Studies on the Rate-limiting Enzyme Component in the Microsomal Monoxygenase System

INCORPORATION OF PURIFIED NADPH-CYTOCHROME c REDUCTASE AND CYTOCHROME P-450 INTO RAT LIVER MICROSOMES*

(Received for publication, September 23, 1977)

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The identity of the rate-limiting enzyme component of the microsomal monoxygenase system has been investigated for six substrates through the incorporation of purified NADPH-cytochrome c reductase into microsomal preparations obtained from untreated, phenobarbital- and 3-methylcholanthrene-treated rats. Incorporation of NADPH-cytochrome c reductase results in rate enhancements which depend on both the microsomal preparation and the substrate examined. These rate enhancements have been interpreted in terms of the variable cytochrome P-450/reductase mole ratios resulting from the multiplicity of cytochrome P-450 species in microsomal preparations.

The rate-limiting enzyme component for benzphetamine metabolism was examined in greater detail with microsomal preparations in which either NADPH-cytochrome c reductase or a cytochrome P-450 species, specific for benzphetamine N-demethylation, was incorporated. A rate enhancement, dependent on both incorporated reductase and cytochrome P-450 components, was observed with microsomes of untreated rats. In contrast, with microsomes derived from phenobarbital-treated rats, an increase in rate was found to depend only on the incorporated reductase component. These data indicate that the rate of benzphetamine N-demethylation is dependent on both enzyme components in microsomes of untreated rats but becomes reductase-limited after phenobarbital induction. The absence of a kinetic isotope effect in studies with a model substrate, N,N-dimethylphtermine, also support this conclusion. In addition, reconstitution studies with the purified enzyme components have been used to substantiate the conclusions drawn from the microsomal system.

Various purified microsomal electron transport proteins have been successfully incorporated into microsomal membranes and upon incorporation function within their respective membrane-bound electron transport systems (1-7). This technique has provided information on the hydrophobic binding nature and the translational properties of these amphipathic proteins as well as their organization within the membrane. In addition to this information, the incorporation of one of the enzyme components of a multicomponent system could also yield information about the rate-limiting enzyme in the membrane-bound system since an increase in concentration of a rate-limiting component would be expected to increase the overall reaction rate observed for the system.

The cytochrome P-450-containing monoxygenase system which is responsible for the oxidation of many lipid-soluble xenobiotics and steroids is one of the multienzyme electron transport systems found in liver microsomes and is composed of two membrane-bound proteins: NADPH-cytochrome c reductase (NADPH-cytochrome P-450 reductase) and cytochrome P-450. The incorporation of either purified cytochrome P-450 or purified NADPH-cytochrome c reductase into microsomal membranes could, therefore, provide information about the rate-limiting enzyme component in monoxygenase reactions catalyzed by this system.

The NADPH-cytochrome c reductase isolated from the liver microsomes of uninduced, phenobarbital- or 3-methylcholanthrene-treated rats cannot be distinguished by either immunologic or catalytic properties (8, 9). Although two electrophoretically distinct forms of the reductase have been recently detected in rats and rabbits, both forms catalyze the reduction of various species of cytochrome P-450 (10). Thus, the influence of incorporated NADPH-cytochrome c reductase on the hydroxylation rate of substrates metabolized by microsomal preparations, obtained from either untreated or treated rats, can be interpreted directly in terms of this electron transport component.

On the other hand, since liver microsomes contain multiple forms of cytochrome P-450 with overlapping but different substrate specificities, the incorporation of either a single species or a mixture of purified cytochrome P-450 species into microsomal membranes would not necessarily represent an increase in an enzyme component common to the microsomal preparation studied. For example, the incorporation of purified cytochrome P-448 from 3-methylcholanthrene-treated rats into microsomes of uninduced rats would not only change the quantity but also the type of major cytochrome P-450 species in the membrane (6). Therefore, the increase in benzo(a)pyrene...
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hydroxylase activity observed following incorporation of cytochrome P-448 can not be interpreted, in this case, to mean that cytochrome P-450 is the rate-limiting component in benzo[a]pyrene metabolism by uninduced microsomal preparations.

We were able, however, to choose one set of conditions in which the effect of the incorporation of a particular species of cytochrome P-450 on the rate of a specific reaction could be interpreted unequivocally. We have recently isolated and purified a species of cytochrome P-450 from the microsomes of rats treated with phenobarbital which has a high turnover number for benzphetamine N-demethylation and appears to be the major species of cytochrome P-450 in microsomes from phenobarbital-treated rats. This highly purified cytochrome P-450 was incorporated into the microsomes from phenobarbital-treated rats and its effect on the rate of benzphetamine N-demethylation determined thus providing a unique opportunity to verify which of the two enzymes, cytochrome P-450 or NADPH-cytochrome P-450 reductase, is rate-limiting during the N-demethylation of benzphetamine by these microsomes.

