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

VIII. Different Catalytic Activities of Rabbit and Rat Cytochromes P-448

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SUMMARY

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Cytochrome P-448 was partially purified from 3-methylcholanthrene-treated rabbits to a specific content of 9-10 nmoles of cytochrome P-448 per milligram of protein. This partially purified rabbit cytochrome P-448 was only 10% as active as rat cytochrome P-448 in catalyzing the hydroxylation of benzo[*a*]pyrene in the presence of NADPH-cytochrome *c* reductase and lipid. Hydroxylation of benzo[*a*]pyrene supported by the rabbit P-448 fraction was much more susceptible to inhibition by 7,8-benzoflavone and diethylaminoethyldiphenylpropyl acetate (SKF 525-A) than was the corresponding P-448 from rat. These results suggest that rabbit cytochrome P-448 is catalytically different from rat cytochrome P-448.

INTRODUCTION

It has become increasingly evident that multiple forms of cytochrome P-450 exist in liver microsomes. Thus cytochrome P-448 from rats treated with 3-MC[1] is different from cytochrome P-450 from untreated or PB-treated rats with respect to CO difference spectrum, ethyl isocyanide difference spectrum, and catalytic activity (1-4). In addition, multiple forms of cytochrome P-450 have been demonstrated in the same animal. For example, based upon different cyanide binding affinities, Comai and Gaylor (5) identified and separated three distinct forms of P-450 in liver microsomes from untreated or induced rats. Glaumann (6) showed that both cytochromes P-448 and P-450 are present in microsomes from 3-MC-treated rats. Several forms of cytochromes P-450 and P-448, differing in their molecular weights, spectral, and/or catalytic properties, have also been separated and partially purified from rat liver microsomes by Ryan *et al.* (7), and from rabbit liver microsomes by Coon *et al.* (8). Furthermore, several investigators

[1] The abbreviations used are: 3/MC, 3-methylcholanthrene; PB, phenobarbital; SKF 525-A, diethylaminoethyldiphenylpropyl acetate.

have demonstrated that the induction of cytochrome P-448 by 3-MC could only be observed in certain species of animals (9) and certain strains of mice (10–12).

In previous papers of this series we demonstrated that cytochrome P-450 from untreated and PB-treated rats and cytochrome P-448 from 3-MC-treated rats have different substrate specificities, and that such specificities reside in the cytochrome fraction rather than in the NADPH–cytochrome *c* reductase or lipid fractions (13, 14). In this paper we report that the partially purified cytochromes P-448 from 3-MC-treated rabbits and rats are catalytically different, despite their similar reduced CO difference spectra.

MATERIALS AND METHODS

Chemicals used in this study were obtained from the following sources: bovine serum albumin, 3-MC, benzo[*a*]pyrene, NADPH, and DEAE-cellulose, Sigma Chemical Company; [4-^{14}C]testosterone, New England Nuclear; SKF 525-A, as a gift from Smith Kline & French; ethylmorphine, Mallinckrodt; hexobarbital, Winthrop Laboratories; 7,8-benzoflavone, Aldrich Chemical Company; Sephadex LH-20, Pharmacia; calcium phosphate gel, Bio-Rad Laboratories; and Emulgen 911, Kao-Atlas Company, Ltd., Japan.

3-MC was administered intraperitoneally to male rats (Long-Evans, 50–55 g, Blue-Spruce Farms, Altamont, N. Y.) and subcutaneously to male rabbits (New Zealand strain, 3 kg, Marlin Farms, Hewitt, N. J.) at a dose of 25 mg/kg for 4 days. The animals were killed 24 hr after the last treatment, and liver microsomes were prepared as previously described (15). The method of solubilization (15) and partial purification (16) of cytochrome P-448 from 3-MC-treated rats has recently been described. The same procedure was used to partially purify cytochrome P-448 from 3-MC-treated rabbits. The concentration of cytochrome P-448 was determined from the reduced CO difference spectrum, using an extinction coefficient of 91 mM^{-1} cm^{-1} (17).

