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Reduction of Amphetamine Hydroxylamine and Other Aliphatic Hydroxylamines by Benzamidoxime Reductase and Human Liver Microsomes

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For the reduction of *N*-hydroxylated derivatives of strongly basic functional groups, such as amidines, guanidines, and aminohydrazones, an oxygen-insensitive liver microsomal system, the benzamidoxime reductase, has been described. To reconstitute the complete activity of the benzamidoxime reductase, the system required cytochrome b_5, NADH-cytochrome b_5-reductase, and the benzamidoxime reductase, a cytochrome P450 enzyme, which has been purified to homogeneity from pig liver. It was not known if this enzyme system was also capable of reducing aliphatic hydroxylamines. The *N*-hydroxylation of aliphatic amines is a well-known metabolic process. It was of interest to study the possibility of benzamidoxime reductase reducing *N*-hydroxylated metabolites of aliphatic amines back to the parent compound. Overall, *N*-hydroxylation and reduction would constitute a futile metabolic cycle. As examples of medicinally relevant compounds, the hydroxylamines of methamphetamine, amphetamine, and *N*-methylamine as model compounds were investigated. Formation of methamphetamine and amphetamine was analyzed by newly developed HPLC methods. All three hydroxylamines were easily reduced by benzamidoxime reductase to their parent amines with reduction rates of 220.6 nmol min^{-1} (mg of protein)$^{-1}$ for methamphetamine, 5.25 nmol min^{-1} (mg of protein)$^{-1}$ for amphetamine, and 153 nmol min^{-1} (mg of protein)$^{-1}$ for *N*-methylhydroxylamine. Administration of synthetic hydroxylamines of amphetamine and methamphetamine to primary rat neuronal cultures produced frank cell toxicity. Compared with amphetamine or the oxime of amphetamine, the hydroxylamines were significantly more toxic to primary neuronal cells. The benzamidoxime reductase is therefore involved in the detoxication of these reactive hydroxylamines.

Introduction

In previous studies, it was shown that *N*-hydroxylated derivatives of strongly basic functional groups were reduced both in vivo and in vitro by a microsomal enzyme system present in all mammalian species (rats, rabbits, pigs, and humans) tested to date (*1*−*4*). An enzyme system that required NADH-cytochrome b_5-reductase, cytochrome b_5, and a third protein component, named the benzamidoxime reductase, was shown to be responsible for the microsomal enzymatic reductions (*5*). The third enzyme component of the reductase system exhibited all the characteristics of a cytochrome P450 enzyme (*5*). Benzamidoxime reductase, isolated from pig liver microsomes, exhibited similarities with other isoenzymes of the cytochrome P450 2D subfamily from other species. Cytochrome P450 2D enzymes from pig have not been described previously (*5*). Benzamidoxime reduction activity in the presence of microsomes from cell lines transfected with cDNAs expressing human cytochrome P450 enzymes obtained from GENTEST Corp. (Woburn, MA) could not be detected (*5*). In previous studies, a pig liver microsomal hydroxylamine reductase was described by Kadlubar and Ziegler (*6*), and that system had many characteristics of the benzamidoxime reductase described herein.

The *N*-oxygenation of alkylamines is a well-known metabolic process (*7*). If *N*-oxygenated metabolites were reduced back to the parent amines, this would constitute another example of a futile metabolic cycle involving oxidation and reduction (Scheme 1).

Methamphetamine (Scheme 1) and amphetamine (Scheme 1) derivatives are used therapeutically for attention hyperactivity deficit disorder in children and as short-term adjunct in exogenous obesity. Amphetamine is also used in the treatment of narcolepsy and in the short-term managment of depression in patients intolerant to the tricyclic antidepressants (*8*).

Metabolism of methamphetamine in humans and animals produces a number of urinary metabolites, including amphetamine, norephedrine, 4-hydroxynorephedrine, *N*-hydroxymethamphetamine and phenylacetone, benzoic acid, and hippuric acid (*9*−*12*).

Microsomal preparations from human livers catalyze the NADPH-dependent *N*-oxygenation of methamphetamine to *N*-hydroxymethamphetamine. It was reported that in animals the *N*-hydroxylation of methamphet-

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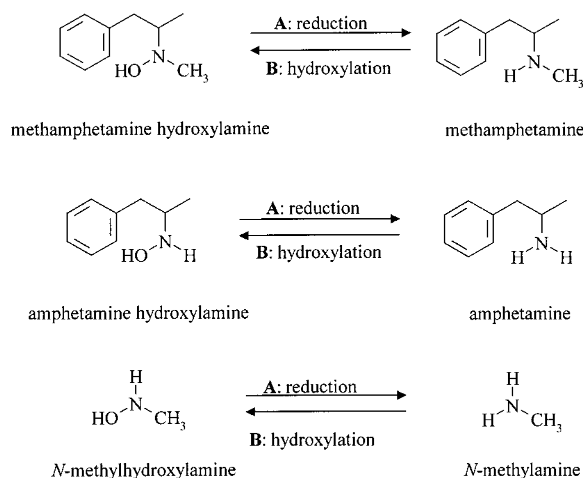
Scheme 1. Metabolic Cycle of the Metabolism of Aliphatic Amines[a]



[a] (A) Reduction by benzamidoxime reductase, composed of cytochrome b_5, NADH-cytochrome b_5-reductase, and a P450 enzyme, the benzamidoxime reductase. (B) Hydroxylation by P450 and the flavin-containing monooxygenases (*12–14, 18*).

amine was catalyzed mainly by the flavin-containing monooxygenase (FMO) system (*13, 14*). Human FMO3 also *N*-oxygenates methamphetamine (*14*). Baba et al. (*12*) showed that the P450[1] system was also capable of methamphetamine *N*-hydroxylation and isolated a P450 enzyme from rat liver that was predominantly involved in the *N*-hydroxylation of methamphetamine.

For the metabolism of amphetamine by P450 and the FMO, four general pathways are well-known, including aromatic hydroxylation, aliphatic hydroxylation, oxidative deamination, and *N*-oxidation (*14–18*).

During our investigations of the metabolism of guanidines, we observed an increase in the extent of P450-mediated *N*-hydroxylation by adding of *N*-methylhydroxylamine to the incubation mixture (*3*). We postulated that *N*-methylhydroxylamine (Scheme 1) inhibited the retroreduction and increased the apparent rate of *N*-hydroxylation to *N*-hydroxylated products. The reduction of *N*-methylhydroxylamine by pig liver microsomes and the hydroxylamine reductase has been described without identifying all the components of the enzyme system (*19*).

The object of the study presented here was to determine if benzamidoxime reductase detoxicates *N*-hydroxylated aliphatic amines by reducing the hydroxylamine to the parent amine. We determined that the hydroxylamine reductase and the benzamidoxime reductase were identical. As model compounds, the hydroxylamines of amphetamine, methamphetamine, and methylamine were examined and found to be good substrates for this reductase system.

