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3,4,5,3',4'-PENTACHLOROBIPHENYL AS A USEFUL INDUCER FOR PURIFICATION OF RAT LIVER MICROSOMAL CYTOCHROME P448

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Summary: An easy purification of rat liver microsomal cytochrome P448 was performed by using 3,4,5,3',4'-pentachlorobiphenyl as an inducer. The cytochrome P448, a high spin form, was purified to 18.1 nmoles/mg protein with a good yield by ω-aminooctyl Sepharose 4B column chromatography followed by a hydroxyapatite column chromatography. This hemoprotein cross-reacted with antibody to cytochrome P448 from β-naphthoflavone-treated rats, but not with antibody to cytochrome P450 from phenobarbital-treated rats at all. The results of amino acid analyses suggested that this cytochrome P448 is similar to cytochrome P448 of 3-methylcholanthrene-treated rats.

Cytochrome P450, a key enzyme of microsomal monooxygenase system, has been implicated in the oxidation of a wide variety of xenobiotics as well as endogenous compounds(1-3). The oxidation activity catalyzed with microsomal monooxygenase system can be increased by a wide range of inducers including PB[2], MC and chlorinated hydrocarbons such as PCBs(4-6). The unusual broad substrate specificity and different susceptibilities to inducers of this enzyme system are explained, at least in part, by the multiplicity of cytochrome P450(7,8). Treatment of rats with MC increases cytochrome P448, which differs from the cytochrome P450 found in untreated or PB-treated rats with respect to its CO-reduced difference spectrum(4), substrate specificity

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[2]Abbreviations used are: PB, phenobarbital; MC, 3-methylcholanthrene; PCB, polychlorinated biphenyl; PenCB, 3,4,5,3',4'-pentachlorobiphenyl; BNF, β-naphthoflavone; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulfate; PB-microsomes, liver microsomes from PB-treated rats; MC-microsomes, liver microsomes from MC-treated rats; PenCB-microsomes, liver microsomes from PenCB-treated rats; BNF-microsomes, liver microsomes from BNF-treated rats; PB-P450, cytochrome P450 from PB-microsomes; MC-P448, cytochrome P448 from MC-microsomes; PenCB-P448, cytochrome P448 from PenCB-microsomes; BNF-P448, cytochrome P448 from BNF-microsomes; anti-P450, antibody to PB-P450; anti-P448, antibody to BNF-P448.

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(9) and ethyl isocyanide difference spectrum(5). Several laboratories have made considerable progress toward the purification and characterization of multiple forms of cytochrome P450 from rat liver(7,8,10-14) and rabbit liver (15-19). However, cytochrome P448 has not been efficiently purified from the liver microsomes of rats treated with MC because of the existence of considerable amounts of cytochrome P450 in MC-microsomes. As previously reported(20), the absorption maximum of CO-reduced difference spectrum of liver microsomes from rats treated with PenCB, one of the most toxic PCB congeners(21), was observed at 448.0 nm while that of MC-microsomes was at 448.5 nm. This was explained by a marked increase in the relative ratio of cytochrome P448 to cytochrome P450 in PenCB-microsomes, suggesting that PenCB might be an excellent inducer for purification of cytochrome P448.

In the present study, the purification of liver microsomal cytochrome P448 from PenCB-treated rats was performed and the purified PenCB-P448 was compared with the purified preparations of PB-P450 and MC-P448 with respect to its amino acid composition.

Materials and Methods

PenCB was synthesized by the Gomberg-Hey reaction as previously reported (22). ω-Aminooctyl Sepharose 4B was prepared from BrCN-activated Sepharose 4B gel and 1,8-diaminooctane by the method as described by Imai and Sato(23). Cholic acid, purchased from Nakarai Chemicals, Ltd., Kyoto, was recrystallized from 50 % ethanol after decolorization with active charcoal. Emulgen 913 was kindly supplied by Kao-Atlas Company, Tokyo. All other chemicals used were of the highest purity commercially available.

