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The Opposing Effects of *N*-Hydroxyamphetamine and *N*-Hydroxyphentermine on the H_2O_2 Generated by Hepatic Cytochrome P-450

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SUMMARY

The effects of *N*-hydroxyphentermine (NOHP) and *N*-hydroxyamphetamine (NOHA) on hydrogen peroxide generated by rat liver microsomes and reconstituted preparations in the presence of NADPH were compared. In microsome incubations, NOHP caused an increase in H_2O_2 levels and NOHA caused a substantial decrease. When the substances were compared for cytochrome P-450-dependent H_2O_2 generation in reconstituted preparations, NOHA at 1 mM blocked generation and NOHP had no effect. NOHP appears to be an uncoupler of the cytochrome P-450 system in microsomes whereas NOHA is a potent inhibitor, presumably because of its ability to form a metabolic intermediate complex. During the course of their effects on O_2 reduction, NOHP and NOHA are themselves undergoing oxidation, NOHP to 2-methyl-2-nitro-1-phenylpropane and NOHA to phenylacetone oxime. The enzymatic natures of these oxidations differ. Thus, two closely related arylalkylhydroxylamines differ substantially in their interaction with cytochrome P-450 systems.

INTRODUCTION

Phentermine (2-methyl-1-phenyl-2-propylamine) and amphetamine (1-phenyl-2-propylamine) are very similar chemically and pharmacologically (1) but are metabolized differently by liver microsomes from different animal species. For example, although both compounds are ring-hydroxylated at micromolar concentrations in rat liver microsomal preparations, very little ring hydroxylation occurs in rabbit preparations (2). At millimolar concentrations both compounds are extensively *N*-oxidized by rabbit liver preparations, but in rat liver only phentermine is *N*-oxidized (3) at an appreciable rate.

Experiments *in vitro* with rat and rabbit liver preparations have shown that the initial *N*-oxidation products of these amines, NOHP[3] and NOHA, are metabolized to different products. NOHP undergoes a four-electron oxidation to the corresponding nitro compound, MNPP (4), and NOHA is converted to PAOx (5), a two-electron oxidation. These hydroxylamines also differ in their interactions with cytochrome P-450. NOHA generates a so-called MI complex with reduced cytochrome P-450 which inhibits the oxidations carried out by this system (6). This MI complex is generated by a number of aliphatic hydroxylamines and is thought to involve the nitrogen atom in the nitroso state (7). NOHP does not form the MI complex (7) and in this respect resembles other hydroxylamines with a tertiary carbon atom adjacent to the nitrogen (8).

This paper describes the results of a study investigating differences in the effects of NOHA and NOHP on the cytochrome P-450-dependent reduction of O_2 to hydrogen peroxide (9). This reduction is the result of electron flow through the cytochrome P-450 catalytic cycle and can involve the participation of substrates (10). The data indicate that NOHA and NOHP have quantitatively different interactions with this cytochrome P-450 function.

MATERIALS AND METHODS

NOHP, NOHA, PAOx, and MNPP (11) were synthesized in our laboratories. NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased

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[3] The abbreviations used are: NOHP, *N*-hydroxyphentermine; NOHA, *N*-hydroxyamphetamine; MNPP, 2-methyl-2-nitro-1-phenylpropane; PAOx, phenylacetone oxime; MI complex, metabolic intermediate complex.

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from Sigma Chemical Company (St. Louis, Mo.). Other chemicals were obtained from common commercial sources.

Preparation of liver microsomes. Livers from 200- to 250-g male Sprague-Dawley rats, which had been pretreated with phenobarbital (80 mg/kg/day i.p. for 3 days and 40 mg/kg on the 4th day), were homogenized in 1.15% KCl (1:4, w/v). The homogenate was centrifuged at 9,000 × *g* for 20 min and the supernatant was processed either by gel filtration (12) or by centrifugation at 88,000 × *g* for 60 min to isolate the microsomal fraction. The microsomal fraction from the gel filtration column was diluted with 50 mM potassium phosphate (pH 7.4) in 1.15% KCl, and the microsomal pellet from the centrifugation was resuspended in 1.15% KCl. The protein concentration, measured by the method of Lowry *et al.* (13), was about 2 mg/ml; crystalline bovine serum albumin was used as the standard.

