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

VI. DIFFERENT SUBSTRATE SPECIFICITIES OF THE CYTOCHROME P450 FRACTIONS FROM CONTROL AND PHENOBARBITAL-TREATED RATS

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SUMMARY

The substrate specificities of the liver microsomal cytochrome P450 fractions from control and phenobarbital-treated immature, male rats were studied with the reconstituted hydroxylation system. In the presence of fixed amounts of the reductase and lipid fractions and various amounts of hemoprotein, the catalytic activities of the cytochrome P450 fractions from control and phenobarbital-treated rats were compared with a variety of substrates. Both fractions were about equally active for the N-demethylation of ethylmorphine and the hydroxylation of testosterone at positions 6β and 7α. However, the cytochrome P450 fraction from phenobarbital-treated rats was much more active than the cytochrome P450 fraction from control rats for the N-demethylation of benzphetamine and chlorcyclizine, the oxidation of pentobarbital, and the hydroxylation of testosterone at position 16α. For the hydroxylation of 3,4-benzpyrene, the cytochrome P450 fraction from control rats was slightly more active than the P450 fraction from phenobarbital-treated rats. These results suggest that the cytochrome P450 fraction from control rats is catalytically different from the cytochrome P450 fraction from phenobarbital-treated rats.

The oxidative metabolism of drugs and steroids is catalyzed by the microsomal enzyme system which has been resolved into three components (1-3): a CO-binding hemoprotein (cytochrome P450), an NADPH-dependent reductase, and a lipid identified as phosphatidylethanolamine (4). The administration of phenobarbital to rats induces the formation of a spectrally distinct hemoprotein, cytochrome P448 (5-10). Using the reconstituted hydroxylation system from liver microsomes (11, 12), we have recently shown that the enzyme systems prepared from PB- or 3-MC-treated immature rats exhibit different substrate specificities, and that such specificities reside primarily in the cytochrome fraction, rather than in the reductase or lipid fraction of liver microsomes (3, 13). These studies indicate that the cytochrome P450 fraction from rats treated with PB is catalytically different from the cytochrome P448 fraction from rats treated with 3-MC. On the other hand, it has been generally assumed that the cytochrome P450 in untreated rats is catalytically identical with the cytochrome P450 in PB-treated rats. Since, in rats, the metabolism of most substrates is enhanced by PB treatment, only quantitative but not qualitative differences between microsomes from control animals and microsomes from PB-treated animals have generally been noted. In addition, a direct comparison of the rates of metabolism of various substrates from control and PB-treated rats is difficult to make due to the differences in levels of both cytochrome P450 and reductase in these microsomal preparations.

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centration of the hemoprotein, we have studied the metabolism of a number of substrates by the reconstituted system. The results presented in this paper show that the P450 fractions from control and PB-treated rats are about equally active for the metabolism of some substrates, but the PB-P450 fraction is far more effective than the control-P450 fraction for the metabolism of other substrates. The results suggest that cytochrome P450 from control rats has different catalytic activity than cytochrome P450 from PB-treated rats.

METHODS

Male Long-Evans rats (from Blue Spruce Farms, Altamont, New York) weighing 50 to 60 g were treated intraperitoneally with PB or 3-MC as previously described (14). Control rats received no treatment. The partial purification of the cytochrome P450 fractions from control and PB-treated rats and the cytochrome P448 fraction from 3-MC-treated rats has recently been described (15). Hemoprotein concentrations were determined by the reduced CO difference spectra (16) using the same extinction coefficient (91 mm⁻¹ cm⁻¹) for all three preparations. When the heme content of these preparations was determined by the pyridine hemochromogen method (16), the extinction coefficient for A₄₃₀₋₄₅₀ or A₄₁₀₋₄₃₀ in the reduced CO difference spectra was found to be within 10% of 91 mm⁻¹ cm⁻¹ (15). The concentrations of the partially purified PB-P450 and P448 ranged from 4.5 to 7.5 nmoles of cytochrome per mg of protein; preparations with a concentration between 4.5 and 5.0 nmoles of cytochrome per mg of protein were used for the present studies. Control-P450 had a concentration of 2.4 nmoles of cytochrome per mg of protein. All three hemoprotein preparations were purified 3-fold with respect to microsomal protein and 20-fold with respect to microsomal phospholipid. The reductase and lipid fractions were prepared from PB-treated rats as previously described (3, 17).

