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MULTIPLE FORMS OF SOLUBILIZED AND PARTIALLY RESOLVED CYTOCHROME P-450 FROM RATS INDUCED BY 2,3,5,2',3',5'- AND 3,4,5,3',4',5'-HEXACHLOROBIPHENYLS

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Summary: Solubilized cytochrome P-450 from control rats and rats treated with two hexachlorobiphenyl (HCB) isomers was resolved into three major fractions (peaks) by anion exchange chromatography at room temperature. 3,4,5,3',4',5'-HCB increased the specific content of cytochrome P-450 in fractions 2 and 3, and induced proteins in these fractions with subunit molecular weights of 52,000 and 55,500 respectively utilizing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 2,3,5,2',3',5'-HCB increased the specific content of fraction 3 and induced a protein in this fraction with a subunit molecular weight of 52,000. These three fractions differed spectrally and catalytically. Our results indicate that 2,3,5,2',3',5'-HCB induced a form of cytochrome P-450 with a subunit molecular weight of 52,000 with a high specific activity toward benzphetamine. 3,4,5,3',4',5'-HCB induced a form of cytochrome P-448 with a subunit molecular weight of 55,500 and high specific activity toward ethoxyresorufin. However, the major portion of the cytochrome induced by 3,4,5,3',4',5'-HCB had a subunit molecular weight of 52,000 and did not metabolize either benzphetamine or ethoxyresorufin. This cytochrome may be another form of cytochrome P-448.

Cytochrome P-450 is the terminal oxidase for the hepatic microsomal mixed function oxygenase system (1). The exact number of subspecies of cytochrome P-450 is not known, but there is considerable evidence for the existence of multiple forms (2,3). Ryan et al. (3) isolated three forms of hepatic cytochrome P-450 from rats with subunit molecular weights on SDS-PAGE of 48,000 (P-450a), 52,000(P-450b), and 56,000(P-450c). Cytochrome P-450b is the major form induced by phenobarbital, while cytochrome P-450c is the major form induced by 3-methylcholanthrene (3-MC). Phenobarbital and 3-MC represent two distinct classes of inducers of hepatic mixed function oxidases (1).

Polychlorinated biphenyls (PCBs) are widespread environmental pollutants. Commercial mixtures of PCBs, such as Aroclor 1254, produce a mixed-type induction of the hepatic mixed-function oxidases which resembles the induction pro-

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duced by a combination of phenobarbital and 3-MC (4). Three forms of cytochrome P-450 have been isolated from rats treated with Aroclor 1254 (3). These forms are immunologically, catalytically, and electrophoretically indistinguishable from the cytochromes purified from phenobarbital (cytochromes P-450a and P-450b) and 3-MC treated rats (cytochromes P-450a and P-450c). It has been reported that individual PCB isomers consist of two classes of inducers, one resembling phenobarbital, and the other 3-MC (5). Ozawa et al. (6) have recently purified a form of cytochrome P-450 from rats treated with 3,4,5,3'4'-pentachlorobiphenyl (an MC-type inducer) to high specific content (18 nmol/mg protein) which cross-reacts with antibody to cytochrome P-448 from β-naphthoflavone treated rats but not with antibody to cytochrome P-450 from phenbarbital treated rats. Using SDS-PAGE, we recently examined the electrophoretic pattern of microsomal proteins induced by PCB isomers representative of the two classes of inducers (7). 3-MC and 3,4,5,3',4',5'-HCB increased a polypeptide with an approximate molecular weight of 54,500, probably representing cytochrome P-450c(P-448), and induced cytochrome P-448 dependent monooxygenase activities. Phenobarbital and 2,3,5,2',3',5'-HCB increased a polypeptide in the 51,000 molecular weight region and induced microsomal benzphetamine N-demethylase activity. However, 3,4,5,3',4',5'-HCB also produced a large increase in a polypeptide band on SDS-PAGE with an approximate molecular weight of 51,500, despite the fact that microsomes from these rats exhibited a marked decrease in benzphetamine N-demethylase activity. The present studies were carried out to examine further the subspecies of cytochrome P-450 induced by 2,3,5,2',3',5'- and 3,4,5,3',4',5'-HCB. A procedure described by Warner and Neims (8) which optimizes recovery was used.