MI complex formation. Metabolite-induced difference spectra were recorded at 25° on an Aminco DW-2 spectrophotometer. A microsomal suspension (5.8 ml, 1 mg of protein per milliliter) in 0.05 M potassium phosphate (pH 7.4) containing NOHA (0.25 mM) and $MgCl_2$ (10 mM) was divided between the cuvettes, and the optical density in the range 390–500 nm was recorded. Water (0.1 ml) was added to the reference cuvette, and the enzyme reaction was started by the addition of 0.1 ml of NADPH solution (12 mM) to the sample cuvette. The change in absorbance, ΔA (455–490 nm), as a function of time was determined by repetitive scanning. The amount of enzyme complexed during the metabolism of NOHA was calculated from the *A* values, and the absorption coefficient for the NOHA complex (ϵ = 65 $mM^{-1} \cdot cm^{-1}$) was determined by the method of Franklin (14).

H_2O_2 and NADPH production. Aliquots (3 ml) of the microsomal suspension (2 mg of protein per milliliter) indicated above, containing the substrate (as indicated in the table and figure legends), 10 mM $MgCl_2$, and 0.2 mM NaN_3, were incubated for various periods of time. The incubations were conducted at 37° and the reaction was started by the addition of 0.1 ml of an NADPH solution (12 mM), giving a final NADPH concentration of 0.383 mM. The reaction was quenched by immersion in an ice bath, and a 0.3-ml aliquot was added to 1.7 ml of 0.6 M trichloroacetic acid; the remainder of the microsomal incubation medium (2.7 ml) was added to 1.8 ml of $HClO_4$ (5.1%). The trichloroacetic acid-precipitated samples were centrifuged (3000 rpm for 10 min), and the clear supernatants were immediately assayed for H_2O_2 by the $Fe(SCN)_3$ assay of Hildebrandt and Roots (15). The H_2O_2 concentrations used for the standard curves were spectrophotometrically standardized at 240 nm (ϵ = 43.6 $M^{-1} \cdot cm^{-1}$). The $HClO_4$-precipitated samples were stored in the refrigerator overnight, and NADP was determined on the following morning according to the method of Sasame and Boyd (16). The ϵ value used to standardize the NADPH solution was 6.22×10^3 $M^{-1} \cdot cm^{-1}$. In those experiments in which metabolism of the hydroxylamines was measured, the reaction was stopped by pouring the contents of the incubation flask into cold (0°) dichloromethane. The products were extracted into the organic solvent, and PAOx (5) and MNPP (4) were analyzed by gas chromatography.

Hydroxylamine concentration and H_2O_2. One milliliter of microsomal suspension (5 mg of protein per milliliter) was added to 4 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 2.0 μmoles of NADP, 25 μmoles of glucose-6-phosphate, 75 μmoles of $MgCl_2$, 2 units of glucose-6-phosphate dehydrogenase, and NOHA or NOHP at the indicated concentrations or in the absence of either. After a 2-min incubation at 37°, the reaction mixtures were assayed for H_2O_2 as above.

Oxygen consumption. Oxygen consumption was measured with a Clark-type oxygen probe (Yellow Springs Instrument Company, Yellow Springs, Ohio) sealed in a temperature-controlled reaction vessel at 37°. The vessel volume of 5 ml contained microsomal protein (1 mg/ml) in 0.1 M potassium phosphate buffer with a substrate concentration of 0.25 mM. The reaction was started after a 2-min preincubation period by the addition of 1 μmole of NADPH. The rate of O_2 uptake was measured by a 2-min interval, and total O_2 was measured from the plateau value.

Purification of enzymes. The major hepatic cytochrome P-450 isozyme inducible in male Long-Evans rats by phenobarbital administration was purified to a specific content of greater than 15 nmoles/mg of protein as described by West *et al.* (17). Cytochrome P-450 was assayed by the method of Omura and Sato (18). NADPH-cytochrome *c* reductase was purified from the same source to a specific activity greater than 35,000 units/mg of protein by DEAE-Sephadex A25 chromatography as described by Dignam and Strobel (19), and affinity chromatography was performed as described by Yasukochi and Masters (20). One unit of NADPH-cytochrome *c* reductase activity is defined as the amount of enzyme catalyzing the reduction of cytochrome *c* at an initial rate of 1 nmole/min at 22° under the conditions of Phillips and Langdon (21).

