MOLECULAR PHARMACOLOGY, 24:146–155

A High-Spin Form of Cytochrome P-450 Highly Purified from Polychlorinated Biphenyl-Treated Rats

Catalytic Characterization and Immunochemical Quantitation in Liver Microsomes

TETSUYA KAMATAKI,[1] KAORI MAEDA,[2] YASUSHI YAMAZOE,[1] NAOMI MATSUDA,[1] KENJI ISHII,[3] AND RYUICHI KATO[1]

Department of Pharmacology, School of Medicine, Keio University, Tokyo 160, and Department of Pharmaceutics, Hoshi Institute of Pharmaceutical Sciences, Tokyo 142, Japan

Received January 10, 1983; Accepted April 1, 1983

SUMMARY

A high-spin form of cytochrome P-450 (termed PCB P-448-H) was purified from liver microsomes of polychlorinated biphenyl (PCB)-treated rats to homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This high-spin form of cytochrome P-450 was distinguishable from a low-spin form of cytochrome P-450 (PCB P-448-L) purified from microsomes of PCB-treated rats by the criteria of molecular weights, peptide mapping, and immunochemical properties. In addition, PCB P-448-H catalyzed the hydroxylation of acetanilide (position 4) and biphenyl (positions 2 and 4), and *N*-hydroxylation of the promutagens 3-amino-1-methyl-5*H*-pyrido(4,3-*b*)indole (Trp-P-2), 2-amino-6-methyl-dipyrido(1,2-*a*:3′,2′-*d*)imidazole (Glu-P-1), 2-aminofluorene, and 4-aminobiphenyl at much faster rates than did PCB P-448-L. These promutagens and aflatoxin B_1 were efficiently metabolized to mutagens by this high-spin form of hemoprotein. Rabbit immunoglobulin G (IgG) raised against PCB P-448-H inhibited the microsomal *O*-depropylation activity of *p*-propoxyaniline. Radial immunodiffusion assay with the IgG showed that PCB P-448-H was one of the major forms of cytochrome P-450 in liver microsomes of PCB-treated rats. On the basis of these results, we propose that this high-spin form of cytochrome P-450 is a key enzyme activating a variety of environmental promutagens in the 9000 × *g* supernatant fraction of PCB-treated rats, which has been widely used as an activation system in routine mutation tests.

INTRODUCTION

Hepatic microsomal cytochrome P-450[4] catalyzes the oxidation and reduction of a number of endogenous and exogenous substrates. Recent studies have shown that there are multiple isozymes of cytochrome P-450 in microsomes. The isozymes are induced by pretreatment of experimental animals with drugs and chemical compounds such as phenobarbital, 3-methylcholanthrene, and PCBs.[5] Among inducers, PCBs are nonspecific inducers of cytochrome P-450, inducing multiple forms of cytochrome P-450. To test the mutagenicity of environmental compounds, the 9000 × *g* supernatant fraction of liver homogenates of PCB-treated rats has been widely used as an activation system. Using the 9000 × *g* supernatant fraction of PCB-treated rats, Sugimura and his co-workers (1) have discovered more than 10 promuta-

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan. Portions of this work were presented at the Fifth International Symposium on Microsomes and Drug Oxidations, Tokyo, July 1981.

[1] Department of Pharmacology, School of Medicine, Keio University.

[2] Department of Pharmaceutics, Hoshi Institute of Pharmaceutical Sciences.

[3] Department of Pharmacology, School of Medicine, Keio University. Present address, Department of Toxicology and Microbiology, Science University of Tokyo.

[4] The term cytochrome P-450 was used to designate any or all forms of liver microsomal cytochrome P-450 and the term cytochrome P-448 was used to designate the specific forms of cytochrome P-450 which show peaks at around 448 nm in carbon monoxide difference spectra.

[5] The abbreviations used are: PCB, polychlorinated biphenyl; Trp-P-1, 3-amino-1,4-dimethyl-5*H*-pyrido(4,3-*b*)indole; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido(4,3-*b*)indole; Glu-P-1, 2-amino-6-methyl-dipyrido(1,2-*a*:3′,2′-*d*)imidazole; Glu-P-2, 2-aminodipyrido(1,2-*a*:3′,2′-*d*)imidazole; IQ, 2-amino-3-methylimidazo(4,5-*f*)quinoline; Buffer A, 10 mM potassium phosphate buffer (pH 7.4) containing 0.5% sodium cholate, 0.2% Emulgen 911, 0.1 mM EDTA, 1.0 mM dithiothreitol, and 20% glycerol; Buffer B, 200 mM potassium phosphate buffer (pH 7.4) containing 0.5% sodium cholate, 0.5% Emulgen 911, 0.1 mM EDTA, 1.0 mM dithiothreitol, and 20% glycerol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography.

0026-895X/83/040146-10$02.00/0

gens in pyrolysates of amino acids and proteins. The mutagenicities of these promutagens are quite dramatic: the mutagenicity of Trp-P-2, as calculated on a microgram basis, is reportedly several hundred times higher than benzo[*a*]pyrene. Previous papers from our laboratory showed that the metabolic activation of Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2 to mutagens is catalyzed by cytochrome P-450 in liver microsomes of PCB-treated rats (2, 3). The metabolic activation of these promutagens was apparently catalyzed by low-spin forms of cytochrome P-448 purified from 3-methylcholanthrene and PCB-treated rats at faster rates than by the other forms of cytochrome P-450 purified from phenobarbital and PCB-treated rats. These results were in accordance with those using microsomes; microsomal activities were induced by pretreatment of the animals with 3-methylcholanthrene and PCB but not with phenobarbital. Despite these results, the activities of the low-spin forms of cytochrome P-448 did not fully account for such high mutagen-producing activities of microsomes from 3-methylcholanthrene- and PCB-treated rats. Thus, in our preliminary communication (4), we raised the possibility that another form of cytochrome P-448 might play a major role in the activation of these promutagens. In the present paper, we confirm that a high-spin form of cytochrome P-448 (PCB P-448-H) purified from microsomes of PCB-treated rats metabolizes these and other well-known promutagens to mutagens at faster rates than a low-spin form of cytochrome P-448 (PCB P-448-L) purified from PCB-treated rats, and that PCB P-448-H is one of the major forms of cytochrome P-450 in liver microsomes of PCB- and 3-methylcholanthrene-treated rats.

MATERIALS AND METHODS

Preparation of microsomes. Male and female rats of the Sprague-Dawley strain (8–11 weeks old) were used throughout this study. PCB (Kaneclor 500), dissolved in corn oil, was given to rats. i.p. 7 days before sacrifice. 3-Methylcholanthrene (dissolved in corn oil) and sodium phenobarbital (dissolved in distilled water) were given i.p. at doses of 40 and 80 mg/kg, respectively, once each day for 3 days, and the animals were deprived of food 18 hr before being killed. Liver microsomes were obtained by differential centrifugation. Microsomes were washed once by resuspension in 1.15% KCl and centrifugation.

Purification of PCB P-448-H and PCB P-448-L from liver microsomes of PCB-treated rats. Liver microsomes (3–3.5 g) from PCB-treated male rats were solubilized with sodium cholate and applied to a column of ω-amino-*n*-octyl Sepharose 4B (5). Cytochrome P-450 was eluted into two peaks (Fractions I and II) by washing of the column with Buffers A and B, respectively, as described in a preceding paper (6). PCB P-448-H was eluted in Fraction II. The pooled Fraction II was dialyzed against Buffer A. The dialyzed sample was applied to a column of Whatman DE-52 (3.2 × 45 cm) equilibrated with Buffer A. The fractions containing PCB P-448-H eluted by washing with Buffer A were pooled, diluted to give a concentration of potassium phosphate of 5 mM, and then applied to the second Whatman DE-52 column (3.2 × 45 cm), equilibrated with 5 mM potassium phosphate containing 20% glycerol and other components as for Buffer A, which is simply referred to as 5 mM Buffer A. The column was washed extensively (2000 ml) with 5 mM Buffer A. Optical density ratios of the absorbance at 393 nm to that at 416 nm were calculated. The fractions with ratios greater than 1.5 were combined and applied to a column of hydroxylapatite (3.5 × 8.5 cm) equilibrated with 5 mM Buffer A. The column was washed with one column volume of 10 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol, then with 300 ml of 100 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol. PCB P-448-H was eluted by increasing the phosphate concentration to 150 mM. The combined fraction (about 60 ml) was diluted 3-fold with 20% glycerol and applied to the second column of hydroxylapatite (3.5 × 5 cm) equilibrated with 50 mM potassium phosphate (pH 7.25) contianing 20% glycerol. The column was washed with one column volume of the same buffer as for the equilibration. PCB P-448-H was eluted by increasing the phosphate concentration to 500 mM. The elutates were combined and dialyzed overnight against 50 mM potassium phosphate (pH 7.4) containing 20% glycerol. A low-spin form of cytochrome P-450 (PCB P-448-L) was purified by a method which will be described in detail elsewhere. The carbon monoxide difference spectrum of PCB P-448-L showed an absorption maximum at 447 nm, and the molecular weight, estimated on SDS-PAGE, was about 56,000. The specific content of PCB P-448-L was 13.5 nmoles/mg of protein.

