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Properties of Purified Liver Microsomal Cytochrome P450 from Rats Treated with the Polychlorinated Biphenyl Mixture Aroclor 1254

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SUMMARY

RYAN, DENE E., THOMAS, PAUL E. & LEVIN, WAYNE (1977) Properties of purified liver microsomal cytochrome P450 from rats treated with the polychlorinated biphenyl mixture Aroclor 1254. *Mol. Pharmacol.*, 13, 521-532.

Hepatic microsomal cytochrome P450 has been purified from rats treated with Aroclor 1254 to a specific content of greater than 19.0 nmoles/mg of protein and compared with purified cytochromes P450 from phenobarbital- and 3-methylcholanthrene-treated rats. Cytochrome P450 from Aroclor 1254-treated rats cannot be distinguished from a mixture of hemeproteins from phenobarbital- and 3-methylcholanthrene-treated rats by its spectral and catalytic properties or its electrophoretic mobility on sodium dodecyl sulfate–polyacrylamide gels. The cross-reactivity of cytochrome P450 from Aroclor 1254-treated rats with antibodies prepared against the hemeproteins from phenobarbital- or 3-methylcholanthrene-treated rats has also been examined. On Ouchterlony double-diffusion plates the cross-reaction of the Aroclor 1254-inducible cytochrome P450 with either antibody is indistinguishable from the reaction of a mixture of hemeproteins from phenobarbital- and 3-methylcholanthrene-treated rats. When the antibody to cytochrome P450 from 3-methylcholanthrene-treated rats is used to inhibit metabolism, no differences are observed between cytochrome P450 from Aroclor 1254-treated rats and a mixture of hemeproteins from phenobarbital- and 3-methylcholanthrene-treated rats. However, the effects of the antibody to cytochrome P450 from phenobarbital-treated rats on the metabolism of 7-ethoxycoumarin and benzo[*a*]pyrene suggest that the hemeprotein preparation from Aroclor 1254-treated rats may differ in certain antigenic properties from a mixture of cytochromes P450 from phenobarbital- and 3-methylcholanthrene-treated rats.

INTRODUCTION

Polychlorinated biphenyls have been used in the production of various commercial products, including paints, plastics, and heat-insulating fluids, and have become widespread environmental pollutants (1-6). Human adipose tissue (7, 8), milk (9), and numerous animal tissues (1, 10-15) have been shown to be affected by PCBs.[1] Several laboratories have reported that PCBs are inducers of the hepatic microsomal enzyme system that metabolizes steroids, fatty acids, and numerous xenobiotics (1, 3, 16-20). Alvares *et al.* (21, 22) have shown that, in the male rat, PCBs may be a unique type of inducer of hepatic cytochrome P450,[2] the terminal oxidase of

[1] The abbreviations used are: PCBs, polychlorinated biphenyls; MC, 3-methylcholanthrene; PB, phenobarbital; SDS, sodium dodecyl sulfate; anti-PB-P450, antibody to cytochrome P450 from PB-treated rats; anti-MC-P448, antibody to cytochrome P450 from MC-treated rats.

[2] The term cytochrome P450 is used in this paper

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this enzyme system. The catalytic properties and the SDS–polyacrylamide gel electrophoretic mobility of the hemeproteins induced by Aroclor 1254, a PCB mixture containing approximately 54% chlorine, had characteristics of both MC- and PB-induced cytochromes P450. The spectral properties of the cytochrome P450 from Aroclor 1254-treated rats, however, were similar to those seen after MC treatment. Therefore the authors suggested that the cytochrome P450 induced by Aroclor 1254 may be a distinct hemeprotein(s) or a mixture of the hemeproteins induced by MC and PB (21).

Recently, the metabolic activation studies of Ames *et al.* (25) have made an examination of the hemeproteins from Aroclor 1254-treated rats of special interest. As pointed out by these authors, it is essential that metabolic activation of chemicals to mutagens in *Salmonella typhimurium* tester strains be performed with microsomal enzymes from induced animals, since a potential mutagen may not be sufficiently metabolized by enzymes from untreated animals. Ames *et al.* (25) suggested that the liver homogenate (9000 × *g* supernatant) from rats treated with Aroclor 1254 may be the preferential source of microsomal enzymes used for metabolic activation of chemicals to mutagenic metabolites in the *S. typhimurium* test system. Therefore an understanding of the cytochrome P450 composition of hepatic microsomes from Aroclor 1254-treated rats is important for the interpretation of metabolic activation studies.

This report describes the purification and characterization of cytochrome P450 from Aroclor 1254-treated rats and compares this purified hemeprotein preparation with those purified from PB- and MC-treated rats. Four different approaches are used to determine whether Aroclor 1254 treatment of rats results in the induction of a distinct hemeprotein(s) or a mixture of the hemeproteins induced by PB and MC. The four criteria examined are spectral characteristics, catalytic properties, electrophoretic mobility on SDS–polyacrylamide gels, and cross-reactivity with antibodies prepared against purified cytochromes P450 from PB- and MC-treated rats.

as a general term to denote any or all forms of liver microsomal cytochrome P450. The hemeprotein(s) induced by treatment of rats with MC has also been termed cytochrome P448 (23) or P_1-450 (24).

METHODS

Purification, Spectral Determinations, and SDS-Gel Electrophoresis

Immature male Long-Evans rats (50–60 g) from Blue Spruce Farms, Altamont, N. Y., were given a single intraperitoneal injection of Aroclor 1254 in corn oil at a dose of 300 mg/kg and killed 4 days later. Cytochrome P450 was purified from hepatic microsomes by the procedure described for the purification of cytochrome P450 from MC- and PB-treated rats (26). Briefly, the method involves solubilization with sodium cholate, two ammonium sulfate fractionations, and calcium phosphate gel adsorption, followed by DEAE-cellulose and CM-cellulose column chromatography in the presence of the non-ionic detergent Emulgen 911. The concentration of cytochrome P450 was determined by the method of Omura and Sato (27) from the CO difference spectrum of dithionite-reduced samples, using an extinction coefficient of 91 mM^{-1} cm^{-1}. All spectral determinations were done on an Aminco DW-2 recording spectrophotometer. Protein was determined by the method of Lowry *et al.* (28), with crystalline bovine serum albumin as the standard. The amino acid composition of cytochrome P450 from Aroclor 1254-treated rats was determined by Worthington Biochemicals Corporation.