The NADPH–cytochrome *c* reductase and lipid fractions were obtained by previously published procedures. After solubilization of the microsomes by treatment with Triton N-101 (18), the reductase was further purified by repeated column chromatography (19). The specific activity of the partially purified reductase was 3900 units/mg of protein, 1 unit of activity being defined as the amount catalyzing the reduction of 1 nmole of cytochrome *c* per minute under the conditions previously described (20). The lipid fraction was obtained from liver microsomes of PB-treated rats by solubilization with deoxycholate and chromatography on DEAE-cellulose (13, 21). Reductase and lipid fractions prepared from PB-treated rats were routinely used in the present study, since it has been demonstrated that these fractions from PB- and 3-MC-treated rats and rabbits are interchangeable (8, 13). In addition, Nebert *et al.* (22) have shown that NADPH–cytochrome *c* reductase and lipid fractions isolated from 3-MC-treated rats and mice are interchangeable for benzo[*a*]pyrene hydroxylation in the reconstituted system.

The hydroxylation of benzo[*a*]pyrene by microsomes and the reconstituted system was determined fluorometrically according to Nebert and Gelboin (23) as described previously (13). The reaction mixture contained 100 μmoles of potassium phosphate buffer (pH 6.8), 3 μmoles of $MgCl_2$, 0.4 μmole of NADPH, 80 nmoles of benzo[*a*]pyrene (added in 0.04 ml of acetone), and the necessary microsomal fraction(s) in a volume of 1.0 ml. Cytochrome P-448 from rats and rabbits, designated fraction B after DEAE-cellulose chromatography (7), was routinely used in the reconstituted system. The mixture was incubated at 37° for 5 min. Activity was expressed as nanomoles of 3-hydroxybenzo[*a*]pyrene formed during 5 min. Testosterone hydroxylation was measured by the method of Jacobson and Kuntzman (24), and the *N*-demethylation of benzphetamine, ethylmorphine, and aminopyrine was determined by measuring the formation of HCHO by the method of Nash (25) as modified by Cochin and Axelrod (26). Protein concentrations

were determined by the method of Lowry *et al.* (27), using bovine serum albumin as the standard.

RESULTS

Partial purification of rabbit P-448 and its spectral properties. The procedure described by Levin *et al.* (16) for the partial purification of rat cytochrome P-448 was used to partially purify rabbit cytochrome P-448. The method included solubilization of sonicated microsomes by treatment with sodium cholate, followed by fractionation with ammonium sulfate precipitation, adsorption on calcium phosphate gel, chromatography on DEAE-cellulose in the presence of the non-ionic detergent Emulgen 911, and, finally, chromatography on Sephadex LH-20 to remove excess detergent. As with the rat preparation, two fractions were obtained by DEAE-cellulose chromatography. About 10% of the heme-protein preparation did not bind to the DEAE-cellulose and was designated fraction A. This heme protein fraction had a low specific content, 2–3 nmoles, of cytochrome P-448 per milligram of protein. The heme protein, designated fraction B, which bound to the DEAE-cellulose was eluted from the column with a buffer containing 50 mM potassium phosphate (pH 7.7), 20% glycerol, and 0.1% Emulgen 911 (w/v). The average specific content of fraction B was 9–10 nmoles of P-448 per milligram of protein; the final over-all recovery from the starting microsomes was 5–8%. In contrast to the rat cytochrome P-448, which was essentially free of NADPH–cytochrome *c* reductase and cytochrome b_5 (16), the final rabbit cytochrome P-448 preparation (fraction B) contained 3–4% cytochrome b_5 and 0.11 unit of NADPH–cytochrome *c* reductase per nanomole of P-448, even though the same procedure was used.

Prior to chromatography on DEAE-cellulose, the absorption maximum of the reduced CO difference spectrum of the rabbit preparation was at 448 nm. After chromatography fractions A and B had absorption maxima at 449 nm and 447 nm, respectively, which are the same absorption maxima exhibited by the rat cytochrome fractions A and B (7).

