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Reconstituted Liver Microsomal Enzyme System That Hydroxylates Drugs, Other Foreign Compounds, and Endogenous Substrates

III. Properties of the Reconstituted 3,4-Benzpyrene Hydroxylase System

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SUMMARY

The hydroxylation of 3,4-benzpyrene by the reconstituted liver microsomal system from rats treated with 3-methylcholanthrene required cytochrome P-448, NADPH-dependent reductase, and lipid for maximal activity. Synthetic phosphatidylcholine could replace the lipid fraction, while detergents such as Triton X-100, sodium deoxycholate, and sodium cholate could only partially replace the lipid fraction. The apparent K_m values of NADPH and NADH were 7.04 μM and 362 μM, respectively.

The apparent K_m of 3,4-benzpyrene was 1.53 μM for the reconstituted system from rats treated with phenobarbital and 2.87 μM for the reconstituted system from rats treated with 3-methylcholanthrene. Although the source of the reductase preparation (from either 3-methylcholanthrene- or phenobarbital-treated rats) did not appear to affect the K_m of 3,4-benzpyrene in the cytochrome P-450-dependent system, the K_m of 3,4-benzpyrene in the cytochrome P-448-dependent system was significantly increased when the reductase from 3-methylcholanthrene-treated rats was replaced with the reductase from rats treated with phenobarbital. In contrast, the lipid fractions from phenobarbital- and 3-methylcholanthrene-treated rats were interchangeable, and thus the source of lipid did not affect the K_m of 3,4-benzpyrene in either of the reconstituted systems.

The cytochrome P-450 fractions from rats treated with phenobarbital was only 10% as active as the cytochrome P-448 fraction in catalyzing the hydroxylation of 3,4-benzpyrene in the presence of reductase and lipid. Cytochrome P-448- and P-450-dependent 3,4-benzpyrene hydroxylations could be selectively affected by various compounds, which suggests—along with other indirect evidence—that the low activity with cytochrome P-450 for 3,4-benzpyrene hydroxylation is due to an inherent property of cytochrome P-450 and is not due to contamination of the cytochrome P-450 preparation with cytochrome P-448.

INTRODUCTION

The hydroxylation of 3,4-benzpyrene in liver microsomes was shown by Conney *et al.* (1, 2) to require NADPH and molecular oxygen. These investigators also described the now widely recognized induction in animals of 3,4-benzpyrene hydroxylase activity by the prior administration of polycyclic hydrocarbons such as 3-methylcholanthrene. Subsequent studies (3–8) have suggested that the increase in 3,4-benzpyrene hydroxylase activity in rats after 3-MC[1] treatment may be related to the formation of a new hemoprotein called cytochrome P-

[1] The abbreviations used are: 3-MC, 3-methylcholanthrene; PB, phenobarbital.

448,[2] so named because it is spectrally distinct from the cytochrome P-450 found in untreated or phenobarbital-treated rats (3, 9, 10). As a result of the induction of its metabolism by polycyclic hydrocarbons, 3,4-benzpyrene has been widely used as a model "3-MC-inducible" substrate, in contrast to the many "PB-inducible" substrates.

Silverman and Talalay (11) were able to solubilize the 3,4-benzpyrene hydroxylase system by treatment of rat liver microsomes with Triton N-101. This preparation was relatively stable and was capable of hydroxylating 3,4-benzpyrene. However, no resolution of this multienzyme system was achieved. Using the technique developed by Lu *et al.* (12–15), we have recently resolved the 3,4-benzpyrene hydroxylase system from rats treated with 3-MC into three fractions, containing cytochrome P-448, NADPH-dependent reductase, and lipid (16). When the metabolism of a variety of substrates by the reconstituted systems prepared from rats treated with either PB or 3-MC was studied, it was found that the substrate specificity for hydroxylation resides primarily in the cytochrome fraction rather than in the reductase or lipid fraction (16–19). Thus, in agreement with induction studies, the cytochrome P-448 fraction was far more active than the cytochrome P-450 fraction for 3,4-benzpyrene hydroxylation. These results were interpreted to indicate that the cytochrome P-448 fraction is catalytically different from the P-450 fraction.

In this paper the properties of the reconstituted and microsomal 3,4-benzpyrene hydroxylase systems are compared. In addition, differences between the reconstituted cytochrome P-448- and P-450-dependent hydroxylations of 3,4-benzpyrene[3] are described.

[2] The cytochrome induced by 3-methylcholanthrene administration to rats has been referred to as P-448 (3), P_1-450 (9), or P-446 (10). In this paper cytochromes P-448 and P-450 will be used to denote the microsomal CO-binding pigments induced by the administration to rats of 3-methylcholanthrene and phenobarbital, respectively.

