Multiple Forms of Cytochrome P-450 in Phenobarbital- and 3-Methylcholanthrene-treated Rats

SEPARATION AND SPECTRAL PROPERTIES*

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SUMMARY

Multiple forms of liver microsomal cytochrome P-450 isolated from immature male rats pretreated with phenobarbital or 3-methylcholanthrene are described. A fraction of low specific content (Fraction A, 1.7 to 4.0 nmol of cytochrome P-450 per mg of protein) and a fraction substantially purified (Fraction B, 9.0 to 11.0 nmol of cytochrome P-450 per mg of protein) are obtained by DEAE-cellulose chromatography of a partially purified cytochrome P-450 preparation in the presence of Emulgen 911.

Shifts in the absorption maxima in the CO-reduced and ethyl isocyanide difference spectra are observed in the fractions derived from 3-methylcholanthrene-treated rats. The fractions derived from phenobarbital-treated rats exhibit different 455:430 ratios and pH intercepts in the ethyl isocyanide difference spectra. The absolute oxidized spectra and n-octylamine binding spectra at room temperature and EPR analysis at the temperature of liquid helium characterize all the fractions, except the Fraction A from 3-methylcholanthrene-treated rats, as low spin ferric hemoproteins. The A hemeprotein fractions from both 3-methylcholanthrene- and phenobarbital-treated rats have poor catalytic activity for the metabolism of benzphetamine and 3,4-benzanthrene- and phenobarbital-treated rats have poor catalytic activity for the metabolism of benzphetamine and 3,4-benzanthrene- and phenobarbital-treated rats have poor catalytic activity for the metabolism of benzphetamine and 3,4-benzanthrene- and phenobarbital-treated rats have poor catalytic activity for the metabolism of benzphetamine and 3,4-benzanthrene- and phenobarbital-treated rats have poor catalytic activity for the metabolism of benzphetamine and 3,4-benzanthrene.

Cytochrome P-450 is the terminal oxidase of the electron transport pathway of hepatic microsomes responsible for the metabolism of steroids, fatty acids, drugs, and xenobiotics (1-4). Substantial evidence has accumulated in recent years that there are multiple forms of this cytochrome (5-8). Most of the evidence has been derived from studies with animals following the administration of various inducers. Thus, cytochrome P-448 in animals treated with polycyclic hydrocarbons, such as 3-methylcholanthrene, differs from cytochrome P-450 in control or phenobarbital-treated animals with respect to several parameters, including CO-difference spectrum (9, 10), ethyl isocyanide difference spectrum (11), substrate specificity (6, 12, 13), and sodium dodecyl sulfate gel electrophoretic pattern (14, 15). These differences have been demonstrated both in intact microsomes and also in partially purified preparations of cytochrome P-450 and P-448. Furthermore, it has been shown that the induction of cytochrome P-448 requires protein synthesis (8, 9, 16, 17), that cytochrome P-448 is synthesized independent of cytochrome P-450 (18), and that induction of cytochrome P-448 by 3-methylcholanthrene in different mouse strains is under genetic control (19-22).

Even though multiple forms of cytochrome P-450 apparently exist in animals treated with different inducers, it has not been definitively established whether multiple forms of cytochrome P-450 exist in liver microsomes obtained from either untreated animals or animals treated with an inducer. The separation and characterization of different forms of cytochrome P-450 from the same animal should provide information essential for an understanding of the mechanism of hydroxylation as well as for the analysis of physical and catalytic properties and the induction phenomena of cytochrome P-450. Recently, Comai and Gaylor (23) have used DEAE-cellulose chromatography to separate three forms of cytochrome P-450 from adult male rat liver microsomes. The three hemoprotein fractions had different affinities for cyanide. Evidence based on CO- and ethyl isocyanide difference spectra is presented below that suggests multiple forms of cytochrome P-450 are present in solubilized preparations prepared from the hepatic microsomes of phenobarbital- or 3-methylcholanthrene-treated immature male rats.

EXPERIMENTAL PROCEDURE

Partial Purification of Cytochromes P-448 and P-450

The procedure for the partial purification of cytochromes P-448 and P-450 from rat liver microsomes has been described in detail elsewhere (24), and only a brief outline of this procedure is included for clarity.

