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A NITRITE-REDUCING ENZYME FROM *Nitrosomonas europaea*

PRELIMINARY CHARACTERIZATION WITH HYDROXYLAMINE AS ELECTRON DONOR

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SUMMARY

1. A soluble enzyme which catalyzes the reduction of nitrite in the presence of hydroxylamine has been extracted from Nitrosomonas cells and partially purified.

2. The hydroxylamine–nitrite reductase was also able to utilize leuco-pyocyanine as an electron donor.

3. The enzyme had a pH optimum for HNO_2 reduction of 5.75 and approximate K_m values for HNO_2 and NH_2OH of 1.6 mM and 0.24 M, respectively.

4. The net evolution of approx. 1 mole of gas and disappearance of 1.8 moles of hydroxylamine occurred per mole of nitrite utilized during the reaction. The compounds N_2O and NO were identified as the products of nitrite reduction.

5. Nitrite reduction by hydroxylamine was inhibited 50% by 2 μM diethyldithiocarbamate, 8 μM α,α'-dipyridyl, $2 \cdot 10^{-4}$ M KCN or 1.5 mM NaN_3.

6. The partially purified enzyme fraction contained mammalian cytochrome *c* oxidase, hydroxylamine oxidase, hydroquinone oxidase and NH_2OH–cytochrome *c* reductase activity and catalyzed the production of HNO_2 from NH_2OH.

7. The rate of O_2 utilization coupled to hydroxylamine oxidation was inhibited in the presence of nitrite, whereas the rate of aerobic hydroxylamine disappearance was stimulated approx. 100% in the presence of nitrite.

8. Hydroxylamine–nitrite reductase (hydroxylamine:nitrite oxidoreductase) appears to involve the combined action of Nitrosomonas hydroxylamine dehydrogenase and nitrite reductase.

INTRODUCTION

Nitrosomonas europaea catalyzes the oxidation of NH_3 to nitrite with hydroxylamine as a probable intermediate[1]. In the presence of O_2, extracts catalyze the conversion of hydroxylamine to nitrite[2], whereas in the absence of O_2 but in the presence of substrate quantities of mammalian cytochrome *c* (ref. 3) or methylene blue[4], hydroxylamine is converted to N_2O or a mixture of N_2O and NO, respectively. Hydroxylamine dehydrogenase partially purified from extracts of both Nitrosomonas

and *Nitrosocystis oceanus* has been shown to catalyze the reduction of 2 moles of mammalian cytochrome *c* per mole of hydroxylamine oxidized indicating that a compound of the oxidation state of NO_2 and N_2O is produced from hydroxylamine[5]. It is thought that the product of hydroxylamine oxidation, perhaps enzyme-bound NO_2, is oxidized to nitrite with the overall stoichiometry of the addition of water followed by the transfer of 2 hydrogen atoms to molecular O_2 (refs. 4, 5).

As reported in the present work, a supernatant fraction of Nitrosomonas extracts contains most of the cell-free ability to catalyze the aerobic oxidation of hydroxylamine to nitrite but also contains an enzyme which catalyzes the disappearance of nitrite in the presence of hydroxylamine. This hydroxylamine:nitrite oxidoreductase has been partially purified and shown to require O_2 and cause the evolution of a net 1 mole of gas (N_2O and NO) per mole of nitrite utilized. Under anaerobic conditions, the enzyme catalyzes the reduction of nitrite by chemically reduced pyocyanine. The enzyme-catalyzed reaction of hydroxylamine, nitrite and O_2 is thought to involve the action of hydroxylamine dehydrogenase and an enzyme which reduces nitrite to NO and N_2O. A preliminary report of this work has been made previously[6].

MATERIALS AND METHODS

Growth of cells

Nitrosomonas cells were grown as described previously in batch cultures[5] or semi-continuously[7]. When grown in batch culture, the nitrite concentration of the growth solution increased from 0.5 mM to 20 mM over the 7–8-day growth period, whereas the nitrite concentration varied daily between 10 and 20 mM in the growth solution of cultures growing semi-continuously. Utilizing either method of growth, the number of bacteria doubled each 24 h during logarithmic phase of growth as indicated by the change of nitrite concentration. The purity of the cultures was determined essentially by the procedure of CLARK AND SCHMIDT[8]. Immediately before harvesting, a 2-ml aliquot of culture solution was removed aseptically and tested for heterotroph contamination by plating on nutrient agar. In addition, a 10-ml aliquot was used to inoculate 5 separate flasks containing 100 ml of one of the following media: I, 0.8 % nutrient broth (Difco Laboratories, Detroit); II, 0.8 % nutrient broth, 1 % proteose peptone (Difco); III, 0.8 % nutrient broth, 0.3 % yeast extract (Difco), 1 % glucose; IV, 10 mg/ml glucose, 5 mg/ml yeast extract; and V, 10 mg/ml glucose, 5 mg/ml yeast extract, 5 mg/ml casein hydrolysate (Nutritional Biochemicals Corporation, Cleveland). Cells which were sedimented from culture solution containing heterotroph contaminants were discarded. Nitrosomonas cells from 90 l of culture solution were harvested by centrifugation in a Sorval continuous flow apparatus at 4° and washed twice by successive suspension and gentle agitation for 20 min and 15 h, respectively, in 200 ml 0.05 M phosphate solution (pH 7.5), followed each time by centrifugation for 20 min at 20000 $\times$ *g*.

Chemicals

Unless otherwise specified, chemicals were obtained from the same sources and prepared by the same procedures as described previously[7]. Buffer solutions were prepared by the procedures described by GOMORI[9]. The compounds NADH (grade III) and α,α'-dipyridyl were obtained from Sigma Chemical Company, St. Louis. Pyo-

cyanine was obtained from Mann Research Labs, New York ("homogeneous by paper chromatography"); hydroquinone and allyl-thiourea from Eastman Chemical Company, Rochester, New York. N_2 ("dry, high purity") was obtained from National Cylinder Gas Company, Chicago, and was made essentially O_2-free by passage through pyrogallol (Fisher Cert. Reagent) as described by STAUFFER[10]. The compounds Na_3AsO_3, diethyldithiocarbamate and EDTA were reagent grade (J. J. Baker Chemical Company, Phillipsburg, N. J.) and KI was reagent grade crystalline from Merck and Company, Rahway, N. J. NaN_3, 3-amino-1,2,4-triazole and atebrin (quinacrine·HCl) were purchased from K and K Laboratories, Plainview, N. Y. Pancreatic deoxyribonuclease (grade I) was obtained from Worthington Biochemical Corporation, Freehold, N. J.