Materials and Methods

Chemicals. Methamphetamine and amphetamine were kindly supplied by P. Rösner (Landeskriminalamt Schleswig-Holstein, Kiel, Germany) or the National Institute on Drug Abuse Drug Supply Program (National Institutes of Health, Rockville, MD). *N*-Methylhydroxylammonium chloride and NADH (disodium salt) as well as all other chemicals and solvents were obtained from E. Merck (Darmstadt, Germany). All chemicals were analytical grade.

Preparation of Methamphetamine Hydroxylamine and Amphetamine Hydroxylamine. The synthesis of amphetamine hydroxylamine and methamphetamine hydroxylamine was as previously described (*14*).

Pig Liver Microsomes. Pig liver microsomes were prepared by fractional acid precipitation according to the procedure of Ziegler and Pettit (*20*) with slight modifications (*21*).

Human Liver Microsomes. Human liver was obtained from the Medical Department of the University of Kiel. Prior consent of the local medical ethics committee and from the donors before removal of the liver pieces was granted. Liver pieces from partial hepatectomies (Chirurgische Klinik der CAU, Kiel, Germany) were minced with knives and washed with 20 mM potassium phosphate buffer containing 0.25 mM sucrose (pH 7.4) at 4 °C. The minced livers were homogenized using a motorized Teflon pestle glass tube homogenizer (Potter S, for 30 mL, Braun Melsungen AG, Melsungen, Germany). After adjustment to pH 7.4, the homogenates were transferred to plastic tubes and centrifuged at 9000*g* for 30 min. The supernatant was carefully decanted and centrifuged at 100000*g* for 60 min. The pellet of the 100000*g* centrifugation was resuspended in phosphate buffer (pH 7.4) and again centrifuged at 100000*g* for 60 min. The supernatant was discarded, and the microsome pellet was resuspended in potasium phosphate buffer (pH 7.4).

The microsomal preparation was stored at −80 °C in aliquots.

Purification of Cytochrome b_5, NADH-Cytochrome b_5-Reductase, and Benzamidoxime Reductase. Cytochrome b_5, NADH-cytochrome b_5-reductase, and benzamidoxime reductase were fractionated by the procedure described by Clement et al. (*5*) with slight modifications. Thesit (Boehringer Mannheim, Mannheim, Germany) was used to solubilize the microsomal proteins in the elution buffers. All purification steps were performed at 4 °C. The solubilized pig liver microsomes were applied to an octyl-sepharose CL 4B (Pharmacia, Freiburg, Germany) column (38 cm × 3.8 cm), and enzymes were eluted as described in detail previously (*5*). The fractions containing NADH-ferricyanide-reductase activity were collected, and the fractions with the highest absorbance at 417 nm (cytochrome b_5) were combined.

Cytochrome b_5. Cytochrome b_5 was purified on a DEAE-cellulose column as described previously (*5*). The final cytochrome b_5 fraction contained 17.22 nmol of cytochrome b_5/mg of protein.

Benzamidoxime Reductase. Benzamidoxime reductase was chromatographed on an anion exchange column by preparative HPLC with a conventional HPLC system (L-6210 Intelligent Pump, 655 A-22 UV detector, D-2500 integrator; Merck/Hitachi, Darmstadt, Germany). Approximately 1 mg of protein was applied to a semipreparative Fractogel EMD TMAE 650 (S) column (150 mm × 10 mm; particle size, 25–40 μm; Merck) that was equilibrated with buffer A [10 mM Tris-acetate (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% (w/v) glycerol, and 0.5% (w/v) Thesit]. After equilibration, fractions were eluted with a one-step increase in the salt concentration up to 0.5 M in buffer A at a flow rate of 1 mL/min. The first fraction, which contained the benzamidoxime reductase, was purified from UDP-glucuronyltransferase on an UDP-hexanolamine-sepharose (Sigma) column (4.5 cm × 1.2 cm) equilibrated with 10 mM Tris-acetate buffer (pH 7.4) and washed with 50 mL of buffer B, consisting of 50 mM KCl and 40 μM phosphatidylcholine in 10 mM Tris-acetate buffer (pH 7.4). The second fraction contained the NADH-cytochrome b_5-reductase.

NADH-Cytochrome b_5-Reductase. NADH-cytochrome b_5-reductase was purified to homogeneity by affinity chromatography on 5′-AMP-Sepharose 4B (Pharmacia) (*22*). The fractions containing the highest NADH-ferricyanide-reductase activity were combined and concentrated, followed by gel filtration (NAP 10, Pharmacia). The specific activity of the purified reductase was 38.1 units/mg of protein.

Detergents were removed from the purified enzymes by shaking the concentrated fractions for 4 h with Calbiosorb (Calbiochem, La Jolla, CA) at 4 °C.

[1] Abbreviations: P450, cytochrome P450; DEAE, diethylaminoethyl; TMAE, trimethylaminoethyl; DLPC, dilaurylphosphatidylcholine.

Analytical Procedures. (1) Protein Concentration. Protein concentrations were measured using the method described by Smith et al. (*23*) with bicinchoninic acid (BCA reagent kit, Pierce Chemical Co., Rockford, IL). All photometric measurements were performed with a Uvicon 930 (Kontron Instruments, Neufahrn, Germany) spectrophotometer.

(2) Cytochrome P450 Concentrations. The P450 concentration was analyzed using the method of Omura and Sato (*24*).

(3) Cytochrome b_5 Concentrations. The cytochrome b_5 concentration was determined by recording the reduced minus the oxidized spectrum (absorbance at 185 mM^{-1} cm^{-1}) as described by Estabrook and Werringloer (*25*).

(4) NADH−Cytochrome b_5-Reductase. NADH-cytochrome b_5-reductase was assessed by its NADH-ferricyanide-reductase activity (1 unit = 1 μmol of reduced ferricyanide/min) as described by Mihara and Sato (*26*).

(5) Benzamidoxime Reductase. Benzamidoxime reductase activity was measured by reduction of benzamidoxime to benzamidine and monitored by HPLC as described by Clement et al. (*5*).

Incubations. (1) Reduction of Methamphetamine Hydroxylamine. Incubations were carried out in a shaking water bath at 37 °C in the presence of oxygen using 1.5 mL reaction vessels. The standard incubation mixture (150 μL) contained the following components: 100 mM potassium phosphate buffer (pH 6.3), 1.9 mM methamphetamine hydroxylamine, 3 μg of benzamidoxime reductase, 0.4 unit of NADH-cytochrome b_5-reductase, 87 pmol of cytochrome b_5, 40 mM DLPC (Sigma), and 2 mM NADH. After preincubation for 3 min at 37 °C, the reactions were started by addition of NADH. The incubation time was 30 min. The reaction was terminated by addition of 150 μL of methanol and by cooling the samples in ice. After centrifugation at 10000*g* and 4 °C, 10 μL aliquots of the supernatant were directly analyzed by HPLC.

