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# Purification and Characterization of Cytochrome P450 Isozymes from β-Naphthoflavone-Induced Adult Hen Liver[1]

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**Cytochromes P450 βNF-A, βNF-B, and βNF-C were purified from β-naphthoflavone-treated adult hens. Cytochrome P450 βNF-A, however, appeared at two places in the purification scheme. They were designated as cytochromes P450 $\beta$NF-$A_1$ and $\beta$NF-$A_2$ for property comparison. The cytochromes $\beta$NF-$A_1$ and $\beta$NF-$A_2$ were induced by both phenobarbital and β-naphthoflavone treatment and were similar to P450 PB-A (previously purified from phenobarbital-induced hen livers) in molecular weights, isoelectric pH, spectral properties, behavior on chromatography columns, catalysis of substrates, immunological cross-reactivity on Ouchterlony plates and by immunoblotting, and $NH_2$-terminal amino acid sequence. However, P450 PB-A differed from $\beta$NF-$A_1$/$\beta$NF-$A_2$ in peptide pattern after partial proteolysis by α-chymotrypsin and *Staphylococcus aureus* $V_8$ protease, and complete digestion of $^{125}$I-labeled cytochromes by trypsin. The cytochrome P450 PB-A also differed from $\beta$NF-$A_1$/$\beta$NF-$A_2$, in that its antibodies cross-reacted with P-450 of normal, PB-, and β-NF-induced rabbit liver microsomes. The cytochromes βNF-B and βNF-C, although immunochemically cross-reactive with each other, were distinct enzymes on the basis of molecular weights, spectral characteristics, isoelectric pH, peptide pattern on partial proteolysis, tryptic peptide pattern, cross-reactivity of their antibodies with other species, and $NH_2$-terminal amino acid sequence. The most notable difference between βNF-B and βNF-C was that the anti-βNF-C IgG completely inhibited *O*-dealkylation of 7-methoxyresorufin and 7-ethoxyresorufin by β-NF-induced microsomes. These activities increased 40- to 50-fold in β-NF-induced microsomes as compared to only 2- to 4-fold in PB-treated hens. The amino-terminal sequences of βNF-B and βNF-C were different from those of mammalian and other nonmammalian species.** © 1990 Academic Press, Inc.

Cytochrome P450 comprises a family of hemoproteins that, in their reduced form, react with CO and exhibit maximum absorbance near 450 or 448 nm. They function in conjunction with NADPH-cytochrome P450 reductase and carry out many biochemical processes including xenobiotic degradation and biosynthesis of steroid hormones, fatty acids, and prostaglandins (1 and references therein). Xenobiotic degradation is a multistep process: The molecule is first hydroxylated, later forms a conjugate with UDP-glucuronic acid, sulfate, or glutathione, and eventually is excreted from the body. Some foreign compounds are also converted into toxic or carcinogenic forms by these hemoproteins during this process (2). Xenobiotic toxicity may increase due to its unusually slow or rapid metabolism by the host mixed-function oxidase system. The functional diversity of such a mixed-function oxidase system is attributable to the presence of a large number of isozymes comprising this family of hemoproteins and their possession of limited but overlapping substrate specificity (3). This has been documented by purification of multiple forms of cytochromes P450 from rat, rabbit, and human livers (4).

The age-, sex-, strain-, and individual-related differences in the metabolism of xenobiotics and steroids have also been attributed to the presence or induction of various cytochrome P450 isozymes (5–7). The population and concentration of cytochrome P450s are greatly influenced by various treatments such as phenobarbital sodium, methylcholanthrene, β-naphthoflavone, pregnenolone-16α-carbonitrile, alcohol, and other chemicals (4, 8). The induction of P450 isozymes by drugs and environmental pollutants predisposes animals to the toxic effects of the same inducer or other environmental pollu-

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tants and carcinogenic agents. The β-naphthoflavone-inducible isozymes (P450IA1, P450IA2) in rat liver are believed to play a key role in the bioactivation of several toxic chemicals and carcinogens (9). These isozymes preferentially metabolize benzo(α)pyrene and polycyclic aromatic hydrocarbons to the mutagenic forms (10). Cytochrome P450-dependent metabolism using purified isozymes has not been explored in β-NF-treated hens.

The adult hen is currently used for evaluating delayed neurotoxic effects of organophosphorous esters and other compounds. This nonmammalian species shows greater sensitivity to neurotoxic aliphatic hydrocarbons (e.g., *n*-hexane, methyl *n*-butyl ketone, 2,5-hexanediol, 2,5-hexanedione) and organophosphorous compounds (e.g., (*O*-4-bromo-2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate, *O*-ethyl-*O*-4-nitrophenyl phenylphosphonothioate) than rodents and some nonhuman primates (11). In a previous study, we purified and characterized phenobarbital-induced P450s from adult hen liver (12). Now, we report the purification and characterization of P450 isozymes induced by a classic polycyclic aromatic hydrocarbon β-naphthoflavone. The availability of purified P450s and their antibodies will aid in studying the role of these cytochromes in the metabolism of various neurotoxic chemicals in adult hens.

## EXPERIMENTAL PROCEDURES

*Chemicals.* Chemicals were obtained from the following sources: sodium cholate, Sepharose 4B, Lubrol PX, α-chymotrypsin, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, aminopyrine, benzo[*a*]pyrene, [U-$^{14}$C]acetanilide (24.1 mCi/mmol), [U-$^{14}$C]aniline hydrochloride (13.6 mCi/mmol) (Sigma Chemical Co., St. Louis, MO); scopoletin (7-hydroxy-6-methoxycoumarin), scoparone (6,7-dimethoxycoumarin), 7-ethoxycoumarin, 7-hydroxycoumarin (Aldrich Chemical Co., Inc., Milwaukee, WI); *Staphylococcus aureus* $V_8$ protease (Miles Laboratories Inc., Naperville, IL); hydroxylapatite (Bio-Gel HT) and electrophoretic reagents (Bio-Rad Laboratories, Richmond, CA); NADPH, ampholines (LKB-Produkter AB, Bromma, Sweden); DE-51, DE-52, DE-53, CM-52 (Whatman BioSystems Ltd., Maidstone, Kent); and $^{125}$I-Protein A (New England Nuclear, Newark, DE).

*Buffers and solutions.* Buffer A: 0.1 M $KH_2PO_4$, pH 7.25, 20% glycerol. Buffer B: 20 mM $KH_2PO_4$, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.3% Lubrol PX. Buffer C: 5 mM $KH_2PO_4$, pH 7.7, 0.1 mM EDTA, 20% glycerol, 0.05% Lubrol PX, 0.2 mM DTT. Buffer D: 5 mM $KH_2PO_4$, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.1% Lubrol PX, 0.2% cholate, 0.2 mM DTT. Buffer E: 5 mM $KH_2PO_4$, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.2 mM DTT. Buffer F: 50 mM $KH_2PO_4$, pH 7.4, 1.0 mM EDTA, 20% glycerol, 0.1 mM DTT, 0.01% sodium cholate. Buffer G: 5 mM $KH_2PO_4$, pH 7.7, 0.1 mM EDTA, 20% glycerol, 0.1% Lubrol PX, 0.2% cholate, 0.2 mM DTT. Buffer H: 5 mM $KH_2PO_4$, pH 6.5, 0.1 mM EDTA, 20% glycerol, 0.2% Lubrol PX, 0.2 mM DTT. Buffer I: 1.5% SDS, 0.375 M Tris·HCl, pH 6.8. Buffer J: 5 mM $KH_2PO_4$, pH 7.4, 0.1 mM EDTA, 20% glycerol.

[3] Abbreviations used: DTT, DL-Dithiothreitol; HA, hydroxylapatite; β-NF, β-naphthoflavone; PB, phenobarbital sodium; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UT, untreated; Pen CB, 3,4,5,3′,4′-pentachlorobiphenyl.

*Isolation and solubilization of microsomes.* Adult leghorn hens, 14 months old and 1.5 ± 0.17 kg (Mean ± SD) were obtained from Featherdown Farm (Raleigh, NC). They were specified pathogen- and medication-free. The hens were intraperitoneally injected with 2% β-naphthoflavone in corn oil (80 mg/kg) for 4 days, anesthesized with carbon dioxide, and sacrificed by decapitation 24 h after the last dose. The birds were kept without feed in the last 24 h, but had free access to water. The microsomes were isolated and solubilized as described by Guengerich and Martin (13) except that homogenization was performed with Polytron and microsomes were suspended during washing with the dounce homogenizer.

*Purification of P450 isozymes.* The microsomal cholate solution from 15 hens was applied on three *n*-octylamino-Sepharose 4B columns (2.5 × 50 cm) equilibrated with buffer A (0.1 M $KH_2PO_4$, pH 7.25, 20% glycerol) containing 0.6% sodium cholate at 4°C at a flow rate of 60 ml/h. The columns were successively eluted with buffer A containing 0.33% cholate and 0.06% Lubrol PX (1200–2000 ml) and then buffer A containing 0.33% cholate and 0.25% Lubrol PX. The fractions eluted with buffer containing 0.06% Lubrol PX were concentrated, diluted with 9 vol of 20% glycerol, and loaded on an HA column (4.4 × 15 cm) equilibrated with buffer B (20 mM $KH_2PO_4$, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.3% Lubrol PX) at 4°C. The flow rate was 90 ml/h. The column was washed with equilibration buffer B containing 20 mM $KH_2PO_4$ (1400 ml), 40 mM $KH_2PO_4$ (1000 ml), 90 mM $KH_2PO_4$ (2000 ml), and 180 mM $KH_2PO_4$ (1200 ml). The eluent was changed when absorbance fell close to that of the eluting buffer. Selected fractions were read at 280 and 417 nm, and were also used for SDS-PAGE. The pooled fractions were concentrated in an Amicon ultrafiltration cell using PM-30 diaflo-membrane. The concentration and dialysis (twice), where necessary, were always performed at 4°C and against 20 vol of the related buffer.