Several investigators have observed a large increase in high-spin heme protein species in rabbit liver microsomes after treatment with 3-MC as determined by both optical and EPR spectroscopy (28–30). Since our rabbit cytochrome P-448 preparation still contained cytochrome b_5, the absolute spectra of the preparations were not studied in detail, but some comments can be made regarding the optical characteristics of the heme protein preparations at different stages during isolation. After DEAE-cellulose chromatography both the A and B fractions appeared to contain high concentrations of a high-spin heme protein species, as evidenced by a broad absorption band between 390 and 420 nm and a peak at 645 nm in the oxidized absolute spectrum. In comparison the rat cytochrome P-448 preparations, before and after DEAE-cellulose column chromatography, were predominantly low-spin heme proteins (31, 32). Preliminary experiments indicated that rabbit cytochrome P-448 can be obtained in a low-spin state (based on a Soret maximum at 417 nm and the absence of a shoulder at 395 nm or a peak at 645 nm) after column chromatography on hydroxylapatite in the presence of 0.1% Emulgen 911 or on DEAE-cellulose with 0.5% Emulgen 911. The absorption maxima of the reduced CO difference spectra of these low-spin and high-spin fractions were identical.

Based on these experiments, it may be concluded that the rabbit P-448 can exist in both high- and low-spin states. The high-spin character of rabbit cytochrome P-448 observed in microsomes and partially purified preparations is probably due to the binding of endogenous substrate(s). This conclusion is also supported by recent studies of Witmer *et al.* (33), in which it was shown that partially purified rabbit cytochrome P-448 could be converted from high- to low-spin states and vice versa by several substrates.

Catalytic activity. Although the induction of benzo[*a*]pyrene hydroxylase activity is associated with the formation of

P-448 in rats and inducible mouse strains (4, 10, 22), Alvares *et al.* (9) and Nebert *et al.* (30) showed that the formation of P-448 in rabbits after 3-MC treatment does not result in a significant increase in benzo[*a*]pyrene hydroxylase activity in microsomal suspensions. As shown in Table 1, the benzo[*a*]pyrene hydroxylase activity of microsomes and the reconstituted system (consisting of P-448, NADPH–cytochrome *c* reductase, and lipid) derived from rats was 10 times the activity of microsomes and the reconstituted system from rabbits, expressed as nanomoles of product formed per minute per milligram of protein. The turnover numbers (nanomoles of product formed per minute per nanomole of cytochrome P-448) obtained with the rat systems were 15-fold greater than those obtained with the rabbit system. It should be noted that the reconstituted system from either rats or rabbits had higher activity than the corresponding microsomes.

TABLE 1

Benzo[a]pyrene hydroxylase activity catalyzed by liver microsomes and reconstituted microsomal hydroxylation system from 3-MC-treated rats and rabbits

The reaction mixtures (final volume, 1.0 ml) contained the usual components and the microsomal fractions as follows: 0.08 mg of microsomal protein from the livers of 3-MC-treated rats or 0.85 mg from 3-MC-treated rabbits; either 0.125 nmole of rat cytochrome P-448 or 0.17 nmole of rabbit cytochrome P-448; 0.05 mg of NADPH–cytochrome *c* reductase (200 units); and 0.1 mg of lipid. Four groups of rats (two in each group) and three rabbits were used for microsomal studies. One hundred rats and four rabbits were used to prepare cytochrome P-448 in the reconstituted system. Each value represents two or more determinations ± standard deviation.

Hydroxylation system	3-Hydroxybenzo[*a*]pyrene formed	
	nmoles/min/mg protein	*nmoles/min/nmole P-448*
Rats		
Microsomes	2.29 ± 0.36	1.39 ± 0.22
Reconstituted system	7.95 ± 0.58	4.36 ± 0.04
Rabbits		
Microsomes	0.28 ± 0.02	0.09 ± 0.01
Reconstituted system	0.74 ± 0.09	0.28 ± 0.04

This difference in benzo[*a*]pyrene hydroxylase activity catalyzed by the rabbit and rat cythchromes P-448 in the reconstituted system is further demonstrated in Fig. 1. There was at least an order of magnitude difference between the benzo[*a*]pyrene hydroxylase activities of rat and rabbit cytochromes P-448 in the presence of fixed amounts of reductase and lipid. Figure 2 is a titration curve showing benzo[*a*]pyrene hydroxylase activity as a function of rat NADPH–cytochrome *c* reductase concentration in the presence of 0.17 nmole of rabbit cytochrome P-448 and 0.1 mg of lipid.

