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SOME PROPERTIES OF A HYDROXYLAMINE OXIDASE FROM *NITROSOMONAS EUROPAEA*

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SUMMARY

A hydroxylamine oxidase derived from *Nitrosomonas europaea* is associated with particles. In the presence of air and a suitable electron acceptor such as mammalian cytochrome *c*, phenazine methosulfate, or methylene blue, hydroxylamine is oxidized to nitrite. Under anaerobic conditions, or with addition of potassium cyanide or CO the particles catalyze the rapid reduction of mammalian cytochrome *c* by hydroxylamine without formation of nitrite. The stoichiometry of this reaction suggests the formation of substrate amounts of a nitrogenous intermediate at the oxidation level of + 1 for N which subsequently is slowly and irreversibly converted to nitrous oxide. Spectroscopic studies showed that the oxidation of hydroxylamine is accompanied by reduction of flavin, and cytochromes *b*, *c*, and *a*. NADH inhibited the rate at which hydroxylamine was oxidized to nitrite. It is suggested that the cytochrome *a* of the particles performs a dual catalytic function: that of a cytochrome oxidase resulting in the formation of water and a final oxidative reaction in the conversion of hydroxylamine to nitrite.

INTRODUCTION

The first experimental evidence that hydroxylamine is utilized by cell suspensions of Nitrosomonas was obtained by LEES[1,2] and HOFMAN AND LEES[3]. Their data supported the earlier postulate of KLUYVER AND DONKER[4] that hydroxylamine is a likely intermediate in the oxidation of ammonia to nitrite.

IMSHENETSKII AND RUBAN[5,6] reported that autolysates of a Nitrosomonas species prepared by incubating cells at 40° for 24 h slowly oxidized ammonia or hydroxylamine after five days incubation. ENGEL AND ALEXANDER[7] prepared sonic extracts of *Nitrosomonas europaea* that oxidized small amounts of either ammonia or hydroxylamine to nitrite but the possibility of whole cells remaining in their crude extracts was not entirely eliminated. The first active cell-free preparations of this organism were described by NICHOLAS AND JONES[8] who showed that the addition of a suitable electron carrier such as mammalian cytochrome *c*, phenazine methosulfate or methylene blue was essential to obtain maximum rates for oxidation of hydroxylamine to nitrite. They found that more hydroxylamine was oxidized than could be

accounted for in the amount of nitrite formed. This paper amplifies a preliminary communication[9] in which it was shown that hydroxylamine oxidase from Nitrosomonas is associated with particles.

EXPERIMENTAL PROCEDURE

Materials

FAD, NADH, Antimycin A, and cytochrome *c* (type III, from horse heart) were obtained from the Sigma Chemical Company, St. Louis, Mo. (U.S.A.) and quinacrine dihydrochloride from the Mann Research Laboratories, New York, N.Y. All other reagents were of analytical grade.

Preparation of particles

Batch cultures of *Nitrosomonas europaea* were grown as described elsewhere[8]. The bacteria were collected and washed free of nitrite with 0.05 M Tris–HCl buffer (pH 7.5) by repeated centrifugation at 15 000 × *g* for 20 min. The washed cells, suspended in four volumes of the buffer were disrupted with the Mullard ultrasonic probe (20 kc/sec) for 20 min. The resulting homogenate was centrifuged at 15 000 × *g* for 20 min to remove cell debris. Microscopic examination and plating of the supernatant fraction (A) confirmed the absence of whole cells. The supernatant fraction (A) was then centrifuged at 105 400 × *g* for 60 min in a Spinco Model L ultracentrifuge. The pellet (B) formed was resuspended in the Tris–HCl buffer and the remaining supernatant fraction was centrifuged further at 105 400 × *g* for 16 h. The resulting pellet (C) was also taken up in the buffer. In air, fractions A to C oxidized hydroxylamine to nitrite in the presence of catalytic amounts of mammalian cytochrome *c*. Under anaerobic conditions, however, the fractions catalyzed the reduction of substrate amounts of mammalian cytochrome *c* by hydroxylamine without formation of nitrite. The above enzymic activities were not detected in the supernatant fraction (D) which remained after centrifuging at 105 400 × *g* for 16 h. Nitrite was not detected in any of the fractions by the sulfanilamide method[8].

