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Properties of a Solubilized Form of the Cytochrome P-450-Containing Mixed-Function Oxidase of Liver Microsomes

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SUMMARY

The carbon monoxide-sensitive liver microsomal mixed-function oxidase has recently been solubilized and resolved into three components: cytochrome P-450, TPNH–cytochrome P-450 reductase, and a heat-stable fraction. Of a variety of compounds tested as possible substrates in the reconstituted rat liver enzyme system, *d*-benzphetamine (*N*-benzyl-*N*,α-dimethylphenylethylamine) was the most active. A series of *n*-alkanes (hexane, heptane, octane, nonane, decane, dodecane, tetradecane, and hexadecane) were shown to serve as substrates, as well as compounds such as cyclohexane, ethylmorphine, hexobarbital, aminopyrine, and norcodeine, which are known to undergo hydroxylation in intact microsomal suspensions. Trilaurin, diolein, and triolein appeared to be active, whereas certain other lipids, including a series of fatty acids, and aniline were not.

In the reconstituted rabbit liver enzyme system, hexane was the most active substrate, followed by cyclohexane and benzphetamine. A series of fatty acids from hexanoate to palmitoleate were found to undergo hydroxylation, in contrast to the results obtained in the rat liver enzyme system.

An absolute requirement for the heat-stable lipid fraction was shown for the hydroxylation of the various drugs as well as of several alkanes. A study of the stoichiometry of benzphetamine demethylation (resulting from hydroxylation of the methyl group and liberation of the resulting hydroxymethyl group as formaldehyde) indicated that equimolar amounts of TPNH and molecular oxygen were consumed and of formaldehyde were produced. The results corresponded to the stoichiometry expected of a mixed-function oxidase, provided that catalase was added to the enzyme system. In the absence of catalase, a significantly greater oxygen uptake was observed.

The K_m of benzphetamine was 1.8×10^{-4} M in the reconstituted enzyme system, and the K_s of benzphetamine, which was unaltered in the absence of the heat-stable lipid fraction, was 5.5×10^{-4} M. Aniline and octane were shown to act as competitive inhibitors with respect to benzphetamine.

INTRODUCTION

As described elsewhere (1–3), we have recently obtained liver cytochrome P-450, the carbon monoxide-binding pigment of microsomes (4), in a solubilized form which retains the ability of the microsome-bound form of this pigment to ω-hydroxylate fatty acids and also exhibits characteristic electron paramagnetic resonance and carbon monoxide and substrate difference spectra. Resolution of the system yielded three frac-

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tions, cytochrome P-450, TPNH–cytochrome P-450 reductase, and a heat-stable component, all of which were required for fatty acid hydroxylation to occur at the maximal rate. The reconstitution of a drug-demethylating system from these components has recently been reported in a brief communication (5). The present paper is concerned with the substrate specificity and other properties of the reconstituted enzyme system and with the stoichiometry of the benzphetamine demethylation reaction.

METHODS

The rate of hydroxylation of various substrates was assayed by TPNH utilization, oxygen uptake, or product formation. The formation of formaldehyde from various *N*- and *O*-methyl compounds was measured by the method of Nash (6) as modified by Cochin and Axelrod (7). The disappearance of TPNH was determined at 340 mμ with a spectrophotometer equipped with a Gilford multiple-sample absorbance recorder (5). TPNH oxidation was found to be linear for at least 10 min; all of the rates reported were determined under such conditions of linearity. Oxygen consumption was measured with a Clark type oxygen electrode used in conjunction with a Sargent SRL recorder; after equilibration of the mixture at 30°, TPNH was added through a capillary slit. In all experiments the temperature was 30°, and semicarbazide was included routinely in the reaction mixtures as a formaldehyde-trapping agent. $MgCl_2$ was added because of the report of Peters and Fouts (8) that it enhances the rate of some microsomal hydroxylation reactions, including that of benzphetamine. In our resolved enzyme system the omission of either Mg^{++} or semicarbazide had no effect on the hydroxylation of benzphetamine as estimated by TPNH oxidation, but the omission of both caused a slight diminution of activity. Phosphate buffer, pH 7.5, was used (as the potassium salt) because it provides the optimal pH for fatty acid hydroxylation in the resolved rabbit liver enzyme system (3). In experiments not presented here, benzphetamine demethylation was shown to have the same pH optimum.

