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†

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ABSTRACT: Eight different forms of cytochrome P-450 (P-450) were purified to electrophoretic homogeneity by a common procedure from liver microsomes of rats treated with phenobarbital or β-naphthoflavone. Antibodies were prepared to seven of these forms in rabbits. The eight P-450s were distinguished by spectral properties of the ferric, ferrous, and ferrous carbonyl forms, apparent monomeric molecular weights, peptide mapping, immunological reactivity as discerned by double-diffusion immunoprecipitin analysis and crossed immunoelectrophoresis, and catalytic activities toward the substrates acetanilide, aminopyrine, aniline, benzo[*a*]-pyrene, *d*-benzphetamine, *N*,*N*-dimethylnitrosamine, 7-ethoxycoumarin, 7-ethoxyresorufin, ethylmorphine, *p*-nitroanisole, testosterone, and (*R*)- and (*S*)-warfarin. Crossed sodium dodecyl sulfate–polyacrylamide gel immunoelectrophoresis was used to estimate the levels of each of the eight forms of P-450 present in the liver microsomes of untreated rats and rats treated with phenobarbital, 5,6-benzoflavone, pregnenolone-16α-carbonitrile, isosafrole, or the polychlorinated biphenyl mixture Aroclor 1254. In each situation, the sum of the levels of these eight P-450s was at least as high as the spectrally determined P-450 content. The results clearly demonstrate that individual forms of P-450 can be induced by different compounds and that a single compound can lower the level of one form of P-450 while inducing one or more other forms of P-450. Catalytic activities toward each of the substrates observed with microsomal preparations are compared to rates predicted on the basis of the content of each of the eight P-450s. These studies provide a basis for further studies on the regulation of individual P-450s, the physical properties of the different P-450s, and the metabolic consequences of changes in the forms of P-450 in rat liver models.

A microsomal monooxygenase system containing P-450[1] as the terminal oxidase catalyzes the oxidation of a variety of endogenous and xenobiotic compounds, many of which are of concern because of their potentially toxic and tumorigenic metabolism-related properties (Wislocki et al., 1980). Extensive evidence has now been gathered to support the hypothesis that P-450 exists in multiple forms and that these multiple forms are true isozymes in the sense that they are different gene products (Guengerich, 1979, and references therein). A number of these forms which occur in the rat, an important model, have been isolated and variously characterized (Agosin et al., 1979; Cheng & Schenkman, 1982; Elshourbagy & Guzelian, 1980; Fisher et al., 1981; Goldstein et al., 1982; Guengerich, 1977, 1978a; Guengerich et al., 1981; Imai, 1979; LeProvost et al., 1981; Masuda-Mikawa et al., 1979; Murakami & Okuda, 1981; Ryan et al., 1979, 1980; Saito & Strobel, 1981; Vlasuk et al., 1982; West et al., 1979; Wolff et al., 1980). Nevertheless, comparison of P-450 preparations isolated in different laboratories has been complicated by demonstration of differences in P-450s due to strain and even colony (Guengerich et al., 1981; Johnson et al., 1982; Vlasuk et al., 1982).

We have modified methods from our laboratory and others [see Guengerich (1977, 1978a) and Guengerich & Martin (1980)] to develop techniques that permit the isolation of eight different electrophoretically homogeneous forms of P-450 from liver microsomes of rats treated with PB or βNF. Antibodies have been raised to seven of these P-450s. We present here a battery of characteristics of these P-450s, all isolated from a single source of rats in a single laboratory. In most cases, the immunologically determined specific contents of these characterized P-450s and theoretical catalytic activities calculated for microsomal preparations (which were developed on the basis of these specific contents) are able to explain the composition of P-450s in liver microsomes isolated from untreated rats and rats treated with several inducing agents. When possible, we have compared these P-450 preparations with those presented in the literature by others.

Experimental Procedures

Chemicals. Unless noted otherwise, chemicals used for treatment of rats or for chromatographic, electrophoretic, proteolytic, immunological, or catalytic studies were from sources described elsewhere (Guengerich, 1977, 1978a,b; Guengerich et al., 1981, 1982) or satisfactory grades were generally commercially available. Aniline was distilled under vacuum (20 mmHg), and *p*-nitroanisole was recrystallized once from 10% aqueous CH_3OH. Water was deionized and then further purified by using a Millipore Milli-Q device (Millipore Corp., Bedford, MA). Hydroxylapatite (Hypatite C) was obtained from Clarkson Chemical Co., Williamsport, PA. DEAE-cellulose (DE-51, DE-52, and DE-53) and CM-cellulose (CM-52) were obtained from Whatman Separation

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[1] Abbreviations: P-450, liver microsomal cytochrome P-450; P-420, cytochrome P-420 (representing denatured forms of P-450); UT, untreated (rats); PB, phenobarbital; βNF, β-naphthoflavone (5,6-benzoflavone); PCN, pregnenolone-16α-carbonitrile; ISF, isosafrole; $NaDodSO_4$, sodium dodecyl sulfate; $LiDodSO_4$, lithium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane. The rationale for nomenclature of the individual P-450s is described in the text.

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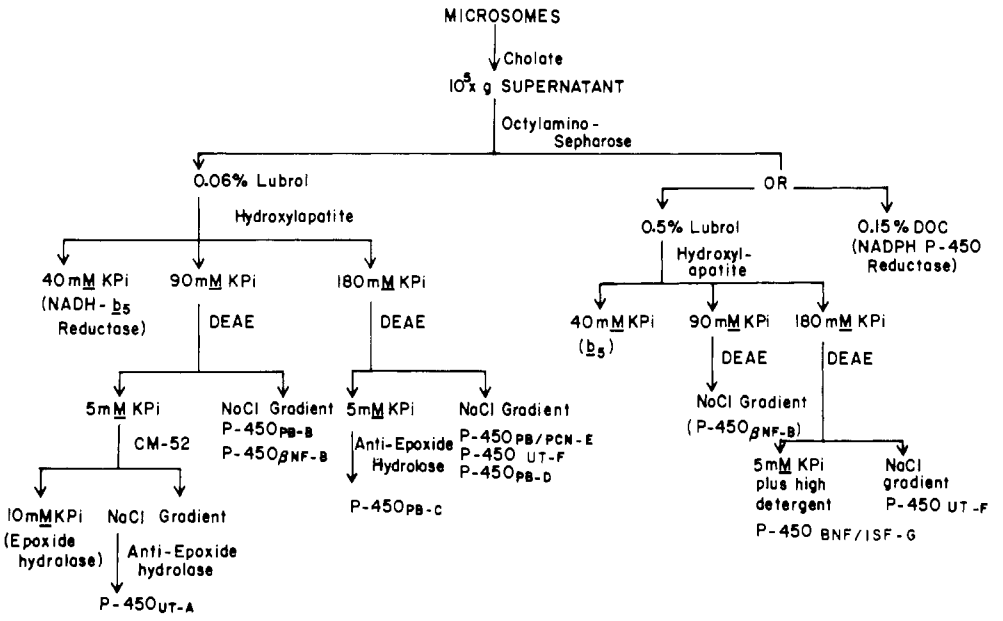

FIGURE 1: Purification scheme for rat liver microsomal cytochromes P-450 and other enzymes. Detailed procedures are given in the text. "Oct 4B" refers to *n*-octylamino-Sepharose 4B and "HA" refers to hydroxylapatite in the designation of various fractions.

Products, Clifton Heights, NJ and treated as described elsewhere prior to use (Elshourbagy & Guzelian, 1980). The IgG fraction of goat antisera raised against hepatic epoxide hydrolase (fraction A) purified from PB-treated rats (Guengerich et al., 1979) was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) by using the procedure of Livingston (1974).

General Assays. Protein concentrations were estimated by using the method of Lowry et al. (1951) with appropriate corrections for buffer components. P-450 concentrations were estimated spectrally with ferrous–carbonyl complex vs. ferrous difference spectra, with an $\epsilon_{450-490}$ value of 91 mM^{-1} cm^{-1} (Omura & Sato, 1964). Estimates of concentrations of individual forms of P-450 in microsomes were made immunologically as described elsewhere (Guengerich et al., 1982). $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5% acrylamide, w/v) was carried out by using the procedure of Laemmli (1970) with staining according to Fairbanks et al. (1971) or Wray et al. (1981). Peptide mapping (Cleveland et al., 1977; Guengerich, 1978b) and double-diffusion immunoprecipitin analysis (Guengerich et al., 1981) techniques are described elsewhere.

Purification of P-450s. Male Sprague-Dawley rats weighing 200–250 g were obtained from Harlan Industries, Indianapolis, IN. Inducers were administered as described elsewhere (Guengerich et al., 1982), and ISF was administered as described by Fisher et al. (1981). The initial portions of the general purification procedure, which were carried out at 4 °C, are described in detail elsewhere (Guengerich, 1977, 1978a; Guengerich & Martin, 1980) and will not be reiterated here. In general, each preparation started with 6000 nmol of P-450 distributed over two 2.5 × 45 cm *n*-octylamino-Sepharose 4B columns. After the "Oct 4B 0.06% Lubrol" P-450 fraction (Guengerich & Martin, 1980) had been eluted (Figure 1), *n*-octylamino-Sepharose 4B columns were further eluted with 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% (v/v) glycerol, 0.33% (w/v) sodium cholate, and 0.5% (w/v) Lubrol PX in the case of microsomes prepared from βNF-treated rats or eluted with 0.1 M potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA, 2 μM FMN, 20% glycerol, 0.35% sodium cholate, and 0.15% (w/v) sodium deoxycholate in the case of microsomes prepared from PB-treated rats (Guengerich & Martin, 1980). In the latter case, the objective was recovery of NADPH–cytochrome P-450 reductase, while with microsomes prepared from βNF-treated rats, the objective was recovery of two forms of P-450 ($P\text{-}450_{UT\text{-}F}$ and $P\text{-}450_{\beta NF/ISF\text{-}G}$). The overall yields of P-450 recovered after the *n*-octylamino-Sepharose 4B step were routinely about 60%.