Step 1 through Step III—Hepatic microsomes prepared as previously described (25) were sonicated and treated with sodium cholate. After centrifugation, the resulting supernatant was fractionated twice with ammonium sulfate and then was adsorbed and eluted from calcium phosphate gel. The fraction eluted from the calcium phosphate gel is referred to as Step III (25).
Step IV—The Step III preparation was treated with the non-ionic detergent Emulgen 911 (1 mg of detergent per mg of protein) and was applied to a DEAE-cellulose column (1.5 X 30 cm) which had been equilibrated with 5.0 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol and 0.1% Emulgen 911. The proteins were eluted with either a linear gradient of potassium chloride (0 to 0.5 M) in the equilibration buffer or a stepwise gradient consisting of one wash with the equilibration buffer followed by a wash with 50 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol and 0.1% Emulgen 911.

By either elution procedure, two fractions of the cytochrome emerged from the column. The first fraction, referred to as A, eluted in the column volume, and the second fraction, B, was eluted with 0.1 M KCl or with 50 mM potassium phosphate buffer (pH 7.7). To minimize conversion to cytochrome P-420, the A and B fractions were routinely prepared by the stepwise procedure.

The excess detergent was removed immediately from both fractions either by passage through a Sephadex LH-20 column (4 X 15 cm) equilibrated and eluted with 10 mM potassium phosphate buffer (pH 7.7) containing 50% glycerol as described by Gaylor and Delwiche (26) or by treatment with Bio-Rad SM-2 beads for 2 hours at 4°C. By either method, approximately 50% of the detergent was removed. No additional detergent could be removed by subjecting the cytochrome fractions to both methods. The Emulgen 911 content was determined in the fractions with the use of [aH]Emulgen 911 (24). The A and B fractions were dialyzed, concentrated by membrane ultrafiltration, and centrifuged. The final samples were stable for at least several months when stored at -15° under N₂.

Approximately 45 to 60% of the cytochrome P-448 in the Step III sample prepared from 3-methylcholanthrene-pretreated rats was recovered from the DEAE-cellulose column. Fraction A represented between 10 and 17% of the cytochrome that eluted from the column, and fraction B represented 83 to 90%. From a Step III preparation from phenobarbital-pretreated rats, the total recovery of cytochrome P-450 from the DEAE-cellulose column ranged between 40 and 60%. Of the cytochrome P-450 that eluted from the column, 26 to 29% was Fraction A and 70 to 75% was Fraction B.

The final A fraction contained 1.0 to 1.6% of the total cytochrome P-450 present in microsomes, and the B fraction contained 3.5 to 4.8%. These values indicate only the recovery of the hemeproteins through the procedure and may not represent the actual amounts of these fractions present in intact microsomes. In addition, it is not known whether other forms of cytochrome P-450 are lost during the purification procedure.

Assay Methods

The spectra were determined using an Aminco DW-2 recording spectrophotometer in the split beam mode. The concentration of cytochrome P-450 or P-448 was determined by the method of Omura and Sato (27) from the CO-difference spectra of dithionite-reduced samples using an extinction coefficient of 91 mm M⁻¹ cm⁻¹ at 414 nm. The spectra were determined using an Aminco DW-2 recording spectrophotometer in the split beam mode. The concentration of cytochrome P-450 or P-448 was determined by the method of Omura and Sato (27) from the CO-difference spectra of dithionite-reduced samples using an extinction coefficient of 91 mm M⁻¹ cm⁻¹ at 414 nm. The concentration of cytochrome P-450 was determined by the method of Lowry et al. (29) using bovine serum albumin as the standard.

