THE INVOLVEMENT OF CYTOCHROME P-448 AND P-450 IN NADH-DEPENDENT O-DEMETHYLATION OF p-NITROANISOLE IN RAT LIVER MICROSOMES

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Abstract—These studies have shown that addition of p-nitroanisole to a reaction mixture containing rat liver microsomes resulted in an increase of the reoxidation rate of NADH-reduced cytochrome b⁵. Fortification of rat liver microsomes with partially purified cytochrome b⁵ produces an increase in both NADPH-dependent and NADH-dependent p-nitroanisole O-demethylation activity. Antiserum to cytochrome P-450 isolated from phenobarbital-treated rat liver microsomes inhibited the NADH-dependent O-demethylation activity as well as the NADPH-dependent O-demethylation activity seen in rat liver microsomes. Addition of either purified cytochrome P-450 or cytochrome P-448 to an incubation mixture containing phenobarbital-treated rat liver microsomes enhanced the NADH-dependent p-nitroanisole O-demethylation activity. These results suggest that NADH-dependent and, in part, NADPH-dependent O-demethylations are catalyzed by cytochrome P-448 and cytochrome P-450 receiving electrons from cytochrome b⁵.

It has been widely accepted that the NADPH-linked electron transport system, especially from NADPH to cytochrome P-450 via NADPH-cytochrome P-450 reductase, plays a major role in drug oxidations in liver microsomes. Recent studies have indicated that NADH is also an electron donor to cytochrome P-450 (1–3). Hrycay and Estabrook (3) reported that cytochrome P-450 receives reducing equivalents from NADH-cytochrome b⁵ reductase and cytochrome b⁵. West et al. (4) have reported that electrons for NADH-dependent hydroxylation of benzo(a)pyrene are transferred from NADH to cytochrome P-448 via cytochrome b⁵. However, little is known of the significance of the electron flow from NADH to cytochrome P-450 with respect to drug hydroxylation.

Shigematsu and coworkers (5, 6) have quite recently demonstrated the possibility that NADH-dependent O-dealkylation of p-nitroanisole and p-nitrophenetole is catalyzed by an enzyme other than cytochrome P-450. Evidence supporting this conclusion was that carbon monoxide-oxygen (8:2) inhibited p-nitroanisole O-demethylation by only 10 to 20%, and enhanced p-nitrophenetole O-deethylation.

In this paper, we report our data that cytochrome P-448 and cytochrome P-450 are also
involved in NADH-dependent O-demethylation of \( p \)-nitroanisole.

**MATERIALS AND METHODS**

Male rats of Crl:CD (SD) strain weighing 100 to 120 g were used throughout this study. The animals, which were maintained on a commercial rat show, CE-2 Nippon Clea Co., Japan, were deprived of food for about 18 hr prior to sacrifice, but were given tap water *ad libitum*. The rats were given either an intraperitoneal injection of 3-methylcholanthrene dissolved in corn oil once a day for 8 successive days or 0.1% sodium phenobarbital in drinking water for 3-days. Sepharose 4B, DEAE-Sephadex (A-50) and CM-Sephadex (C-50) were purchased from Pharmacia Fine Chemicals Co. Hydroxylapatite was purchased from Bio-Rad. NADP, NADH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49, Grade I) were purchased from Boehringer Mannheim. Emulgen 913, a nonionic detergent, was kindly provided by Kao-Atlas Co. All other chemical reagents of analytical grade were purchased from commercial routes and were used without further purification. Unless otherwise stated, values in the tables and figure are the means of duplicate determinations.

**Preparation of \( \omega \)-amino-n-octyl Sepharose 4B**

The \( \omega \)-amino-n-octyl derivative of Sepharose 4B was prepared from cyanogen bromide-activated Sepharose 4B and 1,8-diaminooctane by a method described by Cuatrecasas (7).

**Purification of cytochrome P-450 from phenobarbital-treated rat liver microsomes**

Cytochrome P-450 was purified from phenobarbital-treated rat liver microsomes by a method described by Kamataki *et al.* (8), which is a modification of the method of Imai and Sato (9). The specific content of the cytochrome P-450 preparation was 15.6 nmole/mg protein.

