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Nitrosyl Cyanide, a Putative Metabolic Oxidation Product of the Alcohol-Deterrent Agent Cyanamide

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ABSTRACT. When incubated with catalase/glucose-glucose oxidase, ^{13}C-labeled cyanamide gave rise not only to ^{13}C-labeled cyanide, but also to ^{13}C-labeled CO_2. Moreover, a time-dependent formation of nitrite was observed when cyanamide was oxidized in this system. These results suggested that the initial product of cyanamide oxidation, viz. *N*-hydroxycyanamide, was being further oxidized by catalase/H_2O_2 to nitrosyl cyanide (O=N−C≡N). Theoretically, nitrosyl cyanide can hydrolyze to the four end-products detected in the oxidative metabolism of cyanamide *in vitro*, viz. nitroxyl, cyanide, nitrite, and CO_2. Accordingly, both unlabeled and ^{13}C-labeled nitrosyl cyanide were synthesized by the low temperature (−40 to −50°) nitrosylation of K-(18-crown-6)cyanide with nitrosyl tetrafluoroborate. The product, a faint blue liquid at this temperature, was transferred as a gas to phosphate-buffered solution, pH 7.4, where it was solvolyzed. Analysis of the headspace by gas chromatography showed the presence of N_2O, the dimerization/dehydration product of nitroxyl, while cyanide was detected in the aqueous solution, as measured colorimetrically. [^{13}C]CO_2 was analyzed by GC/MS. An oxidative biotransformation pathway for cyanamide that accounts for all the products detected and involving both *N*-hydroxycyanamide and nitrosyl cyanide as tandem intermediates is proposed. BIOCHEM PHARMACOL 52;1:141–147, 1996.

KEY WORDS. aldehyde dehydrogenase; cyanamide; biotransformation; nitroxyl; nitrosyl cyanide; catalase; nitrite

Cyanamide [$H_2N-C\equiv N$], in the form of its citrated calcium salt, calcium carbimide (Abstem®, Dipsan®), is used as an alcohol-deterrent agent in Europe and Canada, while an aqueous formulation of cyanamide is used in Japan. Cyanamide is not approved for use in the U.S. When ingested with ethanol, cyanamide elicits a multitude of adverse physiological reactions known as the carbimide-ethanol reaction that is presumed to discourage the drinking of alcohol [1]. Cyanamide is known to be bioactivated *in vivo* to an inhibitor of the low K_m hepatic mitochondrial AlDH§ (Class 2) [2, 3], which blocks the metabolism of acetaldehyde, thereby raising its blood levels and provoking the carbimide–ethanol reaction. We [4–6] and others [7] have shown that the enzyme catalase in the presence of H_2O_2 oxidizes cyanamide to *N*-hydroxycyanamide, which spontaneously decomposes to cyanide and nitroxyl (HN=O) (Scheme 1, Paths a and b). Nitroxyl is the active species responsible for the inhibition of the low K_m hepatic mitochondrial AlDH [8].

In the present study, we demonstrated that ^{13}C-labeled cyanamide is metabolized not only to nitroxyl and ^{13}C-labeled cyanide [8], but also to [^{13}C]CO_2. Moreover, a time-dependent formation of *nitrite* suggested that the initially formed *N*-hydroxycyanamide was being metabolized further by catalase/H_2O_2 to nitrosyl cyanide (O=N−C≡N), whose solvolysis would lead to the products observed (Scheme 1). This will be confirmed by the actual synthesis of unlabeled and ^{13}C-labeled nitrosyl cyanide and identification of the hydrolysis products formed in phosphate buffer at pH 7.4.

MATERIALS AND METHODS

Materials

^{13}C-Labeled KCN (99 atom% ^{13}C), cyanamide, bovine liver catalase, glucose, and glucose oxidase were purchased from the Sigma Chemical Co., St. Louis, MO. Uniformly labeled ^{15}N-cyanamide (99 atom% ^{15}N) was purchased from ICON Services Inc., Summit, NJ. Anhydrous acetonitrile, 18-crown-6, and nitrosyl tetrafluoroborate were purchased from the Aldrich Chemical Co., Milwaukee, WI. N_2O standards prepared in helium were purchased from Toll, Minneapolis, MN. The carbon dioxide standard prepared in helium was purchased from the Aldrich Chemical Co. All other chemicals were reagent grade.