Standard assay methods

Nitrite, hydroxylamine and protein were assayed as described previously[5]. Nitrate was assayed by the phenol–disulfonic acid method described by NICHOLAS AND NASON[11] and by reduction of nitrate to nitrite with Zn followed by the assay of nitrite by the diazotization and coupling procedure. The procedure involving Zn reduction was a modification of the procedure of HEWITT AND NICHOLAS[12]. Since the presence of either hydroxylamine or nitrite resulted in color formation following the reaction with Zn, it was necessary to run careful analyses of both those compounds independently. The measurement of O_2 concentration was carried out using a Y.S.I. Oxygen Monitor (Yellow Springs Instrument Company, Yellow Springs, Ohio) with air-saturated solution as a standard. Manometric measurements were carried out in a G.M.E. constant pressure respirometer (Gilson Medical Electronics, Middleton, Wisconsin) and spectrophotometric measurements were made utilizing a Gilford Model 2000 Multiple Sample Absorbance Recorder (Gilford Instruments, Oberlin, Ohio). Mass spectrometer analysis was carried out in a Hitachi–Perkin Elmer R.M.U.-6D mass spectrometer. Difference spectra were measured in a Cary model 15 recording spectrophotometer as described previously[5].

Enzyme assays

Cytochrome oxidase[5], hydroxylamine–cytochrome *c* reductase[5] and glutamate dehydrogenase[7] were measured as described previously. Hydroquinone oxidase was assayed by the decrease in O_2 concentration after the addition of 0.1 ml of 0.1 M hydroquinone to an air-saturated solution containing enzyme in 2.9 ml sodium phosphate (pH 7.0). Correction was made for the slight non-enzymatic utilization of O_2 which was observed in the presence of hydroquinone.

Unless otherwise indicated, hydroxylamine–nitrite reductase was assayed as the rate of nitrite disappearance from a reaction mixture containing 10^{-2} M NH_2OH, 1.0 mM $NaNO_2$, enzyme and 0.05 M citrate–phosphate buffer (pH 6.0). The reaction was carried out in an open test tube which was tilted to a nearly horizontal position and rotated at 20–30 rev./min. Aliquots were taken from the reaction mixture at regular intervals and assayed for nitrite concentration. Correction was made, when necessary, for changes in nitrite concentration in control reaction mixture lacking enzyme. Unless otherwise indicated, the rate of nitrite utilization was constant during the time at least three aliquots were taken from the reaction mixture and was proportional to the amount of enzyme solution added. One unit of activity is defined

as that amount of enzyme catalyzing the disappearance of 1 μmole of nitrite per min.

The rate of aerobic production of nitrite from hydroxylamine was assayed in a reaction mixture containing 1 mM hydroxylamine, enzyme and 0.05 M citrate–phosphate solution (pH 6.0).

RESULTS

Partial purification of hydroxylamine–nitrite reductase

Unless otherwise indicated, all steps of the purification procedure summarized in Table I were carried out at 4°.

Washed Nitrosomonas cells were suspended in a ratio of 0.2 g of cells per ml 0.05 M phosphate solution (pH 7.5) and disrupted by passage 3 times through an Aminco pressure cell under a pressure of 6000 lb/inch^2. The homogenate solution was incubated with a small amount of deoxyribonuclease for 10 min at 37° and was

TABLE I

SUMMARY OF PURIFICATION OF HYDROXYLAMINE–NITRITE REDUCTASE FROM NITROSOMONAS

Fraction	*Activity (units/ml)*	*Protein concn. (mg/ml)*	*Specific activity (units/mg protein × 10^3)*	*Total activity (units)*
1. 20000 × *g* supernatant	0.47 (0.23)*	20	23	55
2. 60000 × *g* supernatant	0.45	10	45	53
3. $(NH_4)_2SO_4$ precipitate	1.4	5.0	280	36
4. DEAE-cellulose eluate	0.12	0.4	300	14

* 0.23 was the measured value of hydroxylamine–nitrite reductase activity. The value of 0.47 was calculated based on the sum of the activity in the 60000 × *g* supernatant and pellet.

centrifuged twice for 20 min at 20000 × *g* to yield a precipitate which was discarded and a red supernatant crude extract (Fraction 1). Fraction 1 was centrifuged at 60000 × *g* for 2 h in a Spinco model L centrifuge to yield a red-brown pellet which was discarded and a clear red supernatant solution (Fraction 2). Fraction 2 contained 97 % of the total cell-free hydroxylamine–nitrite reductase activity. The total units of activity in the 60000 × *g* supernatant and pellet fractions were twice the total units of activity of the 20000 × *g* supernatant fraction indicating that an inhibitor or competing reaction may have been removed by centrifugation. Fraction 2 also contained 70–90 % of the cell-free ability to catalyze the production of nitrite from hydroxylamine.

Solid $(NH_4)_2SO_4$ was added to Fraction 2 in 14 successive additions to bring the total to 0.15, 0.18, 0.21, 0.24, 0.27, 0.30, 0.34, 0.38, 0.42, 0.46, 0.54, 0.58 and 0.62 g $(NH_4)_2SO_4$ per ml of solution. After each addition of $(NH_4)_2SO_4$ sulfate, the solution was allowed to stand for 15 min and the resulting precipitate sedimented by centrifugation for 10 min at 20000 × *g* and resuspended in 5–10 ml of 0.05 M phosphate solution (pH 7.5). All hydroxylamine–nitrite reductase activity was recovered in the $(NH_4)_2SO_4$ fractions. Three deep red fractions precipitating at 0.46, 0.54 and 0.58 g

$(NH_4)_2SO_4$ per ml were pooled to give the $(NH_4)_2SO_4$ precipitate (Fraction 3) containing 70% of the original activity and a specific activity 12-fold greater than Fraction 1. For chromatography on DEAE-cellulose, a 7.5-ml volume of Fraction 3 was twice dialyzed for 1 h against 1 l of 1 mM phosphate solution (pH 7.5) and applied to a 2 cm × 15 cm chromatography column which had been packed with DEAE-cellulose and equilibrated with 1 mM phosphate solution (pH 7.5) as described previously[7]. The hydroxylamine–nitrite reductase activity adhered to the top of the column material in a brownish red band, whereas a peak of pink material containing no activity was eluted from the column with 100 ml of 1 mM phosphate solution (pH 7.5). A linear gradient was then produced by mixing 250-ml volumes of 1 mM phosphate solution (pH 7.5) and 1 mM phosphate, 1 M KCl solution (pH 7.5) to elute three peaks of 280-mμ absorbing material. The red second peak which was eluted in approx. 0.1 M KCl solution contained 50% of the activity applied to the column, whereas activity was not detected in any of the other DEAE-cellulose eluate fractions. The most active fractions were pooled to form the DEAE-cellulose eluate (Fraction 4) containing 25% of the Fraction 1 activity with a specific activity 13-fold greater than Fraction 1. Because of the loss in total units of activity observed during DEAE fractionation, Fraction 3 was used in most of the experiments reported here.