[3] The cytochrome P-450- and P-448-dependent 3,4-benzpyrene hydroxylation systems refer to the reconstituted systems obtained from rats treated with phenobarbital and 3-methylcholanthrene, respectively.

METHODS

Male Long-Evans rats (from Blue Spruce Farms, Altamont, N. Y.), weighing 50–55 g, were treated intraperitoneally with PB (100 mg/kg/day) or 3-MC (25 mg/kg/day) once daily for 3 days. Liver microsomes were prepared as previously described (12). The method of solubilization and partial purification of cytochrome P-448 (from 3-MC-treated rats) and P-450 (from PB-treated rats), and the method of preparation of the lipid fractions, have recently been described (19). Cytochrome P-448 and P-450 concentrations were determined by the method of Omura and Sato (20), using the same extinction coefficient (91 mM^{-1} cm^{-1}) for both cytochromes. When the heme content of these preparations was determined by the pyridine-hemochromogen method (20), the extinction coefficient for $A_{450-490}$ or $A_{448-490}$ was found to be within 10% of 91 mM^{-1} cm^{-1}.

The reductase fraction was prepared from PB microsomes or 3-MC microsomes,[4] as previously described (19), with two major modifications. The microsomes were extracted with acetone kept at room temperature (22°), rather than with acetone chilled to −20°. Warm acetone was more effective than chilled acetone in extracting lipids from microsomes. After sonication and solubilization by sodium deoxycholate of the acetone-treated microsomes, the soluble fraction was subjected to ammonium sulfate fractionation, rather than to DEAE-cellulose column chromatography. Solid ammonium sulfate was added to the clear, yellow, soluble fraction (generally 130 ml from 600 mg of microsomal protein) to 40% saturation. The mixture was stirred for 20 min and centrifuged at 37,000 × *g* for 10 min, and the precipitate was discarded. Additional solid ammonium sulfate was added to the supernatant fluid to 80% saturation; the mixture was again stirred and centrifuged. The resulting precipitate from 40 to 80% saturation was dissolved in about 10 ml of a buffer mixture containing 0.02 M potassium phosphate (pH 7.7), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol, and was dialyzed against

[4] PB microsomes and 3-MC microsomes refer to the microsomes prepared from rats treated with phenobarbital and 3-methylcholanthrene, respectively.

2 liters of the same buffer mixture overnight. The dialyzed sample was centrifuged at 37,000 × *g* for 10 min. The clear, yellow preparation was made 1 mM with respect to dithiothreitol and stored, in small aliquots, under nitrogen at −20°. The final reductase preparations from rats treated with PB or 3-MC generally had the same specific activities (i.e., 500 and 200 units/min/mg of protein, respectively) as those obtained from the original procedure (19). One unit of reductase is defined as the amount catalyzing the reduction of 1.0 nmole of cytochrome *c* per minute under the assay conditions described previously (13). The total recovery was approximately 30% of the total microsomal cytochrome *c*-reducing ability. These reductase preparations were stable for several weeks.

The 3,4-benzpyrene hydroxylase activity of microsomal suspensions, or of the reconstituted system, was determined according to the procedure of Nebert and Gelboin (21), as previously described (19). The reaction mixture, containing 100 μmoles of potassium phosphate buffer (pH 6.8), 3 μmoles of $MgCl_2$, 0.4 μmole of NADPH, 80 nmoles of 3,4-benzpyrene (added in 0.04 ml of acetone), and the necessary microsomal fractions in a final volume of 1.0 ml, was incubated at 37° for 5 min. Protein concentration was determined by the method of Lowry *et al.* (22). 3,4-Benzpyrene, 3-methylcholanthrene, NADPH, and horse heart cytochrome *c* were obtained from Sigma. 7,8-Benzoflavone was purchased from Aldrich. Synthetic lauroylglycerol-3-phosphorylcholine was the generous gift of Drs. M. J. Coon and H. W. Strobel.