The Oct 4B 0.06% Lubrol and Oct 4B 0.5% Lubrol fractions were each diluted 3-fold with a solution of 20% (v/v) glycerol and applied to 5 × 15 cm columns of Hypatite C. The columns were washed successively with about 1000 mL each of 40, 90, and 180 mM potassium phosphate buffers (pH 7.25), each containing 0.1 mM EDTA, 20% (v/v) glycerol, and 0.3% (w/v) Lubrol PX. The peak fractions collected from the 90 and 180 mM washes were individually pooled in each case, concentrated to volumes of approximately 50 mL by ultrafiltration (Amicon PM-30 device, Amicon Corp., Danvers, MA), and dialyzed vs. four changes (8–12 h for each dialysis) of 20 volumes of buffer A [5 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 10% (v/v) glycerol, 0.1% Lubrol PX, and 0.2% sodium cholate]. (For this and subsequent steps, sodium cholate was purchased from Sigma Chemical Co., St. Louis, MO, and was used without recrystallization.) After dialysis, the pH was adjusted to 7.5 with 1 M Tris base, and the conductivity was adjusted, if necessary, to about 800 μΩ by dilution or further dialysis.

Up to 1000 nmol of an individual "Oct 4B 0.06% Lubrol/HA-90 mM", "Oct 4B 0.5% Lubrol/HA-90 mM", "Oct 4B 0.06% Lubrol/HA-180 mM", or "Oct 4B 0.5% Lubrol/HA-180 mM" fraction (Figure 1) was applied at a flow rate of 0.5–1.0 mL min^{-1} to a series of columns of DE-51 (1.5 × 10 cm), DE-52 (1.5 × 15 cm), and DE-53 (1.5 × 25 cm) connected in tandem as suggested by Whatman Separation Products.[2] The series had been previously equilibrated with 700–1000 mL of buffer A. The effluent from the three columns passed through an ISCO UA-5 absorbance monitor (ISCO, Omaha, NE) (both A_{280} and A_{405} were monitored by using the appropriate filters) and was collected in a fraction collector (8-mL aliquots) after the final column. After all of

[2] Bulletin No. AIEC 201, *Advanced Ion Exchange Celluloses Laboratory Manual*, Whatman Chemical Separations Inc., Clifton, NJ.

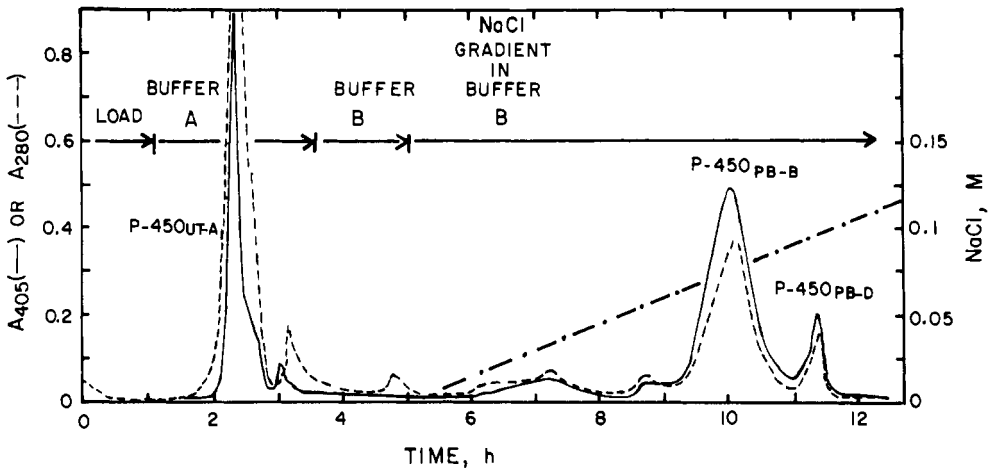

FIGURE 2: Chromatography of P-450 on DEAE-cellulose. A 600-nmol portion of an Oct 4B 0.06% Lubrol/HA-90 mM preparation isolated from PB-treated rats was applied to a system of Whatman DE-51, DE-52, and DE-53 columns which were developed as described in the text to yield $P\text{-}450_{UT\text{-}A}$, $P\text{-}450_{PB\text{-}B}$, and $P\text{-}450_{PB\text{-}D}$.

the sample was loaded, the columns were left in the same arrangement and washed with 150 mL of buffer A and then 150 mL of buffer B [5 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA, 20% glycerol (v/v), 0.2% Lubrol PX, and 0.5% sodium cholate]. The DE-51 column was then removed, and the DE-52 and DE-53 columns (in tandem) were eluted with a linear gradient formed with 250 mL of buffer B and 250 mL of buffer B containing 0.125 M NaCl. The same basic procedure was used to purify all of the isozymes of P-450. Chromatography using *n*-octylamino-Sepharose 4B and hydroxylapatite was carried out at 4 °C, and subsequent steps utilizing DEAE- and CM-cellulose and immunoaffinity chromatography were carried out at 23 °C.

$P\text{-}450_{UT\text{-}A}$. The Oct 4B 0.06% Lubrol/HA-90 mM fractions (Figure 1) from PB-treated rats were used as a source. A hemoprotein fraction moved through the first two columns (DE-51, DE-52), concentrated on the DE-53 column, and moved down the DE-53 column as a tight band when the columns were washed with buffer A. Chromatography of such a fraction is shown in Figure 2, $P\text{-}450_{UT\text{-}A}$ was eluted between 2 and 3 h. $NaDodSO_4$–polyacrylamide gel electrophoresis and double-diffusion immunoprecipitin analysis indicated that epoxide hydrolase was the only major contaminant. The pooled fractions were dialyzed for 16 h against 50 volumes of 10 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM EDTA, 20% glycerol, and 0.2% Lubrol PX and applied to a 1.5 × 15 cm column of CM-52 equilibrated with the same buffer. The column was washed with 200 mL of the equilibration buffer, and elution was carried out with 500 mL of a linear gradient of 0–0.3 M NaCl in the equilibration buffer. The eluate was monitored by using A_{280} and A_{405} measurements. The fractions containing P-450 were pooled and applied to a 1.5 × 15 cm column of goat anti-rat liver epoxide hydrolase (IgG fraction) coupled to Sepharose 4B (Livingston, 1974). The eluate was recycled through the column for 16 h at a rate of 0.5 mL min^{-1} and then collected. Further material was eluted from the column upon washing with 10 mM potassium phosphate buffer (pH 7.4) containing 0.13 M NaCl, 20% glycerol, and 0.2% Lubrol PX. The pooled P-450 fractions were dialyzed for 24 h vs. 50 volumes of 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA, concentrated on an Amicon PM-30 ultrafiltration membrane to a concentration of about 5–10 μM, and turned end-over-end with Bio-Beads SM-2 (0.3 g mL^{-1}) for 3 h at 4 °C. P-450 was recovered by filtration through glass wool or nylon mesh and subsequent centrifugation for 10 min at 10^4g. The resulting supernatant was dialyzed for 16 h against 50 volumes of 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. The resulting preparation was stored at −20 °C in small aliquots. The overall yield of $P\text{-}450_{UT\text{-}A}$, based upon the total P-450 in liver microsomes of PB-treated rats, was about 2–3%.

$P\text{-}450_{PB\text{-}C}$. The Oct 4B 0.06% Lubrol/HA-180 mM fractions from PB-treated rats were used as a source. In the DEAE-cellulose chromatography procedure, the material behaved in the same way as $P\text{-}450_{UT\text{-}A}$; i.e., $P\text{-}450_{PB\text{-}C}$ was eluted from all three columns with buffer A. The hemoprotein fractions were passed through a column of anti-rat liver epoxide hydrolase bound to Sepharose 4B in the manner described for $P\text{-}450_{UT\text{-}A}$, and subsequent steps (dialysis and treatment with Bio-Beads SM-2) were carried out in the same way. The overall yield of $P\text{-}450_{PB\text{-}C}$, based upon the total P-450 in liver microsomes of PB-treated rats, was 2–3%.

$P\text{-}450_{PB\text{-}B}$. The Oct 4B 0.06% Lubrol/HA-90 mM fractions from PB-treated rats were used as the principal source; some $P\text{-}450_{PB\text{-}B}$ could also be recovered from the Oct 4B 0.06% Lubrol/HA-180 mM fractions from PB-treated rats. $P\text{-}450_{PB\text{-}B}$ was retarded by the DE-51 and DE-52 columns during the washing with buffer A. The protein moved completely through the DE-51 column and slowly down the DE-52 column during washing with buffer B. The hemoprotein was eluted as a large peak when the NaCl gradient was applied to the DE-52 and DE-53 columns. In Figure 2, $P\text{-}450_{PB\text{-}B}$ was eluted between 9 and 11 h. The fractions were monitored by using $NaDodSO_4$–polyacrylamide gel electrophoresis, and those fractions that were homogeneous were dialyzed, treated with Bio-Beads SM-2, and stored as in the case of $P\text{-}450_{UT\text{-}A}$. The overall yield, based upon total P-450 in liver microsomes of PB-treated rats, was 3–6%.

