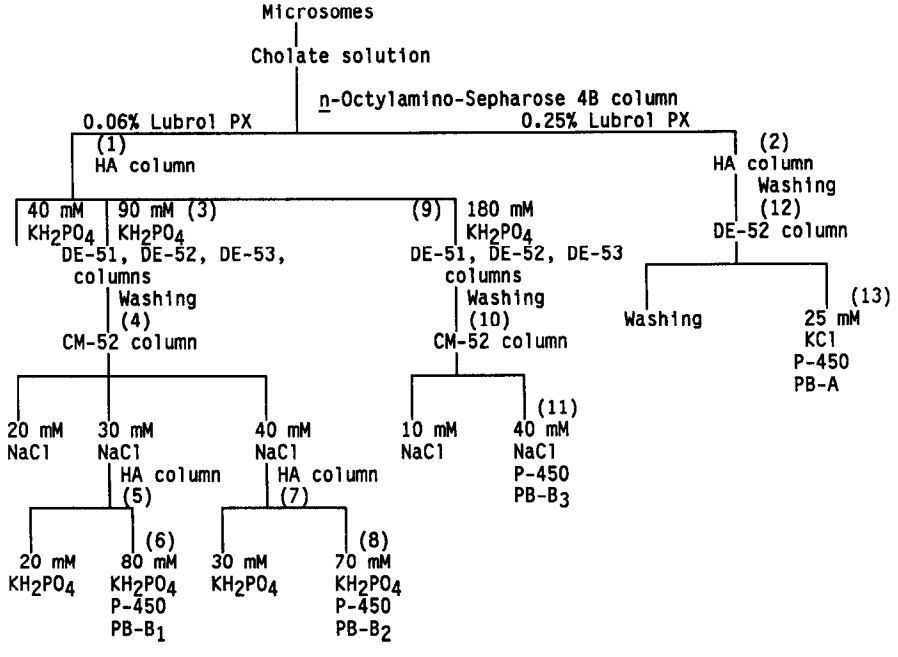

Fig. 1. Scheme for the purification of cytochrome P-450s from phenobarbital-induced hen liver. *n*-octylamino-Sepharose 4B, HA, DE-51, DE-52, DE-53, and CM-52 refer to the material used in column chromatography. The composition of buffers is described in the Materials and Methods section.

dissolved in lysis buffer containing NP-40 and overlaid with 25 μl of solution J before performing NEPHGE at 400 volts for 5 hr (O'Farrell *et al.*, 1977). Second dimension SDS-PAGE was carried out on 7.5% acrylamide gels. The molecular weights of the P-450 isozymes and b_5 were determined from plots of log molecular weight of protein standards versus mobility on SDS-PAGE gels. The protein standards used were phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Peptide mapping and fingerprinting

Peptide mapping of the P-450s was performed by partial hydrolysis with α-chymotrypsin and *Staphylococcus aureus* V8 protease (Cleveland *et al.*, 1977). Twenty-five pmoles of cytochrome P-450 in 25 μl was mixed with 12.5 μl of SDS solution K and heated for 2 min at 100°C. The denatured protein was incubated at 37°C for 30 min with 12.5 μl of α-chymotrypsin (7.5 ng) or *S. aureus* V8 protease (250 ng) solution. The hydrolysis was stopped by adding 2 × SDS sample buffer and heating at 100°C for 3 min (Laemmli, 1970). The samples were electrophoresed and silver-stained as above. Coomassie Blue-stained slices obtained from SDS-PAGE of pure proteins samples (20 pmol) were used for peptide fingerprinting. The cytochrome P-450s in slices were radiolabeled with ^{125}I and used for tryptic fingerprinting as described by Elder *et al.* (1977). The iodination was performed for 1 hr. The samples containing 8×10^5 cpm were electrophoresed at 600 V for about 40 min on cellulose-coated TLC (10 × 20 cm) plates.

N-terminal sequence of proteins

The purified enzymes were dialyzed against deionized water for 2 days, precipitated overnight with 90% ethanol at −20°C, and lyophilized to dryness. They were then subjected to SDS-PAGE and electrophoretic transfer to Immobilon P (PVDF) transfer membrane (Matsudaira, 1987). The proteins were visualized by staining with Coomassie Blue R-250, and analyzed for amino-terminal sequencing by Edman degradation using automatic gas phase sequencer (Applied Biosystems 477A protein sequencer).

Enzyme assays

Reconstitution was performed by incubating cytochrome P-450 (20–100 pmoles), NADPH cytochrome P-450 reductase (3 × pmoles of cytochrome P-450), cytochrome b_5 (1.2 × pmoles of cytochrome P-450), and L-α-dilauroyl-glyceryl-3-phosphoryl-choline (5 μg) in a small volume (200 μl) for 20 min at room temperature. The reaction mixture (1 ml) contained reconstituted enzyme or microsomes, substrate, 100 mM Tris-acetate buffer, pH 7.6,

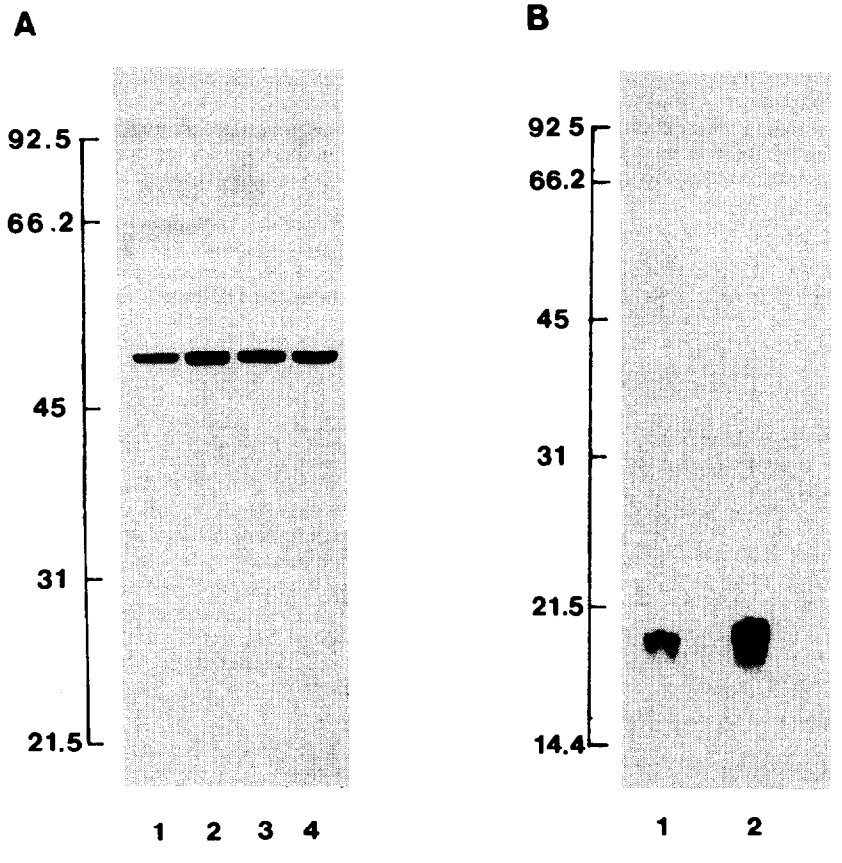

Fig. 2A. SDS-PAGE of cytochrome P-450s on 7.5% acrylamide gels. The gels were stained with alkaline $AgNO_3$ reagent. 1, cytochrome P-450 PB-A; 2, P-450 PB-B_1; 3, P-450 PB-B_2; and 4, P-450 PB-B_3. Each well contained 2–3 pmol of cytochrome P-450.