It might be argued that some type of inhibitor(s) is present in the rabbit P-448 preparation, thereby inhibiting benzo[*a*]pyrene hydroxylase activity. If this were the case, the addition of rabbit cytochrome P-448, with or without boiling, to a reaction mixture containing rat cytochrome P-448, NADPH–cytochrome *c* reductase, and lipid should affect benzo[*a*]pyrene hydroxylase activity. As shown in Table 2, however, the addition of rabbit heme protein did not significantly affect the ability of rat P-448 to hydroxylate benzo[*a*]pyrene. Thus the low rate of benzo[*a*]pyrene hydroxylation catalyzed by rabbit P-448 probably was not due to the presence of an inhibitor in the preparation.

The low benzo[*a*]pyrene hydroxylase activity of rabbit P-448 in microsomes and in the reconstituted system may be due to an inherent property of rabbit P-448, or to the presence of endogenous substrates which inhibit the reaction. In an attempt to differentiate between these two possibilities, the low-spin fractions (as judged by the absence of absorption peaks at 395 nm and 645 nm) obtained by hydroxylapatite chromatography were assayed for their benzo[*a*]pyrene hydroxylase activity, since they should contain less "endogenous substrate." It was found that the rate of benzo[*a*]pyrene hydroxylation catalyzed by the low-spin P-448 fraction was no greater than the rate catalyzed by the high-spin P-448 fraction. Therefore it ap-

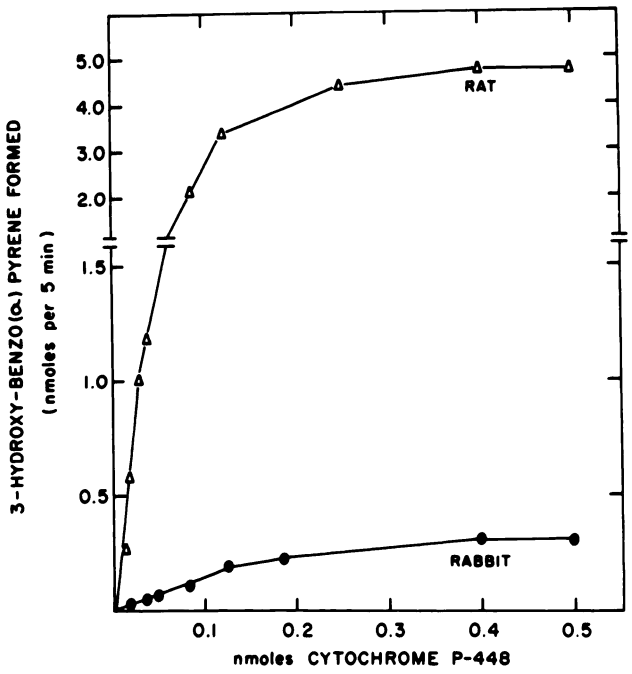

FIG. 1. *Benzo[a]pyrene hydroxylation as a function of rat or rabbit heme protein concentration*
The reaction mixtures (final volume, 1.0 ml) contained the indicated amounts of cytochrome P-448 from 3-MC-treated rats (Δ——Δ) or rabbits (●——●), 0.05 mg of reductase (200 units), and 0.1 mg of lipid. The other components are described in the text.

