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N-Oxidation of Phentermine to *N*-Hydroxyphentermine by a Reconstituted Cytochrome P-450 Oxidase System from Rabbit Liver

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SUMMARY

Previous studies in our laboratory have indicated that the cytochrome P-450 system is involved in the oxidation of phentermine (2-methyl-1-phenyl-2-aminopropane) to *N*-hydroxyphentermine by liver microsomal preparations. In the present study, a reconstituted system which consisted of cytochrome P-450 and NADPH cytochrome P-450 reductase purified from liver microsomes of phenobarbital-induced rabbits was found to oxidize phentermine to *N*-hydroxyphentermine. The reaction was NADPH-dependent and required the presence of both the cytochrome P-450 and reductase preparations. *N*-Hydroxyphentermine was formed 3 times more rapidly in incubation mixtures which contained dilauroyl phosphatidycholine than in those without added phospholipid. The reaction was inhibited several-fold by octylamine. It is concluded that the cytochrome P-450 system is able to catalyze the oxidation of phentermine to *N*-hydroxyphentermine.

Previous studies have shown that microsomal preparations from rabbit liver catalyze the *N*-hydroxylation of phentermine (2-methyl-1-phenyl-2-aminopropane) (1, 2) and amphetamine (2). Catalysis of the *N*-hydroxylation of phentermine by rat liver microsomal preparations has also been shown to occur (1–3). There are two distinct enzyme systems which appear to be active for *N*-oxidation in liver microsomes: the cytochrome P-450 system and a flavin-dependent amine oxidase. Inhibitor and phenobarbital induction studies have indicated that the *N*-hydroxylation of phentermine (2, 3), amphetamine (2, 4), some primary aromatic amines (5), and 2-acetylaminofluorene (6) is catalyzed by the cytochrome P-450 system. Inhibitor studies by others (7–9) have suggested that the cytochrome P-450 system is not involved in *N*-oxidation of tertiary amines.

The purified amine oxidase has been shown to be active for the oxidation of secondary amines to hydroxylamines and of tertiary amines to *N*-oxides (10, 11). The activity of this enzyme is enhanced by primary amines, although they probably are not substrates for *N*-oxidation (10, 12). Reconstituted systems consisting of purified cytochrome P-450 and reductase have been shown to catalyze the *N*-oxidation of 2-acetylaminofluorene (13, 14). However, there apparently have been no reports of *N*-oxidation of aliphatic amines by reconstituted cytochrome P-450 systems. This investigation has been carried out to determine whether the *N*-oxidation of an aliphatic amine which has appeared to be cytochrome P-450-dependent in microsomes could be demonstrated in a reconstituted system.

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Phentermine hydrochloride was a gift of Pennwalt Corporation (Rochester, N. Y.). NOHP[1] was synthesized from phentermine (15). DEAE-cellulose, NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, superoxide dismutase, dilauroyl phosphatidylcholine, sodium cholate, Triton N-101, protamine sulfate, and polyethylene glycol 6000 were purchased from Sigma Chemical Company (St. Louis, Mo.). DEAE-Sephadex and quaternary aminoethyl-Sephadex were purchased from Pharmacia Fine Chemicals (Piscataway, N. J.), 2′,5′-ADP-agarose from P-L Biochemicals (Milwaukee, Wisc.), and Bio-Beads SM-2 and calcium phosphate gel from Bio-Rad Laboratories (Richmond, Calif.).

Washed microsomes were prepared, as previously described (1), from the livers of rabbits which had received injections of phenobarbital (60 mg/kg) on each of the 3 days prior to sacrifice on the 4th day. The primary goals for the cytochrome P-450 purification were to obtain a preparation which contained a majority of the initial cytochrome P-450 isoenzymes in an active state and as low a residual level of nonionic detergent as possible. Others (16–18) have purified cytochrome P-450 to apparent homogeneity by procedures which involved steps performed at room temperature. Since it was desirable to obtain a preparation which contained most of the cytochrome P-450 isoenzymes and because the stability of isoenzymes which might catalyze *N*-oxidation was not known, it was necessary to modify the established procedures for this current study. The procedures of Saito and Strobel (16) were followed for the microsomal solubilization and DEAE-Sephadex A-25 chromatography, except that Triton N-101 was used as the detergent

[1] The abbreviation used is: NOHP, *N*-hydroxyphentermine.

