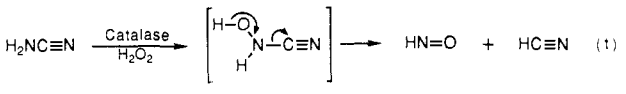
Communications to the Editor

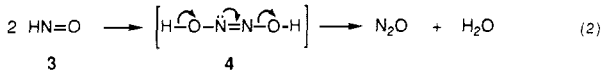
Evidence for Nitroxyl in the Catalase-Mediated Bioactivation of the Alcohol Deterrent Agent Cyanamide

Cyanamide (1), in various pharmaceutical dosage forms, is used as an alcohol deterrent agent in Europe, Canada, and Japan, but not in the U.S. Ethanol ingestion subsequent to 1 elicits a multitude of adverse physiological effects reminiscent of the disulfiram–ethanol reaction (DER),[1] thereby encouraging alcohol avoidance. The biochemical–pharmacological basis for this cyanamide–ethanol reaction is the inhibition of the enzyme aldehyde dehydrogenase (AlDH, EC 1.2.1.3), more specifically, the low-K_m hepatic mitochondrial class II AlDH, resulting in elevated ethanol-derived blood acetaldehyde levels.[2] As purified AlDH is not directly inhibited by 1,[3] it has been suggested that 1 must be bioactivated in vivo.[3a,4] We,[5] and others,[6] have subsequently shown that catalase (and possibly also cytochrome P-450[4c,7]) is the enzyme responsible for the conversion of 1 to an inhibitor of AlDH. On the basis of the observation that cyanide is one of the products of this catalase-mediated oxidation of 1, the intermediacy of *N*-hydroxycyanamide (2) whose decomposition generates nitroxyl (HN=O, nitrosyl hydride, 3), the putative inhibitor of AlDH (eq 1), was postulated.[7,8] Using ^{15}N- and



^{13}C-labeled cyanamide as isotopic tracers for this reaction, we now present persuasive evidence for eq 1 and for the formation of nitroxyl (3) in the oxidative metabolism of 1.

When unlabeled 1 was incubated with bovine liver catalase and a continuous source of H_2O_2 generated from glucose-glucose oxidase, the formation in the headspace (as monitored by GC) of gaseous nitrous oxide, the end product of the dimerization/disproportionation reaction of nitroxyl (3) (eq 2),[9] was observed in a dose-dependent



(Figures 1 and 2) and time-dependent (data not shown) manner. Incubation of uniformly labeled [^{15}N]cyanamide with catalase under the same conditions and analysis by GC/MS of the volatile gaseous product in the headspace[10] gave [^{15}N]N_2O with m/z 46 (relative abundance 100%, Table I). With unlabeled 1, the N_2O had the expected m/z 44. According to eqs 1 and 2, an amino-^{15}N-labeled 1 would be expected to give [^{15}N]N_2O with m/z 46 as the sole isotopically labeled product; however, the nitrous oxide produced in the headspace from [*amino*-^{15}N]-1 had m/z 44, m/z 45, and m/z 46 in relative abundance of 28, 52, and 20%, respectively (Table I). These latter data could readily be explained on the basis of the scrambling of the amino ^{15}N label to the cyano group via a symmetrical carbodiimide intermediate (5)[11] *prior* to oxidative metabolism (Scheme I).[12] Thus, the distribution of the nitrous oxides at m/z 44, 45, and 46 should theoretically be in the ratios of 1:2:1 as experimentally observed.

[^{13}C]Cyanamide was prepared from [^{13}C]thiourea (99 atom % enrichment, Cambridge Isotope Laboratories, Woburn, MA) by stirring a solution of the thiourea in absolute ethanol with $Hg(OAc)_2$. After the removal of the HgS by filtration and concentration of the filtrate, the residue was applied to a silica gel flash column packed in ether. The column was eluted under pressure with ether and the product in the effluent was visualized as spot tests with sodium nitroprusside–potassium ferricyanide reagent. Concentration of the relevant fractions yielded pure [^{13}C]cyanamide as a white solid.

