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Identification of the Sources of Nitrous Oxide Produced by Oxidative and Reductive Processes in *Nitrosomonas europaea*

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1. Cells of *Nitrosomonas europaea* produced N_2O during the oxidation of ammonia and hydroxylamine. 2. The end-product of ammonia oxidation, nitrite, was the predominant source of N_2O in cells. 3. Cells also produced N_2O, but not N_2 gas, by the reduction of nitrite under anaerobic conditions. 4. Hydroxylamine was oxidized by cell-free extracts to yield nitrite and N_2O aerobically, but to yield N_2O and NO anaerobically. 5. Cell extracts reduced nitrite both aerobically and anaerobically to NO and N_2O with hydroxylamine as an electron donor. 6. The relative amounts of NO and N_2O produced during hydroxylamine oxidation and/or nitrite reduction are dependent on the type of artificial electron acceptor utilized. 7. Partially purified hydroxylamine oxidase retained nitrite reductase activity but cytochrome oxidase was absent. 8. There is a close association of hydroxylamine oxidase and nitrite reductase activities in purified preparations.

The nitrifying bacterium *Nitrosomonas europaea* oxidizes ammonia to nitrite with hydroxylamine as an intermediate (Hofman & Lees, 1953; Yoshida & Alexander, 1964). Washed-cell suspensions oxidize hydroxylamine quantitatively to nitrite at low substrate concentrations. Cell-free extracts also oxidize hydroxylamine to nitrite in the presence of an electron acceptor such as phenazine methosulphate or cytochrome *c*, but the recovery as nitrite does not exceed 70% of the hydroxylamine oxidized (Nicholas & Jones, 1960). Homogeneous preparations of hydroxylamine oxidase have been obtained (Rees, 1968*a*) and some molecular properties of this electron-transport particle have been studied (Rees, 1968*b*). Even with the homogeneous enzyme the nitrite stoicheiometry in the presence of phenazine methosulphate did not exceed 70%. With cytochrome *c* as electron acceptor, Falcone *et al.* (1963) demonstrated a stoicheiometry for the initial oxidation of 1 mol of hydroxylamine to 2 mol of cytochrome *c* reduced, thus suggesting a nitrogenous intermediate at the oxidation level of +1 for the N atom. It was also shown that under anaerobic conditions the intermediate was converted into nitrous oxide (N_2O) and the authors postulated that the intermediate was nitroxyl (NOH). In the presence of Methylene Blue, Anderson (1964) observed that cell-free extracts oxidized hydroxylamine anaerobically to give equivalent amounts of N_2O and nitric oxide (NO) estimated by manometric techniques. These results suggested the formation of a second intermediate at the oxidation level of +2 for the nitrogen atom.

An enzyme that reduced nitrite with hydroxylamine as an electron donor (Hooper, 1968) has been partially characterized in cell-free extracts of *Nitrosomonas*. The enzyme was strictly aerobic and nitrite was reduced to a mixture of N_2O and NO. Hooper (1968) concluded, however, that this enzyme system did not function during the normal process of nitrification, although its activity could interfere in enzyme studies with hydroxylamine oxidase.

The present work is concerned with the sources of N_2O and NO produced during the oxidation of hydroxylamine by cell-free extracts and by partially purified hydroxylamine oxidase. The purified enzyme contained an active nitrite-reducing enzyme that also evolved N_2O and NO. The enzyme system was investigated by the use of ^{15}N-labelled substrates and subsequent analysis of the labelled gaseous products in the mass spectrometer. In addition, N_2O was shown to be evolved by washed cells of *Nitrosomonas* primarily as a product of nitrite reduction, and this evolution proceeded both aerobically and anaerobically.