*Cytochrome P450 βNF-$A_1$.* The fractions eluted with buffer A (0.1 M $KH_2PO_4$, pH 7.25, 20% glycerol) containing 0.33% cholate and 0.25% Lubrol PX were concentrated, dialyzed against buffer C (5 mM $KH_2PO_4$, pH 7.7, 0.1 mM EDTA, 20% glycerol, 0.05% Lubrol PX, 0.2 mM DTT), and loaded at room temperature on a DE-52 column (1.5 × 25 cm). The flow rate was 20 ml/h. The column was washed with buffer C and eluted with the same buffer containing a 0–0.15 M KCl linear gradient (250 ml each). The fractions containing cytochrome P450 were pooled, dialyzed against buffer D (5 mM $KH_2PO_4$, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.1% Lubrol PX, 0.2% cholate, 0.2 mM DTT), and loaded on an HA column (1.5 × 25 cm) at 4°C at a flow rate of 20 ml/h. The column was washed and eluted with a 5–100 mM $KH_2PO_4$ gradient (250 each) in the same buffer. The fractions containing pure P450 βNF-$A_1$ were dialyzed against buffer E (5 mM $KH_2PO_4$, pH 7.25, 0.1 mM EDTA, 20% glycerol, 0.2 mM DTT) containing 0.05% Lubrol PX and loaded on a small HA column (10 ml) equilibrated with the dialysis buffer at 4°C. The column was washed with dialysis buffer (50 ml), buffer E containing 0.2% cholate (500 ml), and then eluted with 0.3 M $KH_2PO_4$ in the same buffer. The fractions were pooled, concentrated, dialyzed against buffer F (50 mM $KH_2PO_4$, pH 7.4, 1.0 mM EDTA, 20% glycerol, 0.1 mM DTT, 0.01% sodium cholate), and stored at −70°C.

*Cytochrome P450 βNF-$A_2$.* The fractions obtained during washing of the HA column (4.4 × 15 cm) with buffer B containing 20 mM $KH_2PO_4$ were pooled, concentrated, dialyzed against buffer G (5 mM $KH_2PO_4$, pH 7.7, 0.1 mM EDTA, 20% glycerol, 0.1% Lubrol PX, 0.2% cholate, 0.2 mM DTT), and loaded on a DE-52 column (1.5 × 25 cm) at room temperature and a flow rate of 40 ml/h. The column was washed with buffer G containing 20 mM NaCl and then eluted with a 20–80 mM NaCl linear gradient (250 ml each) in the same buffer. The fractions exhibiting single band on SDS-PAGE were pooled, dialyzed against buffer E containing 0.05% Lubrol PX, and processed for the removal of Lubrol PX as described for βNF-$A_1$. In case pure P450 was not obtained from the DE-52 column, the selected fractions were pooled, dialyzed against buffer C, and loaded on another DE-52 col-

umn at room temperature. This column was washed with buffer C and then eluted with a 0–100 mM NaCl linear gradient in the same buffer.

*Cytochrome P450 βNF-B.* The fractions containing cytochrome P450 and eluted with buffer B containing 90 mM $KH_2PO_4$ from the HA column (4.4 × 15 cm) were pooled, concentrated, dialyzed against buffer G without DTT, and loaded on DE-51, DE-52, and DE-53 columns as described by Guengerich *et al.* (14). The columns were washed with an equilibration buffer, equilibration buffer containing 0.2% Lubrol PX and 0.5% cholate, and then eluted with a 0–0.3 M NaCl gradient (250 ml each) in the latter buffer. The flow rate was 40 ml/h. The fractions containing cytochrome P450 were dialyzed against buffer H (5 mM $KH_2PO_4$, pH 6.5, 0.1 mM EDTA, 20% glycerol, 0.2% Lubrol PX, 0.2 mM DTT) and loaded on a CM-52 column (1.5 × 15 cm) at room temperature. The flow was 20 ml/h. The column was washed with buffer H and eluted with a 0–0.15 M NaCl gradient (250 ml each) in the same buffer. The fractions containing pure P450 were pooled, concentrated, and processed to remove Lubrol PX as above.

*Cytochrome P450 βNF-C.* The fractions eluted from the HA column (4.4 × 15 cm) with buffer B containing 180 mM $KH_2PO_4$ were concentrated, dialyzed against buffer G without DTT, and loaded on a series of three DE-cellulose columns as described above at room temperature. The major portion of cytochrome P450 passed through the column during washing with the dialysis buffer. Selected fractions were pooled, dialyzed against buffer H, and loaded on a CM-52 column (1.5 × 15 cm) as described for βNF-B. This time the column was eluted with a 0–0.1 M NaCl linear gradient in buffer H. The fractions containing pure P450 were dialyzed against buffer D and applied on a 10-ml HA column. Lubrol PX was removed, and P450 eluted as described for βNF-$A_1$.

*Immunocytochemical methods.* The antibodies against pure P450s were raised in New Zealand white rabbits. The cytochrome P450s (0.5 nmol) were injected two times at 1-month intervals after emulsification with 2 vol (1 ml) of complete Fruend's adjuvant. A booster injection was given with the same amount of P450 in incomplete Fruend's adjuvant after 15 days, and then rabbits were bled 8 days following the booster injection. The specificity of antibodies was tested by Ouchterlony double-diffusion immunoprecipitation and immunoblotting (15). Immunodiffusion agar plates (Cooperbiochemical, Inc., Malvern) did not contain ionic or nonionic detergent. The procedure used 3–6 μl of antiserum and 6–12 pmol of cytochrome P450. The immunoprecipitation bands were visualized by amido black staining. The immunoblotting method involved resolution of proteins in polyacrylamide gels, electrophoretic transfer onto nitrocellulose sheets, and development of spots by antiserum (1000–2000 times dilution) and $^{125}$I-protein A treatment as described by Lapadula *et al.* (15).

*Peptide mapping and fingerprinting.* α-Chymotrypsin and *Staphylococcus aureus* $V_8$ protease were used for partial hydrolysis of cytochrome P450s for peptide mapping. Five to twenty-five picomoles of cytochrome P450 in 25 μl was heated for 2 min at 100°C with 12.5 μl of SDS solution I (1.5% SDS, 0.375 M Tris·HCl, pH 6.8). The denatured protein was treated at 37°C with 12.5 μl of 0.005 mg/ml or 0.05 mg/ml α-chymotrypsin or *S. aureus* $V_8$ protease, respectively (16). The peptides were separated in 12.5% polyacrylamide gels and silver stained. The cytochrome P450 bands observed by Coomassie blue staining of SDS–PAGE gels were used for the fingerprinting of pure enzymes (17). In brief, cytochrome P450s in slices were radiolabeled with $^{125}$I, digested completely with trypsin, electrophoresed at 600 V for 40 min on cellulose-coated TLC sheets (10 × 20 cm) in one direction, and chromatographed in another direction.

*Analytical methods.* Protein content was determined by the method of Smith *et al.* (18) using bovine serum albumin as the protein standard. Cytochrome P450 was estimated by the method of Matsubara *et al.* (19) in microsomes, and Omura and Sato (20) at other steps of purification. NADPH-cytochrome P450 reductase was measured by the procedure of Strobel and Dignam (21). SDS–PAGE was performed on 1.5-mm thick gel containing 4 and 7.5% acrylamide in its stacking and resolving portions (22). A linear gradient of 6–15% acrylamide was also used in some experiments. The gels were stained by alkaline silver nitrate reagent (23), and two-dimensional electrophoresis was performed as described by Vlasuk and Walz (24).

*$NH_2$-terminal sequence of proteins.* The cytochrome was dialyzed against deionized water, precipitated overnight with 90% ethanol at 0°C, and lyophilized. The sample was subjected to SDS–PAGE and electrophoretically transfer to Immobilon P (PVDF) transfer membrane (25). The protein was stained with Coomassie blue R-250, and analyzed for $NH_2$-terminal sequencing by Edman degradation using an automatic gas phase sequencer (Applied Biosystems 477A protein sequencer).