MATERIALS AND METHODS: Charles River CD strain male rats (100-150 g) were given a single dose of 3,4,5,3',4',5'-HCB or 2,3,5,2',3',5'-HCB (50 mg/kg) dissolved in cotton seed oil (15 mg/ml) and sacrificed 3 days later. These isomers (>99% pure) were synthesized by the Environmental Chemistry Branch at NIEHS.

Emulgen-911 was a gift from Kao Atlas Co., Ltd., Tokyo 103, Japan. DE-52 was purchased from Whatman, Inc., Clifton, New Jersey. BSA, sodium cholate, and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Solubilization and ion exchange chromatography: Solubilization and ion exchange chromatography of cytochrome P-450 was performed at room temperature essentially as described by Warner and Neims (8), except that the DE-52 column was 15 x 2.5 cm and the amount of cytochrome P-450 chromatographed was 500 nmol. After application of the sample, the column was washed with 100 ml of 10 mM potassium phosphate (pH 7.4), 0.2% Emulgen-911, 0.5% sodium cholate, 20% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol. This was followed by a linear gradient of potassium chloride (0 to 0.25 M) in 500 ml of the same buffer.

Reconstitution of catalytic activity: NADPH-cytochrome c reductase was purified from rat liver microsomes by the method of Yasukochi and Masters (9), except that the eluate from the 2',5'-ADP Sepharose column was concentrated

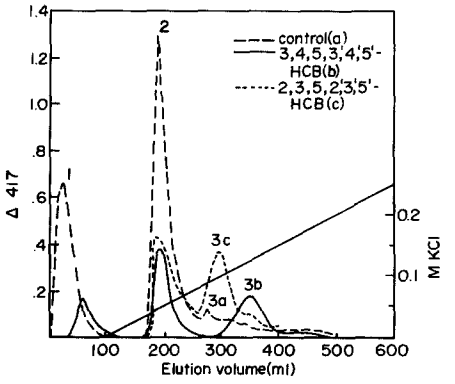

Fig. 1 - Elution profile of solubilized cytochrome P-450 on DE-52 at room temperature. Hepatic microsomes were solubilized in buffered detergent, and 500 nmol of cytochrome P-450 applied to each column. The column was washed with 100 ml of buffered detergent followed by 500 ml of a 0 to 0.25 M KCl gradient.

and purified further on Sephacryl S-200 (10). Reconstitution of benzphetamine N-demethylase activity was performed as described previously (11). The assay mixture contained 0.2 μM cytochrome P-450; 2,000 units/ml NADPH-cytochrome c reductase, 50 μg/ml L-α-phosphatidyl choline (dilauroyl), 100 mM potassium phosphate, pH 7.5; 1 mM benzphetamine, and 0.33 mM NADPH. The cytochrome P-450 was preincubated at 37° for 5 minutes with NADPH-cytochrome c reductase and phosphatidyl choline before the addition of buffer and substrate. The reaction was started by the addition of NADPH, and the disappearance of NADPH followed for 3 minutes. A blank without substrate was subtracted from each value.

Ethoxyresorufin O-deethylase activity was measured essentially as described earlier (8). The assay mixture contained 0.025 μM cytochrome P-450, 1,000 units/ml NADPH-cytochrome c reductase, 50 μg/ml phosphatidyl choline, 72 mM potassium phosphate, pH 7.8, 3 μM ethoxyresorufin, and 0.5 mM NADPH. Cytochrome P-450 was preincubated with reductase and lipid as described above.