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§ *Abbreviation*: AlDH, aldehyde dehydrogenase.

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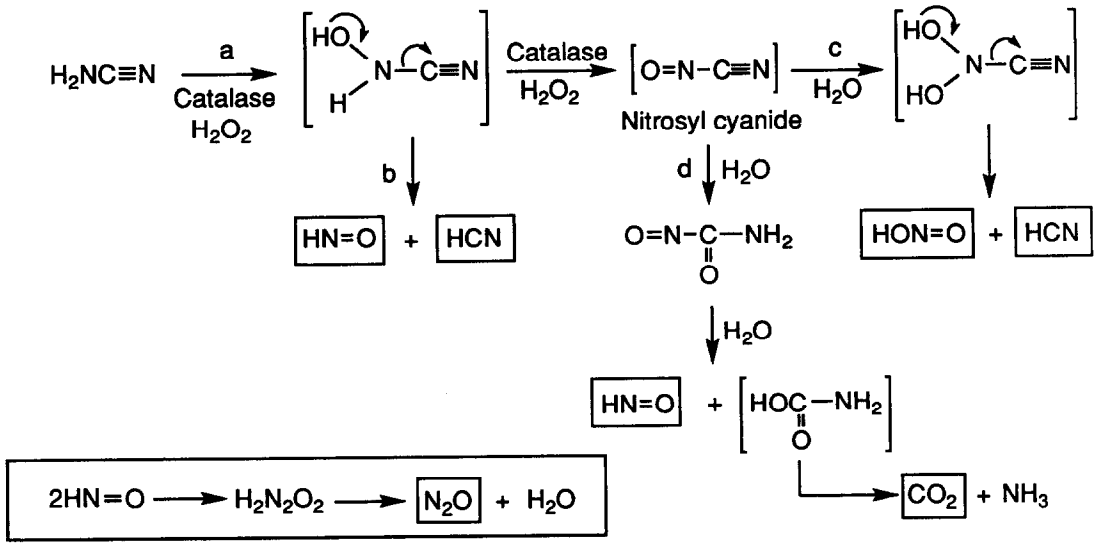

SCHEME 1. Biotransformation pathway for cyanamide mediated by catalase/H_2O_2 *in vitro*. The products identified are enclosed in small rectangles. The dimerization/dehydration reaction of nitroxyl giving N_2O as the final product is shown at the bottom (left) of the scheme.

Synthesis of ^{13}C-Labeled Cyanamide

This labeled cyanamide was prepared from [^{13}C]thiourea as previously described [8].

Synthesis of ^{13}C-Labeled ON-CN and Solvolysis in Phosphate Buffer

[^{13}C]KCN (3.043 mmol), and 18-crown-6 (0.166 mmol) were stirred in anhydrous acetonitrile at room temperature for 10 min. The mixture was cooled in a dry ice/acetonitrile bath for 5 min, and nitrosyl tetrafluoroborate (2.01 mmol) was added all at once. The reaction mixture was stirred for 5 min. The solution was frozen in liquid nitrogen, and the flask was evacuated. Then the system was closed to the vacuum pump, the receiving flask was cooled in liquid nitrogen, and the reaction flask was warmed in dry ice/acetonitrile. The blue-green product (ON-CN) was vacuum transferred from the receiving flask to a 25-mL round-bottom flask containing 4 mL of 100 mM potassium phosphate buffer, pH 7.4, by equilibration. After 30 min, the vacuum was released by the slow introduction of argon. The flask containing the frozen product was removed immediately, sealed with a rubber septum, and kept frozen until assayed for N_2O.

Essentially the same procedure was used to prepare unlabeled ON-CN, which was also hydrolyzed directly.

NMR Analysis of the Products of [^{13}C]Cyanamide Oxidation by Catalase/H_2O_2

Incubations were carried out as previously described [8]. Cyanide and CO_2 were trapped in 0.1 N KOD/D_2O in sealed Erlenmeyer flasks with a suspended center well (Kontes Glass Co., Vineland, NJ) and analyzed by FT-NMR on a Nicolet NT-300WB NMR spectrometer.