*Enzyme assays.* The reconstitution was performed by incubating cytochrome P450 (25–100 pmol) with a 3-fold concentration of NADPH-cytochrome P450 reductase, a 1.2-fold concentration of cytochrome $b_5$, and 10 μg of L-α-dilauroylglyceryl-3-phosphorylcholine in a total volume of 0.2 ml of buffer J (5 mM $KH_2PO_4$, pH 7.4, 0.1 mM EDTA, 20% glycerol). Cytochrome $b_5$ and NADPH-cytochrome P450 reductase were also prepared from adult hen liver as described before (12). The reaction mixture contained microsomes or reconstituted enzyme, substrate, 100 mM Tris·HCl, pH 7.6, NADPH-regenerating system, and any other component given in the related reference. The reaction was started by adding NADPH-regenerating system (10 mM glucose 6-phosphate, 1.0 I.U. of yeast glucose-6-phosphate dehydrogenase, 0.5 mM of NADPH) to the reaction mixture after preincubation at 37°C for 3 min. The concentration and period of incubation are given with each enzyme. The enzymes aminopyrine *N*-demethylase (5 mM, 30 min), benzphetamine *N*-demethylase (1 mM, 30 min), 7-ethoxycoumarin *O*-deethylase (0.5 mM, 10–20 min), 7-methoxyresorufin O-demethylase (0.5 μM), 7-ethoxyresorufin O-deethylase (0.5 μM), and benzo[*a*]pyrene (2 mM, 10 min) were determined as described before (12). Other enzymes such as 4-nitroanisol *O*-demethylase (2 mM, 20 min) (26), 6,7-dimethoxycoumarin *O*-demethylase (0.1 mM, 10 min) (27), aniline hydroxylase (4 mM, 10 min) (28, 29), 2-nitropropane denitrification (25 mM, 10 min) (30), and acetanilide 4-hydroxylase (3 mM, 20 min) (31) were estimated by published procedures. The inhibitor *n*-octylamine was dissolved in water by adjusting pH to 7.0, and *p*-hydroxyphenyl imidazole was added in 50% methanol (25 μl) to the reaction mixture. Immunoinhibition studies were performed by incubating microsomes with anti-P450 IgG for 30 min at room temperature before adding other constituents of the reaction mixture at 37°C.

## RESULTS

The procedure used for the isolation of P450 isozymes from β-NF-induced hen livers is a modification of the method used for the purification of cytochrome P450 isozymes from PB-induced hen livers. The cytochrome P450 βNF-$A_1$ exhibited similarity to PB-A, since it could be purified by the same procedure. However, the purity and particularly the yield increased considerably when the fraction from the *n*-octylamino–Sepharose 4B column was first purified on the DE-52 column followed by HA column chromatography. The former step removed some contaminants as well as reduced Lubrol PX concentration in the P450 fraction. The resulting cytochrome P450 βNF-$A_1$ was retained by the HA column, and eluted as pure protein by the application of a 5–85 mM potassium phosphate gradient in the buffer. The yield and specific content of βNF-$A_1$ was 2% and 13.7 nmol/mg protein, respectively. We again purified a very similar cytochrome P450 (βNF-$A_2$) from the washing of the HA column (Fig. 1, step 3). Its behavior on HA and DE-52 columns was similar to those of βNF-$A_1$ and PB-

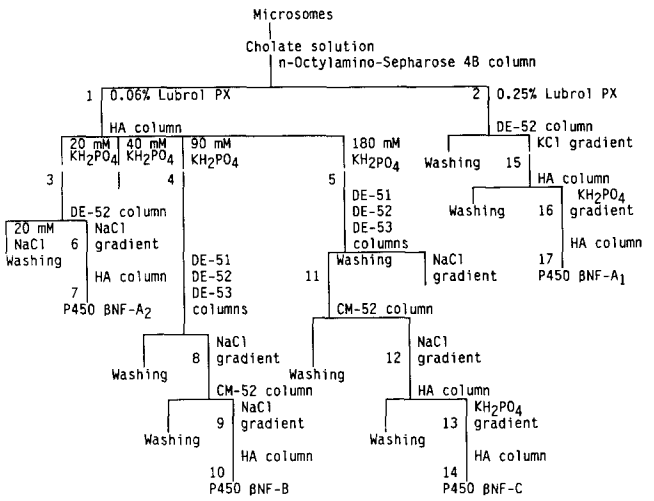


**FIG. 1.** An outline of the procedure used for purification of cytochrome P450 βNF-$A_1$, βNF-$A_2$, βNF-B, and βNF-C.

A on these columns. They were not detained on the HA column at high concentrations of Lubrol PX. The yield and specific content of P450 βNF-$A_2$ after removal of detergent was 0.42% and 21 nmol/mg protein, respectively. Study of various properties, as shown below, suggested that βNF-$A_1$ and βNF-$A_2$ probably represented the same P450. However, they were designated with slightly different names to present data on their various properties.

The cytochrome P450 βNF-B was purified from the fraction eluted with buffer containing 90 mM $KH_2PO_4$ from the HA column (Fig. 1, step 4). In β-NF-treated hens, a greater portion of cytochrome P450 (60%) was retained by the DEAE-cellulose columns, and it could be eluted by applying a 0–0.3 M NaCl gradient in buffer containing 0.2% Lubrol PX and 0.5% cholate. The cytochrome P450 βNF-B was further purified on CM-52 and HA columns. The concentration of Lubrol PX was critical in using CM-52 column for purification of this isozyme. This hemoprotein passed unabsorbed through the CM-52 column if the fractions obtained from the DEAE-cellulose columns were concentrated before being loaded on the column. The yield of isozyme after removal of Lubrol PX was 2.75% and the specific content was 9.6 nmol/mg protein. The loss of heme and partial conversion of cytochrome P450 to P420 during purification were probably responsible for the low specific content of cytochrome P450 βNF-B isozyme.

TABLE I

Summary of Purification of Cytochromes P450 βNF-$A_1$, βNF-$A_2$, βNF-B, and βNF-C from β-Naphthoflavone-Treated Hen Livers

| | | Cytochrome P450 | | |
|---|---|---|---|---|
| | Protein (mg) | Total (nmol) | Specific content (nmol/mg protein) | Recovery (%) |
| Microsomes | 3900 | 6903 | 1.77 | 100 |
| Cholate solution | 3509 | 8506 | 2.42 | 123 |
| *n*-Octylamino-Sepharose 4B column (1) | 568 | 3942 | 6.94 | 57.1 |
| HA column, 20 mM $KH_2PO_4$ (3) | 419 | 413 | 0.98 | 5.98 |
| DE-52 column (6) | — | 49.92 | — | 0.72 |
| HA column (7) | 1.38 | 28.95 | 21.00 | 0.42 |
| P450 βNF-$A_2$ | | | | |
| HA column, 90 mM $KH_2PO_4$ (4) | 363 | 2373 | 6.54 | 34.38 |
| DEAE-cellulose columns (8) | 55.78 | 540 | 9.68 | 7.82 |
| CM-52 column (9) | — | 130 | — | 1.88 |
| HA column (10) | 19.79 | 190 | 9.60 | 2.75 |
| P450 βNF-B | | | | |
| HA column, 180 mM $KH_2PO_4$ (5) | 240 | 1516 | 6.32 | 21.96 |
| DEAE-cellulose columns (11) | 113.7 | 1126 | 9.60 | 16.31 |
| CM-52 column (12) | 39.65 | 712 | 17.96 | 10.31 |
| HA column (13) | — | 510 | — | 7.39 |
| HA column (14) | 26.96 | 480 | 17.80 | 6.95 |
| P450 βNF-C | | | | |
| *n*-Octylamino-Sepharose 4B column (2) | 105.3 | 821 | 7.79 | 11.89 |
| DE-52 column (15) | 21.35 | 271.5 | 12.71 | 3.93 |
| HA column (16) | — | 235.0 | — | 3.40 |
| HA column (17) | 10.29 | 140.6 | 13.66 | 2.04 |
| P450 βNF-$A_1$ | | | | |

*Note.* See Fig. 1 for the numbers in parentheses.

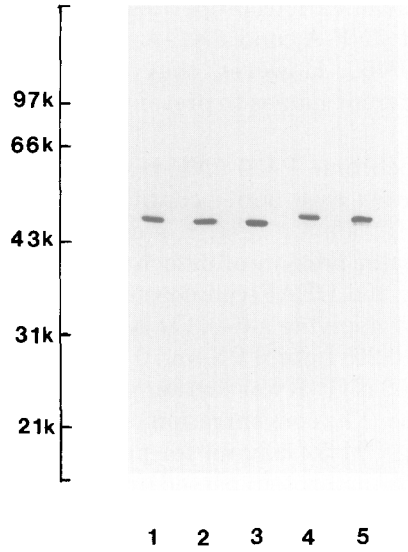


FIG. 2. SDS–PAGE of purified cytochrome P450s. Electrophoresis was performed on discontinuous polyacrylamide gels containing 4 and 7.5% acrylamide in stacking and resolving gels, respectively. The gel was stained by the alkaline silver nitrate method. Lane 1, cytochrome P450 PB-A (3.6 pmol); 2, $\beta$NF-$A_1$ (11 pmol); 3, $\beta$NF-$A_2$ (3 pmol); 4, $\beta$NF-B (3.4 pmol); and 5, $\beta$NF-C (6 pmol).

The cytochrome P450 $\beta$NF-C was purified from the fraction eluted with buffer containing 180 mM $KH_2PO_4$ from the HA column (Fig. 1, step 5). In contrast to $\beta$NF-B, this isozyme passed through the DEAE-cellulose column series during washing and was taken up by the CM-52 columns even at high concentrations of Lubrol PX. The cytochrome P450 $\beta$NF-C was further purified by HA column chromatography. This enzyme completely converted into P420 if 0.2% cholate was not included in the buffer used for removing Lubrol PX from the purified isozyme (Fig. 1, step 14). The yield and specific content of this isozyme was 6.95% and 17.8 nmol/mg protein, respectively (Table I).