Purification of NADPH-cytochrome P-450 reductase and cytochrome b_5. NADPH-cytochrome P-450 reductase was purified from liver microsomes of phenobarbital-treated rats by the method of Yasukochi and Masters (7) with minor modifications. The specific activity of the purified reductase was 50 units/mg of protein. The unit activity of NADPH-cytochrome P-450 reductase was defined as the amount which reduced 1 μmole of cytochrome *c* per minute. Cytochrome b_5 was purified from liver microsomes of untreated male rats as described previously (6). The specific content of cytochrome b_5 ranged from 45 to 47 nmoles/mg of protein. These purified preparations of enzymes were free of any detectable activities of other undesired enzymes in liver microsomes as contaminants.

SDS-PAGE of PCB P-448-H and PCB P-448-L. To determine the molecular weight of PCB P-448-H, and to compare the peptide maps of PCB P-448-H and PCB P-448-L, SDS-PAGE was carried out by the method of Laemmli (8). The concentrations of acrylamide were 8.5% and 15% for the determination of molecular weight and for the comparison of peptide maps, respectively. The limited digestion of cytochrome P-448 was performed by the method essentially as described by Cleveland *et al.* (9). Briefly stated, 50 μg each of the cytochrome preparations were digested with chymotrypsin (0.04 mg/mg of cytochrome P-448 preparation) at 37° for 30 min; peptides were then analyzed on SDS-PAGE.

Preparation of rabbit IgG toward PCB P-448-H. Antibodies to PCB P-448-H were raised in rabbits, and IgG fractions were prepared following the procedure described previously (5). The IgG cross-reacted with PCB P-448-L, as noted by Reik *et al.* (10) with anti-P-450d. Thus, the components of IgG cross-reacting with P-448-L were removed. The IgG fraction was diluted with the same amount of 20 mM potassium phosphate buffer (pH 7.4) containing 0.4 M potassium chloride, 0.4 mM EDTA, and 20% glycerol, and applied on a *n*-octyl Sepharose 4B column (2.5 × 10 cm) on which approximately 200 nmoles of partially purified PCB P-448-L was adsorbed. The partially purified PCB P-448-L preparation was obtained by application of solubilized microsomes from PCB-treated rats to ω-amino-*n*-octyl Sepharose 4B, Whatman DE-52, and hydroxylapatite columns as described elsewhere. The *n*-octyl Sepharose 4B column had been equilibrated and was washed with 10 mM potassium phosphate (pH 7.4) containing 0.2 M potassium chloride, 0.2 mM EDTA, and 20% glycerol. Fractions containing protein eluted near the void volume were combined, concentrated on an Aminco ultrafiltration apparatus equipped with a PM-30 membrane, and dialyzed against 100 times the volume of 5 mM Hepes (pH 7.4.). The IgG preparation was centrifuged at 3000 rpm at 4°. The IgG preparation thus partially purified did not form visible precipitation bands with PCB P-448-L and other cytochrome P-450 preparations purified from liver microsomes of PCB-treated and phenobarbital-treated rats on an Ouchterlony double-diffusion plate (11). However, this IgG reacted to form a fused precipitation band with partially purified cytochrome P-450 eluted earlier than PCB P-448-H at the second Whatman DE-52 column step. This cytochrome P-450 fraction,

a minor fraction, did not contain PCB P-448-H as judged by the absolute spectrum. The IgG thus obtained was used for the immunochemical quantitation of PCB P-448-H in microsomes of variously treated rats.

Assay methods. Protein was determined by the method of Lowry *et al.* (12), with bovine serum albumin as the standard. Cytochrome P-450 was determined according to the method of Omura and Sato (13), except that 20% glycerol and 0.2% Emulgen 911 or 913 were included in all determinations. NADPH-cytochrome P-450 reductase activity was measured by the method of Phillips and Langdon (14). Cytochrome b_5 was determined using sodium dithionite as a reducing reagent.

An incubation mixture (in a final volume of 0.5 ml) for the assay of aromatic ring hydroxylations of benzo[a]pyrene, aniline, coumarin, acetanilide and biphenyl, *O*-deethylation of *p*-nitrophenetole, and *N*-hydroxylations of Trp-P-2, Glu-P-1, 2-aminofluorene, and 4-aminobiphenyl contained 0.1 nmole of cytochrome P-448, 0.5 unit of NADPH-cytochrome P-450 reductase, 15 μg of dilauroyl-L-3-phosphatidylcholine, 1% sodium cholate, 50 mM Hepes (pH 7.4), an NADPH-generating system (0.8 mM NADP, 8.0 mM glucose 6-phosphate, 0.5 unit of glucose 6-phosphate dehydrogenase, and 6.0 mM magnesium chloride), and a substrate. Unless otherwise indicated, the substrate concentrations added to the incubation mixture were as follows: 0.5 mM Trp-P-2 and Glu-P-1, 1.0 mM 2-aminofluorene and 4-aminobiphenyl, 0.08 mM benzo[a]pyrene, 3 mM acetanilide, and 2 mM biphenyl. Incubations were carried out aerobically at 37° for 10 min, except in the assays of oxidations of aniline, *p*-nitrophenetole, and coumarin. In these cases the mixtures were incubated for 15 min. Oxidative metabolism of aniline, *p*-nitrophenetole, and coumarin was measured by determination of *p*-aminophenol (15), *p*-nitrophenol (16), and 7-hydroxycoumarin (17), respectively. Benzo[*a*]pyrene hydroxylase activity was fluorometrically measured as described by Nebert and Gelboin (18). Biphenyl hydroxylations at positions 2 and 4 were measured with HPLC as reported previously (19). The *N*-hydroxylations of Trp-P-2 (20) and Glu-P-1 (3) were measured with HPLC as described in previous papers. Activity of acetanilide 4-hydroxylase was determined with HPLC by a similar method as described previously (21), except that the acetonitrile-treated supernatant fraction of the incubation mixture was directly applied to a reverse-phase column. Peaks of 4-hydroxyacetanilide, 4-aminoacetophenone, and acetanilide were eluted at their retention times of 4.2 min, 9.4 min, and 12.0 min, respectively, when CH_3CN/0.02 M $NH_4H_2PO_4$ (1:5) was used as the mobile phase. *N*-Hydroxylase activities of 2-aminofluorene and 4-aminobiphenyl were measured by HPLC methods similar to that for Trp-P-2 *N*-hydroxylation (21), except that a mobile phase of CH_3CN-H_2O (6:4) was used at a flow rate of 1.2 ml/min. Quantitation was done by UV absorbance measurements at 254 nm, and synthetic *N*-hydroxy compounds were used as standards.