Bovine liver catalase, grades A and B, was obtained from Calbiochem; phospholipids and di- and triglycerides were obtained from the Anspec Company, Ann Arbor, Mich.; and alkanes, from Phillips Petroleum, with the exception of dodecane, tetradecane, and hexadecane, which were obtained from Beckman, and cyclohexane, from Matheson, Coleman, and Bell. *d*-Benzphetamine was kindly donated by Dr. J. W. Hinman of the Upjohn Company; norcodeine, by Dr. T. R. Tephly; and ethylmorphine and aminopyrine, by Dr. H. A. Sasame. The various fatty acids were obtained from the Hormel Institute.

Because of the poor solubility of many of the substrates tested, organic solvents were employed. The alkanes and di- and triglycerides were dissolved in acetone and the fatty acids in 5% acetone and added to the reaction mixtures in amounts such that the final acetone concentration was 1% or less. The phospholipids were dissolved in methanol and added in amounts such that the final methanol concentration was 2% or less. The complete reaction mixtures containing substrates added in this manner remained free of turbidity during the course of the experiments. In control experiments it was shown that these concentrations of methanol and acetone had no effect on the rate of TPNH oxidation in either the presence or absence of benzphetamine. Since all of the drugs were routinely added in aqueous solution, the possible conversion of methanol to formaldehyde was not a problem.

Liver microsomal suspensions were prepared from rabbits induced with phenobarbital or rats induced with phenobarbital and hydrocortisone in the manner described previously (5). The method of solubilization of the enzyme system and resolution by column chromatography into fractions containing the heat-stable lipid, cytochrome P-450, and TPNH–cytochrome P-450 reductase has been described (3). The latter two components were concentrated by the use of alumina Cγ gel.

RESULTS

Substrate specificity. A series of possible substrates was tested with the reconstituted

TABLE 1

Substrate specificity of reconstituted rat liver microsomal enzyme system

TPNH disappearance was determined in reaction mixtures containing 100 μmoles of phosphate buffer (pH 7.5), 5 μmoles of $MgCl_2$, 3 μmoles of semicarbazide, and the following components from rat liver microsomes: reductase fraction (0.09 mg of protein), cytochrome P-450 fraction (0.32 nmole; 0.44 mg of protein), and lipid fraction (0.1 mg), in a final volume of 1.0 ml. The reaction was initiated by the addition of 0.15 μmole of TPNH. The rate with benzphetamine ($\Delta A_{340} = -0.036$/min), corrected for the rate of TPNH oxidation in the absence of the substrate ($\Delta A_{340} = -0.015$/min), gave a calculated rate of 3.4 nmoles/min. The data given represent averages of two or more determinations.

Compound tested	Final concentration	Relative activity
	M	%
Benzphetamine	1.0×10^{-3}	100
Ethylmorphine	1.0×10^{-3}	72
Hexobarbital	1.0×10^{-3}	42
Aminopyrine	1.0×10^{-3}	32
Norcodeine	6.0×10^{-3}	30[a]
Aniline	$0.2\text{–}1.0 \times 10^{-2}$	0[b]
Hexane	5.0×10^{-4}	65
Cyclohexane	1.0×10^{-3}	30
Heptane	5.0×10^{-4}	55
Octane	5.0×10^{-4}	45
Nonane	5.0×10^{-4}	43
Decane	5.0×10^{-4}	55
Dodecane	5.0×10^{-4}	36
Tetradecane	5.0×10^{-4}	14
Hexadecane	5.0×10^{-4}	19
Fatty acids[c]		0
Lecithin	$0.2\text{–}3.4 \times 10^{-4}$	0
Lysolecithin	$0.2\text{–}3.4 \times 10^{-4}$	0
Phosphatidyl-ethanolamine	$0.2\text{–}3.2 \times 10^{-4}$	0[d]
Tricaproin	$2.0\text{–}5.0 \times 10^{-4}$	0
Trilaurin	4.0×10^{-5}	10
Diolein	4.0×10^{-4}	25
Triolein	2.5×10^{-4}	15

[a] Determined by formaldehyde formation, since turbidity prevented using the spectral assay for TPNH disappearance.

[b] Aniline hydroxylation was determined as described by Schenkman *et al.* (9); similar results were obtained when the lipid fraction was omitted.

[c] The fatty acids and their concentrations were the same as in Table 2.