Experimental

Materials

Phenazine methosulphate, bovine serum albumin (fraction V), deoxyribonuclease I, lysozyme and horse heart cytochrome *c* (type II) were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); 8-hydroxyquinoline potassium sulphate, dichlorophenol-indophenol and Methylene Blue were from BDH Chemicals Ltd. (Poole, Dorset, U.K.);

$Na^{15}NO_2$ (31.3 atoms % excess) was lot R.179 from Office National Industriel de l'Azote (Toulouse, France); $^{15}NH_4NO_3$ (64 atoms % excess) was lot J.290-115 from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.); $^{15}NH_2OH,HCl$ (95 atoms % excess) was lot AD-100 from Merck, Sharp and Dohme (Montreal, P.Q., Canada); NH_2OH,HCl (A.R.) was recrystallized twice from methanol–water and dried over P_2O_5; all other reagents were of best purity available. Oxygen, helium, argon, argon+oxygen (80:20) and N_2O (hospital grade) were all obtained from Commonwealth Industrial Gases (Adelaide, Australia); helium containing 0.1% (v/v) oxygen was prepared from cylinder gases.

Methods

Culture of organism. Nitrosomonas europaea (kindly supplied by Dr. Jane Meiklejohn of Rothamsted Experimental Station, U.K.) was grown with forced aeration in 50-litre batch cultures as described by Nicholas & Rao (1964) except the pH was maintained at 7.5 by addition of 20% (w/v) K_2CO_3. After 60h of growth the cells were harvested in a Sorvall RC2 centrifuge at 2°C and 35000*g* by using a continuous-flow rotor. The cells (2.0g wet wt./50 litres) were washed free of nitrite in 0.04M-K_2HPO_4–KH_2PO_4 buffer, pH7.0, containing Ca^{2+} and Mg^{2+} at the same concentration as the culture medium.

Preparation of cell extracts. Cell extracts were prepared by disruption of cells in a French pressure cell (40ml capacity) at 2°C with an Aminco motor-driven press at $1.72\times10^8\ N\cdot m^{-2}$. Washed cells were resuspended at 50mg wet wt./ml in 0.1M-Na_2HPO_4–KH_2PO_4 buffer, pH7.5, containing 5mM-$MgCl_2,6H_2O$ and 6mg of bovine albumin/ml, and were passed once through the pressure cell. The crude cell extracts were centrifuged at 10000*g* for 15min and the supernatant fraction was centrifuged at 144000*g* for 60min (Spinco model L, rotor type 50 Ti). The supernatant (144S fraction) was used in experiments.

A lytic procedure was adopted for the extraction of hydroxylamine oxidase for subsequent purification. Cell lysis was accomplished by osmotic rupture after an EDTA–lysozyme treatment by the method of Rees & Nason (1965).

Washed-cell experiments. Incubation of washed cells was done in matched Warburg flasks (10ml) fitted with ground-glass stopcocks and rubber septa (Suba-seal; Wm. Freeman, Barnsley, Yorks., U.K.) on the side-arm ports. The cells (1.0ml) suspended in 0.1M-Na_2HPO_4–KH_2PO_4 buffer, pH7.5, were placed in the main compartment and the substrates in the side arm. When incubations were in an atmosphere other than air, the flasks were evacuated and flushed four times with the appropriate gas before a final filling, the apparatus described by Elleway *et al.* (1971) being used. After a preincubation of 5min the substrates were tipped and the cells allowed to respire for 30min at 30°C in a shaking water bath (75 oscillations/min). The flasks were then cooled in ice and the gases were analysed immediately either in a model MS2 mass spectrometer (Associated Electrical Industries, Manchester, U.K.) or by gas chromatography.

Chemical methods. (*a*) Nitrite was determined by the Griess Ilosvay colorimetric method in a final volume of 3.0ml, as described by Hewitt & Nicholas (1964). (*b*) Hydroxylamine was determined by a modification of the direct method of Frear & Burrell (1955). The sample (10–150nmol) in an open test tube was made up to 2ml with water and 1ml of 0.05M-Na_2HPO_4–KH_2PO_4 buffer, pH6.8, was added. Then 1ml of aq. 2% (w/v) 8-hydroxyquinoline potassium sulphate was added, and the tube was heated at 60°C for 2min. To the hot solution 1ml of 1M-Na_2CO_3 was added with vigorous shaking. After 15min the E_{705} was measured in a Unicam SP.600 spectrophotometer. Beer's Law was obeyed up to 150nmol of NH_2OH,HCl. The addition of trichloroacetic acid (0.2ml of 10%, w/v) was unnecessary with small quantities of protein, as the final high pH solubilized the protein; however, its inclusion did not interfere with colour development. Ammonia at 4mM final concentration did not interfere with the reaction, but nitrite yielded a yellow chromophore with 8-hydroxyquinoline, which significantly interfered at a final concentration above 2mM. Ethanol totally inhibited colour development. (*c*) Ammonia was assayed by the Russell alkaline phenoxide method, after distillation by the Conway procedure, under the conditions of Wallace & Nicholas (1968). (*d*) Protein was determined by the method of Lowry *et al.* (1951) or, for partially purified enzyme prepara-