Nitroxyl

Nitroxyl formation was monitored by assaying N_2O, its stable dimerization/dehydration product. N_2O was quantitated by headspace gas chromatography on a Hewlett–Packard model 5880A gas chromatograph using thermal conductivity detection essentially as described previously [8]. The reaction flasks were incubated at 37° for 60 min before injecting 0.6 mL of the headspace gas onto the column (Poropak Q, 80/100 mesh, 6 ft × 2 mm glass column; helium carrier gas, 20 mL/min, through dual Poropak Q columns; 40 mL/min auxiliary helium; 30° isothermal). Retention time for N_2O was 2.6 min, and the peak area was used for quantitation. Results are the mean ± SEM of triplicate analyses.

CO_2

CO_2 was quantitated by headspace gas chromatography under the same conditions described above for the N_2O assay. Serum vials containing 1.0 mL of sample and 1.0 mL of distilled deionized water were flushed with helium and stoppered. Acid (0.5 mL of concentrated phosphoric acid) was added through the rubber septum, and an aliquot of the headspace gas (0.6 mL) was assayed for CO_2 after incubation at 37° for 60 min. Retention time for CO_2 was 2.0 min, and the peak area was used for quantitation. Results are the means ± SEM of triplicate analyses.

Cyanide

Cyanide was determined spectrophotometrically by the method of Epstein [9], using known concentrations of cyanide as standards.

Nitrite Formation From Cyanamide

The standard incubation mix contained 50 mM potassium phosphate buffer, pH 7.0; a hydrogen peroxide generating system composed of 10 mM glucose and 100 μg glucose oxidase; and 2.0 mg of bovine liver catalase and 40 mM cyanamide in a total volume of 1.0 mL. The reaction mixtures were preincubated for 5 min (37°); then glucose oxidase was added followed by catalase 15 sec later, and the samples were incubated (37°) for the times indicated. The reactions were stopped by the addition of 0.1 mL of 5.5 N $HClO_4$. The samples were neutralized with 0.2 mL of 3.0 N KOH, allowed to stand for 15 min at 4°, and then centrifuged to remove the precipitated protein and $KClO_4$. The supernatant was removed for nitrite analysis.

Nitrite

Nitrite was quantitated spectrophotometrically using the Greiss reagent [10]. A 0.67-mL aliquot was added to 0.33 mL of the Greiss reagent (equal parts 0.1% aqueous 1-naphthylethylenediamine dihydrochloride and 1% sulfanilic acid in 5% H_3PO_4). After 10 min, the samples were read at 546 nm, and the absorbance values were converted to concentrations based on a standard curve.

Determination of [^{13}C]CO_2 by GC–MS

Analyses were performed on a Kratos model MS-25 GC/mass spectrometer. After heating for 10 min at 55°, a 0.1-mL aliquot of the headspace was injected onto the column (GS-Q capillary column, 0.53 mm i.d. × 30 m; helium carrier gas; flow rate, 1.4 mL/min; column head pressure, 0.2 kg/mL; 31° isothermal; split mode; 290° ion source). The mass spectrometer was operated in electron impact ionization mode and scanned from 17 to 131 a.m.u. with an ionization energy of 70 eV. Unlabeled and ^{13}C-labeled CO_2 were measured by use of limited scans and reconstructed ion profiles at m/z 44, 45, and 46. Percent [^{13}C]CO_2 was calculated by dividing the [^{13}C]CO_2 peak area (m/z 45) by the total CO_2 peak. Results are the means ± SEM of three separate experiments.

Inhibition of AlDH by Isoamylnitrite and Isoamylnitrate

Yeast AlDH was incubated for 10 min at 37° in a primary reaction mixture containing inhibitor (prepared in DMSO and added in 5-μL aliquots), 1.0 mM NAD^+, 0.06 I.U. of yeast AlDH, and 100 mM potassium phosphate buffer, pH 7.4, in a total of 0.1 mL. The primary mixture was preincubated for 5 min at 37°, and the reaction was initiated by the addition of AlDH followed by inhibitor 5 sec later. After the 10-min incubation, a 20-μL aliquot was removed for the determination of remaining AlDH activity as previously described [5].

