Major Isozymes of Rat Liver Microsomal Cytochrome P-450 Involved in the N-Oxidation of N-Isopropyl-\(\alpha\)-(2-methylazo)-p-toluamide, the Azo Derivative of Procarbazine

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ABSTRACT

Seven isozymes of cytochrome P-450 were tested to establish whether they could N-oxidize azoprocarbazine to form the two isomeric azoxy metabolites after optimizing the reconstitution of various purified isozymes with regard to substrate concentration, exogenous lipid, and reduced nicotinamide adenine dinucleotide phosphate-cytochrome c (P-450) reductase concentration. Two isozymes, cytochromes P-450\(_{\text{PCN}}\) (an isozyme present in untreated rats or in rats treated with phenobarbital or \(\beta\)-naphthoflavone) and P-450\(_{\text{NNF}}\) (the major \(\beta\)-naphthoflavone-induced isozyme), had appreciable turnover numbers for the N-oxidation reaction. The product ratio [N-isopropyl-\(\alpha\)-(methyl-ONN-azoxy)-p-toluamide formation relative to N-isopropyl-\(\alpha\)-(methyl-NNO-azoxy)-p-toluamide formation] obtained with cytochrome P-450\(_{\text{PCN}}\) was nearly identical to those values obtained with liver microsomes from untreated and phenobarbital-treated rats. In addition, cytochrome P-450\(_{\text{PCN}}\) and liver microsomes from \(\beta\)-naphthoflavone-treated rats had nearly identical product ratios. Specific inhibitory antibodies to four purified isozymes were used to titrate the N-oxidase activity of liver microsomes from untreated, phenobarbital-, pregnenolone-16a-carbonitrile-, or \(\beta\)-naphthoflavone-treated rats. Anti-cytochrome P-450\(_{\text{PCN}}\) globulin inhibited more than 70 to 90% of the formation of N-isopropyl-\(\alpha\)-(methyl-ONN-azoxy)-p-toluamide in microsomes from untreated, phenobarbital-, and pregnenolone-16a-carbonitrile-treated rats, respectively, but only 20 to 50% of N-isopropyl-\(\alpha\)-(methyl-NNO-azoxy)-p-toluamide formation. A small amount (25 to 30%) of inhibition was observed with anti-cytochrome P-450\(_{\text{NNF}}\) globulin. Anti-cytochrome P-450\(_{\text{PCN}}\) globulin inhibited more than 85% of the synthesis of either azoxy isomer catalyzed by liver microsomes from \(\beta\)-naphthoflavone-treated rats. These results demonstrate that two isozymes are responsible for the oxidative metabolism of azoprocarbazine and can account for the major portion of this N-oxidase activity in liver microsomes from untreated and phenobarbital-, pregnenolone-16a-carbonitrile-, or \(\beta\)-naphthoflavone-treated rats.

INTRODUCTION

The concept that multiple forms of microsomal cytochrome P-450 exist within the organs of a single species was first established by the observations that animal pretreatment with various compounds, termed inducing agents, resulted in alterations in the enzyme activities measured (2), and in the wavelength maxima of the ferrous-carbon monoxide complex of this family of microsomal heme proteins (1, 25). Confirmation of this concept required the efforts of several laboratories to develop methods utilizing specific detergents (16, 24) and chromatographic procedures (11) to facilitate the purification of individual forms of cytochrome P-450 from the organs of animals. Recently, a large number of forms of the heme protein have been purified from the livers of rabbits and rats and characterized with regard to their physicochemical properties and enzyme activities (17). Vlasuk et al. (29) have provided evidence for the existence of a minimum of 8 forms of microsomal cytochrome P-450 in the livers of the Long-Evans rat, using electrophoretic methods. In addition, Guengerich et al. (8) have reported the purification of 8 forms of the cytochrome from livers of Sprague-Dawley rats treated with PB3 and \(\beta\)-NF. The purification of the various isozymes to homogeneity, and subsequent preparation of monoclonal antibodies directed toward the homogenous antigens, allowed the measurement of the amounts of various isozymes in liver microsomes from untreated rats, and from rats pretreated with a number of inducing agents (5, 8, 21, 28).