Table 1. *Aerobic production of N_2O by cells of Nitrosomonas in the presence of different substrates*

Each incubation mixture contained washed cells, the equivalent of 25mg bacterial dry wt. (16.2mg of protein), in a final volume of 1.0ml in 15ml Warburg flasks. Incubation and sampling were as described in the Experimental section.

Substrate		N_2O in gas phase (μmol/30min incubation)
None		0.252
NH_2OH,HCl	(10mM)	2.226
$(NH_4)_2SO_4$	(2.5mM)	2.814
$(NH_4)_2SO_4$	(15mM)	3.108
$NaNO_2$	(20mM)	0.200
$(NH_4)_2SO_4$ + $NaNO_2$	(12.5mM) (20mM)	2.700

tions, by the method of Warburg & Christian (1941).

Analysis of nitrous oxide (N_2O) and nitric oxide (NO). (*a*) Mass spectrometry. The mass spectrometer records mass-to-charge (*m/e*) ratios quantitatively as peaks on chart paper. Peak heights were measured and the background of the mass spectrometer, for each peak, was subtracted. For N_2O produced from unlabelled or ^{15}N-labelled precursors there were three possible combinations of N isotopes: $^{14,14}N_2O$ (*m/e* 44), $^{14,15}N_2O$ (*m/e* 45) and $^{15,15}N_2O$ (*m/e* 46). These three peaks were considered clean since there was no evidence that other molecular species, other than CO_2 (*m/e* 44), contributed to their magnitude. The CO_2 was absorbed into KOH during the incubations. As in subsequent experiments the amount of CO_2 was not observed to alter significantly during the incubation period, a correction value obtained from control incubations without ^{15}N-labelled substrate was subtracted from the peak height for *m/e* 44. Unlabelled nitric oxide (^{14}NO) gave a peak at *m/e* 30 and ^{15}NO at *m/e* 31. These peaks were, however, influenced by associated gases, notably N_2 and N_2O. Nitrogen was not produced under any of the experimental conditions employed in this work and so the influence of the isotope $^{15,15}N_2$ gas (*m/e* 30) did not apply. Nitrous oxide, on the other hand, undergoes some dissociation during ionization in the mass spectrometer and the resulting mass spectrum contained peaks caused by ionized fragments of the molecules as well as those from the parent molecules (Cheng & Bremner, 1965). To determine the necessary corrections a mass spectrum of pure N_2O was made in the MS2 apparatus and gave mass peaks of relative intensity 11.0 and 25.0 at *m/e* 28 and 30, respectively, when the relative intensity at *m/e* 44 was 100.0. Since the N_2O produced in the experiments contained some ^{15}N, ionization fragments appeared at *m/e* 29, 30 and 31. Corrections were made for the peak heights of *m/e* 28, 29, 30 and 31 by using the equations of Cady & Bartholomew (1960). Final results were expressed as peak heights for each type of N-isotope composition of the gas identified. Any isotope effects that may have occurred during the enzymic oxidation and reduction reactions were neglected. There are no results available relevant to isotope-exchange reactions occurring between $^{14,14}N_2O$ and $^{15,15}N_2O$; however, no exchange was observed during a 30min incubation at 30°C, i.e. no increase in *m/e* 45 ($^{14,15}N_2O$) was apparent in sequential gas samples taken from a flask containing only pure labelled and unlabelled N_2O. The corrected peak heights correspond to the corrected ion beam intensities plotted on a chart recorder calibrated at 1.25×10^{-12}A for full-scale deflexion.