Salmonella typhimurium was grown in 0.5% sodium chloride-containing nutrient broth to the log phase. When needed, the organisms were collected by centrifugation and resuspended in the same volume of 5 mM phosphate buffer (pH 7.2) containing 0.9% sodium chloride at a concentration of about 1×10^9 organisms per milliliter. The incubation mixture for the assay of mutagen-producing activities consisted of similar components as for the measurement of drug oxidation activities in a final volume of 0.5 ml, except that 0.5 mg of albumin and 1.0 unit rather than 0.5 unit of glucose 6-phosphate dehydrogenase were added. After the incubation, a portion (20 μl for Trp-P-2 and Glu-P-1, 50 μl for 2-aminofluorene, 20 μl for 4-aminobiphenyl when incubated with PCB P-448-H, and 200 μl for 4-aminobiphenyl with PCB P-448-L) of the mixture was transferred to a tube containing 0.1 ml of the nutrient broth (1×10^8 organisms), 0.4 ml of 100 mM sodium potassium phosphate (pH 7.4) and 2.0 ml of molten soft agar. The resulting mixture was poured into a Petri dish containing 25 ml of minimal glucose agar with 0.1 μmole of L-histidine and 0.1 μmole of biotin. The change in the amount transferred was needed to avoid killing effects due to the parent promutagen and/or the metabolite(s). The number of histidine revertant colonies was counted after incubation for 2 days at 37°. The spontaneous number of revertants was subtracted from the experimental values. When necessary, the *Salmonella*-mutagenesis assay was carried out in a modified manner, considering the labile nature of the metabolities. Thus, promutagens and enzymes were incubated with bacteria cells to trap active mutagens immediately after their production in the incubation mixture. As some components in the nutrient broth inhibited the activity of the cytochrome, the washed bacteria cells prepared as described above were used for this experiment. After the reconstituted system was incubated for 5 min, 9 nmoles of menadione in 0.1 ml of dimethyl sulfoxide were added to terminate the reaction. The resultant mixture was mixed with a soft agar, then poured on a Petri dish. Other assay conditions were the same as reported previously (2). All assays for the activities of PCB P-448-H and PCB P-448-L were conducted in duplicate, and the means of the results shown in the tables.

When inhibition of metabolism by IgG was studied, microsomes (0.35 mg of protein) from PCB-treated rats were preincubated with a desired amount of IgG at 25° for 30 min in the presence of 0.5 unit of purified NADPH-cytochrome P-450 reductase and 100 mM potassium phosphate (pH 7.4) in a final volume of 0.1 ml. To the mixture were added potassium phosphate (pH 7.4) to give a final concentration of 100 mM, 0.1 mM EDTA, 5 mM *p*-propoxyaniline as the substrate, and an NADPH-generating system (0.8 mM NADP, 8 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, and 6 mM magnesium chloride) in a final volume of 1.0 ml. The incubation was started by addition of the NADPH-generating system and was carried out at 37° for 15 min, aerobically. The *O*-depropylation activity of microsomes was estimated by determination of *p*-aminophenol formed during the incubation (15).

To compare the immunoadsorption capacity of microsomes from untreated male and female rats and from male rats treated with phenobarbital, 3-methylcholanthrene, or PCB, rabbit anti-PCB P-448-H IgG (1.8 mg) was incubated in a final volume of 180 μl with 0.6 mg of protein of microsomes in the presence of 100 mM potassium phosphate (pH 7.4) at 25° for 30 min. The microsomes from five rats were pooled and used for the experiments. In another experiment, varying amounts (0–1.8 mg of protein) of microsomes from PCB-treated rats were added. After the preincubation, the mixture was centrifuged at 105,000 × *g* for 30 min. A portion (20 μl) of the resultant supernatant was transferred to a tube containing 0.1 nmole of PCB P-448-H, mixed well, and allowed to stand at 25° for 30 min. To this mixture were added 0.05 nmole of cytochrome b_5, 0.5 unit of NADPH-cytochrome P-450 reductase, 15 μg of dilauroyl-L-3-phosphatidylcholine, 0.04 mM dithiothreitol, 0.02 μM FMN, 50 μM EDTA, the NADPH-generating system as described above, 50 mM Hepes (pH 7.4), and 5 mM *p*-propoxyaniline in a final volume of 1.0 ml. The *O*-depropylase activity of PCB P-448-H that remained uninhibited by IgG was assayed.

To estimate the amounts of PCB remained bound to the purified preparations of PCB P-448-H and PCB P-448-L, components contained in a PCB mixture (Kaneclor 500) were determined using gas chromatography. A 0.2-nmole aliquot of PCB P-448-H or PCB P-448-L was extracted with 3 ml of diethyl ether two times; the organic layer then evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in 50 μl of ethyl acetate. An aliquot (1 μl) was injected into a Hewlett-Packard Model 5840A gas chromatograph equipped with a 63 Ni-EC detector, an automatic sampler, and a glass column [4 feet × 3 mm (inner diameter)] packed with 3% OV-1 on 80/100 mesh Gas-Chrom Q. Argon/methane (95:5, v/v) was used as the carrier gas at a flow rate of 40 ml/min. The column, injector, and detector temperatures were maintained at 175°, 200°, and 200°, respectively. Kaneclor 500, equivalent to 1.0 nmole of pentachlorobiphenyl, was also extracted and injected as the standard.

Immunochemical quantitation of cytochrome P-450 reactive with rabbit anti-PCB P-448-H IgG. Microsomes from untreated male and female rats and from male rats treated with 3-methylcholanthrene, phenobarbital, or PCB were solubilized and applied on a radial immunodiffusion plate prepared by the method essentially described by Thomas *et al.* (22). The concentration of IgG in agarose was 0.4 mg/ml.

Materials. p-Propoxyaniline was purchased from Kanto Chemicals (Tokyo, Japan). *N*-Hydroxy-2-aminofluorene and *N*-hydroxy-4-aminobiphenyl were synthesized by reduction of the corresponding nitro compounds with hydrazine. The *N*-hydroxy compounds were recrys-

tallized with benzene, and structures were established by mass spectrometry. Purities of more than 99% were confirmed by HPLC. *n*-Octyl Sepharose 4B and ω-amino-*n*-octyl Sepharose 4B were synthesized by the method of Cuatrecasas (23). Hydroxylapatite (Bio-Gel HT) was purchased from Bio-Rad Laboratories (Richmond, Calif.). Emulgen 911 and 913 were generous gifts from Kao-Atlas Company (Tokyo, Japan). Trp-P-2 and Glu-P-1 were kindly donated by Dr. T. Sugimura, National Cancer Center Research Institute (Tokyo), and 3-methoxy-4-aminoazobenzene by Drs. M. Degawa and Y. Hashimoto, Tohoku University (Sendai, Japan). *S. typhimurium* TA 98 and TA 100 were kindly donated by Dr. M. Nagao, National Cancer Center Research Institute. Chymotrypsin was purchased from Miles Laboratory, U. K. Other reagents were from commercial sources but were of the highest grade available.

RESULTS

Purification and characterization of PCB P-448-H. As described under Materials and Methods, PCB P-448-H could be reproducibly purified to apparent homogeneity using buffers containing the same or similar components at three of five column steps. A typical result of the purification of PCB P-448-H is shown in Table 1. Cytochrome P-450 was divided into two fractions (Fractions I and II) by elution with Buffers A and B, respectively. The peaks in the carbon monoxide difference spectra were at 450 nm and 448 nm for Fractions I and II, respectively, with recovery of cytochrome P-450 of 30–40% in both Fractions I and II. About 3–5.5% of microsomal cytochrome P-450 could be recovered in final preparations. The specific content of PCB P-448-H ranged from 15.8 to 17.6 nmoles/mg of protein and showed apparent homogeneity as judged by SDS-PAGE. The last hydroxylapatite column step can be eliminated with higher yield and specific content of PCB P-448-H if the removal of excess detergent is not necessary. The molecular weight as estimated on SDS-PAGE was about 54,000. A faint protein band with a higher molecular weight appeared upon storage of the purified preparation at −80°. The oxidized form showed peaks at 645 and 391 nm with no apparent shoulder at around 417 nm, indicating that this form of cytochrome P-450 is in a high-spin state.