$P\text{-}450_{PB\text{-}D}$. The Oct 4B 0.06% Lubrol/HA-180 mM fractions from PB-treated rats were used as the principal enzyme source. The behavior of $P\text{-}450_{PB\text{-}D}$ was similar to that of $P\text{-}450_{PB\text{-}B}$ (vide supra) except that $P\text{-}450_{PB\text{-}D}$ was eluted slightly later than $P\text{-}450_{PB\text{-}B}$ (Figure 2). Some $P\text{-}450_{PB\text{-}D}$ was also recovered from Oct 4B 0.06% Lubrol/HA-90 mM fractions (Figure 2), and some $P\text{-}450_{PB\text{-}B}$ was recovered from the Oct 4B 0.06% Lubrol/HA-180 mM fractions. Fractions were monitored by using $NaDodSO_4$–polyacrylamide gel electrophoresis, and the homogeneous fractions were dialyzed, treated with Bio-Beads SM-2, and stored as in the case of $P\text{-}450_{UT\text{-}A}$. The overall yield, based upon total P-450 in liver microsomes of PB-treated rats, was 1–2%.

P-450$_{\beta NF\text{-}B}$. The Oct 4B 0.06% Lubrol/HA-90 mM and Oct 4B 0.5% Lubrol/HA-90 mM fractions from βNF-treated rats were used as the principal sources of P-450$_{\beta NF\text{-}B}$, although some material could also be recovered from the Oct 4B 0.06% Lubrol/HA-180 mM and Oct 4B 0.5% Lubrol/HA-180 mM fractions obtained from these rats as well. The chromatographic properties of P-450$_{\beta NF\text{-}B}$ in the DEAE-cellulose system were quite similar to those of P-450$_{PB\text{-}B}$ and P-450$_{PB\text{-}D}$, although P-450$_{\beta NF\text{-}B}$ eluted at a slightly higher NaCl concentration (Guengerich & Martin, 1980). Purity of column fractions was monitored by using NaDodSO$_4$–polyacrylamide gel electrophoresis as well as A_{405} and A_{280} measurements. Fractions containing only P-450$_{\beta NF\text{-}B}$ were pooled, dialyzed, treated with Bio-Beads SM-2, and stored as in the case of P-450$_{UT\text{-}A}$. The overall yield of P-450$_{\beta NF\text{-}B}$, based upon total P-450 present in liver microsomes of βNF-treated rats, was 2–4%.

P-450$_{PB/PCN\text{-}E}$. This form of P-450 could be isolated from the Oct 4B 0.06% Lubrol/HA-180 mM fractions obtained from liver microsomes of rats treated with PB or, more conveniently, PCN. P-450$_{PB/PCN\text{-}E}$ bound to the DEAE columns and was eluted from DE-53 in a peak shortly after the NaCl gradient was applied to the DE-52 and DE-53 columns (6–8 h in Figure 2). Fractions were monitored by using NaDodSO$_4$–polyacrylamide gel electrophoresis as well as A_{405} and A_{280} measurements. During the latter portion of the peak, contamination with P-450$_{UT\text{-}F}$ was found. The fractions containing only P-450$_{PB/PCN\text{-}E}$ were pooled, dialyzed, treated with Bio-Beads SM-2, and stored as in the case of P-450$_{UT\text{-}A}$. The overall yield of P-450$_{PB/PCN\text{-}E}$, based on total P-450 in PB microsomes, was about 1%. When microsomes isolated from PCN-treated rats were used as the source, the overall yield (based on total P-450 in PCN-treated rat liver microsomes) was 2–5%.

P-450$_{UT\text{-}F}$. The Oct 4B 0.5% Lubrol/HA-180 mM fractions obtained from βNF-treated rats were the principal sources of P-450$_{UT\text{-}F}$, although some material could also be recovered from the Oct 4B 0.06% Lubrol/HA-180 mM fractions obtained from these animals as well. In the DEAE-cellulose chromatography procedure, P-450$_{UT\text{-}F}$ moved through the DE-51 column and onto the DE-52 column during the wash with buffer B. When the NaCl gradient was applied to the DE-52 and DE-53 columns, P-450$_{UT\text{-}F}$ was eluted in a broad peak from the DE-53 column prior to P-450$_{\beta NF\text{-}B}$. Fractions were monitored by using NaDodSO$_4$–polyacrylamide gel electrophoresis as well as A_{405} and A_{280} measurements. Fractions containing only P-450$_{UT\text{-}F}$ were pooled, dialyzed, treated with Bio-Beads SM-2, and stored as in the case of P-450$_{UT\text{-}A}$. The overall yield of P-450$_{UT\text{-}F}$, based upon the total P-450 in βNF-treated rat liver microsomes, was 0.5–1%.

P-450$_{\beta NF/ISF\text{-}G}$. This isozyme was first purified from the Oct 4B 0.5% Lubrol/HA-180 mM fraction obtained from βNF-treated rats. The chromatographic behavior of P-450$_{\beta NF/ISF\text{-}G}$ in the DEAE-cellulose chromatography system was similar to that of P-450$_{PB/PCN\text{-}E}$. However, the enzyme obtained in this manner was not electrophoretically homogeneous. The fraction recovered from the DE-53 column in the first DEAE-cellulose system was dialyzed vs. buffer A (20 volumes, 12 h) and applied to a 1.5 × 20 cm column of DE-52 equilibrated with 100 mL of buffer A, and then P-450$_{\beta NF/ISF\text{-}G}$ was eluted with buffer B. The overall yield of P-450$_{\beta NF/ISF\text{-}G}$, based upon total P-450 in microsomes of βNF-treated rats, was 0.2–0.5%.

Subsequently, we found after preliminary immunological experiments that P-450$_{\beta NF/ISF\text{-}G}$ could be purified more easily from ISF-treated rats. We found that the Oct 4B 0.5% Lubrol/HA-180 mM fraction was electrophoretically homogeneous and did not require the use of DEAE-cellulose chromatography. The overall yield of P-450$_{\beta NF/ISF\text{-}G}$, based upon total P-450 in liver microsomes of ISF-treated rats, was 5–10%.

In both cases, P-450$_{\beta NF/ISF\text{-}G}$ was dialyzed, treated with Bio-Beads SM-2, and stored as in the case of P-450$_{UT\text{-}A}$.

Purification of Other Microsomal Enzymes. NADH–cytochrome b_5 reductase activity was recovered in the Oct 4B 0.06% Lubrol/HA-40 mM fraction (Figure 1). This enzyme can be further purified by techniques described elsewhere (Guengerich, 1978c; Spatz & Strittmatter, 1973).

Epoxide hydrolase activity was recovered in the void volume of the CM-cellulose column used in the purification of P-450$_{UT\text{-}A}$. At this point the protein was essentially electrophoretically homogeneous. Excess detergent can be removed from the enzyme by hydroxylapatite chromatography (Guengerich et al., 1979) or treatment with Bio-Beads SM-2 (vide supra; Guengerich & Martin, 1980).

If the *n*-octylamino-Sepharose 4B column had not been washed with the 0.5% Lubrol buffer, cytochrome b_5 could be eluted after the 0.15% deoxycholate wash as described by Imai (1976). If the *n*-octylamino Sepharose 4B column was eluted with the 0.5% Lubrol buffer, cytochrome b_5 was found in the 40 and 90 mM phosphate fractions recovered from the hydroxylapatite column. The material bound to DE-51 during the buffer A and B wash procedures and could be eluted with a 0–0.15 M NaCl gradient after the DE-53 column was removed. The cytochrome b_5 fractions can be further purified by repeated DEAE-cellulose and Sephadex G-75 and G-25 chromatography (Guengerich, 1978c; Spatz & Strittmatter, 1971).

NADPH–cytochrome P-450 reductase can be eluted from the *n*-octylamino-Sepharose 4B column with the 0.15% deoxycholate buffer (Imai, 1976) and purified to electrophoretic homogeneity by using 2′,5′-ADP–agarose chromatography (Yasukochi & Masters, 1976) as described in detail elsewhere (Guengerich & Martin, 1980).

Assays of Catalytic Activity. In all cases except with warfarin as the substrate, rat liver microsomes (1.3 mg of protein/mL) or a reconstituted system consisting of the appropriate purified P-450 (0.3–1.0 μM), a 1.5 molar excess of purified NADPH–P-450 reductase, and 30 μM L-α-dilauroylglyceryl-3-phosphorylcholine were mixed with the substrate, 10 mM glucose 6-phosphate, and 1.0 IU of yeast glucose-6-phosphate dehydrogenase mL^{-1} in 100 mM Tris-HCl buffer (pH 7.6) and allowed to equilibrate for 3 min at 37 °C. Reactions were initiated by the addition of 0.5 mM NADPH. The general assay methods are referenced for the individual substrates, along with substrate concentrations and incubation times: warfarin, 0.3 mM, 20 min (Kaminsky et al., 1980); *d*-benzphetamine, 1 mM, 3–10 min (Cochin & Axelrod, 1959; Nash, 1953); 7-ethoxyresorufin, 20 μM, 3–10 min (Burke & Mayer, 1975); 7-ethoxycoumarin, 0.3 mM, 3–10 min (Guengerich, 1978a); benzo[*a*]pyrene, 80 μM, 3–10 min (DePierre et al., 1978); acetanilide, 2 mM, 3–10 min (Guengerich & Martin, 1980); aniline, 8 mM, 30 min (Imai et al., 1966); aminopyrine, 5 mM, 10 min (Cochin & Axelrod, 1959; Nash, 1953); ethylmorphine, 7 mM, 10 min (Lu et al., 1972); *N*,*N*-dimethylnitrosamine, 1 or 100 mM, 30–45 min (Cochin & Axelrod, 1959; Nash, 1953); *p*-nitroanisole, 0.25 mM, continuous spectral assay (Netter & Seidel, 1964). Values obtained in the absence of P-450 were subtracted in all cases, and data are presented as means of duplicate experiments.