Figure 2 shows the SDS–PAGE of cytochromes purified from $\beta$-NF-induced hen livers and cytochrome P450 PB-A purified from PB-treated hen. The purified proteins showed only one major protein band by silver staining. The cytochrome P450 PB-A was included, since it was found to be immunologically similar to $\beta$NF-$A_1$/$\beta$NF-$A_2$. These cytochromes (P450 PB-A, $\beta$NF-$A_1$, $\beta$NF-$A_2$) exhibited the same molecular weights (about 54,000) on gradient (6–15%) and nongradient (7.5%) polyacrylamide gels. The other cytochromes, P450 $\beta$NF-B and $\beta$NF-C, showed some variation in their relative molecular weights. The cytochrome P450 $\beta$-NFC generally showed a slightly lower molecular weight than $\beta$NF-B (56 kDa) on nongradient gels but exactly the same on gradient gels.

The purity and relative positions of the cytochrome P450 isozymes were examined by two-dimensional isoelectric focusing (Fig. 3). Cytochromes P450 PB-A, $\beta$NF-$A_1$, and $\beta$NF-$A_2$ did not separate from each other during two-dimensional isoelectrofocusing, although they demonstrated their purity by showing only one major protein band on polyacrylamide gels. Cytochrome P450 PB-A repeatedly showed more streaking than the immunologically similar cytochromes P450 $\beta$NF-$A_1$ and $\beta$NF-$A_2$, although the same isozyme focused nicely during nonequilibrium pH gradient electrophoresis used in the previous study (12). The other two cytochrome P450 isozymes ($\beta$NF-B, $\beta$NF-C) had different isoelectric points and, therefore, could be separated from each other by two-dimensional isoelectric focusing (Fig. 3). The separation was more distinct when a nongradient gel was used in two-dimensional electrophoresis, since cytochromes P450 $\beta$NF-B and $\beta$NF-C showed some difference in their molecular weights on nongradient gels. These two isozymes showed another lower molecular-weight band on two-dimensional electrophoresis. These bands were artifacts, since they were not observed on one-dimensional SDS–PAGE. The use of gradient gels (6–15%) in two-dimensional electrophoresis decreased the distance between the two bands but did not completely eliminate them.

The spectral studies of purified isozymes demonstrated high-spin states of cytochromes P450 PB-A, $\beta$NF-$A_1$, and $\beta$NF-$A_2$, and low-spin states of cytochromes P450 $\beta$NF-B and $\beta$NF-C. The high-spin hemoproteins showed a shoulder at 415 nm even at high concentration, thus suggesting the presence of low-spin protein at all concentrations. The percentage of low-spin

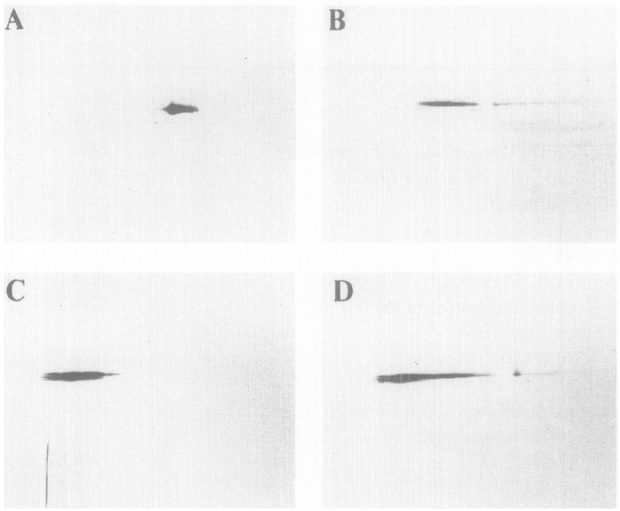


FIG. 3. Two-dimensional electrophoresis of cytochrome P450s. The cytochromes were subjected to isoelectric focusing in one direction and SDS–PAGE on 6–15% polyacrylamide gradient gels in another direction. The anode was on the right hand side during isoelectric focusing and at the bottom during polyacrylamide gel electrophoresis. A, cytochrome P450 PB-A (7.2 pmol) + $\beta$NF-$A_1$ (13.2 pmol) + $\beta$NF-$A_2$ (10 pmol); B, $\beta$NF-B (4.8 pmol); C, $\beta$NF-C (6 pmol); and D, $\beta$NF-B + $\beta$NF-C. The gels were stained by alkaline silver nitrate reagent.

TABLE II

Spectral Properties of Oxidized, Reduced, and $Fe^{2+}$–CO Complex States of Cytochrome P450 Isozymes

| Cytochrome P450 | Oxidized | Reduced | $Fe^{2+}$–CO complex |
|---|---|---|---|
| PB-A | 391 | 410 | 449 |
| | 541 | 554 | 558 |
| | 565 | | |
| βNF-$A_1$ | 394 | 410 | 449 |
| | 530 | 562 | 562 |
| | 566 | | |
| βNF-$A_2$ | 392 | 410 | 450 |
| | 536 | 550 | 556 |
| | 564 | | |
| βNF-B | 414 | 410 | 445 |
| | 532 | 540 | 548 |
| | 564 | | |
| βNF-C | 416 | 410 | 446 |
| | 533 | 539 | 548 |
| | 567 | | |

*Note.* The cytochrome P450 isozymes (P450 PB-A, βNF-$A_1$, βNF-$A_2$, βNF-B, βNF-C) were scanned from 350 to 650 nm in a buffer containing 50 mM $KH_2PO_4$, pH 7.4, 0.1 mM EDTA, and 20% glycerol. The spectrum was recorded in oxidized and reduced cytochrome P450 isozymes and after passing carbon monooxide through the reduced cytochrome P450 isozymes.

states, however, increased with dilution and resulted in a broader peak around 394 nm in dilute solutions of these cytochromes (results not shown). All the purified cytochrome P450s exhibited a soret peak at 410 nm on reduction with dithionite. The soret peak of the $Fe^{2+}$–CO complex was about 449 nm for high-spin hemoproteins and close to 446 nm for low-spin hemoproteins (P450 βNF-B, βNF-C) (Table II). The minor differences in the α and β peaks were not of any significance, since those peaks were very broad and their peak values easily varied under a slight change of conditions.

The primary structures of cytochrome P450 PB-A as well as those purified in this study were compared by peptide mapping of their partial proteolytic products. Comparison of peptides after α-chymotrypsin treatment showed that cytochrome P450 PB-A had several peptide bands that were either not present in cytochromes P450 βNF-$A_1$ and βNF-$A_2$ or present as very faint bands (Fig. 4A). These faint bands in the latter two cytochromes were probably due to the existence of some P450 PB-A (present in phenobarbital-treated hens) in the β-NF-induced hen livers, and its copurification with βNF-$A_1$ and βNF-$A_2$ because of very similar chromatographic properties. Similar behavior of these cytochromes was observed after treatment with *Staphylococcus aureus* $V_8$ protease (Fig. 4B). Hydrolysis by both proteases showed great similarity between cytochromes P450 βNF-$A_1$ and βNF-$A_2$ except two bands in lanes 3 and 6 (Fig. 4), which were deeper in the later cytochrome. However, there

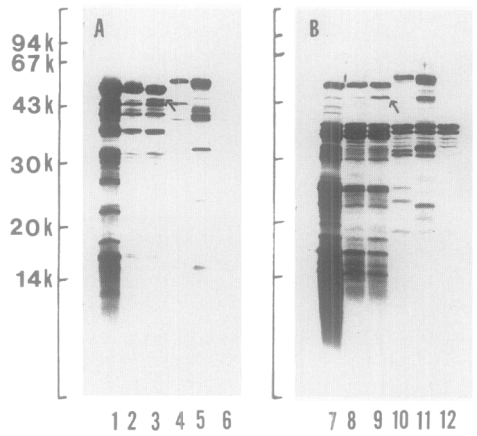


**FIG. 4.** One-dimensional peptide mapping of cytochromes. The cytochromes P450 PB-A (25 pmol) (1, 7), βNF-$A_1$ (25 pmol) (2, 8), βNF-$A_2$ (15 pmol) (3, 9), βNF-B (3 pmol) (4, 10), and βNF-C (14 pmol) (5, 11) were partially hydrolyzed at 37°C for 30 min by α-chymotrypsin (65 ng) or *S. aureus* $V_8$ protease (650 ng). Lane 6, α-chymotrypsin (65 μg) and lane 12, *S. aureus* $V_8$ protease (650 μg). A, α-chymotrypsin and B, *S. aureus* $V_8$ protease. The peptides were heated with SDS–sample buffer and subjected to SDS–PAGE on 12.5% polyacrylamide gels. In some cases a smaller amount of P450 was used to avoid too much of it being left at the origin.

were more marked differences between cytochromes P450 βNF-B and βNF-C, and most of the major peptides present in these two isozymes did not match each other after both types of hydrolysis.