Fig. 2B. SDS-PAGE of cytochrome b_5 on 10% acrylamide gel. Each well contained 30 pmol of cytochrome. Cytochrome b_5 (1) and (2) were obtained at two stages in HA column chromatography. Molecular weight markers used were: phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

NADPH-regenerating system (10 mM glucose-6-phosphate, 1.0 I.U. of yeast glucose-6-phosphate dehydrogenase, 0.5 mM NADPH), and any other component required for a particular enzyme assay. The constituents were allowed to equilibrate for 3 min at 37°C before starting the reactions with NADPH-regenerating system or substrate as described in the given reference. The assay conditions were standardized so that the rate of reaction was linear with time and the amount of P-450 or microsomes. 7-ethoxycoumarin O-deethylase activity was measured by adding 25 μl of 10 mM 7-ethoxycoumarin in 50% methanol to the reaction mixture. The period of incubation was 10–20 min (Greenlee and Poland, 1978). The concentration of aminopyrine and benzphetamine in the N-demethylation assay was 5 mM and 1 mM, respectively. The reaction was started by adding NADPH-regenerating system and the period of incubation was 20–60 min (Udenfriend, 1969; Waxman and Walsh, 1983). Arylhydroxylation activity was measured by using benzo(α)pyrene (2 mM) as the substrate. The reaction was started by adding the NADPH-regenerating system, and the incubation period was 10 min (Nebert and Gelboin, 1968). 7-methoxyresorufin O-demethylation reaction was followed on a fluorospectrophotometer as described by Burke and Mayer (1974). The reaction was initiated by NADPH-regenerating system.

RESULTS

Purification

Purification of P-450 isozymes was initiated by hydrophobic affinity column chromatography on *n*-octylamino-Sepharose 4B columns. The majority of cytochrome P-450 eluted with buffer containing 0.06% Lubrol PX (fraction 1). The peak fractions at 417 nm were pooled and processed for further purification. One faint orange band also moved slowly down the column. This band was eluted by increasing the concentration of Lubrol PX to 0.25% in the same buffer (fraction 2). The hydrophobic affinity column chromatography separated P-450 PB-A from P-450 PB-B (i.e. P-450 PB-B_1, PB-B_2 and PB-B_3) and NADPH-cytochrome P-450 reductase. The purification scheme is shown in Fig. 1, and details are given in the Materials and Methods section. The cytochrome P-450s from *n*-octylamino-Sepharose 4B columns were loaded on hydroxylapatite (HA) columns. Cytochrome P-450 fractions PB-B_1, PB-B_2, and PB-B_2 were taken up by the HA column, whereas P-450 PB-A passed through the column with the washing buffer. Cytochrome P-450 PB-B_3 separated PB-B_1 and PB-B_2 on the HA column. The fraction P-450 PB-B_3 required a higher concentration of potassium phosphate (180 mM) in the buffer than either P-450 PB-B_1 or PB-B_2 (90 mM). Actually, the elution of the cytochrome P-450s never completely stopped while eluting the column with buffer C containing 40, 90 or 180 mM potassium phosphate. The buffer was changed when the absorbance of the effluent at 417 nm dropped to a minimum value.

The separation of P-450, PB-B_1 and PB-B_2 occurred on a CM-52 column. Minor contaminants were removed by a second HA column chromatography of each cytochrome P-450. The summary of purification is given in Table 1. The yields of P-450 PB-A, PB-B_1, PB-B_2, and PB-B_3 were 1.52, 0.68, 0.45, and 0.27% of total P-450 in the microsomes, and their specific contents were 18.4, 14.2, 15.0 and 10.8 nmol per mg protein, respectively. Cytochrome P-450 PB-A was obtained in the largest amount. At each step of purification, we proceeded with the major fractions of P-450, although other fractions also contained some other P-450s. In our earliest attempt, we found one pure cytochrome P-450, designated as P-450 PB-B, in place of PB-B_1 and PB-B_2. In that batch, CM-52 column was eluted with the buffer E containing 0–0.125 M sodium chloride gradient, and the fraction containing P-450 was

Table 1a. Summary of purification of cytochromes P-450 PB-B_1, PB-B_2 and PB-B_3 from phenobarbital-treated hen liver (see Fig. 1 for the numbers in parentheses)

Fraction	Protein (mg)	Cytochrome P-450 Total (nmol)	Specific content (nmol/mg protein)	Recovery (%)
Microsomes	2540	2420	0.95	100
Cholate solution	1742	1845	1.06	76.2
n-octylamino-Sepharose 4B column (1)	437	961	2.20	39.7
HA column 90 mM KH_2PO_4 (3)	138	525	3.80	21.7
DE-cellulose column washing (4)	70	384	5.48	15.9
CM-52 column 30 mM NaCl (5)	2.9	32	11.03	1.32
HA column 80 mM KH_2PO_4 (6) (P-450 PB-B_1)	1.16	16.5	14.2	0.68
CM-52 column 40 mM NaCl (7)	6.4	50	7.81	2.1
HA column 70 mM KH_2PO_4 (8) (P-450 PB-B_2)	0.74	11	14.96	0.45
HA column 180 mM KH_2PO_4 (9)	66	109	1.65	4.50
DE-cellulose column washing (10)	20.4	76	3.72	3.14
CM-52 column 40 mM NaCl (11) (P-450 PB-B_3)	0.61	6.6	10.81	0.27

Table 1b. Summary of purification of cytochrome P-450 PB-A from phenobarbital-treated hen liver (see Fig. 1 for the numbers in parentheses)

Fraction	Protein (mg)	Cytochrome P-450 Total (nmol)	Specific content (nmol/mg protein)	Recovery (%)
Microsomes	2640	5372	2.03	100
Cholate solution	2448	3587	1.46	66.8
n-octylamino-Sepharose 4B column (2)	120	360	3.00	6.70
HA column washing (12)	32	134	4.19	2.49
DE-52 column 25 mM KCl (13) (P-450 PB-A)	4.45	82	18.43	1.52

further purified on a phosphocellulose column. Later, two fractions of cytochrome P-450 (P-450 PB-B_1 and PB-B_2) appeared when the CM-52 column was eluted with buffer E containing 30 and 40 mM sodium chloride, respectively. Amberlite XAD-2 was used to remove detergent from purified isozymes. The recoveries of P-450 PB-A, PB-B_1, PB-B_2, and PB-B_3 after XAD-2 treatment were 98, 95, 91 and 78%, respectively. The cytochrome P-450 PB-A, which was obtained in the largest amount, was also subjected to HA column chromatography to remove most of the remaining Lubrol PX.

SDS-PAGE and NAPHGE

All purified P-450 isozymes gave a single band on discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig 2A). They showed a high degree of electrophoretic homogeneity and the same apparent molecular weight (54 kDa). Since cytochrome P-450 isozymes are basic, they were subjected to nonequilibrium pH gradient electrophoresis in one direction (NEPHGE) and SDS-PAGE in the other (Fig. 3). The homogeneity of cytochrome P-450s was further demonstrated by a single major spot on two-dimensional gels, although most of them (except P-450 PB-A) showed some streaking around their spot. The co-electrophoresis of any two P-450s on two dimensional gels did not show distinct, separate spots on the gels. The minor difference, if any, in their isoelectric points was masked by streaking of the isozymes during NEPHGE.

