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Immunochemical Evidence for Six Forms of Rat Liver Cytochrome P450 Obtained Using Antibodies against Purified Rat Liver Cytochromes P450 and P448

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SUMMARY

THOMAS, PAUL E., LU, ANTHONY Y. H., RYAN, DENE, WEST, SUSAN B., KAWALEK, JOSEPH & LEVIN, WAYNE (1976) Immunochemical evidence for six forms of rat liver cytochrome P450 obtained using antibodies against purified rat liver cytochromes P450 and P448. *Mol. Pharmacol.*, 12, 746-758.

The immune globulin G fraction (anti-PB-P450) isolated from rabbits immunized against purified hepatic cytochrome P450 from phenobarbital-treated rats is quite specific. In Ouchterlony double-diffusion plates, anti-PB-P450 reacts well with its homologous antigen, but poorly and incompletely with purified rat liver cytochrome P448 from 3-methylcholanthrene-treated rats. Purified rabbit cytochrome P450 LM_2 reacts poorly while rabbit cytochrome P448, beef adrenal mitochondrial P450, and *Pseudomonas putida* P450 show no reaction with anti-PB-P450. Using this antibody preparation in conjunction with our previously reported antibody [Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J. & Levin, W. (1976) *J. Biol. Chem.*, 251, 1385-1391] to purified hepatic cytochrome P448 from 3-methylcholanthrene-treated rats (anti-MC-P448), we have detected a total of six different but immunochemically related forms of rat liver cytochrome P450. Anti-MC-P448 cross-reacts with cytochrome P450 in Ouchterlony immunodiffusion plates and gives three heme-staining precipitin bands, but the intensity of these bands suggests that only a portion of the total hemeprotein is precipitated by this antibody. When both antibodies are used in the same immunodiffusion plate, anti-PB-P450 precipitates the hemeprotein which does not cross-react with anti-MC-P448. The presence of four heme-staining precipitin bands is evidence for at least four cytochromes P450 in the purified rat PB-P450 preparation. When anti-PB-P450 cross-reacts with cytochrome P448, less of the hemeprotein is precipitated than with the homologous antigen, which also suggests that one or more forms in the purified rat MC-P448 preparation are not recognized by the heterologous antibody. Again, using both antibodies in the same immunodiffusion plate, there is at least one hemeprotein in the cytochrome P448 preparation that is precipitated by anti-MC-P448 but not by anti-PB-P450. The differential effects of the antibodies on the catalytic activity of the reconstituted system also suggest a multiplicity of cytochromes P450 in the purified preparations obtained from phenobarbital- and 3-methylcholanthrene-treated rats. Thus the immunological and catalytic properties of both purified hemeprotein preparations indicate that the rat is capable of producing at least six immunochemically distinguishable forms of cytochrome P450.

INTRODUCTION

Cytochrome P450, an enzyme present in the endoplasmic reticulum of liver and other tissues, is of considerable interest because of its extremely broad substrate specificity and the modulation of its activity through induction. Substrates metabolized by cytochrome P450 include foreign compounds such as drugs, pesticides, carcinogens, hydrocarbons, and anesthetics, as well as endogenous compounds, including fatty acids and steroids (1–4). Multiple forms of this terminal oxidase have been proposed to explain this broad substrate specificity. Most of the evidence for multiple forms has been derived from studies with microsomes and purified cytochrome P450 preparations from animals treated with different inducers. Treatment of rats with 3-methylcholanthrene, a carcinogenic polycyclic hydrocarbon, gives rise to cytochrome P448, which differs from cytochrome P450 in control or phenobarbital-treated rats with respect to its ethyl isocyanide difference spectrum (5), CO-difference spectrum (6, 7), substrate specificity (8, 9), and sodium dodecyl sulfate–gel electrophoretic pattern (10, 11). Several laboratories have made considerable progress toward the purification and characterization of multiple forms of cytochrome P450 from rabbit liver (12–14) and rat liver (7, 11).

In this paper we report on the production and properties of antibody prepared against cytochrome P450 from phenobarbital-treated rats. This antibody has been used together with antibody prepared against rat cytochrome P448 (15, 16) to demonstrate four antigenically different forms of cytochrome P450 in the rat PB-P450[1] preparation and two different forms in the rat MC-P448 preparation.

[1] The abbreviations used are: PB, phenobarbital; MC, 3-methylcholanthrene; IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; PB-P450, purified cytochrome P450 preparation isolated from livers of phenobarbital-treated rats, and anti-PB-P450, rabbit IgG prepared against it; MC-P448, purified cytochrome P448 preparation isolated from livers of 3-methylcholanthrene-treated rats, and anti-MC-P448, rabbit IgG prepared against it.

METHODS

Enzyme preparation. Hepatic cytochrome P450 from phenobarbital-treated rats and hepatic cytochrome P448 from 3-methylcholanthrene-treated rats (Long-Evans strain, male, 50–60 g, Blue Spruce Farms, Altamont, N.Y.) were detergent-solubilized and purified through step IV (17) or step V (11) as indicated.

Hepatic cytochrome P448 from 3-methylcholanthrene-treated rabbits was solubilized and purified by the method of Kawalek *et al.* (14). Purified hepatic cytochrome P450 LM_2 from phenobarbital-treated rabbits, cytochrome P450 from *Pseudomonas putida,* and beef adrenal mitochondrial cytochrome P450 were kindly given to us by Drs. Minor Coon, Julian Peterson, and David Y. Cooper, respectively.

Hepatic microsomes from phenobarbital-treated male Long-Evans rats were used as the starting material for the purification of NADH–cytochrome b_5 reductase (18), cytochrome b_5 (19), NADPH–cytochrome *c* reductase (20), and the lipid fraction (8).