In this next experiment, methemoglobin was used to sequester and concentrate—as cyanomethemoglobin—the [^{13}C]HCN generated in the reaction of [^{13}C]-1 with bovine liver catalase/glucose-glucose oxidase. Fourier transform ^{13}C NMR analysis of the volatile product released from the methemoglobin on acidification, and trapped in 0.10 N KOD/D_2O in the center well of the incubation flask, showed unequivocal evidence for the presence of [^{13}C]HCN (as [^{13}C]KCN, Figure 3). [^{13}C]HCN was not produced when catalase or glucose-glucose oxidase was omitted from the incubation mixture. The product stoichiometry for this reaction is presently under investigation. In previous experiments, we had shown that the catalase reaction that produced HCN from unlabeled 1, as determined colorimetrically, had an apparent K_m of 172 μM (V_{max} 56.3 nmol of cyanide min^{-1} (mg of protein)$^{-1}$), and was not only time- and concentration-dependent on cyanamide but was sub-

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(10) Technical problems prevented the simultaneous determination of ^{15}N-labeled HCN by GC/MS; hence, the use of [^{13}C]cyanamide in latter experiments (vide infra).
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(12) The supplier of this [*amino*-^{15}N]-1 guaranteed the integrity of the ^{15}N distribution *only* if used immediately after receipt. However, due to circumstances beyond our control, the sample was stored at 0 °C for 6 months before use. The supplier acknowledges that this could cause scrambling of the ^{15}N label to a statistical distribution between the amino and cyano group. [*amino*-^{15}N]-1 is no longer available from this supplier.

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Table I. GC and GC/MS Analyses of the Nitrous Oxide Formed in the Oxidation of ^{15}N-Labeled Cyanamide by Catalase/Glucose-Glucose Oxidase[a]

substrate	GC anal. N_2O formed, nmol ± SEM (n)	GC/MS anal.:[b] m/z (rel abundance) 44	45	46
experiment 1				
U-labeled [^{15}N]cyanamide	538 ± 8 (3)	<1.7	<1.7	100[c]
unlabeled cyanamide	497 ± 5 (3)	100[c]	<0.9	<0.9
		100[c,d]	<0.9[d]	<0.9[d]
experiment 2				
$^{15}NH_2$-labeled cyanamide	186 ± 8[e] (3)	28[f]	52[f]	20[f]
unlabeled cyanamide	440 ± 16 (4)			

[a] Experimental details were as described in the legend to Figure 1, except that the incubation mixtures contained 20 μmol of unlabeled or ^{15}N-labeled cyanamide and the incubations were for one hour. U-Labeled [^{15}N]cyanamide (99 atom %) and $^{15}NH_2$-labeled cyanamide (99 atom %) were purchased from ICON Services, Inc., Summit, NJ. [b] After incubation, the samples were heated for 10 min at 55 °C and a 0.6-mL aliquot of the sample headspace was injected onto the column (Tenax-GC, 60–80 mesh, 2 m × $^1/_8$ in. stainless steel column; He carrier gas, 25 mL/min; 50 °C isothermal; 290 °C ion source; LKB 9000 GC–mass spectrometer). The unlabeled and ^{15}N-labeled nitrous oxides were measured with use of limited mass scans and reconstructed ion profiles at m/z 40, 44, 45, and 46. The lower limits of detectability were as indicated. [c] Only observed peak under these experimental conditions and arbitrarily set to 100%. [d] N_2O standard. [e] Value is lower than the control with unlabeled cyanamide because a significant amount of the cyanamide had dimerized to cyanoguanidine on storage. [f] Average of duplicate determinations.

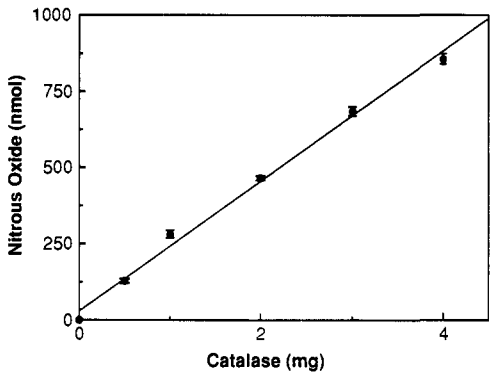


Figure 1. Dependence of N_2O formation (±SEM; n = 3) on catalase concentration. The concentration of cyanamide was 40 mM, and the reactions were analyzed after 1 h. Other details are described as follows. *Incubation Mixture and Reaction Conditions*: The incubation mixture contained potassium phosphate buffer (50 mM, pH 7.0), a continuous H_2O_2 generating system consisting of glucose (10 mM) and glucose oxidase (100 μg, 13.3 units), cyanamide as indicated and bovine liver catalase (2.0 mg, 22000 units, C-40, Sigma Chemical Co., St. Louis, MO) in a total volume of 2.0 mL. The reactions were carried out at 37 °C in 25-mL glass vials equipped with septum seals. The samples were preincubated for 5 min at 37 °C, glucose oxidase was then added followed by catalase 15 s later, and the reaction vessels were sealed. After incubation, the reactions were quenched by the addition of 0.2 mL of 5.5 N perchloric acid through the septa. *Nitrous Oxide Analysis*: N_2O was quantitated by headspace gas chromatography on a Hewlett-Packard Model 5880A gas chromatograph using thermal conductivity detection. N_2O standards were prepared by addition of known amounts of N_2O (9235 ppm prepared in He was purchased from Matheson Gas Products, Chicago, IL) using a gastight syringe (Precision Sampling Corp., Baton Rouge, LA) to samples containing only phosphate buffer and perchloric acid. The samples were stored on ice (4 °C) until analyzed as follows: The sample vials were heated for 10 min at 55 °C, and 0.6 mL of the headspace was injected onto the column (Poropak Q, 80–100 mesh, 6 ft × 2 mm glass column; He carrier gas, 20 mL/min through dual Poropak Q columns, 40 mL/min auxilary He; 30 °C, isothermal).[18] The retention time for N_2O was 1.6 min and peak area was used for quantitation of N_2O.