RESULTS

Separation of Multiple Forms of Cytochrome P-448 and Cytochrome P-450—Fig. 1 illustrates the elution profile of cytochrome P-448 from a DEAE-cellulose column in the presence of Emulgen 911. Forentially 100 mg of Step III protein were treated with Emulgen 911 and applied to the DEAE-cellulose column. The proteins were eluted from the column by a linear gradient of KCl in 5.0 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol and 0.1% Emulgen 911. As monitored by A_{410}, two major fractions of the hemeprotein emerge from the column, one which elutes in the column volume (Fraction A) and one which elutes at 0.1 M KCl (Fraction B). A similar elution pattern is observed if the Step III preparation is obtained from phenobarbital-pretreated rats, as was shown by Levin et al. (24), except that the ratio of the A and B fractions is different.

All fractions have been shown to be devoid of cytochrome b₅ and NADPH-cytochrome c reductase which elute at higher ionic strength (24).

To determine whether the two fractions were interconvertible or artifacts created by the method, each fraction was rechromatographed on a second DEAE column. As shown in Fig. 1, when the A fraction was applied to a second DEAE column and eluted under the same conditions as the initial column, only one fraction of the cytochrome eluted from the second column in the column volume. This result indicated that the A fraction was not contaminated with the B fraction. The B fraction was pooled, dialyzed to remove the KCl, and concentrated, and a portion was applied to a second DEAE-cellulose column and was eluted from the column under the same conditions as the initial column. Only one fraction emerged from the second column at 0.1 M KCl, which suggests that the B fraction was not contaminated with the A fraction and that the two fractions are not interconvertible.

The following conditions are important for the separation of Fractions A and B by column chromatography: (a) If a Step III preparation is applied to a DEAE-cellulose column and eluted in the absence of Emulgen 911, a different profile is observed. The cytochrome P-450 (P-448) elutes as a single broad peak at a higher ionic strength (0.2 to 0.3 M KCl). No separation of the cytochrome b₅ or NADPH-cytochrome c reductase is achieved, nor is there any further purification of the cytochrome P-450 (P-448). Thus, a nonionic detergent is required to separate the two fractions. (b) The separation of the fractions can still be achieved if the Emulgen 911 concentration in the elution buffer is decreased to as low as 0.02% or increased to 0.5%. The separation of the fractions is also achieved if the potassium phosphate buffer is replaced with 0.02 M Tris-HCl buffer, pH 8.2.

(c) If rat liver microsomes are solubilized with Emulgen 911 and...
applied to a DEAE-cellulose column, no separation of the fractions occurs. This result indicates that the prior removal of certain contaminants such as lipid is required to attain the separation. (d) If the anionic exchanger, DEAE-cellulose, is replaced by a cationic exchanger, CM-cellulose, a similar elution profile of the A and B fractions is observed. The proteins were eluted from the column in the presence of 5.0 M potassium phosphate buffer (pH 6.5), 20% glycerol, and 0.1% Emulgen 911 with a linear gradient of KCl (0 to 0.5 M). Therefore, the possibility that the A and B fractions may be separated by a combination of adsorptive properties and ion exchange cannot be excluded.

The specific conditions described under "Experimental Procedure" are optimal for the separation of the fractions, the removal of contaminants, and the minimal conversion of cytochrome P-450 to its inactive form, cytochrome P-420. By the criterion of ion exchange chromatography, the A and B hemeprotein fractions are different.

**CO-reduced Difference Spectra of A and B Fractions**—Fig. 2 illustrates the CO-reduced difference spectra of an A and B fraction isolated from 3-methylcholanthrene-treated rats. The absorption peak in the CO-reduced difference spectrum has been reported to be at 448 nm for the cytochrome induced by polycyclic hydrocarbons (9). Microsomes and the partially purified Step III preparations from 3-methylcholanthrene-pretreated rats exhibited this characteristic maximum (25, 30). The line at 448 nm delineates these maxima when all spectra are recorded on the same sheet. Repeated experiments indicated the absorption peak in the CO-reduced difference spectrum of the A fraction to be at 448.5 to 449.0 nm and of the B fraction to be at 446.8 to 447.0 nm. The absorption peak of the starting preparation (Step III) was intermediate (447.5 to 448.0 nm) to that of the A and B fractions. The small amount of cytochrome P-420 present was equivalent in both fractions. Fig. 2 shows the maximal amount of cytochrome P-420 observed in the fractions from 3-methylcholanthrene-treated rats; routinely, only 3 to 5% of the total hemeprotein was present as cytochrome P-420.

