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Separation and Characterization of Highly Purified Forms of Liver Microsomal Cytochrome P-450 from Rats Treated with Polychlorinated Biphenyls, Phenobarbital, and 3-Methylcholanthrene*

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Highly purified cytochromes P-450, designated P-450$_a$, P-450$_b$, and P-450$_c$, have been isolated from hepatic microsomes of rats treated with the polychlorinated biphenyl mixture Aroclor 1254. Each form shows a single protein-staining band on sodium dodecyl sulfate-polyacrylamide gels with minimum molecular weights of 48,000 (P-450$_a$), 52,000 (P-450$_b$), and 56,000 (P-450$_c$). Cytochromes P-450$_b$ and P-450$_c$ have been obtained from phenobarbital-treated rats, and cytochromes P-450$_b$ and P-450$_c$ were isolated from 3-methylcholanthrene-treated rats. Cytochrome P-450$_b$ is the major hemeprotein inducible by phenobarbital, and cytochrome P-450$_c$ is the predominant hemeprotein induced by 3-methylcholanthrene. The CO-reduced difference spectral peak of cytochrome P-450$_c$ is at 452 nm, P-450$_b$ is at 450 nm, and P-450$_a$ is at 447 nm.

Cytochrome P-450$_c$ preferentially hydroxylates testosterone at the 7α position but has low catalytic activity for the metabolism of benzphetamine, benzo[a]pyrene, 7-ethoxycoumarin, and zoxazolamine. Cytochrome P-450$_b$ catalyzes the hydroxylation of testosterone at the 16α position and the demethylation of benzphetamine most efficiently. Cytochrome P-450$_c$ has very high catalytic activity for benzo[a]pyrene and zoxazolamine hydroxylation and 7-ethoxycoumarin O-dealkylation. Aniline is hydroxylated by the three forms of cytochrome P-450 at comparable rates.

Antisera prepared against cytochrome P-450$_a$, P-450$_b$, or P-450$_c$ do not cross-react with the heterologous antigens in Ouchterlony double diffusion plates. When cytochromes P-450$_a$, P-450$_b$, and P-450$_c$ were subjected to limited proteolysis in the presence of sodium dodecyl sulfate, the resulting peptide maps provided additional evidence that these hemeproteins are structurally distinct. The forms of cytochrome P-450 obtained from phenobarbital- and 3-methylcholanthrene-treated rats are indistinguishable from the corresponding forms from Aroclor 1254-treated rats by all criteria examined.

Polychlorinated biphenyls are widespread environmental pollutants as a result of their use in the manufacture of several commercial products (1–6). Substantial levels of these compounds have been found in both human (7–9) and animal (1, 10–16) tissues. PCBs[1] have been shown to be inducers of the hepatic microsomal enzyme system that metabolizes endogenous substrates such as steroids and fatty acids, and exogenous chemicals including many drugs and carcinogens (1, 3, 17–24).

Aroclor 1254, a polychlorinated biphenyl mixture containing approximately 54% chlorine, is a potent inducer of cytochrome P-450,[2] the terminal oxidase of this enzyme system (27, 28). Alvares *et al.* (27) suggested that the cytochrome P-450 induced in rat liver by Aroclor 1254 was either a distinct *hemeprotein(s)* or a *mixture of the hemeproteins induced by* PB and 3-MC. Recently, we have purified cytochrome P-450 to a specific content greater than 19 nmol/mg of protein from hepatic microsomes of immature male rats administered a single large dose of Aroclor 1254 (29). This highly purified cytochrome P-450 preparation (29) contains multiple forms of the enzyme as do the purified preparations from PB- or 3-MC-treated rats (30–32). By several criteria, including spectral, catalytic, immunological, and electrophoretic properties, the cytochromes P-450 isolated from rats treated with Aroclor 1254 are indistinguishable from a mixture of the hemeproteins induced by PB and 3-MC (29). Alvares and Kappas (33) have recently confirmed these spectral and catalytic observations using partially purified cytochrome P-450 preparations. The relative induction of the various forms of cytochrome P-450 depends upon several factors such as the isomeric content of the PCB mixture (24, 34, 35), the dosage regimen (20), and the species (36) and sex (37) of the animal.

The present report describes the chromatographic separation of three highly purified forms of cytochrome P-450 from Aroclor 1254-treated rats which are referred to as cytochrome P-450,[3] P-450$_b$, and P-450$_c$. Two of these hemeproteins (cytochrome P-450$_a$ and P-450$_b$) have been obtained from PB-*treated rats;* and cytochrome P-450$_c$ and P-450$_c$ were separated from a hemeprotein preparation from 3-MC-treated rats. Cytochrome P-450$_b$ is the major hemeprotein species in liver microsomes from PB-treated rats; and cytochrome P-450$_c$ is the predominant hemeprotein from 3-MC-treated rats. The present investigation was designed to determine: 1) if cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ are distinct hemeproteins; and 2) if each cytochrome obtained from differently treated rats is identical. Several approaches were used to answer these questions. The spectral characteristics, substrate

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[1] The abbreviations used are: PCBs, polychlorinated biphenyls; 3-MC, 3-methylcholanthrene; PB, phenobarbital; SDS, sodium dodecyl sulfate; anti-PB-P-450, antibody to cytochrome P-450 from PB-treated rats; anti-MC-P448, antibody to cytochrome P-460 from 3-MC-treated rats.

[2] The term cytochrome P-450 is used to designate any or all forms of liver microsomal cytochrome P-450. The hemeprotein(s) induced by treatment of rats with 3-MC has also been termed cytochrome P-448 (25) or P$_1$-450 (26).

[3] These separated forms of cytochrome P-450 do not correspond to the heterogeneous Peak A and Peak B hemeprotein preparations previously described (38). The cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ reported in this study, are isolated from the Peak B fraction obtained from Aroclor 1254-, PB-, or 3-MC-treated rats.

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specificity, electrophoretic mobility on SDS-polyacrylamide gels, and immunological properties of each hemeprotein were examined. The peptide fragments of each of the cytochromes after limited proteolysis in the presence of SDS were compared on SDS-polyacrylamide gels according to a recently published procedure (39).

EXPERIMENTAL PROCEDURES

Separation of Multiple Forms of Cytochrome P-450—Immature male Long Evans rats (50 to 60 g, Blue Spruce Farms, Altamont, NY) were administered Aroclor 1254, PB, or 3-MC as described (29, 40). Aroclor 1254 (Lot KC-12-638) was kindly supplied by Monsanto Chemical Co. Hepatic microsomal cytochrome P-450 was partially purified (29, 40) from rats treated with these inducers to a specific content of 9 to 12 nmol/mg of protein based on the protein assay of Lowry *et al.* (41). The final step in the purification procedure (29, 30) was omitted.

Approximately 500 nmol (50 mg of protein) of the partially purified cytochrome P-450 preparation was dialyzed overnight against 6 liters of 5.0 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. After this dialysis, the sample was turbid but clarified upon addition of sodium cholate and Emulgen 911 at final concentrations of 0.5% and 0.2%, respectively (w/v). The sodium cholate was purchased from Calbiochem Co., and the Emulgen 911 was obtained from Kao-Atlas, Ltd. (Japan). The clarified hemeprotein preparation was stirred for 60 min at *room temperature*. The sample was applied to a Whatman DE52 cellulose column (2.4 × 60 cm) equilibrated with 900 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.5% sodium cholate, and 0.2% Emulgen 911. After sample application, the column was washed with 150 ml of the column equilibration buffer. No hemeprotein eluted with this buffer. The cytochrome P-450 was eluted from the column by a linear gradient of sodium chloride (0 to 0.25 M) in 500 ml of the equilibration buffer. *The entire chromatographic procedure was performed at room temperature.* This method is based on a procedure developed by Warner and Neims (42) to separate different cytochrome P-450 fractions from solubilized rat liver microsomes.

