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# CONVERSION OF *N*-HYDROXYAMPHETAMINE TO PHENYLACETONE OXIME BY RAT LIVER MICROSOMES

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**Abstract**—These *in vitro* studies indicate that *N*-oxidation of *N*-hydroxyamphetamine (NOHA) by rat liver homogenates yields phenylacetone oxime (PAOx) as the major metabolite. This oxidation was NADPH and oxygen dependent but was not appreciably increased in microsomes from phenobarbital-pretreated animals. The addition to microsomal incubations of superoxide dismutase (SOD), catalase (CAT), azide or mannitol did not alter the rate of oxidation, suggesting that $O_2^-$, $H_2O_2$, or $OH^\cdot$ are not involved in this process. The reaction was minimally inhibited by a 2:1 ratio of $CO/O_2$, and there was no significant reduction in the formation of product by the presence of diethylaminoethyl diphenylvalerate (SKF-525A) or 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) in micromolar concentrations. Thus, although this NADPH-dependent *N*-oxidation pathway was catalyzed by rat hepatic microsomes, the data suggest that it was not a cytochrome P-450 mediated monooxygenase reaction.

Although phenylacetone oxime (PAOx) has been identified as a metabolite of amphetamine, its role in the metabolic disposition of amphetamine is uncertain. The compound was first identified in studies on the metabolism of amphetamine by rabbit liver 9000 *g* supernatant fraction [1]. In these studies, PAOx was proposed as the precursor of phenylacetone (PA), an *in vivo* and *in vitro* metabolite of amphetamine. Parli and McMahon [2] described the formation of PAOx, *N*-hydroxyamphetamine (NOHA), and PA *in vitro* but proposed that PAOx was formed by a separate path involving the *N*-oxidation of PA imine. In yet another study, Beckett and Al-Sarraj [3] contended, on the basis of chemical stability studies, that PAOx was the decomposition product of *N*-hydroxyamphetamine formed during the analytical workup procedure. Thus, the mechanism of formation of PAOx is a matter of some interest in the metabolism of amphetamine as well as its *N*-hydroxy metabolite.

Following these reports, this laboratory investigated the metabolism of amphetamine by rabbit liver preparations and found that NOHA, PAOx and PA were all metabolically formed and could be assayed under conditions that ensured that no chemical conversion of NOHA to PAOx took place. In contrast to statements describing the stability of NOHA in rabbit liver preparations [1, 2], we found [4] extensive NOHA metabolism to PA, PAOx and 2-nitro-1-phenylpropane (NPP). Furthermore, the data collected in these studies suggested that the conversion of NOHA to PAOx was enzymatically distinct, not sensitive to CO, and not induced in phenobarbital-pretreated animals.

The present report describes results of an investigation of the oxidation of NOHA by rat liver microsomes to PAOx. The study was conducted with rat tissue because of the limited scope of NOHA metabolism, i.e. PAOx was the major metabolite observed. The results showed that the reaction is microsome dependent but probably not P-450 mediated.

## MATERIALS AND METHODS

*Tissue preparations.* Livers excised from freshly killed male Sprague–Dawley rats were homogenized with a Teflon pestle/glass mortar in 3 vol. of ice-cold isotonic KCl (1.15%) solution. In induction studies the rats were injected intraperitoneally with 80 mg of phenobarbital per kg for 3 consecutive days and were killed 24 hr after the last dose. The liver homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant fraction was further centrifuged at 100,000 *g* for 1 hr. The microsomal pellet was resuspended in isotonic KCl as a 25% (whole liver, w/v) homogenate and again centrifuged for 30 min at 100,000 *g*. The resulting pellet was resuspended at a concentration equivalent to 1 g of liver (32 mg protein) per 4 ml. A portion of microsomal suspension equivalent to 0.5 g of liver was incubated in the presence of 2 ml of 0.5 M phosphate buffer (pH 7.4), 0.5 ml of 2.0 mM NOHA solution, an NADPH-generating system consisting of 30 μmoles monosodium glucose-6-phosphate, 12 μmoles $MgCl_2$, 8 I.U. of glucose-6-phosphate dehydrogenase and 2.5 μmoles $NADP^+$, and water to a final volume of 5 ml. Incubations were carried out in 25-ml Erlenmeyer flasks at 37° exposed to air on a Dubnoff shaker for 20 min. The reaction was terminated by immersing the reaction vessel in a 0° water bath. The carbon monoxide inhibition studies were conducted by bubbling $CO/O_2$ (2:1, v/v) through the incubation mixture for 10 min and then quickly sealing with rubber stoppers. When substrate concentrations were varied, the actual volume of substrate added was always 0.5 ml.