**Purification of cytochrome P-448 from 3-methylcholanthrene-treated rat liver microsomes**

Cytochrome P-448 was purified from 3-methylcholanthrene-treated rat liver microsomes by a modification of the method of Hashimoto and Imai (10). The cytochrome P-450 fractions eluted from the \( \omega \)-amino-n-octyl Sepharose 4B column with a buffer containing 100 mM K-phosphate (pH 7.25), 20% glycerol, 0.4% sodium cholate and 0.08% Emulgen 913 were diluted 3-fold with 20% glycerol and applied onto a column of hydroxylapatite (3.0 x 7.0 cm) equilibrated with 33 mM K-phosphate (pH 7.25) containing 20% glycerol. The column was washed with 250 ml of 35 mM K-phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. About 14% of the applied cytochrome P-450 was recovered by washing the column with 100 ml of 100 mM K-phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. The carbon monoxide difference spectrum of the cytochrome P-450 eluted in this manner exhibited a peak at 450 nm, the specific content of the cytochrome P-450 was 5.6 nmole/mg protein and the minimum molecular weight as determined by SDS-polyacrylamide gel electrophoresis (11) was approx. 48,000. About 60% of the applied cytochrome P-450 was eluted by further washing of the column with 150 mM K-phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. The cytochrome
P-450 eluted with 150 mM K-phosphate had a peak in the carbon monoxide difference spectrum of 448 nm and the specific content was 8.6 nmole/mg protein. The combined cytochrome P-448 fractions obtained by washing with 150 mM K-phosphate were diluted 3-fold with 20% glycerol containing 0.2% Emulgen 913. The diluted preparation was then applied on a column of CM-Sephadex (C-50) (2.0 × 10.0 cm) which had been equilibrated with 50 mM K-phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. The column was washed with the same buffer, followed by a small amount of 100 mM K-phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. Cytochrome P-448 was then eluted by washing the column with 200 mM K-phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. The combined sample was dialyzed for 48 hr against 50 mM K-phosphate (pH 7.25) containing 20% glycerol. The sample was applied on a second column of CM-Sephadex (2.0 × 10.0 cm) which had been equilibrated with 50 mM K-phosphate (pH 7.25) containing 20% glycerol. The cytochrome P-448 was obtained by washing the column with 200 mM K-phosphate (pH 7.25) containing 20% glycerol. The results of a typical experiment are shown in Table 1. This cytochrome P-448 had a peak in the carbon monoxide difference spectrum of 448 nm, and was estimated to be a low spin species of cytochrome P-450 as judged from the absolute spectrum (peak at 418 nm and no shoulder at near 398 nm). The minimum molecular weight was estimated to be 52,500 as measured by SDS-polyacrylamide gel electrophoresis. The specific content of this cytochrome P-448 preparation was 16.6 nmole/mg protein.

| Fraction                      | Protein (mg) | TC<sup>a</sup> | SC<sup>b</sup> | Recovery (%) |
|-------------------------------|--------------|----------------|---------------|--------------|
| Cholate-solubilized microsomes| 675          | 2161           | 3.2           | 100          |
| Aminooyctyl column eluate     | 107          | 918            | 8.6           | 42.5         |
| Hydroxyapatite column eluate  | 46.4         | 550.3          | 11.9          | 25.5         |
| 1st CM-Sephadex column eluate | 38.8         | 488.2          | 12.6          | 22.6         |
| 2nd CM-Sephadex column eluate | 22.9         | 381.2          | 16.6          | 17.6         |

<sup>a</sup> Total content of cytochrome P-450 (nmole)

<sup>b</sup> Specific content of cytochrome P-450 (nmole per mg of protein)

Partial purification of cytochrome b<sub>5</sub>

Cytochrome b<sub>5</sub> was partially purified from phenobarbital-treated rat liver microsomes by the method described by Imai (12). The specific content of cytochrome b<sub>5</sub> thus obtained was 28.9 nmole/mg protein, and was free from cytochrome P-450, NADH-cytochrome b<sub>5</sub> reductase and NADPH-cytochrome c reductase. The cytochrome b<sub>5</sub> preparation eluted from the ω-amino-n-octyl Sepharose 4B column was dialyzed against redistilled water for 48 hr and used for the experiment. Cytochrome b<sub>5</sub> was determined by recording the NADH- or dithionite-reduced minus oxidized difference spectrum of cytochrome b<sub>5</sub>. The reductant used depended upon the purification step. All measurements of the difference spectra of
cytochrome b$_2$ and P-450 were conducted using the Aminco recording spectrophotometer, Model DW-2. Cytochrome P-450 was determined by a method described by Omura and Sato (13) using molar extinction coefficient of 91 mM$^{-1}$ cm$^{-1}$ except that 20% glycerol and 0.2% Emulgen 913 were added. The molar extinction coefficient of 185 mM$^{-1}$ cm$^{-1}$ was used for calculating the cytochrome b$_2$ content.