PenCB-microsomes were prepared from the livers of male Wistar rats(about 120 g) pretreated with 10 mg/kg of PenCB on 5 days after a single i.p. injection as previously described(20). After solubilization of the microsomes with sodium cholate, PenCB-P448 was purified according to the method of Hashimoto and Imai(18) with some modifications(see Results and Discussion). PB-P450 from liver microsomes of PB-treated rats(85 mg/kg/day for 7 days) and MC-P448 from liver microsomes of MC-treated rats(25 mg/kg/day for 7 days) were also purified by the methods of Imai and Sato(15) and Hashimoto and Imai (18), respectively, with some modifications. All of the purified cytochromes gave a single protein band on SDS-polyacrylamide gel electrophoresis performed by the method described by Laemmli(24). Anti-P450 and anti-P448 were prepared from the antisera of rabbits immunized with the purified rat liver microsomal PB-P450 and BNF-P448, respectively, as previously reported (20). Cytochrome P450 was estimated as described by Omura and Sato(25). Protein in the presence of interfering materials, such as glycerol, detergents and DTT, was determined by a combination of the methods of Bensadoun and Weinstein(26) and Dulley and Grieve(27). Protein without interfering materials was determined by the usual method of Lowry et al.(28). Quanti-

tative immunoprecipitation analysis was performed by determining the protein, which was precipitated by incubating a mixture of PenCB-P448 and either anti-P450 or anti-P448 at 4° for 24 hr, after washing with phosphate-buffered NaCl as indicated in the legend of Table II. Amino acid analysis was performed with a JEOL JLC-6AH amino acid analyzer according to the method described by Haugen and Coon(29). Half-cystine was determined by performate oxidation, but tryptophan was not determined. Apparent molecular weight of protein subunit was estimated by SDS-polyacrylamide gel electrophoresis in the presence of standard proteins according to the method of Shapiro et al.(30).

Results and Discussion

Liver microsomes from rats treated with each inducer were solubilized with 0.6 % sodium cholate in the presence of 20 % glycerol, 1 mM EDTA, 0.1 mM DTT in 100 mM potassium phosphate buffer(pH 7.25) and fractionated by an affinity column chromatography using ω-aminooctyl Sepharose 4B. This proce-