Cytochrome P-450 was measured by its reduced CO-difference spectrum (12) and protein by the method of Lowry et al. (13).

SDS-polyacrylamide gel electrophoresis: SDS-PAGE was performed on slab gels as described by Laemlli (14), using a 3% stacking gel, pH 6.8 and a 7.5% running gel, pH 8.8. Gels were 1.5 mm thick, and wells 0.5 cm. Sample size was 15 μg of microsomal protein or 5 μg of partially purified cytochrome. Prior to electrophoresis, samples were boiled for 2 minutes in 1% SDS with β-mercaptoethanol. Gels were fixed overnight in methanol: glacial acetic acid: water (10:3:27), stained with 0.2% Coomassie Brilliant Blue R in methanol: glacial acetic acid (10:3) and destained in methanol: glacial acetic acid: water (10:3:27). Phosphorylase a (94,000), bovine serum albumin (68,000), catalase (58,000), glutamic dehydrogenase (53,000), and aldolase (40,000) were used as molecular weight standards.

RESULTS AND DISCUSSION: In this study, solubilized cytochrome P-450 eluted as three major peaks on DE-52 (Fig. 1). The fractions corresponding to these peaks were pooled for further study. A small fourth peak was usually observed as a shoulder on peak 3, but was not resolved sufficiently from peak 3 for further study. Fraction 1 presumably represents cytochrome which did

TABLE 1

Specific Content, Yield and Spectral Properties of Resolved Fractions of Cytochrome P-450 from Contro 2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB Treated Rats

Preparation	Controls			2,3,5,2',3',5'-HCB			3,4,5,3',4',5'-HCB		
	nmol P-450 mg protein	λ_{max}	% Yield	nmol P-450 mg protein	λ_{max}	% Yield	nmol P-450 mg protein	λ_{max}	% Yiel
Microsomes	0.81	450.4	100%	1.74	450.8	100%	3.63	448.6	100
Fraction 1	0.76	450.8	6%	----	-----	0%	2.15	448.8	10
Fraction 2	1.91	449.6	39%	3.31	450.2	29%	7.46	449.0	30
Fraction 3	0.44	449.6	2%	3.12	450.8	20%	3.56	448.0	14
Total recovery			47%			49%			54

not absorb to the column. This fraction was sometimes turbid and represented 0-10% of the cytochrome applied to the column (Table 1). In control microsomes, most of the cytochrome eluted in fraction 2. 3,4,5,3',4',5'-HCB increased the specific content of cytochrome P-450 in fraction 2 (corresponding to peak 2 on the chromatogram). The proportion of cytochrome in this fraction was not increased relative to controls; however, it should be noted that a constant amount of cytochrome P-450 (500 nmol) was applied to each column. 3,4,5,3',4',5'-HCB increased the total amount of cytochrome P-450 eluting in fraction 2 per mg of microsomal protein, since it increased the amount of cytochrome P-450 per mg of microsomal protein. The CO-maximum of peak 2 from 3,4,5,3',4',5'-HCB treated rats was 449 nm. 2,3,5,2',3',5'- and 3,4,5,3',4',5'-HCB increased the specific content and proportion of cytochrome P-450 eluting in fraction 3 (corresponding to peak 3 of the chromatogram)(Table 1). Fraction 3 from 3,4,5,3',4',5'-HCB treated microsomes eluted somewhat later than fraction 3 from 2,3,5,2',3',5'-HCB treated microsomes. Warner and Neims (15) also reported that induction by phenobarbital or β-naphthoflavone (BNF)(a 3-MC type inducer) increased the amount of cytochrome P-450 eluting in peak 3. However, the third peak obtained from phenobarbital-treated animals was spectrally and catalytically different from the third peak obtained from BNF-treated animals, indicating that these fractions contained different cytochromes. In the present experiments, the maximum of the CO-difference spectrum of fraction 3 from 2,3,5,2',3',5'-HCB treated rats (450.8 nm) is also different from that of fraction 3 from 3,4,5,3',4',5'-HCB treated rats (448 nm)(Table 1).