During the elution of the column, A_{417} was monitored by a flow cell in a Gilford 2400 spectrophotometer and conductivity of the eluting buffer was measured continuously with a LDC Conducto Monitor. The order of elution from the column was cytochrome P-450$_a$, P-450$_b$, and P-450$_c$. Approximately 3 to 4% of the cytochrome P-450 applied to the column from Aroclor 1254-, PB-, or 3-MC-treated rats was recovered as cytochrome P-450$_b$. The cytochrome P-450$_b$ recovered from Aroclor 1254-treated rats was approximately 30% of the total hemeprotein applied to the column whereas 60% of the sample applied from PB-treated rats was recovered as cytochrome P-450$_b$. The cytochrome P-450$_c$ recovery was 30% and 60%, respectively, of the applied preparations from Aroclor 1254- and 3-MC-treated rats.

Five grams of Amberlite XAD-2 beads (Rohm and Haas) were added to the cytochrome P-450$_b$ fraction, and the mixture was stirred for 60 min. After removal of the beads by filtration, calcium phosphate gel was added to the hemeprotein at a ratio of 20 mg of gel/*A* unit at 417 nm. The hemeprotein adsorbed to the gel, and the excess detergent remained in the supernatant. Cytochrome P-450$_b$ was eluted from the gel with 10 ml of 0.4 M potassium phosphate buffer (pH 7.4) containing 20% glycerol. The excess detergent was removed from cytochrome P-450$_b$ by treatment with 10 g of XAD-2 beads for 2 h. Cytochrome P-450$_c$ was treated with 10 g of XAD-2 beads for 60 min. The sample was dialyzed for 4 h against 6 liters of 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol after removal of the beads. Calcium phosphate gel was added to the cytochrome P-450$_c$ fraction at a ratio of 5 mg of gel/*A* unit at 417 nm. The hemeprotein was eluted from the gel with 10 ml of 0.4 M potassium phosphate buffer (pH 7.4) containing 20% glycerol. The procedures for removal of detergents from the cytochrome P-450 fractions were conducted at 4°C.

The hemeproteins were dialyzed overnight at 4°C against 6 liters of 0.05 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM dithiothreitol, and 0.1 mM EDTA. Each hemeprotein was concentrated by ultrafiltration through an Amicon PM-30 membrane and stored at −80°C.

The concentration of cytochrome P-450 was determined by the method of Omura and Sato (43) from the CO-reduced difference spectrum using an extinction coefficient of 91 $mM^{-1} cm^{-1}$. Protein was determined by the method of Lowry *et al.* (41) with crystalline bovine serum albumin as the standard. The specific content of the cytochrome P-450$_b$ varied between 12 to 14 nmol/mg of protein whether the hemeprotein was isolated from Aroclor 1254-, PB-, or 3-MC-treated rats. The cytochrome P-450$_b$ from Aroclor 1254- or PB-treated rats was 15 to 17 nmol/mg of protein. The specific content of cytochrome P-450$_c$ from Aroclor 1254- or 3-MC-treated rats was also 15 to 17 nmol/mg of protein. The protein content of the partially purified (40) and purified preparations (29, 30) containing multiple forms of cytochrome P-450 when determined from the amino acid composition has been reported to be approximately 70% of the value by the method of Lowry *et al.* (41). Studies are currently in progress[*] to determine whether any difference in the protein content exists with the separated forms of cytochrome P-450 when determined by the amino acid composition or the standard protein assay. The Emulgen 911 content of each hemeprotein ranged from 0.02 to 0.06 mg/nmol of cytochrome P-450. The recovery of cytochrome P-450$_b$ from the total microsomal cytochrome P-450 from rats treated with any of the inducers was 0.10 to 0.20%. The cytochrome P-450$_b$ and P-450$_c$ were each 1 to 3% of the microsomal cytochrome P-450 when isolated from rats treated with Aroclor 1254, PB, or 3-MC.

Other Assay Methods—An aliquot of each cytochrome was treated with SDS, heated, and electrophoresed on slab gels in the presence of SDS as described by Laemmli (44). The separating gel contained 7.5% acrylamide, was 10 cm long, and 0.75 cm thick. The gels were dried prior to being photographed.

All spectral determinations were done on an Aminco DW-2A recording spectrophotometer. Cytochrome P-450$_b$ was diluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and cytochrome P-450$_c$ or P-450$_a$ was diluted with the same buffer containing 0.1 mM EDTA, 0.5% sodium cholate, and 0.2% Emulgen 911 for all spectral determinations. The heme content of the preparations was determined as the pyridine hemochromogen as described by Falk (45).

The metabolism of aniline (46), benzphetamine (31), benzo[*a*]pyrene (47), 7-ethoxycoumarin (48), zoxazolamine (49), and testosterone (50) were measured as previously described. The catalytic activity of each hemeprotein was assayed under conditions in which metabolism was proportional to hemeprotein concentration and time of incubation. Saturating amounts of NADPH-cytochrome *c* reductase and phosphatidylcholine were used in all experiments. The NADPH-cytochrome *c* reductase was purified to a specific activity of 35,000 to 40,000 units/mg of protein by a combination of the method of Dignam and Strobel (51), and Yasukochi and Masters (52). Briefly, NADPH-cytochrome *c* reductase was solubilized from the hepatic microsomes of PB-treated rats by Renex 690, chromatographed on DEAE Sephadex A-25 (51), and further purified by affinity chromatography on a 2′,5′-ADP-Sepharose 4B column (52). One unit of reductase is defined as that amount catalysing the reduction of 1.0 nmol of cytochrome *c*/min at 22°C assayed by the method of Phillips and Langdon (53). Dilauroylphosphatidylcholine was prepared in 0.02 M Tris-HCl (pH 8.0) containing 1 mM EDTA and sonicated immediately before use.

Antibodies to the purified cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ were produced in sheep by a method previously described (31, 32). Antisera were used in the double diffusion analyses. The Ouchterlony double diffusion plates contained 0.9% agarose and 0.2% Emulgen 911.

The peptide mapping of the different hemeproteins was performed as described by Cleveland *et al.* (39). Each cytochrome was diluted to 0.25 mg of protein/ml in a buffer containing 0.5% SDS and heated for 2 min in a boiling water bath. The final concentration of SDS was at least 2 mg/ml. The hemeproteins were treated with either chymotrypsin (0.04 mg/mg of protein) or *Staphylococcus aureus* V8 protease (0.05 mg/mg of protein) at 37°C for the time indicated in the figure legends. The reaction was stopped by the addition of 2-mercaptoethanol and SDS to final concentrations of 10% and 2%, respectively, followed by heating in a boiling water bath for 2 min. Twenty five microliters (approximately 4.5 μg of protein) of each sample was applied to an SDS-polyacrylamide slab gel. The gel contained 15% acrylamide and was 10 cm long and 1.5 mm thick. Electrophoresis, in the presence of SDS, was performed by the method of Laemmli (44).

[*] Recent studies by this laboratory have indicated that a similar discrepancy in protein content exists with the separated forms of cytochrome P-450. Purified cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ have specific contents (nanomoles per mg protein) of 12 to 17 when protein is estimated by the method of Lowry *et al.* (41) and 16 to 21 when protein is determined by their amino acid compositions.