Assay of hydroxylamine oxidase activity

The reaction mixture, in a final volume of 1 ml, was as follows: 0.2 μmole NH_2OH, 12.6 mμmoles cytochrome *c*, 83 μmoles Tris–HCl buffer, and a suitable aliquot of the enzyme. The pH of the reaction mixture was 7.5. After incubating at 22° for 15 min the nitrite produced was determined by the sulfanilamide method. The quantity of nitrite formed was a linear function of time during the 15-min incubation period. A unit of enzyme activity is defined as the amount that catalyzed the formation of 1 μmole of nitrite in the assay system.

Protein was determined by the Folin method[10]. The amount of cytochrome *c* reduced was followed in a Beckman DU spectrophotometer at 551 mμ. The following molar absorbancy indices (liter/mole/cm) for cytochrome *c* were used: reduced form, $2.81 \cdot 10^4$; oxidized form, $0.90 \cdot 10^4$ (see ref. 11). Other experimental details are given in the legends accompanying the figures and tables.

RESULTS

Hydroxylamine oxidase activity

The distribution of the hydroxylamine oxidase activities in the various fractions is given in Table I. The highest specific activity of the enzyme was found in the particles of Fraction C and none was detected in the supernatant fluid of Fraction D,

TABLE I

HYDROXYLAMINE OXIDASE ACTIVITY OF VARIOUS PARTICULATE FRACTIONS OBTAINED FROM *Nitrosomonas europaea*

Preparative details are given in the text under EXPERIMENTAL PROCEDURE

Fraction	*Volume (ml)*	*Protein (mg/ml)*	*Total units (mμmoles NO_2^-/ 15 min)*	*Specific activity (units/mg protein)*
A (15 000 × *g* for 20 min, supernatant fluid)	18.0	13.6	9550	39.0
B (105 400 × *g* for 60 min, pellet)	5.0	32.3	4860	30.1
C (105 400 × *g* for 16 h, pellet)	6.4	8.5	5580	102.6
D (105 400 × *g* for 16 h, supernatant fluid)	16.8	2.0	0	0

left after centrifuging at 105 400 × *g* for 16 h. The sum of the total enzyme units in Fractions B and C was equivalent to that of the crude extract (Fraction A). The results in Table II show that NADH inhibited hydroxylamine oxidase activity in the particles, suggesting that both substrates when oxidized utilize some common components of the electron transfer chain.

TABLE II

INHIBITION OF HYDROXYLAMINE OXIDASE ACTIVITY OF PARTICLES, DERIVED FROM *Nitrosomonas europaea*, BY NADH

The reaction mixture contained the following in a total volume of 2 ml: oxidized cytochrome *c* (Sigma, Type III, from horse heart), 63 mμmoles; Tris–HCl, 275 μmoles; enzyme (Fraction A), 0.5 mg; and where indicated NH_2OH, 0.2 μmole; NADH, 0.9 μmole; pH of the reaction mixture was 7.5. Incubation at 22° for 15 min.

Experiment number	*Additions*	*Nitrite produced (mμmoles)*	*Per cent inhibition*
1	NH_2OH	59.2	0
2	NH_2OH + NADH	34.9	41

Hydroxylamine—mammalian cytochrome c reductase activity

When particles from Nitrosomonas were incubated with hydroxylamine, mammalian cytochrome *c*, and 10^{-3} M KCN, the cytochrome, after a short lag period, was reduced rapidly. Nitrite was not formed and the cytochrome *c* remained reduced. Similar results were obtained under anaerobic conditions and subsequent introduc-

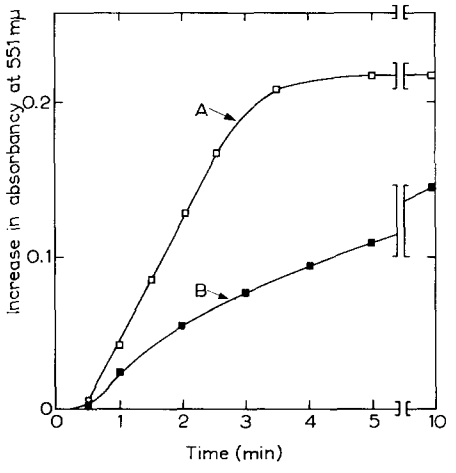


Fig. 1. Hydroxylamine—mammalian cytochrome *c* reductase activity of a particulate preparation from *Nitrosomonas europaea*. The basic reaction mixture contained: KCN, 3 μmoles; Tris–HCl, 245 μmoles; oxidized cytochrome *c* (Sigma, Type III, from horse heart), 63 mμmoles; enzyme (Fraction A), 0.5 mg. The enzyme was preincubated in this mixture for 5 min then 0.2 μmole NH_2OH (Curve A) or 0.9 μmole NADH (Curve B) was added and the rate of reduction of cytochrome *c* was determined by following the rate of change in absorbancy at 551 mμ in a Beckman DU spectrophotometer. Final volume, 3.0 ml; temperature, 22°; length of light path, 1.0 cm; pH, 7.5.