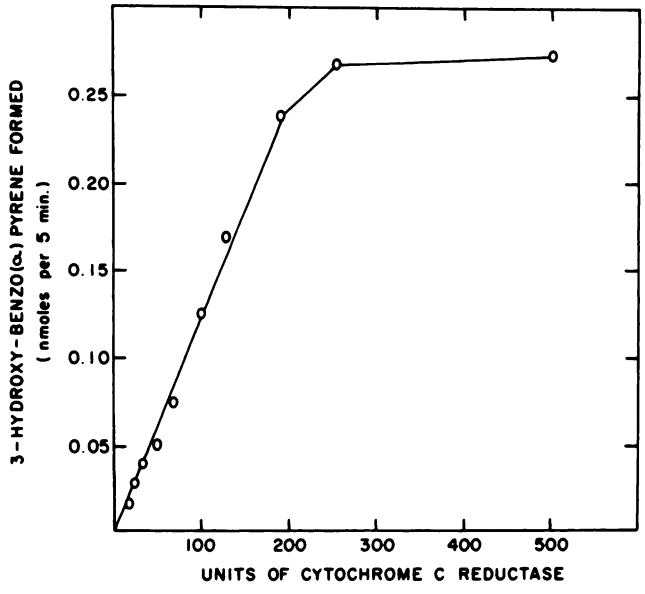

FIG. 2. *Benzo[a]pyrene hydroxylation as a function of NADPH-cytochrome c reductase concentration*
The reaction mixtures (final volume, 1.0 ml) contained fixed amounts of rabbit cytochrome P-448 (0.17 nmole) and lipid (0.1 mg), and variable amounts of NADPH-cytochrome *c* reductase (from PB-treated rats). The other components are described in the text.

TABLE 2

Effect of mixing rat and rabbit cytochromes P-448 on benzo[a]pyrene hydroxylase activity

The reaction mixtures (final volume, 1.0 ml) contained the usual components and the following microsomal fractions: cytochrome P-448, 0.05 mg of NADPH-cytochrome *c* reductase (200 units), and 0.1 mg of lipid. For heat inactivation, the cytochrome fraction was heated in a boiling water bath for 10 min.

Conditions	3-Hydroxybenzo[a]pyrene formed
	nmoles/5 min
1. Rat P-448 (0.04 nmole)	0.90
2. Rat P-448 (0.08 nmole)	1.63
3. Rabbit P-448 (0.08 nmole)	0.10
4. Rat-P-448 (0.04 nmole) + rabbit P-448 (0.08 nmole)	0.88 (88%)[a]
5. Rat P-448 (0.08 nmole) + rabbit P-448 (0.08 nmole)	1.58 (91%)[b]
6. Rat P-448 (0.04 nmole) + boiled rabbit P-448	0.84 (93%)[c]
7. Rat P-448 (0.08 nmole) + boiled rabbit P-448	1.63 (100%)[d]

[a] Percentage of the theoretical sum of lines 1 and 3.
[b] Percentage of the theoretical sum of lines 2 and 3.
[c] Percentage of line 1.
[d] Percentage of line 2.

pears most likely that the low enzymatic activity toward benzo[a]pyrene hydroxylation is an inherent property of the rabbit cytochrome P-448.

Inhibition of benzo[a]pyrene hydroxylation. To demonstrate further the differences between the heme proteins obtained from 3-MC-treated rats and 3-MC-treated rabbits, the susceptibility of benzo[a]pyrene hydroxylation supported by rat and rabbit cytochromes P-448 to inhibition by 7,8-benzoflavone and SKF 525-A was examined. Since the reaction mixtures contained the same amounts of NADPH-cytochrome *c* reductase and lipid, with only the heme proteins being varied, the differential effect obtained with these compounds should reflect the different natures of the heme proteins employed.

As shown in Fig. 3, which is a plot of benzo[a]pyrene hydroxylase activity (percentage of control) as a function of 7,8-benzoflavone concentration, the rabbit P-448-supported benzo[a]pyrene hydroxylation was strongly inhibited by 7,8-benzoflavone. The activity was decreased 80% from control levels at an inhibitor concentration of 0.1 μM. In contrast, the rat P-448-supported reaction was only slightly inhibited (10%) at concentrations less than 10 μM; however, there was marked inhibition (70%) at a 7,8-benzoflavone concentration of 100 μM. The rabbit cytochrome P-448-supported benzo[a]pyrene hydroxylation was also more sensitive to SKF 525-A inhibition than was the rat cytochrome P-448-supported reaction (Fig. 4). The selective effects of 7,8-benzoflavone and SKF 525-A on benzo[a]pyrene hydroxylation strongly suggest that rabbit and rat cytochromes P-448 are catalytically different.

