HYDROXYLATION OF BENZPHETAMINE AND OTHER DRUGS BY A SOLUBILIZED FORM OF CYTOCHROME P-450 FROM LIVER MICROSOMES: LIPID REQUIREMENT FOR DRUG DEMETHYLATION*

Anthony Y. H. Lu,** Henry W. Strobel, and Minor J. Coon

Department of Biological Chemistry
The University of Michigan
Ann Arbor, Michigan

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Summary: A solubilized hepatic microsomal enzyme system previously shown to catalyze the ω-hydroxylation of fatty acids also catalyzes the hydroxylation of drugs. Benzphetamine, aminopyrine, ethylmorphine, hexobarbital, norcodeine, and p-nitroanisole undergo aerobic demethylation in the presence of NADPH and the resolved enzyme system. The required submicrosomal components for benzphetamine demethylation, as determined either by formaldehyde liberation or by NADPH oxidation, are cytochrome P-450, NADPH-cytochrome P-450 reductase, and a heat-stable lipid fraction. Similar requirements were shown for the oxidation of aminopyrine, ethylmorphine, and hexobarbital. Laurate and benzphetamine were found to be mutually inhibitory, as would be expected if a common "methyl hydroxylase" were involved. The solubilized cytochrome P-450 preparation exhibits a difference spectrum in the presence of benzphetamine with a peak at 392 mμ and a trough at 427 mμ and difference spectra with aniline and hexobarbital typical of those obtained with the microsomal bound form of this hemoprotein.

Cytochrome P-450, the carbon monoxide-binding pigment of microsomes (1), serves as a mixed function oxidase effecting the hydroxylation of steroids and the oxidative demethylation and hydroxylation of drugs in animal tissues (2-4). Attempts to solubilize this pigment by a variety of techniques resulted in the extensive formation of P-420 (1), an altered form possessing no catalytic activity. Recent studies in our laboratory on the ω-hydroxylation of fatty acids in liver microsomes led to "solubilization" of the enzyme system by the criterion that enzymatic activity remained in

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** Postdoctoral Fellow, Institute of Science and Technology, The University of Michigan.

the supernatant fraction upon prolonged centrifugation at 105,000 x g (5,6). Resolution of the soluble system yielded the following fractions, all necessary for fatty acid hydroxylation to occur at a maximal rate in the presence of NADPH and molecular oxygen: cytochrome P-450, NADPH-cytochrome P-450 reductase, and a heat-stable component. Such preparations of P-450 exhibit a CO difference spectrum and an electron paramagnetic resonance spectrum (7) similar to those previously attributed to the microsome-bound form. Whereas a soluble form of cytochrome P-450 is functional in camphor hydroxylation in enzyme preparations from Pseudomonas putida (8), the ω-hydroxylation system of Pseudomonas oleovorans does not require this pigment (9).

In the present paper evidence is presented that our "soluble" P-450 preparation combines with drugs, as judged by the resulting difference spectra, and catalyzes their demethy-

Table I

Drug oxidation in resolved enzyme system from liver microsomes

Expt. No.	Substrate	Additions	Formaldehyde formed (nmoles)	% Maximal activity
1	Benzphetamine		234	100
	Aminopyrine		95	41
	Ethylmorphine		85	36
	Hexobarbital		62	26
2	Benzphetamine		152	100
	"	Laurate (10^{-4}M)	135	89
	"	" (4×10^{-4}M)	79	52
	"	" (10^{-3}M)	45	30

The complete reaction mixture contained 150 μmoles of phosphate buffer, pH 7.5, 10 μmoles of $MgCl_2$, 5 μmoles of semicarbazide, 0.5 μmole of NADPH, the drug indicated (1.5 μmoles of benzphetamine or ethylmorphine, 6.0 of aminopyrine, or 4.0 of hexobarbital), cytochrome P-450 (0.56 nmoles), and, in the two experiments, reductase fraction (0.37 and 0.34 mg of protein, respectively), and lipid (0.10 and 0.15 mg, respectively), in a final volume of 1.5 ml. After incubation for 20 min at 30° the reaction mixtures were deproteinized and analyzed for formaldehyde by the method of Nash (10) as modified by Cochin and Axelrod (11).

lation in the presence of other microsomal components. Under optimal conditions benzphetamine is a better substrate than laurate. Of particular interest, a microsomal lipid component is necessary for drug demethylation as well as for fatty acid ω-hydroxylation.

Evidence for the demethylation of various drugs, resulting in the production of formaldehyde, is presented in Table I. The microsomal fractions were prepared as described earlier (7), optimal resolution being obtained by preparing the P-450 and lipid fractions from liver microsomes of rabbits induced with phenobarbital and the reductase from liver microsomes of rats which had been administered 7.5 mg of phenobarbital and 5 mg of hydrocortisone per 100 g of body weight at three 8-hour intervals, resulting in a 4-to 5-fold increase in this enzyme. Of the four N-methyl compounds tested, benzphetamine was hydroxylated most rapidly; the oxidation of this drug in microsomal suspensions has recently been reported by others (12,13). In other experiments not shown here, two O-methyl compounds, norcodeine and p-nitroanisole, were demethylated about 28% as rapidly as benzphetamine. As shown in Expt. 2, laurate is an effective inhibitor of benzphetamine oxidation, and in other experiments laurate hydroxylation was found to be inhibited by the N-methyl compounds (0.01 M) as follows: aminopyrine (13%), ethylmorphine (43%), aniline (54%), benzphetamine (58%), and hexobarbital (63%). Although these results suggest that a single form of P-450 may catalyze both drug and fatty acid hydroxylation, a final conclusion must await purification of this solubilized pigment.