(*b*) Gas chromatography. N_2O was quantitatively analysed together with N_2, CO_2, Ar and O_2 in gas samples by using three columns of molecular-sieve material at elevated, ambient and sub-ambient temperatures. The method used was that of Burford (1969) employing a Shimadzu GC-IC gas chromatograph. Gas samples (5ml) were taken at atmospheric pressure by equilibrating the flask with water by using a syringe. Absolute determination of N_2O in incubations was not possible as this gas is appreciably soluble in aqueous solution (0.121g/100g of water at 20°C and 1atm). Therefore, N_2O production is expressed as μmol present in the gas phase.

Assay of hydroxylamine oxidase. (*a*) Aerobically, the assay was conducted by measuring nitrite formation and hydroxylamine disappearance. The assay mixture, in a 15cm×2.5cm test tube, contained 0.4ml of 0.1M-K_2HPO_4–KH_2PO_4 buffer (pH8.5), 0.02ml (30nmol) of phenazine methosulphate, 0.05ml (0.6μmol) of NH_2OH,HCl, enzyme and water to 0.5ml. The mixture was preincubated for 5min at 30°C with shaking (100 oscillations/min); the reaction was started by adding hydroxylamine. Samples (0.1ml) were removed at known time-intervals for 30min and nitrite and hydroxylamine were deter-

Table 2. *Composition of labelled N_2O produced by cells of Nitrosomonas in the presence of nitrite and $^{15}NH_3$ under an atmosphere of helium and 0.1% oxygen*

Each time-interval was a separate incubation experiment. Each incubation mixture contained the equivalent of 21.0mg bacterial dry wt. (13.6mg of protein), 10μmol of $NaNO_2$ and 20μmol of $^{15}NH_4NO_3$ (64 atoms % excess) in a total volume of 1.0ml.

Incubation time (min)	Corrected peak heights for N_2O			Ratio of peak heights		
	$^{14,14}N_2O$	$^{14,15}N_2O$	$^{15,15}N_2O$	$^{14,14}N_2O$:	$^{14,15}N_2O$:	$^{15,15}N_2O$
5	11.0	0.9	0	1 :	0.082 :	0
10	19.0	2.0	0.5	1 :	0.105 :	0.026
20	33.0	3.0	0.5	1 :	0.091 :	0.015
30	58.0	6.5	0.6	1 :	0.112 :	0.014
Expected ratio assuming all N_2O derived from $^{15}NH_4{}^{14}NO_3$				1 :	3.60 :	3.18

mined. Specific-activity units are defined as μmol of nitrite formed/min per mg of protein.

(*b*) Gas production from $^{15}NH_2OH$ under anaerobic conditions was analysed on the mass spectrometer. The assay was done in single closed-side-arm Warburg flasks (20ml) fitted with ground-glass stopcocks. The main compartment contained phenazine methosulphate (7.2μmol), enzyme and 0.1M-Na_2HPO_4–KH_2PO_4 buffer, pH7.5, to a volume of 2ml. Freshly prepared and neutralized hydroxylamine hydrochloride (3.6μmol) was placed in the side arm. The centre well contained 0.2ml of 2M-KOH absorbed on folded strips of filter paper for the absorption of the CO_2. When included in the reaction mixture, $NaNO_2$ (3.0μmol) was added to the main compartment. For anaerobic experiments the flasks were evacuated to $1.33\times10^{-2}N\cdot m^{-2}$ with a Speedivac 2SC50 pump (Edwards and Co. Ltd., Crawley, U.K.), and for experiments in helium–oxygen atmospheres the flasks were flushed with the gas in an apparatus described by Elleway *et al.* (1971). The reaction mixtures were preincubated for 5min at 30°C before the substrates were tipped in and then incubation was continued for a further 30min before the gas phase above the samples was analysed directly in the mass spectrometer.