The complete digestion of $^{125}I$-labeled cytochrome P450s by trypsin also yielded results similar to those obtained by partial hydrolysis by α-chymotrypsin and *S. aureus* $V_8$ protease. The two-dimensional peptide pattern from cytochrome P450 PB-A was distinct from those of immunologically similar cytochromes P450

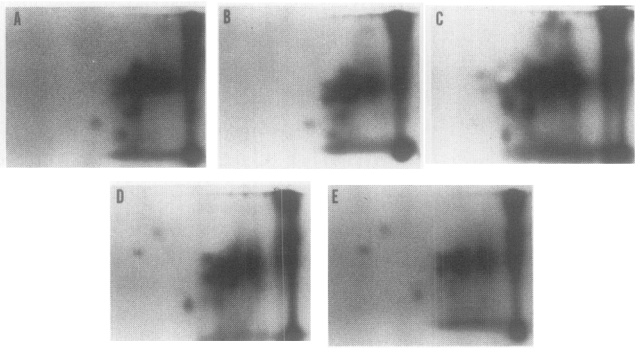


**FIG. 5.** Tryptic peptide fingerprinting of cytochrome P450s. The cytochromes (20 pmol) were electrophoresed on polyacrylamide gels and stained with Coomassie blue. The stained slices were radioiodinated with $^{125}I$, digested overnight with trypsin (50 μg/ml), and subjected to electrophoresis in one direction and chromatography in the other direction on cellulose-coated TLC plates as described under Experimental Procedures. A, cytochrome P450 βNF-$A_1$; B, βNF-$A_2$; C, PB-A; D, βNF-B; and E, βNF-C.

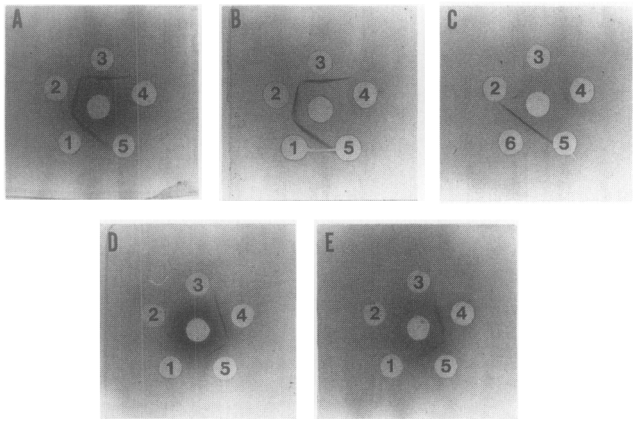


**FIG. 6.** Ouchterlony double-diffusion immunoprecipitation analysis of cytochrome P450 isozymes. The agarose gel used for detecting double-diffusion immunoprecipitation reactions did not contain detergent. The center wells contained anti-serums and the surrounding ones, cytochrome P450s. Center wells: A, anti-$\beta$NF-$A_2$ (2 $\mu$l); B, anti-PB-A (2 $\mu$l); C, anti-PB-$B_1$ (3 $\mu$l); D, anti-$\beta$NF-B (3 $\mu$l); E, anti-$\beta$NF-C (3 $\mu$l). Surrounding wells: 1, P450 PB-A (6 pmol); 2, $\beta$NF-$A_1$ (9 pmol); 3, $\beta$NF-$A_2$ (12 pmol); 4, $\beta$NF-B (9 pmol); 5, $\beta$NF-C (12 pmol); and 6, PB-$B_1$ (6 pmol). The plates were incubated at room temperature for 72 h and stained with amido black.

$\beta$NF-$A_1$ and $\beta$NF-$A_2$, whereas the latter two cytochromes exhibited similar peptide patterns (Fig. 5). The other two immunologically similar cytochromes P450 $\beta$NF-B and $\beta$NF-C also showed similar peptide patterns, but with a few exceptions. There were two overlapping deep spots (in the center cluster of spots) in cytochrome P450 $\beta$NF-B as compared to only one in the case of P450 $\beta$NF-C. In addition, cytochrome P450 $\beta$NF-B yielded more peptides in a separate lane close to the origin.

Double-diffusion immunoprecipitation was used to examine the immunological properties of cytochrome P450 isozymes (Fig. 6). The cytochromes P450 PB-A, $\beta$NF-$A_1$, and $\beta$NF-$A_2$ showed fusion of their precipitation bands with both anti-PB-A and anti-$\beta$NF-$A_2$, and no reaction with anti-P450 PB-$B_1$ (PB-$B_1$ was previously purified from PB-treated hens), anti-$\beta$NF-B, and anti-$\beta$NF-C. On the other hand, cytochromes P450 $\beta$NF-B and $\beta$NF-C formed precipitation bands with both anti-$\beta$NF-B and anti-$\beta$NF-C and exhibited no reaction with anti-PB-A, anti-PB-$B_1$, and anti-$\beta$NF-$A_2$. The antibodies raised against P450 PB-$B_1$ reacted only with homologous cytochrome P450 PB-$B_1$ and not with other cytochrome P450s used in this study. Cytochromes P450 $\beta$NF-B and $\beta$NF-C sometime produced one faint precipitation band with both anti-$\beta$NF-B and anti-$\beta$NF-C. The formation of these faint precipitation bands was probably due to the presence of aggregates of different sizes in these isozymes in detergent-free Ouchterlony plates.

Antiserums raised against cytochrome P450s were used to examine their induction and cross-reactivity with heterologous forms (Fig. 7). Immunoblotting analysis showed that cytochromes P450 $\beta$NF-B and $\beta$NF-C were induced by $\beta$-naphthoflavone and were present only in traces in normal and PB-induced microsomes. The antibodies against P450 $\beta$NF-B and $\beta$NF-C did not react with P450 PB-A and PB-$B_1$ (purified previously from PB-induced hen livers) or P450 $\beta$NF-$A_1$ and $\beta$NF-$A_2$ purified from $\beta$-NF-induced hen livers. However, anti-P450 $\beta$NF-B and anti-P450 $\beta$NF-C cross-reacted with P450 $\beta$NF-C and P450 $\beta$NF-B, respectively. The cross-reaction of anti-P450 $\beta$NF-B with $\beta$NF-C was much stronger than the cross-reaction of anti-P450 $\beta$NF-C with $\beta$NF-B, although the latter is not so clear in the given figure. This suggested that P450 $\beta$NF-B and $\beta$NF-C had some common epitopes that were responsi-

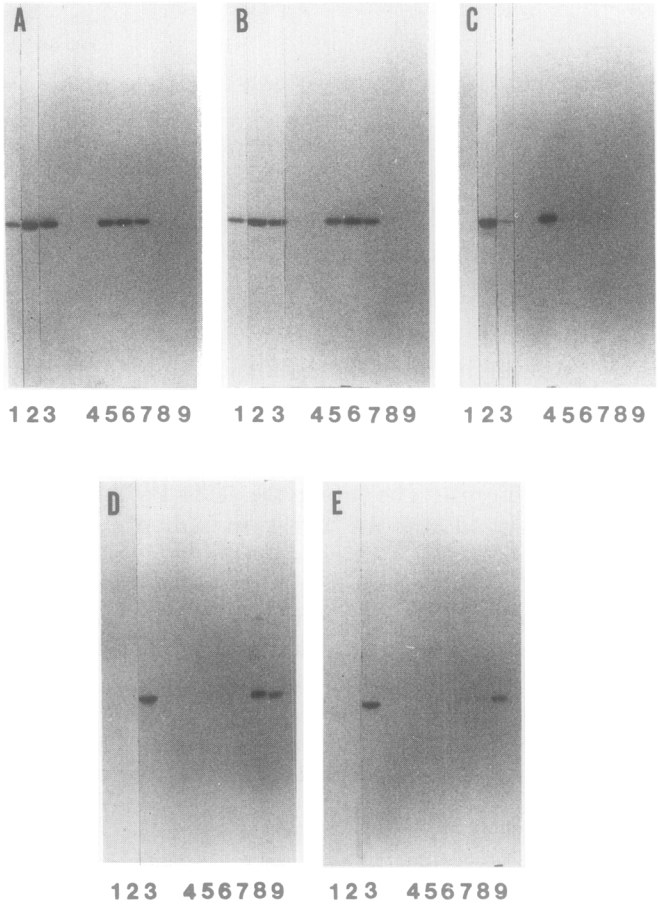


**FIG. 7.** Cross-reactivity of cytochrome P450s and their induction in microsomes. The microsomes or cytochrome P450s were subjected to electrophoresis on polyacrylamide gels and electrophoretically transferred to nitrocellulose sheets. The nitrocellulose sheets were then treated with primary antibodies (1:1000 dilution) and $^{125}$I-protein A as described under Experimental Procedures. The immunopositive reactions were detected by autoradiography in the presence of two intensifying screens. Lane 1, normal microsomes (10 $\mu$g); 2, PB-induced microsomes (10 $\mu$g); 3, $\beta$-NF-induced microsomes (10 $\mu$g); 4, cytochrome P450 PB-$B_1$ (6 pmol); 5, P450 PB-A (6 pmol); 6, P450 $\beta$NF-$A_1$ (11 $\mu$g); 7, P450 $\beta$NF-$A_2$ (3 pmol); 8, P450 $\beta$NF-B (3.4 pmol); and 9, $\beta$NF-C (6 pmol). A, Anti-P450 PB-A; B, anti-P450 $\beta$NF-$A_2$; C, anti-P450 PB-$B_1$; D, anti-P450 $\beta$NF-B; and E, anti-P450 $\beta$NF-C.