Table I: Apparent Molecular Weight Values of P-450s Estimated by Standardized Polyacrylamide Gel Electrophoresis in the Presence of $NaDodSO_4$ or $LiDodSO_4$, Wavelength Maxima of Ferrous–CO Soret Peaks, and Comparison with Other Preparations

P-450	M_r $NaDodSO_4$[a]	M_r $LiDodSO_4$	rel order (from anode) $NaDodSO_4$	rel order (from anode) $LiDodSO_4$	λ_{max} of Fe^{2+}–CO complex (nm)	similar preparations[b]
PB-C	47 500	50 500	1	2a	450	
UT-F	48 500	47 500	2	1	452	P-450a (Ryan et al., 1979)
PB-B	50 000	52 500	3a	4a	450	PB-P_{450} (Elshourbagy & Guzelian, 1980) P-450_{PB} (Harada & Omura, 1981) P-450_4 (Imai, 1979) P-450 (Masuda-Mikawa et al., 1979) P-450_b (Ryan et al., 1979) P-450 "C" (West et al., 1979) P-$450_{PB\text{-}4}$ (Waxman & Walsh, 1982a,b)
PB-D	50 000	52 500	3b	4b	450	P-450_e (Vlasuk et al., 1982) P-$450_{PB\text{-}5}$ (Waxman & Walsh, 1982a,b)
UT-A	50 500	50 500	4	2b	451	
βNF/ISF-G	51 000	53 500	5	3	447	P-450 (Fisher et al., 1981) P-450_{HCB} (Goldstein et al., 1982) P-450_d (Ryan et al., 1980) P-450 II-a (Kamataki et al., 1982)
PB/PCN-E	52 000	51 000	6	5	449	PCN-P_{450} (Elshourbagy & Guzelian, 1980)
βNF-B	54 000	54 000	7	6	447	MC-P_{448} (Elshourbagy & Guzelian, 1980) P-450_{MC} (Harada & Omura, 1981) P-448_2 (Imai, 1979) P-448 (Masuda-Mikawa et al., 1979) P-450_c (Ryan et al., 1979) P-448 (Wolff et al., 1980)

[a] Also see Figure 3. [b] Also see footnote 3.

Results

Electrophoretic Properties. Samples of each of the purified P-450s were electrophoresed together in a discontinuous $NaDodSO_4$–polyacrylamide gel system, as shown in Figure 3. All preparations appeared to be electrophoretically homogeneous as judged by this technique, with the exception of P-$450_{UT\text{-}F}$, which contained a trace of material at M_r 50 500. The apparent monomeric molecular weights, determined by comparison with standard proteins, are presented in Table I. All of the proteins were partially resolved from each other in this system, with the exception of P-$450_{PB\text{-}B}$ and P-$450_{PB\text{-}D}$ [which can be partially separated in another system (Guengerich, 1978a)].

Three points should be made in regard to $NaDodSO_4$–polyacrylamide gel electrophoresis. First, evidence has been presented previously that the monomeric molecular weight values are reasonably valid for P-$450_{PB\text{-}B}$ and P-$450_{\beta NF\text{-}B}$, on the basis of comparison with hydrodynamic measurements (Guengerich & Holladay, 1979). Second, the proximity of migration zones of different P-450s known to be present together in rat liver microsomes, along with the known molecular weight values exhibited by other microsomal proteins, argues against the use of protein-stained electrophoretic patterns in designating microsomal polypeptides as individual P-450s or P-450s in general. For instance, under these conditions, epoxide hydrolase migrated with an apparent molecular weight of 50 500 (identical with P-$450_{UT\text{-}A}$), and FAD-containing monooxygenase migrated with an apparent molecular weight of 54 000 (identical with P-$450_{\beta NF\text{-}B}$) (Dannan & Guengerich, 1982). Moreover, staining of gels for heme is not a particularly useful procedure, as 50–90% of the heme can be lost and heme can migrate from one protein to another (Sinclair et al., 1981; Thomas et al., 1976). The third point is that both the apparent absolute subunit molecular weights and the relative migrations of individual polypeptides can be very sensitive to slight modifications in technique. For instance, changing the counterion from sodium to lithium (Sinclair et al., 1981) affected the order of migration (Table I). The commercial source of $NaDodSO_4$ and the level of alkyl sulfates of varying chain length have already been demonstrated to affect relative migration of proteins during electrophoresis (Swaney et al., 1974). One could name the P-450s in order of electrophoretic mobility, as indicated, in line with nomenclature systems for

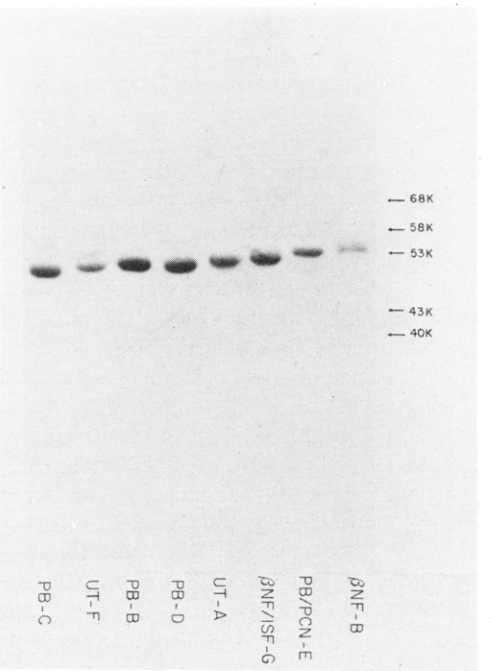

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified P-450s. The anode was at the bottom of the gel, and the gel was stained with alkaline $AgNO_3$ as described elsewhere (Wray et al., 1981). Individual wells contained approximately 1 μg each of standard proteins (molecular weight standards: bovine serum albumin, accepted M_r 68 000; bovine liver catalase, M_r 58 000; *Escherichia coli* L-glutamate dehydrogenase, M_r 53 000; equine liver alcohol dehydrogenase, M_r 43 000; rabbit muscle aldolase, M_r 40 000) or the indicated P-450s.

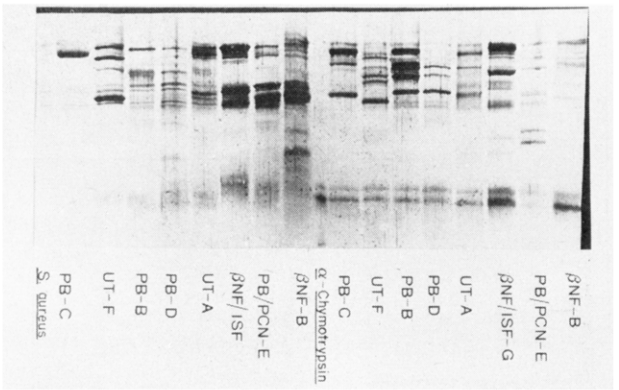

FIGURE 4: Peptide mapping of purified P-450s. One microgram of each P-450 was digested with 0.5 μg of *Staphylococcus aureus* V8 protease (part A) or chymotrypsin (part B) and electrophoresed as described elsewhere (Cleveland et al., 1977; Guengerich, 1978a). The gels were stained with alkaline $AgNO_3$ as described by Wray et al. (1981). The individual P-450s used are designated, and lanes containing only the protease are also marked. The anode was at the bottom of the gel.

soluble proteins which can be readily electrophoresed in the absence of detergents. However, since the nomenclature can be dramatically changed by slight modifications of the technique, we have not advocated such a system. The spectral data presented below indicate that nomenclature of P-450s on the basis of wavelength maxima of ferrous carbonyl Soret bands (e.g., "P-448") is also not particularly useful.

In this work we have decided to retain the same basic system of nomenclature originally used in this laboratory, i.e., designating P-450s by inducer and by a letter arbitrarily based upon the fractionation scheme. Thus, we now add P-$450_{PB/PCN-E}$, P-450_{UT-F}, and P-$450_{\beta NF/ISF-G}$ to the P-450s which were already presented in the literature in varying states of purity (Guengerich, 1977), namely, P-450_{UT-A}, P-450_{PB-B}, P-$450_{\beta NF-B}$, P-450_{PB-C}, and P-450_{PB-D}. Evidence has already been presented elsewhere that P-$450_{\beta NF-B}$ is identical with the major P-450 (P-450_{3MC-B}) (Guengerich, 1977) induced by 3-methylcholanthrene (Gozukara et al., 1982; Guengerich et al., 1981, 1982; Guengerich & Martin, 1980). With the recent availability of reliable immunological techniques for quantitation, individual P-450s can now better be described as "constitutive" (UT in this system) or induced by a certain compound. In general, we have tried to name each P-450 by its most effective inducer(s). In some cases, we term a P-450 with names of two inducers if both inducers have similar efficacies.

Table I also compares the preparations isolated in this laboratory with some of those reported by others. Such comparison is made on the basis of relative electrophoretic mobilities, induction with various agents, spectral properties, general chromatographic behavior, and certain catalytic activities.