Other reactions catalyzed by microsomes from 3-MC-treated rabbits. Since benzo[a]pyrene hydroxylase activity was not induced in microsomes from 3-MC-treated rabbits, an effort was made to determine whether the metabolism of other substrates was enhanced in rabbit liver microsomes after 3-MC treatment. The enzymatic activities of control and 3-MC-treated rabbit liver microsomes with several substrates are compared in Table 3. The reactions included benzo[a]pyrene hydroxylation, the *N*-demethylation of benzphetamine, ethylmorphine, and aminopyrine, and the hydroxylation of testosterone at the 6β, 7α, and 16α positions. Treatment with 3-MC had little or no inductive effect on the metabolism of any of the substrates tested. In fact, for most substrates, there was a decrease in activity after 3-MC treatment. Although benzo[a]pyrene hydroxylase activity was increased approximately 3-fold over controls in microsomes from 3-MC-treated rabbits when compared per milligram of protein, there was little or no increase in turnover number. These results are in agreement with those reported by Nebert *et al.* (30), who demonstrated little or no increase in the *N*-demethylation of 3-methyl-4-methylaminoazobenzene, the *O*-deethylation of 7-ethoxycoumarin, or the *O*-demethylation of *p*-nitroanisole in rabbit liver microsomes after 3-MC treatment. There was no detectable activity with liver microsomes from either 3-MC-treated or untreated rabbits for the hydrox-

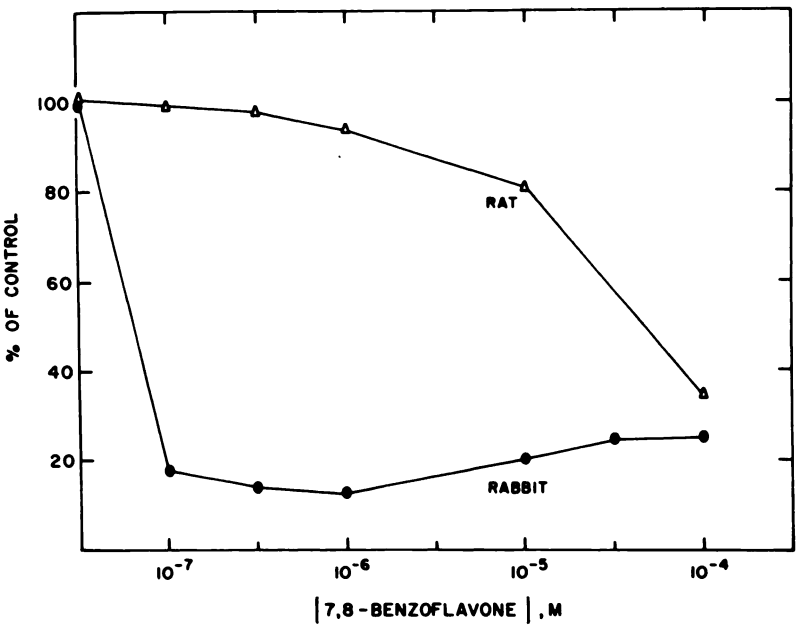

FIG. 3. *Effect of 7,8-benzoflavone on benzo[a]pyrene hydroxylation in reconstituted system using rat and rabbit cytochrome P-448*

The reaction mixtures (final volume, 1.0 ml) contained the indicated microsomal fractions and other components as described in the text. The concentrations of microsomal fractions were the same as described in Fig. 1. One hundred per cent activity using rat P-448 was 2.8 nmoles of 3-hydroxybenzo[a]pyrene formed per 5 min (Δ——Δ); 100% activity using rabbit P-448 was 0.27 nmole of 3-hydroxybenzo[a]pyrene formed per 5 min (●——●).