Spectral properties

The oxidized cytochrome P-450 isozymes showed great similarity in their spectral behaviour (Fig. 4). They had soret maxima at 413.5 or 414.5 nm, and α and β peaks very close to each other (Table 2). However, the oxidized P-450 PB-A could be distinguished from other isozymes on the basis of its α peak and extensive broadening of its soret peak upon

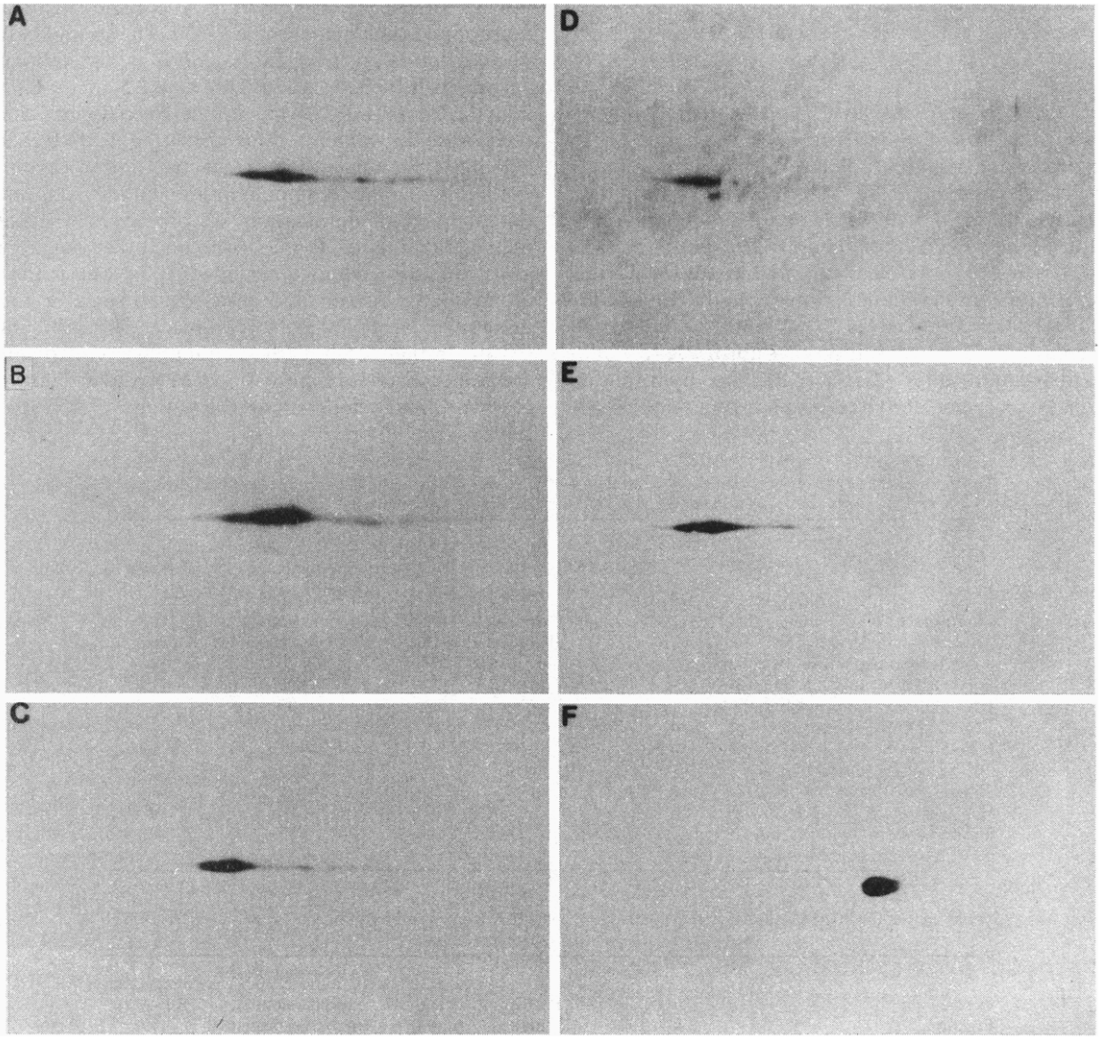

Fig. 3. Two-dimensional electrophoresis of cytochrome P-450s. The samples (2.5–5.0 pmol) were subjected to nonequilibrium pH gradient electrophoresis (NEPHGE) followed by SDS-PAGE on 7.5% acrylamide gel in the other direction. The gels were stained with alkaline $AgNO_3$ reagent. A, P-450 PB-B_1; B, P-450 PB-B_1 + PB-B_2; C, P-450 PB-B_2; D, P-450 PB-B_3; E, P-450 PB-B_1 + PB-B_3; and F, P-450 PB-A. The anode was on the right-hand side during NEPHGE and at the bottom during SDS-PAGE.

Table 2. Spectral properties of hen liver purified P-450s. The value of soret, α and β peaks were derived from the absolute spectra of P-450s in Fig. 4. The status of peak on dilution of the oxidized hemoproteins is given in parentheses

P-450s	Oxidized (nm)	Reduced (nm)	Fe^{2+}-CO complex (nm)
PB-A	413.5 (410.5)	410.5	449.5
	531–533	541.5	551.0
	564.0		
PB-B_1	414.5 (413.5)	413.5	448.5
	533.5	564.0	555.0
	568.0		
PB-B_2	414.5 (413.5)	414.5	449.5
	532.5	532.5	549.0
	568.0	568.0	569.0
PB-B_3	414.5 (413.5)	410.5	448.5
	532.5	473.0	484.5
	569.0	568.0	571.0

dilution (0.5 nmol/ml) (Fig. 4A). The absolute spectrum of oxidized P-450 PB-A (5 nmol/ml) showed a shoulder at about 393 nm indicating that the high-spin form of this enzyme was present in different amounts at different concentrations. The broadening of the peak after dilution may be due to conversion of some low-spin hemoprotein to its high-spin state. There was no apparent broadening of the soret peaks of P-450 PB-B_1, PB-B_2 and PB-B_3, but there was a slight shift (1 nm) in their soret peaks toward the blue region. Cytochrome fractions P-450 PB-B_1, PB-B_2 and PB-B_3 could not be distinguished on the basis of spectra of their oxidized state. Upon reduction with dithionite, P-450 PB-A and PB-B_3 gave soret peak at 410.5 nm, while P-450 PB-B_1 and PB-B_2 gave peak at 413.5 and 414.5 nm, respectively. Thus, the soret peak of fraction P-450 PB-B_3 differed from fractions P-450 PB_1 and PB_2 in their reduced states. The ferrous-carbonyl complexes of all P-450s exhibited the same soret peak (~449 nm). Only cytochrome P-450 PB-A, the major isozyme, was used for the determination of its protoheme content, and its extinction coefficient on the basis of its protoheme