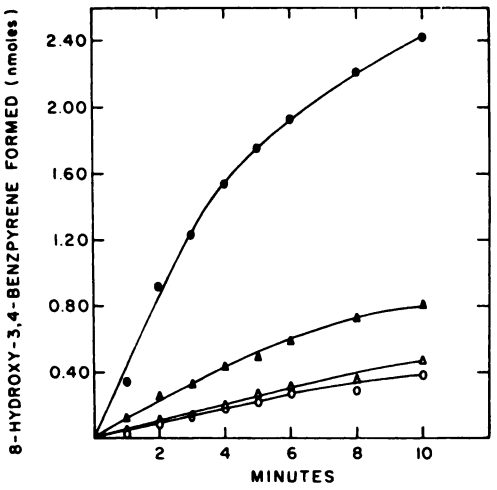

FIG. 1. *3,4-Benzpyrene hydroxylation as a function of incubation time in microsomal and reconstituted systems*

▲, microsomes (3-MC, 0.05 mg of protein containing 0.06 nmole of P-448); △, microsomes (PB, 0.10 mg of protein containing 0.18 nmole of P-450); ●, 0.22 nmole of cytochrome P-448, 0.02 mg of reductase (PB, 12 units), and 0.1 mg of lipid (3-MC); ○, 0.29 nmole of cytochrome P-450, 0.02 mg of reductase (PB, 12 units), and 0.1 mg of lipid (3-MC). PB or 3-MC in parentheses above refers to the fractions prepared from rats treated with either phenobarbital or 3-methylcholanthrene. Other components in the reaction mixtures (final volume, 1.0 ml) were described in the text.

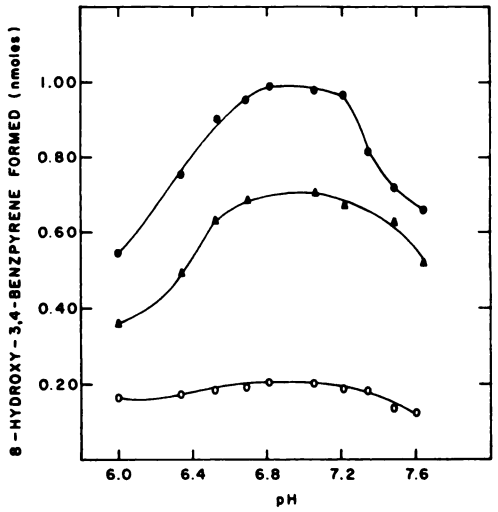

FIG. 2. *3,4-Benzpyrene hydroxylation as a function of pH in microsomal and reconstituted systems*

▲, microsomes (3-MC, 0.05 mg of protein containing 0.06 nmole of P-448); ●, reconstituted system from 3-MC-treated rats (0.22 nmole of cytochrome P-448; 0.04 mg of reductase, 10 units; and 0.1 mg of lipid); ○, reconstituted system from PB-treated rats (0.29 nmole of cytochrome P-450; 0.05 mg of reductase, 23 units; and 0.1 mg of lipid). The final volume was 1.0 ml. The pH values were measured in the complete reaction mixture at 37°.

RESULTS

General properties. Previous studies had established that the hydroxylation of 3,4-benzpyrene by the reconstituted system

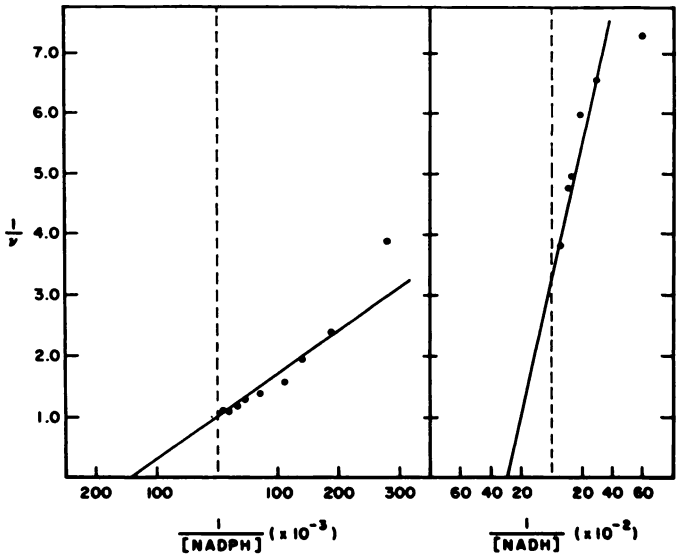

FIG. 3. *Double-reciprocal plot for determination of K_m of NADPH and NADH for 3,4-benzpyrene hydroxylation in the reconstituted system*

The reaction mixtures contained, in addition to the usual components, the following fractions prepared from rats treated with 3-MC: 0.22 nmole of cytochrome P-448, 0.04 mg of reductase (10 units), and 0.1 mg of lipid. The final volume was 1.0 ml. The apparent K_m calculated from the plot was identical with the value obtained with a GE-600 computer, using a FORTRAN program written by W. W. Cleland.

prepared from rats treated with 3-MC required the cytochrome P-448, reductase, and lipid fractions for maximal activity (16). In the determination of the activity of the reconstituted system, it is essential that the three components be added to the incubation flask first and be thoroughly mixed before the buffer and cofactors are added. The results are most consistent when this order of addition is followed. Only one-third to two-thirds of the activity is obtained when the components are added in any other manner. While the mechanism of this phenomenon is not understood at present, the importance of the order of addition of components has been reported for another lipid-requiring enzyme system (23).