[d] Similar results were obtained when either this compound or lauric acid was solubilized by sonication in the absence of an organic solvent.

rat liver system, using the spectral assay described previously (5). The initial rate of TPNH oxidation was determined at a series of substrate concentrations; the values shown in Table 1 are those which were obtained at the concentrations found to be optimal, and are therefore maximal velocities. Benzphetamine proved to be hydroxylated the most rapidly of any of the compounds tested, and significant activity was also obtained with other drugs known to undergo demethylation in intact microsomes, as well as with cyclohexane, a series of *n*-alkanes from hexane to hexadecane, and certain glycerides (trilaurin, diolein, and triolein). On the other hand, aniline, phospholipids, and a series of fatty acids appeared to be inactive. Laurate was not hydroxylated when tested in the absence of acetone or when, under the same conditions, the lipid fraction was omitted. When the more sensi-

TABLE 2

Substrate specificity of reconstituted rabbit liver microsomal enzyme system

The conditions were similar to those already described, except that the following components were obtained from rabbit liver microsomes: reductase fraction (0.21 mg of protein), cytochrome P-450 fraction (0.26 mg of protein; 0.5 nmole), and lipid fraction (0.08 mg). The net rate with benzphetamine, ($\Delta A_{340} = -0.043$/min), corrected for the rate in the absence of substrate ($\Delta A_{340} = -0.016$/min), gave a calculated net rate of 4.0 nmoles/min.

Compound tested	Final concentration	Relative activity[a]
	M	%
Benzphetamine	1.0×10^{-3}	100
Aniline	$1.0\text{–}2.0 \times 10^{-3}$	0
Cyclohexane	1.0×10^{-3}	151
Hexane	5.0×10^{-4}	200
Hexanoate	1.0×10^{-3}	12
Heptanoate	5.0×10^{-3}	8
Octanoate	2.5×10^{-4}	28
Laurate	2.0×10^{-4}	12
Tridecanoate	1.0×10^{-4}	48
Palmitoleate	1.0×10^{-4}	32
Diolein	4.0×10^{-4}	35
Lecithin	$0.8\text{–}1.7 \times 10^{-4}$	0
Lysolecithin	$1.1\text{–}2.3 \times 10^{-4}$	0

[a] Estimated by TPNH disappearance at 340 mμ.

TABLE 3

Lipid requirement for substrate hydroxylation

Rat liver microsomal fractions and various substrates were used as in Table 1. The spectral assay for TPNH disappearance was used, except with norcodeine, for which formaldehyde production was determined.

Substrate	Rate of reaction	
	Lipid present	Lipid absent
	nmoles/min	
Benzphetamine	3.8	0
Hexobarbital	1.7	0
Ethylmorphine	2.7	0.3
Aminopyrine	1.2	0
Norcodeine	1.2	0.2
Hexane	2.0	0
Cyclohexane	0.9	0
Octane	1.8	0

tive radioactive assay (10) was employed, laurate was shown to exhibit low but significant activity in the rat liver system.

A less extensive series of compounds was tested with the reconstituted rabbit liver system (Table 2). *n*-Hexane was the most active substrate, followed by cyclohexane and benzphetamine, and fatty acids from hexanoate to palmitoleate were also attacked at sufficiently rapid rates to be measured spectrophotometrically. Aminopyrine, ethylmorphine, hexobarbital, norcodeine, and *p*-nitroanisole were previously shown to undergo demethylation in the presence of a rabbit liver cytochrome P-450 fraction and a rat liver reductase fraction (5); presumably the substrate specificity was imparted by the cytochrome P-450 fraction. It may be noted that the optimal concentration of cyclohexane was the highest of any of the compounds studied.

It should be emphasized that the substrate-dependent rate of TPNH oxidation appears to be a reliable measure of the rate of hydroxylation in the reconstituted enzyme system. Formaldehyde formation from benzphetamine (see below), ethylmorphine, hexobarbital, and aminopyrine and product formation from typical radioactive alkane and fatty acid substrates were all shown to correspond satisfactorily to TPNH oxidation.

Lipid requirement. The rate of ω-hydroxylation of laurate in the resolved rabbit liver microsomal system was previously shown to be reduced to about 30% in the absence of the lipid fraction (3). More recently, better resolution of the lipid from the enzyme components has been achieved with the rat liver enzyme system by washing the DEAE-cellulose column with buffer before eluting with KCl. For reasons that are not yet clear, however, this modified procedure does not consistently improve the resolution. As shown in Table 3, in the absence of the lipid fraction the demethylation of benzphetamine and other drugs and the hydroxylation of hydrocarbons is reduced to an insignificant level. The rate of benzphetamine hydroxylation was shown to be proportional to the lipid concentration over a limited range (Fig. 1).