Incubations with the purified enzymes. Incubations with purified NADPH-cytochrome *c* reductase or with the reductase and cytochrome P-450 were conducted with NOHA or NOHP as indicated in Table 2. Samples containing the enzymes; 1 μg of phosphatidylcholine; the appropriate hydroxylamine; 0.1 M potassium phosphate buffer (pH 7.4); and an NADPH-generating system consisting of 5 mM glucose-6-phosphate, 10 mM NADP, and glucose-6-phosphate dehydrogenase (0.7 unit/ml) in a total volume of 1.0 ml were incubated for 10 min at 37° before the reaction was stopped by the addition of 0.25 ml of 70% trichloroacetic acid (w/v). The samples were centrifuged for 5 min at 3000 rpm and the clear supernatant was assayed for H_2O_2 (15). NOHA causes a small reduction in the apparent recovery of H_2O_2, and the levels in Table 2 have been corrected for this reduction.

RESULTS

Figure 1 shows the effects of the hydroxylamines on H_2O_2 generation and NADPH consumption at early time points. NOHP causes an over-all increase in H_2O_2 levels and NADPH consumption over substrate-free controls, whereas NOHA causes a decrease over substrate-free

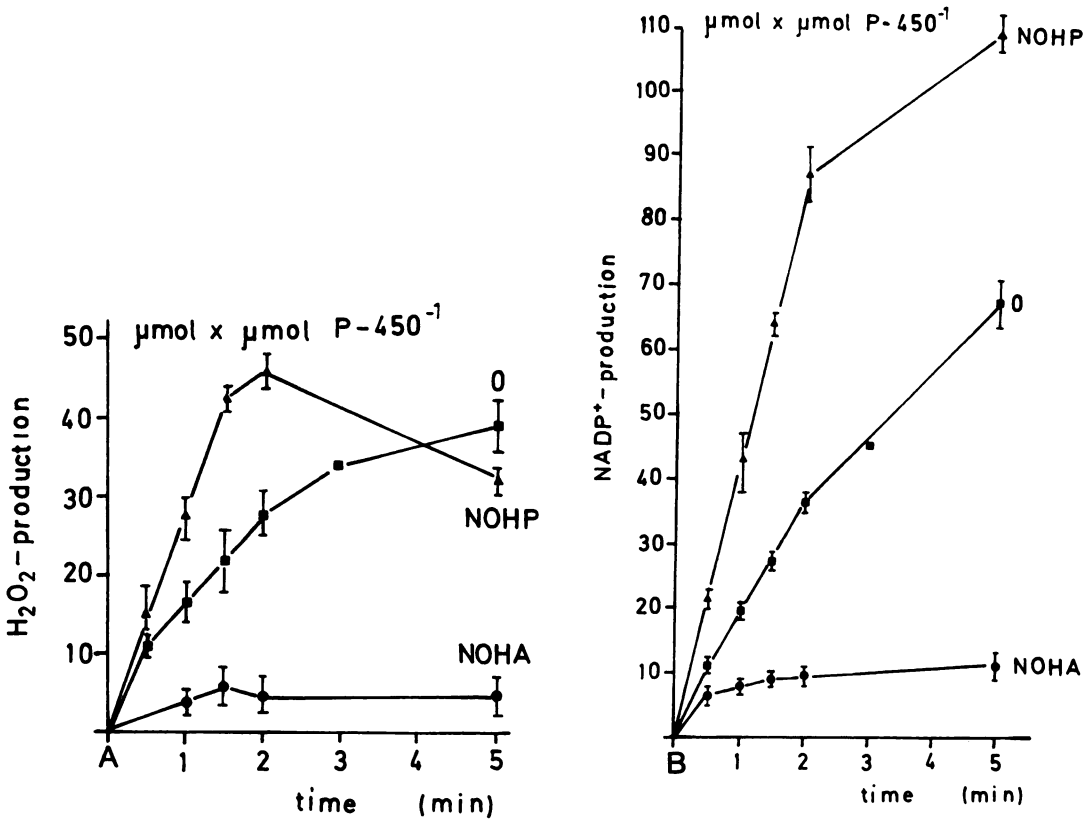

FIG. 1. *H_2O_2 (A) and NADP (B) production during microsomal metabolism*

Microsomes were incubated with NADPH (0.383 µM) for the indicated times in the absence of substrate (■), or in the presence of NOHA (0.25 mM) (●) or NOHP (0.25 mM) (▲). The values for H_2O_2 and for NADP are the mean values ± standard error of the mean of three to five experiments.