Production and purification of antibody. Female New Zealand white rabbits were immunized intradermally along the flanks at 20 or more sites with 200 μg/rabbit of purified cytochrome P450 from phenobarbital-treated rats. The immunogen was diluted in 0.9% NaCl to 1 ml (for each rabbit), and a water-in-oil emulsion was produced by dropwise addition of the diluted immunogen to an equal volume of Freund's complete adjuvant while the oil phase was stirred at room temperature at 18,000 rpm in a VirTis homogenizer equipped with dual 1-cm-diameter blades. The emulsion was relatively stable, as evidenced by its minimal dispersion in water after 5 days at room temperature. Six weeks after immunization, rabbits were boosted with an additional intradermal injection of 40 μg of immunogen in Freund's complete adjuvant. An additional 4–5 weeks elapsed before rabbits were again boosted intravenously with 20 μg of the cytochrome P450 in NaCl but without adjuvant. Rabbits were bled 1 week after the final boost, and sera were collected. Thereafter rabbits were boosted every 4 weeks

with 20 μg of adjuvant-free antigen and bled 1 week later. The IgG fraction from the pooled sera with the highest titer was isolated at 4° by salt fractionation and DEAE-cellulose chromatography as detailed by Thomas *et al.* (15).

Antibody was made in rabbits to cytochrome P448 purified from 3-methylcholanthrene-treated rats, and the IgG fraction was isolated as previously described (15). Control IgG was isolated from control rabbit sera in an identical manner. Protein concentration was estimated by the method of Lowry *et al.* (21) with crystalline bovine albumin as the standard.

Ouchterlony double-diffusion analysis and quantitative immunoprecipitation. The immunodiffusion plates were made with 0.9% Agarose as previously described (15), except that the gel thickness was reduced from 2 mm to 1.5 mm. In some cases plates were stained for heme peroxidase activity, using tetramethylbenzidine–H_2O_2, by the method of Thomas *et al.* (22). Before being stained for peroxidase activity, the plates were first washed for 3–4 days with a solution containing 0.3 M NaCl, 0.05 M K_2HPO_4 (pH 8.0), and 0.015 M sodium azide, followed by several changes of water to remove salts and sodium azide.

Quantitative immunoprecipitation analysis of PB-P450 was performed by allowing tubes containing PB-P450 and either anti-PB-P450 or anti-MC-P448 to stand at 23° for 16 hr before the precipitate was collected by centrifugation. Precipitation was performed in a solution containing 0.4% Emulgen 911, 0.5 M glycine (pH 7.4), 0.043 M NaCl, and 0.015 M sodium azide. Protein in the precipitates was quantitated (21) after washing the precipitates three times with phosphate-buffered NaCl and dissolving the precipitates in 0.1 N NaOH.

Metabolism studies with reconstituted system. All the catalytic activity data were obtained with the same hemeprotein preparations purified through step IV. The specific contents were 17.1 nmoles/mg of protein for rat PB-P450 and 14.7 nmoles/mg of protein for rat MC-P448 (11). The indicated hemeprotein was initially incubated at 23° with the antibody for 5 minutes in a final volume of 0.25 ml in calcium- and magnesium-free phosphate-buffered NaCl at pH 7.4 (23). All tubes were made up to the same final antibody concentration with control antibody. Then the tubes were placed on ice, and NADPH–cytochrome *c* reductase and lipid were added, followed by the necessary cofactors and substrate to a final volume of 1.0 ml for all assays. The amounts of the various components used in each assay are listed in the appropriate figure legends, along with the turnover numbers for each hemeprotein.

Benzphetamine *N*-demethylation was assayed by a radiometric method using [^{14}C]benzphetamine HCl specifically labeled in the *N*-methyl position (15). The assays for the hydroxylation of benzo[*a*]pyrene and testosterone were performed as described by Lu *et al.* (8). The dealkylation of 7-ethoxycoumarin was assayed by the method of Jacobson *et al.* (24).

Materials. Agarose (Sea Kem) was purchased from Marine Colloids, Inc., Rockland, Me. Benzo[*a*]pyrene, NADPH, and crystalline bovine serum albumin were obtained from Sigma Chemical Company. Freund's complete adjuvant was purchased from Grand Island Biological Company; 7-ethoxycoumarin and 7-hydroxycoumarin, from Aldrich Chemical Company; and dilauroylphosphatidylcholine, from Serdary Research Laboratories, London, Ont. [*N-methyl*-^{14}C]Benzphetamine (2.63 mCi/mmole), [^{14}C]formaldehyde (10 mCi/mmole), and [4-^{14}C]testosterone (5.75 mCi/mmole) were obtained from New England Nuclear Corporation. Emulgen 911 was obtained from Kao-Atlas, Ltd., Tokyo. All other chemicals were of the highest purity commercially available.

RESULTS

Ouchterlony and quantitative immunoprecipitation analysis. The specificities of anti-PB-P450 and anti-MC-P448 are shown in Table 1. Neither antibody reacts with three other micosomal electron transport components (NADPH–cytochrome *c* reductase, cytochrome b_5, and NADH–cytochrome b_5 reductase), nor does either antibody recognize cytochromes P450 isolated

TABLE 1

Reactivity of anti-PB-P450 and anti-MC-P448 with several purified proteins in Ouchterlony double-diffusion test

Ouchterlony double-diffusion analysis was performed on plates made with 0.9% Agarose as described in METHODS. The extent of precipitin band formation is recorded on a three-point scale, with +++ indicating the precipitin band formed with the homologous antigen and – indicating no visible precipitation either in the Ouchterlony gel test with or without Emulgen 911 or in the capillary diffusion test.