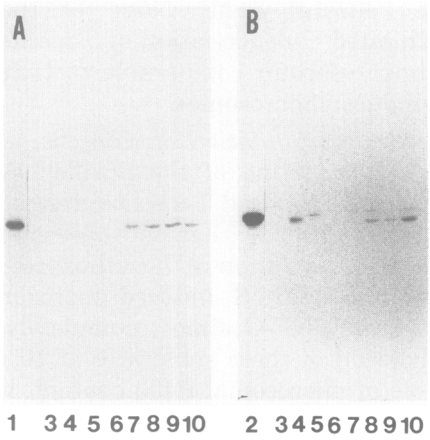


**FIG. 8.** Immunological cross-reactivity of purified cytochrome P450 isozymes with those present in the hepatic microsomes of other species. The liver microsomes (5 μg protein) from (1) PB-treated hens, (2) β-NF-treated hens, (3) normal rats, (4) PB-treated rats, (5) β-NF-treated rats, (6) human, (7) normal rabbits, (8) corn oil-treated rabbits, (9) PB-treated rabbits, and (10) β-NF-treated rabbits were subjected to SDS–PAGE, electrophoretically transferred to nitrocellulose sheets, and developed with primary antiserums and $^{125}$I-protein A as described under Experimental Procedures. A, anti-P450 PB-A; B, anti-βNF-B.

ble for their cross-reactivity. Anti-P450 PB-$B_1$ did not react with cytochrome P450 PB-A, βNF-$A_1$, βNF-$A_2$, βNF-B, or βNF-C. The antiserums raised against cytochromes P450 PB-A and βNF-$A_2$ seemed to react equally well with cytochrome P450 PB-A, βNF-$A_1$, and βNF-$A_2$. The reaction of different antiserums with normal microsomes showed that only cytochromes P450 PB-A and βNF-$A_1$/βNF-$A_2$ were present in normal liver microsomes. The other cytochromes were present only in traces and could not be detected unless autoradiography was performed for comparatively longer periods.

We examined the immunological cross-reactivity of purified P450 isozymes with those present in hepatic microsomes from other species such as rat, mouse, rabbit, cat, and catfish. The results revealed some important differences in immunologically similar cytochrome P450s. Thus, in contrast to anti-P450 βNF-$A_1$/βNF-$A_2$, antiserum against P450 PB-A reacted with protein of the same molecular weight in the microsomes of normal, corn oil-, PB-, and β-NF-treated rabbits (Fig. 8A). Similarly, in contrast to anti-P450 βNF-C, anti-P450 βNF-B slightly cross-reacted with proteins in the range of 45,000 to 60,000 Da from PB- and β-NF-treated rat; normal, PB-, and β-NF-treated rabbit (Fig. 8B); normal, PB-, and β-NF-treated mice; and normal and βNF-treated catfish (data not shown). Also, none of the antiserums reacted with the one available human microsomal sample.

*Amino-terminal sequence.* Cytochromes P450 βNF-B and βNF-C showed distinct amino-terminal sequences, whereas βNF-$A_1$, βNF-$A_2$, and PB-A exhibited the same sequence of their detected amino acid residues (Table III). Cytochromes P450 βNF-B, βNF-$A_1$, βNF-$A_2$, and PB-A had the same first five $NH_2$-terminal residues and had an acidic residue in the second position. However, cytochromes P450 βNF-B and βNF-C did not have the leucine triad in their amino-terminal sequences. Furthermore, the latter also did not contain an acidic residue in its second or third position.

*Effect of cytochrome $b_5$ and inhibitors on catalytic activity.* Since cytochromes P450 PB-A, βNF-$A_1$, and βNF-$A_2$ exhibited the same $NH_2$-terminal sequence of all detected amino acids, we examined further the effect of cytochrome $b_5$ and inhibitors on the metabolism of some substrates. All three P450s showed the same pattern of induction of activity by the addition of cytochrome $b_5$. The induction of activity depended on the nature of the substrate. 7-Ethoxycoumarin *O*-deethylase activity increased about 30-fold, whereas aminopyrine, benzo[*a*]pyrene, and aniline metabolism increased only about 2-fold (Table IV). Aminopyrine *N*-demethylase activity was more sensitive toward metyrapone, *n*-octylamine, and SKF-525A than 7-ethoxycoumarin *O*-deethylation, and the latter showed slightly greater sensitivity toward *p*-hydroxyphenyl imidazole for all the three P450s (Table V). However, none of the inhibitors could distinguish between these cytochromes on the basis of inhibition of 7-ethoxycoumarin *O*-deethylase and aminopyrine *N*-demethylase activities.

*Catalytic activities of microsomes.* Comparison of matabolic activities of microsomes showed that the metabolism of aminopyrine, benzphetamine, 7-ethoxycoumarin, 4-nitroanisole, and aniline was induced by both PB and β-NF treatment of hens (Table VI). However, β-NF treatment greatly increased the metabolism of benzo[*a*]pyrene, acetanilide, 7-methoxyresorufin, and 7-ethoxyresorufin as compared to PB treatment. Acetanilide 4-hydroxylation was induced only by β-NF treatment. The formation of isoscopoletin and scopoletin was not increased by either treatment, but a new unknown metabolite was formed by β-NF-induced microsomes that eluted from the HPLC column between scopoletin and scoparone peaks. The results suggested that β-NF treatment of hens induced P450s βNF-$A_1$/βNF-$A_2$ that were either the same as or similar to P450 PB-A induced by PB treatment.

*Catalytic activities of purified P450s.* Cytochromes P450 PB-A, βNF-$A_1$, and βNF-$A_2$ metabolized all the used substrates except 7-methoxyresorufin and 7-ethoxyresorufin (Table VI). The cytochrome P450 PB-A was the most active of these three enzymes, and was followed by βNF-$A_2$ and βNF-$A_1$ in descending order. Between the other two isozymes βNF-B and βNF-C, the former was more active in the metabolism of aminopyrine, benzphetamine, 4-nitroanisole, aniline, acetanilide, and scoparone, while the latter was of 7-ethoxycou-

TABLE III

Amino-Terminal Sequences of Cytochromes P450 PB-A, βNF-$A_1$, βNF-$A_2$, βNF-B, and βNF-C

| Cytochrome | Residue (yields in pmol) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | | | | | | | 10 | | | | | | | 15 | | | | | | | 20 | | | | | | | 25 | | | | | | | | | | | | 30 | |
| P450 PB-A | M | E | V | T | A | A | L | L | L | F | L | G | L | S | L | V | V | L | L | A | V | R | G | R | | | | | | | | | | | | | | | | |
| | 51 | 57 | 45 | 42 | 74 | 77 | 70 | 73 | 73 | 53 | 60 | 65 | 65 | 13 | 50 | 34 | 39 | 43 | 46 | 32 | 17 | 22 | 37 | 24 | | | | | | | | | | | | | | | | |
| P450 βNF-$A_1$ | M | E | V | T | A | A | L | L | L | F | L | G | L | S | L | V | X | L | X | A | V | R | G | R | G | G | (A) | (G) | | | | | | | | | | | | |
| | 70 | 30 | 53 | 46 | 77 | 62 | 65 | 100 | 82 | 83 | 70 | 71 | 75 | 27 | 70 | 63 | | 93 | | 53 | 47 | —[a] | 49 | —[a] | 55 | 56 | 53 | 50 | | | | | | | | | | | | |
| P450 βNF-$A_2$ | M | E | V | T | A | (A) | L | L | L | F | L | G | L | S | L | V | V | L | L | A | V | R | G | R | G | G | A | G | | | | | | | | | | | | |
| | 24 | 16 | 23 | 13 | 25 | 16 | 21 | 21 | 34 | 28 | 35 | 34 | 19 | 10 | 25 | 19 | 20 | 25 | 28 | 20 | 17 | 24 | 24 | 21 | 23 | 25 | 15 | 22 | | | | | | | | | | | | |
| P450 βNF-B | M | E | V | T | A | V | M | V | T | A | (I) | X | X | G | X | V | | | | | | | | | | | | | | | | | | | | | | | | |
| | 22 | 1 | 6 | 3 | 11 | 15 | 13 | 23 | 4 | 19 | 8 | | | 23 | | 12 | | | | | | | | | | | | | | | | | | | | | | | | |
| P450 βNF-C | A | (A) | G | P | Q | A | A | M | E | Q | A | S | S | P | G | L | I | S | A | X | X | V | L | V | A | (P) | D | T | F | D | | | | | | | | | | |
| | 52 | 33 | 41 | 3 | 18 | 31 | 37 | 19 | 15 | 18 | 30 | 15 | 15 | 6 | 29 | 35 | 24 | 12 | 24 | | | 19 | 32 | 20 | 23 | 6 | 9 | 16 | 14 | 8 | | | | | | | | | | |

*Note.* The cytochromes were subjected to SDS–PAGE and electrophoretically transferred onto Immobilon P (PVDF) transfer membranes. The membranes were stained, destained, and cytochrome P450 spots were used for sequencing as described under Experimental Procedures. The yield for each cycle of the $NH_2$-terminal amino acid sequence is given in pmol. The amino acids represented by X could not be determined and those enclosed in parentheses were not certain.

[a] Peaks not quantitated.

marin and benzo[*a*]pyrene. None of the purified enzymes exhibited *O*-dealkylation of 7-methoxyresorufin or 7-ethoxyresorufin comparable to that observed with β-NF-induced microsomes.