TABLE 1

Purification of PCB P-448-H from liver microsomes of PCB-treated rats

PCB P-448-H was purified according to the method as described under Materials and Methods

Fraction	Protein	Cytochrome P-450		
		Total content	Specific content	Recovery
	mg	*nmoles*	*nmoles/mg*	%
Microsomes	2360	7200	3.1	100
Solubilized supernatant	2100	6900	3.3	96
Aminooctyl column eluate	379	2800	7.4	39
DE-52 (1st) column eluate	84	1250	14.8	17
DE-52 (2nd) column eluate	—[a]	570	—[a]	8
Hydroxylapatite (1st) column eluate	25.0	426	17.0	6
Hydroxylapatite (2nd) column eluate	13.8	218	15.8	3

[a] Not determined because of low concentration of protein in the eluate.

The absorption maximum in the carbon monoxide difference spectrum of PCB P-448-H as well as PCB P-448-L was at 447 nm. PCB P-448-H tended to aggregate when the purified preparation was dialyzed against phosphate buffers with concentrations lower than 50 mM. Peptide maps of highly purified preparations of PCB P-448-H and PCB P-448-L after limited proteolysis with chymotrypsin showed that the peptide pattern was different between PCB P-448-H and PCB-448-L (data not shown). Immunochemical cross-reactivity between anti-PCB P-448-H IgG with PCB P-448-L is shown in Fig. 1. The result shows that the antibody preparation contains a trace amount of IgG reactive with PCB P-448-L. This IgG could be removed by passing the material through a column on which PCB P-448-L had been adsorbed as described under Materials and Methods.

Aromatic hydroxylation activities of PCB P-448-H and PCB P-448-L. Benzo[*a*]pyrene, acetanilide, and biphenyl are substrates widely used for examination of the activities of purified forms of cytochrome P-448. The comparison of the activities of PCB P-448-H and PCB P-448-L in the aromatic hydroxylations of these substrates is shown in Table 2. In accordance with the reported concepts, PCB P-448-L showed a high activity in the hydroxylation of benzo[*a*]pyrene, whereas PCB P-448-H was capable of hydroxylating the same substrate at only a minimal rate. By contrast, PCB P-448-H had higher activities in the hydroxylations of acetanilide at position 4 and biphenyl at positions 2 and 4 than did PCB P-448-L.

N-Hydroxylation activities of PCB P-448-H and PCB P-448-L. Capacities of PCB P-448-H and PCB P-448-L to *N*-hydroxylate Trp-P-2, Glu-P-1, 2-aminofluorene, and 4-aminobiphenyl were compared (Table 2). PCB P-448-H catalyzed the *N*-hydroxylations of these promutagens much faster than did PCB P-448-L. Greater differences between PCB P-448-H and PCB P-448-L were observed in the *N*-hydroxylation of Glu-P-1 and 4-aminobiphenyl. The ratios of the activities of PCB P-448-H to those of PCB P-448-L were 45- and 22-fold for Glu-P-1 and 4-aminobiphenyl, respectively.

Mutagenic activation of promutagens by PCB P-448-H and PCB P-448-L. Trp-P-2, Glu-P-1, 2-aminofluorene, and 4-aminobiphenyl are hydroxylated at *N*-positions as an activation process to show their mutagenicities. Since PCB P-448-H showed higher *N*-hydroxylation activities with these promutagens, this form of cytochrome P-448 was presumed to show higher mutagen-producing activities from these promutagens. As was expected, PCB P-448-H catalyzed mutagenic activation of Trp-P-2, Glu-P-1, 2-aminofluorene, and 4-aminobiphenyl at higher rates than did PCB P-448-L (Table 3). In addition, aflatoxin B_1, which is known to be metabolized to a corresponding epoxide as an active metabolite, was also activated to the mutagen by PCB P-448-H more efficiently than by PCB P-448-L. As expected from the results shown in Table 2, PCB P-448-L rather than PCB P-448-H was active in the mutagenic activation of benzo[*a*]pyrene.

Effects of cytochrome b_5 on drug oxidations and mutagen production catalyzed by PCB P-448-H and PCB P-448-L. In analyzing the NADH synergism of NADPH-dependent *N*-demethylation of ethylmorphine, Hildebrandt and Estabrook (24) demonstrated the electron

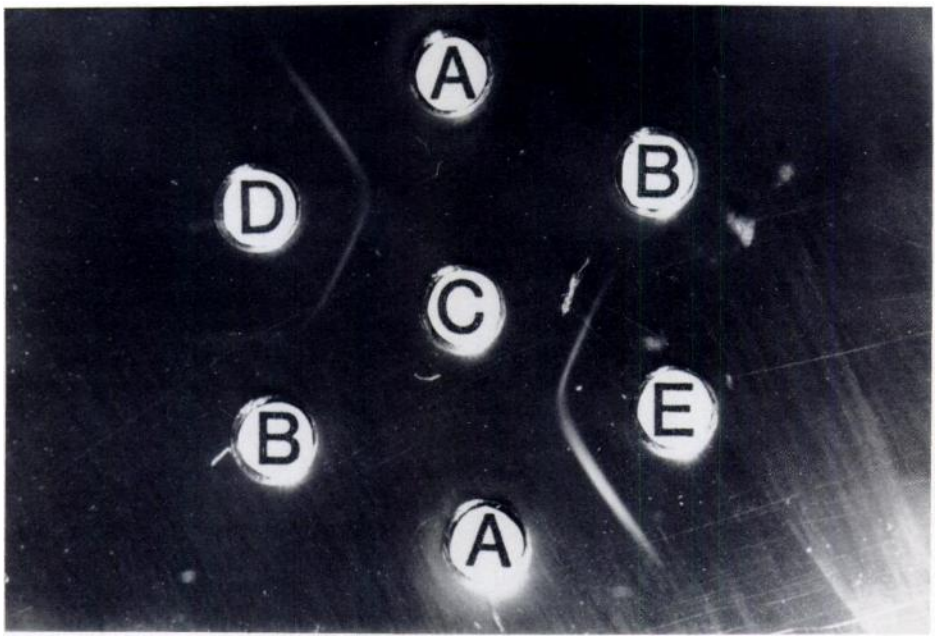

FIG. 1. *Ouchterlony double-diffusion analysis for cross-reactivity of anti-PCB P-448-H IgG with PCB P-448-L*

Purified preparations (10 nmoles/ml) of PCB P-448-H (Well *A*) and PCB P-448-L (well *B*), liver microsomes from PCB-treated rats (3 mg/ml, Well *C*) and the anti-PCB P-448-H IgG preparations before (Well *E*) and after (Well *D*) partial purification were applied to the Ouchterlony double-diffusion plate. The liver microsomes had been treated with 100 mM potassium phosphate (pH 7.25) containing 1% sodium cholate, 0.5% Emulgen 911, 0.9% sodium chloride, and 20% glycerol before application. The amount applied to the wells was 5 μl. The presence of a trace amount of common antigenic site(s) in the PCB P-448-L was assumed from a very faint precipitation band (not clearly visible on the photograph) formed between the Wells *B* and *E*.

transport from cytochrome b_5 to cytochrome P-450. The effects of cytochrome b_5 on drug oxidations and mutagen production catalyzed by PCB P-448-H and PCB P-448-L are shown in Table 4. Cytochrome b_5 enhanced the oxidative metabolism of aniline and *p*-nitrophenetole but not coumarin hydroxylation, in which no significant activity was seen with these cytochromes. As can be seen, the addition of cytochrome b_5 to the incubation system containing either PCB P-448-H or PCB P-448-L increased the number of revertants. The increments by cytochrome b_5 of PCB P-448-H-mediated mutagenic activation of Glu-P-1, 2-aminofluorene, and 3-methoxy-4-aminoazobenzene were 56%, 65%, and 87%, respectively. These values were higher than those of the PCB P-448-L-mediated activations.