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RESULTS AND DISCUSSION

Separation of Highly Purified Forms of Cytochrome P-450—Warner and Neims (42) have used ion exchange column chromatography at room temperature in the presence of sodium cholate and Emulgen 911 to obtain different partially purified fractions of cytochrome P-450 from rat liver microsomes. Our cytochrome P-450 preparations are first partially purified to a specific content of 9 to 12 nmol/mg of protein (40), then treated with detergents and applied to a DEAE-cellulose column. Fig. 1 shows the elution profile of cytochrome P-450 from a DEAE-cellulose column in the presence of sodium cholate and Emulgen 911 when the chromatographic procedure is performed at room temperature. Highly purified forms of cytochrome P-450 are separated by this method when the preparations are isolated from Aroclor 1254-, PB-, or 3-MC-treated rats. However, this protocol does not separate different hemeproteins if conducted at an earlier step in the purification procedure.

No cytochrome P-450 elutes when the column is washed with the equilibration buffer. Cytochrome P-450$_c$ elutes from the column as a small peak with the start of the salt gradient. The specific content of this hemeprotein is 12 to 14 nmol/mg of protein when isolated from Aroclor 1254-, PB-, or 3-MC-treated rats. Although cytochrome P450$_c$ from Aroclor 1254- or 3-MC-treated rats shows only one protein staining band on SDS-polyacrylamide gels, the hemeprotein from PB-treated rats does contain other minor protein bands (Fig. 2). These contaminants are not removed if cytochrome P-450$_c$ is rechromatographed on DEAE-cellulose.

Cytochrome P-450$_b$ is the next hemeprotein to elute from the column with increasing salt concentration. The front of this major peak, shown in Fig. 1, is pooled from preparations isolated from Aroclor 1254-treated rats. Almost the entire peak obtained from PB-treated rats contains only cytochrome P-450$_b$. The peak which elutes at higher ionic strength from Aroclor 1254- or 3-MC-treated rat preparations contains cytochrome P-450$_c$. The specific content of the cytochrome P-450$_b$ or P-450, is 16 to 17 nmol/mg of protein. The hemeprotein from PB- and 3-MC-treated rats elute at the same salt concentration as the corresponding forms of cytochrome P-450 from Aroclor 1254-treated rats.

Immunoaffinity column chromatography, at room temperature in the presence of detergents, can be used to obtain additional amounts of cytochrome P-450. Columns were prepared from the antibodies to multiple forms of purified cytochrome P-450 from PB- or 3-MC-treated rats (31, 32) bound to Sepharose 4B. If a mixture of cytochromes P-450$_b$ and P-450$_c$ was applied to an anti-PB-450 column, the cytochrome P-450$_c$ passed through the column and the cytochrome P-450$_b$ would be retained. The opposite elution pattern would be observed if the mixture were applied to an anti-MC-P450 column, *i.e.* the cytochrome P-450$_b$ was not retained but the cytochrome P-450$_c$ bound to the column. The hemeprotein which passed through the column remained catalytically active; however, the cytochrome P-450 retained by the column could only be recovered as an inactive form since 4 M potassium thiocyanate was used to elute the bound hemeprotein.

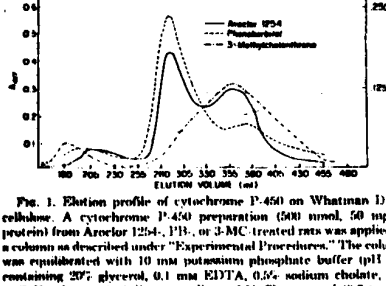

FIG. 1. Elution profile of cytochrome P-450 on Whatman DE52 cellulose. A cytochrome P-450 preparation (500 nmol, 50 mg of protein) from Aroclor 1254-, PB-, or 3-MC-treated rats was applied to a column as described under "Experimental Procedures." The column was equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.5% sodium cholate, and 0.2% Emulgen 911. A linear gradient of NaCl was used (0.0 to 0.25 M) in the same buffer mixture. The entire chromatographic procedure was performed at *room temperature*.



FIG. 2. SDS-polyacrylamide slab gel of cytochrome P-450$_b$, P-450$_c$, and P-450, from Aroclor 1254-, PB-, and 3-MC-treated rats. The different purified hemeproteins were subjected to electrophoresis as described by Laemmli (44). *Well A* contained standards with molecular weights of 58,000, 53,000, 45,000, and 41,000 (top to bottom). *Wells B, F,* and *I* each contained 1.5 μg of the purified preparations from Aroclor 1254-, PB-, and 3-MC-treated rats, respectively, from which the purified forms were isolated. *Wells C, D, E* contained 0.3 μg of cytochrome P-450$_b$, P-450$_c$, and P-450, respectively, from Aroclor 1254-treated rats. *Wells G* and *H* contained 0.3 μg of cytochrome P-450$_b$ (*G*) and P-450$_c$ (*H*) from PB-treated rats. *Wells J* and *K* contained cytochrome P-450$_b$ (*J*) and P-450, (*K*) from 3-MC-treated rats. *Well L* contained a mixture (0.3 μg each) of the cytochromes P-450, from the treated rats. *Well M* contained 0.3 μg of each of the cytochromes P-450$_b$ and *Well N* 0.3 μg of each of the cytochromes P-450$_c$.

Warner and Neims (42) reported that, by Sephadex gel filtration at room temperature, the microsomal fractions obtained from DEAE-cellulose column chromatography had molecular weights of approximately 50,000. When highly purified cytochrome P-450, was subjected to gel filtration at room temperature in the presence of sodium cholate and Emulgen 911, it was excluded from the gel. Therefore, this hemeprotein preparation has a molecular weight much greater than 50,000.

SDS-Polyacrylamide Gel Electrophoresis—Fig. 2 shows an SDS-polyacrylamide gel containing molecular weight standards, the partially purified cytochrome P-450 preparations applied to the DEAE-cellulose columns, and the subsequently separated forms of cytochrome P-450. The preparation isolated from Aroclor 1254-treated rats (*Well B*) contains all the protein-staining bands present in the preparations from PB- and 3-MC-treated rats (*Wells F* and *I*) as was previously reported (29). Since the preparations applied to the column have specific contents of 9 to 12 nmol/mg of protein, indicating lack of homogeneity, some of the bands observed may be proteins which are not cytochrome P-450. The starting prep-

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sections have a protein-staining band with a minimum molecular weight between cytochrome P-450$_b$ and P-450$_c$, and a band which appears just below cytochrome P-450$_b$. The proteins corresponding to these bands elute from the DEAE-cellulose column at intermediate salt concentrations to cytochrome P-450$_b$ and P-450$_c$. Residual amounts of these proteins also elute subsequent to cytochrome P-450$_c$. Preliminary experiments have indicated that the protein-staining band on SDS-polyacrylamide gels located between cytochrome P-450$_b$ and P-450$_c$ is another form of cytochrome P-450. It has not been possible, however, to reproducibly obtain this protein free of cytochrome P-450$_b$ or P-450$_c$.

Approximately the same amount of cytochrome P-450$_b$ is present in the starting preparations from Aroclor 1254-, PB-, or 3-MC-treated rats (*Wells B, F, I*). Only one protein-staining band is observed when the cytochrome P-450$_c$ is isolated from Aroclor 1254- or 3-MC-treated rats (*Wells C* and *J*) illustrating the purity of these samples. However, the electrophoretic profile of this hemeprotein obtained from PB-treated rats (*Well G*) does contain other minor protein-staining bands.

Only one protein-staining band is observed when either cytochrome P-450$_b$ (*Wells D* and *H*) or cytochrome P-450$_c$ (*Wells E* and *K*) are electrophoresed, demonstrating the purity of these hemeproteins. Cytochrome P-450$_b$ is a predominant hemeprotein in the preparation from Aroclor 1254-treated rats (*Well B*) and the major species of cytochrome P-450 in the preparation from PB-treated rats (*Well F*). Cytochrome P-450$_c$ is the major hemeprotein in the preparation from 3-MC-treated rats and a predominant species in the preparation from Aroclor 1254-treated rats.