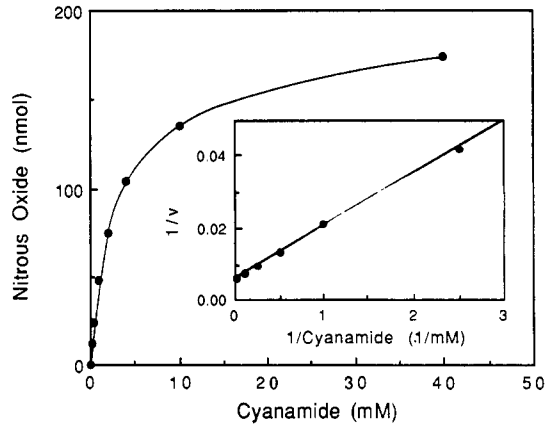
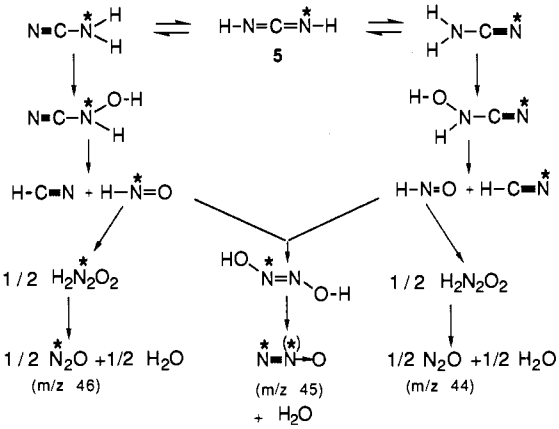


Figure 2. Dependence of N_2O formation on cyanamide concentration. The reactions were carried out as described under Figure 1 using a 3-min incubation time and 4.0 mg of catalase (n = 2). The double-reciprocal plot of the same data is shown in the figure inset. The calculated values for the apparent K_m and V_{max} values for cyanamide were respectively 3.04 mM and 46 mmol of N_2O formed per 3 min per mg of catalase.

Scheme I



ject to inhibition by ethanol.[8]

These results provide support for *N*-hydroxycyanamide (2) as the intermediate in the catalase-mediated oxidative bioactivation of cyanamide (1). Although various peroxidases can catalyze the oxidation of xenobiotic substances in a manner reminiscent of the cytochrome P-450 catalyzed reactions,[13] there is a paucity of information on xenobiotic oxidations catalyzed by catalase in a coupled peroxidatic mode. The oxidation of methanol, ethanol, formaldehyde, azide, hydroxylamine, nitrite, and 3-amino-1*H*-1,2,4-triazole by catalase/H_2O_2,[14] as well as the

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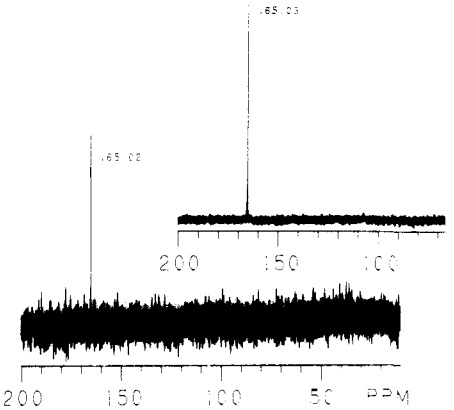