RESULTS

When ^{13}C-labeled cyanamide was incubated with catalase/glucose-glucose oxidase, the latter for the continuous generation of H_2O_2, and the volatile products collected in a well containing 0.1-N KOH/D_2O, ^{13}C-labeled cyanide was detected (as [^{13}C]KCN) by NMR spectroscopy at δ 165.0 ppm (Fig. 1). Another volatile ^{13}C-labeled product was also present at δ 168.2 ppm. This product was identified as [^{13}C]K_2CO_3 by comparison with a sample of K_2CO_3 with naturally abundant ^{13}C (Inset A, Fig. 1), while authentic [^{13}C]KCN had a chemical shift at δ 165.2 ppm (Inset B, Fig. 1). The identity of the two volatile ^{13}C-labeled products trapped in base was therefore confirmed as HCN and CO_2.

The formation of CO_2 as a product of cyanamide metabolism *in vitro* was totally unexpected since we did not detect this product in a previous study [8] (where a different trapping agent was used). This suggested that *N*-hydroxycyanamide may *not* be the penultimate product in the catalase-mediated oxidation of cyanamide. Indeed, the formation of nitrite was also observed when unlabeled cyanamide was incubated with catalase under the same conditions (data not presented). Nitrite formation was dependent on the presence of catalase and H_2O_2, and heat-treated catalase was inactive. Nitrite formation was also time dependent (Fig. 2), but the rate decreased after 60 min. This can be ascribed to the further oxidation of nitrite by catalase [11], as verified in Fig. 3. The amount of nitrite formed from cyanamide decreased as the concentration of cyanamide was increased (data not shown).

Analysis of the above data suggested that *N*-hydroxycyanamide, the initial oxidation product of cyanamide, was being further oxidized by catalase/H_2O_2 to nitrosyl cyanide (O=N–C≡N) and the latter was being hydro-

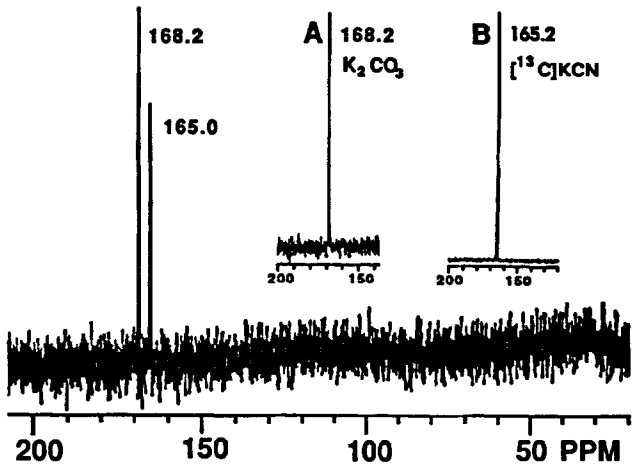

FIG. 1. ^{13}C-NMR spectrum of the [^{13}C]HCN (as [^{13}C]KCN) and [^{13}C]CO_2 (as [^{13}C]K_2CO_3) formed in the oxidation of [^{13}C]H_2NCN by catalase/glucose oxidase. Inset A shows the ^{13}C-spectrum (natural abundance) of K_2CO_3 in 0.1 N KOD/D_2O. Inset B shows authentic [^{13}C]KCN in 0.1 N KOD/D_2O.