Purification of hydroxylamine oxidase. Hydroxylamine oxidase was purified from 144000*g* supernatant fractions obtained after osmotic rupture of washed cells. The conditions of the purification procedure, including separation on 3–20% (w/w) sucrose density gradients, were those described by Rees (1968*a*). Fractions (0.5ml) were collected under gravity and those with highest specific activity were pooled, concentrated against Ficoll (Pharmacia, Uppsala, Sweden) and dialysed overnight at 2°C against 0.1M-Na_2HPO_4–K_2HPO_4 buffer, pH7.5. Purifications of 75-fold over the specific activity of the original 10000*g* supernatant fractions were achieved, with final specific activities between 0.48 and 0.63μmol of nitrite formed/min per mg of protein. The partially purified enzyme was judged to be about 70% pure after flat-sheet polyacrylamide-gel electrophoresis by the method of Reid & Bieleski (1968). Haem-staining by the method of Newton (1969) indicated that two haemoproteins other than hydroxylamine oxidase were present.

Stoicheiometry determinations. Small 10ml flasks fitted with side arms were used. The necks were sealed with serum caps and the side arm with Subaseals. Each incubation mixture contained partially purified enzyme (40μg of protein), phenazine methosulphate (90nmol), $NaNO_2$ (1.5μmol), NH_2OH,HCl (1.8μmol) dissolved in the appropriate buffer in the side arm, and the appropriate buffer (0.1M-Na_2HPO_4–KH_2PO_4) to 1.0ml. The flasks were flushed with helium for 20min and preincubated at 30°C before the contents of the side arm were tipped in. Samples of reaction mixture were removed at timed intervals for the determination of nitrite and hydroxylamine, by using a gas-tight micro-syringe. The reaction rate was linear over 15min and the total loss of hydroxylamine was about 1.0μmol. Identical control incubations, without enzyme, were included so that correction could be made for non-enzymic losses and also to calculate the amounts of the substrates at zero time.

Results

Aerobic gas production by cells of Nitrosomonas

When washed cells were incubated in air for short periods with an oxidizable substrate (ammonia or hydroxylamine) N_2O was produced in significant quantities as detected by gas chromatography (Table 1). The rate of N_2O production was independent of the ammonia concentration between 5 and 30mM. Exogenous nitrite up to 40mM did not affect the N_2O production in the presence of ammonia nor did it lead to a detectable increase in N_2O production when added alone. Aerobic incubations in an argon–oxygen atmosphere did not result in the production of nitrogen gas in addition to N_2O. The production

Table 3. *Composition of labelled N_2O produced by cells of Nitrosomonas in the presence of $^{15}NO_2^-$ and ammonia under an atmosphere of helium and 0.1% oxygen*

Each time-interval was a separate incubation experiment. Each incubation mixture contained the equivalent of 21.0mg bacterial dry wt. (13.6mg of protein), 20μmol of NH_4NO_3 and 10μmol of $Na^{15}NO_2$ (31 atoms % excess) in a total volume of 1.0ml.

Incubation time (min)	Corrected peak heights for N_2O			Ratio of peak heights		
	$^{14,14}N_2O$	$^{14,15}N_2O$	$^{15,15}N_2O$	$^{14,14}N_2O$:	$^{14,15}N_2O$:	$^{15,15}N_2O$
10	14.0	7.4	1.4	2 :	1.10 :	0.2
20	20.0	9.5	2.0	2 :	0.95 :	0.2
30	38.0	16.0	2.9	2 :	0.84 :	0.15
Expected ratio assuming all N_2O derived from $Na^{15}NO_2$				1 :	0.9 :	0.2

of N_2O by washed cells in air was confirmed by gas analysis in the mass spectrometer by using ^{15}N-labelled ammonia, hydroxylamine or nitrite, and $^{14,14}N_2O$, $^{14,15}N_2O$ and $^{15,15}N_2O$ were detected.

The observation that labelled N_2O was derived from $^{15}NO_2^-$ suggested a possible reduction mechanism for its production. The formation of N_2O during oxidation of ammonia and also during nitrite reduction was shown conclusively by incubating cells with either $^{15}NH_3$ plus NO_2^- or $^{15}NO_2^-$ plus NH_3, in an atmosphere of helium and 0.1% oxygen. The results of these experiments are shown in Tables 2 and 3. After 30min of incubation oxygen was still present in the atmosphere. The ratio of the peak heights for N_2O containing the different combinations of N isotopes approximated to the calculated value, assuming all the N_2O was derived from nitrite N. This result was confirmed by the poor correlation between the calculated and observed ratios in the presence of $^{15}NH_3$. The ratios did not alter during the various incubation periods. Most of the gas was derived from nitrite and the remainder from ammonia, thus suggesting an active nitrite reductase system in *Nitrosomonas* cells. No significant changes in gas production were observed in these experiments, using washed cells stored at 4°C for periods of up to 1 week.