A limited number of studies have attempted to establish which isozymes are responsible for the N-oxidase activities of liver microsomal cytochrome P-450. Hlavica and Kehl (10) reported the ability of a partially purified fraction of cytochrome P-450 to catalyze the N-oxidation of \(N,N\)-dimethylaniline to form the N-oxide. The metabolism of some amines has been shown to involve N-hydroxylation reactions catalyzed by the cytochrome P-450-dependent monoxygenase (3, 12, 31). Recently, 2-aminoanthracene was shown to be metabolically activated to a mutagenic species by an isozyme of rabbit liver microsomal cytochrome P-450, Form 4 (18), and 2-aminofluorene was metabolically activated by cytochrome P-450\(_{\text{PPC}}\) to yield a chemical species capable of alkylating DNA (7, 23).

The cancer chemotherapeutic agent procarbazine [N-isopropyl-\(\alpha\)-(2-methylhydrazino)-p-toluamide·HCl], a 1,2-disubstituted hydrazine, is known to be hepatotoxic and carcinogenic (13). A series of metabolic steps have been suggested to activate this compound to a species capable of eliciting its carcinogenic and/ or cytotoxic properties (30). Azoprocarbazine was shown to be the first metabolite formed during oxidative metabolism of procarbazine in liver (4). Recently, we reported the cytochrome P-450-dependent N-oxidation of azoprocarbazine to form 2 azyoxo...
isomers, $^4$ N-isopropyl-$\alpha$-[2-methyl-NNO-azoxy]-p-toluamide and N-isopropyl-$\alpha$-[2-methyl-ONN-azoxy]-p-toluamide (4, 32). The ratio of these products and the rate of their production were markedly affected by pretreatment of Sprague-Dawley rats with either PB or $\beta$NF. With the availability of 8 isozymes of cytochrome P-450 from liver microsomes of Sprague-Dawley rats and of specific antibodies to several of these isozymes (8), we have been able to establish which isozymes are involved in the catalysis of this $N$-oxidation reaction and can account, in large part, for the induction changes and the resultant changes in product ratio.

**MATERIALS AND METHODS**

**Chemicals.** dl-isotonic acid, NADP$,^+$, isocitrate dehydrogenase (type IV), egg $L$-$\alpha$-phosphatidylcholine, egg $L$-$\alpha$-phosphatidylethanolamine, and diacetyl-$L$-$\alpha$-phosphatidylcholine were purchased from the Sigma Chemical Co. (St. Louis, MO). PB was obtained from Merck & Co., Inc. (Rahway, NJ). PCN was obtained from the Upjohn Co. (Kalamazoo, MI), and $\beta$NF was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Procarbazine hydrochloride was a gift from Hoffman-La Roche, NJ, and [ring-$^3$H]procarbazine-HCl was obtained from the Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Azo-procarbazine, its 2 isomeric azoxy derivatives, and [ring-$^1$C]azioprocarbazine (275 $\mu$Ci/mmol) were prepared and purified as described previously (4, 32).

**Microsomes and Purified Proteins.** Male Sprague-Dawley Crl:CD(SD)BR rats (100 to 175 g) were purchased from the Charles River Breeding Laboratories, Inc. (Wilmington, MA) or from Harlan Industries (Indianapolis, IN). The animals were maintained on water and laboratory chow ad libitum. They were given injections of PB in 0.9% NaCl solution (80 mg/kg) or $\beta$NF in corn oil (75 mg/kg) daily for 4 days, and starved 18 hr prior to death. Untreated rats were starved 18 hr prior to death. Microsomal fractions were prepared according to the method of Remmer et al. (22), and the protein concentrations were determined with the method of Lowry et al. (15).

NADPH-cytochrome c (P-450) reductase was prepared as described by Yasukochi and Masters (33), with DEAE-cellulose and 2',5'-ADP-agarose column chromatography. Cytochrome $b_5$ was purified by repeated DEAE-Sephadex chromatography (26). The various isozymes of cytochrome P-450 were purified with n-octylamino-Sepharose 4B column chromatography, followed by column chromatography with hydroxyapatite, DEAE-cellulose, and CM-52 cellulose (8). The isozymes available for these studies included cytochromes P-450$_{ur}$, P-450$_{pr}$, P-450$_{pc}$, and P-450$_{pcF}$, and P-450$_{prF}$, as designated by Guengerich et al. (8). Antibodies to the homogeneous heme proteins were elicited in female New Zealand White rabbits, and preparations of partially purified IgG fractions were prepared as described (9). Specific antibodies were available for cytochrome P-450$_{ub}$, P-450$_{ur}$, P-450$_{pr}$, P-450$_{pc}$, and P-450$_{prF}$. Evidence for the specificity of these antibodies was presented elsewhere (8).