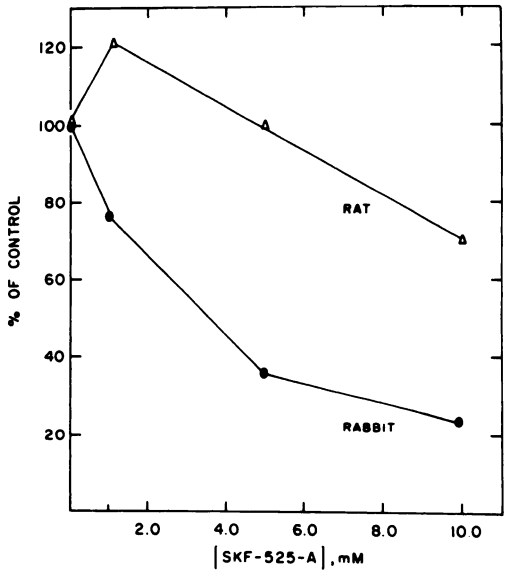

FIG. 4. *Effect of SKF 525-A on benzo[a]pyrene hydroxylation in reconstituted system using rat and rabbit cytochrome P-448*

The reaction mixtures (final volume, 1.0 ml) contained the indicated microsomal fractions and other components as described in the text. The concentrations of microsomal fractions were the same as described in Fig. 1. One hundred per cent activity using rat P-448 (Δ——Δ) was 2.28 nmoles of 3-hydroxybenzo[a]pyrene formed per 5 min; 100% activity using rabbit P-448 (●——●) was 0.28 nmole of 3-hydroxybenzo[a]pyrene formed per 5 min.

ylation of testosterone in the 6β position. On the other hand, Kuntzman *et al.* (34) reported that one of the major metabolites of testosterone in liver microsomes from untreated and 3-MC-treated rats is the 6β-hydroxylation product.

DISCUSSION

Although the induction of benzo[a]-pyrene hydroxylase activity is generally associated with the formation of cytochrome P-448 in rats treated with 3-MC and in inducible mouse strains (4, 10, 22), the present study indicates that the cytochrome P-448 from rabbits treated with 3-MC, either in microsomes or in a partially purified form, has very poor catalytic

TABLE 3

Enzymatic activity of liver microsomes from control and 3-MC-treated rabbits with various substrates

Benzo[*a*]pyrene hydroxylation was assayed as described in Table 1. The *N*-demethylation of benzphetamine, ethylmorphine, and aminopyrine and the hydroxylation of testosterone were assayed according to published procedures (23, 24). The initial rate of the reaction was used to calculate the specific activity and turnover number for all substrates. At least two rabbits were used in each group, and the assays were done in duplicate. Values are ± standard deviations.

Substrate	Enzymatic activity			
	Control		3-MC-treated	
	nmoles/min/mg protein	*nmoles/min/nmole P-450*	*nmoles/min/mg protein*	*nmoles/min/nmole P-448*
Benzo[*a*]pyrene	0.11 ± 0.01	0.07 ± 0.01	0.28 ± 0.02	0.09 ± 0.01
Benzphetamine	8.23 ± 0.10	5.56 ± 0.08	6.38 ± 0.14	2.12 ± 0.05
Ethylmorphine	3.97 ± 0.10	2.68 ± 0.08	4.34 ± 0.10	1.45 ± 0.03
Aminopyrine	6.62 ± 0.14	4.47 ± 0.09	6.64 ± 0.14	2.21 ± 0.05
Testosterone				
6β	0	0	0	0
7α	0.44 ± 0.04	0.30 ± 0.03	0.27 ± 0.04	0.09 ± 0.01
16α	2.09 ± 0.26	1.41 ± 0.18	1.05 ± 0.08	0.35 ± 0.03