*Immunochemical inhibition of catalytic activities in microsomes.* Since none of the purified cytochrome P450s actively metabolized 7-methoxyresorufin and 7-ethoxyresorufin, antibodies against these enzymes were used to inhibit dealkylation of 7-methoxyresorufin and 7-ethoxyresorufin by β-NF-induced microsomes. Anti-cytochrome P450 βNF-C almost completely inhibited the dealkalylation of these substrates at 10 mg of IgG per nanomole of microsomal P450 content, while anti-βNF-B inhibited only slightly at the same concentration of IgG (Fig. 9). Antibodies raised against P450 PB-A, βNF-$A_1$, and βNF-$A_2$ did not show inhibition of dealkylation of 7-methoxyresorufin or 7-ethoxyresorufin by microsomes. The results suggested that cytochrome P450 βNF-C played a major role in the metabolism of these compounds.

Immunoinhibition of microsomal 7-ethoxycoumarin *O*-deethylation by anti-PB-A, anti-βNF-$A_1$, and anti-βNF-$A_2$ was used to differentiate between related cytochrome P450s. All three antibodies showed the same pattern of inhibition and inhibited ~50% and ~40% of 7-ethoxycoumarin *O*-deethylase activity at 2 mg/nmol P450 in PB- and β-NF-induced microsomes, respectively. At higher concentrations, however, IgG from normal rabbit serum strongly inhibited this activity in β-NF-induced microsomes but not in PB-induced microsomes. Thus cytochromes P450 PB-A, βNF-$A_1$, and βNF-$A_2$ could not be differentiated by immunoinhibition of microsomal 7-ethoxycoumarin O-deethylase activity (data not shown).

## DISCUSSION

We previously reported the method of purification of hepatic cytochromes P450 PB-A and PB-B from phenobarbital-treated hens (12). In this report, we present the purification of cytochromes P450 βNF-A, βNF-B, and βNF-C from β-NF-treated adult hens. Cytochrome P450 βNF-A appeared in two fractions that were designated P450 βNF-$A_1$ and βNF-$A_2$ for the purpose of comparing their properties. Cytochrome P450 PB-A previously purified from PB-treated hens was also included in this study, since it was found to be similar to cytochromes P450 βNF-$A_1$ and βNF-$A_2$ and might represent the same cytochrome P450. To the best of our knowledge, there is only one report on the purification of hepatic cytochrome P450 isozymes from 3,4,5,3′,4′-pentachlorobiphenyl (PenCB)-induced 9-day-old chickens (32). Although PenCB is a methylcholanthrene-type inducer, there is a great difference in the age of the chickens used in that study. We used about 18-month-old hens, since they are more sensitive to several neurotoxic chemicals.

TABLE IV

Effect of Cytochrome $b_5$ on the Catalytic Activity of Immunologically Similar Cytochrome P450s (PB-A, βNF-$A_1$, βNF-$A_2$)

| Substrate | PB-A | βNF-$A_1$ | βNF-$A_2$ |
|---|---|---|---|
| | Relative activity $+b_5/-b_5$ | | |
| 7-Ethoxycoumarin | 27.97 | 29.69 | 37.35 |
| Aminopyrine | 2.13 | 1.46 | 1.89 |
| Benzo(α)pyrene | 2.09 | 1.97 | 2.99 |
| Aniline | 2.00 | 1.42 | 1.81 |

*Note.* The catalytic activity of P450s (PB-A, βNF-$A_1$, βNF-$A_2$) was estimated both in the presence and in the absence of cytochrome $b_5$, and the ratio of turnover number of substrate with and without cytochrome $b_5$ was calculated. The standard reconstitution mixture consisted of 20 pmol of P450, 60 pmol of NADPH-cytochrome P450 reductase, 24 pmol of cytochrome $b_5$, and 10 μg of dilauroylphosphatidylcholine in a total volume of 200 μl. The assay conditions are described under Experimental Procedures.

The purification procedure and catalytic properties (e.g., 7-ethoxyresorufin O-deethylation and benzo[*a*]pyrene hydroxylation) suggested that P448H and P448L (32) might correspond to P450 βNF-B and βNF-C, respectively. The cytochrome P448H did not appear to be similar to βNF-$A_1$/βNF-$A_2$, since the former was not present in detectable amounts in untreated chickens, and cytochrome βNF-$A_1$/βNF-$A_2$ (although induced by β-naphthoflavone treatment) was also present in untreated adult hens. Similarly, P450s βNF-B and βNF-C might correspond to P450d and P450c, respectively, in rats (33). Cytochromes P450 βNF-B and βNF-C, however, differed from P448H and P448L in chickens (32) and P450c and P450d in rats (33) in that both βNF-B and βNF-C were low-spin hemoproteins.

Chromatographic, immunoinhibition, catalytic and spectral properties, amino-terminal sequence, and molecular weights suggested that cytochromes P450 βNF-$A_1$/βNF-$A_2$ were identical or very similar to P450 PB-A and were induced by both PB and β-NF treatment. Ryan *et al.* (10) have also reported the induction of cytochrome P450a by three inducers: phenobarbital, 3-methylcholanthrene, and Aroclor 1254.

The other cytochromes P450 βNF-B and βNF-C were not induced by PB treatment. These cytochromes, however, exhibited some anomalous behavior: (i) they showed some difference in their molecular weights on nongradient SDS–PAGE, but not on 6–15% gradient gels; and (ii) they exhibited another faint band of low molecular weight on two-dimensional electrophoresis that was not visible on SDS–PAGE in a single dimension. These isozymes exhibited some immunological similarity by Ouchterlony and immunoblotting analysis. However, compared to Ouchterlony, immunoblotting analysis showed greater difference in the immunological properties of cytochromes P450 βNF-B and βNF-C in that each of them reacted more strongly with the homologous antibody than with anti-βNF-C and anti-βNF-B, respectively. Furthermore, in contrast to anti-βNF-C, anti-βNF-B cross-reacted with proteins in the range of 45,000–60,000 Da from PB- and βNF-treated rats; nor-

TABLE V

Effect of P450 Inhibitors on 7-Ethoxycoumarin *O*-Deethylation and Aminopyrine *N*-Demethylation by Purified Cytochrome P450s (PB-A, βNF-$A_1$, βNF-$A_2$)

| | Percentage of activity in the presence of inhibitor | | | | | |
|---|---|---|---|---|---|---|
| | 7-Ethoxycoumarin *O*-deethylation | | | Aminopyrine *N*-demethylation | | |
| Inhibitor | PB-A | βNF-$A_1$ | βNF-$A_2$ | PB-A | βNF-$A_1$ | βNF-$A_2$ |
| Metyrapone | | | | | | |
| 0.1 mM | 85.15 | 78.29 | 81.24 | 40.76 | 35.79 | 47.78 |
| 0.5 mM | 50.59 | 47.84 | 44.92 | 19.51 | 28.83 | 23.32 |
| *n*-Octylamine | | | | | | |
| 0.2 mM | 72.85 | 67.89 | 66.38 | 27.42 | 30.33 | 31.09 |
| 0.5 mM | 44.46 | 46.82 | 41.02 | 16.97 | 21.43 | 27.31 |
| SKF-525A | | | | | | |
| 0.2 mM | 93.61 | 100.00 | 98.78 | 59.00 | 54.64 | 61.52 |
| P-Hydroxyphenylimidazole | | | | | | |
| 50 μM | 86.87 | 90.09 | 88.91 | 109.91 | 107.10 | 101.96 |
| 0.2 mM | 72.99 | 77.72 | 65.49 | 85.91 | 82.14 | 81.72 |

*Note.* The purified P450s (20 pmol) were reconstituted with 60 pmol of NADPH-cytochrome P450 reductase, 24 pmol of cytochrome $b_5$, and 10 μg of dilauroylphosphatidylcholine in a total volume of 200 μl. The inhibitors were added after the substrate, and the assay conditions are described under Experimental Procedures.

TABLE VI

Turnover Numbers of Some Substrates by Purified Cytochrome P450s, and Control, PB-Induced, β-NF-Induced Hepatic Microsomes