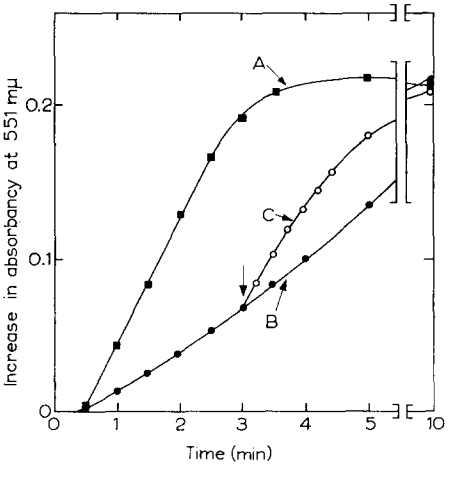


Fig. 2. Inhibition of hydroxylamine oxidation by quinacrine dihydrochloride and its reversal by FAD. The basic reaction mixture was identical to that given in Fig. 1. The enzyme was preincubated in this mixture for 5 min. At the end of this time 0.2 μmole NH_2OH was added to A, B, and C; 5 μmoles quinacrine dihydrochloride were added to B and C. After 3 min (indicated by the vertical arrow) 8.6 μmoles FAD were added to C. Absorbancy changes at 551 mμ for C during the interval 0–3 min were similar to those for B. Final volume, 3.0 ml; temperature, 22°; length of light path, 1.0 cm; pH, 7.5.

tion of air resulted in a rapid oxidation of the reduced cytochrome *c* with nitrite formation. It was also found that CO inhibited nitrite production and the oxidation of reduced cytochrome *c*. When NADH was used instead of hydroxylamine, however, the cytochrome *c* was reduced at an appreciably lower rate as shown in Fig. 1. The oxidation rates for NADPH and succinate, each at 2 μmoles were 8 and 5% of that for hydroxylamine; β-hydroxybutyrate was inactive.

The data in Fig. 2 demonstrate that the inhibition of the oxidation of hydroxylamine by 1.7 mM quinacrine dihydrochloride was reversed by 2.9 mM FAD. Amytal at 4 mM or Antimycin A at 3.33 μg/ml did not affect cytochrome *c* reduction by hydroxylamine, NADH, NADPH, or succinate.

The stoichiometry of cytochrome *c* reduction and hydroxylamine oxidation is shown in Fig. 3. At lower levels of hydroxylamine ($<$ 15 mμmoles) the molar ratio

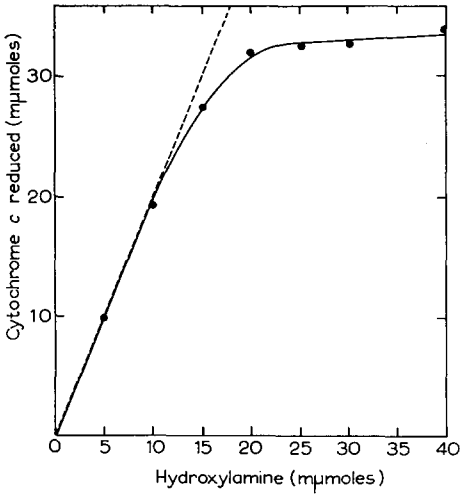


Fig. 3. Reduction of cytochrome *c* by graded amounts of NH_2OH. The final reaction mixture contained: KCN, 3 μmoles; Tris–HCl, 122.5 μmoles; oxidized cytochrome *c* (Sigma, Type III, from horse heart), 63 mμmoles; enzyme (Fraction C), 21.3 μg and NH_2OH in the amounts shown. Final volume, 3.0 ml; temperature, 22°; pH, 7.5. The enzyme was preincubated for 5 min in the mixture in the absence of NH_2OH, then NH_2OH was added and the maximum reduction of cytochrome *c* was determined by following absorbancy at 551 mμ in a Beckman DU spectrophotometer. Under these conditions cytochrome *c* remained reduced and nitrite was not formed.