**Enzyme Assays.** Reaction mixtures with liver microsomes (1 mg/ml) contained 5 mm sodium $d_{l}$-isocitrate, isocitrate dehydrogenase (0.5 IU/ml), 1 mm MgSO$_4$, 0.5 mm NADP$,^+$, 1 mm EDTA, 0.1 $\mu$M Tris-HCl, pH 7.6, and 300 $\mu$M [ring-$^1$C]azioprocarbazine (275 $\mu$Ci/mmol) in a 1-ml volume. After preincubation at 37$^\circ$ for 5 min, the reactions were initiated by addition of azioprocarbazine. Incubation mixtures containing specific antibodies to the various isozymes of the cytochrome were preincubated with the isozymes 5 to 10 min prior to initiation of reactions with azioprocarbazine. Since the reactions were linear with respect to time and protein concentration, a single 10-min incubation time was utilized to measure the effects of the antibodies on metabolism. In order to assess any chemical decomposition of the substrate, separate experiments were performed in the absence of added NADPH. The values for the radioactivity which coeluted with the azoprocarbazine standards were less than 5% of that seen in the presence of NADPH. The specificity and titers of the antibodies were established with ethoxyresorufin (P-450$_{prF}$), benzphetamine (P-450$_{pr}$), and ethylmorphine (P-450$_{ub}$) as substrates. The anti-P-450$_{pr}$ globulin produced more than 85% inhibition of ethoxyresorufin O-deethylase activity (19), at a ratio of 7 to 8 mg microsomal protein from $\beta$NF-treated rats; the other globulins were not inhibitory at or below this ratio. Anti-P-450$_{prF}$ globulin maximally inhibited the benzphetamine N-demethylation activity (20) of liver microsomes from PB-treated rats by 65% at a ratio of 2.0 to 2.5 mg IgG/mg microsomal protein. Similarly, the ethylmorphine N-demethylation activity (20) of liver microsomes from PB-treated rats was maximally inhibited with anti-P-450$_{ub}$ globulin by 40% at a ratio of 0.6 to 1.0 mg IgG/mg microsomal protein. These results with IgG protein to microsomal protein were used to inhibit the conversion of azoprocarbazine to the methyl and benzylazo derivatives catalyzed by liver microsomes from untreated and PB-, $\beta$NF-, or $\beta$NF-pretreated rats.

When purified cytochrome P-450 fractions were used, the microsomal protein was replaced with 0.7 nmol of each of the various fractions of the cytochrome, 0.7 nmol of NADPH-cytochrome c (P-450) reductase, and 30 $\mu$g diacetyl-$L$-$\alpha$-phosphatidylcholine/ml. The reductase, cytochrome, NADPH-regenerating system, and lipid fractions were mixed and preincubated 5 to 10 min prior to addition of azioprocarbazone. The reactions with cytochrome P-450$_{pr}$, P-450$_{prF}$, and P-450$_{ub}$ were linear for up to 20 min. For these cytochromes, reconstitution in vesicles containing egg $L$-$\alpha$-phosphatidylethanolamine and $L$-$\alpha$-phosphatidylcholine (1:2, w/w), with or without cytochrome $b_5$ (0.7 $\mu$M), was performed by the method of Taniguchi et al. (27); the results observed with this procedure were identical to those obtained by the method described above.

The reactions were terminated by extraction with three 2.5-ml portions of benzene (32). The benzene extracts were filtered and dried under a stream of nitrogen. The samples were analyzed by high-performance liquid chromatography with a $\mu$Bondapak CN column (Waters Associates, Milford, MA), and an isocratic solvent system consisting of hexane, methylene chloride, and acetonitrile (86:11:3, v/v). Authentic standards of the azoxy derivatives and $P$-formyl-N-isopropylbenzamide were added to the samples dissolved in the mobile phase, and the eluate was monitored at 254 nm. Fractions were collected, evaporated, and assayed for radioactivity, as described previously (32). The recovery of radioactivity from the column was greater than 92% in all cases.

**RESULTS**

**Catalytic Activity of Purified Isozymes.** Prior to determination of the turnover numbers for the formation of the benzyl- and methylazoxy derivatives of azoprocarbazine catalyzed by the various purified isozymes, the reactions were optimized with regard to the amounts of reductase and phospholipid required. Maximal activity was seen for cytochromes P-450$_{pr}$, P-450$_{prF}$, and P-450$_{ub}$ when diacetyl-$L$-$\alpha$-phosphatidylcholine (30 $\mu$g/ml) was utilized, and an optimal ratio for NADPH-cyto-
chrome c (P-450) reductase to cytochrome P-450\textsubscript{aNF-B} of unity was noted for the formation of the methylazoxy isomer (Chart 1). Similar results were obtained for cytochromes P-450\textsubscript{aNF-B} and P-450\textsubscript{aNF-C}. Use of reconstituted phospholipid vesicles, with or without cytochrome b\textsubscript{5} (0.7 \muM), had little or no effect on the rate of formation of the methylazoxy isomer.