The specific contents of the cytochromes P450 from PB- and MC-treated rats used for all these studies were 15.2 and 16.4 nmoles/mg of protein, respectively, based on their amino acid compositions. These preparations have been shown to be free of NADPH–cytochrome *c* reductase, epoxide hydrase, cytochrome b_5, and lipid, and to be purified more than 98% (per nanomole of hemeprotein) with respect to NADH–cytochrome b_5 reductase (26).

Aliquots of the purified hemeproteins were treated with SDS, heated, and subjected to electrophoresis on SDS–polyacryl-

amide gels according to the procedure described by Laemmli (29). The gels were stained for protein by the method of Fairbanks *et al.* (30).

Catalytic Activity

Benzo[*a*]pyrene hydroxylation (31), benzphetamine *N*-demethylation (32), zoxazolamine hydroxylation (33), 7-ethoxycoumarin *O*-deethylation (34), and testosterone hydroxylation (31) were determined as previously described. All assays were performed under conditions in which metabolism was proportional to hemeprotein concentration and time of incubation and in the presence of saturating amounts of NADPH-cytochrome *c* reductase and phosphatidylcholine. The NADPH-cytochrome *c* reductase was purified by the method of Dignam and Strobel (35) to a specific activity of 35,000–40,000 units/mg of protein. One unit of reductase is defined as the amount catalyzing the reduction of 1.0 nmole of cytochrome *c* per minute at 30° assayed by the method of Phillips and Langdon (36). Dilauroylphosphatidylcholine was prepared in 0.02 M Tris-HCl (pH 8.0) containing 1 mM EDTA and sonicated immediately before use.

Cross-reactivity with Antibodies

Antibodies to purified cytochromes P450 from PB-treated rats and MC-treated rats were prepared as previously described (32, 37). The Ouchterlony double-diffusion plates contained 0.9% agarose and 0.2% Emulgen 911. The immunoprecipitin lines were stained for peroxidase activity, using tetramethylbenzidine and hydrogen peroxide, by the method of Thomas *et al.* (38).

To determine the effects of the antibodies on metabolism by the purified enzyme system, the indicated hemeprotein preparation was incubated with the antibody for 5 min at 23°. All tubes were adjusted to the same final antibody concentration with antibody from untreated rabbits. The final volume for this incubation was adjusted to 0.25 ml with calcium- and magnesium-free phosphate-buffered NaCl at pH 7.4. After the incubation, the tubes were placed on ice, and NADPH-cytochrome *c* reductase and phosphatidylcholine were added, followed by the necessary cofactors and substrate to a final volume of 1.0 ml for all assays.

Materials

Aroclor 1254 (Lot KC-12-638) was kindly supplied by Monsanto Chemical Company. NADPH, cytochrome *c*, crystalline bovine serum albumin, DEAE-cellulose, sodium cholate, benzo[*a*]pyrene, sodium dodecyl sulfate, and 3-methylcholanthrene were obtained from Sigma Chemical Company. Sodium phenobarbital was purchased from Merck & Company. Sephadex LH-20 was purchased from Pharmacia, and CM-52 cellulose was obtained from Whatman Chemical Company. Emulgen 911 was kindly supplied by Kao-Atlas, Ltd. (Japan). The reagents for gel electrophoresis were purchased from Ames Company. [*N-methyl*-^{14}C]Benzphetamine and [4-^{14}C]-testosterone were obtained from New England Nuclear Corporation. 7-Ethoxycoumarin was synthesized for us by Aldrich Chemical Company. [4,6-^{3}H]Zoxazolamine was synthesized as previously described (33).

RESULTS

Purification of Cytochrome P450

Cytochrome P450 was purified from Aroclor 1254-treated rats to a specific content of greater than 14.0 nmoles/mg of protein, based on the protein assay of Lowry *et al.* (28). However, as has been reported for the purified preparations from PB- and MC-treated rats (26), the standard protein assay gives an erroneously high value for the protein content. When determined from the amino acid composition, the protein content is approximately 68–70% of the value obtained from the standard protein assay. Therefore the specific content of the purified cytochrome P450 from Aroclor 1254-treated rats is greater than 19.0 nmoles/mg of protein. The extent of purification and the yield of the hemeproteins from Aroclor 1254-treated rats is similar to that previously reported (26) for the PB- and MC- induced hemeproteins. This purification procedure (26) can be used to obtain highly purified cytochrome P450 from

