RECONSTITUTED LIVER MICROSOMAL ENZYME SYSTEM THAT HYDROXYLATES DRUGS, OTHER FOREIGN COMPOUNDS AND ENDOGENOUS SUBSTRATES. I. Determination of Substrate Specificity by the Cytochrome P-450 and P-448 Fractions

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SUMMARY: The reconstituted microsomal hydroxylation enzyme system from rats treated with phenobarbital exhibited high activity for benzphetamine N-demethylation, but very low activity for 3,4-benzpyrene hydroxylation. However, when the cytochrome P-450 fraction from phenobarbital-treated rats was replaced by the cytochrome P-448 fraction from rats treated with 3-methylcholanthrene, the N-demethylation of benzphetamine was decreased while the hydroxylation of 3,4-benzpyrene was greatly increased. On the other hand, the reconstituted system from 3-methylcholanthrene-treated rats showed good benzpyrene hydroxylase activity which could be greatly decreased if the cytochrome P-448 fraction was replaced by the P-450 fraction from rats treated with phenobarbital. These and other experiments indicate that the substrate specificity of the hydroxylation system resides in the cytochrome fraction rather than in the reductase or lipid fraction, and the data suggest that cytochromes P-450 and P-448 have different catalytic activities.

Cytochrome P-450, the CO-binding pigment of liver microsomes (1-3), has been shown to be the terminal oxidase (4,5) and the substrate-binding site (6,7) for the mixed-function oxidase system which metabolizes a variety of drugs, steroids, fatty acids and other foreign compounds (8,9). The ability of CO and other chemicals to selectively inhibit some microsomal hydroxylation reactions more than others (8, 10,11) suggested that more than one CO-binding hemoprotein may participate in microsomal hydroxylation reactions. Other studies indicated that the CO-binding pigment in animals treated with a polycyclic hydrocarbon such as 3-methylcholanthrene (3-MC) is different from the CO-binding pigment in control animals or in animals treated with phenobarbital (PB) with respect to CO-difference spectrum (12,13), ethyl isocyanide difference spectrum (14) and absorption spectrum (15). In addition,

the relative amounts of two radioactive CO-binding hemoprotein fractions occurring in liver microsomes are altered by 3-MC treatment (16,17).

The liver microsomal hydroxylation enzyme system from PB-treated animals has been solubilized and resolved by Lu et al. into fractions containing cytochrome P-450, NADPH-cytochrome P-450 reductase and lipid (18,19), and all three fractions are essential for the maximal rate of hydroxylation of fatty acids, alkanes and a variety of drugs (19-21). The lipid component has recently been identified as phosphatidylcholine (22). In the study described below, the substrate specificity of the reconstituted hydroxylation enzyme systems prepared from rats treated with PB and 3-MC has been investigated. Benzphetamine and 3,4-benzpyrene were chosen as the substrates because the metabolism of these compounds has been shown to be preferentially induced by PB and 3-MC, respectively (23). Evidence is presented in the present paper that the reconstituted systems from PB- and 3-MC-treated rats have different substrate specificities, and that the specificity resides in the cytochrome fraction rather than in the reductase or lipid fraction.

METHODS: Male Long-Evans rats weighing 50-55 g were injected i.p. with PB (100 mg/kg) or 3-MC (25 mg/kg) once daily for 3 days. The animals were killed 24 hours after the last injection, and liver microsomes were prepared as described elsewhere (18). The lipid fraction was prepared as previously described (18); however, the procedures for the preparation of the reductase and cytochromes P-450 (from PB-treated rats) and P-448 (from 3-MC-treated rats) fractions were modified. The reductase fraction was prepared by extraction of microsomes (1 g of protein in 24 ml of 0.25M sucrose) with 10% aqueous acetone, in a final volume of 600 ml, according to the method of Lester and Fleischer (24). The acetone-treated microsomes were suspended in 105 ml of 0.1M Tris buffer, pH 7.7, containing 10^{-3}M dithiothreitol (DTT), and the suspension (5.7 mg protein/ml) was sonicated, with the temperature maintained below 8°, using a Biosonik III (Bronwill Scientific Division, Rochester, New York) at full output for three 1-min intervals. Sodium deoxycholate was added to a final concentration of 0.5 mg per mg of protein. The

mixture was stirred at 4° for 20 min and then centrifuged at 183,000 xg for 1 hour. The clear yellow supernatant fraction was placed on a DEAE-cellulose column previously equilibrated with 0.1M Tris buffer, pH 7.7, containing 10^{-4}M DTT and 0.05% sodium deoxycholate, and the column was eluted with a linear gradient of KCl as described previously (18). Reductase fractions were then pooled and concentrated by Diaflo membrane ultrafiltration (Amicon Corporation, Lexington, Massachusetts). When prepared in this way, the reductase fraction did not contain cytochrome P-450 or P-448. When assayed by the NADPH-dependent reduction of cytochrome c (25), the final reductase preparations from PB- and 3-MC-treated rats had specific activities (nmole/min/mg protein) of 490 and 260, respectively.