**Preparation of antibody to cytochrome P-450 isolated from phenobarbital-treated rat liver microsomes**

Antiserum to rat liver cytochrome P-450 was obtained using adult female New Zealand rabbits which had been immunized with purified cytochrome P-450 according to the method previously described (8). Using Ouchterlony double diffusion analysis (8), the antiserum thus obtained was found to react with cytochrome P-450 isolated from phenobarbital-treated rat liver microsomes but showed only slight cross reactivity with cytochrome P-448 purified from 3-methylcholanthrene-treated rats.

**Assay of NADH-dependent and NADPH-dependent demethylase activities**

A typical incubation mixture for the assay of NADH-dependent demethylase activities contained a substrate (5 mM), 1 mM NADH, 0.1 M Na,K-phosphate (pH 6.0), 0.1 mM EDTA and rat liver microsomes (1 mg protein) in a final volume of 1.0 ml. When NADPH-dependent demethylation activities were measured, an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 6 mM MgCl$_2$ and 0.045 unit glucose 6-phosphate dehydrogenase) and 0.1 M Na,K-phosphate (pH 7.4) were added to the incubation mixture instead of NADH and 0.1 M Na,K-phosphate (pH 6.0). Incubation were carried out at 37° for 10 min aerobically. Oxidative demethylation activities of N-monomethylaniline, aminopyrine, benzphetamine and ethylmorphine were estimated by determining formaldehyde production using the method of Nash (14). p-Nitroanisole O-demethylation activity was measured by determining the p-nitrophenol formed. In this assay the incubation mixture was deproteinized by the addition of an equal amount of cold 10% trichloroacetic acid and centrifugation. A 1.5 ml aliquot of the supernatant phase was transferred to another tube containing 1.5 ml of 15% sodium carbonate, and the optical density at 410 nm was read. Protein was determined by the method of Lowry et al. (15).

**Measurement of cytochrome b$_2$ reoxidation rate**

A mixture containing 0.2 mM Na$_2$S, 1 mM KCN, 0.1 M Na,K-phosphate (pH 7.4) and phenobarbital-treated rat liver microsomes (1 mg protein/ml of reaction mixture) was placed in a cuvette, and the cytochrome b$_2$ reoxidation rate was measured at 25° by recording the decrease of the absorbance difference between 409 and 424 nm after addition of 1.0 μM NADH. Acetone (30 μM) was added as a control since acetone was used as a solvent of p-nitroanisole.

**Fortification of rat liver microsomes with partially purified cytochrome b$_2$**

Fortification of rat liver microsomes with cytochrome b$_2$ was carried out essentially by the method described by Strittmatter et al. (16). The cytochrome b$_2$ content of the microsomes after fortification was determined by recording the NADH-reduced minus oxidized
difference spectrum of cytochrome b₅. Further details concerning the method are described in the Tables.

RESULTS

The involvement of cytochrome b₅ in the NADH-dependent p-nitroanisole O-demethylation was examined. The results showing the effect of p-nitroanisole on the reoxidation rate of NADH-reduced cytochrome b₅ are shown in Fig. 1. The reoxidation rate increased with the amount of p-nitroanisole added. This result supports the idea that electrons for O-demethylation of p-nitroanisole are transferred via cytochrome b₅. It has been reported that purified cytochrome b₅ can bind to microsomes in vitro and function as an electron carrier (16–18). To further examine the idea that cytochrome b₅ is involved in NADH-dependent O-demethylation of p-nitroanisole, the effect of exogenously added cytochrome b₅ on O-demethylation activity of p-nitroanisole was examined (Table 2). The addition of partially purified cytochrome b₅ to microsomes resulted in an incorporation of the cytochrome b₅ into microsomal membranes. The amount of NADH-reducible cytochrome b₅ incorporated into microsomal membranes increased with the amount of partially purified cytochrome b₅ added. The NADH-dependent O-demethylation activity toward p-nitroanisole also increased when exogenous cytochrome b₅ was added. The NADPH-dependent O-demethylation activity also increased, but to a lesser extent than NADH-dependent activity.