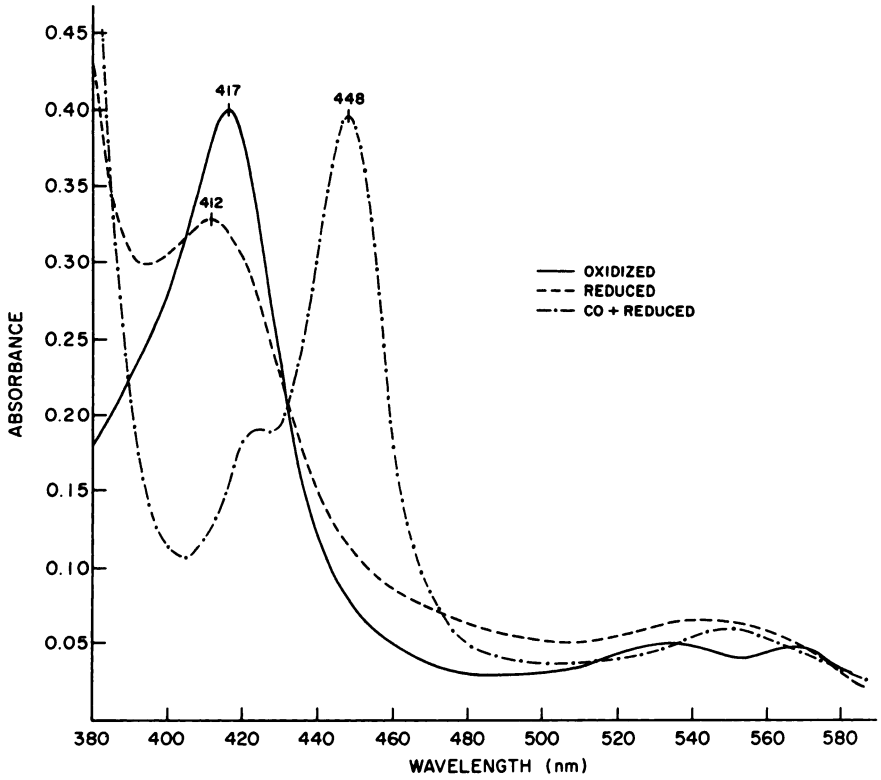

FIG. 1. *Absolute spectra of cytochrome P450 from Aroclor 1254-treated rats*
Cytochrome P450 at a concentration of 2.95 nmoles/ml in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol was used for the spectral determinations. The reference cuvette contained the same buffer mixture.

liver microsomes of rats treated with PB, MC, or Aroclor 1254.

Spectral Properties

Figure 1 shows the absolute spectra of the purified cytochrome P450 from Aroclor 1254-treated rats. The absorption maximum of the oxidized hemeprotein is at 417 nm. The absence of a shoulder between 390 and 400 nm indicates that the preparation is a low-spin ferric hemeprotein. Upon reduction with dithionite, the absorption maximum shifts downward to 412 nm, indicating that the preparation is free of cytochrome b_5 contamination. When reduced in the presence of CO, the preparation has an absorption maximum at 448 nm with a shoulder at 423 nm due to a small amount (less than 10%) of cytochrome P420.

The CO-reduced difference spectra of purified cytochrome P450 from Aroclor 1254-, PB-, and MC-treated rats were compared (results not shown). The absorption maximum of the cytochrome purified from PB-treated rats is at 450 nm, and that purified from MC-treated rats is at 447 nm.[3] Cytochrome P450 from Aroclor 1254-treated rats has an absorption maximum at an intermediate wavelength of 448 nm.

SDS-Polyacrylamide Gel Electrophoresis

The SDS-polyacrylamide gels containing the various purified cytochrome P450 preparations alone and in mixtures are shown in Fig. 2. The molecular weight of the major band of cytochrome P450 from

[3] Liver microsomal cytochrome P450 from rats treated with MC has an absorption peak at 448 nm (23) in the CO-reduced difference spectrum. During the purification procedure, a hemeprotein fraction with an absorption peak at 449 nm is separated by column chromatography (39). The highly purified hemeprotein(s) has an absorption maximum at 447 nm (26).

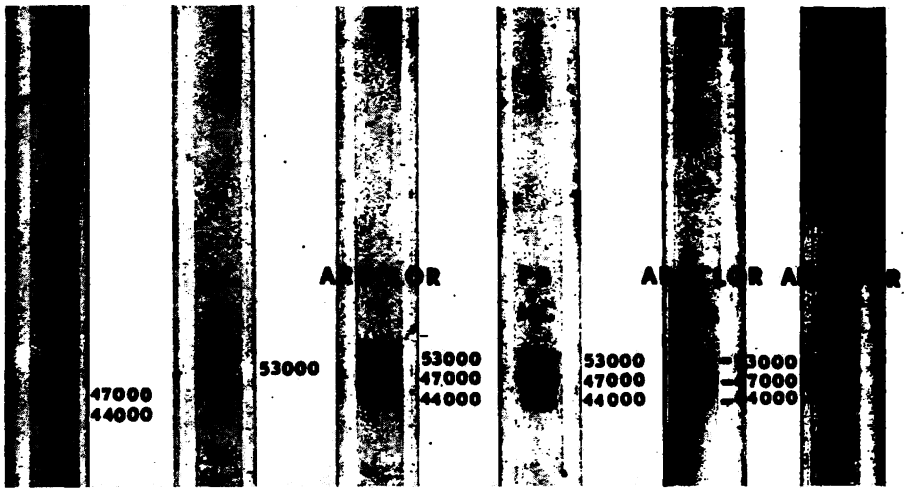

FIG. 2. *SDS-polyacrylamide gels of cytochromes P450 from Aroclor 1254-, PB-, and MC-treated rats*
The hemeproteins were treated with SDS, heated, and subjected to electrophoresis according to Laemmli (29), with 7.5% separation gels. Ten micrograms of each hemeprotein preparation alone and mixtures of 5 μg of each hemeprotein were applied to the gels. The gels were stained for protein by the procedure of Fairbanks et al. (30).

PB-treated rats is 47,000, and that of the major band of the preparation from MC-treated rats is 53,000. The electrophoretic pattern of cytochrome P450 from Aroclor 1254-treated rats shows two major protein bands of approximately equal concentration, with minimum molecular weights of 53,000 and 47,000. Similar results have been obtained using liver microsomes and partially purified cytochrome P450 from Aroclor 1254-treated rats (22). However, the partially purified preparation was only about 25–35% pure, and it is known that other microsomal proteins have molecular weights in the 45,000–55,000 region [e.g., epoxide hydrase (40) and stearyl coenzyme A desaturase (41)].

Figure 2 shows that there are no protein-staining bands other than in the 40,000–60,000 molecular weight region, illustrating the purity of this hemeprotein preparation. The minor band at mol wt 44,000 has been determined to be a hemeprotein in the preparation from PB-treated rats by staining for peroxidase activity.

When cytochrome P450 from PB-treated rats is mixed with the hemeprotein from MC-treated rats, the profile is indistinguishable from that seen on the gel containing the preparation from Aroclor 1254-treated rats. The mixture of the hemeproteins from Aroclor 1254-treated rats and the preparation from PB-treated rats indicates that the major band of lower molecular weight of the Aroclor 1254-induced hemeproteins has the same electrophoretic mobility as the major band of cytochrome P450 from PB-treated rats. When cytochrome P450 from Aroclor 1254-treated rats is mixed with the preparation from MC-induced rats, the major band of the hemeprotein from MC-treated rats has the same molecular weight as the major band of higher molecular weight from Aroclor 1254-treated rats. Therefore the Aroclor 1254-induced cytochrome P450 cannot be distinguished from a mixture of the hemeproteins from PB- and MC-treated rats by this technique.