between the amount of cytochrome *c* reduced and hydroxylamine oxidized is 2. The ratio, however, becomes less than 2 when increased amounts of hydroxylamine were added despite the fact that oxidized cytochrome *c* was present in excess. This was confirmed by increased absorbancy at 551 mμ on adding a trace of sodium dithionite. Thus amounts of hydroxylamine above 15 mμmoles/3 ml reaction mixture inhibit the reduction of cytochrome *c*. The extent of this inhibition is illustrated by the broken curve in Fig. 3 which is a linear extrapolation of the experimental data at the lower hydroxylamine values. The results for amounts of hydroxylamine $<$ 15 mμmoles/3 ml support the following stoichiometry:

$$NH_2OH + 2 \text{ Cytochrome } c\ Fe^{3+} \rightarrow \text{Intermediate}_1 + 2 \text{ Cytochrome } c\ Fe^{2+} \quad (1)$$

The results also demonstrate the formation of substrate amounts of a nitrogenous intermediate, at the oxidation level of + 1 for N, formed from hydroxylamine by loss of two electrons.

Absorption spectra

The spectra for the particulate preparation (Fraction C) presented in Fig. 4 show that the oxidized enzyme has a maximum band at 410 mμ whereas the sodium

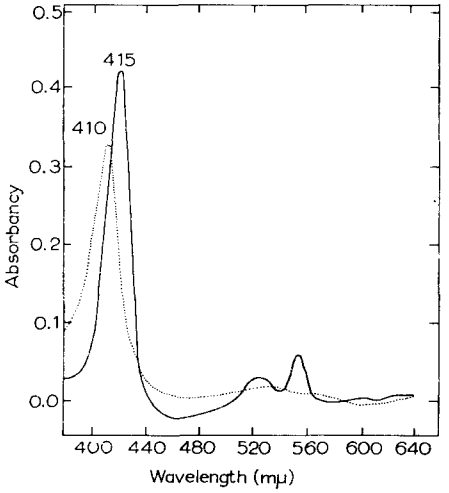


Fig. 4. Absorption spectra of particles containing hydroxylamine oxidase activity. The experimental cuvette contained: Tris–HCl, 50 μmoles; enzyme (Fraction C), 0.85 mg. Final volume, 1.0 ml; temperature, 22°; length of light path, 1.0 cm; pH, 7.5. The reference cuvette contained water. The spectrum of the oxidized enzyme (. . . . .) was determined first and then about 2 mg of sodium dithionite was added and the spectrum of the reduced enzyme (———) was recorded. The specific activity of the enzyme was 102.6 units/mg protein.

dithionite reduced enzyme shows absorption maxima at 600 mμ, 551 mμ, 521 mμ, and 415 mμ corresponding respectively to the α peaks of cytochromes *a* and *c* and their β and Soret bands. In addition there is a shoulder near 560 mμ, indicating the presence of a cytochrome *b*. The decreased absorbancy in the region of 450 mμ, after reduction with sodium dithionite, suggests that flavin is present.

The spectra in Fig. 5 indicate the relative reduction of cytochromes *a*, *b*, and *c* (Fraction C) by various reducing agents. A comparison of the enzyme reduced with sodium dithionite (Curve A), hydroxylamine (Curve B), and NADH (Curve C) indicates that only a small proportion of the total amount of endogenous cytochrome *c* in the particle was enzymically reduced by hydroxylamine or NADH. Hydroxylamine, however, reduced considerably more of the cytochromes *c* and *a* than did NADH. This is in agreement with the finding that NADH oxidase is less active than hydroxylamine oxidase (Fig. 1). Prolonged incubation with either substrate, under anaerobic conditions did not produce a further reduction of the cytochromes *c* and *a* but these were readily oxidized when air was subsequently introduced into the

cuvettes. Thus, cytochromes *a*, *b* and *c* are involved in the transfer of electrons from hydroxylamine or NADH to oxygen.