The minimum molecular weight (±500) of cytochrome P-450$_b$ is 49,000, P-450$_c$ is 52,000, and P-450$_d$ is 56,000. Therefore, cytochromes P-450$_b$, P-450$_c$, and P-450$_d$ are distinct hemeproteins as judged by their mobility on SDS-polyacrylamide gels. Each hemeprotein (cytochrome P-450$_b$, P-450$_c$, or P-450$_d$) isolated from rats treated with different inducers is identical in minimum molecular weight, as shown in Wells L, M, and N.

Fig. 3 shows the electrophoretic profile of molecular weight standards, cytochromes P-450$_b$, P-450$_c$, and P-450$_d$, and liver microsomal preparations from untreated rats, or rats treated with PB, 3-MC, or Aroclor 1254 on SDS-polyacrylamide gels. A mixture of the three highly purified cytochromes from Aroclor 1254-treated rats is in Wells B and K. The rest of the wells contain microsomal preparations from untreated rats (*C, D*) and rats treated with PB (*E, F*), 3-MC (*G, H*), or Aroclor 1254 (*I, J*). Caution must be taken when interpreting the electrophoretic profiles of microsomal preparations since microsomal proteins other than cytochromes P-450 are known to have minimum molecular weights in the 45,000 to 60,000 region (54–56). Fig. 3 shows that cytochrome P-450$_c$ has the same minimum molecular weight as a protein-staining band present in all the microsomal preparations. This band is not dramatically increased in microsomes from rats treated with any of the inducing agents. The protein-staining band located just above the band corresponding in mobility to cytochrome P-450$_b$, has a minimum molecular weight identical with purified epoxide hydrase (54). If purified epoxide hydrase and cytochrome P-450$_c$ are mixed and applied to an SDS-polyacrylamide gel, two protein bands are observed. The band with the lower minimum molecular weight is cytochrome P-450$_c$. PB and Aroclor 1254 significantly induce a protein(s) corresponding in minimum molecular weight to cytochrome P-450$_b$. The protein-staining band with the same migration as cytochrome P-450$_c$ is markedly increased in the microsomal preparations from Aroclor 1254- and 3-MC-treated rats but not following PB treatment. It is of interest to note that PB

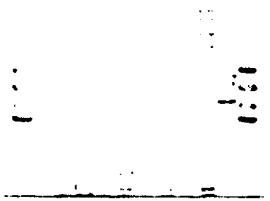

FIG. 3. SDS-polyacrylamide slab gel of cytochrome P-450$_b$, P-450$_c$, and P-450$_d$ from Aroclor 1254-treated rats, and microsomes from rats treated with Aroclor 1254, PB, or 3-MC. All the samples were prepared and electrophoresed by the method of Laemmli (44). *Wells A* and *L* contained standards with molecular weights of 58,000, 53,000, 45,000, and 41,000 (top to bottom). *Wells B* and *K* contained cytochrome P-450$_b$, P-450$_c$, and P-450$_d$ from Aroclor 1254-treated rats (0.4 μg each). *Wells C* and *D* contained 1 and 2 μg, respectively, of microsomes from untreated rats. Microsomes from PB-treated rats are in *Well E* (1 μg) and *F* (2 μg). *Wells G* and *H* contained 1 and 2 μg, respectively, of microsomes from 3-MC-treated rats. Microsomes from Aroclor 1254-treated rats are in *Well I* (1 μg) and *J* (2 μg).

treatment does result in the increase of a protein-staining band with a minimum molecular weight slightly less than cytochrome P-450$_b$.

The administration of a single large dose of Aroclor 1254 to rats results in an increase in all the protein-staining bands in the 45,000 to 60,000 region, as previously reported (28, 29). To determine whether any of these protein bands were the result of proteolysis during the preparation of microsomes, freshly prepared phenylmethylsulfonyl fluoride (in ethanol) was added to the buffer containing livers from Aroclor 1254-treated rats at a final concentration of 0.4 mM. This protease inhibitor has been shown to react with serine residues in the active site of many proteases (57). The livers were homogenized, and microsomes were prepared (40) in the presence of the protease inhibitor (0.1 mM). Cytochrome P-450 was purified (29, 40) from this microsomal preparation containing the protease inhibitor. At each step in the purification procedure, a final concentration of 0.1 mM phenylmethylsulfonyl fluoride was added to the preparation, the preparation was passed through a nucleopore filter (0.4 μm), and all dialysis tubing was autoclaved to prevent bacterial contamination. The SDS-polyacrylamide gel electrophoretic profile of the microsomes and the preparation at each step in the purification procedure was identical with a preparation not treated with the protease inhibitor. Therefore, it does not appear that significant digestion by proteases containing serine in the active site occurs during the preparation of microsomes or purification of cytochrome P-450 by this method.

Spectral Properties—Fig. 4 shows the CO-reduced difference spectra of cytochrome P-450$_b$, P-450$_c$, and P-450$_d$ from Aroclor 1254-treated rats. The spectra of the forms isolated from PB- or 3-MC-treated rats are not shown since the spectral characteristics are identical with the corresponding forms from Aroclor 1254-treated rats. The CO-reduced difference spectral maxima of the three hemeproteins are at different wavelengths. The peak for cytochrome P-450$_b$ is at 452 nm, cytochrome P-450$_c$ is at 450 nm, and cytochrome P-450$_d$ is at 447 nm. The absence of a shoulder at 420 nm indicates

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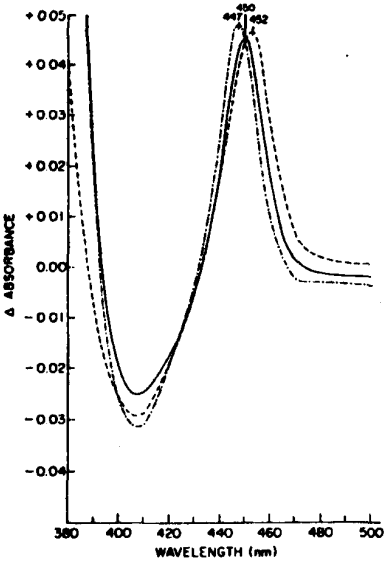

FIG. 4. CO-reduced difference spectra of cytochrome P-450$_n$, P-450$_b$, and P-450$_c$ from Aroclor 1254-treated rats. The spectra were recorded after bubbling CO in the sample cuvette and then adding sodium dithionite to both the sample and reference cuvettes. The concentration of each hemeprotein was 0.5 nmol/ml. Cytochrome P-450$_c$ was in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol. The other cytochromes were in the same buffer mixture which also contained 0.1 mM EDTA, 0.5% sodium cholate, and 0.2% Emulgen 911. The CO-reduced difference spectral peak for cytochrome P-450$_n$ is at 452 nm, P-450$_b$ is at 450 nm, and P-450$_c$ is at 447 nm.

that none of these cytochromes contain significant amounts of cytochrome P-420. The spectrum of cytochrome P-450$_n$ is recorded in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol. The spectra of the other two hemeproteins are recorded in the same buffer mixture which also contains 0.5% sodium cholate and 0.2% Emulgen 911. With time, more cytochrome P-420 develops if the spectra of cytochrome P-450$_b$ or P-450$_c$ are recorded in the absence of these detergents. The presence of both the ionic (sodium cholate) and the nonionic (Emulgen 911) detergents are required for the protection of these hemeproteins against conversion to cytochrome P-420. In contrast, cytochrome P-450$_n$ is less stable in the presence of sodium cholate and Emulgen 911 than in the absence of these detergents. Other investigators (58, 59) have reported that Emulgen 913 protects certain preparations of microsomal and mitochondrial cytochrome P-450 from conversion to its inactive form, cytochrome P-420.