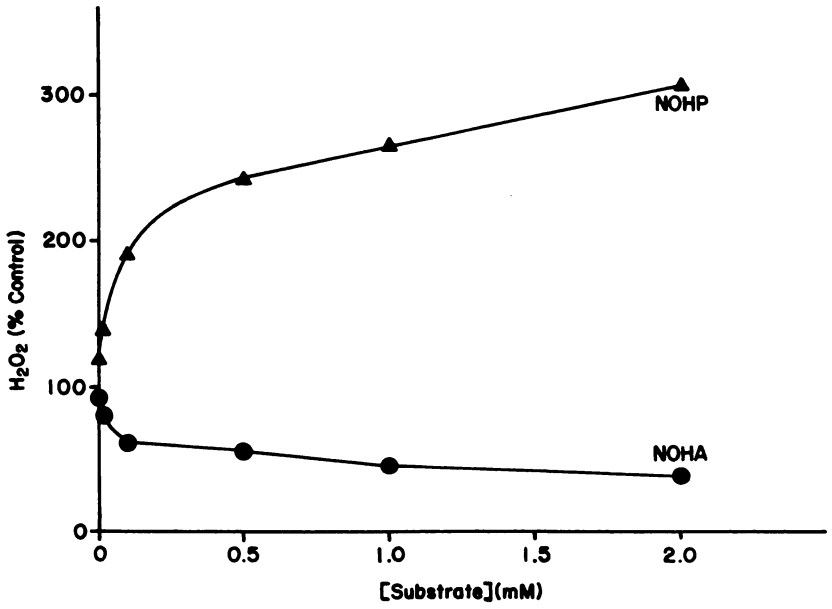

FIG. 2. *Effects of the hydroxylamines on H_2O_2 production*

NOHP (▲) and NOHA (●) at the indicated concentrations were incubated with liver microsomes for 2 min at 37° in the presence of an NADPH-generating system (glucose-6-phosphate and glucose-6-phosphate dehydrogenase). The results are expressed as the percentage of the H_2O_2 formed in the absence of either hydroxylamine (20.8 ± 0.6 nmoles/nmole of cytochrome P-450). The percentage means are contained within the symbols which reflect concentrations of substrate other than zero. The first two symbols indicate substrate concentrations of 5×10^{-3} and 10^{-2} mM.

controls during the initial 2–3min. The changes in levels of NADP and H_2O_2 with time begin to decrease after 2 min for all three conditions, reflecting the consumption of the limiting amount of NADPH. In the presence of NOHP, almost all of the NADPH is consumed within 2 min. In the presence of a saturating amount of NADPH, the stimulating effects of NOHP on H_2O_2 was more dramatic and concentration-dependent. NOHA inhibited H_2O_2 formation in a dose-dependent manner (Fig. 2). Similar effects of the hydroxylamines are seen in microsomal O_2 consumption, where NOHP increases the rate while NOHA decreases the initial rates (Table 1) and reduced O_2 throughout the entire incubation period.

The divergent actions of NOHP and NOHA on H_2O_2 generation were examined in more detail with purified enzyme components of the cytochrome P-450 system. As shown in Table 2, H_2O_2 is generated by both NADPH-cytochrome P-450 reductase and cytochrome P-450. In these reconstitution experiments, the ratio of reductase to cytochrome P-450 is much higher than that in microsomes; thus the contribution of reductase-dependent H_2O_2 formation is higher than that in microsomes. The molar ratio of reductase to cytochrome P-450 in microsomes is 1:20 (22) and in the reconstituted system used here the ratio was varied from about 6:1 to 0.8:1. NOHA, at 1mM, blocked cytochrome P-450-dependent H_2O_2 generation, but NOHP either had no effect or slightly increased H_2O_2 levels. NOHA also slightly decreased reductase-dependent H_2O_2 formation, but its effects were mainly on cytochrome P-450.