Several lines of evidence suggest that the shifts in the CO-reduced difference spectral maxima are not due to the presence of Emulgen 911. (a) The Step III starting material from 3-methylcholanthrene-treated rats exhibited an absorption peak intermediate to that of the A and B fractions. If Emulgen 911 was added to the Step III preparation from either 3-methylcholanthrene- or phenobarbital-treated rats, no shifts in the CO-reduced difference spectra were observed. (b) Although both the A and B fractions isolated from 3-methylcholanthrene-treated rats contained Emulgen 911, the A fraction showed a shift toward a higher wavelength, whereas the B fraction showed a shift toward a lower wavelength. If the spectral changes were due to the presence of detergent, the shifts observed should be in the same direction. (c) If the A and B fractions were prepared from phenobarbital-pretreated animals, no shifts in the CO-reduced difference spectral maxima were observed. These A and B fractions exhibited an absorption peak at 450 nm as indicated by the line at that wavelength in Fig. 2. It should be emphasized that the A fraction from phenobarbital-treated rats contained approximately the same amount of Emulgen 911 as the A fraction from 3-methylcholanthrene-treated rats, and the B fraction from phenobarbital-treated rats contained an equivalent amount of Emulgen 911 as the B fraction from 3-methylcholanthrene-treated rats (Table I). (d) When the spectra were recorded prior to the removal of the excess detergent by Sephadex LH-20, the shifts in maxima described were still observed.

**Ethyl Isocyanide Difference Spectra of A and B Fractions**—As originally described by Imai and Sato (31), ethyl isocyanide binds to reduced cytochrome P-450 to yield two spectral maxima at 455 and 430 nm which exist in a pH-dependent equilibrium. Sladek and Mantering (11) showed that pretreatment of rats with 3-methylcholanthrene resulted in a change in ratio of the 455- and 430-nm peaks as compared to control and phenobarbital-treated rats. Microsomes from untreated or phenobarbital-pretreated animals exhibit the characteristic absorption peaks at 455 and 430 nm, whereas Alvares et al. (16) reported that pretreatment of rats with 3-methylcholanthrene resulted in a change in ratio of the 455- and 430-nm peaks as compared to control and phenobarbital-treated rats. Microsomes from untreated or phenobarbital-pretreated animals exhibit the characteristic absorption peaks at 455 and 430 nm, whereas Alvares et al. (16) reported that pretreatment of rats with 3-methylcholanthrene resulted in a change in ratio of the 455- and 430-nm peaks as compared to control and phenobarbital-treated rats. Microsomes from untreated or phenobarbital-pretreated animals exhibit the characteristic absorption peaks at 455 and 430 nm, whereas Alvares et al. (16) reported that pretreatment of rats with 3-methylcholanthrene resulted in a change in ratio of the 455- and 430-nm peaks as compared to control and phenobarbital-treated rats. Microsomes from untreated or phenobarbital-pretreated animals exhibit the characteristic absorption peaks at 455 and 430 nm, whereas Alvares et al. (16) reported that pretreatment of rats with 3-methylcholanthrene resulted in a change in ratio of the 455- and 430-nm peaks as compared to control and phenobarbital-treated rats. Microsomes from untreated or phenobarbital-pretreated animals exhibit the characteristic absorption peaks at 455 and 430 nm, whereas Alvares et al. (16) reported that pretreatment of rats with 3-methylcholanthrene resulted in a change in ratio of the 455- and 430-nm peaks as compared to control and phenobarbital-treated rats. Microsomes from untreated or phenobarbital-pretreated animals exhibit the characteristic absorption peaks at 455 and 430 nm, whereas Alvares et al. (16) reported that pretreatment of rats with 3-methylcholanthrene resulted in a change in ratio of the 455- and 430-nm peaks as compared to control and phenobarbital-treated rats. Microsomes from untreated or phenobarbital-pretreated animals exhibit the characteristic absorption peaks at 455 and 430 nm, whereas Alvares et al. (16) reported that pretreatment of rats with 3-methylcholanthrene resulted in a change in ratio of the 455- and 430-nm peaks as compared to control and phenobarbital-treated rats.