TABLE II

NITRITE METABOLISM BY NITROSOMONAS CRUDE EXTRACT

Nitrite was assayed as described in *Standard assay methods* in aliquots taken at 5-min intervals from a reaction mixture consisting of 0.05 M phosphate solution (pH 7.5), and the following additions as indicated: 1 mM hydroxylamine; 10^{-4} M $NaNO_2$; 0.7 mg Fraction 2 per ml of reaction; Fraction 2 solution which had been heated at 100° for 10 min.

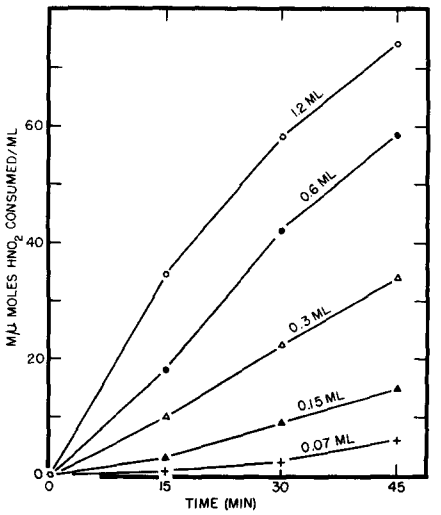
Expt. No.	*Additions*	*ΔNO_2^- (mμmoles/ml reaction per 20 min)*
1	Extract + NH_2OH + $NaNO_2$	−48
2	Extract + NH_2OH	8
3	Extract + $NaNO_2$	0
4	Heated extract + NH_2OH + $NaNO_2$	0
6	NH_2OH + $NaNO_2$	0

Hydroxylamine–nitrite reductase activity in crude extract

As indicated in Table II, the aerobic incubation of Fraction 2 with 1 mM hydroxylamine resulted in the production of nitrite, whereas there was a progressive disappearance of nitrite from an identical reaction mixture which contained 10^{-4} M nitrite at the start of the reaction. Nitrite disappearance was dependent on the presence of both hydroxylamine and enzyme and did not occur if boiled enzyme were used. The rate of nitrite disappearance was constant over 30 min and was proportional to the amount of extract added as shown in Fig. 1. Because KCN was found to stimulate the rate of nitrite disappearance, it was sometimes included in reactions when the crude extract was employed.

Effect of pH on reaction rate

The rate of hydroxylamine-stimulated nitrite disappearance catalyzed by Fraction 2 was greatest at approximately pH 5.75 as shown in Fig. 2. The same dependence of reaction rate on pH was observed for enzyme Fractions 3 and 4. When the pH of the reaction mixture was less than 4.5, a progressive increase in the non-enzymatic reaction of nitrite and hydroxylamine was observed. The probable product of the non-enzymatic reaction is N_2O (ref. 13).



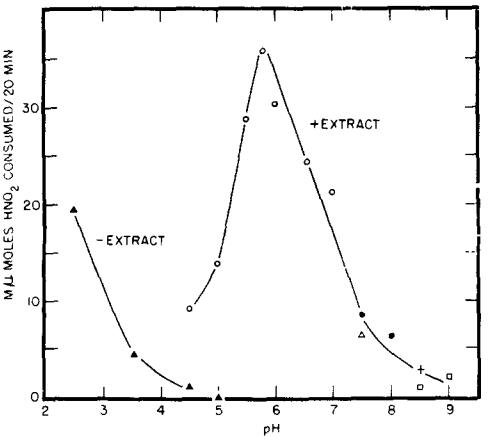


Fig. 1. Effect of varying amounts of Nitrosomonas extract on the rate of hydroxylamine-stimulated nitrite disappearance. The reaction was started by the addition of the indicated volume of Fraction 2 (10 mg protein per ml) to 0.05 M citrate–phosphate solution (pH 6.0) containing $2 \cdot 10^{-4}$ M $NaNO_2$, 1 mM NH_2OH and $3 \cdot 10^{-5}$ M KCN. Aliquots were taken from the reaction mixture at the indicated times and assayed for nitrite as described in *Standard assay methods*.

Fig. 2. Effect of pH and buffer solution on the rate of Nitrosomonas hydroxylamine–nitrite reductase. The rate of nitrite consumption per ml was determined as described in *Standard enzyme assays* in aliquots taken at 0, 20 and 40 min from a 1-ml reaction mixture which contained 10^{-4} M $NaNO_2$; 1 mM NH_2OH; $3 \cdot 10^{-5}$ M KCN; and 0.5 mg of Fraction 2 in 0.05 M citrate–phosphate (○), phosphate (●), Tris (△), glycine (□) or carbonate (+) buffer solution. ▲, citrate–phosphate with no extract added.

Stability of hydroxylamine–nitrite reductase activity

As indicated in Table II, activity was destroyed by heating at 100° for 5 min. Dialysis of the $(NH_4)_2SO_4$ fraction for 8 h against 1000 vol. of 0.05 M phosphate solution (pH 7.5) resulted in no change in the activity of the extract. Fractions 2 or 3 have been stored at −10° for 10 months with essentially no loss in activity.

Effect of varying nitrite or hydroxylamine concentration

As shown in Fig. 3A, the rate of nitrite consumption catalyzed by Nitrosomonas Fraction 3 increased with increasing nitrite concentrations with half the maximum rate occurring at approx. 1.6 mM HNO_2. At concentrations greater than 8 mM, nitrite progressively inhibited the nitrite-utilizing activity so that, assuming that an uninhibited maximum velocity was not observed, the apparent K_m for nitrite was probably

greater than 1.6 mM. The enzyme had a very low apparent affinity for hydroxylamine as indicated in Fig. 3B.

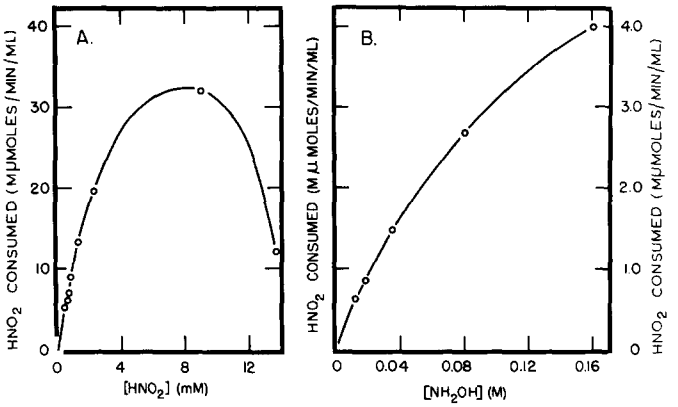


Fig. 3. Effect of varying nitrite or hydroxylamine concentration on the rate of nitrite consumption. The rate of nitrite consumption was assayed in 0.05 M citrate–phosphate solution (pH 6.0) as described in *Standard enzyme assays* except that reaction mixture A contained 0.1 M NH_2OH, 17 μg Fraction 3 per ml and the indicated concentration of $NaNO_2$ and reaction mixture B contained 8 mM $NaNO_2$, 5.5 μg Fraction 3 per ml and the indicated concentration of NH_2OH.