*Analytical procedure.* Metabolites were extracted by transferring the reaction mixture into a 50-ml extraction tube containing 10 ml of cold (0°)

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Table 1. Retention times of relevant compounds*

| Compound | Retention time (min) |
|---|---|
| Amphetamine† | 3.33 |
| Phenylacetone oxime† | 5.48 |
| Phenylacetone | 5.66 |
| Phenylbutanone oxime† | 6.65 |
| *N*-Hydroxyamphetamine† | 7.27 |
| 2-Nitro-1-phenylpropane | 12.79 |

* Retention times are given for pure compounds injected after exposure to the derivatization conditions described in Materials and Methods. The gas chromatographic conditions were: a 2 mm × 2 m glass column packed with 2% GEXE-60 on Gas Chrom Q, operated at 105° with detector and inlet at 200°.

† Mass spectral evidence indicates that these compounds are derivatized.

methylene chloride and 100 μl of 2.5 mM 2-phenylbutanone oxime as an internal standard. Tubes were shaken for 10 min and then centrifuged at 3000 rpm for 10 min. The aqueous layer was discarded by aspiration and approximately 8 ml of the organic layer was pipetted into 15-ml conical screw-capped tubes. The dichloromethane extracts were concentrated by evaporation with a stream of dry nitrogen gas until about 200–250 μl remained. Samples were derivatized by addition of 20 μl of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (Regisil: BSTFA), capping, and heating in 54° water bath for 1 hr.

A 2-μl sample was injected into a Hewlett–Packard (model 5830A) gas chromatograph containing a 2 mm × 2 m silanized glass column of Gas Chrom Q coated with 2% GEXE-60. The operating conditions were as follows: oven temperature, 105°; detector (FID) and injection port temperature, 200°; and carrier gas flow ($N_2$), 15 ml/min.

Standard curves were generated by adding known amounts of metabolites to incubation mixtures and then carrying out the procedures described above, omitting the incubation step. The peak area ratios between products/internal standard were plotted against the concentration added to generate a standard curve for analysis of experimental data. The gas chromatographic analysis was sensitive to less than 50 nmoles of PAOx under these conditions. All protein determinations were measured by the method of Lowry *et al.* [5].

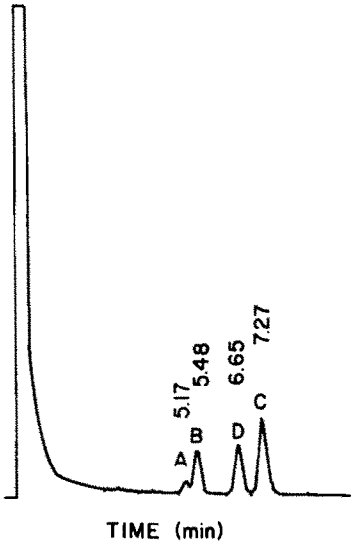


Fig. 1. Gas chromatogram of TMS-derivatized 1-phenyl-2-propanone oxime, isomer mixture, syn (A), anti (B), *N*-hydroxyamphetamine (C), and internal standard (D).

The identification of oxime was achieved by comparing retention times (Table 1; Fig. 1) and mass spectra with those of authentic compounds [6]. The mass spectra were obtained in a Hewlett–Packard (model 5890A) quadrupole mass spectrometer/gas chromatograph system that utilized the same column as that used in the analysis.

*Chemicals.* *R*(−)-NOHA was prepared by oxidation of *R*(−)-amphetamine according to the procedure described in Ref. 7. Sodium azide was purchased form the Aldrich Chemical Co. (Milwaukee, WI). Mannitol was purchased from the J. T. Baker Co. (Phillipsburg, NJ). The following chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO): $NADP^+$, NADPH, glucose-6-phosphate dehydrogenase, albumin, and superoxide dismutase (2500 units/mg). 2,4-Dichloro-6-phenylphenoxyethylamine (DPEA) was a gift of Dr. R. E. McMahon of Lilly Research Laboratories (Indianapolis, IN) and dimethylaminoethyl diphenylvalerate (SKF-525A) was a gift of Smith Kline & French Laboratories (Philadelphia, PA).