Figure 3. ^{13}C NMR spectrum of the [^{13}C]HCN (as [^{13}C]KCN) formed in the oxidation of [^{13}C]cyanamide by catalase/glucose-glucose oxidase. The inset shows authentic [^{13}C]KCN in 0.1 N KOD/D_2O. Incubations were carried out in sealed Erlenmeyer flasks with a suspended center well (Kontes, Vineland, NJ) containing 400 μL of 0.1 N KOD in D_2O in a shaking water bath at 37 °C for 1 h. The incubation mixture consisted of potassium phosphate buffer (100 mM, pD 7.4), bovine liver catalase (4 mg, 56400 units), ^{13}C-free glucose oxidase (0.1 mg, 10.8 units), glucose (10 mM), [^{13}C]cyanamide (93 μmol), and bovine methemoglobin (16.8 mg) in a total volume of 2.0 mL. The reactions were initiated by the addition of glucose oxidase and were quenched by the addition of 0.5 mL of concentrated phosphoric acid through the rubber septum. This released the [^{13}C]HCN bound to methemoglobin for collection in the center well. After further equilibration at 37 °C for 30 min, the reaction mixture was allowed to stand overnight. The contents of the KOD trap from two identical reactions were then combined for determination of [^{13}C]cyanide by FT/NMR on a Nicolet NT-300WB NMR spectrometer. Control incubations lacked either catalase or glucose oxidase.

demethylation of *N,N*-dimethylaniline and aminopyrine by catalase/organic hydroperoxides, are well documented.[15] However, we are unaware of any reactions of catalase comparable to the postulated N-hydroxylation of cyanamide.

All attempts to prepare **2** chemically have so far been unsuccessful due to its instability. However, a stable *N,O*-dibenzoyl derivative of **2** has now been prepared, and this dibenzoyl derivative has been shown to inhibit yeast AlDH in vitro after bioactivation by esterase action intrinsic to this enzyme.[16] Together with data indicating that *C*-nitroso compounds (RN=O) (which can be considered *substituted* nitroxyls) are also good inhibitors of yeast AlDH without bioactivation[17] and that cyanide in concentrations up to 5 mM does not inhibit the enzyme, the present results lend credence to our hypothesis[7,8] that nitroxyl (**3**) produced in the oxidation of **1** is *the* inhibitor of AlDH.

Acknowledgment. Supported by the Department of Veterans Affairs and by ADAMHA Grant 1R01-AA07317. We are indebted to Thomas P. Krick for the GC/MS and the ^{13}C NMR spectra. The GC/MS and NMR facilities are provided by the Biochemistry Department of the University of Minnesota and maintained by the Minnesota Agricultural Experiment Station.

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[†]VA Medical Center.
[‡]University of Minnesota.

H. T. Nagasawa,*[†,‡] E. G. DeMaster,[†] B. Redfern[†]
F. N. Shirota,[†] D. J. W. Goon[‡]
Medical Research Laboratories
VA Medical Center
and Department of Medicinal Chemistry
University of Minnesota
Minneapolis, Minnesota 55417
Received July 20, 1990

Synthesis and Dopamine Receptor Affinity of (*R*)-(−)-2-Fluoro-*N*-*n*-propylnorapomorphine: A Highly Potent and Selective Dopamine D_2 Agonist

(*R*)-(−)-Apomorphine (APO) and its *N*-*n*-propyl analogue (*R*)-(−)-*N*-*n*-propylnorapomorphine (NPA) are considered standard centrally active dopamine (DA) agonists.[1,2] Our past efforts have focused on delineating the portions of the aporphine molecular structure that are critical to interactions with DA receptors and responsible for dopaminergic properties with a goal of developing more potent and selective agonists or antagonists. Previously a series of novel 2-substituted *R*-(−) and *S*-(+) apomorphine derivatives were prepared and evaluated as ligands for DA receptors in mammalian brain. (*R*)-(−)-2-Fluoroapomorphine (2-F-APO), *R*-(−)-2-OCH_3-NPA (**4**), and 2-OH-NPA (**3**) were found to be relatively potent and selective for the D_2 receptor subtype.[3,4] To further elucidate the structural requirements of fluorine-substituted apomorphines for DA receptors, we now report the synthesis and preliminary biological evaluation of (*R*)-(−)-2-fluoro-*N*-*n*-propylnorapomorphine (2-F-NPA, **2**) and its comparison with other analogues for affinity and selectivity to D_1 and D_2 receptor sites in corpus striatum tissue from rat forebrain (Figure 1).

Chemistry

Synthesis of 2-F-NPA (**2**, Figure 1) was achieved by minor modifications of the procedure developed for the synthesis of 2-fluoroapomorphine.[4] The desired starting material for this sequence was the previously reported precursor 2-hydroxy-10,11-(methylenedioxy)-*N*-*n*-propylnoraporphine (**7**), which was prepared in five high-yielding steps from the opium alkaloid thebaine.[3] Conversion of the phenolic group at the 2-position in **7** to the key intermediate, the 2-aminoaporphine **11**, was achieved via a modified Smiles rearrangement reaction[5] (Scheme I).

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