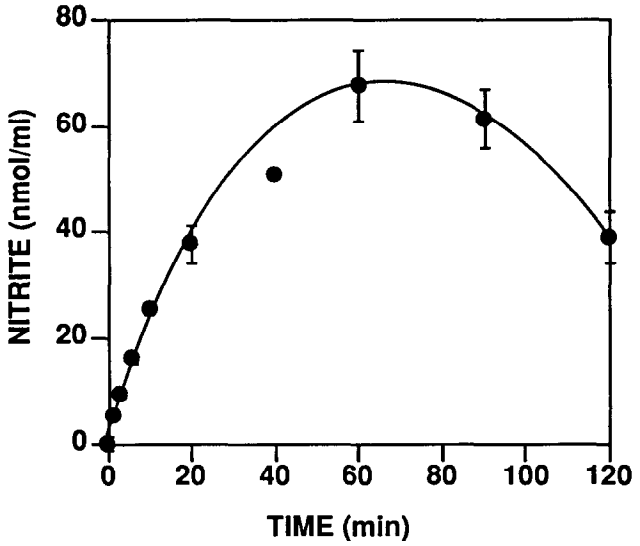

FIG. 2. Nitrite formation from cyanamide over time. Values are the means ± SEM of three determinations with the exception of the determination at 120 min (N = 2). The experimental conditions were as described under Materials and Methods.

lyzed to the products observed, as depicted in Scheme 1 (identified products enclosed in small rectangles).

To test this hypothesis, it was necessary to chemically synthesize ^{13}C-labeled nitrosyl cyanide. Although the preparation of this compound—by the reaction of nitrosyl chloride with unlabeled AgCN or [^{13}C]AgCN—has been reported in the literature [12, 13], the paucity of commercial sources of [^{13}C]AgCN precluded this synthetic route, and the alternative scheme of nitrosylating the readily available [^{13}C]KCN with nitrosyl tetrafluoroborate was implemented. The insolubility of the former in organic solvents required the use of the crown ether reagent, 18-crown-6 [14], to solubilize the cyanide and render it more nucleophilic. Nitrosylation of K-(18-crown-6)cyanide with nitrosyltetrafluoroborate in acetonitrile at low temperature gave nitrosyl cyanide (Equation 1), a faint blue liquid at this temperature [13], which was readily vacuum-transferred as a gas to a phosphate-buffered solution, pH 7.4. The method of synthesis, the color of the product, and its volatility at low temperature [12, 13] indicated that nitrosyl cyanide was, in fact, produced in this reaction. Following solvolysis, nitroxyl was detected as N_2O (Scheme 1, bottom left rectangle) by headspace gas chromatography, while cyanide and nitrite were identified in the aqueous phase by colorimetric analyses specific for these ions.

$$K^+[18\text{-crown-6}]\text{-}CN^- + NOBF_4 \xrightarrow[CH_3CN]{\text{anhydrous}} ON\text{-}CN + KBF_4 \qquad (1)$$

The corresponding ^{13}C-labeled nitrosyl cyanide was prepared from [^{13}C]KCN in an identical manner as above. After hydrolysis in 0.1 M phosphate buffer, pH 7.4, the medium was made alkaline in order to sequester the labeled cyanide as [^{13}C]NaCN and the labeled CO_2 as [^{13}C]Na_2CO_3. Analysis of the headspace showed the presence of N_2O, confirming that nitroxyl was also one of the hydrolysis products of nitrosyl cyanide. The [^{13}C]CO_2 released on acidification was subjected to gas chromatography/mass spectrometry with selective ion monitoring at m/z 44, 45, and 46. Whereas only a trace of m/z 45 peak due to [^{13}C]CO_2 was present when unlabeled nitrosyl cyanide was hydrolyzed (Fig. 4B), this [^{13}C]CO_2 peak from the hydrolysis of ^{13}C-labeled nitrosyl cyanide amounted to 45% of the total CO_2 analyzed (Fig. 4A). A small amount of N_2O was also detected. N_2O and [^{12}C]CO_2, both m/z 44, were readily separable in this system (Fig. 4C).

These data were corroborated by comparison of the electron ionization mass spectrum of the CO_2 liberated from unlabeled nitrosyl cyanide with the spectrum of the CO_2 formed from the hydrolysis of ^{13}C-labeled nitrosyl cyanide (Fig. 5). It can be seen that whereas [^{13}C]CO_2 from the unlabeled compound was essentially undetectable under these conditions (Fig. 5B), substantial amounts of [^{13}C]CO_2 at m/z 45 was produced from the ^{13}C-labeled nitrosyl cyanide (Fig. 5A).

The overall data are summarized in Table 1. The apparent equivalence of [^{13}C]CO_2, nitroxyl, and cyanide liberated from ^{13}C-labeled nitrosyl cyanide appears to be fortuitous since the ratio of nitroxyl/cyanide from the unlabeled compound was 0.34. In fact, since the relative rates of hydrolysis of nitrosyl cyanide via the two pathways involved (Paths c and d, Scheme 1) are not known, such stoichiometry cannot be expected.