The turnover numbers for formation of the azoxy derivatives catalyzed by liver cytochrome P-450 bound to the microsomal membranes from untreated and PB-, PCN- or bNF-pretreated rats are compared to 7 purified cytochrome P-450 isozymes (Table 1). Under the conditions of the reconstitution procedure utilizing dilauroyl-L-α-phosphatidylcholine, only 2 isozymes, cytochrome P-450\textsubscript{aNF-B} and P-450\textsubscript{aNF-C}, showed turnover numbers equal to or greater than the microsomal fractions from which they were derived. The major PB-inducible form, cytochrome P-450\textsubscript{aNF-C}, possessed some activity, but its turnover number was 50% less than that seen with liver microsomes from PB-treated rats. The other isozymes were tested with untreated rat liver microsomes.

Table 1
| Enzyme source | Fraction | Benzylazoxy isomer (nmol product/min/nmol cytochrome) | Methylazoxy isomer (nmol product/min/nmol cytochrome) | Product ratio | Value
|---------------|----------|-----------------------------------------------------|-----------------------------------------------------|--------------|-------
| Untreated rat | Microsomes | 0.05                                                | 0.31                                                | 6.2          |       
|               | P-450\textsubscript{aNF} | <0.01                                               | <0.03                                               | <0.01        |       
| PB-treated rat | Microsomes | 0.10                                                | 0.46                                                | 4.6          |       
|               | P-450\textsubscript{aNF} | 0.08                                                | 0.19                                                | 2.4          |       
|               | P-450\textsubscript{aNF-C} | 0.08                                                | 0.43                                                | 5.6          |       
|               | P-450\textsubscript{aNF-E} | <0.01                                               | <0.03                                               | <0.01        |       
| PCN-treated rat | Microsomes | 0.11                                                | 0.53                                                | 4.8          |       
| βNF-treated rat | Microsomes | 0.05                                                | 1.03                                                | 21           |       
|               | P-450\textsubscript{aNF} | 0.08                                                | 2.26                                                | 28           |       
|               | P-450\textsubscript{aNF-E} | <0.01                                               | <0.03                                               | <0.01        |       

\*Values are the average of 3 experiments, and the standard deviations were less than 15% of the average for all of the liver microsomal preparations and cytochromes P-450\textsubscript{aNF-C}, P-450\textsubscript{aNF-E}, and P-450\textsubscript{aNF}. The other isozymes were tested in 2 separate experiments with identical results.

The product ratio was calculated by dividing the rate of formation of the methylazoxy isomer by the rate of formation of the benzylazoxy isomer.

Antibody Inhibition of Microsomal N-Oxidase Activity of Cytochrome P-450. With the availability of specific antibodies to a number of isozymes of cytochrome P-450, we attempted to titrate the production of either azoxy isomer catalyzed by microsome-bound cytochrome P-450. The effect of various specific antibodies on azoxy isomer production by liver microsomes from untreated rats is shown in Chart 2. Anti-P-450\textsubscript{aNF-C} globulin inhibited the formation of the methylazoxy isomer by more than 70%, and anti-P-450\textsubscript{aNF-E} globulin inhibited methylazoxy isomer formation by less than 20%. Only anti-P-450\textsubscript{aNF-C} was effective against formation of the benzylazoxy isomer, which was inhibited by 35%. Neither anti-P-450\textsubscript{aNF-B} nor P-450\textsubscript{aNF-E} Globulins were inhibitory to the formation of the benzylazoxy isomer.