Role of hydroxylamine in the nitrite-utilizing reaction

The enzyme-catalyzed utilization of nitrite was dependent on the presence of hydroxylamine and ceased, as shown in Fig. 4, when hydroxylamine was no longer present in the reaction mixture. In the experiment shown, 1.5 μmoles of nitrite were utilized in the time that 3 μmoles of hydroxylamine had disappeared from the reaction

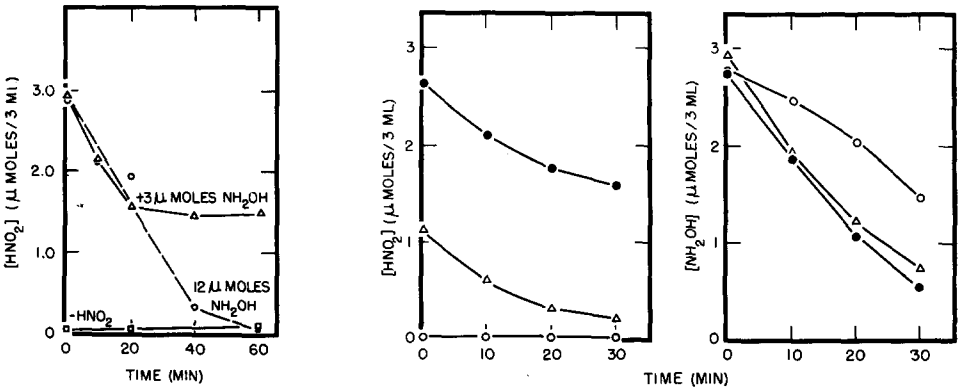


Fig. 4. Nitrite disappearance as a function of time. Nitrite was assayed as described in *Standard assay methods* in aliquots taken at the indicated times from 3-ml reaction mixtures containing 0.05 M citrate–phosphate buffer (pH 6.0) and (△) 1 mM NH_2OH, 1 mM HNO_2, 2 mg Fraction 3; □, 1 mM NH_2OH, HNO_2 absent, 2 mg Fraction 3; ○, 4 mM NH_2OH, 1 mM HNO_2, 0.5 mg Fraction 3.

Fig. 5. Concurrent nitrite and hydroxylamine disappearance catalyzed by Nitrosomonas Fraction 3. Nitrite and hydroxylamine were assayed as described in *Standard assay methods* in aliquots taken from the reaction solution at the time indicated. The 3-ml reaction mixture contained 0.05 M citrate–phosphate buffer (pH 6.0); 2 mg Nitrosomonas $(NH_4)_2SO_4$ precipitate; 0.9 mM HNO_2, 0.9 mM NH_2OH (●); HNO_2 absent, 0.9 mM NH_2OH (○); 0.4 mM HNO_2, 1 mM NH_2OH (△).

mixture, whereas nitrite was completely removed from the medium in the presence of 12 μmoles hydroxylamine. As shown in Fig. 5, in the absence of nitrite, Fraction 3 usually catalyzed the aerobic oxidation of hydroxylamine to a product other than nitrite. In the presence of an excess of nitrite, the rate of hydroxylamine disappearance was greatly increased and remained at a high level. On the other hand, when nitrite had been completely utilized in a reaction mixture containing an excess of hydroxylamine, the rate of hydroxylamine disappearance decreased so that it was approximately equal to the rate of hydroxylamine disappearance in a control reaction lacking nitrite. From experiments such as those shown in Fig. 5, the ratio of the total number of moles of hydroxylamine utilized to the number of moles of nitrite utilized was found to have a value of approx. 1.8. The ratio of nitrite-stimulated hydroxylamine disappearance (the total number of moles of hydroxylamine utilized in the presence of nitrite *minus* the number of moles hydroxylamine utilized in an identical reaction mixture lacking nitrite) to the number of moles of nitrite utilized was close to unity (0.97 $\pm$ 0.03). Aged preparations of Fraction 3 were sometimes unable to oxidize hydroxylamine in the absence of nitrite. In such cases, when nitrite was present in the reaction mixture, the resulting ratio of NH_2OH utilized:HNO_2 utilized was close to 1.0 as shown in Table III.

TABLE III

STOICHIOMETRY OF NH_2OH–HNO_2 REDUCTASE REACTION

The amount of nitrite and hydroxylamine utilized was assayed as described in Fig. 5.

Reaction condition	$-\Delta NH_2OH$*	$-\Delta HNO_2$*
Enzyme, 1 mM HNO_2, 5 mM NH_2OH	950	965
Enzyme, 5 mM NH_2OH	0	0

* mμmoles/ml per 30 min.

Requirement for O_2

As shown in Table IV, the nitrite-utilizing system was inhibited when the reaction was carried out under an atmosphere of N_2. The same result was obtained when the Thunberg vessel was evacuated but not refilled with N_2 and was rotated to promote gas exchange with the reaction solution. The nitrite-utilizing system apparently had a high affinity for O_2 as indicated by the fact that incomplete inhibition was observed unless extreme care was taken in the process of O_2 removal. Attempts to measure stoichiometric O_2 consumption coupled to the NH_2OH–HNO_2 reductase reaction were not successful because it was found that the O_2 electrodes utilized gave positive readings in the presence of NO or N_2O.

Product of nitrite–hydroxylamine reductase

As shown in Fig. 6, the disappearance of nitrite catalyzed by Fraction 3 was accompanied by the evolution of gas at a rate proportional to the amount of enzyme added. Because O_2 was apparently utilized during the course of the reaction, net gas evolution was measured (the difference between the volume of gas produced and the volume of O_2 utilized in the reaction). The net evolution of 1.02 moles of gas per mole

TABLE IV

RFFECT OF ANAEROBIC INCUBATION ON THE RATE OF NITROSOMONAS HYDROXYLAMINE–NITRITE EEDUCTASE

The reaction was carried out in a Thunberg tube containing 0.05 M citrate–phosphate solution (pH 6.0), 10^{-4} M $NaNO_2$ and 0.3 mg Fraction 3 in a total volume of 1.8 ml. The sidearm contained 1.2 μmoles of hydroxylamine in 0.2 ml of citrate–phosphate solution. (Final NH_2OH concentration in the reaction vessel $6 \cdot 10^{-4}$ M.) The vessel was evacuated 5 times to a pressure of 12 mm Hg and refilled each time with N_2. The reaction was started by tipping hydroxylamine into the main vessel and mixing by rotation was accomplished as described in *Standard enzyme assays*. Calculation of the amount of nitrite consumed was based on the difference between the amount remaining in the experimental vessel at 30 min and the amount in the non-pretreated vessel at the start of the reaction.