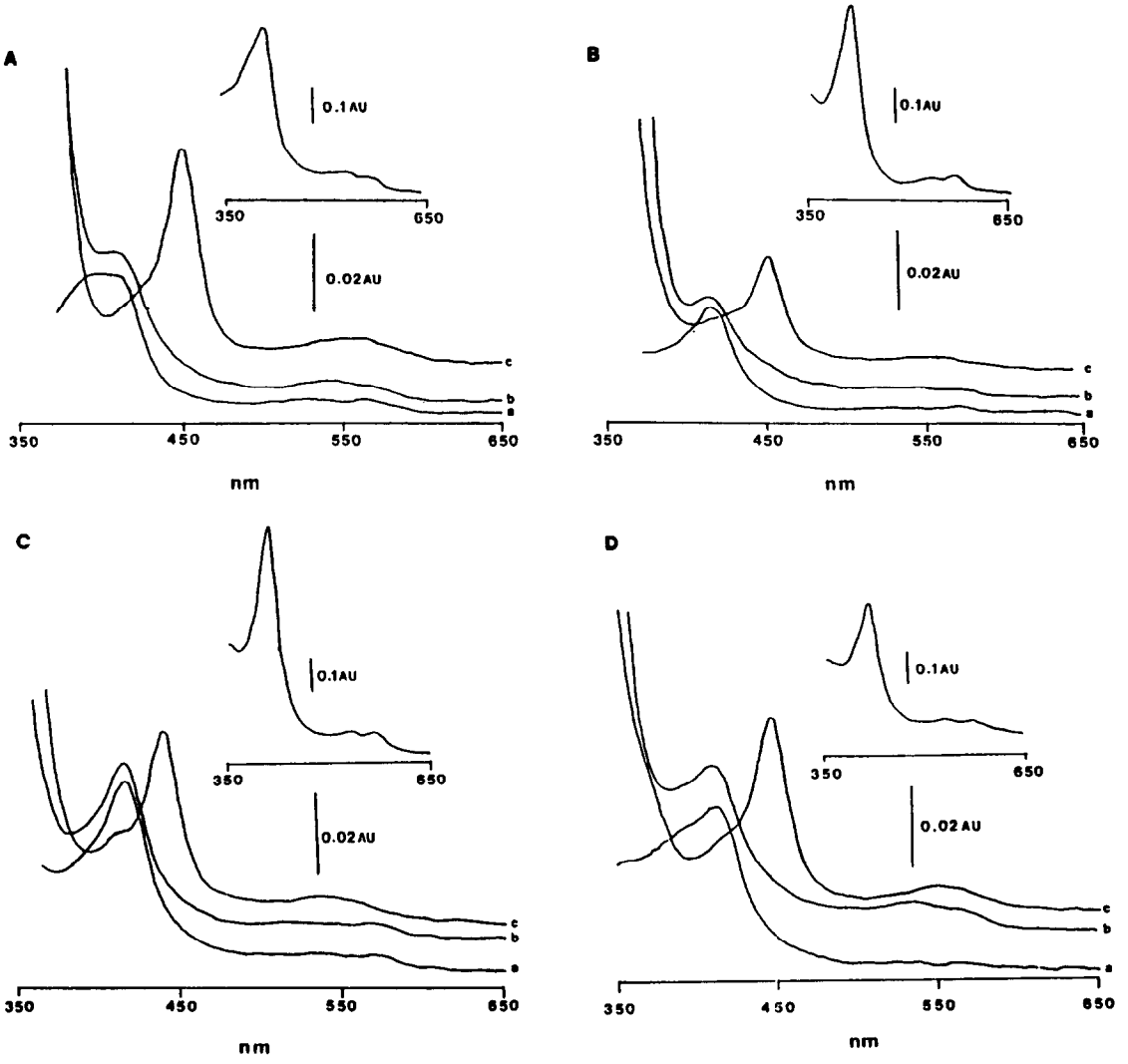

Fig. 4. Absolute spectra of purified P-450s. The hemoproteins were diluted in 10 mM Tris-acetate pH 7.4, 1 mM EDTA, 20% glycerol before recording absolute spectra of oxidized (a), reduced (b) and Fe^{2+}-CO complex (c). Inset in figures gives the absolute spectra of oxidized hemoprotein at higher concentration. A, P-450 PB-A (0.5 μM), inset (4.8 μM); B, P-450 PB-B_1 (0.2 μM), inset (3.9 μM); C, P-450 PB-B2 (0.38 μM), inset (5.0 μM); and D, P-450 PB-B_3 (0.16 μM), inset (3.3 μM).

estimation was 128 cm^{-1} mM^{-1}. However, the concentration of all P-450s was calculated using the molar extinction coefficient of 91 cm^{-1} mM^{-1}. All P-450s showed a slight shoulder at about 420 nm in their reduced-CO complex spectra. This shoulder was further reduced in the difference spectra of their reduced-CO complex versus reduced form, and suggested that only traces of P-420 existed in the purified proteins. Insets in Fig. 4 show the α and β peaks of the oxidized form of concentrated cytochrome P-450s. The position of α, β and soret peaks showed the characteristics of low-spin hemoprotein.

Immunochemical properties

A single immunoprecipitin band was observed when each cytochrome P-450 reacted with its antibody (Fig. 5).The immunoprecipitation band formed by cytochrome fractions P-450 PB-B_1, PB-B_2 and PB-B_3 with either anti-P-450 PB-B or anti-P-450 PB-B_3 showed a line of identity. The absence of spurs demonstrated that P-450 PB-B_1, P-450 PB-B_2 and PB-B_3 had identical antigenic sites. The antibody raised against P-450 PB-A did not immunoprecipitate P-450 PB-B fractions, and antibodies against the latter fractions did not immunoprecipitate P-450 PB-A. The detergents that are supposed to reduce cross-reactivity between antigens were not present in the agar medium of the Ouchterlony plates. Immunoblotting studies showed that antibodies against P-450 PB-B_1, PB-B_2, and PB-B_3 recognized each other but not P-450 PB-A, and vice-versa (Fig. 6A). Ouchterlony double diffusion immunoprecipitation (Fig. 5) and immunoblotting (Fig. 6A) suggested the purification of two distinct cytochrome P-450 isozymes, P-450 PB-A and PB-B. The latter seemed to appear in three cytochrome P-450 fractions. Immunoblotting was further used to check cross-reactivity of hen liver P-450s with those of other species. The results showed that P-450s from hen liver were immunochemically different from P-450s in the livers of normal as well as phenobarbital-treated rat (Fig. 6B), mouse, and cat. The P-450s of hen liver were also immunochemically different from P-450s of liver microsomes of normal catfish. The P-450s in catfish liver are not induced by sodium phenobarbital. The additional bands seen in Fig. 6B were not visible when fresh microsomes were used in initial tests (Fig. 6A). Phenobarbital treatment did not produce new P-450 isozymes, but induced some of those present in the normal hen liver. The induction of these enzymes was approximately six- to seven-fold.

Peptide mapping

Cytochrome P-450 isozymes were partially hydrolyzed by α-chymotrypsin and *Staphylococcus aureus* V8 protease to map peptide fragments that could show structural differences between them. The same amounts of P-450s were used for protease treatment and for loading on the polyacrylamide gels (Fig. 7). The P-450 isozymes were found to be extremely sensitive to α-chymotrypsin digestion. Samples containing about 25 pmol of P-450 were hydrolyzed by 7.5 ng of α-chymotrypsin and 250 ng of *S. aureus* V8 protease for 30 min at 37°C. Cytochrome P-450 PB-A was easily distinguished from other P-450s upon partial hydrolysis by α-chymotrypsin or *S. aureus* V8 protease. The peptide fragments of P-450 PB-B_1, PB-B_2 and PB-B_2 were very similar. The major bands showed no difference. However, some faint bands were seen in the hydrolysis products of one P-450 isozyme and not of another. Thus, bands a and b in lanes 3 and 7 (P-450 PB-B_2) were not seen in adjacent lanes (P-450 PB-B_1 and PB-B_3). Similarly, band c in peptides from P-450 PB-B_3 (lane 8) was not visible in lanes 6 and 7 (P-450 PB-B_1 and P-450 PB-B_2), and band d in lanes 6 and 7 was not visible in lane 8. The peptide patterns of P-450 PB-B_1, PB-B_2 and PB-B_3 illustrated great homology in their primary structures. The primary structures of the P-450s were also compared by two-dimensional peptide fingerprinting (Fig. 8). Cytochrome P-450 PB-A exhibited a unique peptide pattern different from those obtained from P-450 PB-B fractions. The peptide fingerprints of the latter P-450s were nearly identical. Some spots that seemed to distinguish these isozymes from each other were not reproducible.