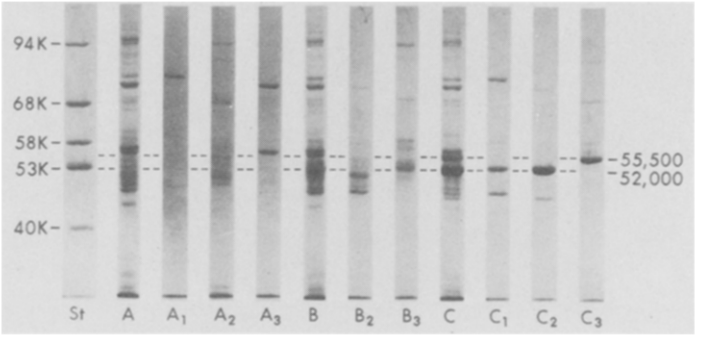

Fig. 2 - SDS-polyacrylamide gel electrophoresis of microsomes and resolved DE-52 fractions from control, 2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB treated rat liver microsomes. Each well contained 5 µg of partially purified fractions or 15 µg of microsomal protein. A = control microsomes, purified fractions designated A_{1-3}; B = 2,3,5,2',3',5'-HCB treated microsomes, B_{2-3} purified fractions; C = 3,4,5,3',4',5'-HCB treated microsomes, C_{1-3} purified fractions. St = standards with the indicated molecular weights.

SDS-PAGE of microsomes and resolved fractions indicate that the major polypeptide in fraction 3 of 2,3,5,2',3',5'-HCB treated microsomes had a subunit molecular weight of 52,000 (Fig. 2), similar to that reported for cytochrome P-450b (3). The major polypeptide in fraction 3 of 3,4,5,3',4',5'-HCB treated microsomes had a subunit molecular weight of 55,500, similar to that reported for cytochrome P-450c (3). Neither of these protein-staining bands were observed in fraction 3 from control microsomes, suggesting that these proteins probably represent cytochrome P-450 which is markedly induced in these fractions (Table 1). Comparable increases were seen in protein bands with these molecular weights in microsomes from 2,3,5,2',3',5'- and 3,4,5,3',4',5'-HCB treated rats. The specific content of cytochrome P-450 in fraction 2 from 3,4,5,3',4',5'-HCB treated rats was 7.5 nmol/mg of protein. The major protein-staining band in this fraction had a subunit molecular weight of 52,000. The intensity of this band was increased markedly compared to fraction 2 from control microsomes. When these three fractions were applied to the gels isochromically (not shown), the 52,000 band in fraction 3 from 2,3,5,2',3',5'-HCB microsomes, the 52,000 band in fraction 2 and the 55,500 band in fraction 3 from 3,4,5,3',4',5'-HCB microsomes were of comparable intensity, further suggesting that these three bands represent the major form of cytochrome P-450 present in these fractions. In contrast, fraction 1 appeared heterogeneous and not significantly purified.

It is interesting that the SDS-PAGE gel profiles of fractions 2 and 3 from 3,4,5,3',4',5'-HCB treated rats appear to indicate a higher purity of the

partially purified cytochrome P-450 than is indicated by the specific contents of these fractions (4-8 nmol/mg). Purified cytochromes P-450$_a$, P-450$_b$, and P-450$_c$ have been shown to have specific contents of 12 to 17 nmol/mg when protein is estimated by the Lowry procedure (3). However, it is difficult to estimate relative amounts of various proteins in gels by visualization with dyes since uptake of dye by different proteins can vary, and Coomassie Blue deviates from Beer's law at low concentrations (16). A number of faint protein bands were consistently observed in fractions 2 and 3 from 3,4,5,3',4',5'-HCB treated rats. Alternatively, some apocytochrome could be present in these fractions.