During these changes in NADPH and H_2O_2 levels, both NOHP and NOHA are metabolized, NOHP to MNPP and NOHA to PAOx (Fig. 3). The time course of MNPP formation from NOHP follows the generation of H_2O_2 by NOHP in that the rate of formation of both products begins to decline at about 3 min when NADPH levels are falling. The oxidation of NOHP to MNPP is indirectly dependent on P-450, since it is dependent on O_2^- that is generated by P-450 (4). The oxidation of NOHA to PAOx is O_2- and NADPH-dependent but does not appear to involve H_2O_2 or O_2^- and is not inhibited by 2-diethylaminoethyl-2,2-diphenylvalerate (SKF 525-A, 0.1–5 mM), 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine (0.05–2 mM), or CN^- (1 mM) (5). Furthermore, phenobarbital pretreatment of the rats had no effect on oxime formation, so the microsomal NOHA to oxime conversion is probably cytochrome P-450-independent (5). Therefore, the metabolic transformations of the two hydroxylamines are enzymatically different.

TABLE 1

Effect of NOHA and NOHP on microsomal oxygen consumption

Oxygen consumption was measured at 37° after addition of NADPH, and the initial rate was measured from the linear portion of time versus uptake tracing. The total O_2 consumption was determined from the plateau value reached in about 4 min. Values represent means ± standard error of the mean of number of sample replicates in parentheses.

	Total O_2	Initial rate of O_2
	μmoles	*nmoles/min/nmoles P-450*
NADPH (1 μmole)	0.460 ± 0.000 (3)	24.9 ± 0.1 (3)
NADPH + NOHP (0.25 mM	0.430 ± 0.012 (3)	57.1 ± 1.8 (3)
NADPH + NOHA (0.25 mM)	0.390 ± 0.010 (3)	16.9 ± 1.5 (3)

TABLE 2

H_2O_2 generation by reconstituted systems in the presence of hydroxylamines

H_2O_2 levels were measured by the $Fe(SCN)_3$ procedure (15) with cytochrome P-450 reductase, cytochrome P-450, and phosphatidylcholine added as indicated under Materials and Methods.

Reductase	Cytochrome P-450	Rate of H_2O_2 formation		
		Control	NOHA (1 mM)	NOHP (1 mM)
units/ml	*nmoles/ml*	*nmoles/min*		
0	0	0.63	0.13	0.76
200	0	2.55	2.93	4.00
500	0	5.38	4.35	5.06
700	0	8.06	6.36	6.85
1000	0	10.04	8.91	10.80
1000	0.05	13.90	6.30	10.40
1000	0.10	15.50	6.36	13.10
1000	0.20	17.90	6.17	18.30
1000	0.30	18.50	6.77	21.50
1000	0.40	17.30	6.64	19.70