Protein	Concentration	Relative extent of precipitin band formation	
		Anti-PB-P450	Anti-MC-P448
Rat liver microsomal proteins			
Cytochrome PB-P450, step IV	20 μM	+++	+
Cytochrome PB-P450, step V	20 μM	+++	+
Cytochrome MC-P448, step IV	20 μM	+	+++
Cytochrome MC-P448, step V	20 μM	+	+++
Cytochrome b_5	20 μM	–	–
NADH-cytochrome b_5 reductase	2 mg/ml	–	–
NADPH-cytochrome *c* reductase	2 mg/ml	–	–
Cytochromes P450 from other sources			
Rabbit liver cytochrome PB-P450 LM_2	20 μM	+	±
Rabbit liver cytochrome MC-P448	20, 40 μM	–	+
Beef adrenal mitochondrial cytochrome P450	20 μM	–	–
P. putida cytochrome P450	20, 50, 100 μM	–	–

from two other sources, *P. putida* and beef adrenal mitochondria. Anti-PB-P450 does cross-react to some extent with rat MC-P448 as well as with rabbit PB-P450 LM_2, but not with rabbit MC-P448. The lack of reaction with many functionally similar proteins, as well as its pattern of cross-reaction, clearly demonstrates the specificity of anti-PB-P450.

To eliminate the possibility that the lack of reactivity in the Ouchterlony double-diffusion test was a result of poor antigen diffusibility due to large molecular weight aggregates, two additional tests were performed on all proteins in Table 1. First, antibody, then antigen, was introduced into a glass capillary at different antigen to antibody ratios, the tube was sealed, and the presence or absence of an immune precipitate was noted during the next 48 hrs. In this capillary test system, gel was not present to hinder diffusion. Second, 0.2% Emulgen 911, a nonionic detergent, was incorporated into the 0.9% Agarose gel plates. In both alternative test systems, those antigens reacting in the standard Ouchterlony test system gave a positive reaction, while those which were negative were also negative in these two tests.

In the course of this study PB-P450 and MC-P448 were further purified to apparent homogeneity (step V) (11). Comparison of the more purified step V with step IV heme-protein preparations using the homologous antibodies revealed no quantitative or qualitative differences (Table 1 and Fig. 1). PB-P450 at both steps IV and V reacts with anti-PB-P450 to give single immunoprecipitin bands which show a line of identity. Rat MC-P448 preparations at steps IV and V also cross-react with anti-PB-P450 to give single distinct bands having a line of identity (Fig. 1). Rat MC-P448, however, gives a markedly less intense precipitin band, suggesting that not all the heme-protein cross-reacts with the antibody. That portion which cross-reacts appears partially identical with rat PB-P450, as judged from the prominent spur at the intersection of the two precipitin bands. Figure 1 also shows the absence of reactivity of rabbit MC-P448 with anti-PB-P450 as well as the poor cross-reaction of this antibody with rabbit PB-P450 LM_2. Rabbit PB-P450 LM_2 shows partial identity with rat PB-P450 (Fig. 1), as well as partial identity with rat MC-P448 upon reaction with anti-PB-P450 (results not shown).

Peroxidase activity of immune precipi-

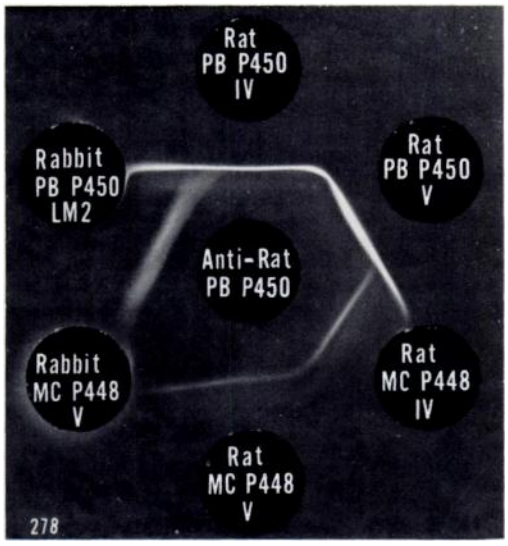

FIG. 1. *Ouchterlony immunodiffusion plate with 0.38 mg of anti-rat PB-P450 in center well*

All wells were 4 mm in diameter and filled with the indicated protein. Numbering clockwise from the top, wells 1 and 2 contained 20 μM rat PB-P450 purified through steps IV and V, respectively. Wells 3 and 4 contained 20 μM rat MC-P448 purified through steps IV and V, respectively. Well 5 was filled with 20 μM rabbit MC-P448 purified through step V, and well 6 was filled with 14 μM rabbit cytochrome P450 LM_2 isolated from PB-treated rabbits.

tates and comparison of Agarose with agar as a gel support medium. To confirm that the precipitin bands observed in Ouchterlony double-diffusion plates contained hemeprotein, the plates were stained for peroxidase activity with tetramethylbenzidine and H_2O_2 (22). Figure 2A shows an unstained Ouchterlony plate with anti-PB-P450 in the central well, while Fig. 2B shows the same plate after staining for peroxidase. It can be clearly seen that the homologous as well as the heterologous precipitin bands contain hemeprotein.

Wells of Ouchterlony plates shown in Fig. 2C and D were filled with the same proteins and quantities and stained for peroxidase at the same time as those shown in Fig. 2B. The only difference between the plates is that agar was used as the gel support in plates C and D, while Agarose was used in plates A and B. As can be seen in Fig. 2C, 0.9% agar diminishes the amount of antigen available for immune complex formation with both the homologous and heterologous antigens. Increasing the agar concentration to 2% (the amount commonly used in commercially available plates) further decreases the amount of antigen diffusing into the gel, as shown by the increased curvature of the immunoprecipitin bands toward the antigen wells and by diminished tetramethylbenzidine–H_2O_2 staining in Fig. 2D. It is probably the negatively charged agaropectin present in agar but absent from Agarose that either binds or in some way hinders free diffusion of these solubilized microsomal hemeproteins. Clearly Agarose is superior to agar as a gel support medium in Ouchterlony double-diffusion analysis with these detergent-solubilized microsomal cytochromes P450, and one should be cautious in interpreting results from agar immunodiffusion plates with these proteins.