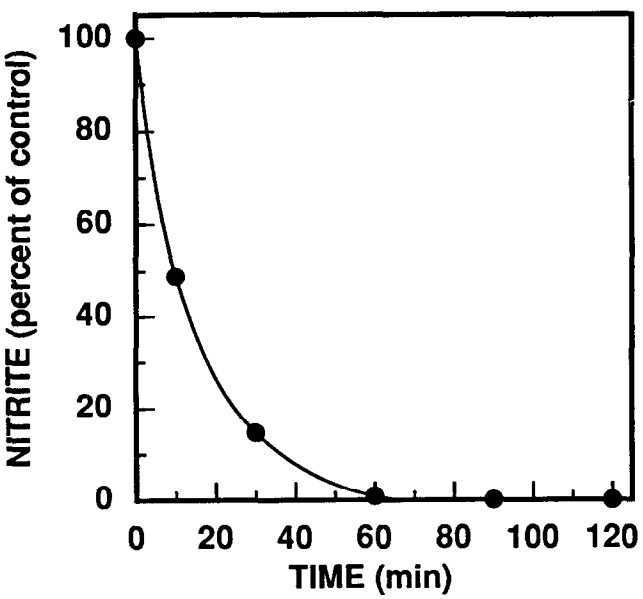

FIG. 3. Oxidation of nitrite by catalase/H_2O_2. The incubation mix and conditions were as described under Materials and Methods for the oxidation of cyanamide to nitrite, except that nitrite (75 μM) replaced cyanamide as substrate (N = 2). In the absence of catalase and glucose/glucose oxidase, nitrate levels were constant over 60 min.

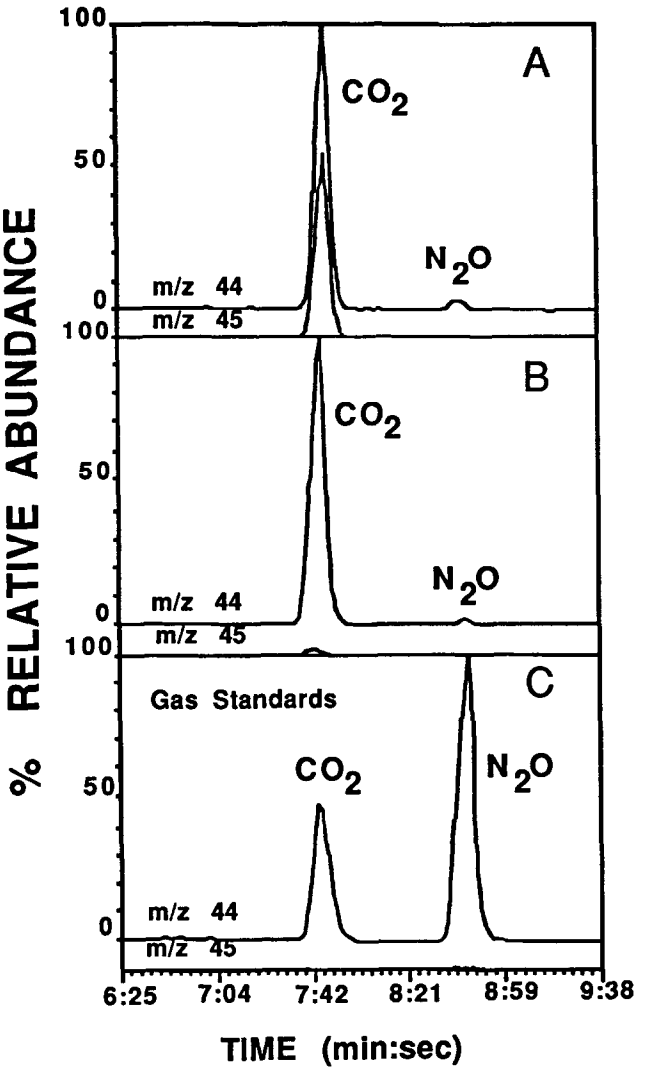

FIG. 4. Ion profiles of the CO_2 liberated from the hydrolysis of ^{13}C-labeled and unlabeled nitrosyl cyanide with selective ion monitoring at *m/z* 44 ([^{12}C]CO_2) and *m/z* 45 ([^{13}C]CO_2). (A) From ^{13}C-labeled ON-CN. (B) From unlabeled ON-CN. (C) Separation of gas standards [^{12}C]CO_2 (*m/z* 44) from N_2O (*m/z* 44) by GC-MS. Retention times were not exactly reproducible due to instrumental parameters.