As shown in Fig. 3, the ethyl isocyanide difference spectral maxima of the A and B hemeprotein fractions from 3-methylcholanthrene-treated rats at pH 7.4 were at 453 and 430 nm and at 451 and 430 nm, respectively. That is, the "455 peak" was shifted to a higher wavelength in the A fraction and to a lower wavelength in the B fraction as compared to the absorption maximum of the Step III preparation which is at an intermediate.  

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**Fig. 1. Chromatography and re-chromatography of partially purified cytochrome P-448 on Sigma DEAE-cellulose.** The Step III preparation from 3-methylcholanthrene-treated rats was treated with Emulgen 911 and was chromatographed on a DEAE-cellulose column (1.5 x 30 cm). The A and B fractions (O---O) were eluted as described under "Results." The rechromatography of each fraction was performed as described under "Results." The elution profile of the A (X---X) and B (O---O) fractions from separate DEAE-cellulose columns is shown.
**Table I**

Properties of partially purified cytochrome P-450 from phenobarbital- and 3-methylcholanthrene-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Step III</th>
<th>Step IV</th>
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<tbody>
<tr>
<td></td>
<td>Fraction A</td>
<td>Fraction B</td>
</tr>
<tr>
<td><strong>Cytochrome P-450 (nmol/mg of protein)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>5-6.5</td>
<td>3-4</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>4.5-5.5</td>
<td>1.7-3</td>
</tr>
<tr>
<td><strong>CO difference spectrum</strong></td>
<td></td>
<td></td>
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<tr>
<td>Phenobarbital</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>447.5</td>
<td>448.7</td>
</tr>
<tr>
<td><strong>Ethyl isocyanide spectrum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>430</td>
<td>430</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>430, 452</td>
<td>450, 455</td>
</tr>
<tr>
<td>455:430 Ratio (pH 7.4)</td>
<td>0.7-0.8</td>
<td>1.8-2.0</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>1.5-1.7</td>
<td>1.7-2.0</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>1.2-1.4</td>
<td>1.8-2.0</td>
</tr>
<tr>
<td>pH intercept</td>
<td>7.70</td>
<td>6.90</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>7.10</td>
<td>7.85</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>6.90</td>
<td>6.85</td>
</tr>
<tr>
<td><strong>Phosphatidylcholine (% of microsomes, per nmol of P-450)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>4-7</td>
<td>12-15</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>5-8</td>
<td>50</td>
</tr>
<tr>
<td><strong>Emulgen 911 (mg/nmol of P-450)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.4-0.5</td>
<td>0.04-0.07</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>0.6-0.7</td>
<td>0.04-0.07</td>
</tr>
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</table>

The relative heights of the 455- and 430-nm absorption maxima are dependent upon the pH at which the ethyl isocyanide binding spectrum is recorded, and the pH at which the peak heights are equal has been referred to as the pH intercept (11, 31). The pH intercept of the spectra obtained with microsomes isolated from untreated or phenobarbital-pretreated animals has been reported to be pH 7.4 to 7.6 and 6.9 from 3-methylcholanthrene-pretreated animals (11, 16). The pH intercepts of the Step III preparation, and the A and B fractions from 3-methylcholanthrene-treated rats were pH 6.90, 7.10, and 6.85, respectively, and the intercepts of the Step III preparation, A fraction, and B fraction from phenobarbital-pretreated rats were pH 7.70, 6.90, and 7.85, respectively (Table I). The Step III preparation in both instances exhibited a pH intercept intermediate to the respective A and B fractions.

The A fraction from phenobarbital-pretreated rats was characterized by a 455:430 ratio of 1.8 to 2.0 at pH 7.4 (Fig. 3, Table I) and a pH intercept at 6.90 (Table I), which was similar to that of cytochrome P-448, but no shifts in the CO-reduced difference spectral maxima or ethyl isocyanide 455 peak were observed. It was observed in all cases that the relative size of the 430 peak did not influence the location of the “455 region” peaks described.
Therefore, this fraction differs not only from the B fraction from phenobarbital-treated rats but also from the cytochrome P-448.