Chart 3 demonstrates the inhibitory effect of anti-P-450\textsubscript{aNF-C} and anti-P-450\textsubscript{aNF-E} globulin on the formation of the methylazoxy isomer by liver microsomes from untreated rats. Open symbols, formation of the methylazoxy isomer; the control value was 0.31 nmol product formed/min/mg microsomal protein. Closed symbols, formation of the benzylazoxy isomer; the control value was 0.05 nmol product formed/min/mg protein. Results were obtained from 3 experiments performed in duplicate, and the standard deviation for the measurements was less than 20% of the average.
azoxy derivatives of procarbazine catalyzed by liver microsomes from PB-treated rats. Anti-P-450<sub>PC</sub> globulin inhibited potently (approximately 85%) the methylazoxy metabolite synthesis, while anti-P-450<sub>PCNF</sub> inhibited the reaction by 20 to 25%. The antibody to P-450<sub>PC</sub> was less effective in blocking benzylazoxy isomer formation, and P-450<sub>PCNF</sub> also inhibited this reaction by 20 to 25%. The antibody to P-450<sub>PC</sub> was not inhibitory to either reaction, while anti-P-450<sub>PNF</sub> was slightly inhibitory to methylazoxy isomer formation at a ratio of 7 mg IgG/mg microsomal protein. Similar results were obtained with liver microsomes from PCN-treated rats (Chart 4), except that the anti-P-450<sub>PC</sub> antibody had even less effect on benzylazoxy synthesis. The formation of the methylazoxy isomer was more potently inhibited by anti-P-450<sub>PC</sub> than was the synthesis of the benzylazoxy isomer in the presence of liver microsomes from PCN-treated rats. The antibodies to P-450<sub>PNF</sub> and P-450<sub>PNF</sub> were without effect on the reactions catalyzed by liver microsomes from PCN-treated rats (data not shown). Separate experiments with purified reconstituted P-450<sub>PC</sub> and anti-P-450<sub>PC</sub> demonstrated that an antibody to heme protein ratios of 2.0 and 6.0 mg/nmol heme protein, the rates of formation of both azoxy isomers were concomitantly inhibited (30 and 60%, respectively).

The N-oxidation of azoprocabarzine to either azoxy isomer by liver microsomes from PNF-treated rats was concomitantly inhibited by anti-P-450<sub>PNF</sub> with increasing ratios of IgG to microsomal protein (Chart 5). The reaction to form either azoxy derivative was maximally inhibited 70 to 80% by this antibody. A small but measurable amount of inhibition of the synthesis of the methylazoxy isomer was caused by the addition of anti-P-450<sub>PC</sub> at a ratio of 1 to 2 mg IgG/mg microsomal protein. These results demonstrate that the major PNF-inducible form of the cytochrome is responsible for this activity in liver microsomes from PNF-pretreated rats, except for a small portion of the formation of the methylazoxy isomer.

DISCUSSION

The realization that the N-oxidation of arylamines, arylamides, and other nitrogenous derivatives may lead to the generation of biologically reactive intermediates has stimulated the study of the enzyme systems capable of catalyzing these reactions (3, 12, 14, 31). Similar processes have been shown to activate metabolically a wide variety of carcinogens and toxic chemicals. The lack of knowledge related to these enzyme systems has led to the development of the procedures for solubilization and purification of the membrane-bound enzymes.

Recently, the existence of at least 8 different forms of cytochrome P-450 in the livers of Sprague-Dawley rats has been demonstrated (8). Specific antibodies raised against the purified enzymes were used to estimate the contents of these isozymes in liver microsomes isolated from untreated and PB- or PNF-induced rats. Four major isozymes can be detected in liver microsomes prepared from untreated rats: P-450<sub>IT</sub> (54%), P-450<sub>PC</sub> (16%), P-450<sub>PCNF</sub> (17%), and P-450<sub>IT</sub> (7%) (percentages were based on the sum of the contents of 8 isozymes). Two of these forms, P-450<sub>PC</sub> and P-450<sub>PCNF</sub>, are induced 2- to 3-fold by treatment of animals with PB, and 2 additional forms, P-450<sub>PC</sub> and P-450<sub>PCNF</sub>, are induced 40- and 20-fold, respectively. Animal treatment with PNF induced 2 liver isozymes, P-450<sub>PNF</sub> (46%), and P-450<sub>PNF</sub> (18%) by 20- to 35-fold.