Ouchterlony results using both anti-PB-P450 and anti-MC-P448. Figures 1 and 2 show that when rat MC-P448 reacts with anti-PB-P450, the resulting precipitin band contains much less protein than the precipitin band formed with the homologous antigen, whether the plates are stained for protein (not shown) or for peroxidase activity (Fig. 2B). Since both hemeproteins are present in identical concentrations and the precipitin band position indicates nearly equivalence conditions, the most likely interpretation for the diminished reactivity of MC-P448 is that it contains a form (or forms) not recognized by anti-PB-P450. This hypothesis was tested in the following way. Anti-MC-P448 and anti-PB-P450 were placed next to each other on an Ouchterlony plate opposite MC-P448. In this arrangement (left half of Fig. 3), both the homologous and heterologous antibodies can "look" at the antigen. A form missed by the heterologous antibody (anti-PB-P450) should be seen by the homologous antibody (anti-MC-P448) and appear as a spur. The predicted spur can be seen in the left half of Fig. 3. We have termed the precipitin band recognized by anti-PB-P450 "ω," and the

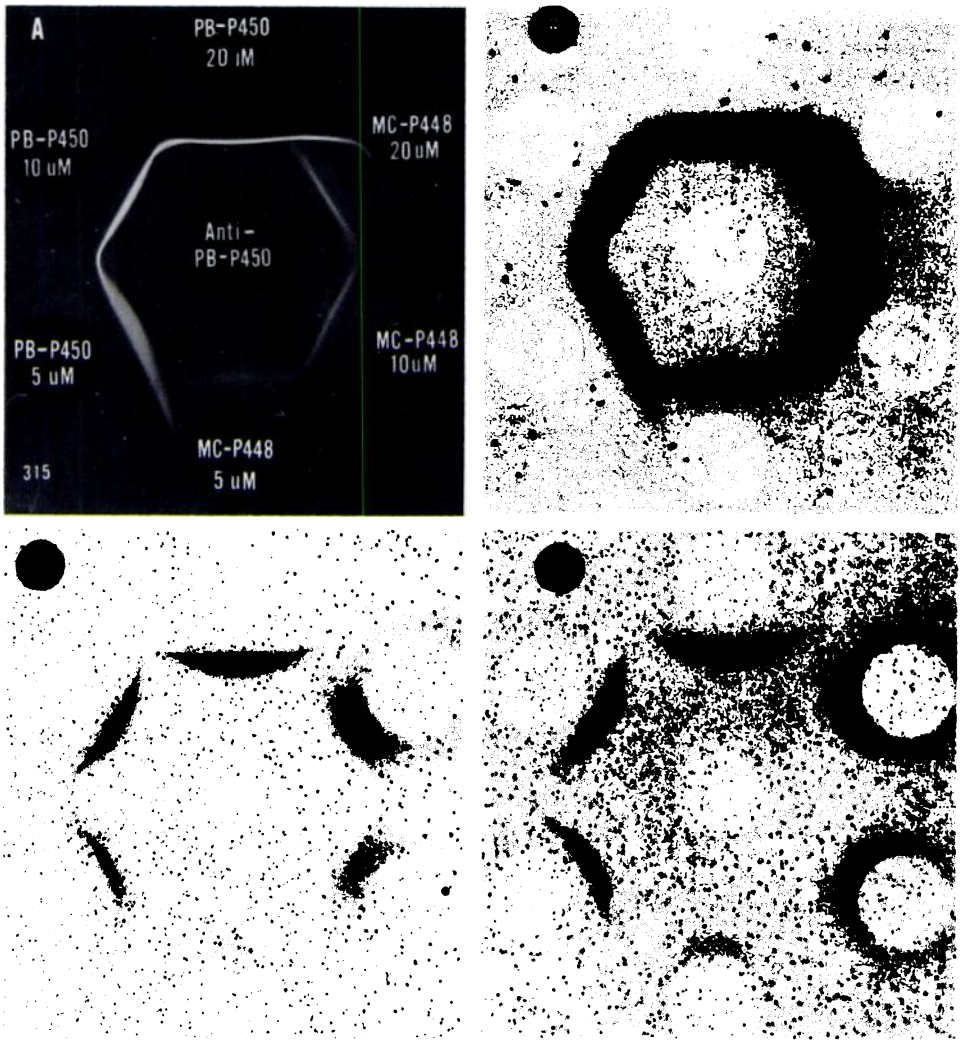

FIG. 2. *Comparison of Agarose and agar as support matrices for Ouchterlony immunodiffusion plates of rat microsomal hemeproteins*

Plates A and B were made with 0.9% Agarose, while plates C and D were made with 0.9% and 2.0% agar, respectively. Plate B is the same plate as A except that it has been stained with H_2O_2–tetramethylbenzidine, as have plates C and D. Peripheral wells of all plates were filled with the same rat proteins, purified through step IV (17), as was plate A. The central wells of all plates were filled with anti-PB-P450.

spur recognized by anti-MC-P448 after anti-PB-P450 has removed those antigens it recognized, as "ψ." The results show that the immunoprecipitin band observed with anti-MC-P448 and its antigen is composed of at least two immunochemically distinguishable but related proteins. The right half of Fig. 3 shows a similar splitting of the homologous precipitin band (anti-PB-P450 vs. PB-P450) by anti-MC-P448. We have previously reported on the presence of three heme-staining bands, labeled α, β, and γ, which result from the cross-reaction of PB-P450 with anti-MC-P448 (15). The α band, not clearly shown here, is best seen with the higher concentrations of PB-P450 (15). (A lower concentration of PB-P450 was used in this plate to prevent blurring of the precipitin band, which occurs when antibody reacts with large

amounts of homologous antigen.) A fourth band, termed δ, which is precipitated by anti-PB-P450 after anti-MC-P448 has removed those antigens it recognizes, can be seen with this arrangement of antibodies. For the most part, the δ band is not recognized by anti-MC-P448, nor is the ψ band recognized by anti-PB-P450; therefore these bands share no antigenic sites. The other bands (α, β, γ, and ω) are recognized by both antibodies and therefore have some common antigenic sites while differing at other antigenic sites. It is unlikely that ω is the same as α, β, or γ, because benzo[a]pyrene hydroxylase activity of PB-P450 is not due to contamination with MC-P448 (8, 25). All bands labeled with Greek letters have peroxidase activity. If step V instead of step IV MC-P448 and PB-P450 are used, the γ band is no longer observable, suggesting that it has been removed by further purification or that its equivalence zone has moved into another immunoprecipitin band.