| Substrate | PB-A | βNF-$A_1$ | βNF-$A_2$ | βNF-B | βNF-C | Control | PB-induced | β-NF-induced |
|---|---|---|---|---|---|---|---|---|
| Aminopyrine | 34.35 | 11.70 | 25.19 | 21.63 | 1.33 | 20.47 ± 4.23 | 26.65 ± 1.87 | 15.55 ± 1.50 |
| | | | | | | (7.76 ± 1.66) | (23.47 ± 4.42) | (24.07 ± 0.67) |
| Benzphetamine | 10.22 | 3.47 | 8.19 | 1.10 | 2.74 | 17.39 ± 4.18 | 28.41 ± 1.24 | 13.41 ± 0.71 |
| | | | | | | (6.62 ± 1.74) | (24.69 ± 2.25) | (20.87 ± 1.49) |
| 7-Ethoxycoumarin | 29.78 | 10.76 | 15.18 | 1.40 | 2.78 | 12.54 ± 2.11 | 10.31 ± 0.14 | 4.93 ± 0.08 |
| | | | | | | (4.78 ± 1.01) | (8.95 ± 1.21) | (7.71 ± 0.90) |
| 2-Nitropropane | 9.14 | 3.80 | 6.52 | 0.32 | 0.28 | 5.58 ± 1.00 | 3.75 ± 0.15 | 1.86 ± 0.22 |
| | | | | | | (2.16 ± 0.61) | (3.27 ± 0.40) | (2.96 ± 0.74) |
| 4-Nitroanisole | 10.66 | 3.83 | 5.92 | 3.25 | 0.92 | 7.70 ± 0.75 | 6.69 ± 0.12 | 5.95 ± 0.53 |
| | | | | | | (3.05 ± 1.00) | (6.84 ± 1.95) | (9.22 ± 0.30) |
| Aniline | 15.44 | 5.87 | 8.42 | 12.64 | 7.45 | 4.15 ± 0.98 | 4.05 ± 0.45 | 4.62 ± 0.21 |
| | | | | | | (1.55 ± 0.28) | (4.28 ± 1.71) | (7.19 ± 0.63) |
| Acetanilide | 25.50 | 11.10 | 23.10 | 14.40 | 5.10 | 10.34 ± 1.23 | 4.50 ± 1.85 | 14.50 ± 4.74 |
| | | | | | | (4.06 ± 1.25) | (3.73 ± 0.99) | (21.84 ± 5.21) |
| Scoparone | 29.85[a] | 5.47 | 10.84 | 1.40 | 0.57 | 6.04 ± 1.00 | 2.63 ± 0.22 | 1.75 ± 0.04 |
| | | | | | | (2.32 ± 0.56) | (2.28 ± 0.28) | (2.75 ± 0.39) |
| | 16.42[b] | 2.79 | 5.73 | 0.57 | ND | 3.96 ± 0.65 | 1.58 ± 0.31 | 1.00 ± 0.21 |
| | | | | | | (1.54 ± 0.45) | (1.38 ± 0.34) | (1.56 ± 0.31) |
| | | | | | | | | 2.51 ± 0.91[c] |
| | | | | | | | | (3.79 ± 1.09) |
| Benzo(α)pyrene | 4.00 | 1.58 | 2.00 | 0.44 | 1.68 | 1.01 ± 0.14 | 1.53 ± 0.65 | 2.43 ± 0.13 |
| | | | | | | (0.40 ± 0.10) | (1.27 ± 0.36) | (3.78 ± 0.32) |
| 7-Methoxyresorufin | 0.04 | ND | 0.05 | 0.01 | ND | 0.04 ± 0.01 | 0.08 ± 0.01 | 0.38 ± 0.05 |
| | | | | | | (0.02 ± 0.00) | (0.07 ± 0.01) | (0.67 ± 0.09) |
| 7-Ethoxyresorufin | 0.04 | ND | 0.02 | 0.03 | 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.23 + 0.03 |
| | | | | | | (0.01 ± 0.00) | (0.01 ± 0.00) | (0.40 ± 0.00) |

*Note.* The pure enzymes (20–100 pmol) were reconstituted at 24–25°C with threefold NADPH-cytochrome P450 reductase, 1.2-fold cytochrome $b_5$, and 10 μg of dilauroylphosphatidylcholine in a total volume of 0.2 ml. The assay conditions are described under Experimental Procedures. The catalytic activities of microsomes are also expressed as nmol of product/min per milligram protein in parentheses. The results are given as means ± SD from three pooled samples of microsomes prepared from five hens.

[a] Isoscopoletin (6-hydroxy-7-methoxycoumarin), [b]scopoletin (7-hydroxy-6-methoxycoumarin) were the products of scoparone, and [c]unknown metabolite.

mal, PB-, and βNF-treated rabbits; normal, PB-, and βNF-treated mice; and normal and βNF-treated catfish. The distinction between cytochromes P450 βNF-B and βNF-C was further confirmed by their different amino-terminal sequences and partial separation by two-dimensional electrophoresis. Similar cross-reactivity between βNF/ISF-G and βNF-B, and P450c and P450d have been reported by Guengerich *et al.* (14) and Ryan *et al.* (33), respectively.

Cytochromes P450 PB-A, βNF-$A_1$, and βNF-$A_2$ could not be unambiguously distinguished from each other on the basis of following characteristics: molecular weights, isoelectric focusing, $NH_2$-terminal amino acid sequence, immunoinhibition studies using PB- and β-NF-induced microsomal enzymes, inhibition of 7-ethoxycoumarin O-deethylase and aminopyrine *N*-demethylase activities by inhibitors such as metyrapone, *n*-octylamine, SKF 525-A, and *p*-hydroxyphenyl imidazole, and the effect of cytochrome $b_5$ on the metabolism of some substrates (e.g., 7-ethoxycoumarin, aminopyrine, benzo[*a*]pyrene, and aniline). However, some characteristics of these P450s suggested that there might be some minor difference in the primary structures of βNF-$A_1$/βNF-$A_2$ and PB-A away from the amino-terminal sequence: (i) in contrast to anti-βNF-$A_1$/βNF-$A_2$, anti-PB-A cross-reacted with the P450 present in rabbit microsomes as shown by immunoblotting analysis; and (ii) one-dimensional peptide maps after partial proteolysis and two-dimensional peptide maps after complete proteolysis of PB-A were completely different from those of βNF-$A_1$/βNF-$A_2$. The cytochromes P450 βNF-$A_1$ and βNF-$A_2$ were similar to each other in all respects except that they appeared at different places during chromatographic separation and had some minor differences in their spectral properties and catalytic activities. The appearance of cytochrome at two places has also been noticed by other investigators (14) and may be due to association of some P450 βNF-A with other proteins.

The cytochromes P450 βNF-$A_1$, βNF-$A_2$, and PB-A, induced by both PB and β-NF treatment, exhibited a soret peak of a ferrous–carbonyl complex at ~449 nm, whereas isozymes P450 β-NFB and β-NFC, induced only

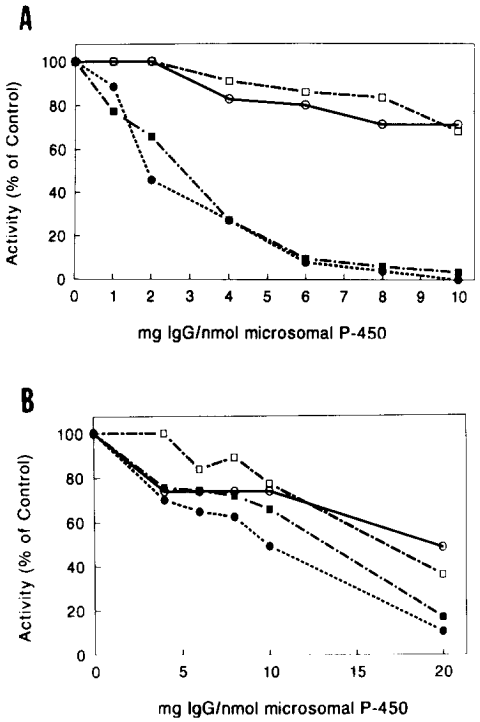


**FIG. 9.** Immunoinhibition of microsomal 7-methoxyresorufin and 7-ethoxyresorufin *O*-dealkylase activity. The β-NF-induced microsomes (50 pmol) were incubated for 30 min with various concentrations of anti-βNF-B (B) or anti-βNF-C IgG (A) at room temperature. This was followed by estimation of *O*-dealkylase activity as described under Experimental Procedures. The open symbols represent incubation of microsomes with preimmunized serum IgG and closed symbols, with anti-P450 IgG. (□, ■), 7-ethoxyresorufin *O*-deethylase activity and (○, ●), 7-methoxyresorufin *O*-demethylase activity.

by β-NF, exhibited a peak at ~446 nm. The polycyclic aromatic hydrocarbon-induced cytochrome P450s generally exhibit ferrous–carbonyl complex peaks at about 448 nm (14, 32). The cytochromes P450 β-NFB and β-NFC did not exhibit *O*-dealkylation of 7-methoxyresorufin and 7-ethoxyresorufin in a reconstitution system in contrast to a 40- to 55-fold increase of this activity in β-NF-induced microsomes. However, immunoinhibition studies using anti-βNF-$A_1$/βNF-$A_2$, anti-βNF-B, and anti-βNF-C suggested that cytochrome P450 βNF-C was the major enzyme catalyzing *O*-alkylation of 7-methoxyresorufin and 7-ethoxyresorufin, and the latter property was somehow lost during purification of P450 βNF-C. Some inhibition observed by anti-βNF-B could probably be attributed to its cross-reactivity with βNF-C. Similar observations have been reported by Dutton and Parkinson (34) on *O*-dealkylation of 7-pentoxyresorufin by cytochrome P450b purified from PB-treated rat liver microsomes. The N-terminal amino acid sequence of cytochromes P450 PB-A/βNF-$A_1$/βNF-$A_2$ is identical to that of fraction 3 purified from ethanol-treated 10-day-old chicken by Sinclair *et al.* (35). However, the $NH_2$-terminal sequences of purified cytochrome P450s did not show any similarity with those sequenced through cDNA libraries from chick embryos (36), 3-day-old chick (37), or ovaries from 9-week-old chick (38). The purified cytochrome P450s were also different in their amino-terminal sequence from other mammalian or nonmammalian species (4).

We have purified and characterized three cytochrome P450 isozymes from β-naphthoflavone-treated adult hens. Cytochromes P450 βNF-$A_1$/βNF-$A_2$ were identical or very similar to PB-induced PB-A, since all of them exhibited almost the same amino-terminal sequence, mobilities during one- and two-dimensional electrophoresis, and other properties. The other two immunochemically related cytochrome P450 βNF-B and βNF-C were distinct isozymes with different N-terminal amino acid sequence, and several biophysical and biochemical properties. The amino-terminal sequences of P450 βNF-B and βNF-C were also different from other cytochrome P450s purified from mammalian or nonmammalian species (4). The purification of these P450s and availability of antiserums will be helpful in studying their role in the metabolism of various environmental neurotoxins.

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