In view of the large number of substrates metabolized by the liver microsomal enzymes and the numerous forms of cytochrome P-450 present in the microsomes, we have considered that the rate-limiting enzyme component may not be the same for all substrates and for microsomes isolated from animals treated with different inducers. We have therefore examined the reaction rates of six substrates representing eight different reactions following the incorporation of NADPH-cytochrome P-450 reductase into microsomal membranes. Among the six substrates, only the N-demethylation of benzphetamine was examined in detail after the incorporation of either NADPH-cytochrome P-450 reductase or cytochrome P-450 into liver microsomes.

**EXPERIMENTAL PROCEDURES**

Preparation of Enzyme Components—Liver microsomes from uninduced, phenobarbital-treated (75 mg/kg/day, intraperitoneally for 3 days) and 3-methylcholanthrene-treated (25 mg/kg/day, intraperitoneally for 3 days) male, immature, Long-Evans rats were obtained by standard procedures (11). The NADPH-cytochrome c reductase was solubilized from the microsomes of phenobarbital-treated rats with Renex 890 and chromatographed on a DEAE-Sephadex A-25 column as described by Dignam and Strobel (12). The reductase was further purified by affinity chromatography on a 2',5'-ADP-Sepharose 4B column as described by Yaukoshi and Masters (13). Potassium phosphate buffer (0.2 M, pH 7.7) containing 20% glycerol, 0.4 mM EDTA, 0.2 mM dithiothreitol, and 0.05% deoxycholate was used to wash nonspecifically bound proteins and the Renex detergent from the affinity column until the absorbance at 280 nm in the effluent was less than 0.02. The reductase was then eluted with 1 mM 2'-AMP and concentrated over an Amicon XM50 membrane. The reductase was then passed twice through a Sephadex G-50 column previously equilibrated with 50 mM phosphate buffer, pH 7.7, to remove the 2'-AMP and essentially all of the deoxycholate.

The residual deoxycholate was less than 0.05% mg/mg of protein when measured radiometrically with [H14C]deoxycholate. The final preparations had a specific activity greater than 35,000 units/mg of protein. One unit of NADPH-cytochrome reductase activity is defined as the amount of enzyme catalyzing the reduction of cytochrome c at an initial rate of 1 nmol/min at 22° under the assay conditions of Phillipps and Langdon (14).

The cytochrome P-450 species purified from phenobarbital-treated rats had a specific content of 12 to 15 nmol/mg of protein and exhibited a single protein band on SDS-polyacrylamide gel electrophoresis. Details of the purification method will be published elsewhere.

**Incorporation of NADPH-cytochrome P-450 reductase and Cytochrome P-450 into Microsomes—NADPH-cytochrome P-450 reductase activity**

| COMPOUND | R1 | R2 |
|----------|----|----|
| IIa      | CH3 | CH3 |
| IIb      | CH3 | CH3 |
| IIIa     | CD3 | CD3 |
| IIIb     | CH3 | H  |

Stable isotope studies—The rate of N-demethylation of N,N-diethylphentermine (1-phenyl-2-methyl-2-(N,N-diethylamino)-phenylpropylamine, IIa in Table I) was measured by gas chromatography/mass spectrometry analysis of the product, N,N-diethylphenylpiperidine, IIIa. Similarly, the rate of N-demethylation of hexafluorodimethylphenylpiperidine, IIb, was measured by formation of product, IIIb. The rates of formation of Products IIIa and IIIb were determined in separate incubations of IIa and IIb using IIIa and IIIb, respectively, as internal standards. Standard curves for the products were obtained by varying the concentration of each product while maintaining a fixed concentration of the opposite isotope as an internal standard.

A Finnigan quadrupole mass spectrometer (model 3200) coupled to a Finnigan gas chromatograph (model 9500) employing a silanized glass column (5 feet x 2 mm inside diameter) packed with 5% OV-17 on 100 to 120 mesh Gas-Chrom Q was used. The retention times for the trifluoroacetyl derivatives of IIa and IIIb were 80 s with a column temperature of 140°. Selected ion monitoring by means of a Finnigan PROMIMA™ permitted simultaneous measurements of m/z 260 and 263 corresponding to the M+ ions of the trifluoroacetyl derivatives of IIa and IIIb, respectively, when isobutane was used as a reagent gas.