Cytochrome P-450 and P-448 fractions were prepared by suspending liver microsomes (900 mg of protein) from PB- and 3-MC-treated rats in 61 ml of a mixture containing 14 ml of glycerol, 3.5 ml of 2M Tris (pH 7.7), 0.7 ml of 0.1M DTT and water. The suspension was sonicated for four 30-sec intervals, with the temperature below 8°. Nine ml of 10% sodium cholate solution was added, and the mixture was stirred at 4° for 20 min. Solid ammonium sulfate was added to 43% saturation and then to 50% saturation. The precipitate from 43-50% saturation was dissolved in 10 ml of 0.1M Tris (pH 7.7), containing 10^{-4}M DTT and 20% glycerol, and dialyzed overnight in the same buffer. The dialyzed hemoprotein preparation was diluted to 50 ml with the Tris buffer mixture, and solid ammonium sulfate was added to 45% saturation and then 50% saturation. The resulting precipitate from 0-45% saturation was dissolved in buffer and used for enzyme assay, although considerable amounts of cytochrome P-450 or P-448 were occasionally recovered in the 45-50% saturation preparation. The 0-45% fraction generally had a specific activity of 1.5-2.5 nmoles of P-450 or P-448 per mg of protein. When prepared in this way, the cytochrome P-450 and P-448 fraction contained insignificant amounts of reductase activity.

Benzphetamine N-demethylation was assayed by following the rate of NADPH oxidation as described earlier (20), since it has been shown that the rate of benzphetamine-dependent NADPH oxidation corresponds to the rate of formaldehyde forma-

tion (21). 3,4-Benzpyrene hydroxylation was measured by the procedures described by Nebert and Gelboin (26), with slight modification. Cytochromes P-450 and P-448 were determined by the method of Omura and Sato (3).

RESULTS AND DISCUSSION: The hydroxylation of 3,4-benzpyrene by the reconstituted system prepared from 3-MC-treated rats was found to require the cytochrome P-448, reductase, and lipid fractions for maximal activity (Table I). When the cytochrome P-448 fraction was replaced by an approximately equivalent amount (Table I), or a 5-fold higher amount of the cytochrome P-450 fraction obtained from PB-treated rats, 3,4-benzpyrene hydroxylation activity was markedly decreased. In experiments not shown here, it was found that synthetic lauroyl glycerol-3-phosphorylcholine could replace the crude lipid fraction in supporting 3,4-benzpyrene hydroxylation in the presence of the cytochrome P-448 and reductase fractions. Thus, it appears that the lipid requirement for the 3-MC-induced benzpyrene hydroxylase system is the same as that reported by Strobel et al. (22) for the PB-induced hydroxylase system.

Table I

3,4-Benzpyrene Hydroxylation by the Reconstituted System from Rats Treated with 3-MC

Fractions	nmoles 8-Hydroxy-3,4-benzpyrene formed
Reductase (3-MC) + lipid (3-MC)	0.04
Reductase (3-MC) + P-448 (3-MC)	0.07
P-448 (3-MC) + lipid (3-MC)	0.10
Reductase (3-MC) + lipid (3-MC) + P-448 (3-MC)	0.66
Reductase (3-MC) + lipid (3-MC) + P-450 (PB)	0.05

The reaction mixture, in a final volume of 1.0 ml, contained 100 µmoles of potassium phosphate buffer (pH 7.4), 3 µmoles of $MgCl_2$, 2 mg bovine serum albumin, 0.4 µmoles of NADPH, 80 nmoles of 3,4-benzpyrene, and the following microsomal components: from 3-MC-treated rats—reductase fraction (0.04 mg of protein), P-448 fraction (0.05 nmole), and lipid fraction (0.1 mg of lipid); from PB-treated rats—P-450 fraction (0.04 nmole). The reaction mixture was incubated at 30° for 10 min. 8-Hydroxy-3,4-benzpyrene was determined by the method of Nebert and Gelboin (26).