Immunoadsorption of anti-PCB P-448-H IgG to liver microsomes from PCB-treated male rats. To determine whether or not a hydrophilic site of PCB P-448-H is exposed to the surface of microsomal membranes, the inhibition by the IgG of the *O*-depropylation activity was measured using liver microsomes of PCB-treated rats (Fig. 2). The *p*-propoxyaniline *O*-depropylation activity was inhibited with the increase in the added IgG. The maximal inhibition by about 35% was attainable by excess IgG. This indicates that at least some of the antigenic

TABLE 2

Comparison of the activities of PCB P-448-H and PCB P-448-L to hydroxylate foreign compounds, including promutagens

Hydroxylase activities of PCB P-448-H and PCB P-448-L were reconstituted with a purified preparation of NADPH-cytochrome P-450 reductase and phospholipid as described under Materials and Methods

Substrate	PCB P-448-H	PCB P-448-L	PCB P-448-H/ PCB P-448-L
	nmoles/nmole P-448/min		
Benzo[*a*]pyrene, 3-OH	0.071	4.330	0.02
Acetanilide, 4-OH	14.91	9.28	1.61
Biphenyl			
2-OH	2.39	1.05	2.19
4-OH	2.17	1.35	1.61
Trp-P-2, *N*-OH	2.09	0.70	2.99
Glu-P-1, *N*-OH	3.93	0.09	44.7
2-Aminofluorene, *N*-OH	16.30	2.14	7.62
4-Aminobiphenyl, *N*-OH	38.10	1.70	22.4

TABLE 3

Mutagenic activation of promutagens by PCB P-448-H and PCB P-448-L

In the case with Trp-P-2, Glu-P-1, 2-aminofluorene, and 4-aminobiphenyl, each of these promutagens at a concentration of 0.5 mM was added to a reconstituted system containing PCB P-448-H or PCB P-448-L and other necessary components. After incubation for 10 min, a portion was transferred to a tube containing bacteria cells in the soft agar as described under Materials and Methods. In the case of aflatoxin B_1 and benzo[*a*]pyrene, the washed bacteria cells were added to the reconstituted system containing a substrate, the cytochrome, and the reductase.

Promutagens	Concentration	Tester strain	PCB P-448-H	PCB P-448-L
	mM		*rev.* $\times 10^{-3}$*/nmole P-448*	
Trp-P-2	0.5	TA 98	7810	3650
Glu-P-1	0.5	TA 98	5090	180
2-Aminofluorene	0.5	TA 98	211.4	63.1
4-Aminobiphenyl	0.5	TA 98	11.50	1.58
Aflatoxin B_1	0.002	TA 100	8.17	1.73
Benzo[*a*]pyrene	0.04	TA 100	0.84	2.22

TABLE 4

Effects of cytochrome b_5 on activities of PCB P-448-H and PCB P-448-L

When oxidations of aniline, *p*-nitrophenetole, and coumarin were assayed, 0.04 mM dithiothreitol, 0.02 μM FMN, and 50 μM EDTA were added to the incubation mixture in addition to other necessary components described under Materials and Methods in a final volume of 1.0 ml rather than 0.5 ml. When the mutagen-producing activities of cytochrome P-448 were measured, washed bacteria cells of *Salmonella typhimurium* TA 98 were added to an incubation mixture containing a reconstituted system and a promutagen. The mean number of revertants was subtracted. All assays were carried out in duplicate, and the mean values are presented. Other experimental conditions are as described under Materials and Methods.

Substrates	Concentration	PCB P-448-H			PCB P-448-L		
		$-b_5$	$+b_5$[a]	Change (%)	$-b_5$	$+b_5$[a]	Change (%)
	mM	*nmoles metabolite/nmole P-448/min*					
Aniline	5.0	0.85	2.66	+213	0.33	0.36	+9
p-Nitrophenetole	5.0	3.28	8.40	+156	12.39	15.62	+26
Coumarin	0.5	0.01	0.01	0	0.03	0.03	0
		Rev. $\times 10^{-3}$*/nmole P-448*					
Glu-P-1	0.02	126	197	+56	1.89	2.33	+23
2-Aminofluorene	0.2	12.7	21.0	+65	5.67	7.33	+29
3-Methoxy-4-aminoazobenzene	0.2	3.74	6.07	+87	2.68	4.36	+63

[a] 0.05 nmole.

sites of PCB P-448-H are exposed to the surface of microsomal membranes. In the reconstituted system, PCB P-448-H showed higher *O*-depropylation activity than did PCB P-448-L: the turnover numbers of PCB P-448-H and PCB P-448-L in the presence of cytochrome b_5 were 7.15 and 1.21 nmoles/nmole of cytochrome P-448 per minute, respectively. Despite the higher activity of PCB P-448-H, the anti-PCB P-448-H IgG inhibited the microsomal activity by only 35%, probably suggesting that the remaining activity (about 65% of total microsomal activity) is due to the other forms of cytochrome P-450. To estimate roughly the amount of PCB P-448-H in liver microsomes capable of adsorbing the anti-PCB P-448-H IgG, a fixed amount of the anti-PCB P-448-H IgG was added to tubes which contained varying amounts of microsomes from PCB-treated rats. The IgG not adsorbed to microsomes was collected by centrifugation and assayed by addition to a reconstituted system containing PCB P-448-H and *p*-propoxyaniline. As shown in Table 5, the inhibition by the IgG of the activity of PCB P-448-H was decreased by the pretreatment of the IgG with microsomes. The reversal of the inhibition reached plateau level: about 30% of the activity of PCB P-448-H remained even in the use of excess microsomes. The pretreatment of IgG with microsomes did not result in the solubilization of cytochrome P-450, since no detectable cytochrome P-450 was present in the supernatant IgG fraction after centrifugation.

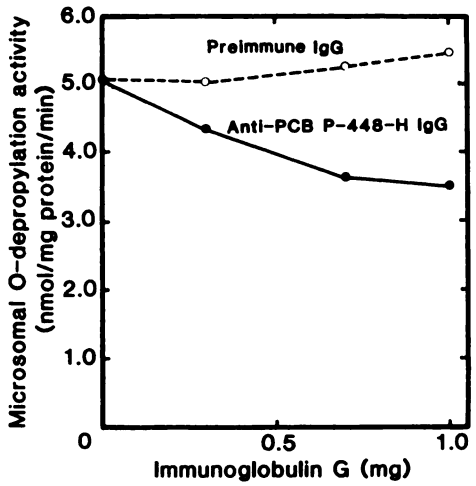

FIG. 2. *Inhibition by anti-PCB P-448-H IgG of p-propoxyaniline O-depropylation activity of liver microsomes from PCB-treated rats*

To microsomes from PCB-treated rats were added the indicated amounts of anti-PCB P-448-H IgG, and the activity was measured as described under Materials and Methods.

Immunoadsorption assay for PCB P-448-H in liver microsomes from untreated male and female rats and variously treated male rats. As mentioned above, most of the antibodies to PCB P-448-H capable of inhibiting the activity of PCB P-448-H were bound to microsomal membranes. Thus, this method was used to estimate approximately the amounts of PCB P-448-H in microsomes of untreated male and female rats and male rats pretreated with phenobarbital, 3-methylcholanthrene, or PCB (Table 5). When the IgG untreated with microsomes was added, the *O*-depropylation activity of PCB P-448-H was inhibited by 90.8%. On addition of microsomes from untreated and phenobarbital-treated male rats, no apparent reversal of the inhibition was observed. Female microsomes adsorbed the IgG to a small level. Microsomes from 3-methylcolanthrene- and PCB-treated rats showed higher capacities to adsorb the IgG, resulting in the marked reversal of the *O*-depropylation activity of PCB P-448-H. These results indicate that PCB P-448-H is not only induced rather specifically by 3-methylcholanthrene and PCB but exists in microsomes from untreated female rats. In agreement with this finding, we have observed a fraction containing a high-spin form of cytochrome P-448 in eluates from the DE-52 column when microsomes from untreated female rats were used instead of those from PCB-treated rats.

Radial immunodiffusion assay for PCB P-448-H in liver microsomes of untreated male and female rats and male rats treated with phenobarbital, 3-methylcholanthrene, and PCB. To confirm these results described above, the content in microsomes of PCB P-448-H was assayed by radial immunodiffusion analysis. As shown in Table 6, PCB and 3-methylcholanthrene induced cytochrome P-450 to be immunochemically reactive with the anti-PCB P-448-H IgG. The amounts of PCB P-448-H

TABLE 5

Comparison of capacity to adsorb anti-PCB P-448H IgG of microsomes from untreated male and female rats and from male rats treated with phenobarbital, 3-methylcholanthrene, and PCB

IgG from unimmunized rabbits and rabbits immunized with PCB P-448-H was adsorbed to indicated amounts of liver microsomes from untreated or variously treated rats. After centrifugation, a portion of the supernatant was added to PCB P-448-H, and the capacity of the cytochrome which remained uninhibited was measured using *p*-propoxyaniline as a substrate.