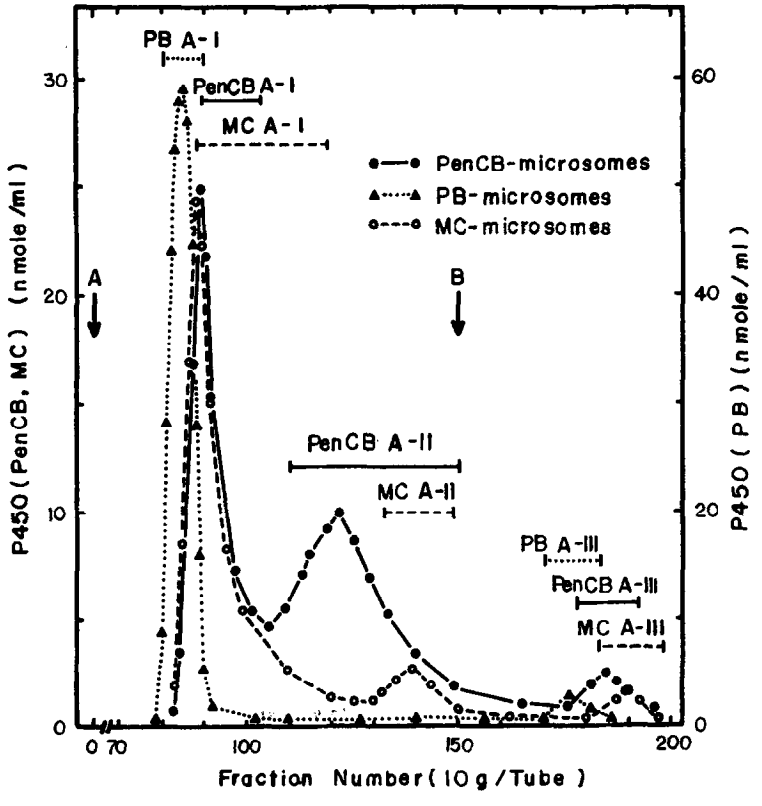

Fig. 1. Elution patterns of liver microsomal cytochromes P450 from rats treated with PenCB, PB and MC on ω-aminooctyl Sepharose 4B column chromatography. The experimental conditions are described in the text. Elution was carried out with the buffer containing 0.08 %(A) and 0.2 % Emulgen(B) from the points indicated. The amounts of cytochromes P450 applied to the column(2.8×47 cm) were as follows: PenCB-microsomes, 7400 nmoles; PB-microsomes, 7990 nmoles; MC-microsomes, 6630 nmoles. Horizontal bar at each peak indicates the range of fractions pooled for further purification.

dure could separate cytochrome P450 from cytochrome b5 and NADPH-cytochrome c reductase. As shown in Fig. 1, three cytochrome P450 fractions(PenCB A-I, PenCB A-II and PenCB A-III) were obtained from PenCB-microsomes at this step. Similarly MC-microsomes produced three peaks(MC A-I, MC A-II and MC A-III) whereas PB-microsomes gave two peaks(PB A-I and PB A-III). A-Is and A-IIs were eluted with 100 mM potassium phosphate buffer(pH 7.25) containing 20 % glycerol, 0.1 mM DTT, 0.4 % sodium cholate and 0.08 % Emulgen 913, and A-IIIs were eluted with the same buffer except that Emulgen concentration was increased to 0.2 %. The absorption maxima of CO-reduced difference spectra of PenCB A-I(a low spin form), PenCB A-II(a high spin form) and PenCB A-III (a low spin form) were observed at 447.5 nm, 447.0 nm and 447.1 nm, respectively. The yield of cytochrome P448 in PenCB A-II was 3 times greater than that in MC A-II and its specific content was also increased by 25 % as compared with that of MC A-II(Table I). Further purifications of the affinity column eluates were performed by a hydroxyapatite column chromato-

Table I

Purification of liver microsomal cytochromes P450 from rats treated with PenCB, PB and MC

Preparation		Specific content of cytochrome P450 (nmoles/mg protein)		
		PenCB	PB	MC
Solubilized supernatant		3.12(100)*	3.41(100)	1.85(100)
ω-Aminooctyl Sepharose 4B eluate	A-I	6.19(18)	7.31(41)	4.65(19)
	A-II	10.2 (15)	–	8.18(5)
	A-III	6.72(0.2)	–	5.54(4)
Hydroxyapatite eluate	H-I	12.9 (6)	13.6 (14)	–
	H-II	16.2 (11) [18.1 (2)]**	–	14.2 (0.8) [15.0 (0.3)]
	H-III	7.46(0.1)	–	–

* The value in parenthesis represents the yield(%) of pooled fraction.
** The value in bracket indicates the specific content and yield of the most purified fraction.

graphy, but no further purification of MC A-I, MC A-III and PB A-III was performed. The hydroxyapatite column eluates of PenCB A-I, PenCB A-II, PenCB A-III, MC A-II and PB A-I were designated as PenCB H-I, PenGB H-II, PenCB H-III, MC H-II and PB H-I, respectively. All of these fractions was eluted with 150 mM potassium phosphate buffer containing 20 % glycerol, 0.1 mM DTT and 0.2 % Emulgen 913 after washing with 100 mM potassium phosphate buffer containing the same components. The results of the purifications of liver microsomal cytochrome P450 from PenCB-, MC- and PB-treated rats are summarized in Table I. The absolute absorption maxima(nm) of cytochrome P448 in PenCB H-II were as follows: oxidized, 391, 512, 538; reduced, 417, 542.