Number of trials	*Pretreatment*	*Reaction condition*	*Average amount of nitrite consumed (mμmoles/30 min)*
3	None	Open vessel, mixing by rotation	57
6	Evacuated, gassed with N_2	Open vessel, mixing by rotation	59
9	Evacuated, gassed with N_2	Closed vessel, stationary	5

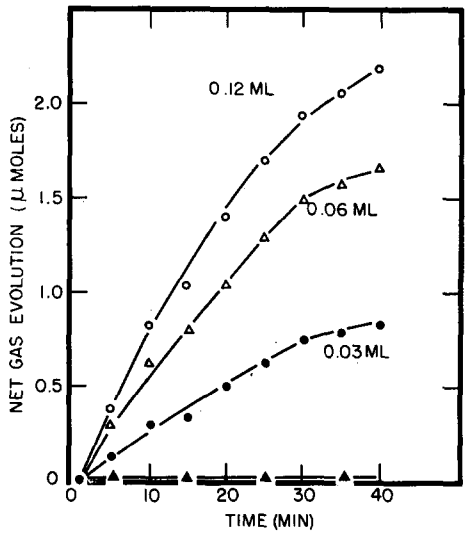


Fig. 6. The net production of gas in the reaction of nitrite and hydroxylamine catalyzed by Nitrosomonas Fraction 3. The reaction was started by tipping the indicated volume of Fraction 3 (5 mg/ml) from the sidearm of a Warburg manometric vessel into the main compartment which contained 1 mM $NaNO_2$ and 2 mM NH_2OH in 2.8 ml 0.05 M citrate–phosphate buffer (pH 6.0). ▲, either HNO_2 or Fraction 3 absent. The value of net gas evolution has not been corrected for the solubility of product gas in the buffer solution.

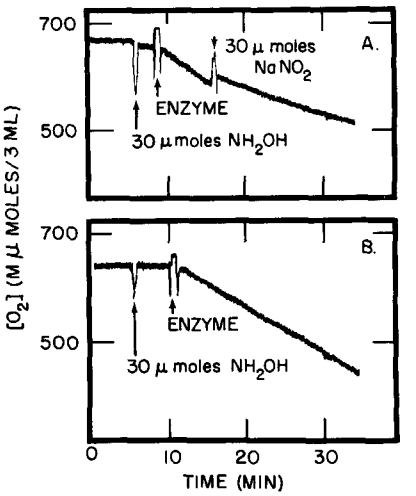


Fig. 7. Inhibition by HNO_2 of NH_2OH-stimulated O_2 consumption as catalyzed by Nitrosomonas Fraction 3. To two YSI Oxygen Analyzer vessels (A, B) were added 3 ml 0.05 M citrate–phosphate solution (pH 6.0), 30 μmoles NH_2OH and 1 mg Fraction 3 at the times indicated. The change in O_2 concentration was recorded. In addition, 30 μmoles of $NaNO_2$ were added to vessel A at the time indicated.

of nitrite utilized occurred when nitrite was determined either at the end of the reaction as indicated in Table V, or at several time points in aliquots taken from duplicate reactions during the course of gas evolution. In a complete reaction mixture containing

TABLE V

RATIO OF NET GAS EVOLUTION AND CONCURRENT NITRITE DISAPPEARANCE

The reaction carried out as described in Fig. 6 was terminated by pipetting a 0.2-ml aliquot of the reaction mixture into sulfanilic acid for nitrite assay. The amount of nitrite utilized was based on the difference between the amount remaining in the experimental vessel and the amount remaining in control vessels lacking either enzyme or hydroxylamine. The values given represent the average of 23 tials.

Time of reaction (min)	*Gas evolved (μmoles)*	*Nitrite utilized (μmoles)*	*Moles gas evolved: moles HNO_2 utilized*
52	2.36	2.32	1.02 ± 0.11

an excess of hydroxylamine, gas production ceased after a quantity of gas had been evolved which was equal on a molar basis to the amount of nitrite originally present in the reaction mixture. The net evolution of gas was zero in a reaction mixture lacking either nitrite, enzyme or hydroxylamine.

The product of the hydroxylamine–nitrite reductase reaction was identified as a mixture of N_2O and NO by mass spectrometer analysis as shown in Table VI. To minimize the interference of air with the gas analysis but in consideration of the O_2 requirement for the reaction, experiments were carried out under an atmosphere of He containing 4 mm of O_2. When correction was made for gas production in control vessels and gas solubility, the ratio of N_2O:NO was 13 and the ratio of NH_2OH:HNO_2

TABLE VI

PRODUCT OF HYDROXYLAMINE–NITRITE REDUCTASE REACTION

A 12.5-ml Thunberg tube containing 15 μmoles of $NaNO_2$, 10 μg Fraction 3 and 3.0 ml 0.05 M citrate–phosphate buffer (pH 6.0) in the main compartment and 15 μmoles NH_2OH and 0.4 ml citrate–phosphate buffer in the sidearm was chilled on ice and evacuated 3 times to 250 μ pressure. The tube was refilled with He after the first evacuation and He containing 0.55% O_2 after the second and third evacuations. The reaction was started by tipping NH_2OH from the sidearm and carried out with agitation for 60 min. Immediately after transfer of the gas with a Tepler pump for mass spectrometer analysis, aliquots were taken from the reaction mixture and analyzed for HNO_2 and NH_2OH as described in *Standard assay procedures*. Changes in HNO_2 and NH_2OH were based on a comparison with aliquots taken from identical reaction mixtures at the start of the reaction. The amount of each gas is expressed relative to the peak height of Ar. The greatest errors in gas analysis are of the order of 3%.

Reaction mixture	*Amount of gas produced*			*Total HNO_2 utilized (μmoles)*	*Total NH_2OH utilized (μmoles)*
	N_2	*NO*	*N_2O*		
	Peak heights relative to Ar				
Complete	26	0.84	12	12.7	13.4
	22	1.5	22	11.6	13.9
Control *minus* enzyme	28	—	0.19	2.73	0
	29	0.032	0.89	2.52	0
Control *minus* HNO_2	22	0.061	1.3	—	0
	24	0.073	1.3	—	0

utilized was 1.4. In most experiments, the N_2O:NO ratio was higher than 13 although it was once observed to be as low as 2.5. In the experiment reported in Table VI, NH_2OH was not oxidized in the absence of HNO_2. Where NH_2OH oxidation was observed in the absence of HNO_2, the product was identified as N_2O. In one experiment, analysis of the infrared spectrum of the gas evolved in the NH_2OH–HNO_2 reductase reaction indicated the presence of N_2O and not NO.