The rate of the reaction was linear for approximately 5 min, with either microsomes or the reconstituted systems containing cytochrome P-448 or P-450 (Fig. 1). Both the microsomal and the reconstituted enzyme systems exhibited the same broad pH optimum, which extended from 6.7 to 7.2 (Fig. 2). NADH was only about 20% as active as NADPH in supporting the reaction with microsomes (2). With the reconstituted system, NADH at saturating levels was only 30% as active as NADPH. The apparent K_m values of NADPH and NADH were found to be 7.04 μM and 362 μM, respectively (Fig. 3). In the presence of 0.2 mM NADH and 0.08 mM NADPH, NADH inhibited the NADPH-supported hydroxylation of 3,4-benzpyrene by 13%—rather than enhancing it—as has been reported for NADPH-supported aminopyrine metabolism in microsomes (24).

Alvares *et al.* (25) reported that bovine serum albumin enhanced 3,4-benzpyrene hydroxylase activity when low microsomal protein concentrations were used. With the reconstituted system, as well as our microsomal preparations, albumin (0.5–2 mg/ml) has been found to have no effect on the reaction rate. The stimulatory effect of bovine serum albumin may be due to the quality of the water used for assay. Hayakawa and Udenfriend[5] have observed that the stimulatory effect of albumin on 3,4-benzpyrene hydroxylation in rat liver microsomes was not consistent. In addition, 0.1 mM EDTA

[5] Personal communication.

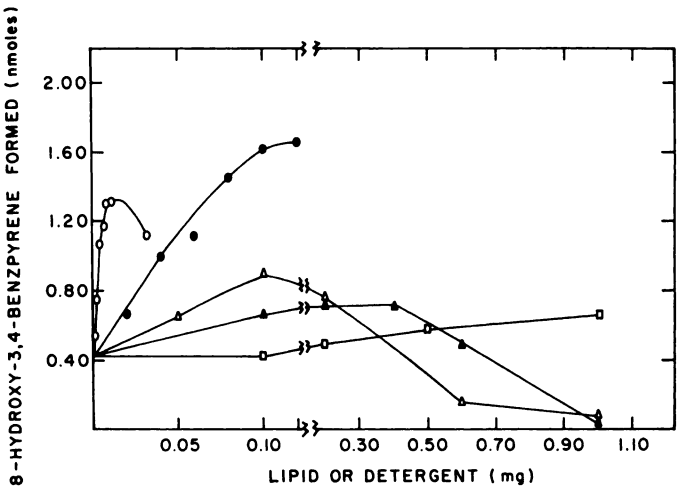

FIG. 4. *3,4-Benzpyrene hydroxylation as a function of lipid or detergent concentration in reconstituted system*

The standard reaction mixtures contained 0.26 nmole of cytochrome P-448, 0.03 mg of reductase (3-MC, 5 units), and the indicated amounts of lipid or detergent. ●, crude lipid fraction from 3-MC-treated rats; ○, synthetic lauroylglycerol-3-phosphorylcholine; ▲, sodium deoxycholate; △, Triton X-100; □, sodium cholate. The final volume was 1.0 ml.

was as effective as 1 mg of albumin per milliliter. In contrast to the Mg^{2+}-dependent hydroxylation of 3,4-benzpyrene in the hamster fetus cell culture system of Nebert and Gelboin (21), magnesium ions are not required for the reaction.

Lipid requirement. Phosphatidylcholine has recently been identified as the active lipid component required for the hydroxylation of fatty acids, hydrocarbons, and a number of drugs (26). As shown in Fig. 4, rather low concentrations of synthetic lauroylglycerol-3-phosphorylcholine could replace the crude lipid fraction in supporting 3,4-benzpyrene hydroxylation. In this particular experiment, lauroylglycerol-3-phosphorylcholine was about 80% as active as the crude lipid fraction. In earlier experiments, not shown here, the same synthetic phospholipid was found to be as effective as the crude lipid fraction. In addition, certain detergents could also partially replace the lipid fraction. For example, Triton X-100 and sodium deoxycholate slightly stimulated the reaction rate at low concentrations, but were strongly inhibitory at higher concentrations. Sodium cholate stimulated the reaction slightly at rather high concentrations. The same pattern of stimulation and inhibition by Triton X-100, sodium deoxycholate, and sodium cholate was also observed with the reconstituted system obtained from PB-treated rats. Recently Triton X-100 has also been shown to stimulate the ω-hydroxylation of fatty acids in the reconstituted system from porcine kidney cortex microsomes (27).

