STABLE ISOTOPE STUDIES ON THE OXIDATION OF AMMONIA TO HYDROXYLAMINE BY *NITROSOMONAS EUROPAEA*

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1. Introduction

It has been proposed that the oxidation of ammonia to nitrite by *Nitrosomonas* involves the following steps:

$$NH_4^+ + \tfrac{1}{2}O_2 \xrightarrow{-2e} NH_2OH + H^+$$

$$\Delta F = +4 \text{ kcal} \quad (1)$$

$$NH_2OH + O_2 \xrightarrow{-4e} NO_2^- + H^+ + H_2O$$

$$\Delta F = -69 \text{ kcal} \quad (2)$$

Lees [1] and Hofman and Lees [2] observed that hydroxylamine accumulated when ammonia was oxidised by intact cells in the presence of hydrazine. Hydroxylamine was first oxidised with I_2 to nitrite and determined colorimetrically. These results were confirmed by Yoshida and Alexander [3], who detected hydroxylamine by thin-layer chromatography [4]. Both these procedures, however, may not be specific for hydroxylamine. We now describe a method whereby hydroxylamine produced during the oxidation of ammonia by *Nitrosomonas* is isolated directly as an oxime. Using this technique we have employed stable isotopes to show that $^{15}NH_4Cl$ is oxidised to $^{15}NH_2OH$ and that the oxygen of hydroxylamine is derived from $^{18}O_2$ and not from $H_2{}^{18}O$.

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2. Materials and methods

2.1. *Chemicals*

All chemicals used were AR grade. $^{18}O_2$ (99 atom% excess) and $H_2{}^{18}O$ (80.64 atom% excess) were supplied by Yeda R and D Co. (Kiryat Weizmann Rehovot). $^{15}NH_4Cl$ (33 atom% excess) was purchased from M. W. Hardy and Co., London EC2.

2.2. *Culture of bacterium*

Cultures of *Nitrosomonas europaea* (kindly supplied by Dr Jane Meiklejohn of Rothamsted Experimental Station, England) were grown in 40 l batches to 77 μg (wet wt)/ml of culture in the following medium (g/l): $(NH_4)_2SO_4$, 4; KH_2PO_4, 0.5; $CaCl_2$ 2 H_2O, 0.004; $MgSO_4$ 7 H_2O, 0.05; chelated iron (methyldiamine bis-orthohydroxyphenylacetic acid), 0.0001; $CuSO_4$, 0.00002. Cultures were sparged with sterile air at 30°C. The pH was continuously adjusted during growth to 7.5 with sterile 20% (w/v) K_2CO_3 using a pH stat unit. During the exponential growth phase (72 h), cultures were harvested at 4°C at 27 000 × *g* at 18 l/h flowrate in a Sorvall superspeed RC-2B centrifuge fitted with a continuous flow rotor (Ivan Sorvall Inc., Norwalk, CT). The cells were washed twice with 0.05 M Tris–HCl buffer (pH 7.8) to remove nitrite. Washed cells were suspended at 400 mg (wet wt)/ml in a 50 mM Tris–HCl buffer (pH 7.8).

2.3. *Preparation of oxime*

Hydroxylamine hydrochloride (100 mg, 1.44 mmol) was added to 160 μl of cyclohexanone (1.6 mmol) in 0.15 M K_2HPO_4 buffer (pH 6.8) in a test tube and

shaken by a vortex stirrer at room temperature for 90 min. The oxime was extracted from the buffer with 25, 15 and 15 ml of diethylether, respectively. The ether extracts were combined and dried over anhydrous Na_2SO_4 for 3 h, then evaporated to dryness. The residue was crystallised from petroleum ether (b.p. 30–40°C) m.p. 90°C. The purity of the oxime was checked by thin-layer chromatography on silica gel G using ethyl acetate–benzene (20:80, v/v) as a solvent system [5].

2.4. *Determination of hydroxylamine*

Hydroxylamine was determined by the method in [4] using 8-hydroxyquinoline. The A_{705} was measured in a Shimadzu (QU-50) spectrophotometer.

2.5. *Hydroxylamine production*

Cells (150 mg) washed with water to remove nitrite, then with 0.15 M hydrazine sulphate (adjusted to pH 7.0) were added to a 30 mM hydrazine solution contained in a 250 ml Erlenmeyer flask. The reaction was started by adding 10 mmol ammonium chloride, then 1 ml aliquots were taken at intervals and centrifuged at 15 000 × *g* for 10 min. The supernatant was then assayed for hydroxylamine.

2.6. *Conversion of oxime to dinitrogen gas*

Oxime (5–7 mg) was transferred to a microkjeldahl flask and digested in 5 ml 36 N H_2SO_4 containing 0.2 g HgO. The ammonia produced was distilled under alkaline conditions into boric acid and concentrated to 2 ml after addition of one drop of N . H_2SO_4. The sample was transferred into one of the limbs of a Rittenberg tube and alkaline hypobromite added to the other. The tube was affixed to a mass-spectrometer vacuum system and rigorously evacuated to 10^{-7} mm Hg. The contents of the Rittenberg tube were then mixed to generate N_2 gas from ammonia. The tube was immersed in liquid nitrogen to freeze out water vapour and nitrogen oxides. The gas was then introduced into an evacuated expansion flask and passed into the mass spectrometer.