Linear standard curves were obtained by plotting the ratio of m/z 263 to m/z 260.
290 and 263 against the concentration of IIIa and the ratio of m/e 265 and 260 against the concentration of IIIb. To ensure the accuracy and reproducibility of the result, two or three methods were employed to measure the kinetic isotope effect, \( k_{HN}/k_{Ch} \). The isotope effect was computed as the ratio of the rates of formation of IIIa and IIIb. Equal aliquots of the separate incubations of IIa and IIb were pooled, no internal standard was added and the ratio of products IIIa to IIIb was taken as a measure of \( k_{HN}/k_{Ch} \). 3. Equal concentrations of Substrates IIa and IIb were co-incubated and the ratio of products IIIa to IIIb used as a measure of the kinetic isotope effect. All three methods gave essentially the same results.

The reaction mixture (1.0 ml) was composed of the following: microsomes containing 1 nmol of cytochrome P-450 or P-448, 0.01 \( \mu \)mol of EDTA, 0.1 \( \mu \)mol of phosphate buffer, pH 7.4, 5 \( \mu \)mol of glucose-6-phosphate, 1 \( \mu \)mol of NADP\(^+\), and 0.5 \( \mu \)mol of glucose-6-phosphate dehydrogenase. The reactions were initiated by the addition of Substrates IIa and IIb (1 \( \mu \)mol) in either individually or as an equimolar mixture. The mixtures were incubated for 10 min at 37\(^\circ\)C and the reaction terminated by the addition of 0.5 ml of 17% perchloric acid. Internal standards (25 nmol) were appropriately added and the mixtures were made alkaline with 0.5 ml of 5.5 N NaOH. The products and internal standards were extracted into 1.5 ml of hexane and a 1.0 ml aliquot of the hexane layer transferred to a 12-ml conical test tube. Trifluoroacetic anhydride (50 \( \mu \)l) was added to derivatize the secondary amine products. The samples were stored overnight at 4\(^\circ\)C to permit complete derivatization and then concentrated to a volume of 50 to 100 \( \mu \)l by evaporation of most of the solvent under a stream of dry nitrogen. Two to five microliter volumes were injected into the gas chromatography/mass spectrometry system.

Other Assays—The alkali-extractable metabolites of benzo(a)pyrene were determined by the method of Nebert and Gelboin (17). The 7a, 16a-, and 6β-hydroxytestosterone metabolites were determined radiochemically by the method of Lu et al. (18). The O-demethylation of 7α-hydroxycoumarin to 7-hydroxycoumarin was determined spectrophotofluorimetrically by the method of Ullrich et al. (19) as modified by Jacobson et al. (20). Benzphetamine and ethylmorphine \( N \)-demethylation were assayed colorimetrically as the lutidine derivative of the product, formaldehyde, by the procedure of Nash (21). In some experiments, benzphetamine \( N \)-demethylation was assayed radiochemically by the method of Thomas et al. (22). Protein was determined by the method of Lowry et al. (23) using bovine serum albumin as a standard.

Materials—Ethylmorphine hydrochloride was purchased from Mallinkrodt Chemical Works, St. Louis, Mo., and \( (4\,-^1\mathrm{C}) \)testosterone (5.8 mCi/mmol), \( (\mathrm{H})\)deoxycholic acid, and \( (\mathrm{C})\)benzphetamine (2.6 mCi/mmol) were obtained from New England Nuclear Corp., Boston. Benzphetamine hydrochloride was a generous gift of Dr. F. F. Sun of the Upjohn Co., Kalamazoo, Mich. Benzo[a]pyrene and 3-methylcholanthrene were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium phenobarbital was purchased from Merck and Co., Rahway, N. J., and 7-ethoxycoumarin and 7-hydroxycoumarin were obtained from Aldrich Chemical Co., Milwaukee, Wis. The gas chromatography/mass spectrometry packing, 3% OV-17 on Gas-Chrom Q (100 to 120 mesh) was obtained from Applied Science Laboratories, Inc., State College, Pa., and DEA-SE-Sephadex A-25 from Pharmacia Fine Chemicals, Piscataway, N. J. Dilauroylglyceryl-3-phosphorylcholine was purchased from Serdary Research Laboratories, Ontario, Canada. Renex 690 was obtained from ICI America, Inc., Wilmington, Del. Phentermine hydrochloride was a generous gift of Dr. Arthur K. Cho of the University of California, Los Angeles.