(2) Human Liver Microsomal Reduction of Methamphetamine Hydroxylamine. Incubations were carried out as described above but with addition of 30 μg of human liver microsomes instead of cytochrome b_5, NADH-cytochrome b_5-reductase, DLPC, and benzamidoxime reductase.

(3) Reduction of Amphetamine Hydroxylamine. Incubations were carried out in a shaking water bath at 37 °C in the presence of oxygen using 1.5 mL reaction vessels. The standard incubation mixture (150 μL) contained the following components: 100 mM potassium phosphate buffer (pH 6.3), 0.5 mM amphetamine hydroxylamine, 2.5 μg of benzamidoxime reductase, 0.33 unit of NADH-cytochrome b_5-reductase, 72.5 pmol of cytochrome b_5, 40 mM DLPC (Sigma), and 2 mM NADH. After preincubation for 3 min at 37 °C, the reactions were started by addition of NADH. The incubation time was 30 min. The reaction was terminated by addition of 150 μL of methanol and by cooling the samples in ice. After centrifugation at 10000*g* and 4 °C, 10 μL aliquots of the supernatant were directly analyzed by HPLC.

(4) Human Liver Microsomal Reduction of Amphetamine Hydroxylamine. Incubations were performed as described above but with addition of 30 μg of human liver microsomes instead of cytochrome b_5, NADH-cytochrome b_5-reductase, DLPC, and benzamidoxime reductase.

(5) Reduction of *N*-Methylhydroxylamine. Incubations were carried out in a shaking water bath at 37 °C in the presence of oxygen using 1.5 mL reaction vessels. The standard incubation mixture (300 μL) contained the following components: 50 mM Tris-acetate buffer (pH 6.3), 0.2 mM *N*-methylhydroxylamine, 5 μg of benzamidoxime reductase, 0.5 unit of NADH-cytochrome b_5-reductase, 100 pmol of cytochrome b_5, 40 mM DLPC (Sigma), 500 units/mL superoxide dismutase, and 1 mM NADH. After preincubation for 2 min at 37 °C, the reactions were started by addition of NADH. The incubation time was 15 min. The reaction was terminated by the addition of 300 μL of methanol and cooling the samples in ice. After centrifugation at 10000*g* and 4 °C, the disappearance of the hydroxylamine was assessed by UV/vis spectrophotometry.

(6) Microsomal Reduction of *N*-Methylhydroxylamine. Incubations were performed as described above but with addition of 30 μg of human liver microsomes instead of cytochrome b_5, NADH-cytochrome b_5-reductase, DLPC, and benzamidoxime reductase.

HPLC. (1) Methamphetamine Hydroxylamine. The resulting clear supernatants from incubations were analyzed using high-performance liquid chromatography (616 HPLC pump and 600S controller from Waters, Milford, CT) equipped with a variable-wavelength UV detector (Waters 486 TAD) set at 220 nm and an autosampler (Waters 717 plus WISP). The areas under the peak were integrated with the EZChrom Chromatography Data System (version 6.7, Scientific Software Inc., San Ramon, CA). Separation and quantification were performed at room temperature on an Aluspher RP-select B-column (244 mm × 4 mm; particle size, 5 μm; Merck). The mobile phase was 0.025 M NaOH/methanol (70:30, v/v) and was passed through the column at a rate of 1.0 mL/min. The injected sample volume was 10 μL. Solvents used in the analysis were filtered through a Sartolon membrane filter (0.45 μm, Sartorius AG, Goettingen, Germany) and degassed by bubbling with helium or sonication. For the determination of the recovery efficiency and the detection limit of the metabolite methamphetamine, known concentrations of the synthetic reference substance (from 14 to 90 μM) dissolved in the mobile phase were measured. The standard curves were linear over this range with correlation coefficients of 0.998. The detection limit was about 5 μM which corresponded to a rate of reduction of 1.17 nmol of methamphetamine min^{-1} (mg of benzamidoxime reductase)$^{-1}$. The retention times were 5.8 ± 0.5 min for methamphetamine hydroxylamine and 7.6 ± 0.5 min for methamphetamine.

(2) Amphetamine Hydroxylamine. Separation and quantification were carried out as described above for methamphetamine hydroxylamine. The mobile phase was 0.025 M NaOH/methanol (90:10, v/v). Standard curves at the levels from 63 μM to 0.71 mM amphetamine were constructed and found to be linear over this range with correlation coefficients of 0.964. The recovery rate of amphetamine from incubation mixtures without adding cofactor was 91.5 ± 2.5% ($N = 32$).

The detection limit was about 57 μM, which corresponded to a rate of reduction of 0.32 nmol of amphetamine min^{-1} (mg of benzamidoxime reductase)$^{-1}$. The retention times were 5.2 ± 0.5 min for amphetamine hydroxylamine and 7.5 ± 0.5 min for amphetamine.

UV/Vis Spectrophotometry. *N*-Methylhydroxylamine. The resulting clear supernatant was analyzed using the indirect spectrophotometric analysis of *N*-methylhydroxylamine as described by Kadlubar et al. (*19*). An aliquot of 250 μL of the clear supernatant was diluted with ethanol to afford a volume of 450 μL. Sodium acetate buffer (200 μL, 1 mM, pH 4.6), 200 μL of a 10 mM ethanolic bathophenanthrolin solution, and 50 μL of a 10 mM ferrinitrate solution in sodium acetate buffer were added. After exactly 3 min, 100 μL of a 10 mM EDTA solution in sodium acetate buffer was added. Within 2 min, the solution was assessed with a wavelength set at 535 nm and compared with a solution of 50 mM Tris buffer (pH 6.3) and methanol (1:1, v/v) instead of the aliquot. Standard curves in the range from 25 μM to 0.15 mM *N*-methylhydroxylamine were constructed and found to be linear over this range with correlation coefficients of 0.9954.