3. Results and discussion

Oximes formed by interacting hydroxylamine with

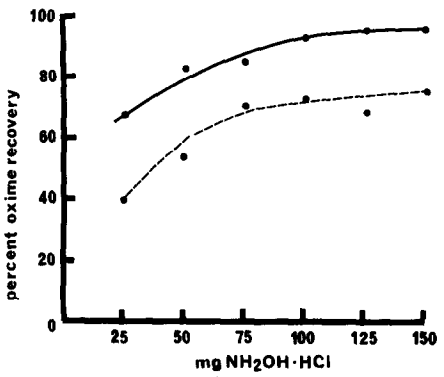

Fig.1. Oxime recovery. Hydroxylamine hydrochloride and cyclohexanone (1:1.1, molar ratios) were added to 0.15 M KH_2PO_4 buffer (pH 6.8) and mixed by a vortex stirrer for 90 min. Oxime was extracted with 25, 15 and 15 ml diethylether, respectively. Ether extracts were combined and dried over anhydrous Na_2SO_4 for 3 h. Solvent was evaporated and the residue was crystallized from petroleum ether (b.p. 30–40°C). Final volume of the reaction mixture: (a) 10 ml (●——●); (b) 100 ml (●- - -●).

ketones and aldehydes usually need aqueous alcohol to complete the reaction but the recovery of the oxime from the reaction mixture is incomplete. Keto-acids also react with hydroxylamine in aqueous solution to form the corresponding oximes, but these compounds are too soluble in water to give a good recovery on extraction into hydrophobic solvents.

However, we find that cyclohexanone reacts with hydroxylamine in microquantities in aqueous solution at pH 6.8 to form oxime with a reasonably good

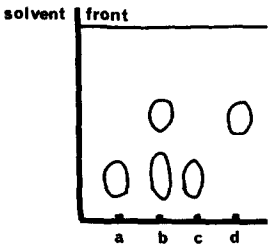

Fig.2. Purity of oxime determined by thin-layer chromatography. Oxime dissolved in ethanol was spotting onto the plates. These chromatographs were developed in ethyl acetate:benzene (20:80, v/v) and areas were detected by placing the plates in an iodine-saturated atmosphere. Oximes prepared at the following NH_2OH–HCl/cyclohexanone molar ratios: (a) 1:1.1, (b) 1:1.2, (c) 1:1.1 in the presence of 30 mM hydrazine sulphate; (d) cyclohexanone.

recovery in ether. The recovery of the oxime varied from 40–95%, depending upon the concentration of the reactants and their molar ratios as shown in fig.1. Hydroxylamine : cyclohexanone at molar ratio of 1:1.1 resulted in optimum production of the oxime. Mass spectrometric analysis of the crystallized oxime prepared in the presence of 30 mM hydrazine showed no contamination of cyclohexanone-hydrazide. Molar ratios of cyclohexanone > 1:1.1 gave improved yields, but unreacted cyclohexanone could not be removed by repeated crystallization of oxime from petroleum ether, fig.2.

The oxidation of NH_4Cl by intact cells is optimal at pH 7.5, but hydroxylamine produced at this pH, decomposes fairly rapidly. Cells were therefore incubated with NH_4Cl in the presence of 30 mM hydrazine at pH 7.0 at 30°C and 1 ml aliquots were withdrawn at various time intervals, centrifuged and supernatant fractions assayed for hydroxylamine. The optimum period of incubation was found to be between 30–40 min as shown in fig.3 and maximal hydroxylamine production occurred at 0.1 M NH_4Cl (fig.4).

Washed cells (150 mg) were incubated for 30 min with 0.1 M $^{15}NH_4Cl$ (33 atom% excess) and 30 mM NH_2NH_2 in a 100 ml total vol. in a 250 ml Erlenmeyer flask in a reciprocating waterbath at 30°C. The reaction mixture was then centrifuged at 15 000 × *g* for

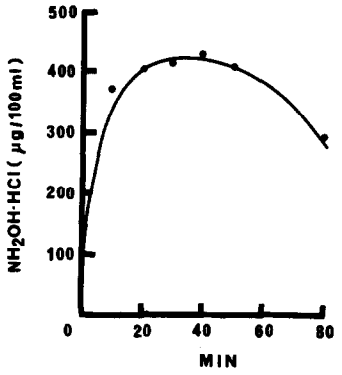

Fig.3. Time course for hydroxylamine production. The reaction mixture contained 150 mg washed cells, 3 mmol hydrazine sulphate (pH adjusted to 7.0 with 10 N NaOH) in 100 ml final vol. in a 250 ml Erlenmeyer flask. The reaction was started by adding 10 mmol/NH_4Cl. The flasks were shaken continuously at 30°C and 1 ml aliquots were withdrawn at various time intervals, centrifuged and the supernatant fraction assayed for hydroxylamine, as in section 2.