To determine whether or not cytochrome P-450 was involved in NADH-dependent p-nitroanisole O-demethylation, the effect of cytochrome P-450 antibody on the O-demethylation activity was examined. As is shown in Table 3, the addition of antisera to the incubation mixture resulted in an inhibition of both the NADH-dependent and the NADPH-dependent O-demethylation activity. However, the extent of inhibition by the cytochrome P-450 antisera was less in the case of NADH-dependent as compared to NADPH-

![Fig. 1. Effect of p-nitroanisole (in 30 μl acetone) on the reoxidation rate of NADH-reduced cytochrome b₅ in phenobarbital-treated rat liver microsomes. Values obtained at each time point given in the Figure were reduced by the amount obtained using acetone (30 μl) alone. The reoxidation rate of NADH reduced cytochrome b₅ in the presence or absence of acetone (30 μl) was 0.373 and 0.168 nmole cytochrome b₅ oxidized/mg protein/min, respectively.](image-url)
dependent activity. Since it has been demonstrated that there are immunologically distinguishable multiple cytochrome P-450 species in liver microsomes (19, 20), it seemed possible to assume that the difference between NADH-dependent and NADPH-dependent O-demethylation in the extent of inhibition by the antiserum is due to a difference in the cytochrome P-450 species involved in the reactions. Yang and Strickhart (21) have reported that addition of purified cytochrome P-448 to a microsomal preparation enhanced benzo(a)pyrene hydroxylation activity. The results suggested that purified cytochrome P-448 was incorporated into the microsomal membranes and that this cytochrome P-448 could function in benzo(a)pyrene hydroxylation. The effects of addition of purified cytochrome P-450 or cytochrome P-448 to rat liver microsomes on NADH-dependent and NADPH-dependent O-demethylation activities are shown in Table 4. The addition of cytochrome

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**Table 2.** Effect of exogeneously added cytochrome b₅ on NADPH-dependent and NADH-dependent O-demethylation activities of p-nitroanisole in phenobarbital-treated rat liver microsomes

| Group                  | Microsomal cyt. b₅ fortification (nmole/mg protein) | p-Nitroanisole O-demethylation (nmole/mg protein 10 min) |
|------------------------|-----------------------------------------------------|--------------------------------------------------------|
| Control                | 0.60                                                | 21.7 (100)                                             |
| Cyt. b₅ (6.96 nmole)   | 0.94                                                | 28.9 (133)                                             |
| + Cyt. b₅ (26.12 nmole)| 1.46                                                | 23.4 (108)                                             |

Liver microsomes (105 mg protein) were separated into three flasks. The flasks were incubated at 37° for 30 min after addition of 0, 6.96 or 26.12 nmole of partially purified cytochrome b₅ and 0.1 M Na, K-phosphate (pH 7.4). The microsomal suspensions were centrifuged at 105,000 g for 1 hr. The microsomes sedimented by the centrifugation were resuspended in 0.1 M Na, K-phosphate (pH 7.4) and centrifuged at 105,000 g for 30 min. The washed microsomes were suspended in twice distilled water and used for the assay of p-nitroanisole O-demethylation activity and cytochrome b₅ content.

**Table 3.** Effect of antiserum to cytochrome P-450, isolated from phenobarbital-treated rat liver microsomes, on the NADPH-dependent and NADH-dependent O-demethylation of p-nitroanisole in phenobarbital-treated rat liver microsomes

| Addition                  | NADPH | NADH |
|---------------------------|-------|------|
| Control serum (35 µl)     | 31.2  | 7.0  |
| Anti cyt. P-450 serum (35 µl) | 25.1 | 5.9  |
| Inhibition (%)            | 19.6  | 15.7 |
| Control serum (105 µl)    | 28.9  | 6.8  |
| Anti cyt. P-450 serum (105 µl) | 18.3 | 5.4  |
| Inhibition (%)            | 36.7  | 20.6 |

Antiserum obtained from immune rabbits (amounts shown in the parentheses) was added to the incubation mixture containing liver microsomes from phenobarbital-treated rats and other necessary components. Serum from preimmune rabbits was added as a control. For other experimental conditions, see MATERIALS AND METHODS.
P-450 did not appreciably change the NADPH-dependent activity of \( p \)-nitroanisole O-demethylation but increased by about 14\(^{\circ} \) the NADH-dependent activity. The addition of purified cytochrome P-448 to the reaction mixture slightly increased the NADPH-dependent O-demethylation activity while the activity was increased by about 22\(^{\circ} \) in the NADH-dependent reaction. Therefore, the addition of the purified cytochrome preparations produced greater enhancement of the O-demethylation activity in the NADH-dependent reaction than in the NADPH-dependent reaction. In addition, it is noteworthy that cytochrome P-448 is a preferential species of cytochrome P-450 in the enhancement of