**Absolute Oxidized Spectra of A and B Fractions**—The absolute oxidized spectra of the A and B hemeprotein fractions from 3-methylcholanthrene-treated rats are shown in Fig. 4. The oxidized form of the A fraction had absorption peaks at 395, 414, 530, 564, and 642 nm; the oxidized form of the B fraction had absorption peaks at 417, 535, and 568 nm. The absolute oxidized spectra characterize the A fraction as containing a high amount of high spin ferric hemeprotein and the B fraction as a low spin ferric hemeprotein at room temperature. If 3-methylcholanthrene was added to the B fraction, new absorption maxima appeared at 395, 413, and 642 nm, similar to that seen with the A fraction from 3-methylcholanthrene-treated rats. Schenkman et al. (34) have reported similar spectral shifts in the “apparent absolute spectra” upon the addition of substrates to microsomal suspensions. Therefore, the high spin component of this A fraction may represent a substrate-bound species of the hemeprotein. The A and B fractions from 3-methylcholanthrene-treated rats had different absorption peaks in the oxidized spectra but not in the reduced spectra. Upon reduction of either hemeprotein fraction with dithionite, the absorption maxima shifted to 411 and 542 nm (not shown).

**n-Octylamine Binding Spectra**—The position of the difference
n-Octylamine binding spectra of the Step III preparation, A fraction, and the B fraction from 3-methylcholanthrene-pretreated rats. The spectra were recorded as described under "Experimental Procedure," after the addition of n-octylamine (final concentration 1.0 mM) to the sample cuvette and the addition of an equal volume of buffer-glycerol to the reference cuvette.

spectra produced by the binding of n-octylamine to the oxidized hemeprotein has been reported to be an estimate of the ratio of high spin to low spin ferric cytochrome P-450 (28). The n-octylamine binding spectra of the Step III preparation, A fraction, and B fraction from 3-methylcholanthrene-pretreated rats is shown in Fig. 5. The spectrum of the Step III preparation (maximum 431 nm, minimum 411 nm) exhibited a high spin to low spin ratio of 0.077, indicating that 7 to 8% of the hemeprotein was present as the high spin species which correlated well with the value calculated from EPR analysis (35). The spectrum of the A fraction (maximum 429 nm, minimum 392 nm) was characterized by a high spin to low spin ratio of 0.57 which would indicate that 57% of the hemeprotein was present as the high spin form at room temperature. EPR analysis indicated that 25 to 40% of this hemeprotein fraction was present as the high spin species at the temperature of liquid helium (36). The spectrum of the B fraction (maximum 431 nm, minimum 411 nm) was characterized by a high spin to low spin ratio of 0.009, indicating that this fraction contained negligible amounts of the high spin form. Stern et al. (36) have calculated from EPR analysis the amount of high spin species present in this fraction to be <1% of the total hemeprotein.

These results suggested that the small amount of high spin species present in the Step III preparation from 3-methylcholanthrene-pretreated rats was selectively eluted in the A fraction. The B fraction, partially purified to 9 to 11 nmol per mg of protein, contained a negligible amount of the high spin species. However, this fraction still retained the substrate specificity as well as the peak shifts toward lower wavelengths in the CO-reduced and ethyl isocyanide difference spectra characteristic of microsomes from 3-methylcholanthrene-pretreated rats.

Cyanide Binding Constants—Cyanide binds with oxidized cytochrome P-450 to produce a difference spectrum characterized by a maximum at 442 nm and a minimum at 405 nm. Comai and Gaylor (23) recently have reported the separation on DEAE-cellulose of three forms of cytochrome P-450 from protease and detergent-treated rat liver microsomes based on their differential affinity for cyanide. The $K_m$ values reported were 0.5 mM (Form I), 1.6 mM (Form II), and 4.9 mM (Form III). Pretreatment of the rats with microsomal enzyme inducers was shown to alter the relative amount of each form. The binding constants of the Step III preparation, the A fraction, and the B fraction from 3-methylcholanthrene-pretreated rats were 1.3 and 5.2 mM; 1.09 and 5.6 mM; and 0.94 and 9.6 mM, respectively. The binding constants of the Step III preparation, the A fraction, and B fraction from phenobarbital-treated rats were 1.89 and 6.18 mM; 1.57 and 7.27 mM; and 2.16 and 6.81 mM, respectively. In each case, the Hofste plot was curvilinear and only two binding constants were obtained. The Form I fraction was absent from all of the preparations, and the amount of the other forms varied. It was apparent that the fractions obtained by our procedure did not correspond to the forms separated by Comai and Gaylor (23).