Similar conclusions were reached when the hydroxylation reaction was determined by following the disappearance of NADPH at 340 mμ (Fig. 1). Benzphetamine was clearly the best substrate, with the other drugs being hydroxylated at lower, but significant rates. When determined under similar conditions, with benzphetamine as the substrate, NADPH disappearance and formaldehyde liberation occurred in the molar ratio of 0.9, conforming to the stoichiometry expected of a mixed function oxidase.

The demethylation of benzphetamine was found to require the same three microsomal components as does laurate ω-hydroxyla-

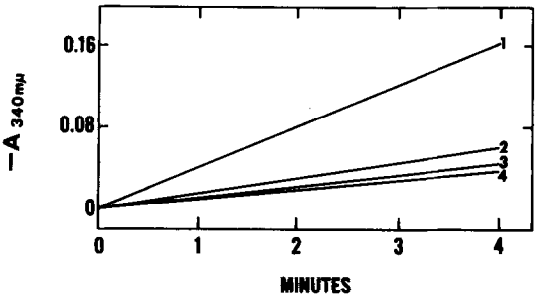

Fig. 1. NADPH disappearance as a function of time in reaction mixtures similar to those described in the legend to Table I, but in a final volume of 1.0 ml. The reaction mixtures contained P-450 (0.18 nmole), reductase (0.074 mg of protein), and lipid (0.1 mg). After the final addition of NADPH, the reaction mixtures were allowed to equilibrate for 2 min at 30°, and the absorbance at 340 mμ was then recorded at the same temperature with a spectrophotometer equipped with a Gilford multiple sample absorbance recorder. Curves 1 to 4 were obtained with 1 mM benzphetamine, 5 mM aminopyrine (or 2 mM hexobarbital), 1 mM ethylmorphine, and no substrate, respectively.

tion, as shown in Table II. Similar results were obtained whether NADPH oxidation or formaldehyde formation was determined. Because of the presence of small amounts of P-450 in the reduc-

Table II

Requirements for benzphetamine hydroxylation

System	Formaldehyde formed (% maximal activity)	NADPH oxidized (% maximal activity)
Complete	100	100
No P-450 fraction	31	14
No reductase fraction	0	0
No lipid fraction	12	3

NADPH disappearance was estimated spectrophotometrically at 340 mμ and formaldehyde formation was measured by the Nash method in reaction mixtures containing 1 mM benzphetamine but otherwise similar to those described in the legend to Table I.

tase fraction, a complete dependence on P-450 was not observed. In similar experiments not shown here, the hydroxylation of aminopyrine, hexobarbital, and ethylmorphine was also found to require the presence of the lipid component.

As reported by others, hepatic microsomal bound P-450 exhibits spectral shifts upon the addition of certain hydroxylatable substrates (14-16). We have recently described a Type I difference spectrum resulting from the addition of laurate to the soluble P-450 preparation (7). Upon the addition of benzphetamine, as shown in Fig. 2, the soluble preparation gave a difference spectrum with a peak at about 392 mμ and a trough at 427 mμ which may also be classified as Type I. The difference spectra recorded with hexobarbital and with aniline are typical of those obtained with microsomal suspensions. These results indicate that our P-450 preparation retains the binding properties of the microsomal form.

The cytochrome P-450-containing hydroxylation system of liver microsomes has unusually broad specificity, acting on fatty acids (5,7,17,18), alkanes (7,17), various steroids (19-21), carcinogenic polycyclic hydrocarbons (22), and a host of drugs and related substances (22,23). Although the existence of multiple distinct forms would account for the broad

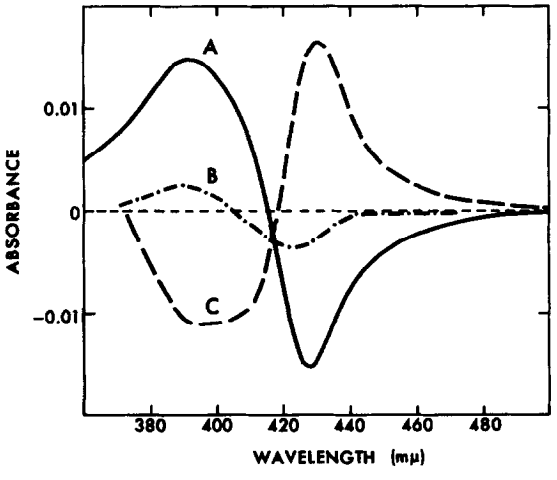

Fig. 2. Difference spectra due to the presence of 5×10^{-4} M benzphetamine (Curve A), 5×10^{-3} M hexobarbital (Curve B), or 5×10^{-3} M aniline (Curve C) in a soluble preparation of cytochrome P-450 (1.5 nmoles; 0.91 mg of protein per ml) in phosphate buffer, pH 7.7.

specificity, no more than one form of P-450 has been identified with certainty by spectral methods (24,25). Evidence that such factors as sex, age, species, and drug induction have similar effects on the steroid- and drug-metabolizing activities of liver microsomes has been presented by Kuntzman et al. (26). Moreover, kinetic data have been reported by Rubin et al. (27) showing that drugs are mutually competititive and by Tephly and Mannering (28) indicating that steroids competitively inhibit the oxidation of drugs. More recently, George and Tephly (29) have reported that different rate-controlling events occur in the N- and O-dealkylation of morphine analogs, but that both require cytochrome P-450. The evidence in the present paper that drug demethylation and fatty acid ω-hydroxylation require the same submicrosomal components also provides no indication for more than one form of P-450 or "methyl hydroxylase". The possibility may also be considered that lipids affect the specificity of cytochrome P-450. The identity and role of the microsomal lipid factor (or factors) required for the hydroxylation of fatty acids and drugs in the resolved microsomal system are presently under investigation.

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