Quantitative immunoprecipitation technique was used to learn the ratio of cytochrome P448 to cytochrome P450 in the samples from PenCB-microsomes at each purification step(Table II). Solubilized supernatant of liver microsomes from PenCB-treated rats consisted of about 31 % of cytochrome

Table II

Quantitative immunoprecipitation of cytochromes P450 and P448 in PenCB-microsomes using anti-P450 and anti-P448

Antibody	Solubilized supernatant	ω-Aminooctyl Sepharose 4B eluate			Hydroxyapatite eluate
		A-I	A-II	A-III	H-II
Anti-P450	31*	52	5	0	0
Anti-P448	64	43	90	100	100

The preparations containing 2 nmoles of cytochrome P450 were incubated with varying amounts of either anti-P450(0-52.3 mg protein) or anti-P448(0-33.7 mg protein) in 10 mM phosphate-buffered 0.9 % NaCl(pH 7.5) containing 0.5 % sodium cholate(final volume, 1.0 ml) at 25° for 1 hr and then at 4° for 24 hr. The precipitates were collected by centrifugation and washed with 5 ml of the same saline 4 times. Protein in the precipitates was quantitated by the method of Lowry et al.(28). In order to blank out the protein due to the antibody added, the value obtained from the incubation system without antigen was subtracted from the value of the complete system. Calibration of the precipitated antigen was made by using a calibration curve obtained from varying amounts of purified PB-P450 or BNF-P448 under the same conditions.

*The value represents percent of antigen precipitated with the respective antibody.

Table III

Amino acid compositions of purified cytochromes P450

Amino acid	Number of residues per molecule		
	PenCB-P448	PB-P450	MC-P448
Lysine	34	27	41
Histidine	13	14	12
Arginine	24	25	21
Asx	50	40	47
Threonine	30	25	29
Serine	31	29	49
Glx	49	44	37
Proline	31	30	28
Glycine	31	32	36
Alanine	23	23	26
Valine	38	24	31
Methionine	7	10	5
Isoleucine	27	25	24
Leucine	51	53	45
Tyrosine	11	15	11
Phenylalanine	34	34	28
Cys(O_3H)	6	6	6
(Tryptophan)*	—	—	—
Total**	490	456	476
Polypeptide molecular weight	53,500	52,000	53,500

The specific contents(nmoles/ mg protein) of purified cytochromes P450 analyzed were as follows: PenCB-P448 (PenCB H-II), 18.1; PB-P450(CM-Sephadex eluate), 15.2; MC-P448(MC H-II), 15.0.

* Not determined.
**Number of tryptophan was omitted.

P450 and 64 % of cytochrome P448. PenCB A-III and PenCB H-II did not contain any cytochrome P450, but PenCB A-I and PenCB A-II contained 52 % and 5 % of cytochrome P450, respectively. These results indicate that a homogeneous cytochrome P448 in a high spin form(PenCB H-II) can be easily obtained from PenCB-treated rat liver microsomes without any further purification procedure, that is, CM-Sephadex column chromatography as described by Hashimoto and Imai(18). As shown in Table III, the amino acid composi-

tions of both cytochromes P448 were similar to each other although minor differences were shown with respect to Lys, Ser and Glx. These differences are possibly resulted from a little impurity of MC-P448 analyzed, considering from the fact that the amino acid composition of PenCB-P448 is quite similar to that of MC-P448 reported by Guengerich(12). In addition, the previous study on the immunochemical reactivity of the liver microsomes of rats treated with either MC or PenCB has suggested that MC-P448 and PenCB-P448 contained a common antigenic moiety against anti-P448(20).

In conclusion, the present study clearly indicates that PenCB is an excellent inducer for purifying cytochrome P448, which is similar to MC-P448 with respect to its amino acid composition and immunochemical properties.

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