Neuronal Cell Culture and Amphetamine and Methamphetamine Hydroxylamine Toxicity. Primary neuronal material was obtained from two 15−17-day-old rat embryos. Appropriate approval from the institutional animal care and use committee was obtained for this study. The pregnant rats were anesthetized with ether, and the embryos were removed. The whole brain was next removed with forceps, and the brain was dissected. Because of the ease and yield of cell culturing, the studies were carried out with cerebral cortex. After removal of the thalmus, the cerebral cortex was dispersed and passed through 80 μm nylon mesh into Eagle's modified essential medium containing 30 mM glucose and 5 μg of insulin/mL,

Table 1. In Vitro Reduction of Methamphetamine Hydroxylamine to Methamphetamine by Benzamidoxime Reductase and Human and Pig Liver Microsomes[a]

preparation	composition	N^b	nmol of methamphetamine $\min^{-1}$ (mg of protein)$^{-1}$
benzamidoxime reductase	complete mixture	8	220.6 ± 27.2
	without NADH	4	7.3 ± 2.6
	without benzamidoxime reductase	4	8.4 ± 1.2
	without methamphetamine hydroxylamine	4	ND[c]
pig liver microsomes	complete mixture	8	11.0 ± 0.4
	without NADH	4	1.9 ± 0.2
	without microsomes	4	2.0 ± 0.3
	without methamphetamine hydroxylamine	4	ND[c]
human liver microsomes	complete mixture	8	7.3 ± 0.5
	without NADH	4	1.2 ± 0.5
	without microsomes	4	1.3 ± 0.3
	without methamphetamine hydroxylamine	4	ND[c]

[a] A complete incubation mixture for optimized conditions consisted of 1.9 mM methamphetamine hydroxylamine, 3 μg of benzamidoxime reductase, 0.4 unit of NADH-cytochrome b_5-reductase, 87 pmol of cytochrome b_5, 40 mM DLPC (Sigma), and 2 mM NADH, in 150 μL of 100 mM potassium phosphate buffer (pH 6.3). Incubation mixtures with microsomes consisted of 30 μg of microsomal protein, as described in Materials and Methods. Data are means ± the standard error from *N* different determinations; $p < 0.05$. [b] Number of determinations. [c] Not detected.

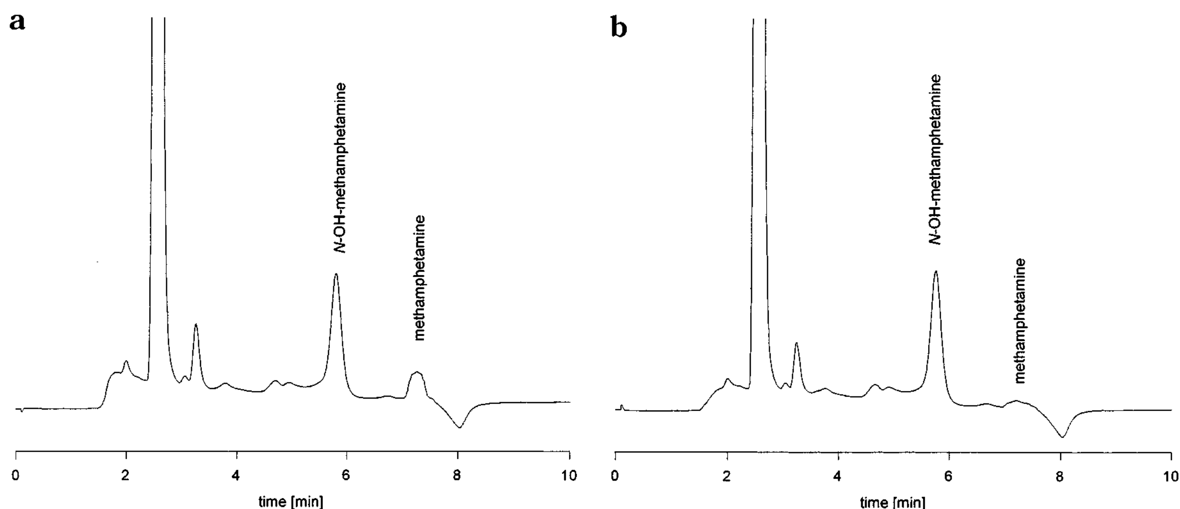


Figure 1. Representative HPLC chromatogram of the incubation of methamphetamine hydroxylamine with benzamidoxime reductase. The incubation mixtures were as described in Materials and Methods: (a) complete incubation mixture and (b) incubation mixture without NADH.

250 000 IU/L penicillin, 0.5% streptomycin, and 20% fetal calf serum (v/v) supplemented with extra agents (*27*). The cells were maintained in a humidified atmosphere. The NUNC A/S Petri dishes that were used were precoated with sterile poly(L-lysine) in boric acid/NaOH at pH 8.4. Neuronal cells were initially plated at a density of 0.1−0.5 million cells/mL in polylysinated 12-well plates. After cultivation for 1 day, the medium was changed, and after 4−5 days, the fetal calf serum level was changed from 20 to 10%. Also, after 4−5 days, Ara-c (10^{-5} M) was added to suppress the growth of astroglia (*28*). Using this procedure, about 80−85% of the amount of neurons in culture from prenatal brain were obtained (*29*, *30*). The cells were characterized with respect to cellular content by morphological inspection. The cell viability of isolated neurons was checked by lactate dehydrogenase release and trypan blue exclusion.

Results

Reduction of Methamphetamine Hydroxylamine. The relative rates of in vitro reduction of methamphetamine hydroxylamine to methamphetamine by the benzamidoxime reductase and by microsomal fractions from pig and human livers are listed in Table 1. A representative HPLC chromatogram recorded after the incubation of methamphetamine hydroxylamine with the benzamidoxime reductase shown in Figure 1 showed that the retention time for the metabolite (7.6 min) agreed with that of the synthetic material. Addition of the reference substrate to the incubation mixture gave rise to an increase in the area of the metabolite peak. This was also reproduced when the HPLC eluent was varied (data not shown). The chromatogram of the incubation mixture in the absence of cofactor (NADH) (Figure 1) exhibited a smaller peak for methamphetamine. The apparent reductase activity in control incubations without NADH resulted from impurities of the substrate methamphetamine hydroxylamine with methamphetamine and from chemical reduction under the conditions of the incubation (data not shown). For incubation times of 30 min, the enzymatic reduction of methamphetamine hydroxylamine proceeded linearly (data not shown).

The species dependency (Table 1) revealed that hydroxylamine reduction was more pronounced in pig liver while appreciably lower conversion rates were observed in human liver preparations.

The reduction of methamphetamine hydroxylamine in completely reconstituted systems and NADH obeyed Michaelis−Menten kinetics. The kinetic data are summarized in Table 2.