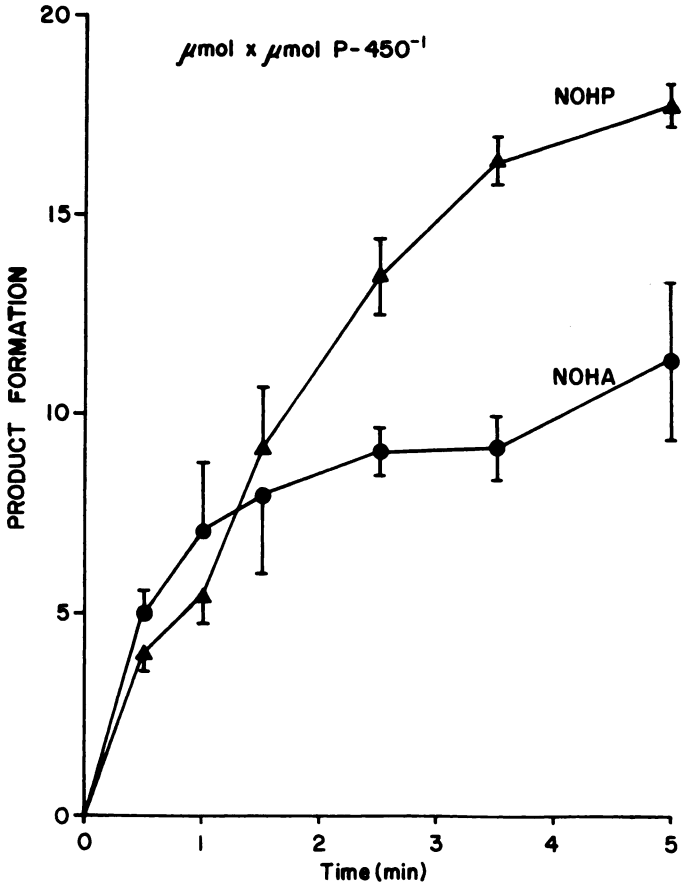

FIG. 3. *Metabolism of NOHP and NOHA in liver microsomes*

NOHA (0.25 mM) or NOHP (0.25 mM) was incubated with microsomes and NADPH (0.383 mM) under conditions described under Materials and Methods. The products of the reactions, phenylacetone oxime (●) from NOHA and 2-methyl-2-nitro-1-phenylpropane (▲) from NOHP, were measured by gas chromatographic analysis.

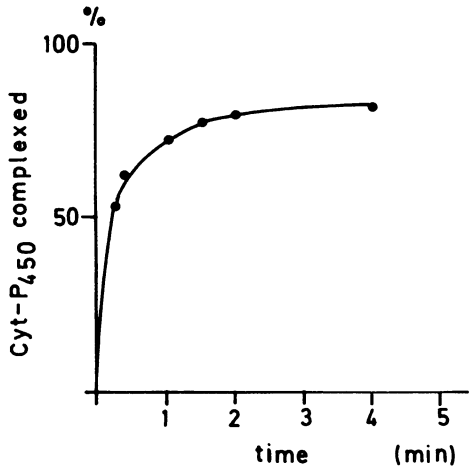

FIG. 4. *Cytochrome P-450 MI complex formation as a function of time during NADPH-dependent microsomal metabolism of NOHA (250 μM)*

One representative experiment of five is shown. The standard deviation at any time point for the five experiments was less than 15%.

The interaction of NOHA with reduced and oxidized cytochrome P-450 is also different from that of NOHP. At 0.25 mM, NOHA rapidly forms the 455-nm MI complex with reduced cytochome P-450 (Fig. 4), whereas NOHP does not. Upon interaction with oxidized microsomal P-450, NOHA exhibits a modified Type II spectrum and NOHP a weak Type I spectrum (Fig. 5).

DISCUSSION

Liver microsomes and the reconstituted cytochrome P-450 systems are thought to generate H_2O_2 by an NADPH-dependent process that either reduces oxygen to superoxide (23), which then dismutates to H_2O_2 or by a direct two-electron reduction of oxygen by cytochrome P-450 (9). The generation of H_2O_2 can be increased by some substrates of the cytochrome P-450 system, such as benzphetamine, that uncouple the electron flow from its normal path in substrate oxidation to the direct reduction of oxygen (9, 10). There are also inhibitors of the cytochrome P-450 system, such as metyrapone (9), that reduce electron flow and thereby inhibit H_2O_2 production. The data presented here indicate that NOHP and NOHA are examples of a cytochrome P-450-dependent H_2O_2 uncoupler and an inhibitor, respectively. This phenomenon is even more dramatic in experiments with microsomes of untreated rats, as 1 mM NOHP causes a 3-fold increase in H_2O_2 production (4), whereas 1 mM NOHA caused a complete inhibition of H_2O_2 (data not shown). The observed H_2O_2 levels determined in the presence of NOHP in the present study are net levels after some consumption of O_2^- by NOHP that may occur during its conversion to MNPP.