## RESULTS

The incubation mixture of NOHA with rat liver microsomes was examined for the presence of PA, amphetamine and NPP metabolites that had been observed in experiments with rabbit liver preparations [4], but none of the compounds were detected. Material balance studies indicated that PAOx accounted for at least 90 per cent of the consumed substrate so that with the rat liver preparations oxime is the major metabolite. The conversion was protein dependent and linear with time to 20 min. Experiments designed to localize the site of the conversion indicated (Table 2) that most, if not all, of the activity resides in the microsomal fractions. NOHA was stable in the presence of nonenzymatic protein as evidenced by its incubation with the 100,000 *g* supernatant fraction.

In a separate experiment, *R*(−)-amphetamine (1.0 mM) was incubated with rat liver microsomes to determine whether it was metabolized. No evidence for its metabolic consumption could be obtained either from measurement of amphetamine itself or the *N*-oxidized metabolites described above. Amphetamine does undergo ring hydroxylation in these preparations but only at low (10 μM) substrate concentrations as it exhibits substrate inhibition at 0.1 mM [8].

PAOx formation from NOHA was oxygen and NADPH dependent (Table 3) with NADH providing partial activity. Oxidized pyridine nucleotides had little activity and, in experiments not shown, pyrazole at 1.0 mM had no effect on the reaction.

Inhibitors of cytochrome P-450 were not consistent in their effects on the reaction (Table 4). SKF-525A

Table 2. Oxidation of *N*-hydroxyamphetamine to phenylacetone oxime liver fractions*

| Fraction | Activity | |
|---|---|---|
| | nmoles · min$^{-1}$ · (mg protein)$^{-1}$ ± S.E. | Per cent of microsomal activity |
| 9000 *g* Supernatant | 0.74 ± 0.02 | 35 |
| Mitochondria | 0.72 ± 0.02 | 34 |
| Microsomes | 2.11 ± 0.05 | 100 |
| 100,000 *g* Supernatant | 0.03 ± 0.01 | 1.3 |

* The indicated subcellular fractions were separated by differential centrifugation of rat liver homogenate. Aliquots of the fractions equivalent to 0.5 g of whole rat liver were incubated with NOHA (0.2 mM) and an NADPH-generating system for 20 min and analyzed as indicated in Materials and Methods ($N = 9$).

Table 3. Properties of oxime formation*

| | Condition | Activity (per cent control) |
|---|---|---|
| Microsomes | + NADPH generating system (in air) | 100 |
| | + NADPH generating system (in $N_2$) | 12.9 |
| | + NADP (0.86 mM) | 26 |
| | + NADH (0.86 mM) | 40 |

* Microsomes were incubated with NOHA (0.2 mM) in the presence of the indicated cofactors for 20 min, and levels of PAOx were determined as described in Materials and Methods. The results are expressed as per cent of control (31.65 nmoles · min$^{-1}$ · g$^{-1}$ ± 0.75).

at concentrations of 0.1 to 5.0 mM had no effect and DPEA inhibited at 0.5 mM. Carbon monoxide inhibited the reaction but only in high concentrations, reflecting the oxygen dependency of the reaction rather than heme involvement. Pretreatment of the animals with phenobarbital under conditions that increase levels of P-450 2-fold [9] had only a slight effect on the activity of microsomes (Table 4). The reaction exhibited saturation kinetics with half-maximal velocity occurring at a substrate concentration of 0.3 mM.

Table 4. Effects of cytochrome P-450 modifiers on rat liver microsomal oxidation of *N*-hydroxyamphetamine*

| Chemical | | Activity (per cent control) |
|---|---|---|
| SKF-525A | (0.1 mM) | 99 ± 1.5 |
| | (0.5 mM) | 98 ± 3.6 |
| | (1.0 mM) | 100 ± 2.5 |
| | (5.0 mM) | 96 ± 2.9 |
| DPEA | (0.05 mM) | 91 ± 3.9 |
| | (0.2 mM) | 85 ± 1.0 |
| | (0.5 mM) | 67 ± 5.0 |
| | (2.0 mM) | 43 ± 6.5 |
| NaCN | (1 mM) | 100 ± 2.7 |
| PB pretreatment | | 114 ± 4.2 |
| $CO/O_2$ | (2:1) | 83 ± 7.0 |
| | (4:1) | 77 ± 7.6) |
| | (8:1) | 70 ± 5.2 |
| | (16:1) | 60 ± 1.9 |

* Microsomes and cofactors were incubated with NOHA (0.2 mM) for 20 min at 37° and levels of oxime were measured as described in Materials and Methods. The rats pretreated with phenobarbital were given daily doses of 80 mg/kg, i.p., for 3 days and killed 24 hr after the last dose. The results are expressed as per cent of oxime formed in the absence of inhibitor in incubations with tissue from untreated animals (42.4 ± 2 nmoles/mg) and represent data from nine experiments.