The metabolic activation of arylamines and hydrazine/azo compounds has been suggested to involve the N-oxidation reactions catalyzed by cytochrome P-450 (3, 4, 30-32). Little is known about the specific isozymes of the cytochrome which could account for these reactions. Frederick et al. (7) and Robertson et al. (23) have established that one of these isozymes, P-450<sub>IT</sub>, has a high turnover with regard to the N-hydroxylation of 2-aminofluorene. The major PB-inducible isozyme of the cytochrome (P-450<sub>PC</sub>) did not catalyze a significant portion of this reaction (23). Our results, using azoprocabarzine as a substrate for the cytochrome P-450-dependent N-oxidation to form 2 isomeric azoxy derivatives, have also shown that the isozyme, P-450<sub>PC</sub>, can nearly account for all of the formation of azoxy isomers in liver microsomes from PNF-treated rats. For example, about 45% of the cytochrome P-450 in these microsomes is P-450<sub>PC</sub> (8), and our results demonstrate a turnover number for microsomes of 1.03 nmol methylazoxy isomer formed/min/nmol total cytochrome. The theoretical turnover number could be calculated to be 1.03 nmol/min/nmol ÷ 0.45 or 2.29 nmol product/min/nmol cytochrome P-450<sub>PC</sub>, if only P-450<sub>PC</sub> were functioning in the microsomes. Surprisingly, this value is almost identical to that obtained with the purified, reconstituted cyto-
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The situation with liver microsomes from untreated or PB-treated rats is more complex. The experiments with antibodies to the purified cytochromes indicate that P-450_{PB-C} accounts for nearly all of the synthesis of the methylazoxyl isomer in untreated rat liver microsomes, but only 30 to 40% of the benzylazoxyl isomer formation. A small portion of the formation of the methylazoxyl isomer appears to be due to P-450_{PB-E}. With liver microsomes from PB- or PCN-treated rats, P-450_{PB-C} and P-450_{PB-PHEN} were shown to be involved in the synthesis of both isomers, based on antibody inhibition studies. However, if one utilizes the turnover numbers obtained with purified P-450_{PB-C} or P-450_{PB-PHEN}, and the levels of these isozymes in microsomes from untreated and PB-treated rats to calculate theoretical rates of metabolism of azoprocabazine in the microsomes, these isozymes do not account quantitatively for the observed microsomal activities. The theoretical activities of P-450_{PB-C} and P-450_{PB-PHEN} for the formation of some of the hydroxylated warfarin derivatives were considerably lower than the actual values obtained with intact liver microsomes isolated from PB- or PCN-treated rats (8). In addition, Elshourbagy and Guzelian (6) have demonstrated that P-450_{PB-PHEN} is not as active after purification as expected from its apparent catalytic activity in liver microsomes. These results suggest that some purified forms of the cytochrome may not be maximally active when reconstituted in the manner described in this and other reports (5, 8).

The results with the inhibitory antibodies strongly suggest that the major isozymes of cytochrome P-450 which are involved in N-oxidation of azoprocabazine by Sprague-Dawley rat liver microsomes are P-450_{PB-C}, P-450_{PB-E}, and perhaps P-450_{PB-PHEN}. The induction seen after treatment of animals with PB or βNF can be accounted for by the known increases in the content of these forms in the various liver microsomal fractions. Of interest is the fact that, while the purified isozymes from PB-treated rats did not yield turnover numbers sufficient to account for the observed activity in microsomes, the change in product ratios of the 2 azoxy derivatives seen during induction by PB or βNF can be accounted for by the specific increases in the microsomal content of P-450_{PB-C} and P-450_{PB-E}. For example, liver microsomes prepared from untreated rats have a methylazoxyl:benzylazoxyl formation ratio of 6.2, which is nearly identical to the product ratio of 5.6 seen with the major N-oxidizing species present in untreated microsomes, P-450_{PB-C} (Table 1). Similarly, the ratio seen with microsomes isolated from PB-treated animals is 4.6 and is in agreement with the observation that P-450_{PB-C} is the major species involved in azoxy synthesis. Upon treatment of rats with βNF, the ratio obtained from liver microsomes is 21, which is nearly identical to that obtained with cytochrome P-450_{PB-E} (i.e., 28). Based on the results of Frederick et al. (7), Robertson et al. (23), and this report, P-450_{PB-C} and P-450_{PB-E} most likely are the major isozymes of cytochrome P-450 which N-oxidize 2-aminofluorene and azoprocabazine. These conclusions are based on the turnover numbers of the purified isozymes, the titration of azoxy formation by inhibitory antibodies to specific cytochromes, and the changes of the product ratios obtained with liver microsomes upon pretreatment of animals with PB and βNF in relation to the ratios obtained with the pure reconstituted isozymes. However, this generalization may not be valid for all arylamines, as the N-hydroxylation of 2-naphthylamine appears to be preferentially catalyzed by P-450_{PB-E}. Further studies using a larger choice of substrates will be required to prove unambiguously whether these 2 major forms of the cytochrome are unique for the N-oxygenation reactions catalyzed by cytochrome P-450.

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