DISCUSSION

Tracer studies using ^{13}C-labeled cyanamide revealed that not only was the metabolically produced cyanide labeled, ^{13}C-labeled carbon dioxide was also detected as one of the end-products of the oxidative metabolism of cyanamide catalyzed by catalase/H_2O_2. In earlier studies using ^{15}N-labeled cyanamide [8], we had established that the label on both [U^{15}N]H_2NCN and amino-labeled [^{15}N]H_2NCN were incorporated into [^{15}N]HNO (measured as [^{15}N]N_2O), suggesting that Scheme 1, Paths a and b, with *N*-hydroxycyanamide as intermediate, was a viable metabolic pathway. However, in view of the identification of nitrite and CO_2 as additional products of cyanamide metabolism, a unifying mechanism was needed wherein the overall process would accommodate *all* of these products.

TABLE 1. Solvolysis of ^{13}C-labeled and unlabeled nitrosyl cyanide in phosphate buffer, pH 7.4

Product	^{13}C-Labeled ON-CN (μmol)	Unlabeled ON-CN (μmol)
[^{13}C]CO_2	18.17 ± 0.22*	0.29 ± 0.01*
Nitroxyl	14.27 ± 0.27	2.66 ± 0.03
Cyanide	14.18 ± 0.97	7.80 ± 0.37

Results are means ± SEM (N = 3).

* Percent [^{13}C]CO_2 in the CO_2 peak after the solvolysis of ^{13}C-labeled ON-CN was 36.92 ± 0.86, while solvolysis of unlabeled ON-CN gave only 1.17 ± 0.02.

By invoking a further oxidation of *N*-hydroxycyanamide to nitrosyl cyanide followed by solvolysis of the latter, the formation of the four products identified (enclosed in small rectangles in Scheme 1) can be rationalized. Indeed, when unlabeled and ^{13}C-labeled nitrosyl cyanide were prepared and hydrolyzed in phosphate buffer at pH 7.4, three products identified from the oxidation of cyanamide itself, viz. nitroxyl, cyanide, and CO_2, were shown to be formed with their origin clearly traced to nitrosyl cyanide. The identification of nitrite following the solvolysis of nitrosyl cyanide was equivocal (⩾100% of expected levels, data not presented) because the presence of the latter interfered with the Greiss test (*vide infra*). The present results, in conjunction with the previous tracer studies [8], offer support for Scheme 1 as the overall metabolic path taken by cyanamide.

The extraordinarily high nitrite levels found can be explained, in part, as follows. The assumption had been made—without having any precedent for comparison—that nitrosyl cyanide would be hydrolyzed completely at pH 7.4 under the conditions of the experiment (see Materials and Methods). However, this assumption is probably incor-

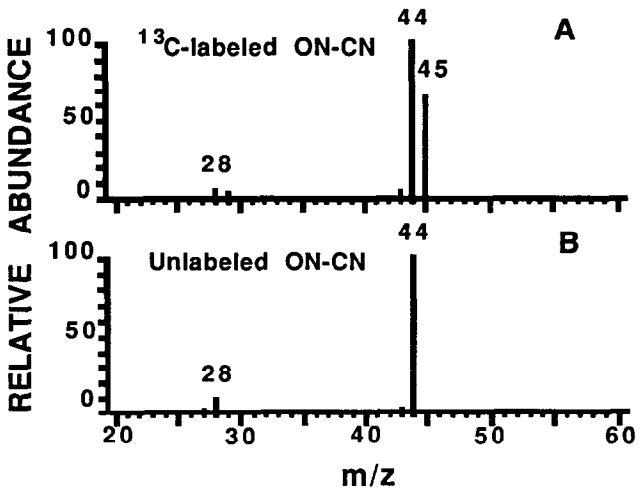

FIG. 5. Electron ionization mass spectra of the CO_2 peaks from the hydrolysis of: (A) ^{13}C-labeled ON-CN and (B) unlabeled ON-CN.