Inhibition studies. The presence of both cytochromes P-450 and P-448 in liver microsomes has been suggested by a number of investigators (28, 29). Since the cytochrome P-448 and P-450 fractions used in the present study were not highly purified, and the existing assay method (mainly the use of the CO difference spectrum) cannot precisely determine the percentage of each cytochrome in a preparation, the relative amounts of cross-contamination in the cytochrome fractions are not known. Until the enzymes have been totally purified, only indirect means are available to determine whether the low activity of the cytochrome P-450 fraction for 3,4-benzpyrene hydroxylation is due to the P-450 itself or to the presence of small amounts of P-448 in the cytochrome P-450 preparation. One approach employed in these studies was to show a selective effect on either the cytochrome P-448- or P-450-dependent 3,4-benzpyrene hydroxylation reaction by various compounds. 7,8-Benzo-

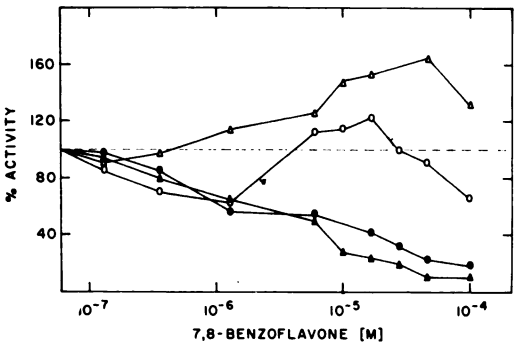

FIG. 5. *Effect of 7,8-benzoflavone on 3,4-benzpyrene hydroxylation in microsomal and reconstituted systems*

▲, microsomes (3-MC, 0.05 mg of protein; 100% activity, 0.56 nmole/5 min); △, microsomes (PB, 0.08 mg of protein; 100% activity, 0.21 nmole/5 min); ●, reconstituted system from 3-MC-treated rats (0.22 nmole of cytochrome P-448, 0.09 mg of protein; 0.04 mg of reductase, 10 units; and 0.1 mg of lipid; 100% activity, 1.17 nmoles/5 min); ○, reconstituted system from PB-treated rats (0.29 nmole of cytochrome P-450, 0.09 mg of protein; 0.04 mg of reductase, 17 units; and 0.1 mg of lipid; 100% activity, 0.30 nmole/5 min). The final volume was 1.0 ml.

flavone, aminopyrine, and SKF 525-A (diethylaminoethyl diphenylpropylacetate) were selected because they have previously been shown to have a selective effect on 3,4-benzpyrene hydroxylation in microsomes from rats treated with PB or 3-MC (7, 30, 31).

As shown in Fig. 5, 7,8-benzoflavone strongly inhibited 3,4-benzpyrene hydroxylation in 3-MC microsomes as well as in the cytochrome P-448-dependent reconstituted system. On the other hand, in PB microsomes, as well as in the cytochrome P-450-dependent system, this compound inhibited the reaction slightly at low concentrations but stimulated the reaction at higher concentrations. Aminopyrine, a substrate of the microsomal hydroxylation system, did not affect either the cytochrome P-448 or the 3-MC microsomal system, but inhibited both the cytochrome P-450 and the PB-microsomal systems (Fig. 6). Although the effect of a compound on the PB microsomal and the cytochrome P-450-dependent reconstituted systems differed quantitatively (as shown in Figs. 5 and 6), the direction of the effect was the same with both PB systems. The effect of both compounds on the 3-MC microsomal and cytochrome P-448 systems was more internally consistent. The studies with 7,8-benzoflavone and aminopyrine were repeated, using the same reductase and lipid fractions and varying only the hemoprotein. The results were the same as reported for the reconstituted system from PB- or 3-MC-treated rats, indicating that the observed differential effect is due to the hemoprotein, rather than to the reductase or lipid. SKF 525-A at a concentration of 2 mM inhibited the cytochrome P-450-dependent 3,4-benzpyrene hydroxylation by 48% but the cytochrome P-448 system by only 9%. At this same concentration, SKF 525-A inhibited 3,4-benzpyrene hydroxylation in PB and 3-MC microsomes by 90% and 60%, respectively. Both reconstituted systems could be inhibited to a greater extent by increasing the concentration of SKF 525-A. Thus the microsomal 3,4-benzpyrene hydroxylation system was more sensitive to inhibition by SKF 525-A than the reconstituted system. These selective effects strongly suggest that