Catalytic Activity

Several laboratories have reported that the microsomal metabolism of a variety of substrates is increased following treatment with PCBs. For example, the hydroxylation of benzo[*a*]pyrene (21), aniline (12, 18), biphenyl (42), acetanilide (43), and pentobarbital (19) and the demethylation of azo dyes (44), ethylmorphine (18, 19), aminopyrine (12), and *p*-nitroanisole

(45) have been shown to be elevated in livers from animals treated with PCBs. Table 1 shows the catalytic activity of purified cytochromes P450 from Aroclor 1254-, PB-, and MC-treated rats. The catalytic activity was determined in the presence of saturating amounts of NADPH–cytochrome *c* reductase and phosphatidylcholine.

As is apparent from the relative turnover numbers, the purified cytochrome P450 from PB-treated rats preferentially catalyzes benzphetamine demethylation and testosterone 16α-hydroxylation. The hemeprotein from MC-treated rats metabolizes zoxazolamine, benzo[*a*]pyrene, and 7-ethoxycoumarin more efficiently than PB-induced cytochrome P450. Although the hydroxylation of testosterone at the 6β position appears to be greatest with the MC-induced hemeprotein, this result must be interpreted with caution. Most of the activity for this hydroxylation pathway is lost with purification, and, in our assay, the blank in this region is very high.

With each substrate assayed, the turnover number for the hemeproteins from Aroclor 1254-treated rats is intermediate between those obtained with the PB- and MC-induced cytochromes P450 and is similar to the turnover number for an equal mixture of PB- and MC-induced hemeproteins. The differences in turnover numbers between the Aroclor 1254-induced cytochrome P450 and the mixture of PB- and MC-induced hemeproteins may reflect differences in the amount of an Aroclor 1254-induced hemeprotein most active in the metabolism of a particular substrate. For example, the hydroxylation of benzo[*a*]pyrene is greater with the preparation from Aroclor 1254-treated rats than with the equal mixture of PB- and MC-induced hemeproteins. The Aroclor 1254-induced cytochrome P450 may contain more of the hemeprotein(s) responsible for the hydroxylation of benzo[*a*]pyrene than is present in the equal mixture. Alternatively, the purified cytochrome P450 preparation from Aroclor 1254-treated rats may contain a form(s) of cytochrome P450 more active for the metabolism of benzo[*a*]pyrene than is present in the mixture of PB- and MC-induced hemeproteins.

TABLE 1

Catalytic activity of purified cytochromes P450 from PB-, MC-, and Aroclor 1254-treated rats

The cytochromes P450 were purified as described in METHODS. The turnover numbers were calculated from the enzymatic activity in the presence of saturating amounts of NADPH–cytochrome *c* reductase and phosphatidylcholine. No activity was observed in the absence of hemeprotein or NADPH–cytochrome *c* reductase. All assays were performed as described in METHODS.

Substrate	Turnover No.			
	PB-P450	MC-P450	Aroclor-P450	PB + MC-P450[a]
	nmoles/min/nmole hemeprotein			
Benzphetamine	49.4	3.4	22.7	23.9
Zoxazolamine	1.5	18.8	11.2	ND[b]
Benzo[*a*]pyrene	0.2	7.7	4.2	2.7
7-Ethoxycoumarin	5.7	98.6	52.6	45.8
Testosterone				
7α-Hydroxy	0.7	0.6	0.6	ND
16α-Hydroxy	1.4	0.01	0.5	ND
6β-Hydroxy	0.02	0.3	0.2	ND

[a] Half the concentration of each hemeprotein was mixed with half the concentration of the other hemeprotein preparation to yield the same final concentration used in the assays of each cytochrome P450 preparation alone.

[b] Not determined.

Cross-reactivity with Antibodies

Ouchterlony double-diffusion analyses. Figures 3 and 4 show Ouchterlony double-diffusion plates containing the antibodies to cytochrome P450 from PB- and MC-treated rats and the various purified hemeprotein preparations. The immunoprecipitin lines have been stained for peroxidase activity using tetramethylbenzidine and hydrogen peroxide (38).

In Fig. 3A the antibody to cytochrome P450 from PB-treated rats was placed in the center well. The homologous antigen (cytochrome P450 from PB-treated rats) reacts with the antibody to a much greater degree than the same concentration of the heterologous antigen (cytochrome P450 from MC-treated rats), as previously reported (37). The cross-reactivity of the preparation from Aroclor 1254-treated rats is indistinguishable from the equal mix-

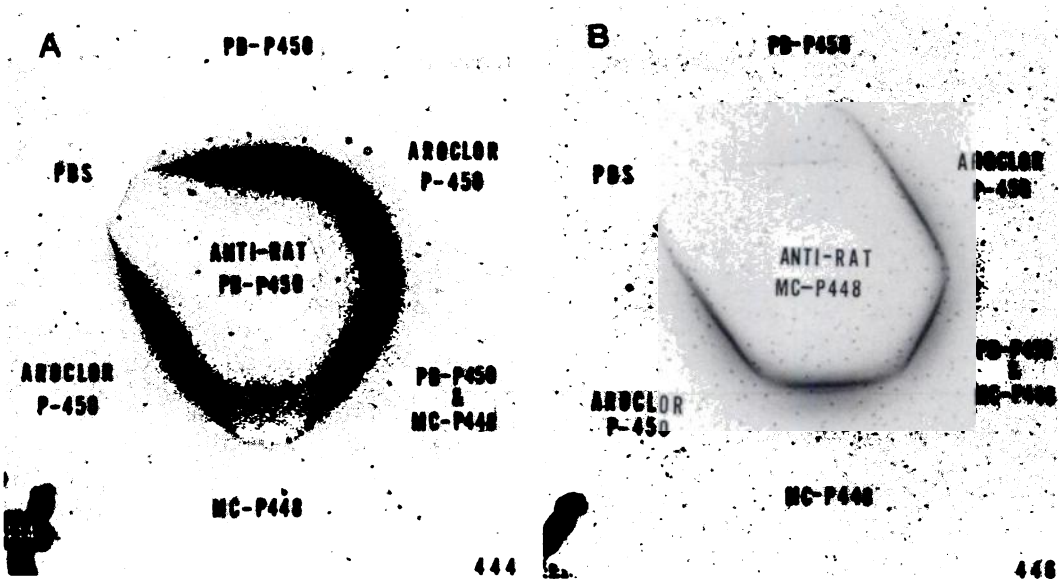

FIG. 3. *Ouchterlony double-diffusion analyses of cytochromes P450 from Aroclor 1254-, PB-, and MC-treated rats*