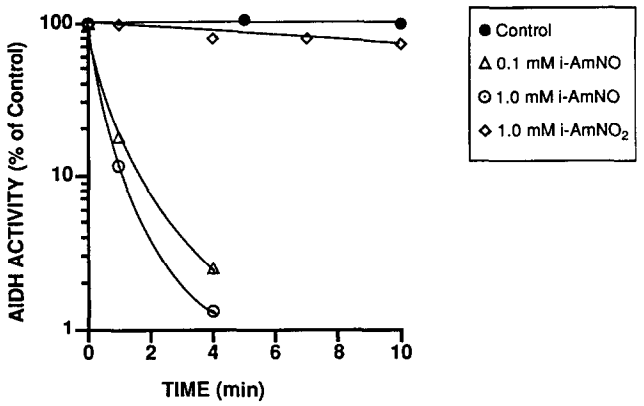

FIG. 6. Inhibition of AlDH by isoamyl nitrite (i-AmNO) and isoamyl nitrate (i-AmNO$_2$). Specific activity of yeast AlDH = 3.6 units/mg protein (pH 7.4), using benzaldehyde as substrate. Results are from a single run (N = 1) and represent typical time courses for these concentrations of i-AmNO and i-AmNO$_2$.

rect. The fact that [^{13}C]CO_2 liberated from [^{13}C]ON-CN amounted to only ~1% of the theoretically possible value suggests that the rate of hydrolysis under these conditions was considerably slower than anticipated, and that a large amount of nitrosyl cyanide must still have remained unhydrolyzed. Based on its structural similarity to nitrosyl benzenesulfonyl ($C_6H_6SO_2NO$) which is one of the most powerful nitrosylating agents known [15], the unhydrolyzed nitrosyl cyanide would also be expected to be a nitrosylating agent and would, therefore, compromise the Greiss test for nitrite.*

On the basis of the above data, the molecular mechanism involved in AlDH inhibition by cyanamide metabolism must be reassessed, and consideration must be given to the fact that not only can the active site of the enzyme be altered by reaction with HNO†, *nitrosylation* of the active site by nitrosyl cyanide is also possible. Since the active site amino acid of AlDH is known to be Cys-302 [16, 17], reaction of nitroxyl with this sulfhydryl group of the enzyme would produce an RSNHOH moiety, while nitrosylation would give an enzyme thionitrite, RSN=O. That S-nitrosylation of AlDH leads to profound enzyme inhibition is indicated by the data of Fig. 6, which shows that isoamyl nitrite, an organic S-nitrosylating agent similar to *t*-butylnitrite [18], rapidly inhibited yeast AlDH when incubated with the enzyme. In contrast, isoamyl*nitrate*, which is not a nitrosylating agent, was inactive.

In summary, the oxidation of cyanamide catalyzed by catalase/H_2O_2 leads to the generation of N_2O, cyanide, nitrite, and CO_2 as products, and by chemical deduction (Scheme 1), ammonia (which was not analyzed). The formation of these products can be rationalized by invoking both *N*-hydroxycyanamide and nitrosyl cyanide as tandem intermediates in the oxidation of cyanamide by catalase/H_2O_2. Both can generate nitroxyl, an inhibitor of AlDH, while nitrosyl cyanide may itself inhibit the enzyme by virtue of its nitrosylating propensity.

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* Another possibility is that the nitroso carbamoyl intermediate (Scheme 1, Path d) is the active nitrosylating species. The fact that the blue color of nitrosyl cyanide was dissipated immediately when dissolved in the phosphate buffer is supportive of this latter possibility. However, more chemical evidence on the solvolytic stability of nitrosyl cyanide needs to be provided to resolve this question. We hydrolyzed nitrosyl cyanide *in situ* immediately after preparation because of its known explosive properties on purification [12].

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