Reduction of Amphetamine Hydroxylamine. The reduction of amphetamine hydroxylamine to amphetamine in vitro by the highly purified benzamidoxime

Table 2. Kinetic Data for the Reduction of Methamphetamine Hydroxylamine, Amphetamine Hydroxylamine, and *N*-Methylhydroxylamine by Benzamidoxime Reductase and Human Liver Microsomes

	K_m (mM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]	V_{max}/K_m [L min^{-1} (mg of protein)$^{-1}$]
methamphetamine hydroxylamine by benzamidoxime reductase	5.32	193.3	3.63×10^{-5}
amphetamine hydroxylamine by benzamidoxime reductase	0.49	4.37	8.92×10^{-6}
amphetamine hydroxylamine by human liver microsomes	0.76	4.01	5.26×10^{-6}
N-methylhydroxylamine by benzamidoxime reductase (first phase)	0.21	60.24	2.87×10^{-4}
N-methylhydroxylamine by benzamidoxime reductase (second phase)	0.57	722.2	1.27×10^{-3}

Table 3. In Vitro Reduction of Amphetamine Hydroxylamine to Amphetamine by Benzamidoxime Reductase and Human and Pig Liver Microsomes[a]

preparation	composition	N[b]	nmol of amphetamine min^{-1} (mg of protein)$^{-1}$
benzamidoxime reductase	complete mixture	8	5.25 ± 0.09
	without NADH	4	0.8 ± 0.31
	without benzamidoxine reductase	4	ND[c]
	without amphetamine hydroxylamine	4	ND[c]
pig liver microsomes	complete mixture	8	3.01 ± 0.42
	without NADH	4	1.41 ± 0.02
	without microsomes	4	1.0 ± 0.30
	without amphetamine hydroxylamine	4	ND[c]
human liver microsomes	complete mixture	8	4.09 ± 0.11
	without NADH	4	2.0 ± 0.06
	without microsomes	4	1.98 ± 0.29
	without amphetamine hydroxylamine	4	ND[c]

[a] A complete incubation mixture for optimized conditions consisted of 0.5 mM amphetamine hydroxylamine, 2.5 μg of benzamidoxime reductase, 0.33 unit of NADH-cytochrome b_5-reductase, 72.5 pmol of cytochrome b_5, 40 mM DLPC (Sigma), and 2 mM NADH, in 150 μL of 100 mM potassium phosphate buffer (pH 6.3). Incubation mixtures with microsomes consisted of 30 μg of microsomal protein, as described in Materials and Methods. Data are means ± the standard error from N different determinations; $p < 0.05$. [b] Number of determinations. [c] Not detected.

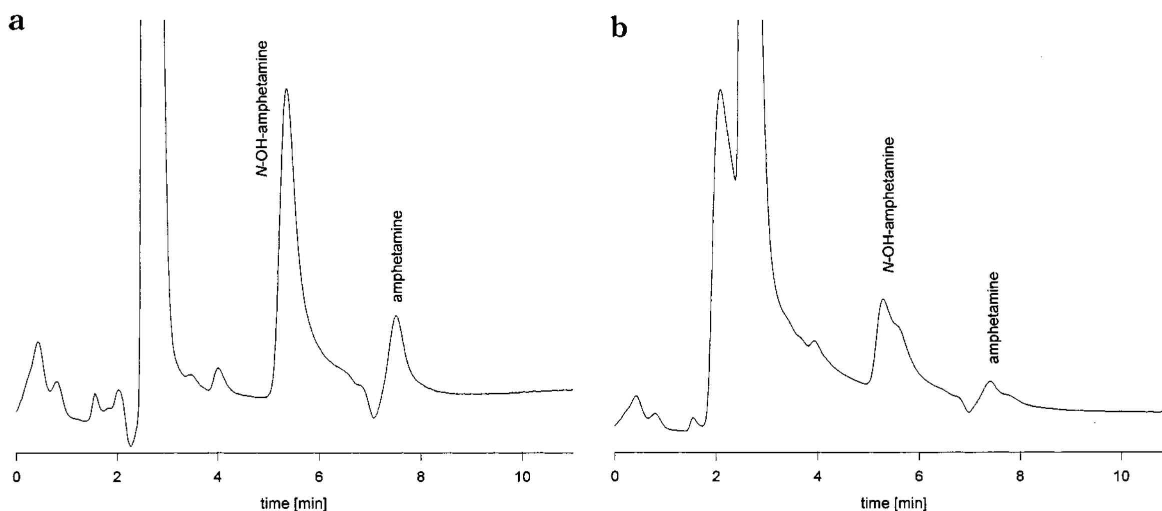


Figure 2. Representative HPLC chromatogram of the incubation of amphetamine hydroxylamine with benzamidoxime reductase. The incubation mixtures were as described in Materials and Methods: (a) complete incubation mixture and (b) incubation mixture without NADH.

reductase and by microsomal fractions from pig and human livers has been demonstrated (Table 3). A representative HPLC chromatogram recorded after the incubation of amphetamine hydroxylamine with the benzamidoxime reductase is shown in Figure 2. The retention time for the metabolite (7.5 min) agreed with that of the authentic synthetic material. Addition of the reference substrate to the incubation mixture gave rise to an increase in the area of the metabolite peak when the HPLC eluent was varied (data not shown). The chromatogram of the incubation mixture in the absence of cofactor (NADH) (Figure 2) exhibited a smaller peak for amphetamine. The apparent reductase activity in control incubations without NADH resulted from impurities of the substrate amphetamine hydroxylamine with amphetamine and from chemical reduction under the conditions of the incubation (data not shown). It is known that in particular primary hydroxylamines are usually quite unstable compounds. Thus, the chemical formation of amphetamine could not be avoided completely. For incubation times of 45 min, the reaction proceeded linearly and exhibited a pH optimum at 5.1 (data not shown).

The reduction of amphetamine hydroxylamine in completely reconstituted systems and NADH obeyed Michaelis−Menten kinetics. The kinetic data are summarized in Table 2.

The reduction of amphetamine hydroxylamine in complete systems with human liver microsomes and NADH also obeyed Michaelis−Menten kinetics (Table 2).

The species dependency (Table 3) revealed that amphetamine hydroxylamine reduction was more pronounced in human liver microsomes while appreciably lower conversion rates were observed in pig liver mi-

Table 4. In Vitro Reduction of *N*-Methylhydroxylamine to *N*-Methylamine by Benzamidoxime Reductase and Human Liver Microsomes[a]

preparation	composition	*N*[b]	ratio of *N*-reduction of *N*-methylhydroxylamine [nmol min^{-1} (mg of protein)$^{-1}$]
benzamidoxime reductase	complete mixture	8	153 ± 15
	without NADH	4	ND[c]
	without benzamidoxine reductase	4	ND[c]
	without *N*-methylhydroxylamine	4	ND[c]
human liver microsomes	complete mixture	8	24.7 ± 1.71
	without NADH	4	2.0 ± 0.03
	without microsomes	4	ND[c]
	without *N*-methylhydroxylamine	4	ND[c]

[a] A complete incubation mixture for optimized conditions consisted of 0.2 mM *N*-methylhydroxylamine, 5 μg of benzamidoxime reductase, 0.5 unit of NADH-cytochrome b_5-reductase, 100 pmol of cytochrome b_5, 40 mM DLPC (Sigma), 1 mM NADH, and 500 units/mL superoxide dismutase, in 150 μL of 100 mM potassium phosphate buffer (pH 6.3). Incubation mixtures with microsomes consisted of 30 μg of microsomal protein, as described in Materials and Methods. Data are means ± the standard error from *N* different determinations; $p < 0.05$. [b] Number of determinations. [c] Not detected.