Although the major protein in fraction 2 of 3,4,5,3',4',5'-HCB treated microsomes had a subunit molecular weight similar to that of fraction 3 of 2,3,5,2',3',5'-HCB treated microsomes, they differed with respect to their substrate specificity. Fraction 3 from 2,3,5,2',3',5'-HCB treated rats metabolized benzphetamine at a relatively high rate (Table 2), as would be expected for cytochrome P-450b (3). However, we were unable to reconstitute benzphetamine N-demethylase activity using fraction 2 from 3,4,5,3',4',5'-HCB treated rats. Fraction 3 from 3,4,5,3',4',5'-HCB treated rats did not metabolize benzphetamine, but it had a high specific activity for ethoxyresorufin, as would be expected for cytochrome P-448 (15). Surprisingly, fraction 2 from 3,4,5,3',4',5'-HCB treated microsomes did not metabolize either ethoxyresorufin or benzphetamine. These data with partially purified fractions are compatible with results obtained with intact microsomes, which show that 2,3,5,2',3',5'-HCB induces benzphetamine N-demethylase (4-fold) and ethoxyresorufin O-deethylase (2-fold), while 3,4,5,3',4',5'-HCB increases ethoxyresorufin O-deethylase (80-fold) but decreases benzphetamine N-demethylase markedly (to 10% of controls) (7).

Cytochrome P-448 is only one of a number of proteins which are induced by the 3-MC class of inducers (16). Coordinate expression of a number of enzymes occurs through derepression of a common gene locus. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), which is believed to interact with the same receptor as 3,4,5,3',4',5'-HCB and 3-MC (17) has been shown to induce two species of cytochrome P-448 in the rabbit (18). Only one form of cytochrome P-448 has been isolated in the rat (3). Our data provide preliminary evidence that 3,4,5,3',4',5'-HCB induces two subspecies of cytochrome P-448 in the rat. One form has spectral electrophoretic, and enzymatic properties similar to those reported for cytochrome P-448(P-450c). The other form has a subunit molecular weight of 52,000, but does not metabolize either benzphetamine or ethoxyresorufin, substrates preferentially metabolized by cytochromes P-450b and P-450c. Ryan et al. (19) have recently isolated a cyto-

TABLE 2

Catalytic activity of resolved fractions of cytochrome P-450 from control, 2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB treated rats

	Benzphetamine N-demethylase activity		
	nmol NADPH oxidized/min/nmol P-450		
	Control	2,3,5,2',3',5'-HCB	3,4,5,3',4',5'-HCB
Fraction 1	0.0 (12.8)[a]	----	29.0 (11.2)
Fraction 2	20.8 (19.1)	38.6 (12.0)	0.0 (17.5)
Fraction 3	41.5 (19.1)	62.3 (12.8)	7.5 (10.4)
	Ethoxyresorufin O-deethylase activity		
	nmol resorufin formed/min/nmol P-450		
	Control	2,3,5,2',3',5'-HCB	3,4,5,3',4',5'-HCB
Fraction 1	0.00	----	0.11
Fraction 2	0.00	0.09	0.00
Fraction 3	0.11	0.00	10.90

[a]Blank values representing the amount of NADPH oxidized by each Fraction without added substrate (Figures in parentheses) were subtracted from each value.

chrome with a molecular weight of 52,000 from isosafrole treated rats which did not metabolize a number of substrates metabolized by cytochromes P-450b or P-450c. This is the first report that PCBs induce a form of cytochrome P-450 with these properties. In contrast to 3,4,5,3',4',5'-HCB, 2,3,5,2',3',5'-HCB induces a subspecies of cytochrome P-450 with a subunit molecular weight of 52,000 which metabolizes benzphetamine. Its electrophoretic catalytic and spectral properties are consistent with those reported for cytochrome P-450b (3). Therefore, PCBs appear to induce at least three distinct subspecies of cytochrome P-450.

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