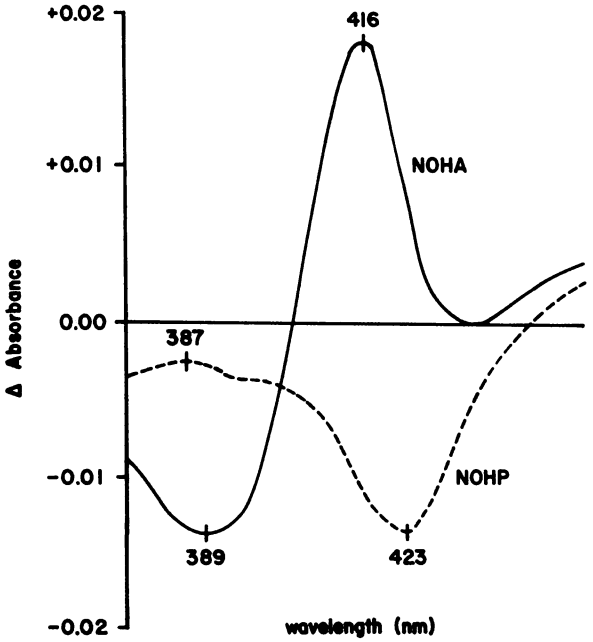

FIG. 5. *Aerobic difference spectra of liver microsomes in the presence of NOHA and NOHP*

NOHA or NOHP (10 μl) was added to cuvettes containing 5.8 ml of microsomal suspension (1 mg of protein per milliliter). The difference spectra between substrate and distilled water were determined over the range 350–500 nm. The final concentrations of NOHA and NOHP were 0.8 mM and 0.6 mM, respectively.

The differences in actions of NOHA and NOHP on cytochrome P-450-dependent O_2 reduction reflect the differences in their interaction with cytochrome P-450. NOHA forms the MI complex, which is irreversible and has been shown to block oxidation reactions mediated by cytochrome P-450 (6). The present data suggest that the MI complex can also affect the reduction of O_2 catalyzed by cytochrome P-450. NOHP and phentermine itself do not form the complexes (7); instead, NOHP appears to promote the reduction of oxygen.

The two hydroxylamines appear to interact differently with oxidized P-450 as shown by the microsome substrate-binding spectra. NOHA generates a reverse Type I or modified Type II spectrum with cytochrome P-450 which has been interpreted (24) to indicate an interaction with the heme portion of cytochrome P-450. The Type I spectrum generated by NOHP, on the other hand, reflects an interaction with the hydrophobic region of the cytochrome, so that the hydrocarbon residue interacts with cytochrome P-450 instead of the amine function.

Thus, NOHA and NOHP appear to be interacting with the cytochrome P-450 heme protein with different orientations. The NOHA orientation results in a ligand that is inhibitory to cytochrome P-450 function, and the NOHP orientation makes the —NHOH functionality available for a chemical reaction. NOHP is oxidized to the corresponding nitro compound by cytochrome P-450-generated superoxide, and the remaining noncomplexed NOHA is oxidized to the oxime by a non-cytochrome P-450 enzyme system present in microsomes.

Although substrate specificity is common for most enzymes, an outstanding characteristic of cytochrome P-450 enzymes viewed as a group is their nonspecificity. However, the present studies show that the presence of more than one methyl group on an α-carbon has a profound effect on the interaction of aliphatic *N*-hydroxylamines with cytochrome P-450. The additional methyl group of phentermine does not have an appreciable effect on either pK_a or partition coefficient (25), so that very little change in physical properties occur. Chemically, the absence of the α-hydrogen in NOHP prevents formation of oxime, but since the nitroso state is responsible

(7, 8) for MI complex formation, the inability of NOHP to form an oxime should be expected to favor the complex. For these reasons, the steric effect of the additional methyl group which has been previously proposed (8) to account for the inability of aliphatic nitrogen compounds with a tertiary α-carbon to form MI complexes is a likely explanation here. Indeed, compounds with trialkyl-substituted carbon atoms in the α-position to heteroatoms are, in general, refractory toward coordination with cytochrome P-450 iron through the heteroatom (8). These results suggest that there is a critical steric or bulk factor around the heme in the isozyme(s) of cytochrome P-450 responsible for *N*-oxidation that control the orientation of potential substrates. This steric constraint is of interest in the opportunities it provides for structural manipulation of metabolic pathways and for studies of nitrogen-cytochrome P-450 interactions.

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