Table II

3,4-Benzpyrene Hydroxylation by the Reconstituted System from Rats Treated with PB

Fractions	nmoles 8-Hydroxy-3,4-benzpyrene formed
Reductase (PB) + lipid (PB)	0.01
Reductase (PB) + lipid (PB) + P-450 (PB)	0.07
Reductase (PB) + lipid (PB) + P-448 (3-MC)	1.13

Assay conditions were similar to those described in Table I. The amounts of microsomal components used were as follows: from PB-treated rats—reductase fraction (0.05 mg of protein), lipid fraction (0.1 mg of lipid), and P-450 fraction (0.10 nmole); from 3-MC-treated rats—P-448 fraction (0.12 nmole).

Further evidence indicating that the cytochrome fraction is responsible for the substrate specificity is shown in Table II. It can be seen that the reconstituted system from PB-treated rats exhibited very low activity using 3,4-benzpyrene as substrate. A marked increase in 3,4-benzpyrene hydroxylation activity, however, was observed when a comparable amount of cytochrome P-448 was used instead of P-450. In confirmation of an earlier report (20), benzphetamine N-demethylation by the reconstituted system prepared from PB-treated rats was found to require cytochrome P-450, NADPH-dependent reductase and a lipid fraction (Table III). No activity was observed when any one of these components was omitted from the reaction mixture. When the cytochrome P-450 fraction was replaced by an equal amount of the cytochrome P-448 fraction prepared from 3-MC-treated rats, the N-demethylation of benzphetamine was markedly decreased. This low activity was not significantly increased—even by a 10-fold increase in P-448 concentration. Additional studies revealed that the cytochrome P-448 fraction was not inhibitory when added to the reaction mixture in the presence of the cytochrome P-450 fraction.

Evidence presented in this paper shows that the reconstituted hydroxylation enzyme system from rats treated with PB exhibited high activity for benzphetamine

Table III

Benzphetamine N-Demethylation by the Reconstituted System from Rats Treated with PB

Fractions	nmoles NADPH oxidized per min at 30°
Reductase (PB) + lipid (PB)	<0.30
Reductase (PB) + P-450 (PB)	<0.30
P-450 (PB) + lipid (PB)	<0.30
Reductase (PB) + lipid (PB) + P-450 (PB)	3.23
Reductase (PB) + lipid (PB) + P-448 (3-MC)	0.97

The reaction mixture, in a final volume of 1.0 ml, contained 100 μmoles of potassium phosphate buffer (pH 7.4), 3 μmoles of $MgCl_2$, 1 μmole of benzphetamine, 0.1 μmole of NADPH, and the following microsomal components: from PB-treated rats—reductase fraction (0.25 mg of protein), P-450 fraction (0.21 nmole), and lipid fraction (0.1 mg of lipid); from 3-MC treated rats—P-448 fraction (0.22 nmole). The reaction was initiated by the addition of NADPH. The above activities have been corrected for the rate of NADPH oxidation in the absence of benzphetamine.

N-demethylation but very low activity for 3,4-benzpyrene hydroxylation. Replacement of the cytochrome P-450 fraction by the cytochrome P-448 fraction from rats treated with 3-MC resulted in a marked decrease in benzphetamine N-demethylation and a large increase in 3,4-benzpyrene hydroxylation. Conversely, the reconstituted system from 3-MC-treated rats showed high 3,4-benzpyrene hydroxylase activity, which was greatly decreased when the cytochrome P-448 fraction was replaced by the P-450 fraction from rats treated with PB. Preliminary studies indicated that, in the presence of cytocyrome P-450 or P-448, the reductase and lipid fractions prepared from rats treated with PB or 3-MC were interchangeable in supporting 3,4-benzpyrene hydroxylation and benzphetamine N-demethylation. These findings indicate that the substrate specificity of the hydroxylation system resides in the cytochrome fraction rather than in the reductase or lipid fraction. The data also suggest that cytochromes P-450 and P-448 in liver microsomes have different catalytic activities. Interestingly, Jefcoate *et al.* recently reported that two cytochrome P-450 fractions in adrenal cortex mitochondria possess different catalytic activities (27). Definitive information on the cata-

lytic activities of cytochromes in the liver and adrenal gland, however, must await further purification of the hemoproteins.

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