Peptide Mapping. The electrophoretogram presented in Figure 4 indicates that the various P-450s were readily distinguished by using the technique of Cleveland et al. (1977), with the exception of P-450_{PB-B} and P-450_{PB-D}, when digestion was carried out with either *Staphylococcus aureus* V8 protease or α-chymotrypsin.

One report in the literature suggests that some of the rabbit liver P-450s may be glycoproteins (Haugen & Coon, 1976). With the assistance of Dr. C. Hellerqvist of our institution, samples of P-450_{PB-B} and P-$450_{\beta NF-B}$ were hydrolyzed in acid, and methylated carbohydrate residues were analyzed by using capillary-column gas-liquid chromatography. In neither case were any residues detected at levels greater than 0.2 nmol (nmol of P-450)$^{-1}$. Further, neither P-450_{PB-B} nor P-$450_{\beta NF-B}$ was bound to a column of agarose-bound concanavalin A. Thus, we do not believe that these two particular P-450s are glycoproteins. The presence of carbohydrate and other posttranslational modifications in other P-450s remain to be determined.

Spectral Properties. Contamination of the P-450s with P-420 (≤8%) was minimal except in the case of P-$450_{PB/PCN-E}$ (17%), which showed some degradation during purification and storage. The RZ values (ratio of absorbance to Soret maximum to A_{280}) ranged from 0.7 (P-450_{UT-A}) to 1.3 (P-450_{PB-B}) in the samples which were examined in this study. These values can be used as an index to purification because UV-absorbing materials were not introduced during isolation procedures. While these values vary, depending upon content of aromatic amino acids, all of the values were in the neighborhood of unity. The specific contents of P-450 heme per milligram of protein observed for the various preparations (8.0 nmol of P-$450_{\beta NF/ISF-G}$ to 17.5 nmol for P-450_{PB-C}) are probably also influenced by heme loss, as well as some deviation from ideality in the protein estimations.

All of the oxidized P-450s had Soret maxima at 416–417 nm, and ε ranged from 125 to 162 mM^{-1} cm^{-1}, with the exception of P-$450_{\beta NF/ISF-G}$ (λ_{max} 392 nm, ε 81 mM^{-1} cm^{-1}). The ferric β peak was found at 533–536 nm and the α peak at 566–574 nm, except in the case of P-$450_{\beta NF/ISF-G}$, which had a nondescript α,β region and showed a spin-associated peak at 646 nm (ε 4.7 mM^{-1} cm^{-1}). The wavelength maxima of the ferrous Soret bands were found between 410 and 420 nm, with extinction coefficients ranging from 91 to 116 mM^{-1} cm^{-1}. The Soret wavelength maxima of the ferrous carbonyl complexes of the P-450s were found in the range 447–452 nm (Table I).

The spectral properties of P-$450_{\beta NF/ISF-G}$, which appears to exist predominantly in the high-spin state, are particularly worth noting. Spectra of preparations isolated from βNF- and ISF-treated rats were essentially identical, and we feel that this P-450 is similar to those in preparations of others isolated from rats treated with other compounds (Table I). The ferrous form of the preparation isolated from ISF-treated rats contained a small shoulder at 455 nm (cf. Ryan et al., 1980; Fisher et al., 1981), although the A_{455}/A_{413} ratio was only 0.44 even without special measures to remove any bound ligand. White & Coon (1982) have recently reported that alcohols influence the conversion of rabbit P-450_{LM-4} from a high- to low-spin iron configuration and have interpreted their results to mean that an oxygen compound (hydroxyl) is a ligand in low-spin P-450s. We also found that the addition of 0.25 M 1-butanol to P-$450_{\beta NF/ISF-G}$ caused the conversion of about 80% of the high-spin iron to the low-spin state.

Immunological Properties. The techniques of double-diffusion immunoprecipitin analysis and particularly crossed immunoelectrophoresis using $NaDodSO_4$–polyacrylamide gel electrophoresis and peroxidase staining were used to characterize the immunological properties of the P-450s.

In the immunodiffusion experiments (data not shown), the antibody raised to P-450_{PB-B} recognized it and P-450_{PB-D}, and a pattern of fusion was observed for the precipitin lines formed by these P-450s with the antibody. Antisera to P-$450_{PB/PCN-E}$, P-$450_{\beta NF-B}$, P-450_{PB-C}, and P-450_{UT-F} recognized only the homologous antigens and formed single lines. Anti-P-$450_{\beta NF/ISF-G}$ recognized both P-$450_{\beta NF/ISF-G}$ and P-$450_{\beta NF-B}$. Crude anti-P-450_{UT-A} recognized only P-450_{UT-A} of all the P-450s examined but also produced a line with epoxide hydrolase, which was a trace contaminant in some P-450_{UT-A}

preparations even after treatment with a column of bound anti-epoxide hydrolase (Figure 1). Anti-epoxide hydrolase was removed from the antiserum by the addition of epoxide hydrolase (5 mg/mL antiserum) and precipitation. In each case the lines formed by the reaction of the purified antigen, and the appropriate preparation of detergent-solubilized rat liver microsomes formed a pattern of fusion.

Purified antigens and rat liver microsomes were electrophoresed in $NaDodSO_4$–polyacrylamide gels, and resolved proteins were transferred to sheets of nitrocellulose (Towbin et al., 1979). The sheets were treated sequentially with a rabbit anti-P-450 preparation, goat anti-rabbit IgG, horseradish peroxidase/rabbit anti-horseradish peroxidase complex, and 3,3′-diaminobenzidine/H_2O_2 to visualize brown bands, corresponding to proteins recognized by the antibody, on a light background (Glass et al., 1981; Guengerich et al., 1982). 4-Chloro-1-naphthol can be used in place of 3,3′-diaminobenzidine to obtain similar results. In the case of each antibody, the eight individual purified P-450s and the microsomes prepared from the rats treated in various ways were examined (Figure 5).

Anti-P-$450_{PB\text{-}B}$ recognized P-$450_{PB\text{-}B}$, P-$450_{PB\text{-}D}$, and proteins in microsomes with the same apparent molecular weight. Anti-P-$450_{PB/PCN\text{-}E}$ and anti-P-$450_{PB\text{-}C}$ recognized only the homologous antigens in the purified samples and microsomes. Anti-P-$450_{UT\text{-}A}$ recognized only P-$450_{UT\text{-}A}$ and a microsomal protein of the same molecular weight. (Epoxide hydrolase has the same molecular weight, and the removal of anti-epoxide hydrolase has been discussed above; purified epoxide hydrolase was not stained in this system after such treatment.) Anti-P-$450_{UT\text{-}F}$ recognized P-$450_{UT\text{-}F}$ and a similar protein in microsomes. A low molecular weight band ($M_r \sim 30\,000$) was also observed when resolved microsomes were stained. This band corresponded in migration distance to a trace contaminant in the particular antigen used (but not other preparations) and is not believed to be related to the P-450. Its migration relative to that of P-$450_{UT\text{-}F}$ does not cause interference in making qualitative or quantitative comparisons in this system. Anti-P-$450_{\beta NF\text{-}B}$ preparations, in general, recognized only P-$450_{\beta NF\text{-}B}$ and a similar protein in microsomes, as shown in these (Figure 5) and previous experiments (Guengerich et al., 1982). Some of the antisera also appeared to recognize P-$450_{\beta NF/ISF\text{-}G}$, and these antisera could be rendered more specific by cross-adsorption with immobilized liver microsomes obtained from ISF-treated rats. Anti-P-$450_{\beta NF/ISF\text{-}G}$ preparations recognized a single band in the purified antigen or microsomes obtained from ISF-treated rats. However, anti-P-$450_{\beta NF/ISF\text{-}G}$ also recognized P-$450_{\beta NF\text{-}B}$ and (to a lesser extent) P-$450_{PB\text{-}C}$, even though these proteins were clearly not present at detectable levels in the antigen. The crossed immunoelectrophoresis results show the usefulness of the technique in establishing cross-reactivities of various antisera, particularly when unknown proteins must be considered for potential antigenic response.

Inducibility. Clearly the overlap among the spectral, catalytic, and electrophoretic properties of the individual P-450s renders these techniques of limited value in studying the induction of individual species of P-450. In addition, basing relative levels of individual P-450s upon recoveries during chromatography is not feasible unless homogeneous isoenzymes can be isolated in nearly quantitative yield. The most useful methods available now involve immunochemical quantitation of individual forms of P-450. We utilized densitometry of peroxidase-stained transfer gels (vide supra) as described elsewhere (Guengerich et al., 1982). Concentrations of P-450 in the standards, used to prepare a standard curve on each individual transfer sheet, were based upon spectrally determined P-450 (plus P-420), so that the estimates of concentrations of each form present in microsomes (Table II) probably represent minimum values. Errors in protein estimation can introduce errors in the reported levels of individual P-450s, but comparisons of total immunologically determined P-450 with spectrally determined P-450 are not affected, as both values rely on the same protein determination.

Table II: Immunological Estimation of Individual Forms of P-450 in Liver Microsomes of Rats Treated with Various Compounds

form of P-450	nmol/mg of protein using inducing agent					
	none	PB	βNF	PCN	ISF	Aroclor 1254
Immunologically Determined Content of Each Form of P-450						
UT-A	1.20	0.49	0.33	0.33	0.33	0.27
PB-B	0.03	1.27	0.04	0.10	0.12	1.29
βNF-B	0.04	0.04	1.41	0.06	0.13	1.45
PB-C	0.36	0.69	0.26	0.31	0.34	0.36
PB-D	0.07	0.92	0.04	0.09	0.18	1.46
PB/PCN-E	0.39	1.06	0.33	1.32	0.48	0.77
UT-F	0.15	0.10	0.12	0.08	0.11	0.15
βNF/ISF-G	<0.03	<0.03	0.57	<0.03	0.97	1.23
total	2.24	4.57	3.10	2.29	2.66	6.98
Spectrally Determined Content of Total P-450 plus P-420						
	1.10	2.39	1.69	1.86	1.11[a] 1.29[b]	4.28

[a] Estimated before removal of endogenous ligand. [b] Estimated after treatment with cyclohexane according to Ryan et al. (1980). Such treatment was without effect on purified P-$450_{\beta NF/ISF\text{-}G}$.