The following stoichiometry was calculated utilizing the N_2O:NO ratio and the molar changes in NH_2OH and HNO_2 from the experiment shown in Table VI: 2.22 O_2 + 13.6 NH_2OH + 9.6 HNO_2 → 0.88 NO + 11.2 N_2O + 25.2 H_2O. The molar ratio of net gas evolved: HNO_2 utilized in this reaction is calculated to be 1.03. The gas:HNO_2 ratio measured in other experiments (Table V) is 1.1 ± 0.12 after correction is made for the solubility of N_2O and NO.

The gas produced in the enzymatic reaction of nitrite with hydroxylamine was not CO_2 as indicated by the fact that net gas evolution was not affected by the presence of 0.2 ml 10% KOH and a folded 2 cm × 2.4 cm piece of filter paper in the center well of the Warburg flask. Nitrate was not detected as a product of the nitrite-utilizing reaction.

Other enzyme activities in Fraction 3

Fraction 3 catalyzed the aerobic production of nitrite from hydroxylamine most rapidly at pH 6.0. Under the conditions of assay, 0.2 mole or less of HNO_2 was produced per mole of NH_2OH oxidized indicating that the product of hydroxylamine oxidation was predominantly N_2O rather than HNO_2. On the basis of the number of moles of nitrite metabolized per mg protein of Fraction 3, the rate of nitrite disappearance was approx. 10^3 times greater than the rate of aerobic nitrite production from hydroxylamine utilizing the reaction conditions appropriate for each enzyme activity.

In addition to its assay by the disappearance of hydroxylamine (Fig. 5), hydroxylamine oxidase was assayed by following the utilization of O_2 in the presence

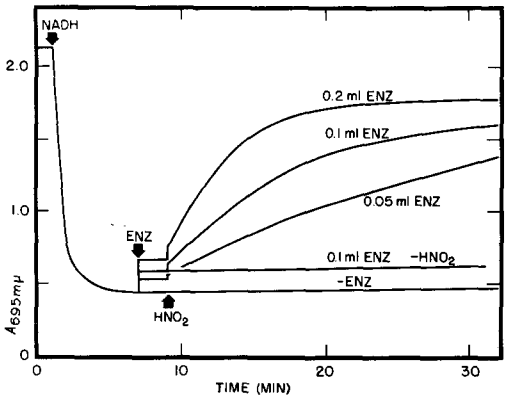


Fig. 8. Nitrite-stimulated oxidation of chemically reduced pyocyanine catalyzed by Nitrosomonas Fraction 3. A rubber-stoppered Thunberg cuvette containing 1.5 μmoles pyocyanine in 2.8 ml 0.05 M citrate–phosphate solution (pH 6.0) was twice evacuated and refilled with nitrogen. NADH was injected (1.2 μmoles) and the pyocyanine reduction followed to completion by the change in absorbance at 695 mμ. The indicated volumes of Fraction 3 (15 mg/ml) and 10 μmoles of $NaNO_2$ were then injected and the changes in absorbance recorded.

of enzyme and hydroxylamine. As shown in Fig. 7, the apparent rate of O_2 utilization coupled to the enzyme-catalyzed oxidation of hydroxylamine was 50% inhibited by the presence of 10 mM $NaNO_2$, whereas nitrite stimulated the utilization of hydroxylamine as shown in Fig. 5 and did not significantly inhibit nitrite utilization at a concentration of 10 mM (Fig. 3).

In addition to hydroxylamine oxidase, hydroxylamine–nitrite reductase, and nitrite production from hydroxylamine, Fraction 3 possessed hydroxylamine–mammalian cytochrome *c* reductase, mammalian cytochrome *c* oxidase, hydroquinone oxidase, and pyocyanine–HNO_2 reductase activity. Hydroxylamine–cytochrome *c* reductase activity was assayed in 0.05 M citrate–phosphate (pH 6.0), 1 mM hydroxylamine, $8 \cdot 10^{-5}$ M cytochrome *c* although the pH value for optimum activity is 9.6 (ref. 5). The rate of cytochrome *c* reduction by NH_2OH was stimulated 10–30% in the presence of 1 mM KNO_2. In the presence of 1 mM HNO_2, the specific activity of hydroxylamine–cytochrome *c* reductase in Fraction 3 expressed as μmoles cytochrome *c* reduced per min per mg protein was approximately twice the specific activity of nitrite uptake assayed in the absence of mammalian cytochrome *c* and expressed as μmoles nitrite reduced per min per mg protein. The maximum rate of hydroxylamine–cytochrome *c* reductase was thus great enough for it to have participated as an electron donor in the hydroxylamine–nitrite reductase reaction.

Preliminary observations indicated that fractions collected during various steps of the purification procedure which had high quantities of one enzyme activity also had relatively high quantities of the other four of the following five enzyme activities: NH_2OH–HNO_2 reductase; HNO_2 production from NH_2OH; NH_2OH–cytochrome *c* reductase; pyocyanine–nitrite reductase; and hydroquinone oxidase.

Electron donors other than hydroxylamine

Reduced mammalian cytochrome *c* oxidase activity of Fraction 3 was not affected by the presence of 1 mM HNO_2 suggesting that reduced mammalian cytochrome *c* was unable to serve as an electron donor for nitrite reduction.

Pyocyanine which had been reduced chemically with NADH was able to act as an electron donor for enzymatic nitrite reduction. As shown in Fig. 8, $5 \cdot 10^{-4}$ M pyocyanine and 0.05 M citrate–phosphate solution (pH 6.0) contained in an anaerobic Thurnberg cuvette was rapidly reduced by NADH. The subsequent addition of either enzyme or $NaNO_2$ did not result in the reoxidation of pyocyanine, but the addition of both enzyme and $NaNO_2$ caused a rapid increase in absorbance. The rate of increase in absorbance was proportional to the amount of Nitrosomonas extract added and to the amount of $NaNO_2$ present. Preliminary results indicate that the product of the enzymatic reduction of nitrite by pyocyanine was a mixture of NO and N_2O.