In plate A the center well contained 15 μl of anti-PB-P450, and in plate B, 15 μl of anti-MC-P448. In both plates the final concentration of each hemeprotein preparation alone was 8 μM, and the mixture contained a 4 μM concentration of each cytochrome P450. MC-P448 refers to the hemeprotein preparation from MC-treated rats, and PB-P450 denotes the cytochrome P450 from PB-treated rats. The wells labeled PBS contained 15 μl of calcium- and magnesium-free phosphate-buffered NaCl at pH 7.4. The immunoprecipitin lines were stained for peroxidase activity by the method of Thomas *et al.* (38).

ture of hemeproteins from PB- and MC-treated rats. There is a line of identity among the immunoprecipitin lines formed from PB-induced, Aroclor 1254-induced, and the mixture of PB- and MC-induced hemeproteins. The lines of identity and the absence of spurs at the intersections of these lines suggest that the reacting species in each case is identical. Similar results were obtained when the antibody to cytochrome P450 from MC-treated rats (anti-MC-P448) was placed in the center well (Fig. 3B). The reaction of cytochrome P450 from MC-treated rats is much greater than the cross-reaction of the hemeprotein preparation from PB-treated rats (32). The cytochrome P450 from Aroclor 1254-treated rats and the mixture of PB- and MC-induced hemeproteins also cross-react with this antibody in an identical manner. The lines of identity of the immunoprecipitin lines formed from the reaction of the MC-induced, Aroclor 1254-induced, and the mixture of MC- and PB-induced hemeproteins and the absence of spurs at the intersections of these lines indicate that the same cross-reacting species is present in each preparation.

Figure 4 shows a double-diffusion plate containing both antibodies, Aroclor 1254-induced cytochrome P450 and an equal mixture of the PB- and MC-induced hemeproteins. The reaction of each antibody with the hemeproteins from Aroclor 1254-treated rats is indistinguishable from the reaction of the mixture of hemeproteins. The spurs indicate that there are forms of the Aroclor 1254-induced cytochrome P450 and the mixture of hemeproteins which are recognized by one antibody but not by the other antibody.

Effect of antibodies on metabolism. Previously, it has been shown (32, 37) that anti-PB-P450 and anti-MC-P448 can differentially inhibit the metabolism of various substrates by the purified reconstituted system containing cytochrome P450, NADPH–cytochrome *c* reductase, and phosphatidylcholine. The pattern of inhibition has been reported to depend on the

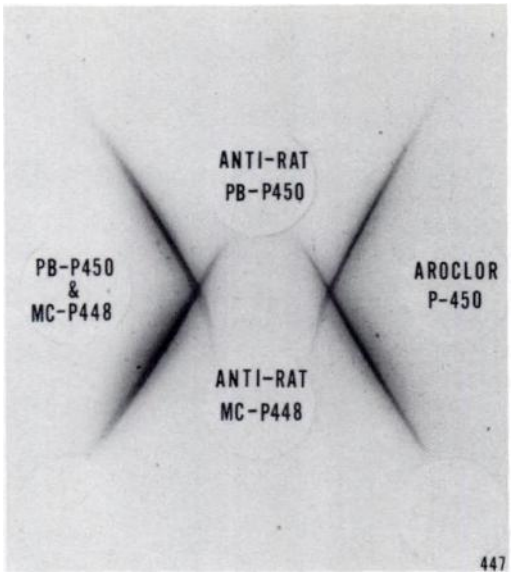

FIG. 4. *Cross-reaction of cytochrome P450 from Aroclor 1254-treated rats and a mixture of PB- and MC-inducible hemeproteins with anti-PB-P450 and anti-MC-P448*

The upper well of this Ouchterlony plate contained 15 μl of anti-PB-P450, and the lower well contained 15 μl of anti-MC-P448. The PB- and MC-inducible hemeproteins (PB-P450 and MC-P448) were each present at a final concentration of 4 μM. The concentration of cytochrome P450 from Aroclor 1254-treated rats was 8 μM. The immunoprecipitin lines were stained as described (38).

antibody, substrate, and the source of the purified cytochrome (32, 37). Figures 5 and 6 show the effects of these antibodies on the metabolism of different substrates supported by the hemeproteins from PB-, MC-, and Aroclor 1254-treated rats, and an equal mixture of the PB- and MC-induced hemeproteins.

Figure 5 shows the effects of each antibody on the deethylation of 7-ethoxycoumarin, a reaction which is catalyzed most efficiently by cytochrome P450 from MC-treated rats (Table 1). When anti-MC-P448 is used to inhibit metabolism (left side), the reactions supported by the hemeproteins from MC- and Aroclor 1254-treated rats and an equal mixture of hemeproteins from PB- and MC-treated rats are completely inhibited by 5 mg of antibody per nanomole of hemeprotein. However, when the reaction is supported by cytochrome P450 from PB-treated rats, more than 50% of the activity remains. The mixture of PB- and MC-induced hemeproteins is inhibited most dramatically, probably because most of the activity for this substrate resides in the MC-induced hemeprotein In addition, the ratio of antibody to the cytochrome P450 from MC-treated rats in the mixture is double that in the assays with this hemeprotein preparation alone.