RESULTS

Incorporation of NADPH-cytochrome c Reductase and Cytochrome P-450 into Microsomal Membranes

The recovery of cytochrome P-450 or P-448, NADPH-cytochrome c reductase and total microsomal protein during the reductase incorporation process and subsequent washings are summarized in Table II. Recovery of cytochrome P-450 or P-448 was essentially quantitative and unaffected by the incorporated reductase. The recovery of NADPH-cytochrome c reductase and total microsomal protein was about 70 to 80%. The parallel loss in NADPH-cytochrome c reductase activity and total microsomal protein are reflected in the similarity between the reductase specific activity in control samples that were not treated with added reductase and the values obtained before incorporation. The specific content of cytochromes P-450 and P-448 were slightly elevated after incorporation.

### Table II

| Pretreatment | Specific activity of NADPH-cytochrome c reductase | Specific content Cytochrome P-450 | Per cent recovery after reductase incorporation | Cytochrome P-450 | NADPH-cytochrome c reductase | Protein |
|--------------|-----------------------------------------------|---------------------------------|-----------------------------------------------|-----------------|-----------------------------|---------|
|              | units/mg                                      | nmol/mg                         |                                               |                  |                             |         |
| Untreated    |                                               |                                 |                                               |                  |                             |         |
| Before incorporation | 268                      | 0.91                           | 88                                            | 73               | 71                          |         |
| After incorporation |                                      |                                 | 107                                          | 78               |                             |         |
| Control      |                                               |                                 |                                               |                  |                             |         |
| + reductase  | 1427                                          | 1.18                           |                                               |                  |                             |         |
| % change     | 450                                           | 12.0                           |                                               |                  |                             |         |
| Phenobarbital|                                               |                                 |                                               |                  |                             |         |
| Before incorporation | 359                      | 2.05                           | 90                                            | 74               | 71                          |         |
| After incorporation |                                      |                                 | 97                                            | 73               |                             |         |
| Control      |                                               |                                 |                                               |                  |                             |         |
| + reductase  | 1708                                          | 2.50                           |                                               |                  |                             |         |
| % change     | 378                                           | 2.0                            |                                               |                  |                             |         |
| 3-Methylcholanthrene|                                    |                                 |                                               |                  |                             |         |
| Before incorporation | 210                      | 1.90                           | 99                                            | 76               | 70                          |         |
| After incorporation |                                      |                                 | 98                                            | 74               |                             |         |
| Control      |                                               |                                 |                                               |                  |                             |         |
| + reductase  | 1405                                          | 2.21                           |                                               |                  |                             |         |
| % change     | 560                                           | -6.0                           |                                               |                  |                             |         |

Before incorporation samples were not treated with added reductase and the values obtained before incorporation. The specific content of cytochromes P-450 and P-448 were slightly elevated after incorporation.
Since NADPH-cytochrome c reductase represents less than 1% of the total microsomal protein a 5- to 10-fold increase in this component following incorporation would result in an insignificant (<6%) increase in total microsomal protein. Thus, all microsomal rate data obtained in this study are expressed per mg of protein.

The amount of NADPH-cytochrome c reductase incorporated into the microsomal membrane was quite marked (380 to 560% in Table II, 1000% in Table III, Experiment 2) as assessed by the increase in NADPH-cytochrome c reductase activity associated with the microsomes. In addition, the incorporated reductase is tightly associated with the microsomal pellet since repeated washings with buffered, 0.5 M KCl solutions do not reduce the NADPH-cytochrome c reductase activity sedimenting with the microsomes (data not shown). The rate of reduction of cytochrome P-450 increases by about 2.5- to 4-fold after incorporation (Fig. 1) indicating that the incorporated NADPH-cytochrome c reductase couples in the electron transport from NADPH to cytochrome P-450.

The major form of cytochrome P-450 purified from rats treated with phenobarbital can also be incorporated into microsomes and couples with the microsomal NADPH-cytochrome c reductase (Fig. 1) in agreement with the earlier observations of Yang (6). In microsomes isolated from phenobarbital-induced rats, this results in no change in the initial rate of NADPH-dependent cytochrome P-450 reduction but does result in an increase in the total amount of cytochrome P 450 reduced after the initial linear phase. Furthermore, an increase in the total cytochrome P-450, after incorporation, is evident from the increase in the AA450 absorbance following reduction by sodium dithionite.