Analysis of the particles (Fraction C) for heme components by the pyridine hemochromogen method[12] and total flavin by difference spectra[13], gave the following

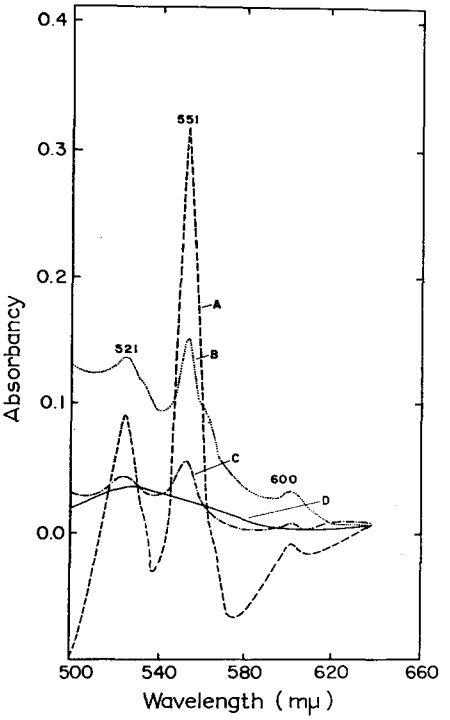


Fig. 5. Difference spectra of particles containing hydroxylamine oxidase. Curve A, spectrum of the sodium dithionite-reduced enzyme minus oxidized enzyme; Curve B, spectrum of the hydroxylamine-reduced enzyme minus oxidized enzyme; Curve C, spectrum of the NADH-reduced enzyme minus oxidized enzyme; Curve D, base line (oxidized minus oxidized enzyme). The experimental cuvette contained: Tris–HCl, 100 μmoles; and enzyme (Fraction C), 17.0 mg. The reference cuvette was identical to the experimental cuvette. The spectra were determined under anaerobic conditions with cuvettes equipped with Thunberg attachments. The gas phase was nitrogen which was passed through chromous chloride to remove oxygen. After recording Curve D (oxidized minus oxidized) an excess of the reducing agent was tipped into the experimental cuvette from a side arm, and the difference spectrum was recorded. Final volume, 2.0 ml; temperature, 22°; length of light path, 1.0 cm; pH, 7.5. The specific activity of the enzyme was 102.6 units/mg protein.

results in mμmoles/mg protein: cytochrome *c*, 3.82; cytochrome *b*, 0.34; cytochrome *a*, 0.50; and flavin, 0.30.

Electron paramagnetic resonance spectra of these particles clearly show that they contain a copper protein with a signal centered around gauss = 2.1 and a hyperfine structure of Cu^{2+} (see ref. 14). On addition of hydroxylamine the copper signal was shown to unbergo complex changes which strongly suggest that the metal is involved in the oxidase system[14].

Nature of the intermediate between hydroxylamine and nitrite

When particles (Fraction C) were incubated anaerobically (under N_2) with hydroxylamine and methylene blue or phenazine methosulfate in a Warburg apparatus a gas was evolved slowly. The gas was analyzed in a recording mass spectrometer from mass 1 to 60 and the main nitrogenous component occurred at mass 44. The ratio of mass 30/44 in the sample was compared with an authentic specimen of N_2O and the agreement is close as shown in Table III. Nitrous oxide was the only

TABLE III

MASS SPECTROGRAPHIC ANALYSIS OF GAS PRODUCED DURING ANAEROBIC INCUBATION OF PARTICLES WITH HYDROXYLAMINE

The final reaction mixture contained in a total volume of 5 ml the following: NH_2OH, 100 μmoles; Tris–HCl, 230 μmoles; methylene blue, 45 μmoles; enzyme (Fraction C), 4 mg. The temperature was 22°, the pH, 7.5. The reaction was initiated by addition of NH_2OH and was conducted under a vacuum for 90 min. The gas formed during this interval was collected and analyzed in the mass spectrometer.

Gas sample	*Peak heights**		*Ratio 30/44*
	Mass 30 (cm)	*Mass 44 (cm)*	
N_2O	402	2280	0.186
Experimental	405	2370	0.181

* Analysis was performed with a Consolidated Electrodynamics Corp. mass spectrometer, Model 21–620. The various peak heights were manually measured and multiplied by the proper attenuation factor to give the values in the table.

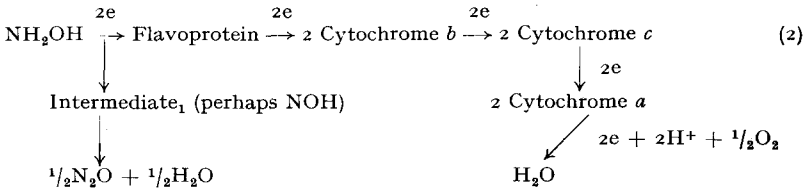
gas detected in this reaction. The background scans showed the complete absence of peaks corresponding to mass 30 and mass 44, and an experiment with boiled enzyme produced insufficient gas for analysis.