The reaction was not sensitive to superoxide dismutase or catalase (Table 5) at concentrations far in excess of those necessary to completely inhibit the oxidation of *N*-hydroxyphentermine (NOHP) to the corresponding nitro compound [10]. Attempts to stabilize peroxide or peroxy radicals with azide or mannitol, respectively, were also without effect.

## DISCUSSION

The results described for NOHA metabolism by rat liver microsomes are consistent with observations made with rabbit liver preparations [11] that indicated that NOHA is not oxidized to PAOx by a P-450 dependent system. The metabolism of NOHA by the rabbit is complex, generating PA, NPP and PAOx. In contrast, NOHA to PAOx conversion is the major pathway in the rat.

Table 5. Effects of agents affecting active oxygen on the rate of formation of oxime in rat liver microsomes*

| Agent | Activity (per cent control) |
|---|---|
| Superoxide dismutase (0.2 mg) | 89 ± 2.9 |
| Catalase (2 mg) | 97 ± 1.0 |
| Sodium azide (1 mM) | 89 ± 1.4 |
| Mannitol (3 mM) | 95 ± 1.5 |

* NOHA (0.2 mM) was incubated with microsomes in the presence of the indicated reagents for 20 min under conditions described in Materials and Methods. The levels of oxime formed are expressed as per cent of control (31.7 nmoles · min$^{-1}$ · g$^{-1}$).

The oxidation of NOHA to PAOx is a 2-electron change, equivalent to the oxidation of amphetamine to NOHA. Several pathways for this conversion are possible, some of which have been eliminated by this study. The oxime is the thermodynamically stable 2-electron oxidation product of NOHA. The other 2-electron oxidation product, NPP, could rearrange to PAOx after being formed by a P-450 mediated reaction [12]. The nitroso compound has been proposed [12, 13] to be the ligand in the metabolic intermediate (MI) complex with cytochrome P-450 formed by amines. This inhibitory complex [14] can be formed either by *N*-oxidation of amphetamine or NOHA, or by reduction of the corresponding nitro compound. Because of P-450 involvement, complex formation and, therefore, nitroso compound formation from NOHA are induced by phenobarbital pretreatment and inhibited by carbon monoxide [14], which is not consistent with the insensitivity of NOHA to PAOx conversion to these agents. Another cytochrome P-450 dependent pathway has been suggested by Parli *et al.* [15] who proposed that PAOx could form by *N*-oxidation of phenylacetone imine [16]. This pathway is also inconsistent with lack of inhibition by SKF-525A, poor inhibition by DEPA, and minimal induction by phenobarbital observed here since their enzyme system was extremely sensitive to these agents [15]. For example, at 5 mM DPEA, imine formation was inhibited by 95 per cent and phenobarbital induction increased formation by 500 per cent.

The possible involvement of the flavin dependent monooxygenase was examined with dithiothreitol as an inhibitor, but prior work had also shown NOHA to be stable in the presence of purified amine oxidase [17]. A dehydrogenase reaction is also unlikely since NAD and NADP were not effective as cofactors, and pyrazole at high concentrations did not inhibit the reaction.

The conversion of NOHA to PAOx was shown to occur in a xanthine/xanthine oxidase mixture [18]. Since this system generates superoxide, microsome generated superoxide was also considered a possible pathway and high levels of superoxide dismutase were added, but this enzyme had no effect on the reaction. Superoxide dismutase can effectively block the microsomal oxidation of *N*-hydroxyphentermine to 2-methyl-2-nitro-1-phenylpropane which is another related *N*-oxidation reaction that utilizes superoxide [19]. These studies indicate that the conversion of NOHA to PAOx appears to be catalyzed by an enzyme system that differs from the more common nitrogen oxidation enzymes. The properties of the system as characterized in rat liver preparations parallel those found in rabbit. However, the role of this enzyme in the formation of PAOx from amphetamine is still uncertain.

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