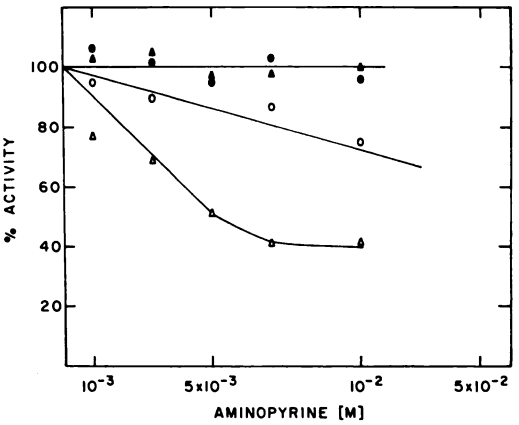

FIG. 6. *Effect of aminopyrine on 3,4-benzpyrene hydroxylation in microsomal (▲, 3-MC; △, PB) and reconstituted (●, 3-MC; ○, PB) systems*

The concentrations of microsomes and various fractions used were the same as described in Fig. 5. The activities for 100% were 0.48, 0.17, 0.90, and 0.24 nmole/5 min for the 3-MC microsomal, PB microsomal, P-448 reconstituted, and P-450 reconstituted systems, respectively. The final volume was 1.0 ml.

TABLE 1

Effects of various compounds on 3,4-benzpyrene hydroxylation by reconstituted systems from PB- and 3-MC-treated rats

The reaction mixtures (final volume, 1.0 ml) contained the usual components and the microsomal fractions as follows: the reconstituted system from 3-MC-treated rats (P-448 system) consisted of 0.43 nmole of cytochrome P-448, 0.07 mg of reductase (10 units), and 0.1 mg of lipid; the reconstituted system from PB-treated rats (P-450 system) contained 0.58 nmole of cytochrome P-450, 0.05 mg of reductase (23 units), and 0.1 mg of lipid. The activities for 100% were 1.34 and 0.26 nmoles/5 min for the P-448 and P-450 systems, respectively. For compounds which were dissolved in methanol, a control containing the same volume of methanol (0.05 ml) was included. The activities for 100% in these controls were 1.51 and 0.32 nmoles/5 min for the P-448 and P-450 systems, respectively.

Compound	Final concentration	Activity	
		P-448 system	P-450 system
	M	%	%
None		100	100
EDTA	10^{-2}	112	100
KCN	10^{-2}	104	90
Cytochrome *c*	10^{-6}	51	53
	10^{-4}	21	15
Menadione[a]	5×10^{-6}	97	99
	5×10^{-5}	8	6
N-Ethylmaleimide	10^{-4}	67	68
Iodoacetamide	10^{-3}	30	56
	10^{-2}	11	22
p-Hydroxymercuribenzoate	5×10^{-5}	5	4
8-Hydroxyquinoline[a]	5×10^{-5}	22	21
1,10-Phenanthroline[a]	5×10^{-3}	38	39
α,α'-Dipyridyl[a]	5×10^{-3}	55	55
Diethyldithiocarbamate[a]	5×10^{-3}	80	91
Sodium azide	10^{-3}	103	90
	5×10^{-3}	98	72
	10^{-2}	92	61

[a] Dissolved in methanol.

the hydroxylation of 3,4-benzpyrene by the cytochrome P-450- and P-448-dependent reconstituted systems is catalyzed by different enzymes, i.e., the two different cytochromes.

The effects of a variety of other inhibitors on the reconstituted cytochrome P-448- and P-450-dependent 3,4-benzpyrene hydroxylation systems are summarized in Table 1. No selective effect was found on either the cytochrome P-448- or P-450-dependent system with any of the following compounds. As expected for the microsomal hydroxylation system, EDTA and KCN had no effect, even at very high concentrations. Cytochrome *c* and menadione strongly inhibited the reaction, presumably by serving as electron acceptors for the reductase. Among the sulfhydryl-binding reagents tested, *p*-hydroxymercuribenzoate was the most potent inhibitor. Metal-binding agents such as 8-hydroxyquinoline, 1,10-phenanthroline, diethyldithiocarbamate, and α,α'-dipyridyl also inhibited the systems. The only compound which appeared to have a selective effect was sodium azide. At concentrations of 5 and 10 mM, sodium azide did not affect the cytochrome P-448 system but inhibited the cytochrome P-450 system by 28% and 39%, respectively.