The hydroxylation of pentobarbital (18) and 3,4-benzpyrene (3, 19), the N-demethylation of chlorcyclizine (3), and the hydroxylation of testosterone at positions 6α, 7α, and 16α (3) were determined according to previously published procedures. The N-demethylation of benzphetamine and ethylmorphine were assayed by following the rate of NADPH oxidation (1). The rate of NADPH oxidation has previously been shown to be equal to the rate of formaldehyde formation (2).

To determine the rate of metabolism of various substrates, fixed amounts of reductase and lipid, and variable amounts of control-P450 and PB-450 were incubated with substrates, buffers, and cofactors. Thus, reductase and lipid were in excess at low hemoprotein concentrations but limiting at high hemoprotein concentrations. For the purpose of comparison, cytochrome P448 was also assayed for its ability to metabolize various substrates. Although at low concentrations of hemoprotein (up to 0.2 nmoles per ml), no catalytic activity was observed in the absence of added reductase, the hemoprotein fractions were not entirely free of reductase, and a small but measurable amount of metabolism was observed without the addition of reductase when a large amount of hemoprotein (0.5 to 1.0 nmoles per ml) was used. Therefore, at the high concentrations of hemoprotein, the reaction rates were corrected by subtracting the activity obtained in the absence of reductase from the activity obtained in the presence of reductase. When this correction was made, hydroxylation activity increased with increasing concentrations of hemoprotein, reached a maximum, and plateaued.

RESULTS

Hydroxylation of Testosterone—The hemoprotein fractions prepared from control, PB-, and 3-MC-treated rats showed different specificities for the hydroxylation of testosterone at positions 6α, 7α, and 16α (Fig. 1). All three fractions were equally active for 6α-hydroxylation at all concentrations tested. The cytochrome P448 fraction was more active than the other two fractions for 7α-hydroxylation, while the PB-P450 fraction was far more active than the control-P450 or P448 fraction for the hydroxylation of testosterone at position 16α. Since the activities were determined in the presence of fixed amounts of the reductase and lipid fractions and only the cytochrome fractions were varied, the differences in activity of the control-P450, PB-P450, and P448 fractions for the hydroxylation of testosterone at positions 6α, 7α, and 16α reflect differences in catalytic activities of the three hemoprotein fractions.

N-Demethylation of Benzphetamine and Ethylmorphine—Previous studies have shown that the PB-P450 fraction was much more active than the P448 fraction for the N-demethylation of benzphetamine (3). The results plotted in Fig. 2 confirm our earlier findings and also show that the control-P450 fraction was considerably less active than the PB-P450 fraction, but consistently more active than the cytochrome P448 fraction for benzphetamine N-demethylation. In contrast, all three hemoprotein fractions were approximately equally active for the N-demethylation of ethylmorphine.

Hydroxylation of Pentobarbital and 3,4-Benzpyrene and N-Demethylation of Chlorcyclizine—Table I shows that the PB-P450 fraction was considerably more active than the control-P450 fraction for pentobarbital hydroxylation, while the control-P450 fraction was slightly, but consistently, more effective than the PB-P450 fraction for the hydroxylation of 3,4-benzpyrene at all concentrations tested. For the N-demethylation of chlorcyclizine, the PB-P450 fraction was more active than the control-P450 fraction. As was reported previously (3, 20), the cytochrome P448 fraction was very active for 3,4-benzpyrene hydroxylation, very poor for pentobarbital hydroxylation, but moderately active for chlorcyclizine N-demethylation.
When from PB-treated rats. No hydroxylation activity was detected of hemoproteins. The reaction mixture was preincubated at 37° for 3 min and the reaction was initiated by the addition of NADPH. Activity was determined by following the rate of substrate-dependent NADPH oxidation at 37° (1). Control, PB, and 3-MC refer to the hemoprotein fractions prepared from untreated, PB-treated, and 3-MC-treated rats, respectively. Both the reductase and lipid fractions were prepared from PB-treated rats.