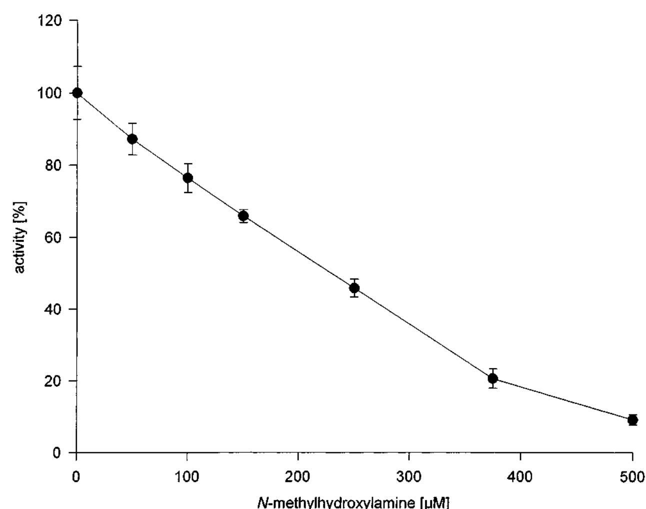


Figure 3. Effect of different concentrations of *N*-methylhydroxylamine on the reduction of amphetamine hydroxylamine by benzamidoxime reductase. The incubation mixtures were as described in Materials and Methods in the presence of different concentrations of *N*-methylhydroxylamine. The amount of amphetamine in the absence of *N*-methylhydroxylamine under control conditions was taken to be 100%. Data are means ± the standard error from four determinations.

crosomes. The effect of increasing concentrations of *N*-methylhydroxylamine in the incubation mixtures is shown in Figure 3.

Reduction of *N*-Methylhydroxylamine. The reduction of *N*-methylhydroxylamine to methylamine in vitro by the highly purified benzamidoxime reductase and by microsomal fractions from pig liver is described in Table 4.

In incubation mixtures in the absence of cofactor (NADH) or benzamidoxime reductase, no reduction of *N*-methylhydroxylamine could be detected. For incubation times of 45 min, the reaction proceeded linearly.

The reduction of *N*-methylhydroxylamine in completely reconstituted systems and NADH exhibited a biphasic course. The kinetic data are summarized in Table 2.

The species dependency (Table 4) revealed that *N*-methylhydroxylamine reduction was more pronounced in humans while appreciably lower conversion rates were observed in pig liver microsomes (*19*). The effect of increasing concentrations of *N*-methylhydroxylamine in the incubation mixtures of benzamidoxime reduction is shown in Figure 4. The substrate dependency for *N*-methylhydroxylamine reduction is shown in Figure 5.

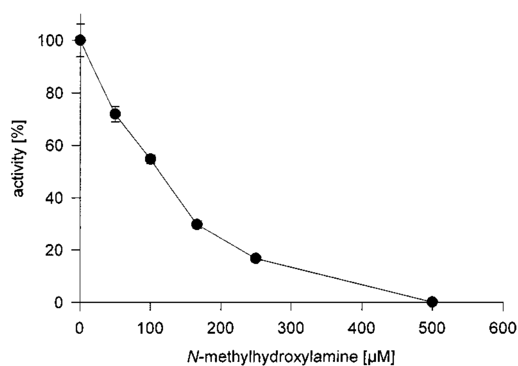


Figure 4. Effect of different concentrations of *N*-methylhydroxylamine on the reduction of benzamidoxime to benzamidine by benzamidoxime reductase. The incubation mixtures were as described in Materials and Methods in the presence of different concentrations of *N*-methylhydroxylamine. The amount of benzamidine in the absence of *N*-methylhydroxylamine under control conditions was taken to be 100%. Data are means ± the standard error from four determinations.

The effect of increasing concentrations of benzamidoxime for the *N*-methylhydroxylamine reduction and the substrate dependency are shown in Figures 6 and 7, respectively. Kinetic data are summarized in Table 2.

Neuronal Cell Culture and Amphetamine and Methamphetamine Hydroxylamine Toxicity. Young (i.e., 5-day-old) cultures containing astrocytes, oligodendrocytes, and microglia were incubated in the presence of test compounds for 16 h. Panel A of Figure 8 shows cells sham-treated with vehicle (i.e., ethanol). Panel B shows cell cultures treated with 0.6 mM amphetamine hydroxylamine and panel D cell cultures treated with 0.6 mM methamphetamine hydroxylamine. Although the primary cultures were not fully differentiated, the data show a striking effect for the compounds that were administered. At 0.6 mM, both amphetamine hydroxylamine and methamphetamine hydroxylamine caused frank cell toxicity. Panels C and D of Figure 8 show massive cell death and cytotoxicity.

At a comparable dose and incubation time, amphetamine and methamphetamine showed some detectable cell toxicity but considerably less than their corresponding hydroxylamines (data not shown). Amphetamine oxime was virtually nontoxic at doses that far exceeded that of the hydroxylamines. Compared with healthy control cells (panel A), cells treated with 1.0 mM amphetamine oxime did not show detectable cell toxicity.

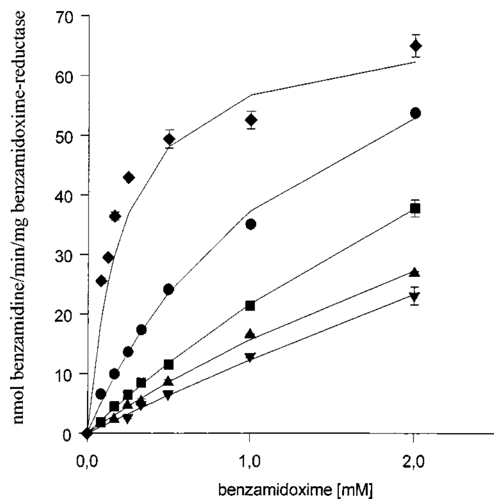


Figure 5. Direct plot of the reduction of benzamidoxime to benzamidine by benzamidoxime reductase and its dependence on different concentrations of *N*-methylhydroxylamine. The incubation mixtures were as described in Materials and Methods in the presence of different concentrations of *N*-methylhydroxylamine. Data are means ± the standard error from four determinations: (◆) no *N*-methylhydroxylamine, (●) 50 μM *N*-methylhydroxylamine, (■) 100 μM *N*-methylhydroxylamine, (▲) 150 μM *N*-methylhydroxylamine, and (▼) 200 μM *N*-methylhydroxylamine.