Anaerobic gas production from cells of Nitrosomonas

Cells stored for 24h were incubated in an atmosphere of pure argon with nitrite in the presence and absence of ammonia (Table 4). The amount of N_2O produced was 1.5–2 times that from ammonia under aerobic conditions. Moreover, the inclusion of ammonia with nitrite did not increase the anaerobic production of N_2O. The composition of N_2O produced anaerobically by whole cells in the presence of nitrite or ammonia or both substrates is shown in Table 5. In the presence of $^{15}NH_3$ there was no incorporation of label into N_2O although ammonia was lost from the incubation medium. Ammonia was also lost in the presence of nitrite; however, the N_2O production was increased eightfold and was mainly derived from nitrite, which itself was utilized. When $^{15}NO_2^-$ was incubated alone it was utilized and the composition of the N_2O reflected this, in that the calculated and observed ratios were similar. The cells were able to reduce nitrite to N_2O under anaerobic conditions during these short incubation periods.

Oxidation of hydroxylamine by 144S fractions of Nitrosomonas

The lability of the ammonia oxidase activity in cell-free extracts, even in the presence of bovine serum albumin (Suzuki & Kwok, 1970), prevented the detection of N_2O during the oxidation of ammonia. Cell extracts with added bovine serum albumin possessed hydroxylamine oxidase activity and this activity was greatly stimulated on addition of the electron acceptor phenazine methosulphate (Nicholas & Jones, 1960). Under aerobic conditions (helium–oxygen) oxidation of $^{15}NH_2OH$ by 144S fractions resulted in the production of N_2O. When phenazine methosulphate was included in the incubation mixture, in addition to a stimulation of activity both NO and N_2O were evolved (Table 6). Under anaerobic conditions there was no oxidation of hydroxylamine unless phenazine methosulphate was included (Table 7). The main gaseous product was NO although substantial amounts of N_2O were also evolved. No nitrite was formed under anaerobic conditions as molecular oxygen is necessary for its formation.

Hydroxylamine-dependent nitrite reductase activity in the 144S fraction

The presence of a nitrite-reducing enzyme that uses hydroxylamine as an electron donor, which was described by Hooper (1968) in cell extracts of *Nitrosomonas*, was confirmed, and it was apparent from his results that the gaseous products of nitrite reduction were identical with those of hydroxylamine oxidation. The use of ^{15}N-labelled substrates enabled the identification of the sources of gas evolution in 144S

Table 4. *Aerobic and anaerobic production of N_2O by cells of Nitrosomonas in the presence of different substrates*

Each incubation mixture with washed cells contained the equivalent of 25mg bacterial dry wt. (16.2mg of protein) in 1.0ml final volume in 10ml Warburg flasks. Incubation and sampling were as described in the Experimental section.

Substrate concn. (mM)	Atmosphere	N_2O in gas phase (nmol/30min incubation)
None	Argon/oxygen	26.0
$(NH_4)_2SO_4$ (25mM)	Argon/oxygen	307.0
$(NH_4)_2SO_4$ (25mM) +$NaNO_2$ (20mM)	Argon	671.0
$NaNO_2$ (20mM)	Argon	685.0

Table 5. *Composition of labelled N_2O produced by anaerobic incubation of cells of Nitrosomonas with $^{15}NO_2^-$ and $^{15}NH_3$*

Washed cells (25 mg dry wt., 16.2 mg of protein) were incubated for 30 min with the appropriate substrate in an atmosphere of argon under the conditions described in the Experimental section. After gas sampling the cell suspension was centrifuged and nitrite and ammonia were determined in samples of the supernatant fraction.