**TABLE I**

Effect of different hemoprotein fractions on rates of hydroxylation of pentobarbital and 3,4-benzpyrene and N-demethylation of chlorcyclizine

| Substrate                | Hemoprotein concentration | Control-P450 | PB-P450 | 3-MC-P448 |
|--------------------------|---------------------------|--------------|---------|-----------|
|                          | nmole/ml                  | nmole chased | nmole metabolite formed | nmole metabolite formed |
| Pentobarbital            | 0.5                       | 0.10         | 1.00    | 0.06      |
| 3,4-Benzpyrene           | 0.08                      | 0.16         | 0.07    | 0.69      |
| Chlorcyclizine           | 0.13                      | 0.23         | 0.14    | 0.93      |

Although the control-P450 fraction was only slightly more active than the PB-P450 fraction for 3,4-benzpyrene hydroxylation, these two reactions could be differentially affected by 7,8-benzoflavone and SKF-525A. As shown in Fig. 3, with the control-P450 fraction, the reaction was inhibited by 7,8-benzoflavone while with the PB-P450 fraction, 3,4-benzpyrene hydroxylation was either slightly stimulated or slightly inhibited, depending on the concentration of 7,8-benzoflavone added. These results were surprising since the hydroxylation of 3,4-benzpyrene was stimulated by 7,8-benzoflavone in microsomes from both control and PB-treated rats (21). We have no explanation for this difference between the microsomal and reconstituted systems. In contrast, SKF-525A at a concentration of $4 \times 10^{-4}$M inhibited the PB-P450-supported hydroxylation of 3,4-benzpyrene by 60%, but only inhibited the control-P450-supported reaction by 15%.

**DISCUSSION**

Since the amounts of the reductase and lipid were kept constant, it is apparent from the studies described above that the control-P450, PB-P450, and 3-MC-P448 fractions have different substrate specificities. The relative catalytic activities of the control-P450, PB-P450, and P448 fractions from immature, male rats for various substrates in the presence of reductase and lipid are summarized in Table II. The relative activities were calculated from either the maximal activity obtained with each substrate (where the hemoprotein concentration is in excess and the reductase and lipid are limiting) or the activity obtained with each substrate at 0.1 μm hemoprotein (where the hemoprotein concentration is limiting), setting the control-P450 value equal to 100%. From this table, it can be seen that the relative activities of the three hemoprotein fractions depended upon the particular substrate studied. The need to use a variety of substrates to establish differences in catalytic activity between various hemoprotein fractions is therefore evident. For some substrates, all three hemoprotein fractions were equally active, whereas for other substrates, either the PB-P450 or the P448 fraction was much more active. Although these results strongly suggest that the control-P450, PB-P450, and 3-MC-P448 have different catalytic activity, the possibility cannot be excluded that a substance other than P450 in the various partially purified preparations contributes to the different substrate specificities of the three hemoprotein fractions.

The possibility that several CO-binding pigments with different catalytic activities occur in liver microsomes has been one of the most extensively studied aspects of the microsomal mixed function oxidase system. Studies in recent years with microsomal suspensions have provided evidence to support the possibility...
For each substrate, the activity was determined in the presence of fixed amounts of lipid and reductase but variable amounts of hemoprotein. The relative activity was then calculated by setting the maximal activity (where the hemoprotein was in excess) obtained with control-P450 for each substrate equal to 100%. The values in parentheses were calculated by setting the activity at 0.1 μM hemoprotein (where the hemoprotein concentration was limiting) obtained with control-P450 for each substrate equal to 100%. Data obtained from Figs. 1 and 2 and Table I were used for the calculations.