Liver microsomes of untreated rats contain substantial levels of P-$450_{UT\text{-}A}$. The level of P-$450_{UT\text{-}A}$ was decreased only slightly by treatment with PB, when one considers that under such conditions liver weight and protein usually increase by roughly 30%. All of the other compounds (βNF, PCN, ISF, or Aroclor 1254) dramatically lowered the level of P-$450_{UT\text{-}A}$. The results can be seen qualitatively in Figure 5. This appears to be the first case in which a decrease in the level of a given P-450 (apoprotein) has been clearly demonstrated during the induction of others. P-$450_{\beta NF\text{-}B}$ was found at very low levels in untreated animals and was only induced to a significant extent by βNF or Aroclor 1254. P-$450_{UT\text{-}F}$ was found at similar levels in all of the rats. P-$450_{\beta NF/ISF\text{-}G}$ was essentially not present in untreated rats and was induced with βNF but more markedly with Aroclor 1254 or ISF. P-$450_{PB/PCN\text{-}E}$ was induced by either PB or PCN. Considerable levels of P-$450_{PB\text{-}C}$ and P-$450_{PB/PCN\text{-}E}$ were present in untreated animals, and the extent of induction with PB was not as dramatic as in the cases of P-$450_{PB\text{-}B}$ and P-$450_{PB\text{-}D}$. P-$450_{PB\text{-}C}$ was induced by PB only to the extent of 2-fold. P-$450_{PB\text{-}C}$ was not induced by PCN or by Aroclor 1254.

The data we originally presented on the levels of P-$450_{PB\text{-}B}$ (Guengerich et al., 1982) did not take cross-reactivity with P-$450_{PB\text{-}D}$ into consideration. In the present study, we resolved P-$450_{PB\text{-}B}$ and P-$450_{PB\text{-}D}$ by isoelectric focusing in polyacrylamide gels under denaturing conditions (Vlasuk et al., 1982) and used similar transfer and immunochemical techniques. Briefly, focusing was carried out in the wells of a 0.15 × 10 × 15 slab gel. The polyacrylamide gel was polymerized by using 1 μg of riboflavin/mL and fluorescent light, and the addition of solubilizing buffer at the cathode was omitted. Other conditions were essentially identical with those of Vlasuk et al. (1982). Resolved proteins were electrophoretically transferred to a nitrocellulose sheet [placed next to the *cathode*

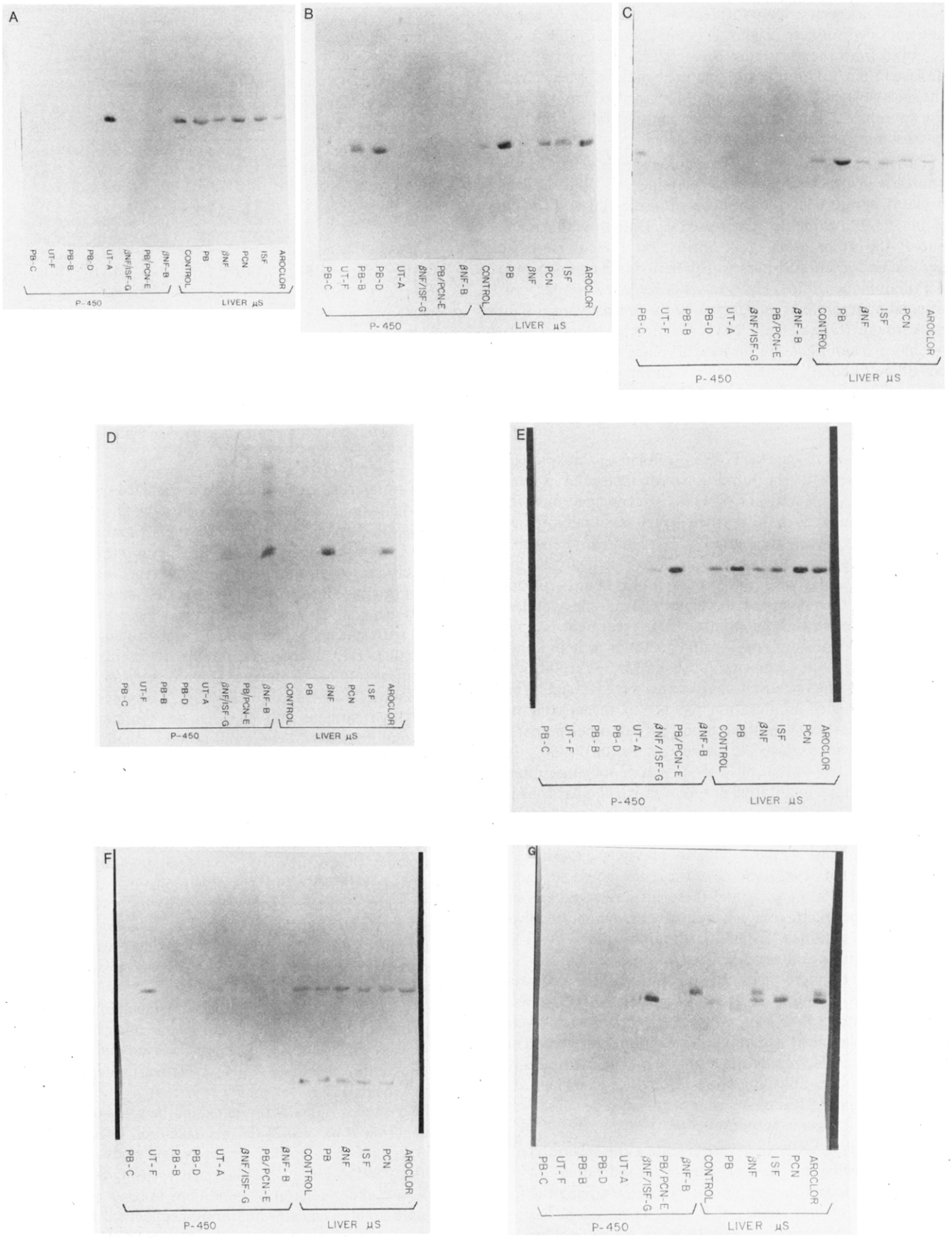

FIGURE 5: Immunological staining of $NaDodSO_4$–polyacrylamide gel electrophoretograms after transfer to nitrocellulose. One picomole of each of the purified proteins or 2 μg of each of the indicated preparations of microsomes (μS) were electrophoresed in a single, separate slab gel. The resolved proteins were electrophoretically transferred to nitrocellulose sheets which were stained by using the indicated antisera (at a dilution of 1/100 to 1/200) (Glass et al., 1981; Guengerich et al., 1982; Towbin et al., 1979). Sheets were stained by using (part A) anti-P-450$_{UT-A}$, (part B) anti-P-450$_{PB-B}$, (part C) anti-P-450$_{PB-C}$, (part D) anti-P-450$_{\beta NF-B}$, (part E) anti-P-450$_{PB/PCN-E}$, (part F) anti-P-450$_{UT-F}$, and (part G) anti-P-450$_{\beta NF/ISF-G}$.

of a transfer device (Glass et al., 1981)] in 1% (v/v) acetic acid. The current setting was 200 mA, the transfer time was 3 h, and the temperature was 10 °C. Visualization of the nitrocellulose sheets using rabbit anti-P-450$_{PB-B}$ and the peroxidase method (Glass et al., 1981) revealed two distinct bands in microsomes. The more basic band was P-450$_{PB-B}$ (apparent pI = 5.91), and the more acidic band was P-450$_{PB-D}$ (apparent pI = 5.78), in line with the DEAE-cellulose chromatographic separation (Figure 2). The ratio of two P-450s was similar in all of the microsomal preparations except those from untreated rats in which P-450$_{PB-D}$ was dominant. Both P-450$_{PB-B}$ and P-450$_{PB-D}$ were induced to large extents by PB or Aroclor 1254 but not by any of the other compounds.

In every case the sum of the immunochemically determined concentrations of individual P-450s was greater than that of the spectrally determined levels.

We also investigated microsomes prepared from a number of extrahepatic tissues from untreated rats and rats treated with PB or βNF. The microsomes were examined with the crossed immunoelectrophoretic method using anti-P-450$_{PB-B}$, anti-P-450$_{\beta NF-B}$, anti-P-450$_{UT-A}$, and anti-P-450$_{PB/PCN-E}$. A protein corresponding to P-450$_{PB-B}$ or P-450$_{PB-D}$ was found in lung microsomes regardless of the treatment of the animals, and the data obtained were similar to those presented elsewhere (Guengerich et al., 1982). Data for protein corresponding to P-450$_{\beta NF-B}$ in kidney microsomes prepared from βNF-treated rats were also similar to those previously reported (Guengerich et al., 1982). In addition, a protein corresponding to P-450$_{\beta NF-B}$ was quantitated in thymus microsomes (4 pmol/mg of protein) and stomach microsomes (21 pmol/mg of protein) of βNF-treated rats. The four proteins to which the antibodies were raised were not present at levels of 2 pmol/mg of protein in the spleen, thymus, kidney, heart, stomach, testis, pancreas, brain, and duodenal microsomes prepared from untreated, PB-treated, or βNF-treated rats. Thus, the P-450s under consideration here are primarily hepatic proteins, although these P-450s or highly similar P-450s can be found in some extrahepatic tissues, albeit at lower levels. More sensitive techniques may reveal small populations of these enzymes which are localized in regions important for certain physiological mechanisms and zonal toxicities (Baron et al., 1981).