Microsomes added	Amount of microsomes	IgG	*O*-Depropylation activity of PCB P-448-H[a]	Activity
	mg		*nmoles/nmole P-448/min*	%
None	—	Preimmune	15.2	100
None	—	Anti-PCB P-448-H	1.4	9.2
PCB-treated	0.3	Anti-PCB P-448-H	6.9	45.4
PCB-treated	0.6	Anti-PCB P-448-H	10.0	65.8
PCB-treated	1.2	Anti-PCB P-448-H	10.5	69.1
PCB-treated	1.8	Anti-PCB P-448-H	10.4	68.4
Untreated males	0.6	Anti-PCB P-448-H	1.4	9.2
Untreated females	0.6	Anti-PCB P-448-H	2.4	15.8
Phenobarbital-treated	0.6	Anti-PCB P-448-H	1.2	7.9
3-Methylcholanthrene-treated	0.6	Anti-PCB P-448-H	8.0	52.6

[a] Formation of *p*-aminophenol from *p*-propoxyaniline.

thus estimated accounted for about 50% and 20% of the total cytochrome P-450 in microsomes of PCB- and 3-methylcholanthrene-treated rats, respectively. In accordance with the results shown above, liver microsomes from female rats contained 0.048 nmole/mg of protein of cytochrome P-450 immunochemically reactive with the IgG. This was 8.4% of the total cytochrome P-450 in microsomes. Furthermore, microsomes from untreated and phenobarbital-treated male rats contained only trace amounts of PCB P-448-H.

DISCUSSION

Cytochrome P-450 often shows a characteristic spectral change upon binding with a substrate. The spectral change has been known to be caused by a change in a spin state of the cytochrome. In this study, we purified PCB P-448-H which was in a high-spin state. Thus, it seemed reasonable to assume that the high-spin state resulted from binding of a low spin cytochrome with PCB given to the rats to induce cytochrome P-450. However, as shown in Fig. 3, only trace amounts of the PCB components remained bound to the cytochrome: the amounts of the components of PCB were calculated to be less than 0.01 nmole/nmole of PCB P-448-H. Furthermore, we observed approximately the same amount of remnant PCB in the purified preparation of PCB P-448-L, which is in a low-spin state. These results indicate that the high-spin state of PCB P-448-H is not caused by the bound PCB.

A high-spin form of cytochrome P-448 has been purified to a homogeneity from microsomes of rabbits. Unlike a low-spin form of cytochrome P-448 purified from microsomes of 3-methylcholanthrene-treated rats, the purified preparation from rabbits shows catalytic activities

TABLE 6

Radial immunodiffusion assay of rat liver microsomes for content of PCB P-448-H using purified anti-PCB P-448-H IgG-impregnated agarose gels

Liver microsomes from untreated and variously treated rats were solubilized with detergents at the concentrations indicated. The solubilized microsomes were applied to a gel for assay of the amount of the cytochrome reactive with anti-PCB P-448-H IgG. The total content of cytochrome P-450 in these microsomes was determined by reduced carbon monoxide difference spectra.

Microsomes	Concentration of protein	PCB P-448-H	Cytochrome P-450	$\frac{\text{PCB P-448-H}}{\text{cytochrome P-450}} \times 100$
	mg/ml	*nmoles/mg*	*nmoles/mg*	
Untreated males	5.0	0.012	0.99	1.2
Untreated females	5.0	0.048	0.57	8.4
Phenobarbital-treated	5.0	0.030	2.62	1.1
3-Methylcholanthrene-treated	5.0	0.24	1.27	18.9
PCB-treated	1.0	1.53	2.86	53.5

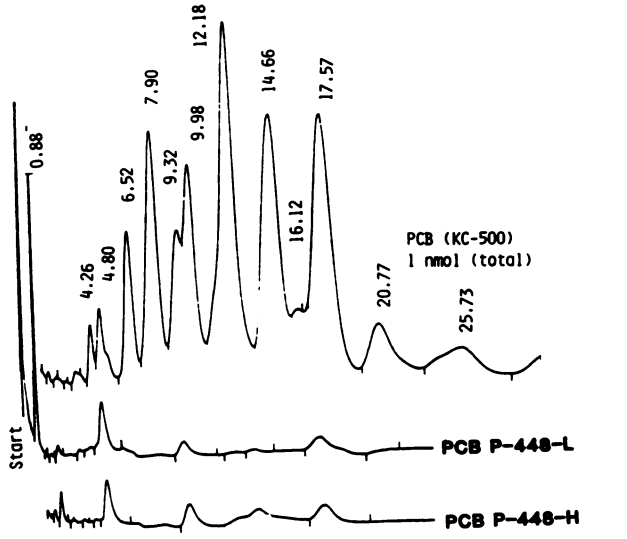

FIG. 3. *Analysis with gas chromatography of PCB remaining in the purified preparations of PCB P-448-H and PCB P-448-L*

Experimental details are described under Materials and Methods. *Numbers* in the *upper part* represent retention times of the components of the PCB mixture. A peak with a retention time of 4.80 min is due to a component in the solvent used for the extraction of PCB from purified preparations.

with only limited substrates such as 2-acetylaminofluorene (25). Our recent experiments have shown that 2-acetylaminofluorene as well as 2-aminofluorene is activated to a mutagen(s) by PCB P-448-H and the high-spin form of cytochrome P-448 from rabbits more efficiently than by PCB P-448-L.[6]

The higher *N*-hydroxylation and mutagen-producing activities of PCB P-448-H suggested that this form of cytochrome P-448 plays central roles in the activation of numerous amino group-containing compounds. This is under examination in our laboratory; however, our recent preliminary experiments have shown that the IQ which was isolated by Sugimura and his co-workers (1) from extracts of broiled sardine and heated beef is also rather specifically activated to a mutagen(s). The mutagenicity of IQ has been reported to be about 1000 times as high as that of benzo[*a*]pyrene.

As reported previously (2), PCB P-448-L and microsomes from PCB-treated rats catalyzed mutagenic activation of Trp-P-2 at similar rates on the basis of nanomoles of cytochrome P-450. Considering the presence of multiple forms of cytochrome P-450 in microsomes of PCB-treated rats, the specific activity of the purified enzyme as been expected to be higher than observed. These results led us to look for another form of cytochrome P-450 having higher activity in the mutagenic activation of Trp-P-2. As shown in Table 3, we found that PCB P-448-H as about 2 times higher activity in Trp-P-2 activation than did PCB P-448-L. Thus, this form was expected to be the major form involved in the activation.

Regarding the modifier to the activities of PCB P-448-H, we found that cytochrome b_5 enhanced PCB P-448-H-mediated mutagenic activation of promutagens (Table 4), although the degrees of the enhancement varied among the promutagens employed. The significance of cytochrome b_5 in the metabolic activation of promutagens to mutagens should be studied extensively with other forms by cytochrome P-450 and a wide variety of promutagens. One cannot confirm that a certain form of cytochrome P-450 is not capable of activating a promutagen without performing experiments in the presence of cytochrome b_5 in the reconstituted system, since cytochrome b_5 is obligatorily required in some oxidation reactions depending on the substrates and forms of cytochrome P-450.