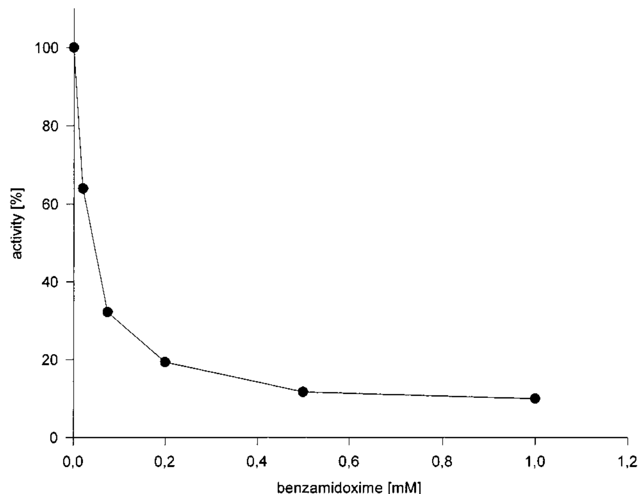


Figure 6. Effect of different concentrations of benzamidoxime on the reduction of *N*-methylhydroxylamine by benzamidoxime reductase. The incubation mixtures were as described in Materials and Methods in the presence of different concentrations of benzamidoxime. The rate of *N*-methylhydroxylamine conversion in the absence of benzamidoxime under control conditions was taken to be 100%. Data are means ± the standard error from four determinations.

Discussion

The objective of this study was to investigate the reduction of aliphatic hydroxylamines to their parent amines by the hepatic benzamidoxime reductase system. On the basis of previous investigations (*5*), the enzyme system is composed of cytochrome b_5, NADH-cytochrome b_5-reductase, and the benzamidoxime reductase. For the reduction of benzamidoxime to benzamidine (*5*), NADH is preferred over NADPH as a cofactor, so the same cofactor was used in the work presented here. The reductions of methamphetamine hydroxylamine, amphetamine hydroxylamine, and *N*-methylhydroxylamine have been carried out for the first time by this enzyme system.

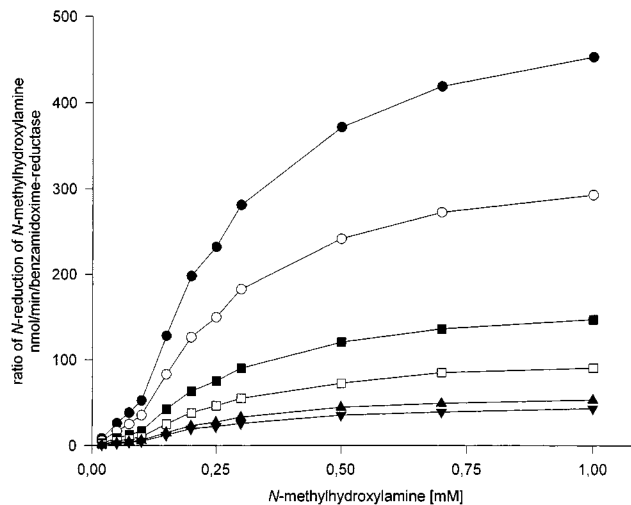


Figure 7. Direct plot of the reduction of *N*-methylhydroxylamine by benzamidoxime reductase and its dependence on different concentrations of benzamidoxime. The incubation mixtures were as described in Materials and Methods in the presence of different concentrations of benzamidoxime. Data are means ± the standard error from four determinations: (●) no benzamidoxime, (○) 0.02 mM benzamidoxime, (■) 0.075 mM benzamidoxime, (□) 0.2 mM benzamidoxime, (▲) 0.5 mM benzamidoxime, and (▼) 1.0 mM benzamidoxime.

Methamphetamine and amphetamine have a high potential for abuse and a significant human toxic potential. The relative rate of hydroxylamine metabolite formation and the relative rate of hydroxylamine reduction to parent amine may be important in the overall toxicity of these compounds (*31*).

While limited, the effect of amphetamine hydroxylamine and methamphetamine hydroxylamine on primary rat brain cell cultures clearly shows that these metabolites are cytotoxic at the concentrations that were used (Figure 8). At the concentrations that were studied, it appeared that methamphetamine hydroxylamine showed greater cell toxicity than amphetamine hydroxylamine. However, the kinetics of the onset of cell toxicity for each compound may be different, and additional studies are needed to clarify this point. It is notable that amphetamine oxime is virtually nontoxic at the dose that was examined. This result is in keeping with the hypothesis that oxime formation is a metabolic detoxication pathway, converting toxic hydroxylamines into nontoxic oximes (*14*). At comparable doses, the parent compounds amphetamine and methamphetamine showed significantly less cytotoxicity. Thus, retroreduction of amphetamine hydroxylamine or methamphetamine hydroxylamine to the amine also represents a detoxication pathway.

The formation of the amines from the reactive metabolites of methamphetamine hydroxylamine and amphetamine hydroxylamine was confirmed in each case by means of an HPLC system coupled to a UV detector (Figures 1 and 2). The reduction of *N*-methylhydroxylamine was analyzed using an indirect spectrophotometric analysis (*19*).

Methamphetamine hydroxylamine, amphetamine hydroxylamine, and *N*-methylhydroxylamine were also reduced by liver microsomes from pigs and humans (Tables 1, 3, and 4). Methamphetamine hydroxylamine was efficiently metabolized by pig liver microsomes, but amphetamine hydroxylamine is more efficiently reduced by human liver microsomes than by pig liver microsomes. In the presence of pig liver microsomes, Kadlubar et al.