Stoichiometry of benzphetamine demethylation. The data in Table 4 show the stoichiometry of the hydroxylation reaction with benzphetamine as the substrate. Essentially equimolar amounts of TPNH and O_2 were consumed and of formaldehyde were produced, provided that catalase was added to the reaction mixture. In the absence of catalase similar results were obtained for TPNH disappearance and formaldehyde formation, but the oxygen consumption was high. The reason for this effect of catalase is not yet known, but it is evident from the data obtained that catalase had no significant effect on the rates determined in the

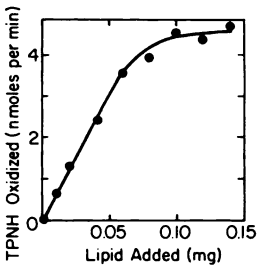

FIG. 1. *Benzphetamine hydroxylation as a function of concentration of lipid fraction added*

The reaction mixtures contained, in addition to the usual components, 1 μmole of benzphetamine and the following rat liver microsomal fractions: reductase (0.15 mg of protein), cytochrome P-450 (0.26 nmole), and the indicated amounts of lipid.

TABLE 4

Stoichiometry of benzphetamine hydroxylation

Reaction mixtures containing 300 μmoles of phosphate buffer (pH 7.5), 10 μmoles of semicarbazide, 20 μmoles of $MgCl_2$, 3 μmoles of benzphetamine, rabbit liver cytochrome P-450 fraction (1.6 nmoles; 0.88 mg of protein), rabbit liver lipid fraction (0.18 mg), and rat liver reductase fraction (0.3 mg of protein) were incubated at 30° for at least 2 min before the addition of TPNH (1 μmole) to initiate the reaction; the final volume was 3.0 ml. The formation of formaldehyde and the disappearance of TPNH and O_2 were shown to be linear with time for the entire incubation. Where indicated, catalase (Calbiochem, grade B; 40 μg of protein) was added prior to TPNH; similar results were obtained with crystalline catalase (4 μg). The data are averages of at least two determinations.

Reaction component determined	Catalase present	Increase or decrease in components			Ratio relative to TPNH utilization
		Benzphetamine present	Benzphetamine absent	Net change	
		nmoles/min			
Experiment 1					
Formaldehyde	−	+21.3	+3.6	+17.7	0.9
TPNH	−	−26.3	−6.0	−20.3	1.0
O_2	−	−47.5	−18.8	−28.7	1.4
Experiment 2					
Formaldehyde	+	+21.6	+4.1	+17.5	0.9
TPNH	+	−26.8	−6.8	−20.0	1.0
O_2	+	−35.0	−16.1	−18.9	0.9

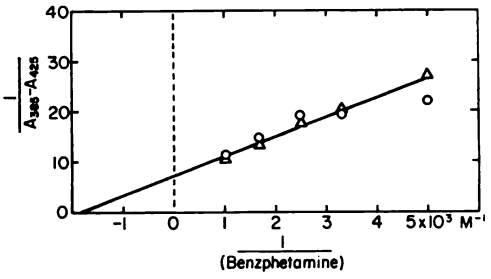

FIG. 2. *Inverse plot for determination of K_s of benzphetamine*

The magnitude of the spectral change induced by varying concentrations of benzphetamine was determined at room temperature as A_{385} minus A_{425} in the presence of the rat liver cytochrome P-450 fraction (2.1 nmoles; 6.1 mg of protein) and 100 μmoles of phosphate buffer, pH 7.5, in either the presence (○) or absence (△) of the rat liver lipid fraction (0.4 mg). The final volume was 1.0 ml. The K_s of benzphetamine is 5.5×10^{-4} M.

absence of benzphetamine. Rat and rabbit liver enzyme components were found to be interchangeable in the experiments establishing the stoichiometry of the reaction.

Kinetic constants and effects of inhibitors. As reported previously (5), benzphetamine gives a type I difference spectrum with the resolved cytochrome P-450 fraction. The effect of the benzphetamine concentration on the magnitude of the difference spectrum, determined as $A_{385} - A_{425}$, is shown in the inverse plot in Fig. 2. From these data the K_s of benzphetamine was found to be 5.5×10^{-4} M, and it should be noted that the presence of the lipid fraction had no effect on the magnitude of this dissociation constant. The K_m of benzphetamine was found to be 1.8×10^{-4} M in the complete hydroxylation assay system (Figs. 3 and 4). These constants obtained in the resolved enzyme system are similar in magnitude, although not identical, to those recently obtained by Peters and Fouts[1] for benzphetamine in rat liver microsomal suspensions; these investigators found that magnesium ions brought about a slight decrease in the apparent K_s and a slight increase in the apparent K_m of this substrate.