N-terminal amino acid sequence analysis

The cytochrome P-450 PB-A exhibited an amino-terminal sequence different from the other three

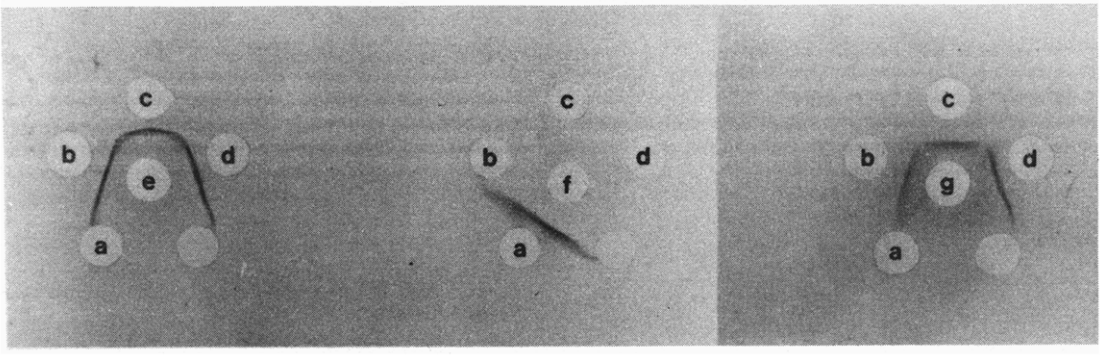

Fig. 5. Ouchterlony double immunodiffusion analysis of P-450s. The central wells contained anti-P-450 PB-B, 3 μl (e); anti-P-450 PB-A, 3 μl (f), and anti-P-450 PB-B_3, 6 μl (g). The peripheral wells contained 6 pmol of P-450 PB-A, (a); PB-B_1, (b); PB-B_2, (c); and PB-B_3, (d). The agar gel in the plates did not contain any detergent. The plates were incubated for 72 hr at room temperature under humid atmosphere and stained with amido black.

Table 3. N-terminal amino acid sequence of cytochrome P-450s

Cytochrome	Residue 1				5				10				15				20	
P-450 PB-A	M	E	V	T	A	A	L	L	L	F	L	G	L	S	L	V	V	L	L
P-450 PB-4*	M	E	P	S	I	L	L	L	L	A	L	L	V	G	F	L	L	L	L
P-450 PB-B_1	M	L	L	L	G	A	A	S	V	V	L	L	V	(T)	V	A	(R)	L	L
P-450 PB-B_2	M	L	L	L	G	A	A	S	V	V	L	L	V	T	V	A	X	L	L
P-450 PB-B_3	M	L	L	L	G	A	A	S	V	V	L	L	V	T	V	A	X	L	L
PB15†	M	D	F	L	G	L	P	T	I	L	L	L	V	C	I	S	C	L	L

*PB15: phenobarbital-induced chick embryo liver cytochrome P-450 (Nebert *et al.*, 1989; Hobbs *et al.*, 1986).
†P-450 PB-4: phenobarbital-induced rat liver cytochrome P-450 (Waxman and Walsh, 1982).

P-450 fractions (Table 3). The latter three P-450s exhibited almost the same sequence up to 19 amino acid residues and did not have serine or acidic residue at second or third position, that is present in cytochrome P-450 PB-A.

Catalytic activities

The conditions for all the assays were standardized with the major isozyme P-450 PB-A. The activities of all the purified cytochromes were compared under the same conditions, although some might not have had optimum conditions for their catalytic activities. Cytochrome b_5 was also included in the reconstitution system since it increased the metabolic activity of P-450 PB-A several times (5- to 16-fold). Further increases in the concentration of cytochrome b_5 did not affect the metabolic rate of the reactions. The maximum increase was observed with O-deethylation of 7-ethoxycoumarin. The metabolisms of benzphetamine and aminopyrine were increased by about five- and six-fold, respectively. Thus, stimulation of the catalytic activity of P-450 was dependent upon the substrate used for the metabolic study. The purified isozymes exhibited overlapping substrate specificities for the substrates used in study (Table 4a). Nearly all the substrates were metabolized in descending order by P-450 PB-A, PB-B_3, PB-B_1 and PB-B_2. 7-methoxy-resorufin O-demethylase activity was detectable only in P-450 PB-B_1. Removal of Lubrol PX by HA column chromatography greatly enhanced (2–13 times) the catalytic activity of cytochrome P-450 PB-A. The yield cytochrome P-450 PB-B fractions was too low to be used for HA column chromatography.

The comparison of the catalytic activity of microsomes (nmol/min/nmol) and P-450 PB-A suggested that the latter might be primarily responsible for the metabolism of aminopyrine, 7-ethoxycoumarin, and benzo(α)pyrene in PB-treated hen liver (Table 4b). These substrates were more rapidly metabolized by P-450 PB-A than microsomes. On the other hand, cytochrome P-450 PB-B fractions might be more active in carrying out the N-demethylation of benzphetamine, since the latter was more actively metabolized by microsomes than P-450 PB-A. The catalytic activity of P-450 PB-B_1, PB-B_2 and PB-B_3 was lower than those of P-450 PB-A and microsomes. This might be due to the presence of some Lubrol PX in these preparations.

Cytochrome b_5

Cytochrome b_5 was also purified to electrophoretic homogeneity from phenobarbital-induced hen liver (Fig. 2B). Pure b_5 eluted in two peaks from HA column. The molecular weights of two proteins eluted with buffer containing 20 and 30 mM KH_2PO_4 were 17,200 and 17,500, respectively.

Table 4a. Mono-oxidase activity of cytochrome P-450s from phenobarbital-treated hen liver. The activities are given in nmol/min/nmol P-450. The values in parentheses represent the activity of P-450 PB-A after removal of Lubrol-PX by hydroxylapatite column chromatography. The assay conditions are described in the Materials and Methods section

Substrate	P-450 PB-A	P-450 PB-B_1	P-450 PB-B_2	P-450 PB-B_3
Benzphetamine	4.75 (10.22)	2.83	2.63	3.12
7-methoxy-resorufin	ND (0.018)	0.0005	ND	ND
Aminopyrine	5.32 (34.35)	1.84	1.87	2.36
7-ethoxy-coumarin	2.8 (29.78)	0.024	0.021	0.058
Benzo(α)pyrene	0.31 (4.00)	0.009	0.012	0.016

Table 4b. Mono-oxidase activity of liver microsomes from normal and PB-treated hens. The results are expresseed as mean ± SD from three hens. The assay conditions are described in the Materials and Methods section

Substrate	Normal microsomes		PB-induced microsomes	
	nmol/min per mg protein	nmol/min per nmol P-450	nmol/min per mg protein	nmol/min per nmol P-450
Benzphetamine	6.62 ± 1.74	17.39 ± 4.18	24.69 ± 2.25	28.41 ± 1.24
7-methoxy-resorufin	0.017 ± 0.005	0.045 ± 0.010	0.069 ± 0.015	0.077 ± 0.010
Aminopyrine	7.76 ± 1.66	20.47 ± 4.23	23.47 ± 4.42	26.65 ± 1.87
7-ethoxy-coumarin	4.78 ± 1.01	12.54 ± 2.11	8.95 ± 1.21	10.31 ± 1.14
Benzo(a)pyrene	0.40 ± 0.10	1.01 ± 0.14	1.27 ± 0.36	1.53 ± 0.65

DISCUSSION

In this study, we have developed a technique to purify P-450 isozymes from phenobarbital-treated adult hen livers. All P-450s purified from adult hen exhibited the same apparent molecular weight on SDS-PAGE (54 kDa). The isolation of some P-450 isozymes having the same molecular weight has been reported in other species. Thus, P-450 PB-B and PB-D (50 kDa) from phenobarbital-treated rat liver (Guengerich *et al.*, 1982), P-450 UT-2 and UT-3 (50 kDa), UT-4, UT-5 and UT-6 (48 kDa) from