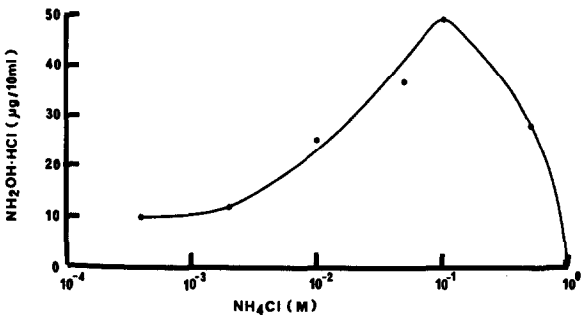

Fig.4. Effect of NH_4^+ concentration on hydroxylamine production. The reaction mixture contained 20 mg washed cells, 0.3 mmol hydrazine sulphate (adjusted to pH 7.0 with 10 N NaOH) in a 10 ml final vol. in a 25 ml Erlenmeyer flask. The reaction was started by adding various amounts of NH_4Cl as indicated. The flasks were shaken continuously in a reciprocator bath at 30°C and 1 ml aliquots withdrawn after 30 min were centrifuged and assayed for hydroxylamine, as in section 2.

15 min and hydroxylamine was determined in 1 ml aliquot of the supernatant. Then 2 g KH_2PO_4 was added to the rest of the supernatant, followed by 100 mg hydroxylamine–HCl and 160 μl cyclohexanone and the reaction mixture shaken at 30°C for 90 min in a reciprocating waterbath. The oxime was isolated, crystallized and converted to N_2 gas, as in section 2. The ^{15}N enrichment was determined by measuring mass 28, 29 and 30, representing ^{14}N–^{14}N, ^{14}N–^{15}N and ^{15}N–^{15}N, respectively, using the Micromass 602C mass spectrometer, AEI, Manchester.

Experiments were also done under similar conditions with ^{18}O-labelled compounds except that the cells were evacuated for 5 min using a rotary vacuum

Table 1
The incorporation of stable isotopes into hydroxylamine produced by the oxidation of NH_4Cl by washed cells of *Nitrosomonas*

Source of stable isotope	Atom% excess	Atom% excess incorporated into oxime	Percent of theoretical value
$^{15}NH_4Cl$	33.0	0.12	96
$^{18}O_2$	99.0	0.34	93
$H_2{}^{18}O$	80.6	0	0

Experimental details given in the text

pump and exposed to an atmosphere of He–$^{18}O_2$ (99 atom% excess) (80:20) in one case and ^{18}O enriched water (80.64 atom% excess) in the other case. The oximes produced were crystallized 3 times from petroleum ether and analysed in a Hewlett-Packard GC MS Model 5992B mass spectrometer.

The results in table 1 show that the ^{15}N enrichment in NH_2OH is 96% of the theoretically expected value, thus indicating that ammonia is directly oxidised to hydroxylamine by *Nitrosomonas*, so that hydrazine makes little or no contribution to its production. Dilution factors for hydroxylamine, calculated from both the colorimetric method and the incorporation of ^{15}N into the oxime, were used to determine the percent incorporation of $^{18}O_2$ into oxime. These values were found to be 93% and 97%, respectively, based on experiments done simultaneously with the same cell suspension. The $^{18}O_2$ experiment shows that the oxygen of hydroxylamine is derived from air and not from water.

These results are in agreement with thermodynamic data and the findings of Suzuki and Kwok [6], who postulated that the oxidation of ammonia by *Nitrosomonas* is catalysed by an oxygenase. Rees and Nason [7] on the basis of ^{18}O isotope studies also concluded that during the oxidation of ammonia to nitrite, one of the two oxygen atoms of nitrite was derived from air. However, it must be pointed out that they isolated nitrite and not hydroxylamine from the reaction mixture. Two atoms of oxygen in nitrite can exchange with water. On the other hand, the oxygen atom of both hydroxylamine and oxime are covalently linked to nitrogen and are, therefore, not exchangeable with water. Thus the results for the incorporation of $^{15}NH_4Cl$ and $^{18}O_2$ into oximes are more reliable.

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References

[1] Lees, H. (1952) Nature 169, 156.
[2] Hofman, T. and Lees, H. (1953) Biochem. J. 54, 579–583.
[3] Yoshida, T. and Alexander, M. (1964) Can. J. Microbiol. 10, 923–926.
[4] Frear, D. S. and Burrell, R. C. (1955) Anal. Chem. 27, 1664–1665.
[5] Hranisavljevic-Jakovljevic, M., Pejkovic-Tadic, I. and Stojiljkovic, A. (1963) J. Chromatog. 12, 70–73.
[6] Suzuki, I. and Kwok, S. C. (1969) J. Bacteriol. 99, 897–898.
[7] Rees, M. K. and Nason, A. (1966) Biochim. Biophys. Acta, 113, 398–401.