The effect of octane on the velocity of benzphetamine hydroxylation is shown in the inverse plot in Fig. 3. Formaldehyde formation was determined in such experiments, rather than TPNH disappearance, since the latter method would measure the hydroxylation of the added octane as well as that of benzphetamine. The results indicate

[1] J. R. Fouts, personal communication.

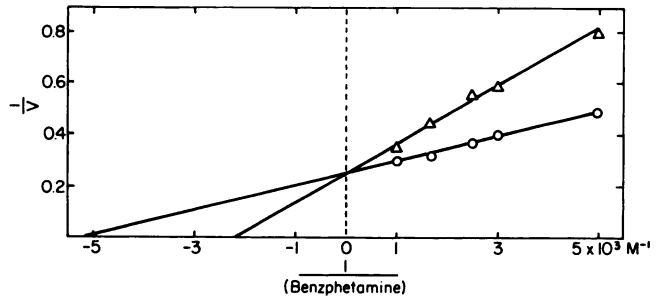

FIG. 3. *Inverse plot showing effect of octane on velocity of benzphetamine hydroxylation*
Formaldehyde formation was determined in the presence (△) or absence (○) of 6×10^{-4} M octane in reaction mixtures containing, in addition to the usual components, the following rat liver microsomal fractions: reductase (0.2 mg of protein), cytochrome P-450 (0.21 nmole; C.51 mg of protein), and lipid (0.12 mg). The velocity is expressed as nanomoles of formaldehyde formed per minute. The K_m of benzphetamine and the K_i of octane were found to be 1.9×10^{-4} M and 4.4×10^{-4} M, respectively.

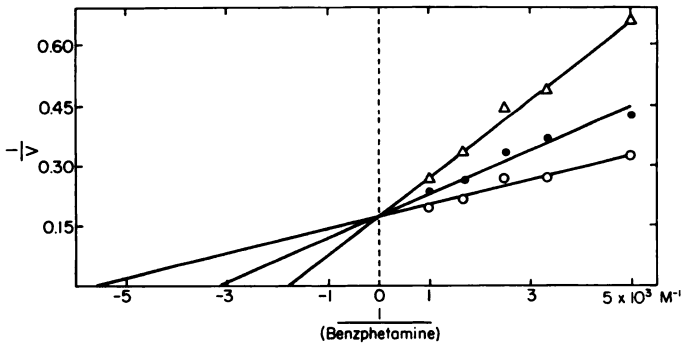

FIG. 4. *Inverse plot indicating effect of aniline on velocity of benzphetamine hydroxylation*
The benzphetamine-dependent disappearance of TPNH was determined in the absence (○) or presence of 5.0×10^{-4} M (●) or 2.0×10^{-3} M (△) aniline in reaction mixtures containing, in addition to the other usual components, the following rat liver microsomal fractions: reductase (0.16 mg of protein), lipid (0.1 mg), and cytochrome P-450 (0.13 nmole; 0.2 mg of protein). The velocity is expressed as nanomoles of TPNH oxidized per minute. The K_m of benzphetamine and the K_i of aniline were found to be 1.8×10^{-4} M and 8.0×10^{-4} M, respectively.

that octane is a competitive inhibitor with respect to benzphetamine. Since aniline is not hydroxylated in the reconstituted enzyme system, the rate of TPNH oxidation could be used to determine the effect of this compound on the rate of benzphetamine hydroxylation. The inverse plot of the data obtained (Fig. 4) indicates that aniline, like octane, behaves as a competitive inhibitor with respect to benzphetamine.

DISCUSSION

The results presented show that the solubilized and reconstituted enzyme system exhibits activity toward a variety of drugs, hydrocarbons, and fatty acids. With the exception of a loss of activity toward aniline (tested in the rat liver system only) and a much decreased activity toward fatty acids in the rat liver system (but not in that from rabbit liver), the solubilized form of cytochrome P-450 evidently retains the catalytic activity of the particulate, microsomal form of this pigment. Liver microsomal suspensions have been shown to hydroxylate fatty acids (1–3, 11–13), various steroids (14–17), carcinogenic polycyclic hydrocarbons (18), and a variety of drugs and related substances (18, 19). We have previously reported that octane hydroxylation occurs in the reconstituted microsomal enzyme system (3, 20). Spectral data showing the interaction of