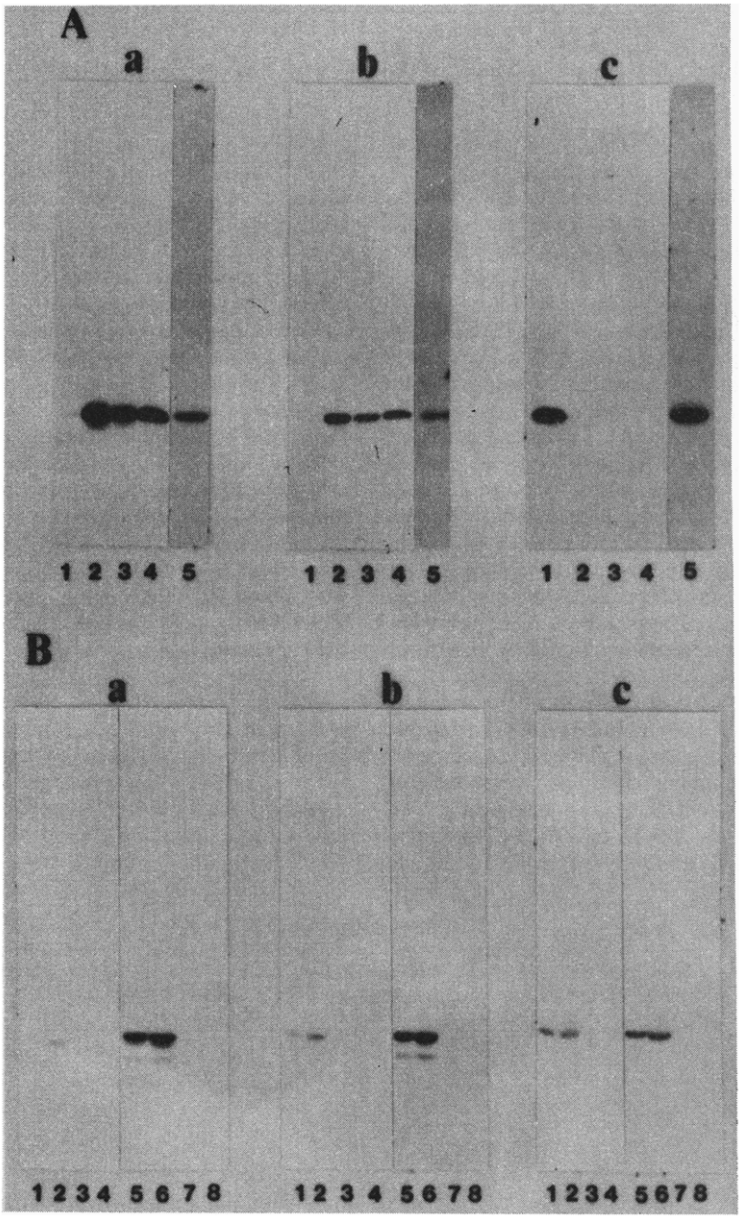

Fig. 6A. Immunoblotting analysis of purified P-450s and microsomes. The purified proteins and microsomes from phenobarbital-induced hen liver were subjected to SDS-PAGE on 7.5% acrylamide gels and transferred to nitrocellulose sheets (0.2 μm) at 100 V for 1 hr. The sheets were immunostained using antiserum against P-450s and ^{125}I-protein A as described in the Materials and Methods section. 1, P-450 PB-A, 2.9 pmol; 2, P-450 PB-B_1, 2.3 pmol; 3, P-450 PB-B_2, 2 pmol; 4, P-450 PB-B_3, 2.6 pmol; and 5, microsomes (28 μg protein).

Fig. 6B. Immunochemical cross-reactivity of P-450s in different animal species by immunoblotting technique. The experiments were performed as described in the legend to Fig. 6A, and the samples contained 5 or 10 μg of microsomal protein from phenobarbital-treated animals and 10 or 25 μg of microsomal protein from normal animals. Lanes 1, 3 (10 μg) and 2, 4 (25 μg) of microsomal proteins from normal animals; lanes 5, 7 (5 μg) and 6, 8 (10 μg) of microsomal protein from phenobarbital-treated animals. Lanes 1, 2, 5, 6, microsomes from hen liver and 3, 4, 7, 8, microsomes from rat. a, anti-P-450 PB-B, 1:1000 dilution; b, anti-P-450 PB-B_3, 1:1000 dilution; and c, anti-P-450 PB-A, 1:2000 dilution.

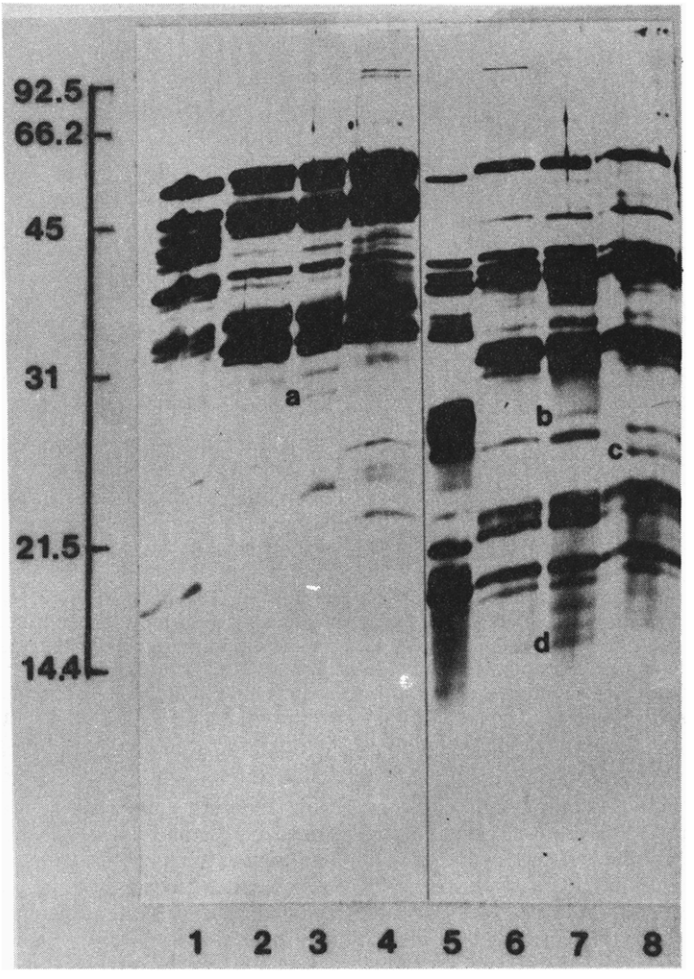

Fig. 7. Peptide mapping of cytochrome P-450s. Twenty-five pmol of each P-450 isozyme was partially hydrolyzed by α-chymotrypsin (7.5 ng) and *S. aureus* V8 protease (250 ng) for 30 min. The peptides were separated by SDS-PAGE on 12.5% acrylamide and stained by alkaline $AgNO_3$. Lanes 1–4, treatment with α-chymotrypsin; lanes 5–8, treatment with *S. aureus* V8 protease; lanes 1 and 5, P-450 PB-A; lanes 2 and 6, P-450 PB-B_1; lanes 3 and 7, P-450 PB-B_2; and lanes 4 and 8, P-450 PB-B_3.

untreated rat liver (Funae and Imaoka, 1987), and P-450 LM-4a and LM-4b (53 kDa) from β-naphthoflavone-treated rabbit liver (Guengerich, 1977) have the same molecular weight. The result that cytochrome P-450s from hen liver did not separate on two-dimensional isoelectric-polyacrylamide gel electrophoresis is consistent with the findings for some other species (Ryan *et al.*, 1982; Vlasuk *et al.*, 1982). Cytochrome P-450s separated by isoelectric focusing show only minor difference in their isoelectric points (Ryan *et al.*, 1982; Vlasuk *et al.*, 1982). The lack of separation of P-450 isozymes might be attributed to their close pI values and streaking by cytochrome fractions P-450 PB-B_1, PB-B_2 and PB-B_3. Similar streaking of some P-450s and not of others from the same animal source has been noted by other investigators (Ryan *et al.*, 1982; Wang *et al.*, 1983).

The broadening of the soret peak of P-450 PB-A toward the blue region upon dilution was an indication of partial conversion of P-450 to its high-spin state. A similar broadening is reported for oxidized P-450 LM-4 purified from β-naphthoflavone-treated rabbits (Haugen and Coon, 1976). The soret peak of oxidized P-450s from hen liver occurred at ~414 nm, while in rat, rabbit, and dog it occurs at 416–418 nm (Haugen and Coon, 1976; Guengerich *et al.*, 1982; Larrey *et al.*, 1984; Duignan *et al.*, 1987). However, soret peak values (~449 nm) of ferrous-carbonyl complexes of all P-450s from hen liver compared well with those reported in other PB-induced animal species (Haughen and Coon, 1976; Guengerich *et al.*, 1982; Larrey *et al.*, 1984).