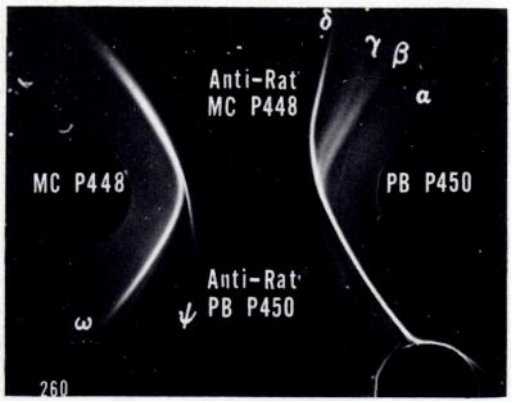

FIG. 3. *Ouchterlony immunodiffusion plate using both antibodies and both rat hemeprotein preparations purified through step IV*

The left well contained 20 μM MC-P448, and the right well contained 20 μM PB-P450. High-contrast film (Kodak 4155) was used to photograph this plate, in order to enhance the intensity of the α, β, and γ bands relative to the δ band. As a consequence, the magnitude of cross-reaction of MC-P448 with anti-PB-P450 compared with its reaction with the homologous antibody is exaggerated relative to that shown in Fig. 1.

Ouchterlony double-diffusion analysis is capable of showing only gross quantitative differences, such as the large difference in the amount of PB-P450 precipitated by anti-PB-P450 compared to the amount precipitated by anti-MC-P448. Quantitative immunoprecipitation is another technique which can be used to study difference in reactivity of the two antibodies with PB-P450. In Fig. 4 the concentration of PB-P450 was kept low (0.2 nmole/tube) in order to examine the region of the curve at high antibody to hemeprotein ratios. With both the homologous and heterologous antibodies, the amount of protein precipitated is nearly maximal at 50 mg of anti-

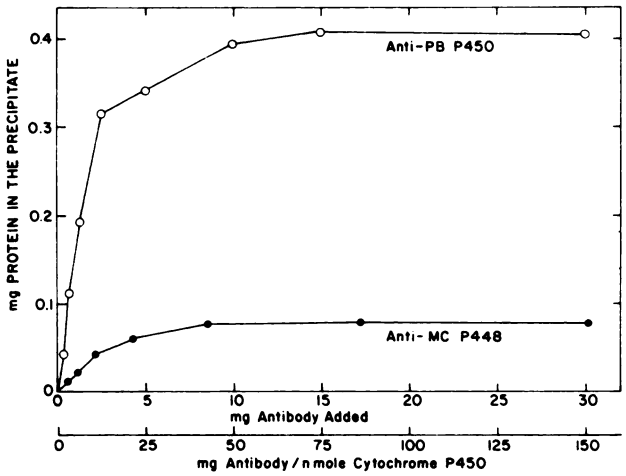

FIG. 4. *Quantitative immunoprecipitation of rat PB-P450 by anti-PB-P450 and anti-MC-P448*

Precipitation was carried out at 23° in the presence of 0.4% Emulgen 911, as given under METHODS. Each tube contained 0.2 nmole of PB-P450.

body per nanomole of PB-P450. In both cases further addition of antibody results in no further increase in the immunoprecipitate, indicating that the number of sites recognized by anti-MC-P448 never approaches that recognized by the homologous antibody. The maximum amount of protein precipitated with anti-MC-P448 is only 20% of that precipitated with anti-PB-P450.

Effect of anti-PB-P450 on metabolism by reconstituted PB-P450 and MC-P448 systems. The *N*-demethylation of benzphetamine is inhibited more by the anti-PB-P450 antibody when the reaction is supported by the homologous hemeprotein PB-P450 then when supported by MC-P448 (Fig. 5; hemeprotein turnover numbers appear in the figure legend). The reaction is completely inhibited at 4 mg of antibody per nanomole of PB-P450, whereas the MC-P448-supported demethylation is only 50% inhibited at this ratio. In the MC-P448-supported reaction, increasing the antibody to hemeprotein ratio beyond 4 mg/nmole results in an additional 20% inhibition but causes no further inhibition at even higher antibody concentrations, suggesting that some of the MC-P448-supported metabolism is anti-PB-P450-insensitive.

Ethoxycoumarin *O*-deethylation (Fig. 6) is also more sensitive to antibody inhibition when catalyzed by PB-P450. At 12 mg of antibody per nanomole of hemeprotein, there is complete inhibition of the PB-P450-supported reaction but only 42% inhibition of the MC-P448-supported reaction. Only slightly more inhibition of the MC-P448-supported reaction is obtained with an 8-fold increase in antibody concentration, again suggesting that some of the MC-P448-dependent activity is insensitive to anti-PB-P450. Since very low concentrations of MC-P448 or PB-P450 were used in this assay, the highest antibody to hemeprotein ratio used is much greater (100 mg/nmole) than that used in the other reactions described (20 or 40 mg/nmole).