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instead of Renex 690 and the concentration of Tris-HCl in the solubilization step was 100 mM. The cytochrome P-450 fraction which eluted in the wash buffer from the column was precipitated with polyethylene glycol 6000 as described by Saito and Strobel (16) and applied to a DEAE-cellulose column equilibrated with 10 mM potassium phosphate buffer (pH 7.4) which contained 1 mM EDTA, 0.1 mM dithiothreitol, 20% (v/v) glycerol, 0.5% sodium cholate, and 0.15% (w/v) Triton N-101. This step (and those which follow) was performed at 4°. Cytochrome P-450 eluted in the wash buffer while cytochrome P-420 remained bound on the column. The cytochrome P-450 column fractions were pooled, and Triton N-101 was removed by treatment with Bio-Beads SM-2 and chromatography on quaternary aminoethyl Sephadex A-25 by a slight modification of the procedure described by West *et al.* (17). Thus, chromatography was performed at 4° immediately after treatment with Bio-Beads, the wash step with nonionic detergent was omitted, and 0.3 M KCl was used to elute cytochrome P-450 instead of potassium phosphate. The cytochrome P-450 fraction was dialyzed to reduce the concentration of sodium cholate. The specific content of the final preparation was 5.5 nmoles/mg of protein and the level of Triton N-101 was less than 5.1 μg/nmole of cytochrome P-450 when estimated by the method of Garewal (19).

NADPH-cytochrome P-450 reductase was eluted from the DEAE-Sephadex A-25 column with a 0.08–0.3 M KCl gradient and further purified by 2′,5′-ADP-agarose affinity chromatography by procedures similar to those of Yasukochi and Masters (20). The final reductase preparation contained 11.8 nmoles of reductase per milligram of protein and gave only one major band upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Triton N-101 could not be detected in the preparation.

A typical incubation mixture contained 1 nmole of cytochrome P-450, 1 nmole of cytochrome P-450 reductase, 500 μmoles of KPO_4 (pH 7.4), 6 μmoles of $MgCl_2$, 60 μg of dilauroyl phosphatidylcholine, 60 μg of superoxide dismutase, 1.3 μmoles of NADP, 15 μmoles of glucose-6-phosphate, 6 units of glucose-6-phosphate dehydrogenase, and 15 μmoles of phentermine in a final volume of 3 ml. Superoxide dismutase was added to inhibit the superoxide-dependent oxidation of NOHP (21). All components except phentermine were added and the mixture was incubated at 37° for 5 min. Reactions were then started by the addition of phentermine. Production of NOHP was shown to be linear for at least 45 min, and this was chosen as the typical incubation time. The reaction was terminated by the addition of cold CH_2Cl_2. Isolation and quantitation of NOHP were identical with procedures described previously (3) except that the column temperature for gas chromatography was 125° and the internal standard was 1-phenyl-2-butanone.

Mass spectrometry was performed on a Hewlett-Packard 5981A gas chromatography-mass spectrometry system which contained a selected ion-monitoring and microprocessor system. Protein was determined by the method of Bradford (22).

That the reconstituted cytochrome P-450 system from rabbit liver microsomes was active for the catalysis of *N*-hydroxylation of phentermine was indicated by the presence of a compound with a gas chromatography retention time identical with that of *N*-hydroxyphentermine trifluoroacetamide in the processed extract of the incubation mixture. The identity of this compound was further investigated by mass spectrometry. The mass spectra of this compound and of authentic *N*-hydroxyphentermine trifluoroacetamide are shown in Fig. 1. On the basis of work by Lindeke and Cho (23) and by Cho *et al.* (24), the tentative fragment assignments shown in Fig. 2 were made. The presence of a fragment of m/e 266 is indicative that diderivatization by trifluoroacetic anhydride had occurred. According to the tentative identification of the fragments and the similarity of the two spectra, it was concluded that the compound was indeed NOHP.

In Table 1 it is shown that there was no detectable formation of NOHP when cytochrome P-450, cytochrome P-450 reductase, or NADPH was omitted from the system. The omission of dilauroyl phosphatidylcholine resulted in a 3-fold decrease in *N*-hydroxylase activity. When benzphetamine *N*-demethylation was studied in a reconstituted system consisting of these same enzymatic preparations, an 8-fold decrease in activity resulted when dilauroyl phosphatidylcholine was omitted from an otherwise complete system. This result provided further evidence that the enzymatic preparations were free of nonionic detergent. As shown in Table 1, the inclusion of Triton N-101 in the incubation mixture at a final concentration of 0.02% resulted in a 3.6-fold decrease in activity. Inclusion of 2 mM octylamine, an inhibitor of the cytochrome P-450 system and an activator of the flavin amine