**Table 4. Effect of addition of either purified cytochrome P-450 or cytochrome P-448 on NADPH-dependent and NADH-dependent O-demethylation activity toward p-nitroanisole using phenobarbital-treated rat liver microsomes**

| Addition           | NADPH (nmole/mg protein/10 min) | NADH (nmole/mg protein/10 min) |
|--------------------|--------------------------------|--------------------------------|
| Buffer             | 25.6                           | 5.1                            |
| Cytochrome P-450 (5 nmoles) | 25.0                         | 5.8                            |
| Change (\(^{\circ} \)) | 2.3                          | 13.7                           |
| Buffer             | 25.9                           | 6.8                            |
| Cytochrome P-448 (5 nmoles) | 27.0                         | 8.3                            |
| Change (\(^{\circ} \)) | 4.2                          | 22.1                           |

Purified cytochrome P-450 or cytochrome P-448 (5 nmoles) was added to the incubation mixture containing liver microsomes from phenobarbital-treated rats and all necessary components (see MATERIALS AND METHODS). Since the final samples of cytochrome P-450 and cytochrome P-448 contained 20\(^{\circ} \) glycerol and K-phosphate (pH 7.25), the corresponding amounts of the buffer solution were added as controls. The incubation mixture was preincubated at 37 \(^{\circ} \) for 30 min before starting the reaction by addition of NADH or an NADPH-generating system.

**Table 5. Effect of addition of purified cytochrome P-450 and cytochrome P-448 on NADPH-dependent and NADH-dependent O-demethylation activities of p-nitroanisole in phenobarbital-treated rat liver microsomes in the presence of fortified-cytochrome b**

| Addition           | NADPH (nmole/mg protein/10 min) | NADH (nmole/mg protein/10 min) |
|--------------------|--------------------------------|--------------------------------|
| Buffer             | 23.7                           | 10.0                           |
| Cytochrome P-450 (5 nmoles) | 30.1                         | 13.7                           |
| Change (\(^{\circ} \)) | 6.4                          | 37.0                           |
| Buffer             | 25.1                           | 10.9                           |
| Cytochrome P-448 (5 nmoles) | 32.4                         | 16.3                           |
| Change (\(^{\circ} \)) | 29.2                          | 49.9                           |

Liver microsomes (70 mg protein) from phenobarbital-treated rats were incubated at 37 \(^{\circ} \) for 30 min with partially purified cytochrome b (55.7 nmoles) and 0.1 M Na, K-phosphate (pH 7.4). The microsomes were washed once as described in Table 2. The washed microsomes were used for the assay of the O-demethylation activity. The incubation mixture was preincubated for 5 min after addition of purified preparations of cytochrome P-450 and cytochrome P-448. K-phosphate (pH 7.25) containing 20\(^{\circ} \) glycerol was added as a control.
NADH-dependent O-demethylase activity. The increase in p-nitroanisole O-demethylation seen on fortification of microsomes with cytochrome P-448 was less than that seen on fortification with cytochrome b5. This suggested that the amount of cytochrome b5 is the rate limiting factor in the NADH-dependent activity. The effect of addition of purified cytochrome P-450 or cytochrome P-448 on O-demethylation activity was also examined using cytochrome b5 fortified microsomes (Table 5). The fortification with cytochrome b5 produced a greater enhancement of p-nitroanisole O-demethylation activity due to addition of the purified cytochromes P-450 and P-448. The addition of cytochrome P-448 was more effective than that of cytochrome P-450 in the enhancement of the NADH-dependent O-demethylation activity.

In Table 6, the reaction velocities of NADH-dependent and NADPH-dependent demethylation of p-nitroanisole, N-monomethylaniline, aminopyrine, benzphetamine and ethylmorphine are compared using 3-methylcholanthrene-treated rat liver microsomes. With all substrates the NADPH-dependent reaction rates were higher than the NADH-dependent rates. Higher activities were seen for N-monomethylaniline and p-nitroanisole than for other substrates in NADH-dependent reactions.