The effect of anti-PB-P450 on the MC-P448- and PB-P450-supported hydroxylation of benzo[*a*]pyrene is shown in Fig. 7. In contrast to the former two inhibition patterns, the PB-P450-supported hydroxylation is not completely inhibited by anti-PB-P450. However, maximal inhibition oc-

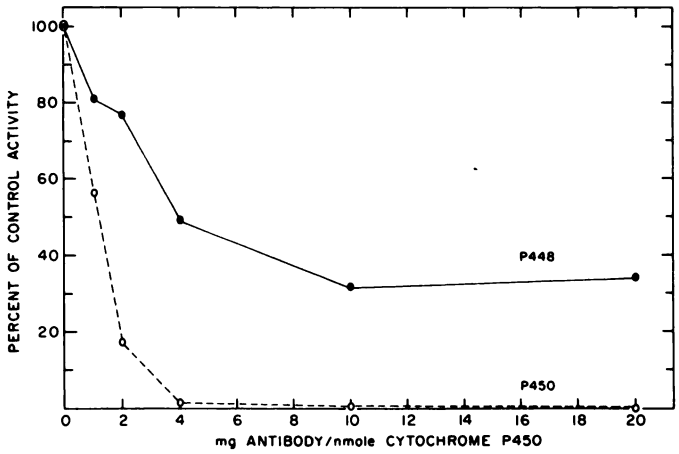

FIG. 5. *Effect of varying concentration of anti-PB-P450 on benzphetamine N-demethylation catalyzed by reconstituted rat liver microsomal system*

The incubation contained the following components in a total volume of 1 ml: 0.25 nmole of PB-P450 or MC-P448, 200 units of NADPH-cytochrome *c* reductase (1 unit of activity equals 1 nmole of cytochrome *c* reduced per minute at 25°), 120 μg of crude rat liver microsomal lipid (8), 0.7 unit of glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate, 0.5 mM NADPH, 10 mM semicarbazide, 1 mM [^{14}C]-benzphetamine (200,000 cpm/tube), 10 mM $MgCl_2$, and 0.1 M potassium phosphate, pH 7.4. Substrate was added to start the reaction, and tubes were incubated at 37° for 5 min for PB-P450 and for 15 min for MC-P448. The turnover numbers for PB-P450 and MC-P448 were 40 and 3.2 nmoles of formaldehyde formed per nanomole of cytochrome per minute, respectively.

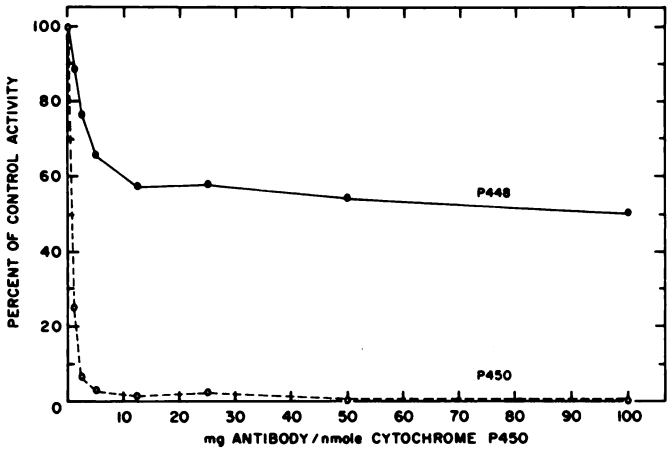

FIG. 6. *Effect of varying concentration of anti-PB-P450 on ethoxycoumarin O-deethylation catalyzed by reconstituted rat liver microsomal system*

The incubation mixture contained the following components in a final volume of 1 ml: 20 pmoles of PB-P450 or MC-P448, 250 units of NADPH-cytochrome *c* reductase, 20 μg of dilaurylphosphatidylcholine, 0.5 mM NADPH, 0.3 mM ethoxycoumarin, 3 mM $MgCl_2$, and 50 mM potassium phosphate, pH 7.4. Tubes containing PB-P450 and MC-P448 were incubated at 37° for 15 and 5 min, respectively. The turnover numbers, in nanomoles of 7-hydroxycoumarin formed per nanomole of cytochrome per minute, were 6.6 for PB-P450 and 34 for MC-P448.

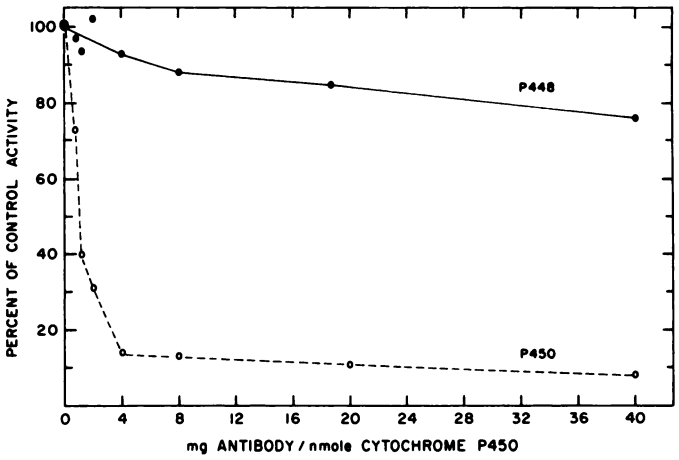

FIG. 7. *Effect of varying concentration of anti-rat PB-P450 on benzo[a]pyrene hydroxylation catalyzed by reconstituted rat liver microsomal system*

The incubation mixture contained the following components in a final volume of 1 ml: 0.13 nmole of rat PB-P450 or MC-P448, 200 units of NADPH-cytochrome *c* reductase, 100 μg of crude rat liver microsomal lipid, 0.4 mM NADPH, 0.08 mM benzo[*a*]pyrene, 0.8 mg of bovine serum albumin, 3 mM $MgCl_2$, and 0.1 M potassium phosphate, pH 6.8. Assays were initiated by the addition of substrate and incubated at 37° for 5 min. The turnover numbers, in nanomoles of benzo[*a*]pyrene hydroxylated per nanomole of cytochrome per minute, were 0.19 for PB-P450 and 3.9 for MC-P448.

curs at 4 mg of antibody per nanomole, which is the same ratio observed for the two previous substrates. Unlike the previous two reactions, the MC-P448-supported reaction is much less sensitive to antibody inhibition, and no comparable inhibition maximum is observed. In this reaction, as in the previous two reactions, the homologous antigen is more sensitive to antibody inhibition.