Catalytic Activities. The data on the comparisons among purified P-450s and among microsomal preparations are presented in Table III. The data on differences in specific activities among microsomal preparations, after treatments of rats, largely reflect many previous observations and will be treated primarily only in the context of individual forms of P-450 studied by other techniques.

Differences between the purified P-450s can be clearly seen. *p*-Nitroanisole O-demethylation was catalyzed primarily by P-450$_{PB-B}$ and P-450$_{\beta NF-B}$. P-450$_{PB-B}$ was the form most active in *d*-benzphetamine N-demethylation and aminopyrine N-demethylation, although several other forms of P-450 showed substantial activity. P-450$_{\beta NF-B}$ and P-450$_{PB-B}$ were most active in 7-ethoxycoumarin O-deethylation, with significant activity also exhibited by other forms. P-450$_{PB-B}$ was also active in demethylating ethylmorphine, although P-450$_{PB-C}$ and P-450$_{UT-A}$ were more active. P-450$_{\beta NF-B}$ was an order of magnitude more active than any other form of P-450 in catalyzing 7-ethoxyresorufin O-deethylation and acetanilide hydroxylation. P-450$_{\beta NF-B}$ was the form most active in catalyzing the oxidation of benzo[*a*]pyrene to total phenolic products. The differences among P-450 isozymes are more dramatic when individual benzo[*a*]pyrene metabolites are considered (Gozukara et al., 1982). P-450$_{\beta NF/ISF-G}$ and P-450$_{PB-B}$ were the forms most active in catalyzing aniline hydroxylation, although P-450$_{UT-A}$, P-450$_{\beta NF-B}$, and P-450$_{PB-C}$ also showed substantial activity. *N*,*N*-Dimethylnitrosamine activity was not particularly specific with either high or low substrate concentrations, although several forms of P-450 appeared rather active when compared to microsomes.

Data are also shown for regioselective hydroxylation of both enantiomers of warfarin. P-450$_{UT-A}$ was most active in forming the 9,10-olefin [from (*R*)-warfarin]. This P-450 preferentially metabolized both the *R* and *S* isomers to 4′-hydroxy- and 6-hydroxywarfarin. P-450$_{PB-B}$ preferentially metabolized (*R*)-warfarin to 4′-hydroxywarfarin, but this activity was not as efficient as with P-450$_{UT-A}$. P-450$_{\beta NF-B}$ extensively metabolized both (*R*)-warfarin and (*S*)-warfarin at the 6 and 8 positions. P-450$_{PB-C}$ yielded the 7-hydroxy derivatives of both (*R*)-warfarin and (*S*)-warfarin. P-450$_{PB/PCN-E}$ can be considered primarily an (*R*)-warfarin 10-hydroxylase. The remaining three forms of P-450 (P-450$_{PB-D}$, P-450$_{UT-F}$, and P-450$_{\beta NF/ISF-G}$) did not metabolize warfarin in an extensive or in a characteristic manner.

In collaboration with Drs. D. J. Waxman and C. Walsh of the Massachusetts Institute of Technology, we examined the metabolism of testosterone by the eight forms of P-450. The observed metabolite patterns were essentially indistinguishable from those observed with their analogous isozymes (Waxman et al., 1982).[3] P-450$_{UT-F}$ yielded essentially only the 7α-alcohol. P-450$_{PB-B}$ produced roughly equivalent amounts of three metabolites, the 16α- and 16β-alcohols and androstenedione, the latter of which was presumably derived by dehydration of 17-hydroxytestosterone. P-450$_{UT-A}$ yielded roughly equivalent amounts of the 16α- and 2α-alcohol derivatives of testosterone as well as androstenedione. P-450$_{\beta NF-B}$ produced essentially only the 6β-alcohol. P-450$_{PB-C}$ produced small amounts of the 16α- and 16β-alcohols and androstenedione, and P-450$_{\beta NF/ISF-G}$ produced small amounts of products comigrating with the 15α-, 16α-, 7α-, and 6β-alcohols. P-450$_{PB-D}$ and P-450$_{PB/PCN-E}$ had very little activity toward testosterone.

Discussion

Techniques have been developed for the isolation of eight different forms of rat liver microsomal P-450 from PB- and βNF-treated rats. However, some of the forms are present in larger quantities in animals treated with other compounds (e.g., P-450$_{PB/PCN-E}$ in PCN-treated rats and P-450$_{\beta NF/ISF-G}$ in ISF-treated rats). This is the most extensive collection of isozymes of rat P-450 obtained to date in a single laboratory. These P-450s probably account for the bulk of the P-450 activity of liver microsomes prepared from rats treated with the inducing agents used here. This battery of P-450s, and antibodies raised to them, provides a substantial basis to initiate investigations to answer questions about the control, mechanism of specificity, and catalytic mechanism of P-450s. While other forms of P-450 undoubtedly exist in rat liver in addition to these eight, purification of additional variants will probably only be useful for determining specific isozymes that metabolize certain other endogenous and xenobiotic compounds. Important questions, such as the total number of P-450s which

[3] On the basis of these catalytic testosterone activities (vide infra) and direct comparisons of electrophoretic mobilities in $NaDodSO_4$–polyacrylamide gels, our P-450$_{PB-C}$, P-450$_{UT-A}$, P-450$_{UT-F}$, and P-450$_{PB-B}$ preparations appear to correspond to the P-450 preparations which are designated PB-1, PB-2, PB-3, and PB-4, respectively, elsewhere by Drs. D. J. Waxman and C. Walsh (Waxman & Walsh, 1982a,b; Waxman et al., 1982).

Table III: Metabolism of Various Compounds by Rat Liver Microsomes and Purified P-450s

substrate	rate: purified P-450 [nmol of product min^{-1} (nmol of P-450)$^{-1}$]								rat liver microsomes [nmol min^{-1} (mg of protein)$^{-1}$][a]					
	UT-A	PB-B	βNF-B	PB-C	PB-D	PB/PCN-E	UT-F	βNF/ISF-G	UT	PB	βNF	PCN	ISF	Aroclor 1254
none (oxidase activity)	32	56	38	22	9	18	47	61	9	33	12	24	17	32
p-nitroanisole	<0.5	14	23	<0.5	<0.5	<0.5	<0.5	1.2	0.47 (0.90)	4.8 (19)	2.7 (34)	0.41 (2.7)	2.2 (5.7)	7.2 (53)
d-benzphetamine	29	72	13	16	3	2	8	11	5.3 (45)	19 (122)	3.3 (43)	7.5 (25)	7.9 (38)	15 (143)
aminopyrine	17	85	22	12	7	9	11	29	5.9 (33)	11 (142)	4.6 (64)	9.4 (34)	6.9 (57)	8.9 (198)
7-ethoxycoumarin	2.4	9.2	27	<0.1	<0.1	0.8	<0.1	3.6	0.5 (4.4)	2.2 (15)	6.8 (41)	0.8 (4.2)	2.3 (9.0)	12 (57)
ethylmorphine	44	24	5	54	3	2	5	7	9.7 (75)	22 (95)	5.2 (42)	20 (38)	20 (45)	14 (81)
7-ethoxyresorufin	<0.01	<0.01	6.9	<0.01	<0.01	<0.01	<0.01	0.52	0.055 (0.2)	0.087 (0.2)	2.1 (9.9)	0.025 (0.4)	0.044 (1.3)	3.4 (10.6)
acetanilide	6	4	46	3	3	5	4	4	1.2 (13)	2.2 (20)	5.4 (71)	2.5 (28)	3.2 (16)	7.9 (85)
benzo[*a*]pyrene	3.9	3.3	9.7	<0.5	3.0	1.3	0.8	2.1	0.8 (5.9)	1.3 (10.5)	4.2 (17)	0.8 (4.0)	1.8 (5.9)	4.6 (24)
aniline	1.3	3.9	1.3	1.2	0.2	0.2	0.3	4.1	0.53 (2.1)	0.80 (6.8)	0.52 (5.1)	0.61 (1.4)	1.8 (5.7)	1.2 (13.5)
N,*N*-dimethylnitrosamine[b]														
0.1 M	7.2	8.0	4.0	4.3	6.2	2.2	3.5	4.3	1.8 (12)	2.2 (28)	2.0 (13)	1.2 (8.5)	2.6 (12)	2.5 (29)
1 mM	3.1	3.6	3.6	1.7	3.6	1.8	0.5	1.8	0.29 (5.6)	0.13 (13)	0.16 (8.4)	0.11 (4.8)	0.48 (5.8)	0.11 (17)
(*R*)-warfarin[c]														
9,10-dehydro	0.04	0.01	0.01	0.01	0.01	0.01	<0.005	<0.005	0.04 (0.04)	0.19 (0.06)	0.02 (0.02)	0.18 (0.02)	0.10 (0.01)	0.08 (0.03)
4′-OH	0.43	0.13	0.03	0.06	0.06	0.02	<0.005	0.01	0.16 (0.53)	0.71 (0.44)	0.09 (0.20)	0.16 (0.18)	0.19 (0.16)	0.22 (0.35)
6-OH	0.55	0.04	1.37	0.14	0.03	0.01	<0.005	0.01	0.10 (0.76)	0.56 (0.47)	0.31 (2.25)	0.18 (0.31)	0.19 (0.40)	0.88 (2.23)
7-OH	0.06	0.02	0.31	0.80	0.01	0.01	<0.005	<0.005	0.22 (0.37)	1.87 (0.62)	0.14 (0.65)	0.27 (0.30)	0.17 (0.32)	0.19 (0.78)
8-OH	0.01	0.01	1.90	0.03	<0.005	<0.005	<0.005	0.01	0.03 (0.09)	0.29 (0.10)	0.58 (2.68)	0.03 (0.12)	0.21 (0.25)	1.52 (2.77)
10-OH	0.16	0.02	0.01	0.01	0.01	0.09	<0.005	<0.005	0.05 (0.23)	0.32 (0.23)	0.02 (0.09)	0.54 (0.17)	0.12 (0.09)	0.22 (0.15)
(*S*)-warfarin														
9,10-dehydro	0.01	0.01	<0.005	0.01	<0.005	0.01	<0.005	<0.005	0.11 (0.01)	0.34 (0.2)	0.07 (0)	0.31 (0)	0.23 (0)	0.15 (0.02)
4′-OH	0.74	0.03	0.04	0.16	0.03	0.02	0.01	0.01	0.28 (0.94)	0.28 (0.53)	0.12 (0.33)	0.23 (0.31)	0.20 (0.29)	0.11 (0.35)
6-OH	0.49	0.02	1.13	0.20	0.02	0.01	0.01	0.01	0.17 (0.69)	0.42 (0.46)	0.28 (1.80)	0.20 (0.30)	0.16 (0.37)	0.57 (1.87)
7-OH	0.01	0.02	0.23	1.17	0.01	0.02	<0.005	<0.005	0.13 (0.44)	0.89 (0.86)	0.10 (0.63)	0.19 (0.40)	0.11 (0.50)	0.17 (0.78)
8-OH	0.02	<0.005	0.32	0.10	<0.005	<0.005	<0.005	<0.005	0.02 (0.07)	0.14 (0.09)	0.09 (0.40)	0.02 (0.05)	0.03 (0.08)	0.16 (0.50)
10-OH	0.13	<0.005	<0.005	<0.005	0.01	0.02	0.004	<0.005	0.02 (0.15)	0.08 (0.08)	0.01 (0.04)	0.15 (0.06)	0.04 (0.04)	0.07 (0.04)