hexane with rat liver microsomal cytochrome P-450 (21) and evidence for the occurrence of heptane oxidation in the microsomal fraction of rat liver (11) and of hexadecane oxidation in the microsomal fraction of guinea pig intestinal mucosa (22) have been reported by others. During the course of the present investigation, papers appeared by Ichihara *et al.* (23, 24) describing the oxidation of long-chain alkanes in mouse liver microsomal suspensions. Ullrich (25) has recently reported that cyclohexane is converted to cyclohexanol by rat liver microsomes, and has clearly shown the involvement of cytochrome P-450 in the reaction.

The requirement for a heat-stable lipid fraction for reconstitution of an active enzyme system, previously demonstrated for fatty acid ω-hydroxylation (3) and benzphetamine demethylation (5), has now been shown for the hydroxylation of hydrocarbons as well as several other drugs. The function of the lipid fraction remains to be established; no lipid-dependent change in the rate or extent of cytochrome P-450 reduction or in the K_s of laurate was observed previously (3), or in the K_s of benzphetamine in the present investigation. Although deoxycholate does not replace the lipid fraction, the possibility remains that an impurity in the deoxycholate used to aid in the solubilization of the enzyme system is the active component, or one of the active components, of the heat-stable, chloroform-soluble fraction obtained by column chromatography of the system on DEAE-cellulose.

The competitive inhibition observed when octane or aniline is added to the reconstituted enzyme system in the presence of benzphetamine as the substrate suggests a common binding site for these compounds. Kinetic data obtained with microsomal suspensions have been reported previously by Rubin *et al.* (26), showing that certain drugs are mutually competitive; by Tephly and Mannering (27), showing that steroids competitively inhibit the oxidation of drugs; and by Wada *et al.* (28), indicating that prednisolone and hydrocortisone competitively inhibit aniline hydroxylation and aminopyrine demethylation. Although the existence of multiple forms of cytochrome P-450 would account for the broad substrate specificity, no more than one form has been identified with certainty by spectral methods (29, 30). Recently, Leibman *et al.* (31) have studied the effects of certain substrates upon the difference spectra produced by the binding of other substrates to liver microsomes and have concluded that a single cytochrome P-450 may be capable of interacting in different ways with type I and type II substrates. It seems unlikely, from the evidence now available, that the remarkably broad specificity of cytochrome P-450 is due to the occurrence of many distinct forms of this hemoprotein. Evidence is now available, however, that the administration of 3-methylcholanthrene to animals induces the synthesis of a spectrally distinct form of P-450 involved in the hydroxylation of aromatic carcinogens (32–36). An alternative explanation to the problem of specificity is that cytochrome P-450 possesses multiple sites. The mutually competitive inhibition which has been observed between substrates is in accord with the hypothesis that this hemoprotein possesses multiple catalytic sites, provided that these also function as effector sites, thereby giving allosteric control. For example, substrate A, in binding to catalytic-effector site A', may, by promoting a conformational change, inhibit the binding of substrate B at catalytic-effector site B'. Conversely, substrate B, upon binding at catalytic-effector site B', may prevent the binding of A at site A'. The individual sites might be group-specific—one binding the methyl groups of fatty acids and alkanes, another binding those drugs containing *N*- and *O*-methyl groups, another binding aniline, and so forth. Attempts are now in progress to purify the resolved form of cytochrome P-450 in order to determine how many distinct forms exist and whether allosteric control is a feature of this hemoprotein.

The difficulties involved in determining the stoichiometry of drug hydroxylation in microsomal suspensions have been summarized in a review by Gillette (19). To our knowledge the only previous evidence for the equivalence of formaldehyde formation and O_2 and TPNH utilization is that of Orrenius (37), who studied aminopyrine oxidation in rat liver microsomal suspen-

sions. More recently, however, Estabrook and Cohen (38) have reported the utilization of 2 moles of TPNH per mole of formaldehyde formed from aminopyrine in microsomes. The stoichiometry of benzphetamine demethylation, which we have determined in the reconstituted enzyme system, corresponds to that of a hydroxylation or monooxygenation reaction, as follows.

$$R_2NCH_3 + TPNH + H^+ + O_2 \rightarrow R_2NH + CH_2O + TPN^+ + H_2O$$

The effect of catalase in lowering the apparent oxygen uptake indicates an additional variable which may complicate similar studies with intact microsomes.

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