Among the substrates examined, testosterone is unique in that hydroxylation in three different positions can be followed simultaneously. All three hydroxylation reactions are similarly inhibited in the PB-P450-supported reaction (Fig. 8A). Maximal inhibition occurs between 2 and 4 mg of anti-PB-P450 per nanomole, which approximates that seen using the other substrates. In contrast, when MC-P448 is used to support hydroxylation of testosterone (Fig. 8B), the pattern of inhibition is not the same at the three positions. The formation of the 6β-hydroxylated product is inhibited minimally, while hydroxylation at the 7α- and 16α-positions is inhibited more than 90%. These results are consistent with at least two immunologically distinct forms of cytochrome in the MC-P448 preparation.

The results of anti-PB-P450 inhibition of catalytic activity can be summarized as follows. (a) Maximal inhibition is observed when the homologous antigen is used to

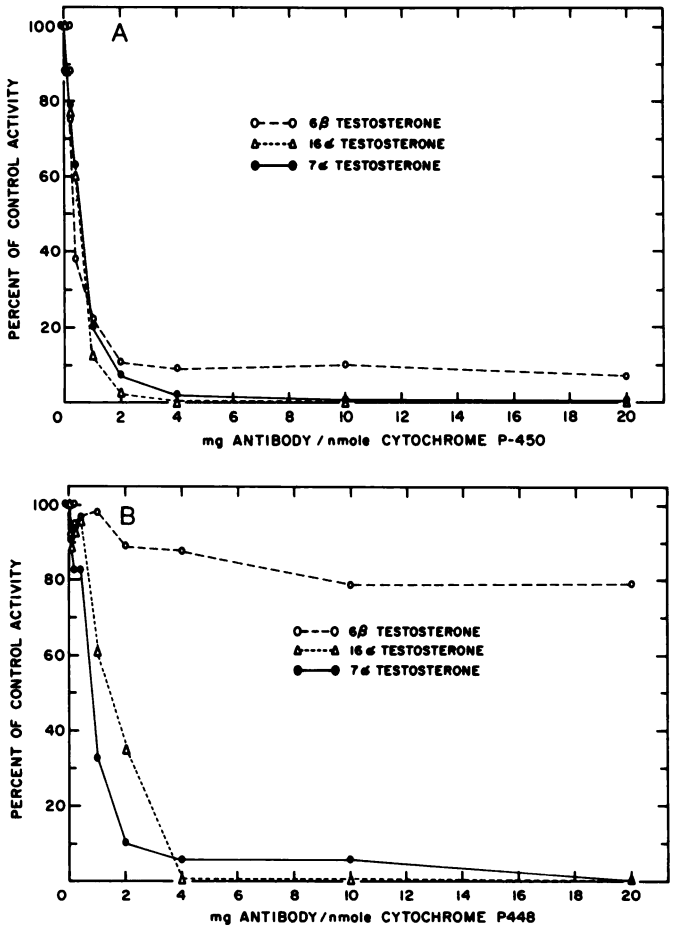

FIG. 8. *Effect of varying anti-PB-P450 concentration on testosterone hydroxylation catalyzed by rat PB-P450 (plate A) and rat MC-P448 (plate B) in reconstituted system*

The reaction mixture, with a final volume of 1 ml, contained the following components: 0.25 nmole of PB-P450 or MC-P448, 300 units of NADPH-cytochrome *c* reductase, 120 μg of crude rat liver microsomal lipid, 0.5 mM NADPH, 125 μM [^{14}C]testosterone, 5 mM glucose-6-phosphate, 0.7 units of glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$, and 0.1 M potassium phosphate, pH 7.4. Assays were initiated by the addition of testosterone and incubated for 10 min at 37°. Turnover numbers, in nanomoles of testosterone hydroxylated per nanomole of cytochrome per minute, were 0.97 for 7α-, 1.5 for 16α-, and 0.12 for 6β-positions using PB-P450, and 0.69 for 7α-, 0.17 for 16α-, and 0.25 for 6β-positions using MC-P448.

support metabolism. (b) Not only is metabolism supported by the heterologous antigen inhibited to a lesser extent, but in most cases a portion of the activity is resistant to inhibition. (c) That portion of the activity which is resistant to inhibition by antibody is dependent on the substrate used. These results indicate that the portion of the catalytic activity which is resistant to inhibition should be inhibited if the homologous antibody is used. Thus the effects of both antibodies, independently and in combination, on the metabolism of benzo[*a*]pyrene and benzphetamine were examined (Table 2). As was shown in Fig. 5, benzphetamine *N*-demethylation is inhibited more efficiently by anti-PB-P450 when the reaction is supported by PB-P450. Inhibition of the PB-P450-supported *N*-demethylation reaction (Table 2) by anti-MC-P448 is poor. At a ratio of 17.2 mg of anti-MC-P448 per nanomole of PB-P450, only 23% inhibition is observed, while a mixture of both antibodies (3.5 mg of anti-MC-P448 and 3.5 mg of anti-PB-P450 per nanomole) gives complete inhibition. When MC-P448 is used to support benzo[*a*]pyrene hydroxylation, it can be seen that anti-PB-P450 is a poor inhibitor of the reaction (Table 2) compared with anti-MC-P448. Only 8% inhibition is seen with 6.9 mg of anti-PB-P450 per nanomole of MC-P448, but 36% inhibition is obtained with 3.5 mg of anti-MC-P448 and 3.5 mg of anti-PB-P450 per nanomole of hemeprotein. These results show that the catalytic activity which is resistant to inhibition by the heterologous antibody is inhibited by the homologous antibody, demonstrating both the specificity of the two antibody preparations and the inherent differences between the two hemeprotein preparations.

TABLE 2

Inhibition of catalytic activity by anti-PB-P450 and anti-MC-P448

Both assays were performed using the reconstituted system. The conditions for the benzphetamine assay are given in the legend to Fig. 5, and the conditions for the benzo[*a*]pyrene assay are given in the legend to Fig. 7. When both antibodies were used together, they were first mixed and then the hemeprotein was added. The antibody and antigen were allowed to react for 5 min at 23° before being returned to ice for addition of the remaining reaction components. The turnover numbers for the uninhibited reactions, in nanomoles of product per minute per nanomole of cytochrome, were 37 for benzphetamine *N*-demethylation and 3.6 for benzo[*a*]pyrene hydroxylation.