Kinetic constants. In confirmation of earlier reports by Alvares *et al.* (5, 7), the apparent K_m of 3,4-benzpyrene in 3-MC microsomes (1.13 μM) was found to be considerably lower than that in PB microsomes (7.70 μM) (Fig. 7). However, when the

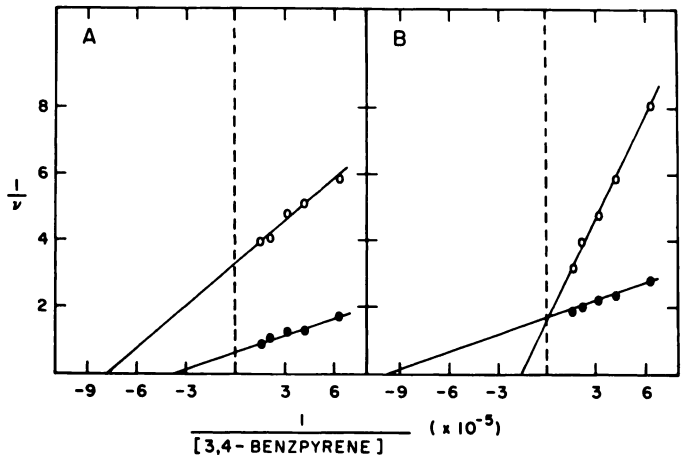

FIG. 7. *Double-reciprocal plot for determination of K_m of 3,4-benzpyrene in reconstituted (A, ●, 3-MC; ○, PB) and microsomal (B, ●, 3-MC; ○, PB) systems*

The concentrations of microsomes and various fractions used were the same as described in Fig. 5. The final volume was 1.0 ml. Each point represents duplicate determinations. The apparent K_m calculated from the plot was identical with the value obtained with a GE-600 computer, using a FORTRAN program written by W. W. Cleland.

reconstituted system was used to determine these kinetic constants, it was found that the apparent K_m of 3,4-benzpyrene in the system prepared from PB-treated rats (1.53 μM) was lower than that obtained from 3-MC-treated rats (2.87 μM), as shown in Fig. 7. The maximal activity of the cytochrome P-450-dependent reaction (0.005 nmole/unit of reductase per minute) was approximately 10% of the cytochrome P-448-dependent reaction (0.05 nmole/unit of reductase per minute when the activity was expressed as nanomoles of product formed per unit of reductase per minute. The maximal velocity of the reaction was expressed per unit of reductase, beause the specific activity (nanomoles of cytochrome *c* reduced per minute per milligram of protein) of the reductase fraction from PB-treated rats is generally twice as great as that of the reductase fraction from 3-MC-treated rats.

The effect of each component on the Michaelis constant is summarized in Table 2. As shown in experiments A and C, the lipid fractions from PB- and 3-MC-treated rats were interchangeable and did not affect the K_m of 3,4-benzpyrene in either of the reconstituted systems. With the cytochrome P-448-supported 3,4-benzpyrene hydroxylation, the replacement of reductase from 3-MC-treated rats by reductase from PB-treated rats caused a 3-fold increase in K_m (experiment A vs. experiment B), while the maximal velocity was not significantly changed (0.04 vs. 0.05 nmole/unit of reductase per minute), which is consistent with our earlier report that the source of reductase and lipid did not affect the maximal activity of the cytochrome P-448-supported 3,4-benzpyrene hydroxylation (19). In contrast, the use of reductase from 3-MC-treated rats in the cytochrome P-450-dependent system did not significantly change either the apparent K_m (experiment D vs. experiment C) or the maximal velocity (0.005 nmole/unit of reductase per minute). Thus, while the source of the reductase did not significantly affect the maximal velocity, it seemed to play an important role—along with the cytochrome fraction—in determining the K_m of 3,4-benzpyrene in the cytochrome P-448-supported reconstituted system.

Experiments B and C in Table 2 were designed to determine the apparent K_m of 3,4-benzpyrene under conditions in which the same reductase and lipid fractions were used but the cytochrome was different. Both the K_m and maximal velocity with cyto-

TABLE 2

Kinetic constants of 3,4-benzpyrene

The reaction mixtures contained the indicated microsomal fractions and other components, as described in the text, for the assay of 3,4-benzpyrene hydroxylation. The concentration of microsomal fractions used was similar to that described in Fig. 7. K_m was determined with a GE-600 computer, using a FORTRAN program written by W. W. Cleland. Each value represents two or more determinations, and each 3,4-benzpyrene concentration was done in duplicate.