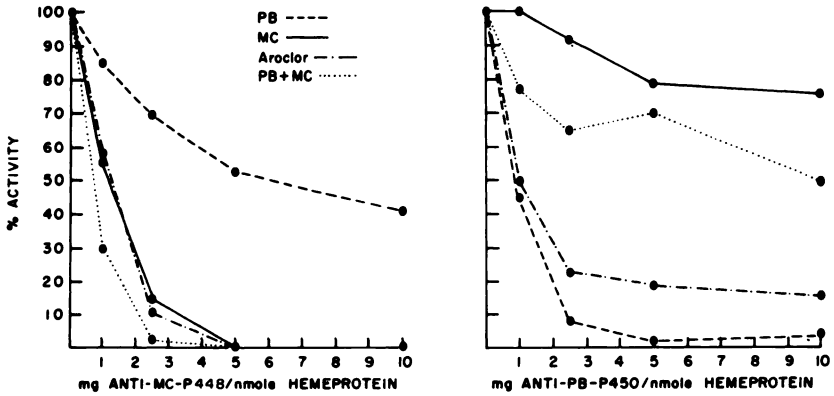

FIG. 5. *Effect of anti-MC-P448 and anti-PB-P450 on 7-ethoxycoumarin metabolism*

The incubation mixture contained the following components in a final volume of 1 ml: 0.02 nmole of the indicated cytochrome P450 preparation, 300 units of NADPH–cytochrome *c* reductase, 20 μg of dilauroylphosphatidylcholine, 0.5 mM NADPH, 0.3 mM 7-ethoxycoumarin, 3 mM $MgCl_2$, and 50 mM potassium phosphate buffer, pH 7.4. The assays of the mixture of PB- and MC-inducible hemeproteins contained 0.01 nmole of each cytochrome P450. The assays containing only cytochrome P450 from PB-treated rats were incubated for 15 min and the other assays were incubated for 5 min. All incubations were performed at 37°.

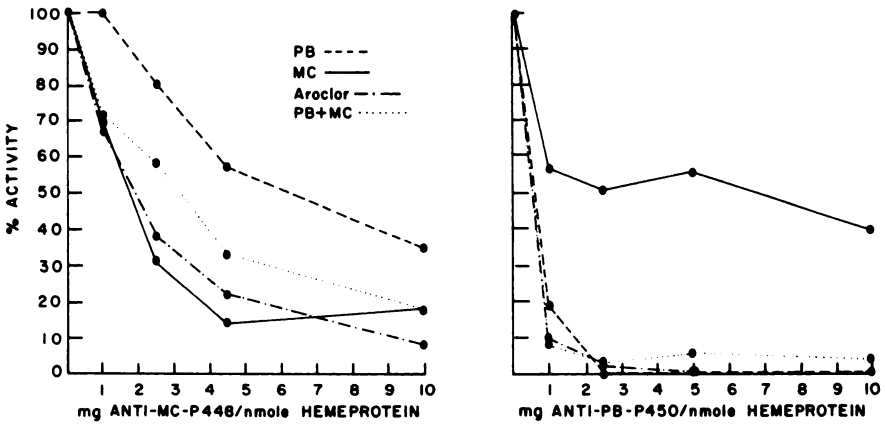

FIG. 6. *Effect of anti-MC-P448 and anti-PB-P450 on benzphetamine demethylation*
The incubation mixture contained the following components in a total of 1 ml: 0.1 nmole of the indicated cytochrome P450, 400 units of NADPH–cytochrome *c* reductase, 10 μg of dilauroylphosphatidylcholine (30 μg in the assays of the hemeprotein from MC-treated rats), 5 mM glucose 6-phosphate, 0.7 unit of glucose 6-phosphate dehydrogenase, 0.5 mM NADPH, 10 mM semicarbazide, 1 mM[^{14}C]benzphetamine (approximately 200,000 cpm/tube), 10 mM $MgCl_2$, and 0.1 M potassium phosphate buffer, pH 7.4. The assays of the mixture of PB- and MC-inducible cytochromes P450 contained 0.05 nmole of each hemeprotein preparation. The assays containing only cytochrome P450 from MC-treated rats were incubated for 15 min and the other assays were incubated for 5 min. All incubations were performed at 37°.

The inhibition patterns observed are very similar when the mixture of PB- and MC-induced hemeproteins or the Aroclor 1254-induced hemeproteins are used to catalyze the reaction. Therefore the cytochrome P450 from Aroclor 1254-treated rats cannot be distinguished from a mixture of PB- and MC-induced cytochromes P450 with this antibody and substrate.

When anti-PB-P450 is used to inhibit 7-ethoxycoumarin metabolism (right side of Fig. 5), very different results are obtained. The metabolism catalyzed by cytochrome P450 from PB-treated rats is almost completely inhibited whereas that catalyzed by the hemeprotein from MC-treated rats is minimally affected. These inhibition patterns would be expected, since the anti-PB-P450 is more specific for its homologous antigen than the heterologous antigen (37). Metabolism by an equal mixture of the PB- and MC-induced hemeproteins is not substantially inhibited, since most of the activity for this substrate resides in the MC-induced hemeprotein. However, the activity catalyzed by the Aroclor 1254-induced cytochrome P450 is inhibited dramatically and does not parallel the mixture of PB- and MC-induced hemeproteins.

The effects of each antibody on the hydroxylation of benzo[*a*]pyrene by the purified hemeproteins from PB-, MC-, and Aroclor 1254-treated rats and an equal mixture of the PB- and MC-induced hemeproteins (data not shown) were very similar to the effects on 7-ethoxycoumarin metabolism. The inhibition of benzo[*a*]pyrene hydroxylation by anti-MC-P448 is very similar when the Aroclor 1254- or the mixture of PB- and MC-induced hemeproteins catalyze the reaction. Whereas the metabolism by the mixture of PB- and MC-induced hemeproteins is not inhibited by anti-PB-P450, there is dramatic inhibition of the activity supported by the Aroclor 1254-induced cytochrome P450. Therefore the hemeproteins induced by Aroclor 1254 can be distinguished from a mixture of the PB- and MC-induced hemeproteins by anti-PB-P450 in the metabolism of benzo[*a*]pyrene and 7-ethoxycoumarin.