Reaction	Anti-PB-P450	Anti-MC-P448	Inhibition
	mg antibody/nmole cytochrome P450		%
PB-P450-supported benzphetamine *N*-demethylation		3.5	1
		6.9	19
		12.1	24
		17.2	23
	3.5	3.5	99
	6.9	3.5	100
MC-P448-supported benzo[*a*]pyrene hydroxylation	3.5		6
	6.9		8
	17.6		19
	34.4		18
	3.5	3.5	36
	3.5	13.6	76
	3.5	31.2	87
	0	31.2	89

DISCUSSION

The antibody prepared against purified rat PB-P450 has been shown to be specific by its lack of cross-reaction or poor cross-reactivity with purified cytochromes P450 from other sources and its lack of recognition of three other proteins involved in the rat liver drug-metabolizing electron transport system. Anti-PB-P450 specificity is also demonstrated by the formation of a single immunoprecipitin band when tested by Ouchterlony analysis against its homologous antigen. Since it is specific, anti-rat PB-P450 can be used as was anti-MC-P448 (15) to give information on immunochemical relatedness or nonrelatedness of different forms of cytochrome P450 in the same or different animal species.

Rabbit cytochrome P448 cross-reacts poorly with the antibody prepared against rat cytochrome P448, suggesting that these hemeproteins have different antigenic sites (15). Likewise, these two hemeproteins have different cross-reactivities with anti-PB-P450, again suggesting different antigenic sites on these proteins. Kawalek *et al.* (14) have shown that these proteins also differ markedly in catalytic activity as well as molecular weight on

SDS–polyacrylamide gels. Thus rabbit cytochrome P448 and rat cytochrome P448 have been shown to be different hemeproteins as judged not only by their different catalytic and physical properties, but also by their different immunological properties. Similarly, rabbit PB-P450 LM_2 is immunochemically related to, but different from rat PB-P450. Rabbit PB-P450 LM_2 cross-reacts poorly with anti-rat PB-P450 and, based on a partial line of identity with rat PB-P450, shares some but not all antigenic sites with rat PB-P450. In addition, rabbit PB-P450 LM_2 (13) and rabbit MC-P448 (14) can also be shown to differ immunochemically from one another, based on their patterns of cross-reaction with the rat antibodies.

As can be seen from the results of this study with anti-PB-P450 and our previous study with anti-MC-P448 (15), rat MC-P448 and rat PB-P450 have different immunological properties, although they are immunochemically related, a property which has been used to show the presence of multiple forms of cytochrome P450 in each hemeprotein preparation. Each of these hemeprotein preparations contains more than one form of cytochrome, as indicated by the precipitin bands observed using the heterologous antibody alone or in combination with the homologous antibody. In Fig. 3 the small ψ spur seen with MC-P448 and the large δ spur seen with PB-P450 indicate that the cross-reaction in Ouchterlony plates of either antigen with the heterologous antibody is incomplete. In each case the precipitin band formed on Ouchterolony plates with the homologous antibody represents complete precipitation of all the hemeprotein, since quantitative immunoprecipitation studies have shown that all of the hemeprotein is precipitated by the homologous antibody. In addition, quantitative immunoprecipitation of rat PB-P450 indicated that the heterologous antibody recognizes only a small portion of the total hemeprotein.

We believe that the purified cytochrome MC-P448 and PB-P450 preparations contain a total of at least six different forms of cytochrome P450, for the following reasons. (a) Ouchterlony double-diffusion analyses show six immunochemically related precipitin bands, all of which are hemeproteins as judged by peroxidase activity. It is doubtful that functionally unrelated proteins (i.e., proteins other than cytochrome P450) would share antigenic sites. (b) The extent of inhibition of catalytic activity in the reconstituted system is dependent not only on the antibody used, but also on the substrate used. If each hemeprotein preparation were composed of one distinct hemeprotein, the inhibition pattern should be dependent only on the antibody used, not on the substrate studied. However, the inhibition of catalytic activity was found to be dependent on both the antibody and substrate examined. For example, anti-PB-P450 inhibition of MC-P448-supported demethylation of benzphetamine is inhibited to a greater extent than the hydroxylation of benzo[a]pyrene, while ethoxycoumarin O-deethylation is inhibited to an intermediate extent. Another example of dissimilar inhibition with anti-PB-P450 is seen in the MC-P488-supported hydroxylation of testosterone in three different positions. In this case hydroxylation at the 6β-position of testosterone is relatively resistant to inhibition by anti-PB-P450, whereas hydroxylation at the 16α- and 7α-positions is markedly inhibited. These results suggest that there are at least two forms of hemeprotein in the MC-P448 preparation, as do the results using both antibodies on Ouchterlony plates. As might be anticipated, and as can be seen from these results on the inhibition of metabolism, there is more variation in inhibition pattern with the heterologous antigen (MC-P448) than with the homologous antigen (PB-P450). In our previous study of anti-MC-P448 (15), more variation in the inhibition pattern was also observed when the heterologous antigen (PB-P450) was used. The pattern of inhibition of PB-P450-supported hydroxylation of testosterone by anti-MC-P448 indicated that there were at least three antigenically dissimilar hemeproteins in the PB-P450 preparation. Recently Welton *et al.* (26) also presented immunological evidence for multiple forms of cytochrome P450 in rat liver microsomes. They de-

scribed an antibody to a trypsin-resistant, partially purified cytochrome P450 preparation from phenobarbital-treated rats which did not cross-react with two other microsomal hemeproteins as demonstrated by SDS-gel electrophoresis. In conclusion, both Ouchterlony data and inhibition of catalytic activity by anti-PB-P450 and anti-MC-P448 show that there are multiple forms of cytochrome P450 in animals treated with different inducers as well as in rats treated with the same inducer.

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