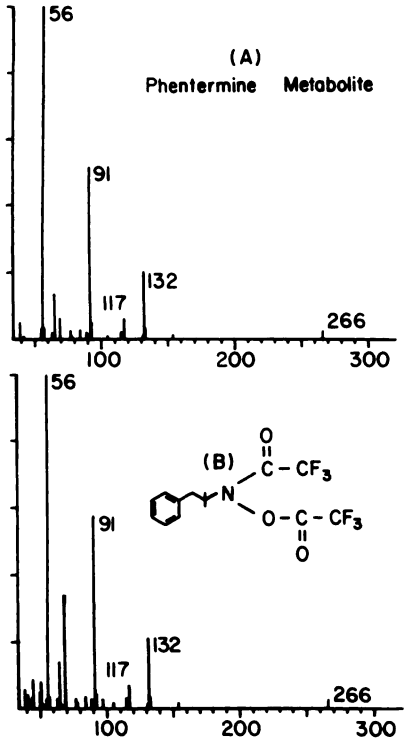

FIG. 1. *Mass spectra of trifluoroacetic anhydride derivatives*
A, 70-Ev mass spectrum of trifluoroacetic anhydride derivative of a metabolite tentatively identified as NOHP; B, 70-eV mass spectrum of authentic trifluoroacetic anhydride derivative of NOHP [$C_6H_5CH_2C(CH_3)_2NHOTFA$].

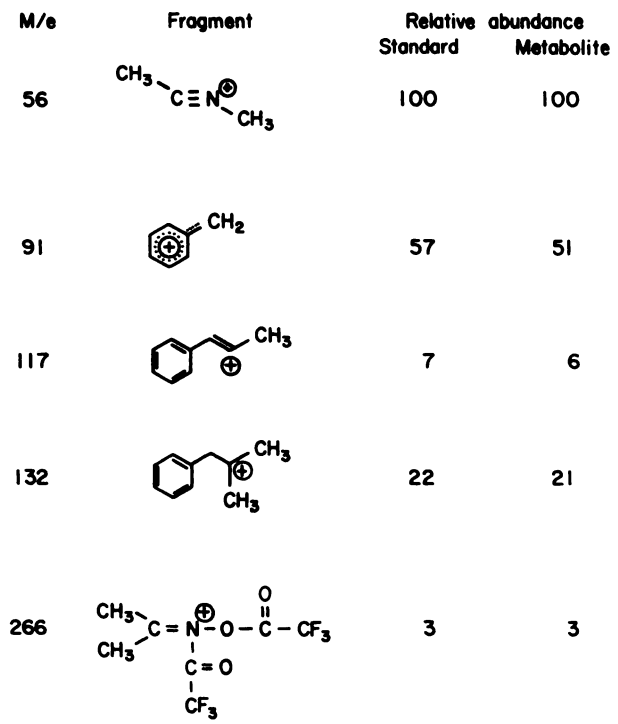

FIG. 2. *Tentative structural assignments for major mass spectral fragments*

oxidase (10, 11), resulted in an 81% inhibition of NOHP synthesis.

The dependence of this reaction on the cytochrome P-450 system was established, since the inclusion of both the cytochrome P-450 and reductase preparations was required for activity. If contaminating flavin amine oxidase or another oxidative enzyme were actually responsible for catalysis of the reaction, a similar level of activity with one of the two preparations alone, as found in the complete reconstituted system, would have been expected. When similar incubations were performed with cytochrome P-450 and reductase preparations obtained from liver microsomes of phenobarbital-induced rats, activity was observed also only when both components for the oxidase system were present (data not shown). The results obtained when either dilauroyl phosphatidylcholine was omitted or octylamine was added to the incubation mixture provide further evidence that the cytochrome P-450 system, and not a trace contaminant of the flavin amine oxidase, catalyzed the reaction. The large inhibition which resulted in the presence of only 0.02% Triton N-101 suggests that *N*-hydroxylation of primary amines in reconstituted cytochrome P-450 systems may be very sensitive to inhibition by nonionic detergents.

The results presented in this communication confirm indications from previous studies (1–3, 21) that cytochrome P-450 is involved in the catalysis of *N*-hydroxylation of phentermine. Further studies are necessary to determine whether the N-oxidation of other primary amines which has been demonstrated in microsomes can also be catalyzed by reconstituted cytochrome P-450 oxidase systems.

TABLE 1

Reconstitution of rabbit cytochrome P-450 oxidase system

Incubations were performed for 45 min as described in the text. Abbreviations: reductase, NADPH-cytochrome P-450 reductase; P-450, cytochrome P-450; NADPH, an NADPH-generating system consisting of 0.43 mM NADP, 5 mM glucose-6-phosphate, 2 mM $MgCl_2$, and glucose-6-phosphate dehydrogenase (2 units/ml); PC: L-α-dilauroyl phosphatidycholine; ND, not detectable; *n*, number of separate determinations.

Components	Additions	*n*	nmoles of NOHP/min/nmole of cytochrome P-450 ± SD
Reductase, PC, NADPH	None	4	ND
P-450, PC, NADPH	None	3	ND
P-450, reductase, PC	None	3	ND
P-450, reductase, NADPH	None	3	0.23 ± 0.07
P-450, reductase, NADPH, PC	None	4	0.73 ± 0.02
P-450, reductase, NADPH, PC	0.02% Triton N-101	2	0.2 ± 0.02
P-450, reductase, NADPH, PC	2 mM Octylamine	2	0.14 ± 0.01

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