In recent years, antibodies raised to highly purified preparations of cytochrome P-450 have been used for quantitation of the specific form of cytochrome P-450 in crude mixtures (11, 22). However, as pointed out by Dus *et al.* (29) and by others (26), many of these antibodies have recognized forms of cytochrome P-450 other than the antigen used for immunization. As mentioned under Materials and Methods, our crude IgG preparation cross-reacted with PCB P-448-L, which is probably identical with P-450*c*, and other forms of cytochrome P-450 involving a form probably identical with P-450*b*.[6] However, this IgG reacted with a partially purified cytochrome P-450 preparation eluted earlier from the second DE-52 column to form a precipitation band. The precipitation band apparently fused with a band formed between PCB P-448-H and the IgG (Fig. 4). This cytochrome P-450 preparation, with a specific content of about 3 nmoles/mg of protein, did not contain PCB P-448-H as judged by the absolute spectrum. Application of the IgG to an octyl Sepharose 4B column, on which the PCB P-450 fraction had been adsorbed, did not allow elution of the IgG capable of reacting with PCB P-448-H on Ouchterlony double-diffusion analysis. Supporting this, immunoprecipitation bands formed by the PCB P-450 fraction and PCB P-448-H with the IgG disappeared concomitantly, when varying amounts of PCB P-450 fraction were previously mixed and centrifuged and the resultant supernatant was assayed with the double-diffusion method for the remaining anti-PCB P-448-H IgG. These results indicate that the PCB P-450 fraction contains cytochrome P-450 with essentially the same antigenic sites as PCB P-448-H. Thus, one form(s) of cytochrome P-450 in the PCB P-450 fraction and PCB P-448-H is indistinguishable with these immunochemical experiments. Since only a trace amount of cytochrome P-450 reactive with anti-PCB P-448-H IgG was found in microsomes of untreated male rats, it can be confirmed that a form(s) of cytochrome P-450 in the PCB P-450 fraction having antigenic sites in common with PCB P-448-H is induced by PCB and 3-methylcholanthrene in male rats.

The anti-PCB P-448-H IgG formed precipitation bands with PCB P-448-H, the PCB P-450 fraction and microsomes from PCB-treated rats: the precipitation bands fused with each other (Fig. 4). This result indicates that most of the cytochrome P-450 capable of reacting with anti-PCB P-448-H IgG in microsomes of PCB-treated rats can be accounted for by PCB P-448-H and a minor form(s) of cytochrome P-450 in the PCB P-450 fraction.

The roles of two forms of cytochrome P-450 in the mutagenic activation of promutagens have been examined with the use of crude antibodies raised to the cytochrome preparations purified from microsomes of phenobarbital- or 3-methylcholanthrene-treated rats. Thus, Watanabe *et al.* (30) demonstrated that mutagenic activations of promutagens by the 9000 × *g* supernatant fraction of liver homogenates from PCB (Kaneclor 500)-treated rats were inhibited differently by addition of the antibodies depending on the promutagen employed. They confirmed that almost all of the promutagen-producing activities in the 9000 × *g* supernatant fraction could be accounted for by these two forms of cytochrome

[6] High-spin forms of cytochrome P-448 have been purified from rats treated with isosafrole (26), 3,4,5,3′,4′-pentachlorobiphenyl (27), and 3,4,5,3′,4′,5′-hexachlorobiphenyl (28). The catalytic activities of these high-spin forms of cytochrome P-448 have not been fully understood, and no comparison could be made. To confirm the possible identity of these purified forms of cytochrome P-448, we sent our purified preparation of PCB P-448 H and the antibodies to the cytochrome to Dr. W. Levin, of Hoffmann-La Roche Inc. (Nutley, N. J.), and Dr. Levin compared the properties of P-450d and P-448-H. According to Dr. Levin's results, these preparations showed very similar molecular weights as judged on SDS-PAGE and immunochemical cross-reactivities. Therefore, it can be concluded that P-450d and PCB P-448-H are identical molecular species. The PCB P-448-L used in these experiments is assumed to be the same form as those which have been reported by other laboratories, as judged by the property to adsorb the DE-52 column tightly, its adsorption peak at 447 nm in a carbon monoxide difference spectrum, a high activity in benzo[*a*]pyrene hydroxylation upon reconstitution, and an apparent molecular weight of 56,000.

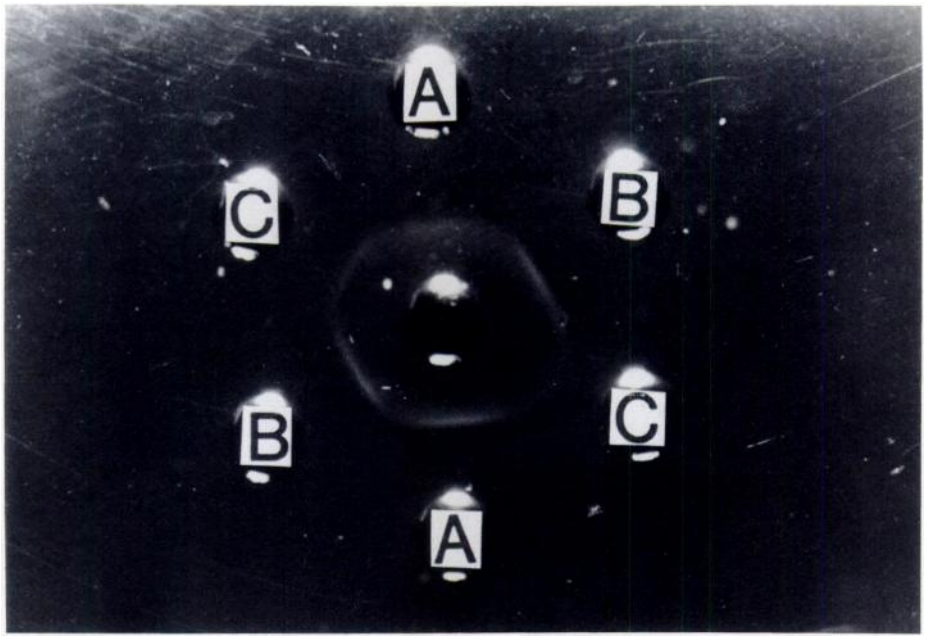

FIG. 4. *Ouchterlony double-diffusion analysis of cytochrome P-450 reactive with anti-PCB P-448-H IgG*
The Ouchterlony double-diffusion analysis plates were prepared as described by Thomas *et al.* (22). Purified preparations of PCB P-448-H and PCB P-450 fraction (10 nmoles/ml) and liver microsomes from PCB-treated rats (1 mg/ml) in 100 mM potassium phosphate (pH 7.25) containing 1% sodium cholate, 0.5% Emulgen 911, 0.9% sodium chloride, and 20% glycerol were applied to the wells (5 μl). Well *A*, PCB P-448-H; Well *B*, PCB P-450 fraction; Well *C*, liver microsomes; *center well*, anti-PCB P-448-H IgG (20 mg/ml).

P-450. In addition, they proposed that, among the mutagenic activations they examined, the activation of Trp-P-2, Glu-P-1, or IQ was catalyzed almost exclusively by cytochrome P-448 which was in a low-spin state. As judged by the spin state and other evidence, their preparation can be assumed to be identical with PCB P-448-L and P-450c as described above. Their results are not in agreement with our hypothesis that Trp-P-2 and other promutagens in pyrolysates are activated mainly by PCB P-448-H. In accordance with our hypothesis, our preliminary experiments have shown that anti-PCB P-448-H antibodies previously treated by immunoadsorption inhibit the majority of mutagenic activation of Glu-P-1 and some other mutagenic activation catalyzed by liver microsomes from PCB-treated rats. The exact reason for the discrepancy is not known at present, but it is most likely that their antibodies to cytochrome P-448 cross-react with PCB P-448-H.

As regards the immunochemical quantitation, the IgG may react with both apo- and holoenzymes. Thus, another explanation for the remarkably large amount of PCB P-448-H in microsomes of PCB-treated rats was that PCB-induced microsomes contain some apo-PCB P-448-H protein. However, this did not seem to be the case, since the apparent amount of microsomal cytochrome P-450 as determined by carbon monoxide difference spectra was increased only by about 13% by the pretreatment of liver homogenates with hemin (not shown). However, the possibility that some apo-cytochrome P-450 is unable to reconstitute with externally added hemin cannot be ruled out.

The results showing that about 50% of the total cytochrome P-450 in microsomes of PCB-treated rats could be accounted for by a cytochrome P-450 immunochemically reactive with anti-PCB P-448-H IgG had been unexpected. Thomas *et al.* (11) reported that Aroclor 1254 induces P-450b and P-450c^6 almost equally, and the sum of the amounts of P-450b and P-450c accounted for about 75% of the total cytochrome P-450 in liver microsomes. The induction of cytochrome P-450 by polychlorinated biphenyl derivatives has been shown to vary, depending on the number and the position of chlorine atoms in the biphenyl rings. Since the components of the derivatives in Aroclor 1254 and Kaneclor 500 may not be quite the same, it can also be assumed that Kaneclor 500 induced PCB P-448-H to a greater extent.