Catalytic Activity—The A fractions from both phenobarbital- and 3-methylcholanthrene-pretreated rats had poor catalytic activity toward both benzphetamine and benzo[a]pyrene in comparison to the catalytic activity of more purified B fractions. The B fractions retain the substrate specificity of the microsomes from which they were derived (24). The low activity of the A fractions may be due either to the high concentration of detergent still present in these fractions which may be inhibitory or to an inherent property of these hemeprotein fractions. No conclusions regarding the low catalytic activity of the A fractions can be drawn at this time.

DISCUSSION

In this report evidence is presented which indicates the presence of two forms of cytochrome P-450 (Fractions A and B) in the livers of phenobarbital-treated rats and the presence of two forms of cytochrome P-448 (Fractions A and B) in the livers of 3-methylcholanthrene-treated rats. Each hemeprotein fraction possesses distinct spectral properties. Table I presents a summary of the properties of the A and B hemeprotein fractions obtained from rats pretreated with either phenobarbital or 3-methylcholanthrene. A fraction of low specific content (Fraction A) and a fraction substantially purified (Fraction B) are derived from a partially purified Step III preparation by DEAE-cellulose chromatography in the presence of Emulgen 911. The two hemeprotein fractions derived from 3-methylcholanthrene-pretreated rats differ most significantly in the location of the absorption peaks in the CO-reduced spectrum and the ethyl isocyanide difference spectrum. Whereas the two hemeprotein fractions from phenobarbital-pretreated rats do not exhibit shifts in absorption maxima, substantial differences are observed in the ethyl isocyanide difference spectra with respect to ratios and pH intercepts. The spectral differences among the various fractions observed, however, did not relate to the Emulgen 911 or phosphatidylcholine concentration. The data summarized in Table I indicate that the four hemeprotein fractions obtained from phenobarbital- and 3-methylcholanthrene-pretreated rats are different. Preliminary experiments indicated that the two hemeprotein fractions obtained from control rats may resemble the fractions from phenobarbital-pretreated rats.

A preliminary experiment was conducted to determine whether one of the two hemeprotein fractions from rats pretreated with
reported the existence of three spectrally distinguishable forms of cytochrome P-450. δ-[3H]Aminolevulinic acid, a precursor of heme, was injected into control rats. Sixteen hours later the administration of 3-methylcholanthrene was begun. By this procedure, only the control cytochrome P-450 would be labeled; the cytochrome P-448 induced by 3-methylcholanthrene would not be labeled. After 3 days of 3-methylcholanthrene treatment, the rats were killed. The livers obtained were processed through the entire method. Radioactivity was present in the A and B fraction, suggesting that a small amount of control P-450 remained in both fractions. These data suggest that neither fraction selectively contained control cytochrome P-450. Moreover, the presence of the control cytochrome P-450 in both fractions suggested that the presence of this hemoprotein was not responsible for the spectral changes observed. Both fractions, which differ in spectral characteristics, probably contain hemoprotein specifically induced by 3-methylcholanthrene.

The question of how spectrally distinct forms of cytochrome P-450 in solubilized preparations relate to the cytochrome P-450 composition of intact microsomes may be posed. It should be noted that the recovery of the A and B hemeprotein fractions may not reflect the amount of these hemeproteins present in microsomes. Furthermore, it is unknown whether the A and B fractions contain multiple hemeproteins with different substrate specificities. Several studies with intact microsomes have suggested that the presence of this hemeprotein was not responsible for the spectral changes observed. Both fractions, which differ in spectral characteristics, probably contain hemoprotein specifically induced by 3-methylcholanthrene.

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