### TABLE III

**Effect of incorporated NADPH-cytochrome c reductase on catalytic activity**

NADPH-cytochrome c reductase was incorporated into microsome and the catalytic activity measured for various substrates as described under "Experimental Procedures."

| Pretreatment          | Testosterone |          |          |
|-----------------------|--------------|----------|----------|
|                       | 7α-OH        | 16α-OH   | 6β-OH    |
| **Experiment 1**      |              |          |          |
| Untreated             |              |          |          |
| Control               | 0.23         | 0.07     | 1.1      |
| + reductase           | 0.25         | 0.08     | 1.3      |
| % change              | 9            | 15       | 15       |
| Phenobarbital         |              |          |          |
| Control               | 0.54         | 0.39     | 2.39     |
| + reductase           | 0.85         | 0.73     | 4.0      |
| % change              | 57           | 86       | 67       |
| 3-Methylcholanthrene  |              |          |          |
| Control               | 0.56         | 0.08     | 1.58     |
| + reductase           | 1.15         | 0.08     | 2.90     |
| % change              | 105          | 20       | 64       |
| **Experiment 2**      |              |          |          |
| Untreated             |              |          |          |
| Control               | 14.2         | 6.51     | 0.30     |
| + reductase           | 16.2         | 15.9     | 0.72     |
| % change              | 14           | 144      | 140      |

a. 4- to 6-fold increase in reductase specific activity was obtained after incorporation in microsomes used in Experiment 1.
b. 10-fold increase in reductase specific activity was obtained after incorporation in microsomes used in Experiment 2.

dimethylphthlenermine N-demethylase activity (Experiment 1) In contrast, a larger rate increase (60 to 145%) is observed for these substrates when microsomes of phenobarbital-treated rats are employed. Reductase incorporation into microsomes of 3-methylcholanthrene-treated rats results in an increase in the 7α- and 6β-hydroxylation of testosterone but has essentially no effect in 16α-hydroxytestosterone formation. Dimethylphthlenermine N-demethylase activity is moderately (50%) increased.

The metabolism of ethylmorphine, benzphetamine, benzo[a]pyrene, and ethoxycoumarin are all moderately increased (40 to 50%) following reductase incorporation into microsomes of untreated rats. In contrast, the metabolic activities are all markedly increased, except for benzol[a]pyrene, when reductase is incorporated into microsomes of 3-methylcholanthrene-treated rats.

When the reductase activity in microsomes of untreated rats is increased from 4- to 6-fold (Experiment 1) to 10-fold as in Experiment 2, a further stimulation in rate is observed for benzphetamine, benzo[a]pyrene, and ethoxycoumarin metab-
contain multiple forms of cytochrome P-450 with overlapping action on the rate of substrate metabolism. Increasing the concentration of the cytochrome P-450 species responsible for benzphetamine N-demethylation further substantiates the catalytic dependence on the hydroxylation of benzphetamine with a turnover number equal to or greater than that observed for microsomal preparations obtained from phenobarbital-treated rats.

Table IV summarizes three separate experiments in which both NADPH-cytochrome c reductase and the major form of cytochrome P-450 purified from phenobarbital-treated rats were separately incorporated into microsomes from untreated and phenobarbital-treated rats. The NADPH-cytochrome c reductase specific activity is essentially unaltered (<20%) when cytochrome P-450 is incorporated into microsomes from either source although the specific content of cytochrome P-450 is increased by 40 to 100%. In contrast, the reductase activity is markedly increased (250 to 500%) in both microsomal preparations after reductase incorporation while the cytochrome P-450-specific content is essentially unaltered (<12%).

The incorporation of either cytochrome P-450 or NADPH-cytochrome c reductase moderately increases benzphetamine N-demethylation activity in microsomes derived from untreated rats. Yang and Strickhart have previously reported a moderate enhancement of benzphetamine N-demethylation following the incorporation of cytochrome P-450 into the sonicated microsomes of untreated rats. This pattern, however, has not been observed in identical experiments using microsomes obtained from phenobarbital-treated rats. Reduction incorporation markedly increases benzphetamine N-demethylation activity while cytochrome P-450 incorporation has no effect. These data further substantiate the hypothesis that the rate-determining enzyme in benzphetamine N-demethylation is the reductase component in microsomes obtained from phenobarbital-treated rats.

In contrast, microsomes derived from untreated rats demonstrate a moderate rate dependence on both the reductase and cytochrome P-450 components. The rate increase, however, following cytochrome P-450 incorporation into microsomes of untreated rats cannot be interpreted as unequivocally as after reductase incorporation since incorporation of the cytochrome P-450 species induced after phenobarbital treatment may not represent an increase in the native cytochrome P-450 species responsible for benzphetamine N-demethylation.

Studies with the Purified, Reconstituted Monooxygenase System

Three conditions may exist in microsomes containing two enzymes acting in series during the hydroxylation of a substrate.