Sensitivity to inhibitors

As shown in Table VII, NH_2OH–HNO_2 reductase activity catalyzed by Fraction 3 was inhibited by the compounds atebrin, 3-amino-1,2,4-triazole and EDTA only at high concentrations and was not inhibited by 2 mM 1-allyl-2-thiourea. On the other hand, the involvement of iron and copper ions in the action of the enzyme was indicated by the sensitivity of the enzyme to chelating agents. Approx. 50% inhibition was caused to 1.5 mM NaN_3, $3 \cdot 10^{-4}$ M KCN, 8 μM α,α'-dipyridyl or 2 μM diethyldithiocarbamate. A significant stimulation of the rate of nitrite utilization was

TABLE VII

EFFECT OF INHIBITORS ON THE RATE OF HYDROXYLAMINE–NITRITE REDUCTASE

For KCN inhibition the rate of nitrite utilization was assayed essentially as described in *Standard enzyme assays* in a 3-ml reaction mixture containing 0.05 M citrate–phosphate solution (pH 6.0), 1 mM NH_2OH, 1 mM $NaNO_2$, 2 mg Fraction 3 and KCN at the indicated concentrations. For testing the effect of the other compounds, a 2-ml reaction mixture contained 0.05 M citrate–phosphate solution (pH 6.0), 0.01 M NH_2OH, 1 mM $NaNO_2$, 15 μg enzyme protein and the compounds at the indicated concentrations.

Compound	*Concentration (M)*	*Rate of nitrite utilization (% of control)*
Atebrin*	0.01	71
3-Amino-1,2,4-triazole*	0.08	39
EDTA*	0.07	42
1-Allyl-2-thiourea	$2 \cdot 10^{-3}$	100
NaN_3	$1.5 \cdot 10^{-3}$	43
KCN	$5 \cdot 10^{-4}$	23
	$2 \cdot 10^{-5}$	150
α,α'-Dipyridyl*,**	$4 \cdot 10^{-5}$	0
	$8 \cdot 10^{-6}$	50
	$3 \cdot 10^{-7}$	82
Diethyl dithiocarbamate	$6 \cdot 10^{-6}$	0
	$2 \cdot 10^{-6}$	45
	$2 \cdot 10^{-7}$	120

* Certain inhibitors were incubated with the enzyme for 20 min before NH_2OH and $NaNO_2$ were added. Preincubation was not inhibitory.

** Reaction mixtures containing α,α'-dipyridyl contained a maximum of 0.95% ethanol. Ethanol was not inhibitory.

observed in the presence of KCN or diethyl dithiocarbamate at concentrations less than 10^{-4} M or 1 μM, respectively.

Inhibition of hydroxylamine–nitrite reductase by the iron-sequestering agents KCN or α,α'-dipyridyl was not reversed by separation of inhibitor from the enzyme by dialysis, whereas inhibition by the copper-sequestering agents Chelex-100 or diethyl dithiocarbamate was partly reversed by this procedure. Dialysis of Fraction 3 for 8 h against 100 vol. of 0.01 M KCN or 10 g/60 ml Bio-Rad Chelex-100 (iminodiacetic acid-substituted polystyrene, Bio-Rad Laboratories, Richmond, Calif.) followed by four separate changes of phosphate buffer resulted in 100% or 33% inhibition of hydroxylamine–nitrite reductase activity, respectively. Enzyme activity was not restored by the inclusion of 1 mM $CuSO_4$, Na_2MoO_4, $MgCl_2$, $CoCl_2$, $ZnCl_2$, $CaCl_2$, $MnCl_2$ or $FeCl_3$ in the assay mixture. Incubation of Fraction 3 for 45 min in the presence of 10^{-4} M diethyl dithiocarbamate or 10^{-5} M α,α'-dipyridyl resulted in inhibition of hydroxylamine–nitrite reductase activity by 80% or more. When the inhibitor was removed from the enzyme solution by dialysis for 45 min against two changes of 100 vol. of buffer solution, the inhibition of α,α'-dipyridyl increased to 100%, whereas the inhibition by diethyl dithiocarbamate decreased to 20%. Fraction 3 which had been treated in this manner with α,α'-dipyridyl was not reactivated by either aerobic or anaerobic preincubation in the presence of 10^{-4} M FAD, FMN, or $FeSO_4$.

DISCUSSION

Table VIII summarizes the properties of the enzyme system referred to as NH_2OH–HNO_2 reductase (NH_2OH:HNO_2 oxidoreductase) which catalyzes the reaction of NH_2OH, HNO_2 and O_2 to produce N_2O, NO and H_2O. As determined here, the ratio of moles N_2O produced:moles NO produced, the number of moles NH_2OH and HNO_2 utilized and the ratio of net moles gas produced:moles HNO_2 utilized are all consistent with this formulation of the reaction.

For purposes of discussion and further investigation, the reaction can be divided into two steps. In the first step, which can take place in the absence of nitrite, hydroxylamine is oxidized to a compound such as NO_2 with the concomitant reduction of a bacterial electron acceptor (NH_2OH + oxidized electron carrier → 1/2 N_2O + 1/2 H_2O + reduced electron carrier). NO_2 spontaneously dimerizes to $H_2N_2O_2$ which decomposes to form N_2O and water. The enzyme catalyzing this step may be the same as (1) hydroxylamine oxidase where O_2 serves as electron acceptor or (2) hydroxylamine dehydrogenase with enzyme-bound cytochrome transferring electrons to an acceptor such as mammalian cytochrome *c*. Although the apparent K_m for NH_2OH of hydroxylamine:nitrite oxidoreductase assayed at pH 6.0 is several orders of magnitude greater than the K_m for NH_2OH of hydroxylamine:cytochrome *c* oxidoreductase assayed at pH 9.6, it is possible that this discrepancy is due to the difference in the pH of the assay solution. The gas produced in the hydroxylamine oxidase reaction has been identified as N_2O by mass spectrometry. Under anaerobic conditions, extracts of Nitrosomonas have previously been shown to catalyze the

TABLE VIII

SUMMARY OF NITROSOMONAS NH_2OH–HNO_2 REDUCTASE

Property	*Description*
Physical state	In supernatant solution of extracts centrifuged for 2 h at 60000 × *g*. Purified 13-fold by precipitation with $(NH_4)_2SO_4$ and chromatography on DEAE-cellulose
Heat sensitivity	Inactivated by heating at 100° for 10 min
pH optimum	5.75
Substrates required	NH_2OH, HNO_2, O_2
K_m for NH_2OH	Approx. 0.25 M
K_m for HNO_2	Approx. 1.6 mM
Net moles gas evolved per mole HNO_2 utilized	1.02 ± 0.11
Product of reaction	80–90% N_2O; 10–20% NO
Electron donor other than NH_2OH	Leuco-pyocyanine
Other enzyme activities in partially purified fraction	Hydroxylamine oxidase Production of HNO_2 from NH_2OH Cytochrome *c* oxidase Hydroquinone oxidase NH_2OH–cytochrome *c* reductase
Metal-binding agents causing 50% inhibition	$2 \cdot 10^{-6}$ M diethyl dithiocarbamate $8 \cdot 10^{-6}$ M α,α′-dipyridyl $2 \cdot 10^{-4}$ M KCN $1.5 \cdot 10^{-3}$ M NaN_3

oxidation of hydroxylamine to N_2O in the presence of substrate quantities of mammalian cytochrome *c* (ref. 3) or to a mixture of N_2O and NO in the presence of methylene blue[4]. In the presence of O_2, hydroxylamine oxidase produces 1 mole of N_2O per mole of O_2 utilized so that the net change in gas volume is zero as was observed here.