Fig. 5 shows the absolute spectra of cytochrome P-450$_n$, P-450$_b$, and P-450$_c$ from Aroclor 1254-treated rats. The concentrations of cytochrome P-450 used, as listed in the figure legend, were determined from the CO-reduced difference spectra. The oxidized spectra show that the Soret peaks for each form of oxidized cytochrome P-450 are at the same wavelength. The absence of a shoulder between 390 and 400 nm indicates that cytochrome P-450$_c$ (*Plate a*) and cytochrome P-450$_n$ (*Plate c*) are low spin ferric hemeproteins at room temperature. The absolute oxidized spectrum of cytochrome P-450$_b$ (*Plate b*) has a broader peak in the 417 nm region

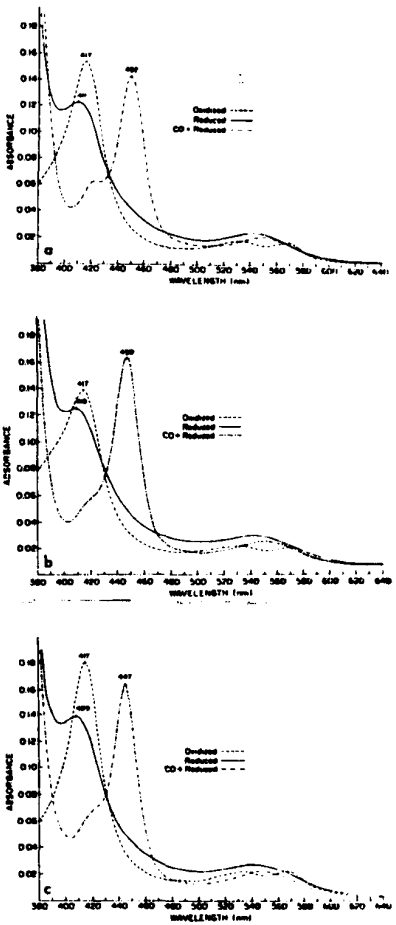

FIG. 5. Absolute spectra of cytochrome P-450$_n$, P-450$_b$, and P-450$_c$ from Aroclor 1254-treated rats. The oxidized, reduced, and reduced + CO spectra were recorded with the reference cuvette containing the same buffer mixture as the sample. *Plate a* shows the absolute spectra of cytochrome P-450$_c$ at 1.2 nmol/ml in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol. The absolute spectra of cytochrome P-450$_b$ (*Plate b*) and cytochrome P-450$_n$ (*Plate c*) were recorded at 1.4 nmol/ml in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.5% sodium cholate, and 0.2% Emulgen 911.

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whether the hemeprotein is obtained from Aroclor 1254- or PB-treated rats. Cytochrome P-450$_b$ may contain a high spin component although purified preparations of cytochrome P-450 from either PB- or Aroclor 1254-treated rats (29, 30, 40) are usually low spin ferric hemeproteins. The broader absorbance peak in the 417 nm region of the oxidized spectrum of cytochrome P-450$_b$ is not the result of the method used for removing detergent. If detergents are removed from this hemeprotein by calcium phosphate gel adsorption rather than treatment with XAD-2 beads, no change in the oxidized spectrum of cytochrome P-450$_b$ is observed.

When the hemeproteins are reduced by sodium dithionite, the Soret maxima shift downward. The reduced Soret peak of cytochromes P-450$_b$, P-450$_e$, and P-450 are at 409 to 411 nm. When reduced in the presence of CO, cytochromes P-450$_b$, P-450$_e$, and P-450 have spectral maxima at 452 nm, 450 nm and 447 nm, respectively.

Table I summarizes the spectral properties of cytochromes P-450$_b$, P-450$_e$, and P-450$_c$. The absorbance maxima for the three oxidized hemeproteins are identical; however, the extinction of cytochrome P-450$_b$ is lower than the other cytochromes. The reduced spectra of the hemeproteins are similar, although reduced cytochrome P-450$_b$ has a lower extinction than the other hemeproteins. The CO-reduced absolute spectral maxima are different for the three cytochromes but the extinctions are very similar. The CO-reduced absorption maxima of the three cytochromes indicate that they are different hemeproteins. The spectral properties of the cytochromes P-450 from Aroclor 1254-treated rats are identical to the corresponding forms from PB- or 3-MC-treated rats.

Catalytic Activity—Table II shows the catalytic activity of cytochromes P-450$_b$, P-450$_e$, and P-450, from Aroclor 1254-, PB-, and 3-MC-treated rats toward several substrates. Although cytochrome P-450, from Aroclor 1254- or 3-MC-treated rats metabolizes aniline most efficiently, all the forms of cytochrome P-450 catalyze the hydroxylation of aniline at comparable rates. Microsomes isolated from rats which were untreated or treated with PB- or 3-MC catalyze the hydroxylation of aniline similarly, with turnover numbers of 2.6 to 2.8 nmol of product formed/min/nmol of cytochrome P-450. The metabolism of this substrate per nmol of cytochrome P-450 increases 2- to 3-fold with purification and separation of these purified forms of cytochrome P-450.

A dramatic difference in the rate of metabolism of benzphetamine is observed among the different cytochromes P-450. The turnover numbers obtained when cytochromes P-450$_b$ are used to catalyze the reaction are 40 times greater than when cytochromes P-450, are used. The cytochromes P-450$_b$ demethylate benzphetamine at a slower rate than cytochromes P-450$_e$. The turnover numbers for the demethylation of benzphetamine catalyzed by cytochromes P-450$_b$ are approximately 20 times greater than the rate at which microsomes from PB-treated rats catalyze the metabolism of this substrate (60). Cytochrome P-450$_b$ from Aroclor 1254- or PB-treated rats catalyzes this reaction with approximately equal efficiency (Table II). To obtain the turnover numbers listed for this substrate, it was necessary to include sodium cholate in the reconstituted system. At a cytochrome P-450 concentration of 0.05 nmol, NADPH-cytochrome *c* reductase concentration of 750 units and phosphatidylcholine concentration of 10 μg/ml, maximum activity was obtained in the presence of 0.5 mg of sodium cholate. The turnover number for the demethylation of benzphetamine by cytochrome P-450$_b$ in the absence of sodium cholate, ranged from 40 to 50 nmol of product formed/min/nmol of cytochrome P-450. The presence of sodium cholate is also necessary to obtain the maximum rate of benzphetamine metabolism when cytochromes P-450$_e$ or P-450, are used to catalyze the reaction, although the stimulatory effect of cholate is not as dramatic. It is not necessary to include sodium cholate in the assays for the other substrates to obtain maximal activity.

As is apparent from Table II, cytochromes P-450, preferentially catalyze *O*-dealkylation of 7-ethoxycoumarin and hydroxylation of benzo[*a*]pyrene and zoxazolamine. Cytochrome P-450, from Aroclor 1254- or 3-MC-treated rats catalyzes the metabolism of benzo[*a*]pyrene with approximately equal efficiency, at a rate which is 14 times greater than the rate at which microsomes from 3-MC-treated rats catalyze the hydroxylation of this substrate (60). Cytochromes P-450$_e$ and P-450$_b$, from rats treated with any of the inducers, have very low catalytic activity toward benzo[*a*]pyrene.