**DISCUSSION**

Using rat liver microsomes, we observed that the ratio of NADH-dependent p-nitroanisole O-demethylation activity to NADPH-dependent activity was 0.23 (Table 6). Recent studies in this laboratory have also indicated there is a marked species difference in the ratio of these activities. We observed a ratio of 0.71 ± 0.16 (mean ± SD, n = 6) using rabbit liver microsomes.

It is of interest that the electron transport chain from NADH to cytochrome P-450 via cytochrome b5 plays a role in drug oxidations. Oshino et al. (22) demonstrated that NADPH-cytochrome P-450 reductase transfers electrons to cytochrome b5 as well as to cytochrome P-450. Thus, electrons transferred from NADPH to cytochrome b5 via NADPH-cytochrome P-450 reductase must also be utilized, at least in part, for reducing cytochrome P-450. In fact, our results (Table 2) indicate that exogenously added cytochrome b5 enhances NADPH-
dependent as well as NADH-dependent O-demethylation activity. Shigematsu and co-workers (5, 6) have suggested that an enzyme other than cytochrome P-450 is involved in NADH-dependent O-dealkylation of p-nitroanisole. However, the results of this study indicate the NADH-dependent demethylation of p-nitroanisole is, in fact, catalyzed by cytochrome P-450. Shigematsu et al. proposed the existence of a new enzyme based on evidence that NADH-dependent O-dealkylation of these substrates was insensitive to carbon monoxide. However, the possibility that certain species of cytochrome P-450 are insensitive to carbon monoxide in the presence of adequate oxygen has been suggested. Thus, Thorgeirsson et al. (23) reported that N-hydroxylation activity of 2-acetylaminofluorene was inhibited by carbon monoxide but that it was necessary to use 90% carbon monoxide. Niwaguchi (personal communication) partially purified a cytochrome P-450 species which catalyzed lysergic acid (LSD) indole ring hydroxylation. He noted that one of the cytochrome P-450 species (cytochrome P-448 species) having a high activity for the reaction was insensitive to carbon monoxide in the presence of oxygen. Further, we have reported that not all of the microsomal cytochrome P-450 bound carbon monoxide in the presence of oxygen when NADPH was used as an electron donor (24). From these results it appears likely that one or more species of cytochrome P-450, which is somewhat carbon monoxide-insensitive, is capable of accepting electron more readily from cytochrome b_5 than other species. In support of this hypothesis, the results shown in Tables 3 to 5 indicated that cytochrome P-448 purified as described in MATERIAL AND METHODS was perhaps more active than cytochrome P-450 from phenobarbital-treated rats in accepting electrons from cytochrome b_5. Therefore, it appears possible that cytochrome P-450 is involved in NADH-dependent O-demethylation reaction. In this study we did not determine the amounts of cytochrome P-450 or P-448 incorporated into the microsomal membrane after the preincubation. Therefore, it is possible that the difference in the ability of these cytochrome species to enhance p-nitroanisole O-demethylation is due to the difference in the amount bound to microsomes.

The fortification of microsomes with cytochrome b_5 produced a greater enhancement of the NADH-dependent activity than the addition of either cytochrome P-450 or cytochrome P-448 in the absence of fortified cytochrome b_5. This fact suggests that cytochrome b_5 is a rate limiting factor in the NADH-dependent cytochrome P-450-mediated reaction.

A recent study using a reconstituted system which contained purified NADPH-cytochrome P-450 reductase, dilauroyl-L-3-phosphatidylcholine and cytochrome P-450 from phenobarbital-treated rat liver or cytochrome P-448 from 3-methylcholanthrene-treated rats has indicated that the cytochrome P-448 system has much higher activity than the cytochrome P-450 system for p-nitroanisole O-demethylation (not shown). The results shown in Table 3 revealed that antiserum of cytochrome P-450 preferentially inhibited the NADPH-dependent demethylation. These results suggest that in microsomes, cytochrome P-450 may accept electron more readily than cytochrome P-448 from NADPH-cytochrome P-450 reductase, whereas cytochrome P-448 may accept electron from cytochrome b_5 more readily than cytochrome P-450. In support of this hypothesis it has been reported that the NADPH-
cytochrome P-450 reductase is the rate limiting enzyme in the electron transport system from NADPH to cytochrome P-450 via NADPH-cytochrome P-450 reductase (25). In addition, West and Lu (26) reported that the addition of purified cytochrome P-450 to the incubation mixture containing purified cytochrome P-448 resulted in an inhibition of cytochrome P-448-mediated benzo(a)pyrene hydroxylation activity.

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