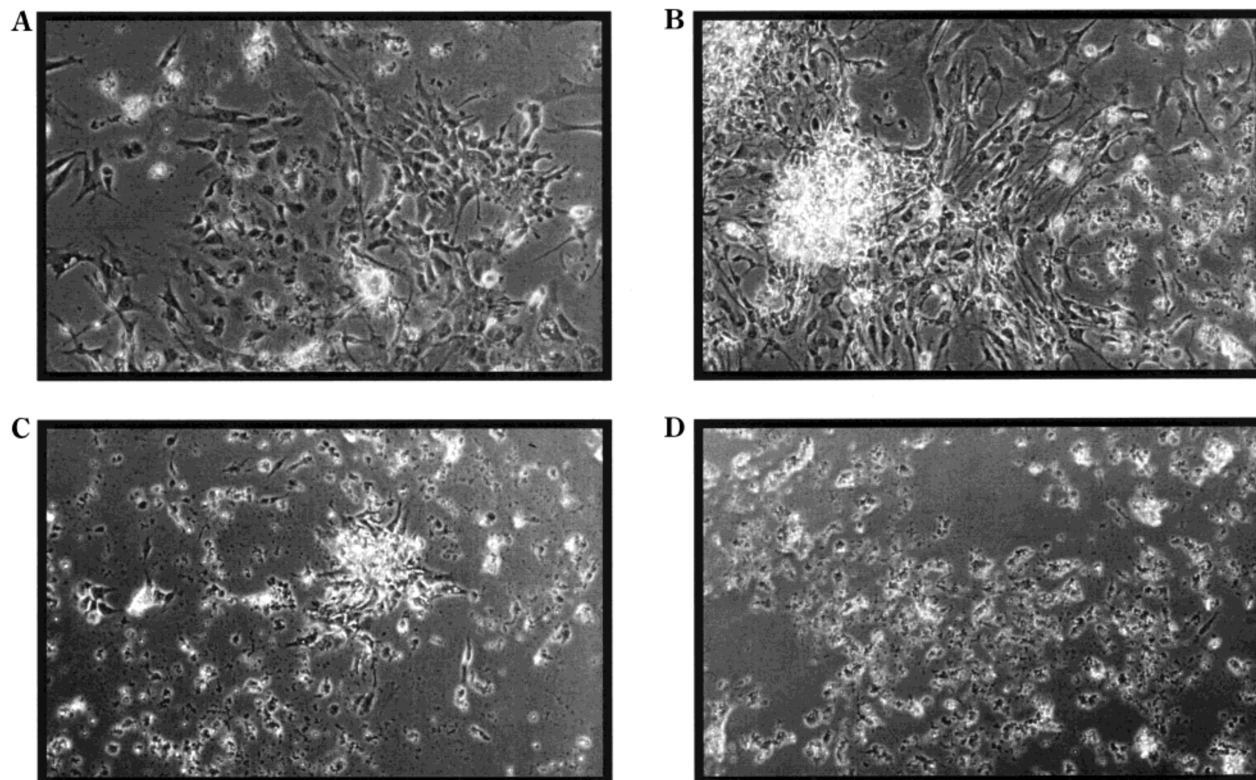


Figure 8. Effect of 0.6 mM amphetamine hydroxylamine (C) or 0.6 mM methamphetamine hydroxylamine (D) on primary rat brain cell cultures. Compared to vehicle-treated control cells (A), cells treated with amphetamine hydroxylamine and methamphetamine hydroxylamine showed massive cell toxicity and cell death. Amphetamine oxime administered to cell cultures at a concentration of 1.0 mM did not show detectable cell toxicity (B).

(*19*) observed a rate of reduction of 16.0 ± 0.5 nmol min^{-1} (mg of protein)$^{-1}$ for *N*-methylhydroxylamine. We conclude that *N*-methylhydroxylamine is more efficiently reduced by human liver microsomes than by pig liver microsomes.

The V_{max}/K_m ratios for the reduction of amphetamine hydroxylamine by the highly purified benzamidoxime reductase and human liver microsomes (Table 2) were lower than the V_{max}/K_m value for the reduction of benzamidoxime to benzamidine [$V_{max}/K_m = 5.3 \times 10^{-4}$ L min^{-1} (mg of bezamidoxime reductase)$^{-1}$] in the same systems (*5*).

The ratios of the reduction by benzamidoxime reductase were significantly higher than the value observed for the reduction by human liver microsomes. The values for the reduction of *N*-methylhydroxylamine were in the same range as those for the reduction of benzamidoxime by benzamidoxime reductase (*5*). In comparison, the level of reduction of benzamidoxime (*5*) was 140% of that of methamphetamine hydroxylamine. In contrast, the level of reduction of amphetamine hydroxylamine was only about 4% of that of benzamidoxime.

The V_{max}/K_m value for the reduction of amphetamine hydroxylamine by benzamidoxime reductase was higher than that for the reduction by human liver microsomes (Table 2). The value for the reduction of amphetamine hydroxylamine was significantly lower than that for the reduction of methamphetamine hydroxylamine. Although both V_{max}/K_m values were lower than the ratio for the reduction of benzamidoxime [$V_{max}/K_m = 5.3 \times 10^{-4}$ L min^{-1} (mg of benzamidoxime reductase)$^{-1}$] (*5*), they were comparable with those of other enzymatic reductions, including that with xanthine oxidase (*32*).

The addition of *N*-methylhydroxylamine in increasing concentrations to incubation mixtures of amphetamine hydroxylamine (Figure 3) and benzamidoxime (Figure 4) effected a corresponding decrease in the rates of conversion. In accordance with studies with *N*-hydroxydebrisoquine (*3*) and guanoxabenz (*4*), the presence of *N*-methylhydroxylamine probably causes inhibition of these reductions.

The kinetic data for the reduction of benzamidoxime to benzamidine in the presence of increasing concentrations of *N*-methylhydroxylamine (Figure 5) and the resulting K_{iu} value of 91.7 μM and the K_{ic} value of 61.1 μM showed all the characteristics of a mixed noncompetitive enzyme inhibition.

The kinetic data for the reduction of *N*-methylhydroxylamine in the presence of increasing concentrations of benzamidoxime (Figure 7) and the resulting *K* values (K_{iu1} = 102.8 μM, K_{ic1} = 6.94 mM, K_{iu2} = 16.9 μM, and K_{ic2} = 49.61 μM) also showed the characteristics of a mixed noncompetitive enzyme inhibition.

According to Kadlubar and Ziegler (*7*), the enzyme system consists of cytochrome b_5, NADH-cytochrome b_5-reductase, and a third unidentified protein. We isolated a P450 2D enzyme from pig liver which in combination with cytochrome b_5 and its reductase reduced benzamidoxime very efficently (*5*) and as shown here also reduces aliphatic hydroxylamines.

The question arose as to which P450 enzyme is involved in the hydroxylamine reduction. In pig liver, it is P450 2D (*5*). However, human microsomes from donors deficient in 2D6 were also able to reduce benzamidoxime (*5*). Studies with inhibitors did not give a hint as to which human P450 enzyme is involved. The reduction of benz-

amidoxime could not be detected with microsomes from cell lines transfected with cDNAs expressing human P450 enzymes (P450 1A1, P450 1A2, P450 2A6, P450 2B6, P450 2C8, P450 2C9, P450 2C19, P450 2D6-Val, P450 2D6-Met, P450 2E1, P450 3A4, and P450 4A11) (*5*). This suggested that a human P450 enzyme not described so far or one that is usually involved in the metabolism of endogenous compounds may be responsible, and the isolation of this enzyme from human liver is the subject of further studies.

The reductive system of the benzamidoxime reductase converts aliphatic hydroxylamines to their corresponding amines more effectively than the oxidative system can convert the amines to the hydroxylamines. Under steady state conditions, the situation in vivo may be that the nontoxic aliphatic amines exist predominantly in the organism.

In this study, we show that the benzamidoxime reductase is involved in the detoxication of reactive hydroxylamines and may be an important path of protection for humans.

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