Condition A - The reductase component is much less saturating with respect to cytochrome P-450. By using saturating cofactor and substrate concentrations, kinetically zero order rate conditions are established for these components. This is essentially the condition for a coupled enzyme assay for the reductase enzyme and will result in the rate of hydroxylation being proportional to the amount of reductase enzyme in the system and independent of an increase in the cytochrome P-450 concentration.

Condition B - Neither component is present in excess with respect to the other. Under this condition, the substrate hydroxylation rate will be influenced by a change in concen-
Effect of incorporated NADPH-cytochrome c reductase and cytochrome P-450, on benzphetamine N-demethylase activity

NADPH-cytochrome c reductase and cytochrome P-450 were incorporated into microsomes and benzphetamine N-demethylase activity assayed radiometrically as described under "Experimental Procedures."

| Pretreatment | NADPH-cytochrome c reductase experiment No. | Cytochrome P-450 experiment No. | Benzphetamine rate experiment No. |
|--------------|------------------------------------------|-------------------------------|-----------------------------------|
|              | 1  | 2  | 3  | 1  | 2  | 3  | 1  | 2  | 3  |
| Untreated    |    |    |    |    |    |    |    |    |    |
| Control      | 408| 326| 283| 1.09| 1.44| 1.49| 5.9| 4.3| 3.3|
| +P450        | 328| 313| 292| 2.18| 2.34| 2.65| 7.2| 6.0| 5.4|
| % change     | -20| -4 | 3  | 100 | 63  | 78  | 22 | 40 | 64 |
| Control      | 422| 340| 247| 1.11| 1.34| 1.15| 5.2| 4.3| 3.1|
| +reductase   | 1550| 1156| 898| 1.01| 1.48| 1.35| 10.3| 6.0| 4.3|
| % change     | 301| 251| 264| -9  | 10  | 9   | 98 | 40 | 39 |
| Phenobarbital| 521| 383| 367| 2.68| 2.61| 3.5 | 26.4| 25.6| 21.5|
| Control      | 412| 305| 377| 3.74| 3.90| 5.63| 21.8| 24.6| 23.1|
| +P450        | -21| -20| 3  | 40  | 49  | 61  | -17 | -4 | 7 |
| Control      | 422| 322| 370| 2.34| 2.59| 2.80| 24.1| 26.6| 24.2|
| +reductase   | 2230| 1405| 2215| 2.06| 2.84| 2.94| 49.8| 55.2| 58.1|
| % change     | 428| 336| 499| -12 | 10  | 5   | 107| 108| 140|

FIG. 2. NADPH-cytochrome c reductase titration of cytochrome P-450 in a reconstituted enzyme system. Cytochrome P-450 (0.1 nmol) isolated from rats treated with phenobarbital and dilauroylglyeryl-3-phosphorylcholine (25 μg) were incubated for 5 min at 37°C with [14C]benzphetamine (1 μmol), an NADPH-generating system and various concentrations of NADPH-cytochrome c reductase in a total volume of 1.0 ml. The [14C]formaldehyde formed was assayed by the method of Thomas et al. (22).

TABLE IV

Rate-limiting Enzyme in Microsomal P-450 Hydroxylase System

Stable Isotope Studies

A primary kinetic isotope effect in chemical reactions is generally taken as evidence for carbon-hydrogen bond cleavage during the rate-limiting step (38). Similarly, isotope effects have been reported for some substrates in microsomal cytochrome P-450-catalyzed reactions (27-32) and have been interpreted to indicate that carbon-hydrogen bond cleavage occurs during the slow step in the overall hydroxylation reaction. A kinetic isotope effect would presumably be noocel...
ated with a cytochrome P 450 dependent reaction step rather than an NADPH-cytochrome c reductase-dependent step since the cytochrome has been shown to be the terminal oxidase in this system (33) and the site of substrate binding (34).

Northrop (35) has suggested that the absence of a kinetic isotope effect in a multistep enzyme-catalyzed reaction may not be sufficient evidence for the absence of a rate-limiting carbon-hydrogen bond cleavage step. The large number of kinetic parameters entering into a rate equation for a multistep reaction may suppress the isotope effect in reactions in which carbon-hydrogen bond cleavage is, nevertheless, the slowest step.

The N-demethylation of the deuterium and protium isotopes of N,N-dimethylphenylpiperidine (Table I, Compounds IIa and IIb) was therefore, first examined in a reconstituted system to determine whether an isotope effect could be demonstrated in either a reductase-limited system (Figs. 2 and 3, Condition A) or a cytochrome-limited system (Condition C). Table V summarizes an experiment in which Substrates IIa and IIb were separately incubated with a reconstituted system composed of the cytochrome P 450 isolated from phenobarbital-treated rats, phospholipid, and a rate-limiting amount of reductase (20 units) or a near-saturating amount of reductase (624 units).