According to Eqn. 1 substrate amounts of an intermediate are formed simultaneously with cytochrome *c* reduction. This intermediate(s) is presumably at the oxidation level of + 1 for N and under anaerobic conditions it degrades slowly to N_2O. The latter is not utilized by the enzyme since it neither reduces cytochrome *c* anaerobically nor is it oxidized to nitrite in air. When the reaction mixture containing enzyme, hydroxylamine, and cytochrome *c* was incubated in a system subjected to intermittent evacuation over a period of 90 min, the subsequent introduction of air resulted in the oxidation of the reduced cytochrome *c* but nitrite was not formed. Thus, some product of hydroxylamine oxidation was removed from the reaction mixture. The subsequent addition of hydroxylamine to this system in air, however, resulted in nitrite production.

DISCUSSION

It is significant that particles from Nitrosomonas which have an active hydroxylamine oxidase activity also contain a full complement of respiratory carriers including flavin, cytochromes *a*, *b*, and *c*. Evidence that these function when hydroxyl-

amine is oxidized has been presented in this paper. A possible scheme to account for the experimental data is as follows:



(2)

$$\text{Intermediate}_1 + \text{Carrier-oxidized} \rightarrow \text{Intermediate}_2 + \text{Carrier-reduced} \quad (3)$$

$$\text{Carrier-reduced} + \text{Cytochrome } a\text{-oxidized} \rightarrow \text{Carrier-oxidized} + \text{Cytochrome } a\text{-reduced} \quad (4)$$

$$\text{Intermediate}_2 + \text{Cytochrome } a\text{-reduced} + {}^1/_2O_2 \rightarrow NO_2^- + \text{Cytochrome } a\text{-oxidized} \quad (5)$$

In this interpretation the first step (Eqn. 2) involves the dehydrogenation of hydroxylamine which, under anaerobic conditions, results in the reduction of the respiratory chain as far as cytochrome *a* and the simultaneous formation of a nitrogenous intermediate which is at the oxidation level of +1 for N. With anaerobic conditions this intermediate is converted slowly to N_2O, but in air it is oxidized to nitrite presumably by mediation of the electron transport chain. The experimental data suggest that cytochrome *a* in this organism may have a dual function: oxidizing reduced cytochrome *c* with formation of water as shown in Eqn. 2 and transferring electrons from intermediate$_1$ resulting in formation of nitrite as shown by Eqns. 3, 4 and 5. It is possible that intermediate$_1$ reacts directly with cytochrome *a*, as shown in Eqn. 6 which would substitute for Eqns. 3, 4 and 5 in the above scheme.

$$\text{Intermediate}_1 + {}^1/_2O_2 \xrightarrow{\text{Cytochrome } a} NO_2^- \quad (6)$$

The suggestion of a dual role for cytochrome *a* is not unique since it has been reported that a purified nitrite reductase from *Pseudomonas aeruginosa* also has a cytochrome oxidase activity[15,16]. It is of interest that the nitrite reductase enzyme contains copper[15] as does the hydroxylamine oxidase described in this paper. The latter enzyme was shown to undergo complex changes in its electron paramagnetic resonance spectrum on addition of hydroxylamine[14]. These results suggest that the metal functions in the hydroxylamine oxidase as it does in nitrite reductase.

One interesting feature is the high cytochrome *c* content of the particles, which exceeds by a factor of 10 the amount of other components of the respiratory chain. Much cytochrome *c* is leached out of the particles during preparation and perhaps this fact may account for the necessity of adding suitable carriers such as mammalian cytochrome *c*, phenazine methosulfate, or methylene blue for oxidation of hydroxylamine.

ALEEM *et al.*[17] have proposed that nitrohydroxylamine is the intermediate formed in the oxidation of hydroxylamine by an initial oxidative condensation of hydroxylamine and nitrite as follows:

$$NH_2OH + HNO_2 + {}^1/_2O_2 \rightarrow NO_2 \cdot NHOH + H_2O \quad (7)$$

Our experiments show, however, that substrate amounts of intermediate are formed (Eqn. 1) under anaerobic conditions in the absence of nitrite as determined by the sensitive sulfanilamide reaction.

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