Since the inhibition of 7-ethoxycoumarin and benzo[*a*]pyrene metabolism by anti-PB-P450 was the only evidence obtained thus far indicating that the cytochrome P450 from Aroclor 1254-treated rats might differ from a mixture of cytochromes P450 from PB- and MC-treated

rats, the effects of the antibodies on benzphetamine demethylation (Fig. 6) were also examined. The hemeprotein(s) from MC-treated rats is most active for the metabolism of 7-ethoxycoumarin and benzo[a]pyrene, whereas cytochrome P450 from PB-treated rats preferentially demethylates benzphetamine (Table 1).

Figure 6 shows the effects of anti-MC-P448 and anti-PB-P450 on the demethylation of benzphetamine by the various purified hemeprotein preparations. As expected, when anti-MC-P448 is used to inhibit metabolism (left side), the hemeprotein from MC-treated rats is substantially more inhibited than the cytochrome P450 from PB-treated rats (32). Demethylation supported by the cytochrome P450 from Aroclor 1254-treated rats or the equal mixture of PB- and MC-induced hemeproteins is inhibited to an intermediate, but fairly similar, extent. Therefore anti-MC-P448 cannot distinguish the hemeproteins from Aroclor 1254-treated rats from the mixture of PB- and MC-induced hemeproteins in the metabolism of 7-ethoxycoumarin, benzo[a]pyrene, or benzphetamine.

As previously reported (37), anti-PB-P450 inhibits the demethylation of benzphetamine supported by cytochrome P450 from PB-treated rats much more dramatically than the metabolism by the hemeprotein from MC-treated rats (Fig. 6, right side). The catalytic activity of the Aroclor 1254-induced cytochrome P450 and the mixture of PB- and MC-induced hemeproteins is completely inhibited by anti-PB-P450. At a ratio of 2.5 mg of antibody per nanomole of hemeprotein, only the cytochrome P450 from MC-treated rats retains metabolic activity. The inhibition curves of the Aroclor 1254-induced cytochrome P450 and the mixture of PB- and MC-induced hemeproteins are very similar. Therefore, with a substrate most efficiently metabolized by cytochrome P450 from PB-treated rats, anti-PB-P450 cannot distinguish the Aroclor 1254-induced hemeproteins from an equal mixture of the PB- and MC-induced hemeproteins.

DISCUSSION

Treatment of male rats with the polychlorinated biphenyl mixture Aroclor 1254 has been reported to result in the induction of either a distinct liver microsomal cytochrome P450 or a mixture of the hemeproteins induced by PB and MC (21). The spectral, catalytic, and electrophoretic properties of purified cytochrome P450 from Aroclor 1254-treated rats indicate that this polychlorinated biphenyl mixture induces a mixture of hemeproteins similar to those induced by PB and MC. The percentage chlorination of the PCBs (18, 46) and the dose administered (19, 45–48), as well as the species (49) and sex (50) of the animal, may influence the relative induction of the hemeproteins. After the single large dose of Aroclor 1254 used in these experiments, slightly more of the hemeprotein(s) induced by MC appears to be present than the hemeprotein(s) induced by PB. For example, if there had been equal induction of the PB- and MC- inducible hemeproteins, the absorption maximum of the CO-reduced spectrum would have been at 448.5 nm rather than 448 nm. Furthermore, the turnover numbers obtained using cytochrome P450 from Aroclor 1254-treated rats would have been exactly the same as the values for the equal mixture of PB- and MC-induced hemeproteins.

The cross-reactivity of cytochrome P450 from Aroclor 1254-treated rats with anti-PB-P450 and anti-MC-P448 is indistinguishable from a mixture of highly purified PB- and MC-induced hemeproteins on Ouchterlony double-diffusion plates. Anti-MC-P448 has the same inhibitory effect on the metabolism of 7-ethoxycoumarin, benzo[a]pyrene, and benzphetamine whether the reactions are supported by cytochrome P450 from Aroclor 1254-treated rats or a mixture of cytochromes P450 from MC- and PB-treated rats. However, the effects of anti-PB-P450 on metabolism suggest that the preparation from Aroclor 1254-treated rats may differ from the mixture of hemeproteins induced by PB and MC. When the demethylation of benzphetamine is studied, the anti-PB-P450 has a similar effect on the reactions catalyzed by Aroclor 1254-induced cytochrome P450 and the mixture of PB- and MC-induced hemeproteins. When the metabolism of two substrates (7-ethoxycoumarin and benzo[a]-

pyrene) most efficiently metabolized by the hemeprotein(s) from MC-treated rats is studied, very different results are obtained. Anti-PB-P450 minimally affects the activity of the mixture of PB- and MC-induced cytochrome P450 but substantially inhibits metabolism by the Aroclor 1254-induced hemeproteins. These results suggest that cytochrome P450 from rats treated with Aroclor 1254 differs antigenically from a mixture of hemeproteins from PB- and MC-treated rats. The immunological differences are related to anti-PB-P450 and the metabolism of substrates most efficiently catalyzed by cytochrome P450 from MC-treated rats.

It is well established that the preparations purified from PB- and MC-treated rats by the procedure described (26) contain multiple forms of cytochrome P450 (26, 32, 37). By several criteria the cytochrome P450 from rats treated with Aroclor 1254 is indistinguishable from a mixture of the hemeproteins induced by PB and MC. However, there may be a form of cytochrome P450 present in a small amount in the preparation from Aroclor 1254-treated rats which is very active for the metabolism of benzo[*a*]pyrene and 7-ethoxycoumarin and sensitive to inhibition by anti-PB-P450.