7-Ethoxycoumarin is metabolized at comparable rates by cytochrome P-450, from Aroclor 1254- or 3-MC-treated rats. These rates are approximately 4 times greater than the rate at which microsomal preparations from rats treated with 3-MC catalyze the *O*-dealkylation of 7-ethoxycoumarin (60). Cytochromes P-450$_e$ isolated from rats treated with Aroclor 1254, PB, or 3-MC catalyze the metabolism of 7-ethoxycoumarin very poorly. Cytochrome P-450$_b$ from Aroclor 1254- or

TABLE I

Spectral properties of purified forms of rat liver cytochrome P-450

The various hemeproteins were isolated as described under "Experimental Procedures." The heme content of the preparations was determined by the method of Falk (45). The wavelength maxima were determined from the absolute spectra at hemeprotein concentrations of 1.2 to 1.4 nmol/ml.

Source of microsomes	Cytochrome	Oxidized		Reduced		CO reduced	
		λ_{max}	ϵ	λ_{max}	ϵ	λ_{max}	ϵ
		nm	$\frac{mM}{cm}^{-1}$	nm	$\frac{mM}{cm}^{-1}$	nm	$\frac{mM}{cm}^{-1}$
Aroclor 1254, PB, or 3-MC	P-450$_b$	417	125	411	100	452	116
		533	14	540	21	547	17
		567	14				
Aroclor 1254 or PB	P-450$_e$	417	98	410	85	450	114
		533	13	540	20	548	17
		567	12				
Aroclor 1254 or 3-MC	P-450,	417	127	409	98	447	117
		533	14	540	20	546	17
		567	16				

TABLE II

Catalytic activity of various purified forms of rat liver cytochrome P-450

The cytochromes P-450 were purified as described under "Experimental Procedures." The catalytic activity is expressed as nanomoles of product formed/min/nmol of cytochrome. Metabolism was measured in the presence of saturating amounts of NADPH-cytochrome *c* reductase and phosphatidylcholine.

Substrate	Aroclor 1254			Phenobarbital		3-Methylcholanthrene	
	P-450$_b$	P-450$_e$	P-450,	P-450$_b$	P-450$_e$	P-450,	P-450
Aniline	5.1	6.7	9.3	5.1	7.7	6.3	10.6
Benzphetamine	3.2	176.5	5.1	2.2	216.6	2.6	5.0
Benzo[*a*]pyrene	0.1	0.5	19.2	0.04	0.2	0.3	24.5
7-Ethoxycoumarin	0.6	12.1	48.5	0.2	13.9	1.1	67.5
Zoxazolamine	0.9	2.4	27.9	0.5	2.2	2.0	29.7
Testosterone							
7α-OH	4.4	≤0.10	≤0.10	4.1	≤0.10	5.4	≤0.10
16α-OH	≤0.05	2.4	≤0.05	≤0.05	1.9	≤0.05	≤0.05
6β-OH	≤0.10	≤0.10	0.35	≤0.10	≤0.10	≤0.10	0.36

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PB-treated rats metabolizes 7-ethoxycoumarin with turnover numbers of 12 to 14 nmol of product formed/min/nmol of P-450. These turnover numbers are approximately 5 times the value obtained with microsomal preparations from PB-treated rats (60).

Table II shows that cytochromes P-450, catalyze the hydroxylation of zoxazolamine more efficiently than cytochromes P-450, or P-450$_b$. The turnover number obtained when the reaction is catalyzed by cytochrome P-450, from Aroclor 1254-treated rats (27.9 nmol of product formed/min/nmol of cytochrome P-450) is similar to that obtained when cytochrome P-450, is isolated from 3-MC-treated rats (29.7 nmol of product formed/min/nmol of cytochrome P-450). Hydroxylation of zoxazolamine by cytochrome P-450, occurs at a rate approximately 5 times greater than the rate at which this substrate is metabolized by microsomal preparations from 3-MC-treated rats (49). Cytochromes P-450, and P-450$_b$ catalyze the metabolism of zoxazolamine at a very low rate relative to the rate of hydroxylation catalyzed by cytochrome P-450. It was necessary, however, to preincubate the reconstituted system (cytochrome P-450$_c$, NADPH-cytochrome *c* reductase, phospholipid) with zoxazolamine in the absence of NADPH to obtain linear conditions of metabolism. With this substrate, the reaction was started by the addition of NADPH. The optimal preincubation time was determined to be 10 min at 37°C. Preincubation of cytochrome P-450$_c$ or P-450$_b$ with the substrate did not change the turnover number obtained.

The hydroxylation of testosterone at the 7α, 6β, and 16α positions catalyzed by the various forms of cytochrome P-450 is shown in Table II. The hydroxylation of testosterone at the 7α position is the only metabolic pathway that we observed to be catalyzed by cytochrome P-450, but not by the other cytochromes P-450. The turnover number for hydroxylation in the 7α position ranges between 4 to 6 nmol of product formed/min/nmol of cytochrome P-450 whether cytochrome P-450, is obtained from Aroclor 1254-, PB-, or 3-MC-treated rats. This value is dramatically increased (10-fold) over the turnover numbers obtained for the hydroxylation of testosterone at the 7α position by microsomal preparations. Microsomal preparations from untreated rats or rats treated with PB, 3-MC, or Aroclor 1254 catalyze the hydroxylation of testosterone at the 7α position comparably, with turnover numbers of 0.45 to 0.64 nmol of product formed/min/nmol of cytochrome P-450.

Cytochromes P-450, preferentially catalyze the hydroxylation of testosterone at the 16α position. Cytochrome P-450$_b$ from either Aroclor 1254- or PB-treated rats catalyze the hydroxylation of testosterone at the 16α position at comparable rates (1.9 to 2.4 nmol of product formed/min/nmol of cytochrome P-450). The turnover numbers for the hydroxylation of testosterone at the 16α position catalyzed by cytochrome P-450$_b$ are approximately 2- to 3-fold greater than the rate at which microsomes from PB-treated rats metabolize testosterone at this position. There is negligible metabolism of testosterone at the 16α position catalyzed by either cytochrome P-450, or P-450$_c$.

Although the hydroxylation of testosterone at the 6β position is greatest when catalyzed by cytochrome P-450$_c$, this observation must be interpreted with caution. Cytochrome P-450, catalyzes the 6β-hydroxylation of testosterone at a rate which is only 10 to 20% of that obtained with liver microsomes. It is possible that the major form of cytochrome P-450 responsible for this hydroxylation reaction is lost during purification.

In summary, Table II shows that the substrate specificities of cytochromes P-450$_c$, P-450$_b$, and P-450, are very different. Cytochrome P-450$_c$ preferentially catalyzes the hydroxylation of testosterone at the 7α position. The hydroxylation of testosterone at the 16α position and the demethylation of benzphetamine are catalyzed most efficiently by cytochrome P-450$_b$. Cytochrome P-450, has very high catalytic activity for the metabolism of benzo[*a*]pyrene, 7-ethoxycoumarin, and zoxazolamine. Aniline is metabolized at comparable rates by all three isolated forms of cytochrome P-450. The substrate specificities of the cytochromes P-450$_c$, P-450$_b$, and P-450, isolated from Aroclor 1254-treated rats are the same as the specificities of the corresponding hemeproteins from PB- and 3-MC-treated rats.

Reactivity with Antibodies—Antibodies were produced in sheep against cytochromes P-450$_c$, P-450$_b$, and P-450, from Aroclor 1254-treated rats by a previously described method (31, 32). Fig. 6 shows the reactivity of these antisera with the various forms of cytochrome P-450 in Ouchterlony double diffusion plates. In *Plate A*, antisera to cytochrome P-450$_c$ from Aroclor 1254-treated rats are in Wells 1 and 8. Wells 2, 6, and 7 contain cytochrome P-450$_c$ from Aroclor 1254-, 3-MC-, and PB-treated rats, respectively, at the same heme-protein concentration (1.7 μM). There is a single immunoprecipitin band observed when each cytochrome P-450$_c$ reacts with the antibody. The immunoprecipitin bands produced by the reaction of the antibody with cytochrome P-450$_c$ isolated from rats treated with different inducers form a line of identity. The absence of spurs at the intersections of these lines indicates that the cytochromes P-450$_c$ have identical antigenic sites. Neither cytochrome P-450$_b$ (*Wells 4* and *5*) nor cytochrome P-450, (*Wells 3, 9,* and *10*) cross-react with the antibody to cytochrome P-450$_c$. Cytochromes P-450$_b$ were present at 5.0 μM, and cytochromes P-450, were present at 4.2 μM. Therefore, the heterologous antigens appear to be immunologically different from cytochrome P-450$_c$.