The electron transport chain of hydroxylamine oxidase probably consists of flavin, iron, a *b*- and *c*-type cytochrome[5] and a soluble, CO-binding cytochrome of the *o*-type[14] as terminal oxidase. Measurements of absorption spectra indicate the presence of a CO-binding cytochrome and cytochromes of the *b*- and *c*-type in the fractions containing NH_2OH–HNO_2 reductase activity. Hydroxylamine–nitrite reductase assayed at pH 6.0 was inhibited by atebrin at concentrations 100 times greater than those inhibitory to hydroxylamine–cytochrome *c* reductase assayed at pH 9.6 (ref. 5). If hydroxylamine dehydrogenase is involved in hydroxylamine–nitrite reductase activity, the discrepancy in sensitivity to atebrin indicates that either (1) NH_2OH dehydrogenase is most sensitive to atebrin at high pH values or (2) low concentrations of atebrin inhibit the transfer of electrons from bacterial enzyme to mammalian cytochrome *c*.

In the hypothesized second step of NH_2OH–HNO_2 reductase, nitrite added to the reaction mixture is reduced to N_2O, NO and water by a bacterial electron donor (HNO_2 + reduced electron carrier → N_2O + NO + H_2O + oxidized electron carrier). As demonstrated here, leuco-pyocyanine is also apparently able to reduce the bacterial electron carrier. The fact that N_2O is always produced in much greater quantity than NO in the reduction of nitrite by hydroxylamine suggests that NO is an intermediate in nitrite reduction (*i.e.* HONO → NO^+ → NO → NO^- → HNO). Under circumstances where hydroxylamine dehydrogenase does not utilize O_2 as an electron acceptor and nitrite is reduced to N_2O, the NH_2OH–HNO_2 reductase reaction has the overall stoichiometry: NH_2OH + HNO_2 → N_2O + 2 H_2O. In this case, the molar ratio of NH_2OH utilized:HNO_2 utilized and net gas produced:HNO_2 utilized would be 1.0 which is consistent with the value reported here.

That the nitrite-reducing enzyme is identical with the bacterial terminal oxidase is suggested by (1) inhibition of nitrite utilization by KCN, NaN_3, α,α′-dipyridyl or diethyl dithiocarbamate, (2) inhibition by nitrite of hydroxylamine-dependent O_2 uptake, and (3) presence of mammalian cytochrome *c* oxidase and hydroquinone oxidase in fractions containing nitrite reductase activity. It is also possible that nitrite reductase and Nitrosomonas terminal oxidase are separate enzymes which compete with each other for electrons originating from NH_2OH or leuco-pyocyanine. Assuming that the terminal oxidase was sensitive to lesser concentrations of KCN than nitrite reductase, the competition of two separate enzymes might explain the observed KCN stimulation of aerobic nitrite utilization. The stimulation by nitrite of hydroxylamine utilization as catalyzed by hydroxylamine oxidase is interpreted in terms of this model to indicate that terminal oxidase activity is rate-limiting for hydroxylamine oxidation and that the rate of nitrite reduction is greater than the rate of reduction of O_2.

The apparent requirement of Nitrosomonas hydroxylamine–nitrite reductase for O_2 formed the basis for the earlier proposal that (1) hydroxylamine oxidation involves the removal of two electrons by two separate electron carriers with each electron carrier being reoxidized by one of two separate terminal oxidases and (2) only the second oxidase is able to function as a nitrite reductase[6]. According to this

model, O_2 must act as a substrate for the first oxidase in order for nitrite utilization to occur. It has not been possible to measure stoichiometric O_2 utilization coupled to nitrite reduction and irreversible inactivation of the enzyme has been observed in the presence of NH_2OH under an atmosphere of N_2. Anaerobic inactivation of the reduced enzyme rather than the need for substrate quantities of O_2 thus seems to best explain the O_2 requirement of NH_2OH–HNO_2 reductase at this time. It is possible that a product of the reaction such as NO is inhibitory because of its ability to bind transition metal complexes[15] and heme proteins such as hemoglobin[16], catalase[17] and cytochrome *c* (ref. 18).

The hydroxylamine–nitrite reductase reaction apparently does not occur during the normal process of nitrification by Nitrosomonas. Under the conditions of assay used in this work, at pH 6 or 7.5, whole cells were not observed to catalyze the disappearance of nitrite in the presence of hydroxylamine. Preliminary results indicate that the specific activity of hydroxylamine–nitrite reductase from cells grown in nitrite at a concentration of 5–10 mM is the same as that from cells grown in concentrations of 10–20 mM suggesting that the activity is not an adaptation to detoxify the growth medium. Clearly NH_2OH–nitrite reductase activity can interfere with studies concerning the enzymatic production of nitrite from NH_3 or hydroxylamine. As shown here, the reaction occurs at pH 6 or 7.5 at a significant rate relative to that of cell-free nitrite production. It is possible that the apparent production of NO from NH_2OH which had been observed in Nitrosomonas extracts[4] was in fact due to the reduction by Nitrosomonas nitrite reductase of nitrite produced from hydroxylamine.

The nitrite-utilizing activity reported here has properties in common with the copper-requiring "denitrifying enzyme" crystallized by IWASAKI *et al.*[19] from *Pseudomonas denitrificans*. The latter enzyme catalyzes the reaction of hydroxylamine and nitrite to produce N_2O and is sensitive to inhibition by KCN and diethyl dithiocarbamate. Based on the inhibition of Nitrosomonas hydroxylamine–nitrite reductase by copper-binding agents (diethyl dithiocarbamate or iminodiacetic acid-substituted polystyrene) or iron-binding agents (α,α'-dipyridyl or KCN), copper or iron ions are involved in the action of the enzyme. The Nitrosomonas nitrite-reducing activity also has some properties in common with the nitrite reductase from *Pseudomonas aeruginosa* studied by YAMANAKA and co-workers (see ref. 20), although the *P. aeruginosa* enzyme apparently produces NO rather than N_2O. The Pseudomonas enzyme has KCN-sensitive cytochrome oxidase activity and utilizes Pseudomonas cytochrome *c*-551, leuco-pyocyanine, or hydroquinone as electron donors for nitrite reduction. The pH optimum for nitrite reduction is 6.0 or 6.6 using hydroquinone or Pseudomonas cytochrome *c*-551, respectively, as electron donors.

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