[a] Theoretical rate in parentheses. For calculation of the theoretical rate for each activity, the immunologically determined specific content of each form of P-450 in the microsomes (Table II) was multiplied by the turnover number found for that isolated P-450 in the reconstituted system and the products were summated. [b] The substrate concentration was 0.1 M or 1 mM as indicated. Incubation vials were capped (using Teflon liners) to prevent evaporation of the substrate. Because of the length of the incubations, catalase (1 μg/mL) was also added to consume H_2O_2 which is produced and can destroy P-450 heme (Guengerich, 1978c). [c] Each positional warfarin product was estimated individually.

exist (Nebert, 1979) and the nature of the catalytic mechanism of P-450, will probably not be answered by the purification of more forms of P-450, even to the extent of an order of magnitude.

In Table I we have made an attempt to compare our preparations with those of others. Caution is advised against making strict comparisons, because of strain (Guengerich et al., 1981) and colony (Vlasuk et al., 1982) variations in P-450s among rats, as well as rabbits (Johnson et al., 1982). There are some minor differences from the other preparations in the literature, but this cross-listing is intended to serve as a general guide to the reader for comparison of the forms of rat P-450. In this regard we stress that absolute monomeric molecular weight values, normal spectral properties (i.e., Soret absorption maxima of the ferrous carbonyl complex in the range 449–451 nm), and relatively medium to low activities with substrates that yield a single product do not provide a firm basis for making comparisons. One would expect the greatest differences between strains and colonies in untreated rats. We are unable to compare any of our eight isozymes to the P-450 preparations of Agosin et al. (1979), Cheng & Schenkman (1982), or LeProvost et al. (1981), which were isolated from untreated animals, as the characteristics of these preparations are not differentiated from other P-450s enough to provide a basis for comparison.

Several observations can be derived from the substrate specificities of the isozymes presented in Table III. First, determination of induction of individual P-450s on the basis of activities is tenuous, because of the lack of unique specificities of the P-450s and the induction and repression of multiple forms of P-450 by the same compound. Moreover, the complexity of the expression of the activities of a number of P-450s in microsomal preparations suggests caution in making discrete conclusions on the basis of even a bank of activities (Lang et al., 1981). Another point to be made is that certain types of reactions (i.e., N-dealkylation, O-dealkylation, and aromatic ring hydroxylation) are catalyzed by different P-450s and cannot be grouped together with an individual P-450 or a common inducer.

Data are presented in Table III for the summation of catalytic activities of individual P-450s in comparison to the activities observed with microsomal preparations. In most cases, the data suggest that the bulk of microsomal activity can be accounted for in terms of the activities of the isolated P-450s. In some cases the theoretical rate, based on activities of purified isozymes, is an order of magnitude greater than that actually observed with microsomes. These results suggest that factors such as the level of NADPH–P-450 reductase or its rate of interaction with P-450 limit metabolism in microsomes. However, in some cases the activity in microsomes cannot be accounted for by the eight preparations. Such comparisons are not completely valid in that the NADPH–P-450 reductase concentration is optimized in the reconstituted system but not in the case of the microsomes. Nevertheless, the comparisons are of use in evaluating the validity of the reconstitution methods and the recovery of P-450s responsible for certain catalytic activities. The results of the warfarin experiments are of particular interest in comparing reconstituted and microsomal systems. Similar stereoselectivity of hydroxylation occurred in both systems, e.g., 8-hydroxylation by P-450$_{\beta NF\text{-}B}$ and microsomes isolated from βNF-treated rats and 10-hydroxylation by P-450$_{PB/PCN\text{-}E}$ and microsomes isolated from PB- or PCN-treated rats. Some forms of P-450 were more active in the reconstituted systems than in microsomes, e.g., P-450$_{UT\text{-}A}$ (especially as a 4′-hydroxylase) and P-450$_{\beta NF\text{-}B}$ (especially as a 6- or 8-hydroxylase). Qualitatively, the microsomal preparations metabolize warfarin and many other substrates in a manner which could be predicted from a knowledge of the metabolizing activities of the isozymes that are present in the microsomal preparations.

Specific antibodies complement other techniques in answering questions about induction and control of P-450s. The particular technique used here provides a check on antibody specificity in terms of apparent monomeric molecular weight. Nevertheless, even then, two P-450s may appear indistinguishable, and isoelectric focusing was used to separate P-450$_{PB\text{-}B}$ and P-450$_{PB\text{-}D}$. Thus, to be most cautious, we should perhaps still regard each of the eight isolated P-450s as a subset that may or may not contain only one P-450, since it is not possible to unambiguously prove that a P-450 is truly homogeneous or "pure". The inducibility data (Table II) indicate that P-450 isozymes may respond differently on administration of common inducers. PB induced four forms of P-450 (P-450$_{PB\text{-}B}$, P-450$_{PB\text{-}C}$, P-450$_{PB\text{-}D}$, and P-450$_{PB/PCN\text{-}E}$) to varying extents and lowered the level of one (P-450$_{UT\text{-}A}$). βNF induced two forms of P-450 (P-450$_{\beta NF\text{-}B}$ and P-450$_{\beta NF/ISF\text{-}G}$) and lowered the level of one (P-450$_{UT\text{-}A}$). PCN and ISF each induced essentially one form of P-450 (P-450$_{PB/PCN\text{-}E}$ or P-450$_{\beta NF/ISF\text{-}G}$, respectively) and lowered the level of another (P-450$_{UT\text{-}A}$). However, the forms of P-450 induced by these two latter compounds are also induced by others (PB and βNF). Since Aroclor 1254 is a mixture of individual congeners, its effects can be described in terms of mixtures of inducing agents. However, both the immunological and catalytic (warfarin 7-hydroxylase) data suggest that Aroclor 1254 did not induce P-450$_{PB\text{-}C}$ while PB did. We have ruled out a number of technical factors responsible for the discrepancy between the sums of the immunologically determined P-450 levels and the levels estimated by spectral analysis (vide supra). Our present view is that microsomes can, at least under certain conditions, contain significant levels of apo-P-450. As a corollary, heme synthesis and insertion into apo-P-450 may limit levels of P-450.

Finally, a great number of questions remain to be answered. What is the source of the functional differences among these isozymes? More specifically, what features of P-450s control substrate specificity and specificity toward different sites within a common substrate? What are the comparative rates of synthesis and degradation of these individual P-450s, and are differences appreciable in terms of function? Which P-450s contribute to the metabolism of compounds of environmental concern? Finally, how closely related are the P-450s? The physical similarity of P-450$_{PB\text{-}B}$ and P-450$_{PB\text{-}D}$ is quite striking, although the two enzymes can be clearly separated on the basis of net charge and one is clearly more active than the other toward most, though not all, substrates tested (Table III). The data also suggest some common antigenic sites for at least some of the P-450s (i.e., P-450$_{\beta NF\text{-}B}$ and P-450$_{\beta NF/ISF\text{-}G}$; P-450$_{PB\text{-}B}$ and P-450$_{PB\text{-}D}$; Figure 5), and therefore, characterization of these epitopes may be useful in understanding basic features of P-450s.

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