### Table II

Relative catalytic activities of control P450, PB-P450, and 3-MC-P450 fractions from immature, male rats

| Substrate          | Relative activity |
|--------------------|------------------|
|                    | Control-P450 | PB-P450 | 3-MC P450 |
| 3,4-Benzpyrene     | 100          | 70 (60) | 390 (400) |
| Benzphetamine      | 100          | 250 (180) | 50 (50) |
| Ethylmorphine      | 100          | 100 (130) | 100 (110) |
| Pentobarbitalb     | 100          | 1000 | 60 |
| Chlorcyclazine     | 100          | 220 | 60 |
| Anilineb           | 100          | 120 | 170 |
| Testosteroneb      | 100          | 100 (120) | 100 (70) |
| 16α-Hydroxylationb | 100          | 100 (90) | 200 (120) |
| 16α-Hydroxylationb | 100          | 550 (440) | 50 (50) |

- Only one hemoprotein concentration (0.5 μM) was used.
- Only one hemoprotein concentration (0.3 μM) was used. Data were taken from Reference 21a.

that multiple forms of cytochrome P450 exist in liver microsomes (22–27). The hydroxylation of testosterone at positions 6α, 7α, and 16α in rat liver microsomes occurs by a cytochrome P450 dependent system, and the three hydroxylation reactions are under different regulatory control (22, 23). In microsomes from control rats, the three hydroxylation reactions had different patterns of development with age. In addition, chloroform strongly inhibited the 16α-hydroxylation of testosterone but only slightly inhibited hydroxylation at positions 6β and 7α. Chronic treatment of rats with PB and 3-MC also increased the 6β-, 7α-, and 16α-hydroxylations of testosterone by liver microsomes to varying degrees. Although the hydroxylation of testosterone at all three positions in microsomes from control rats was inhibited by CO, and the inhibition by CO of all three reactions was relieved maximally by monochromatic light at or near 450 nm, the ratio of CO:O2 needed for 50% inhibition of testosterone hydroxylation at positions 6β, 7α, and 16α was very different. These results suggest that different forms of cytochrome P450 with different sensitivities to CO participate in the 6β-, 7α-, and 16α-hydroxylations of testosterone.

A number of other studies have also shown some differences between the liver microsomes isolated from untreated and PB-treated animals, but it is uncertain from these studies whether different cytochrome P450s were responsible for the differences. For example, N-demethylation of metharbital in liver microsomes from PB-treated rats was 50-fold faster than the N demethylation in microsomes from control animals (28). This increased metabolism could not be explained solely on the basis of increased levels of reductase and P450. Guarino et al. (29) found that in PB-treated rats, the Km and Ks values for aniline and liver microsomes were predominantly different from the Km and Ks values for aniline in microsomes from control rats. Wiebel et al. (21) reported that acetone slightly stimulated 3,4-benzpyrene hydroxylation in microsomes from control rats, but inhibited the hydroxylation of 3,4-benzpyrene in microsomes from PB-treated rats. Based on kinetic analysis, it has been suggested that different enzyme systems may be responsible for the metabolism of several drugs in microsomes from control and PB-treated rats (25, 30, 31). The ratios of the specific activities for the N-demethylation of (+) and (−) methylphenobarbital, and the metabolism of (+) and (−) hexobarbital by microsomes from control and PB-treated rats were found to be significantly different (26, 27). The hydroxylation of nortriptyline and desmethylimipramine in microsomes was significantly decreased in rats treated with PB, even though cytochrome P450 content was increased (32). In addition, a small but significant change in half-life of cytochrome P450 after PB-treatment has been reported (33). The ratio of the 450 to 430 nm absorption peaks in the ethyl isocyanide difference spectrum is slightly, but consistently lower in untreated rats than in PB-treated rats (34). These results and the data presented in the present paper suggest that multiple CO-binding hemoproteins—each with a different substrate specificity—exist in liver microsomes, but a complete separation and purification of the various P450 species are needed to firmly establish such a conclusion.

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