activity toward benzo[*a*]pyrene hydroxylation. In addition, the *N*-demethylation of ethylmorphine, benzphetamine, and aminopyrine and the hydroxylation of testosterone in the 7α and 16α positions were not enhanced in liver microsomes from 3-MC-treated rabbits. These results are in agreement with those of Alvares *et al.* (9), who observed that benzo[*a*]pyrene hydroxylase activity was not increased in liver microsomes from 3-MC-treated rabbits even though cytochrome P-448 could be shown to be present, as well as with the studies of Nebert *et al.* (30), who showed that 3-MC treatment did not induce aryl hydrocarbon hydroxylase, *O*-deethylase, *O*-demethylase, or *N*-demethylase activities in rabbit liver microsomes. The formation of cytochrome P-448 was also observed by Miyake *et al.* (35) in the Morris hepatoma line 7777 after 3-MC treatment. However, the microsomes from those cells had no measurable aryl hydrocarbon hydroxylase activity and the *N*-demethylation of aminopyrine and benzphetamine occurred at a very low rate. Based on these results, two possible conclusions are that (a) 3-MC induce a P-448 in rabbit liver microsomes which has poor catalytic activity toward all substrates, or (b) rabbit cytochrome P-448 actively metabolizes some substrate(s) but the identity of this compound(s) is not now known.

Microsomal studies using optical and EPR spectroscopy (28–30) suggest that cytochrome P-448 from 3-MC-treated rabbits exists predominantly as a high-spin heme protein. On the other hand, cytochrome P-448 from 3-MC-treated rats, either in microsomes or in a partially purified form, has been shown to be predominantly low-spin (31, 32, 36). Thus either the rabbit P-448 is a high-spin heme protein or perhaps this high-spin state is due to the presence of endogenous substrate(s). The latter possibility is more likely, since rabbit cytochrome P-448 can be isolated in either a high-spin or a low-spin state, depending on the purification procedure used. Furthermore, Witmer *et al.* (33) demonstrated the conversion from a high-spin to a low-spin form, and vice versa, of partially purified rabbit cytochromes P-448 and P-450.

The relatively slow rate of benzo[*a*]-pyrene hydroxylation catalyzed by rabbit P-448 compared to rat P-448 is probably an inherent property of the rabbit cytochrome. The conclusion that rabbit and rat cytochromes P-448 are catalytically different is also based on the differential effect of 7,8-benzoflavone and SKF 525-A on the hydroxylation of benzo[*a*]pyrene catalyzed by these two cytochromes. In addition, the hydroxylation of testosterone at the 6β and 7α positions is enhanced in liver mi-

crosomes from 3-MC-treated rats (34), whereas in rabbits 7α-hydroxylation is decreased after 3-MC treatment and 6β-hydroxylation is not detectable before or after 3-MC-treatment. Based on these differences in enzymatic activity and susceptibility to the effects of various inhibitors, we conclude that these two hemoproteins represent different forms of cytochrome P-448. Rabbit P-448 may simply be a less active heme protein. The low activity does not appear to be due to the presence of an inhibitor(s) in the preparation, since in mixing experiments rabbit P-448 did not inhibit rat P-448-supported benzo[*a*]pyrene hydroxylation. The high-spin state of rabbit P-448 did not affect the catalytic activity, since benzo[*a*]pyrene hydroxylase activity obtained with the low-spin form was no greater than activity with the high-spin form. This is consistent with the observations of Nebert and Kon (37) that the addition of a small amount of acetone (0.19 M) to liver microsomes from 3-MC-treated mice essentially eliminated the high-spin EPR signal without affecting benzo[*a*]pyrene hydroxylase activity.

NOTE ADDED IN PROOF: Besides having a different minimum molecular weight as determined by SDS gel electrophoresis, the rabbit P-488 showed very poor reactivity with the antibody prepared against the rat P-488.

Rabbit cytochrome P-448 has now been purified to near homogeneity. This purified rabbit P-448 has a minimum molecular weight different from rat P-448 as determined by sodium dodecyl sulfate-gel electrophoresis. In addition, it reacts very poorly with the antibody prepared against the rat P-448. These results further support our conclusion that rat P-448 and rabbit P-448 represent two different proteins.

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