The cytochromes P-450 PB-B_1, PB-B_2 and PB-B_3 from hen liver could not be distinguished on the basis of immunochemical properties. In contrast to rooster P-450 (Oron and Bar-Nun, 1984), none of the anti-hen P-450s reacted with normal or PB-induced P-450s in rat liver. In addition, they did not show cross-reactivity with P-450s of mice, cat and catfish. The absence of any reaction between anti-hen P-450s and P-450s of both rat and mice is in agreement with the report that rat and mice P-450s are immunochemically very similar (Guengerich *et al.*, 1982a). This is also supported by the NH2-terminal sequence of these P-450s that did not show homology with any one of them.

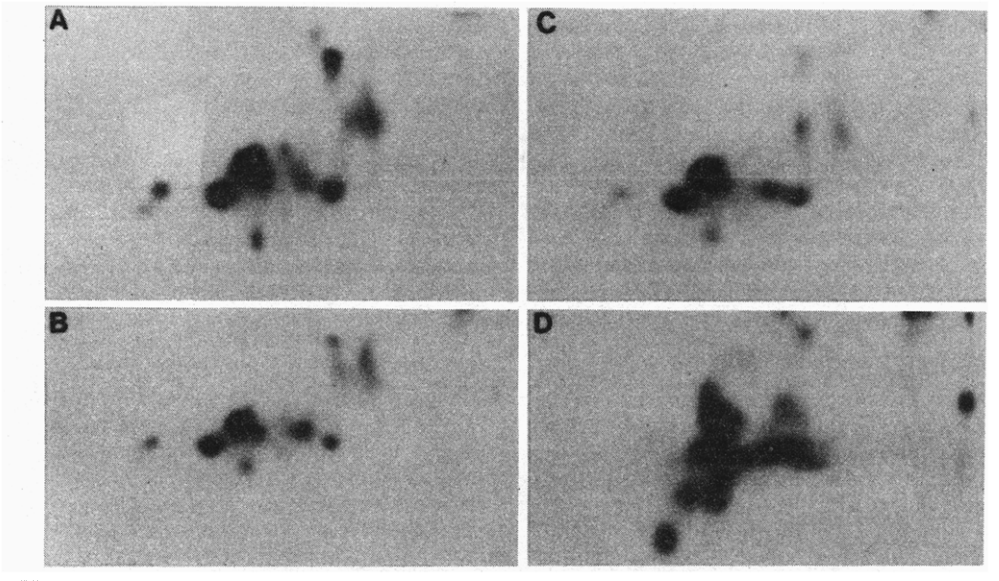

Fig. 8. Tryptic peptide fingerprinting of cytochrome P-450s. The purified P-450s (20 pmol) were electrophoresed by SDS-PAGE on 7.5% acrylamide gels and stained with Coomassie blue. The stained gel slices containing P-450s were radioiodinated with ^{125}I and digested overnight with trypsin (50 μg/ml). The tryptic peptides were electrophoresed on cellulose-coated TLC plates (10 × 20 cm) in one direction and chromatographed in the other as described in the Materials and Methods section. The TLC plates were autoradiographed with two Dupont intensifying screens. A, P-450 PB-B_1; B, P-450 PB-B_2; C, P-450 PB-B_3; and D, P-450 PB-A.

The immunochemically identical cytochromes P-450 PB-B_1, PB-B_2 and PB-B_3 showed some minor differences in their peptide pattern upon partial hydrolysis by α-chymotrypsin and *S. aureus* V8 protease, but these differing peptides formed only faint bands on the polyacrylamide gels. All the major peptides bands were exactly the same in these fractions. Furthermore, two-dimensional fingerprints or tryptic peptides did not show any distinct differences between these immunochemically identical P-450s.

The N-terminal sequence of P-450 isozymes of three immunologically similar P-450s was almost the same up to 19 amino acid residues. Their N-terminal also did not have any serine or acidic residue at the second or third position, as has usually been found in other P-450s. The cytochromes P-450 PB-B_1, PB-B_2 and PB-B_3 showed about 40% homology with the N-terminal sequence deduced from chick liver P-450 cDNA clones (Hobbs *et al.*, 1986). These clones were isolated from livers of 2-allyl-2-isopropylacetamide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine-induced 18-day chick embryos. Similarly, P-450 PB-A showed 40% homology with rat P-450 PB-4 (Waxman and Walsh, 1982). Both the amino-terminal sequences of purified P-450s possessed the familiar leucine triad and differed from other so-far listed P-450s (Nebert *et al.*, 1989).

The immunochemically similar cytochromes (P-450 PB-B_1, PB-B_2 and PB-B_3), which appeared at different steps of purification, could not be unambiguously distinguished from each other on the basis of above characteristics. It seemed as if all these immunochemically similar P-450s formed one cytochrome P-450 (i.e. P-450 PB-B), and appeared in three fractions during purification because of their association with some other proteins, or for some other reason. However, it could not be ruled out that these P-450s had minor differences in their primary structure that were responsible for their different chromatographic behaviour, and some differences in their spectral properties.

Cytochrome b_5 increased the catalytic activity of P-450 PB-A several fold (5- to 16-fold). This suggested that the mono-oxidase system in phenobarbital-induced hen liver was more dependent on cytochrome b_5 than in other species (Haugen and Coon, 1976; Guengerich *et al.*, 1982; Waxman and Walsh, 1982; Duignan *et al.*, 1987). The catalytic activity of P-450 PB-A increased 2–13 times on removing the Lubrol PX detergent by HA column chromatography. The low catalytic activity of other purified isozymes was probably related to the presence of Lubrol PX in the final preparation. Since the yield of three immunochemically similar P-450s was less and the loss of enzyme by HA column chromatography was great, they could not be made detergent-free by the latter process. Immunoinhibition studies using antibodies against two distinct isozymes (P-450 PB-A and PB-B) would be of great use in defining the role of these P-450s in the metabolism of different substrates.

This report describes the purification and characteristics of at least two cytochrome P-450 isozymes (P-450 PB-A and PB-B) from phenobarbital-treated adult hen liver. Both the isozymes were used to raise two highly specific P-450 antibodies that gave a single band with microsomes by immunoblotting. Both the purified P-450 isozymes had the same molecular weight, but could be easily distinguished by the following characteristics: chromatographic behaviour, spectral analysis, peptide mapping, tryptic peptide

mapping, immunochemical properties, and amino-terminal sequencing. In contrast, cytochromes P-450 PB-B_1, PB-B_2 and PB-B_3 were found to be very similar in most of their properties and amino-terminal sequence. At present it seemed that these cytochromes represented one cytochrome P-450, i.e. P-450 PB-B. However, further work comprising (1) regioselective or stereoselective metabolism of substrates such as steroid hormones (Waxman and Walsh, 1983; Duignan *et al.*, 1987), and R- and S-warfarin (Guengerich *et al.*, 1982), (2) isolation of mRNAs synthesizing these cytochromes, and (3) amino acid sequencing would be required to reveal any difference between these cytochromes. After microsomal enzyme, cytochrome b_5 was also purified by a simple method. Actually, two pure fractions of cytochrome b_5 were obtained. Only the major fraction of b_5 was used in this study. Immuno-inhibition experiments using antibodies against purified P-450s would probably be of great help in revealing the role of these isozymes in the metabolism of various substrates by microsomes.

REFERENCES

Abou-Donia M. B. (1981) Organophosphorous ester-induced delayed neurotoxicity. *Ann. Rev. Pharmacol. Toxicol.* **21,** 511–548.