Expt	Microsomal component used	K_m
		$\mu M \pm SE$
A	P-448 (3-MC) + reductase (3-MC) + lipid (PB or 3-MC)	2.87 ± 0.03
B	P-448 (3-MC) + reductase (PB) + lipid (PB or 3-MC)	9.03 ± 0.44[a]
C	P-450 (PB) + reductase (PB) + lipid (PB or 3-MC)	1.53 ± 0.20[a]
D	P-450 (PB) + reductase (3-MC) + lipid (PB)	1.00[b]
E	3-MC microsomes	1.13 ± 0.10
	PB microsomes	7.70 ± 1.29[c]

[a] The values are significantly different from the value obtained from experiment A ($p < 0.01$).

[b] K_m value represents only one determination.

[c] This value is significantly different from the value in the 3-MC microsomal system ($p < 0.05$).

chrome P-450 were significantly lower than the values obtained with cytochrome P-448.

DISCUSSION

The hydroxylation of 3,4-benzpyrene is similar to the hydroxylation of many other endogenous and foreign substrates of the microsomal hydroxylation system, being catalyzed by a multienzyme system consisting of hemoprotein, reductase, and lipid. A major difference is that 3,4-benzpyrene hydroxylation is preferentially catalyzed by cytochrome P-448, whereas the hydroxylation of most other substrates is preferentially catalyzed by cytochrome P-450. This observation is consistent with the results of induction studies with microsomes, which revealed that PB stimulates the hydroxylation of many substrates and increases the cytochrome P-450 content of microsomes, while 3-MC stimulates the hydroxylation of only a few substrates (such as 3,4-benzpyrene) and induces cytochrome P-448 (32–34).

The apparent K_m of 3,4-benzpyrene in 3-MC microsomes was lower than that in PB microsomes, whereas the apparent K_m of 3,4-benzpyrene in the reconstituted system prepared from 3-MC treated rats was higher than that in the reconstituted system prepared from PB-treated rats. A similar change in the Michaelis constant has been observed upon the solubilization and purification of several other membrane-bound enzyme systems (35, 36). This change is generally attributed to the change in environment of the enzyme caused by the disruption of the membrane.

The results of the kinetic studies reported in this paper suggest that the reductase isolated from PB-treated rats may differ from the reductase isolated from 3-MC-treated rats. Although the source of the reductase preparation did not appear to affect the maximal velocity of the reaction, the apparent K_m of 3,4-benzpyrene in the cytochrome P-448-dependent system—but not in the cytochrome P-450-dependent system—was significantly increased when the reductase from 3-MC-treated rats was replaced with the reductase from rats treated with PB. On the other hand, the lipid, phosphatidylcholine, appeared to be the same from both sources, since the replacement of one by the other did not affect the K_m or maximal velocity.

If the same reductase and lipid fractions were used and only the hemoprotein was varied, the apparent K_m of 3,4-benzpyrene with cytochrome P-450 was significantly different (lower) than that with cytochrome P-448. This finding is subject to at least two interpretations: either 3,4-benzpyrene hydroxylation is catalyzed by two distinct cytochromes, and thus the apparent K_m is

different, or the low activity with cytochrome P-450 is due to the presence of small amounts of cytochrome P-448 in the preparation. Since cytochrome P-450 has been shown to inhibit the cytochrome P-448-dependent 3,4-benzpyrene hydroxylation (19), the apparent K_m should be different with the two cytochrome fractions. However, inhibition by cytochrome P-450 should result in an increase, rather than a decrease, in apparent K_m. In fact, when similar experiments were done at pH values other than the optimal pH (6.8), the apparent K_m of 3,4-benzpyrene with cytochrome P-450 was as much as 10 times lower than that obtained with cytochrome P-448. Thus the second interpretation is unlikely. Consistent with the differential effect of various compounds on the reconstituted P-450- and P-448-containing systems, these results indicate that the hydroxylation of 3,4-benzpyrene by the cytochrome P-450- and P-448-containing reconstituted systems is catalyzed by catalytically different cytochromes. The low activity of cytochrome P-450 for 3,4-benzpyrene hydroxylation is probably an inherent property of this cytochrome and is not due to the presence of small amounts of P-448 in the preparation.

Since the cytochrome P-450 and P-448 fractions were prepared by the same procedure, and since there was no evidence for the selective removal of either of these cytochromes during their isolation, as judged by the same recovery of cytochrome in both preparations, there is very little, if any, cytochrome P-448 in rats treated with PB. Sladek and Mannering (37) have recently suggested a similar conclusion.

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