Although the presence of such a large quantity of PCB P-448-H in liver microsomes from PCB-treated rats cannot be fully confirmed as yet, PCB P-448-H must be one of the major forms of cytochrome P-450 in the microsomes because it has been purified with a recovery ranging from 3% to 5.5%. Therefore, it can be confirmed that PCB P-448-H plays important roles in the activation of mutagenic substances in the liver 9000 × *g* supernatant fraction widely used as an activation system in routine mutation tests.

ACKNOWLEDGMENTS

The authors are grateful to Drs. W. Levin and A. H. Conney, of Hoffmann-La Roche Inc. (Nutley, N. J.), for kindly examining the identity between PCB P-448-H and P-450*d*. The authors are also indebted to Dr. P. W. Beatty, of Shell Development Company, for his kind help in the preparation of the manuscript.

REFERENCES

1. Sugimura, T., K. Wakabayashi, M. Yamada, M. Nagao, and T. Fujino. Activation of chemicals to proximal carcinogens, in *Mechanisms of Toxicity and Hazard Evaluation* (B. Holmstedt, R. Lauwerys, M. Mercier, and M. Roberfroid, eds.). Elsevier/North-Holland Biomedical Press, Amsterdam, 205–217 (1980).

2. Ishii, K., M. Ando, T. Kamataki, and R. Kato. Metabolic activation of mutagenic tryptophan pyrolysis products (Trp-P-1 and Trp-P-2) by a purified cytochrome P-450-dependent monooxygenase system. *Cancer Lett.* **9**:271–276 (1980).
3. Ishii, K., Y. Yamazoe, T. Kamataki, and R. Kato. Metabolic activation of glutamic acid pyrolysis products, Glu-P-1 and Glu-P-2, by purified cytochrome P-450. *Chem.-Biol. Interact.* **38**:1–13 (1981).
4. Kamataki, T., Y. Yamazoe, S. Mita, and R. Kato. Mutagenic activation and covalent binding of 3-amino-1-methyl-5*H*-pyrido(4,3-*b*)indole (Trp-P-2) by microsomal and reconstituted cytochrome P-450 system, in *Microsomes, Drug Oxidations and Drug Toxicity* (R. Sato, and R. Kato, eds.). Japanese Scientific Societies Press, Tokyo, and Wiley-Interscience, New York, 463–470 (1982).
5. Kamataki, T., D. H. Belcher, and R. A. Neal. Studies of the metabolism of diethyl *p*-nitrophenyl phosphorothionate (parathion) and benzphetamine using an apparently homogeneous preparation of rat liver cytochrome P-450 antibody preparation. *Mol. Pharmacol.* **12**:921–932 (1976).
6. Kamataki, T., K. Maeda, Y. Yamazoe, T. Nagai, and R. Kato. Partial purification and characterization of cytochrome P-450 responsible for the occurrence of sex difference in drug metabolism in the rat. *Biochem. Biophys. Res. Commun.* **103**:1–7 (1981).
7. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
8. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
9. Cleveland, D. W., S. G. Fischer, M. W. Kirscher, and U. K. Laemmli. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**:1102–1106 (1977).
10. Reik, L. M., W. Levin, D. E. Ryan, and P. E. Thomas. Immunochemical relatedness of rat hepatic microsomal cytochromes P-450c and P-450d. *J. Biol. Chem.* **257**:3950–3957 (1982).
11. Thomas, P. E., L. M. Reik, D. E. Ryan, and W. Levin. Regulation of three forms of cytochrome P-450 and epoxide hydrolase in rat liver microsomes: effects of age, sex and induction. *J. Biol. Chem.* **256**:1044–1052 (1981).
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
13. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
14. Phillips, A. H., and R. G. Langdon. Hepatic triphosphopyridine nucleotide-cytochrome *c* reductase: isolation, characterization and kinetic studies. *J. Biol. Chem.* **237**:2652–2660 (1962).
15. Imai, Y., A. Ito, and R. Sato. Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J. Biochem.* **60**:417–428 (1966).
16. Kamataki, T., M. Kitada, H. Shigematsu, and H. Kitagawa. The involvement of cytochrome P-448 and P-450 in NADH-dependent *O*-demethylation of *p*-nitroanisole in rat liver microsomes. *Jpn. J. Pharmacol.* **29**:191–201 (1979).
17. Aitio, A. A simple and sensitive assay of 7-ethoxycoumarin deethylation. *Anal. Biochem.* **85**:488–491 (1978).
18. Nebert, D. W., and H. V. Gelboin. Substrate-induced microsomal aryl hydrocarbon hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J. Biol. Chem.* **243**:6242–6249 (1968).
19. Yamazoe, Y., T. Kamataki, and R. Kato. A sensitive method for determination of biphenyl hydroxylase activities using high-performance liquid chromatography. *Anal. Biochem.* **111**:126–130 (1981).
20. Yamazoe, Y., K. Ishii, T. Kamataki, and R. Kato. Structural elucidation of a mutagenic metabolite of 3-amino-1-methyl-5*H*-pyrido(4,3-*b*)indole. *Drug Metab. Dispos.* **9**:292–296 (1981).
21. Yamazoe, Y., K. Ishii, T. Kamataki, and R. Kato. Isolation and characterization of active metabolites of tryptophan-pyrolysate mutagen, Trp-P-2, formed by rat liver microsomes. *Chem.-Biol. Interact.* **30**:125–138 (1980).
22. Thomas, P. E., D. Korzeniowski, D. Ryan, and W. Levin. Preparation of monospecific antibodies against two forms of rat liver cytochrome P-450 and quantitation of these antigens in microsomes. *Arch. Biochem. Biophys.* **192**:524–532 (1979).
23. Cuatrecasas, P. Protein purification by affinity chromatography: derivatization of agarose and polyacrylamide beads. *J. Biol. Chem.* **245**:3059–3065 (1970).
24. Hildebrandt, A., and R. Estabrook. Evidence for the participation of cytochrome b_5 in hepatic microsomal mixed-function oxidation reactions. *Arch. Biochem. Biophys.* **143**:66–79 (1971).
25. Johnson, E. F., D. S. Levitt, U. Muller-Eberhard, and S. S. Thorgeirsson. Catalysis of divergent pathways of 2-acetylaminofluorene metabolism by multiple forms of cytochrome P-450. *Cancer Res.* **40**:4456–4459 (1980).
26. Ryan, D. E., P. E. Thomas, and W. Levin. Hepatic microsomal cytochrome P-450 from rats treated with isosafrole: purification and characterization of four enzymic forms. *J. Biol. Chem.* **255**:7941–7955 (1980).
27. Ozawa, N., S. Yoshihara, K. Kawano, Y. Okada, and H. Yoshimura. 3,4,5,3′,4′-Pentachlorobiphenyl as a useful inducer for purification of rat liver microsomal cytochrome P-448. *Biochem. Biophys. Res. Commun.* **91**:1140–1147 (1979).
28. Goldstein, J. A., P. Linko, M. I. Luster, and D. W. Sundheimer. Purification and characterization of a second form of hepatic cytochrome P-448 from rats treated with a pure polychlorinated biphenyl isomer. *J. Biol. Chem.* **257**:2702–2707 (1982).
29. Dus, K. M., W. J. Litchfield, P. J. Hippenmeyer, J. A. Bumpus, O. Obidia, V. Spitsberg, and C. R. Jefcoate. Comparative immunochemical studies of cytochrome $P\text{-}450_{CAM}$ of *Pseudomonas putida* and of cytochrome P-450scc of bovine adrenal mitochondria. *Eur. J. Biochem.* **111**:307–314 (1980).
30. Watanabe, J., K. Kawajiri, H. Yonekawa, M. Nagao, and Y. Tagashira. Immunochemical analysis of the roles of two major types of cytochrome P-450 in mutagenesis of compounds isolated from pyrolysates. *Biochem. Biophys. Res. Commun.* **104**:193–199 (1982).

Send reprint requests to: Dr. Tetsuya Kamataki, Department of Pharmacology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.