In Fig. 6, Plate B, antisera to cytochrome P-450$_b$ from Aroclor 1254-treated rats are in Wells 1 and 8. Wells 2 and 7 contain cytochrome P-450$_b$ (5.0 μM) from Aroclor 1254- and PB-treated rats, respectively. An intense immunoprecipitin band is observed when either cytochrome P-450$_b$ reacts with the antibody. There is a line of identity between these immunoprecipitin bands indicating that cytochromes P-450$_b$ from Aroclor 1254- and PB-treated rats are antigenically identical. Cytochrome P-450$_c$ isolated from rats treated with different inducers (*Wells 3, 4, 9, 10*) does not cross-react with the antibody to cytochrome P-450$_b$. The antibody to cytochrome P-450$_b$ from Aroclor 1254-treated rats does not recognize cytochrome P-450$_c$ from either Aroclor 1254- or 3-MC-treated rats.

Wells 1 and 8 of Fig. 6, *Plate C*, contain antisera to cytochrome P-450, from Aroclor 1254-treated rats, and Well 7 contains the antigen. The titer of antibody produced against cytochrome P-450, is not as high as the titer of the other antibodies. However, it is apparent in *Plate C* that cytochrome P-450, isolated from rats treated with Aroclor 1254 is antigenically identical with cytochrome P-450, obtained from 3-MC-treated rats (*Well 6*). Cytochrome P-450$_c$ (*Wells 4, 5, 9, 10*) and cytochrome P-450$_b$ (*Wells 2* and *3*) from rats treated with various inducers do not cross-react with the antibody to cytochrome P-450$_c$, indicating that they have different antigenic sites.

It is evident from these results that cytochrome P-450$_c$, P-450$_b$, and P-450, have distinct immunologic properties. However, the three hemeproteins isolated from Aroclor 1254-treated rats are antigenically identical with the corresponding cytochromes P-450 from PB- and 3-MC-treated rats.

Peptide Mapping—Figs. 7 to 10 show the SDS-gel electrophoretic profiles of the various forms of cytochrome P-450

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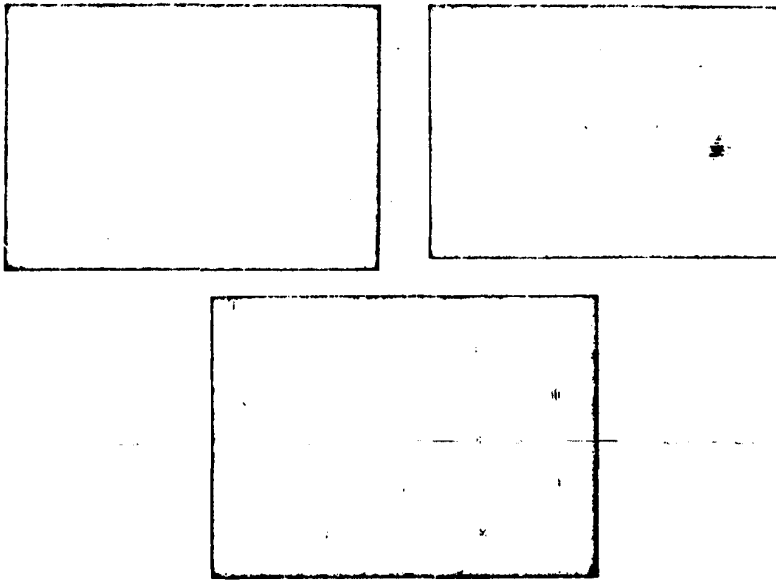

FIG. 6. Ouchterlony double diffusion analyses of cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ from Aroclor 1254-, PB-, and 3-MC-treated rats. *Plate A, Wells 1* and *8* each contained 12 μl of antisera to cytochrome P-450$_c$ from Aroclor 1254-treated rats. Cytochrome P-450$_c$ (1.7 μM) from Aroclor 1254-treated rats was in *Well 2*, from 3-MC-treated rats in *Well 6* and from PB-treated rats in *Well 7*. *Wells 4* and *5* each contained cytochrome P-450$_c$ at a concentration of 5.0 μM from PB- and Aroclor 1254-treated rats, respectively. Cytochrome P-450$_b$ (4.2 μM) was in *Wells 3, 9*, and *10*, from Aroclor 1254-treated rats (*Wells 3*, and *9*) and 3-MC-treated rats (*Well 10*). *Wells 11* and *12* contained phosphate-buffered saline at pH 7.4. *Plate B, Wells 1* and *8* and 12 μl of antisera to cytochrome P-450$_a$ from Aroclor 1254-treated rats. *Wells 2* and *7* contained cytochrome P-450$_a$ (5.0 μM) from Aroclor 1254- and PB-treated rats, respectively. Cytochrome P-450$_a$ (1.7 μM) from PB-treated rats is in *Well 3*, and from Aroclor 1254-treated rats in *Well 4* and from 3-MC-treated rats in *Well 10*. *Wells 5* and *6* each contained cytochrome P-450$_b$ at a concentration of 4.2 μM from 3-MC-treated rats (*Well 5*) and Aroclor 1254-treated rats (*Well 6*). *Wells 11* and *12* contained phosphate-buffered saline at pH 7.4. *Plate C, Wells 1* and *8* each contained 12 μl of antisera to cytochrome P-450$_b$ from Aroclor 1254-treated rats. *Wells 6* and *7* contained cytochrome P-450$_b$ (2.0 μM) from 3-MC-treated rats (*Well 6*) and Aroclor 1254-treated rats (*Well 7*). Cytochrome P-450$_a$ (1.7 μM) from PB-treated rats was in *Wells 4* and *9*, from Aroclor 1254-treated rats in *Well 5* and from 3-MC-treated rats in *Well 10*. *Wells 2* and *3* each contained cytochrome P-450$_c$ at a concentration of 5.0 μM from Aroclor 1254- and PB-treated rats, respectively. *Wells 11* and *12* contained phosphate-buffered saline at pH 7.4.

after limited proteolysis in the presence of SDS according to the method of Cleveland *et al.* (39). The effects of chymotrypsin, which cleaves adjacent to aromatic amino acids, and *S. aureus* V8 protease, which cleaves adjacent to glutamic acid and aspartic acid residues, on the SDS-gel profile of the cytochromes P-450 are shown in Figs. 7 and 8. Figs. 9 and 10 show the peptide fragments generated by treatment of the various cytochromes P-450 with these proteases for different time periods. To visualize the peptides produced by proteolysis, 15% acrylamide gels were used. Cytochrome P-450$_c$, with a minimum molecular weight in the 50,000 region, is the intense protein-staining band near the top of the gel. If either protease is applied to the SDS-gel at the concentration used for limited proteolysis of the hemeproteins, no protein staining bands are observed. Cytochrome P-450$_b$, isolated from PB-treated rats was not included in these studies since, as shown in Fig. 2, this preparation contains other minor protein-staining bands on SDS-polyacrylamide gels.