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REFERENCES

1. Risebrough, R. W., Rieche, P., Peakall, D. B., Herman, S. G. & Kirven, M. N. (1968) *Nature*, 220, 1098–1102.
2. Gustafson, C. G. (1970) *Environ. Sci. Technol.*, 4, 814–819.
3. Peakall, D. B. & Lincer, J. L. (1970) *BioScience*, 20, 958.
4. Hammond, A. L. (1972) *Science*, 175, 155–156.
5. Nisbet, I. C. T. & Sarofim, A. F. (1972) *Environ. Health Perspect.*, 1, 21.
6. (1976) *J. Am. Med. Assoc.*, 235, 1655–1656.
7. Biros, F. J., Walker, A. C. & Medbery, A. (1970) *Bull. Environ. Contam. Toxicol.*, 5, 317–323.
8. Price, H. A. & Welch, R. L. (1972) *Environ. Health Perspect.*, 1, 73–78.
9. Acker, L. & Schulte, E. (1970) *Naturwissenschaften*, 57, 497.
10. Jensen, S., Johnels, A. G., Olsson, M. & Otterlind, G. (1969) *Nature*, 224, 247–250.
11. Koeman, J. H., Ten Noever deBrauw, M. C. & deVos, R. H. (1969) *Nature*, 221, 1126–1128.
12. Villeneuve, D. C., Grant, D. L., Phillips, W. E. J., Clark, M. L. & Clegg, D. J. (1971) *Bull. Environ. Contam. Toxicol.*, 6, 120–128.
13. Bache, C. A., Serum, J. W., Youngs, W. D. & Lisk, D. J. (1972) *Science*, 177, 1191.
14. Allen, J. R. & Abrahamson, L. J. (1973) *Arch. Environ. Contam. Toxicol.*, 1, 265–280.
15. Bruckner, J. V., Khanna, K. L. & Cornish, H. H. (1974) *Toxicol. Appl. Pharmacol.*, 28, 189–199.
16. Street, J. C., Urry, F. M., Wagstaff, D. J. & Blau, A. D. (1969) *Abstr. 158th Meet. Am. Chem. Soc. (Sept.)*.
17. Benthe, H. F., Schmoldt, A. & Schmidt, H. (1972) *Arch. Toxikol.*, 29, 97–106.
18. Bickers, D. R., Harber, L. C., Kappas, A. & Alvares, A. P. (1972) *Res. Commun. Chem. Pathol. Pharmacol.*, 3, 505–512.
19. Litterest, C. L., Farber, T. M., Baker, A. M. & Van Loon, E. J. (1972) *Toxicol. Appl. Pharmacol.*, 23, 112–122.
20. Nowicki, H. G. & Norman, A. W. (1972) *Steroids*, 19, 85–99.
21. Alvares, A. P., Bickers, D. R. & Kappas, A. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, 70, 1321–1325.
22. Alvares, A. P. & Siekevitz, P. (1973) *Biochem. Biophys. Res. Commun.*, 54, 923–929.
23. Alvares, A. P. Schilling, G., Levin, W. & Kuntzman, R. (1967) *Biochem. Biophys. Res. Commun.*, 29, 521–526.
24. Sladek, N. E. & Mannering, G. J. (1966) *Biochem. Biophys. Res. Commun.*, 24, 668–674.
25. Ames, B. N., McCann, J. & Yamasaki, E. (1975) *Mutat. Res.*, 31, 347–364.
26. Ryan, D., Lu, A. Y. H., Kawalek, J., West, S. B. & Levin, W. (1975) *Biochem. Biophys. Res. Commun.*, 64, 1134–1141.
27. Omura T. & Sato, R. (1964) *J. Biol. Chem.*, 239, 2379–2385.
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
29. Laemmli, U. K. (1970) *Nature*, 227, 680–685.
30. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry*, 10, 2606–2617.
31. Lu, A. Y. H., Kuntzman, R., West, S., Jacobson, M. & Conney, A. H. (1972) *J. Biol. Chem.*, 247, 1727–1734.
32. Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. & Levin, W. (1976) *J. Biol. Chem.*, 251, 1385–1391.
33. Tomaszewski, J. E., Jerina, D. M., Levin W. &

Conney, A. H. (1976) *Arch. Biochem. Biophys.*, 176, 788–798.
34. Jacobson, M., Levin, W., Poppers, P. J., Wood, A. W. & Conney, A. H. (1974) *Clin. Pharmacol. Ther.*, 16, 701–710.
35. Dignam, J. D. & Strobel, H. W. (1975) *Biochem. Biophys. Res. Commun.*, 63, 845–852.
36. Phillips, A. H. & Langdon, R. G. (1962) *J. Biol. Chem.*, 237, 2652–2660.
37. Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. & Levin, W. (1976) *Mol. Pharmacol.*, 12, 746–758.
38. Thomas, P. E., Ryan, D. & Levin, W. (1976) *Anal. Biochem.*, 75, 168–176.
39. Ryan, D., Lu, A. Y. H., West, S. & Levin, W. (1975) *J. Biol. Chem.*, 250, 2157–2163.
40. Lu, A. Y. H., Ryan, D., Jerina, D. M., Daly, J. W. & Levin, W. (1975) *J. Biol. Chem.*, 250, 8283–8288.
41. Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B. & Redline, R. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, 71, 4565–4569.
42. Turner, J. C. & Green, R. S. (1974) *Bull. Environ. Contam. Toxicol.*, 12, 669–671.
43. Bruckner, J. V., Khanna, K. L. & Cornish, H. H. (1973) *Toxicol. Appl. Pharmacol.*, 24, 434–448.
44. Argus, M. F., Bryant, G. M., Pastor, K. M. & Arcos, J. C. (1975) *Cancer Res.*, 35, 1574–1579.
45. Chen, T. S. & DuBois, K. P. (1973) *Toxicol. Appl. Pharmacol.*, 26, 504–512.
46. Schmoldt, A., Benthe, H. F. & Fruhling, R. (1974) *Arch. Toxikol.* 32, 69–81.
47. Litterest, C. L. & van Loon, E. J. (1974) *Bull. Environ. Contam. Toxicol.*, 11, 206–212.
48. Bruckner, J. V., Khanna, K. L. & Cornish, H. H. (1974) *Food Cosmet. Toxicol.*, 12, 323–330.
49. Wolff, T. & Hesse, S. (1976) *Naunyn-Schmiedebergs Arch. Pharmacol.*, 293, 51–52.
50. Alvares, A. P. & Kappas, A. (1976) *Hoppe-Seylers Z. Physiol. Chem.*, 357, 1019–1020.