Abou-Donia M. B., Lapadula D. M., Campbell G. and Timmons P. R. (1985) The synergism of *n*-hexane-induced neurotoxicity by methyl butyl ketone following subchronic (90 days) inhalation in hens: induction of hepatic microsomal cytochrome P-450. *Toxicol. Appl. Pharmacol.* **81,** 1–16.

Brooker J. D., Srivastava G., Borthwick I. A., May B. K. and Elliott W. H. (1983) Evidence that 2-allyl-2-isopropyl-acetamide, phenobarbital, and 3,5-diethoxycarbonyl-1,14-dihydrocollidine induce the same cytochrome P450 mRNA in chick embryo liver. *Eur. J. Biochem.* **136,** 327–332.

Burke M. D. and Mayer R. T. (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methyl-cholanthrene. *Drug Metab. Dispos.* **2,** 583–588.

Cleveland D. W., Fischer S. G., Kirschner M. W. and Laemmli U. K. (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252,** 1102–1106.

Duignan D. B., Sipes I. G., Leonard T. B. and Halpert J. R. (1987) Purification and characterization of the dog hepatic cytochrome P-450 isozyme responsible for the metabolism of 2,2′, 4,4′, 5,5′-hexachlorobiphenyl. *Arch. Biochem. biophys.* **255,** 290–303.

Elder J. H., Pickett R. A., Hampton J. and Lerner R. A. (1977) Radioiodination of proteins in single polyacrylamide gel slices. *J. Biol. Chem.* **252,** 6510–6515.

Falk J. K. (1964) Haems. In *Porphyrins and Metalloporphyrins.* Falk J. E. (ed.), Vol. 2, pp. 181–182. Elsevier, New York.

Funae Y. and Imaoka S. (1987) Purification and characterization of liver microsomal cytochrome P-450 from untreated male rats. *Biochim. Biophys. Acta* **926,** 349–358.

Greenlee W. F. and Poland A. (1978) An improved assay of 7-ethoxycoumarin O-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Pharmacol. Expt. Ther.* **205,** 596–605.

Guengerich F. P. (1977) Separation and purification of multiple forms of microsomal cytochrome P-450. *J. Biol. Chem.* **252,** 3970–3979.

Guengerich F. P., Dannan G. A., Wright S. T., Martin M. V. and Kaminsky L. S. (1982) Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21,** 6019–6030.

Guengerich F. P. and Martin M. V. (1980) Purification of cytochrome P-450, NADPH-cytochrome P-450 reductase, and epoxide hydratase from a single preparation of rat liver microsomes. *Arch. Biochem. Biophys.* **205,** 365–379.

Guengerich F. P., Wang P. and Davidson N. K. (1982a) Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry* **21,** 1698–1706.

Hansen L. G. (1984) Biotransformation of organophosphorous compounds relative to delayed neurotoxicity. In *Delayed Neurotoxicity,* Cranmer J. M. and Hixson E. J. (eds), pp. 72–86. Intox Press, Arkansas.

Haugen D. A. and Coon M. J. (1976) Properties of electrophoretically homogenous phenobarbital-inducible and β-naphthoflavone-inducible forms of liver microsomal cytochrome P-450. *J. Biol. Chem.* **251,** 7929–7939.

Hobbs A. A., Mattschoss L. A., May B. K., Williams K. E. and Elliott W. H. (1986) The cDNA and protein sequence of a phenobarbital-induced chicken cytochrome P-450. *J. Biol. Chem.* **261,** 9444–9449.

Laemmli U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lapadula D. M., Irwin R. D., Suwita E. and Abou-Donia M. B. (1986) Cross-linking of neurofilament proteins of rat spinal cord *in vivo* after administration of 2,5-hexanedione. *J. Neurochem.* **46,** 1843–1850.

Larrey D., Distlerath L. M., Dannan G. A., Wilkinson G. R. and Guengerich F. P. (1984) Purification and characterization of the rat liver microsomal cytochrome P-450 involved in the hydroxylation of debrisoquine, a prototype for genetic variation in oxidative drug metabolism. *Biochemistry* **23,** 2787–2795.

Matsubara T., Koike M., Touchi A., Tochino Y. and Sugeno K. (1976) Quantitative determination of cytochrome P-450 in rat liver homogenate. *Anal. Biochem.* **75,** 596–603.

Matsudaira P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262,** 10,035–10,038.

Nebert D. W. and Gelboin H. V. (1968) Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. *J. Biol. Chem.* **243,** 6242–6249.

Nebert D. W., Nelson D. R., Adesnik M., Coon M. J., Estabrook R. W., Gonzalez F. J., Guengerich F. P., Gunsalus I. C., Johnson E. F., Kemper B., Levin W., Phillips I. R., Sato R. and Waterman M. R. (1989) The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8,** 1–13.

O'Farrell P. Z., Goodman H. M. and O'Farrell P. H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12,** 1133–1142.

Omura T. and Sato R. (1964) The carbon monoxide-binding pigment of liver microsomes. 1. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239,** 2370–2378.

Oron L. and Bar-Nun S. (1984) Immunological evidence for phenobarbital-stimulated synthesis of cytochrome P-450 in primary cultures of chick embryo hepatocytes. *Biochim. Biophys. Acta* **805,** 291–299.

Ryan D. E., Wood A. W., Thomas P. E., Walz F. G. Jr, Yuan P. M., Shively J. E. and Levin W. (1982)

Comparisons of highly purified hepatic microsomal cytochromes P-450 from Holtzman and Long–Evans rats. *Biochim. Biophys. Acta* **709,** 273–283.

Sinclair P., Frezza J., Sinclair J., Bement W., Haugen S., Healey J. and Bonkovsky H. (1989) Immunochemical detection of different isozymes of cytochrome P-450 induced in chick hepatocyte cultures. *Biochem. J.* **258,** 237–245.

Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J. and Klenk D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150,** 76–85.

Strittmatter P., Flemming P., Connors M. and Corcoran D. (1978) Purification of cytochrome b_5. *Methods Enzymol.* **52,** 97–101.

Strobel H. W. and Dignam J. D. (1978) Purification and properties of NADPH-cytochrome P-450 reductase. *Methods Enzymol.* **52,** 89–96.

Udenfriend S. (1969) *Fluorescence Assay in Biology and Medicine*, Vol. 2, pp. 352–353. Academic Press, London.

Vlasuk G. P., Ghrayeb J., Ryan D. E., Reik L., Thomas P. E., Levin W. and Walz F. G. Jr (1982) Multiplicity, strain differences, and topology of phenobarbital-induced cytochrome P-450 in rat liver microsomes. *Biochemistry* **21,** 789–798.

Wang P. P., Beaune P., Kaminsky L. S., Dannan G. A., Kadlubar F. F., Larrey D. and Guengerich F. P. (1983) Purification and characterization of six cytochrome P-450 isoenzymes from human liver microsomes. *Biochemistry* **22,** 5375–5383.

Waxman D. J., Ko A. and Walsh C. (1983) Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J. Biol. Chem.* **258,** 11,937–11,947.

Waxman D. J. and Walsh C. (1982) Phenobarbital-induced rat liver cytochrome P-450. Purification and characterization of two closely related isozymic forms. *J. Biol. Chem.* **257,** 10,446–10,457.

Waxman D. J. and Walsh C. (1983) Cytochrome P-450 isozyme 1 from phenobarbital-induced rat liver: purification, characterization and interactions with metyrapone and cytochrome b_5. *Biochemistry* **22,** 4846–4855.

Wislocki P. G., Miwa G. T. and Lu A. Y. H. (1980) Reactions catalyzed by the cytochrome P-450 system. In *Enzymatic Basis of Detoxification*, Jakoby W. B. (ed.), Vol. 1, pp. 135–182. Academic Press, New York.

Wray W., Boulikas T., Wray V. P. and Hancock R. (1981) Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118,** 197–203.