The isotope effect, \( k_{IIa}/k_{IIb} \), was determined by measuring the rates of formation of Products IIIa and IIIb. The ratio of these two rates, \( v(IIa)/v(IIb) \), is a measure of the isotope effect. Alternatively, equal aliquots from incubations of substrates IIa and IIb were pooled together and the ratio of products, IIIa/IIIb, used as a measure of the observed isotope effect.

Under reductase-limiting conditions, the rate of N-demethylation of IIa and IIb were essentially equal, resulting in an insignificant isotope effect when expressed either as a ratio of rates or as a ratio of products. When the reductase was elevated to levels approaching saturation with respect to this component, the rate of N-demethylation increased by approximately 10-fold for both substrates IIa and IIb. The relative increase for IIa, however, was significantly greater than for IIb, resulting in an isotope effect of 1.21 to 1.37. Thus, a kinetic isotope effect can be demonstrated for this system only at or near conditions in which the rate is dependent on the cytochrome P 450 component alone.

Since the demethylation of N,N-dimethylphenylpiperidine catalyzed by liver microsomes of phenobarbital- and 3-methylcholanthrene-treated rats are NADPH-cytochrome c reductase-dependent (Table III), a primary kinetic isotope effect would not be expected for this substrate. The effect of reductase incorporation into microsomes on the isotope effect is summarized in Table V. No significant isotope effect is ob-

**Table V**

Isotope effect studies with N,N-dimethylphenylpiperidine in reconstituted and microsomal enzyme systems

The rate of N-demethylation of the deuterium (IIb in Table I) and protium (IIa in Table I) isotopes of N,N-dimethylphenylpiperidine and the ratio of the two isotopic products were determined in separate and co-incubations of IIa and IIb by chemical ionization-gas chromatography/mass spectrometry as described under "Experimental Procedures."

| Enzyme System       | Rate          | Isotope effect |
|---------------------|---------------|----------------|
|                     | IIa | IIb | \( V(IIa)/V(IIb) \) | Pooled | Co-incubation IIa & IIb |
| Reconstituted system| \( \text{nmol/min/mg} \) |               |               |                   |
| Cytochrome P 450 (0.1 nmol) | 0.285 | 0.279 | 1.02 | 1.05 |
| + Reductase (20 units) | 3.06  | 2.54  | 1.21 | 1.37 |
| + Reductase (624 units) | 980  | 810   |       |       |
| % change |       |       | 980  | 810   |
| Microsomal system   |               |               |                   |
| Phenobarbital       | 2.1 | 2.0 | 1.0 | 1.0 |
| + Reductase | 5.0 | 4.6 | 1.1 | 1.1 |
| % change   | 140 | 130 |       |       |
| 3-Methylcholanthrene | 0.39 | 0.34 | 1.1 | 1.0 |
| + Reductase | 0.63 | 0.62 | 1.0 | 1.1 |
| % change   | 60  | 80   |       |       |
observed for microsomes derived from either phenobarbital- or 3-methylcholanthrene-treated rats. This is consistent with slow turnover of cytochrome P-450. Under the conditions employed, a reductase-limited reaction since an isotope effect can only be demonstrated for a cytochrome P-450-limited condition with the purified enzymes. In addition, there is a marked (60 to 140%) rate increase for this substrate after reductase incorporation. This increase in rate, however, does not result in the genesis of a significant isotope effect as demonstrated in the reconstituted enzyme system. The relative increase in reductase/cytochrome P-450 ratio after reductase incorporation is presumably insufficient to saturate the cytochrome component under the conditions employed.

**Discussion**

The cytochrome P-450-dependent monooxygenase system is of considerable interest to biochemists and pharmacologists because of the diverse nature of the substrates metabolized by this multicomponent enzyme system. Of central importance in describing the biochemical mechanism involved during substrate hydroxylation by a multicomponent system is the site of the rate-determining step. Numerous investigations have attempted to define the rate-limiting step in reactions catalyzed by the microsomal cytochrome P-450 system in terms of experimental parameters such as cytochrome P-450 reduction (36-40), energies of activation of component steps (41, 42) and kinetic isotope effect measurements (27-31).