Fig. 7 illustrates the electrophoretic profile of the various cytochromes P-450 after treatment with chymotrypsin (0.04 mg of protease/mg of protein) for 10 min at 37°C in the presence of SDS. Wells A, C, E, G, I, and K contain the untreated cytochromes, and Wells B, D, F, H, J, and L contain the proteolytic digests. Limited proteolysis of cytochrome P-450$_a$, whether isolated from Aroclor 1254 (*Well B*)- or 3-MC (*Well D*)-treated rats yield identical cleavage products. The cytochrome P-450$_b$ digests from rats treated with Aroclor 1254 (*Well F*) and PB (*Well H*) cannot be distinguished from each other. The peptides generated by chymotrypsin cleavage of cytochrome P-450$_c$ are the same whether this hemoprotein is obtained from Aroclor 1254- or 3-MC-treated rats (*Wells J* and *L*). Fig. 7 also clearly shows that the peptides generated are very different, depending on whether cytochrome P-450$_a$, P-450$_b$, or P-450$_c$ is used as the substrate. There is also a significant difference in the sensitivity of the three forms of cytochrome P-450 to proteolysis by chymotrypsin in the presence of SDS. Cytochrome P-450$_c$ is more susceptible to digestion by chymotrypsin than are either cytochromes P-450$_a$ or P-450$_b$, as judged by the loss of the original protein staining band and the appearance of protein-staining bands at lower

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FIG. 7. Peptide mapping of cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ from Aroclor 1254-, PB-, and 3-MC-treated rats on an SDS-polyacrylamide gel after treatment with chymotrypsin. Each cytochrome P-450 was treated with chymotrypsin (0.04 mg/mg of protein) for 10 min at 37°C in the presence of SDS as described (39). Twenty-five microliters (4.5 μg of protein) of each sample was applied to the gel. *Wells A, C, E, G, I,* and *K* contained the hemeproteins with no protease treatment. *Wells B, D, F, H, J,* and *L* contained the cytochromes after limited proteolysis. Cytochrome P-450$_c$ from Aroclor 1254-treated rats was in *Wells A* and *B*, from 3-MC-treated rats in *Wells C* and *D*. *Wells E, F, G,* and *H* contained cytochrome P-450$_b$ from Aroclor 1254-treated rats (*E, F*) and PB-treated rats (*G, H*). Cytochrome P-450$_a$ from Aroclor 1254-treated rats was in *Wells I* and *J* and from 3-MC-treated rats in *Wells K* and *L*.

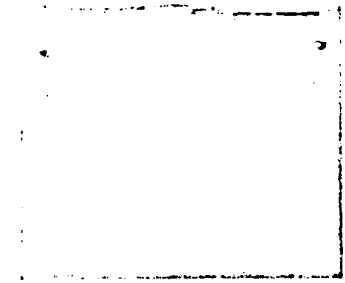

FIG. 8. SDS-polyacrylamide slab gel containing cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ from Aroclor 1254-, PB-, and 3-MC-treated rats after limited proteolysis by *S. aureus* V8 protease. The hemeproteins were treated with the protease (0.05 mg/mg of protein) for 10 min at 37°C in the presence of SDS (39). Each well contained 4.5 μg of protein. *Wells A, C, E, G, I,* and *K* contained the untreated hemeproteins. *Wells B, D, F, H, J,* and *L* contained the cytochromes after treatment with the protease. Cytochrome P-450$_c$ from Aroclor 1254-treated rats was in *Wells A* and *B*, from 3-MC-treated rats in *Wells C* and *D*. Cytochrome P-450$_b$ was in *Wells E, F, G, H*: from Aroclor 1254-treated rats (*E, F*) and PB-treated rats (*G, H*). Cytochrome P-450$_a$ from Aroclor 1254-treated rats was in *Wells I* and *J* and from 3-MC-treated rats in *Wells K* and *L*.

molecular weight regions.

The SDS-gel electrophoretic profile after proteolysis of the various cytochromes P-450 by *S. aureus* V8 protease is shown in Fig. 8. Each hemeprotein had been treated with the protease (0.05 mg of protease/mg of protein) for 10 min at 37°C in the presence of SDS. The peptide digests of cytochromes P-450$_b$ (*Wells B* and *D*) are different than the digests of cytochromes P-450$_a$ (*Wells F* and *H*) or cytochromes P-450$_c$ (*Wells J* and *L*). However, the cleavage products of the hemeproteins isolated from Aroclor 1254-treated rats are identical with the products of the corresponding hemeproteins from PB- and 3-MC-treated rats. In contrast to the results presented in Fig. 7, all the forms of cytochrome P-450 studied appear equally susceptible to digestion by the *S. aureus* V8 protease. It is unlikely that the differences in the peptide digests of cytochromes P-450$_a$, P-450$_b$, and P-450$_c$ are due to conformational differences in the native hemeproteins since the cytochromes are treated with SDS and heated prior to proteolysis.

Figs. 9 and 10 show the SDS-gel electrophoretic profiles of cytochromes P-450$_a$, P-450$_b$, and P-450$_c$ after treatment with the proteases at 37°C for different time periods. Only one cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ are illustrated, since the corresponding hemeproteins from rats treated with different inducers yield identical fragments. At each time period, with either chymotrypsin (Fig. 9) or *S. aureus* V8 protease (Fig. 10), the digests of cytochrome P-450$_a$ are different than

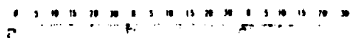

FIG. 9. Time course of proteolysis by chymotrypsin of cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ from Aroclor 1254-treated rats. Each hemeprotein (0.25 mg of protein/ml) was treated with chymotrypsin (0.04 mg/mg of protein) at 37°C for the times indicated (min) on the figure. Each well contained 4.5 μg of the hemeprotein. From left to right, the cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ series were applied. The method of Cleveland *et al.* (39) was used for the entire procedure.

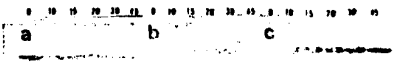

FIG. 10. Time course of the digestion of cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ from Aroclor 1254-treated rats by *S. aureus* V8 protease. Each cytochrome (0.25 mg of protein/ml) was treated with the protease (0.05 mg/mg of protein) as described (39) at 37°C for the time (*min*) indicated. Each well contained 4.5 μg of the cytochrome. From *left* to *right*, the cytochrome P-450$_a$, P-450$_b$, and P-450$_c$ series were applied to the gel.

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the digests of cytochromes P-450, or P-450, . With each protease treatment, increasing time of incubation yields a loss in the protein-staining band at a minimum molecular weight of approximately 50,000 with increasing protein staining at lower molecular weights. However, many of the peptide fragments are stable throughout the time periods studied. The SDS-gel profile after proteolysis of the cytochromes by chymotrypsin or *S. aureus* V8 protease is extremely reproducible. These studies provide additional evidence that cytochrome P-450, P-450, , and P-450, are different hemeproteins.

In summary, the present report has described the separation of three different forms of highly purified cytochrome P-450 from rats treated with the polychlorinated biphenyl mixture, Aroclor 1254. The corresponding hemeproteins have also been obtained from PB- and 3-MC-treated rats. Several approaches have been utilized to characterize cytochrome P-450, , P-450, , and P-450, . These hemeproteins differ in minimum molecular weight on SDS-gels, spectral properties, substrate specificity, and in immunological characteristics. Furthermore, different peptide fragments are generated when these cytochromes are subjected to limited proteolysis in the presence of SDS. By these five criteria, the forms of cytochrome P-450 isolated from rats treated with Aroclor 1254 are different from one another and identical with the corresponding hemeproteins from PB- or 3-MC-treated rats.

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Note Added in Proof—After submission of this manuscript, a report by Guengerich (61) has described differences in electrophoretic profiles of proteolytic digests of several rat liver microsomal cytochromes P-450. The peptide mapping procedure of Cleveland *et al.* (29), used in our studies, was also employed by Guengerich.

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