In few of these studies have factors which are known to perturb the microsomal system, such as age and species variations, type of enzyme-inducing agent and substrate employed, been considered in the interpretation of results. We have, for example, demonstrated that phenobarbital induction alters the rate dependence of benzphetamine N-demethylation from a case in which both cytochrome P-450 and NADPH-cytochrome P-450 reductase concentrations alter the observed rate (untreated microsomes) to a case in which the rate is dependent only on the reductase component (Table IV). In addition, ethylmorphine N-demethylase activity is reductase-dependent in microsomes of phenobarbital-treated rats but relatively independent of reductase incorporation in microsomes of untreated rats (Table III). This suggests that the cytochrome P-450 component may limit ethylmorphine N-demethylation with microsomes of untreated rats. The kinetic isotope effect observed by Thompson and Holtzman (29) for this substrate with microsomes of untreated rats is consistent with this proposal. Nevertheless, considerable controversy and apparent discrepancies exist in the literature concerning the rate-limiting step in microsomal hydroxylation reactions and therefore conclusions about this process must be made with caution.

Three parameters have been measured in this study to determine whether cytochrome P-450 or NADPH-cytochrome c reductase limit substrate hydroxylation reactions with microsomal systems obtained from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats. These parameters are 1) rate effects with increasing NADPH-cytochrome c reductase concentrations, 2) rate effects with increasing cytochrome P-450 concentration, and 3) substrate kinetic isotope effect. We have also defined the relationship between these parameters and changes in the rate-limiting step during substrate hydroxylation in a reconstituted system composed of cytochrome P-450, synthetic phospholipid, and NADPH-cytochrome c reductase. Thus a change in the rate-limiting component can be demonstrated in the reconstituted system during the titration of cytochrome P 450 with NADPH cytochrome c reductase (Figs. 2 and 3, Conditions A and C).

Cytochrome P-450 has been shown to be the terminal oxidase (33) and confers the substrate specificity to this system (18). In the present study, we have extended the properties associated with this protein to include the site of carbon-hydrogen bond cleavage during substrate hydroxylation (Table V). A kinetic isotope effect is only observed under conditions in which the reductase to cytochrome P-450 mole ratio exceeds 2:1. Under this condition, the rate is zero order with respect to reductase concentration and first order with respect to cytochrome P 450 concentration (Figs. 2 and 3, Condition C). With the exception of ethylmorphine, none of the microsomal preparations or substrates examined can be described by this condition since zero order rate dependence on incorporated reductase is not observed, or a kinetic isotope effect is not demonstrated, or both, even after reductase incorporation (Table V).

Microsomes isolated from untreated rats show a rate dependence for both reductase and cytochrome P-450 components when benzphetamine is used as a substrate (Table IV). This relation also occurs in the reconstituted system during the transition from an NADPH-cytochrome c reductase-dependent reaction to a cytochrome P-450-dependent reaction (Figs. 2 and 3, Condition B). The rate dependence on both enzyme components may therefore represent a condition in which the reaction rates catalyzed by each enzyme are nearly equivalent. Alternatively, it may represent an increase in a cytochrome P-450 species which normally is not significantly responsible for the benzphetamine N-demethylation activity in microsomes of untreated rats.

Under conditions in which the reductase/cytochrome ratio is low, the reaction rate is limited by the reductase component and is independent of increases in cytochrome P-450 concentration (Figs. 2 and 3, Condition A). This is clearly the case with microsomes derived from phenobarbital-treated rats (Table III) where the reductase/cytochrome P-450 ratio has been estimated to be about 1:20 (10).

The rate-limiting step during substrate hydroxylation has been most completely studied in the microsomal system derived from phenobarbital-treated rats. Matsubara et al. (40) have shown that the reduction of the cytochrome P-450-substrate complex by the first electron occurs at a much greater rate than substrate metabolism. Thus, the slow step must occur after this NADPH-cytochrome P reductase-catalyzed step. This conclusion is supported by the work of Estabrook et al. (43) and Guengerich et al. (44) who have demonstrated a spectral intermediate thought to be the reduced cytochrome P-450-substrate-oxygen complex in both the microsomal and reconstituted enzyme systems. Since this intermediate could be demonstrated under steady state conditions, a slow step occurring after oxygen complexation is indicated. A rate-limiting transfer of the second electron during the reaction cycle is entirely compatible with these observations. The rate dependence on NADPH-cytochrome c reductase, observed in this study, does not differentiate between these two sites of reductase involvement. Therefore, in view of these observations, the rate-limiting step in benzphetamine N-demethylation may involve the transfer of the second electron.

**Acknowledgments** — We wish to thank Dr. William Garland and Ms. Barbara Hodshon for the gas chromatography/mass
spectrometry analysis used in the stable isotope studies and Dr. Allan Conney for the critical review of this manuscript. We also wish to thank Mrs. Peggy Althoff for the typing of this manuscript.

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1929
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G T Miwa, S B West and A Y Lu

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