Substrate (μmol)	Corrected peak heights for N_2O			Nitrite produced (+) or utilized (−) (μmol)	Ammonia produced (+) or utilized (−) (μmol)	Ratio of N_2O peak heights and calculated ratio					
	$^{14,14}N_2O$	$^{14,15}N_2O$	$^{15,15}N_2O$			$^{14,14}N_2O$	:	$^{14,15}N_2O$	:	$^{15,15}N_2O$
None	4	0	0	+0.114	0					
$^{15}NH_4NO_3$ (29.1)	5.5	0	0	+0.216	−5.5					
$Na^{15}NO_2$ (7.76)	26	11	2.4	−6.18	0	Observed	2	: 0.85	:	0.185
						Calculated	1	: 0.90	:	0.20
$^{15}NH_4NO_3$ (29.1)	41	0.7	0	−4.92	−4.3	Observed	1	: 0.018	:	0
$+NaNO_2$ (8.36)						Calculated	1	: 3.60	:	3.18

fractions and results of such experiments are included in Tables 6 and 7. In an atmosphere of helium–oxygen the addition of nitrite to the incubation mixture in the presence or absence of phenazine methosulphate resulted in the increase in the peak heights of ^{14}NO, $^{14,14}N_2O$ and $^{14,15}N_2O$. The most notable increase was in the amount of mixed-label N_2O produced. Similar results were obtained in anaerobiosis (Table 7); however, most of the gas from nitrite was ^{14}NO. Without phenazine methosulphate but in the presence of nitrite, substantial amounts of N_2O were produced; most was again the mixed-label N_2O. These results were confirmed in experiments with hydroxylamine and $^{15}NO_2^-$ as substrates, and suggested an integrated reaction mechanism for hydroxylamine oxidation and nitrite reduction.

Influence of electron acceptors on gas production by 144S fractions

Electron acceptors other than phenazine methosulphate have been shown to mediate hydroxylamine oxidase activity; these include ferricyanide, Methylene Blue, dichlorophenol-indophenol and mammalian cytochrome *c* (Nicholas & Jones, 1960). These acceptors were all effective in gas production during $^{15}NH_2OH$ oxidation by 144S fractions in anaerobiosis (Table 8). As shown, the same acceptors also mediated hydroxylamine-dependent nitrite reductase in 144S fractions. However, the use of different electron acceptors caused variation in the ratio of NO to N_2O produced during the incubations. Phenazine methosulphate and ferricyanide both showed a consistently greater production of NO than N_2O, whereas dichlorophenol-indophenol and Methylene Blue favoured a greater production of N_2O. Exactly the same pattern was observed with NO and N_2O production from added nitrite. Ferrocyanide (3.5 mM) was an effective electron donor for the reduction of nitrite by 144S fraction under anaerobic conditions, but negligible nitrite reduction was observed with partially purified hydroxylamine oxidase. Chemically reduced phenazine methosulphate (1.5 mM) was also an effective electron donor for nitrite reduction with either 144S fraction or partially purified hydroxylamine oxidase. The reduction of nitrite was not mediated by NADH (1.5 mM) or NADPH (1.5 mM) in 144S fractions.

Gas production by partially purified hydroxylamine oxidase

Without the addition of an electron acceptor a 75-fold purified hydroxylamine oxidase (described in the Experimental section) did not oxidize $^{15}NH_2OH$ aerobically. In the presence of phenazine methosulphate and under anaerobic conditions the gases produced during the oxidation of $^{15}NH_2OH$ were NO and N_2O, as for 144S fractions (Table 9).

Table 6. *Gaseous products formed during aerobic oxidation of $^{15}NH_2OH$ by 144S fraction under helium plus 0.1% oxygen*

Each incubation flask contained: Na_2HPO_4–KH_2PO_4 buffer, pH7.5, 150μmol; $MgCl_2,6H_2O$, 7.5μmol; 144S fraction (6mg of protein) containing 9mg of bovine serum albumin; phenazine methosulphate 7.2μmol where appropriate; $NaNO_2$, 3.0μmol where appropriate; and $^{15}NH_2OH$, HCl, 3.6μmol in the side arm (total volume of reaction mixture 2.0ml). The centre well contained 0.2ml of 2M-KOH. Conditions for incubation and gas analysis were as described in the Experimental section.

Experiment	Corrected peak heights for NO and N_2O				
	^{14}NO	^{15}NO	$^{14,14}N_2O$	$^{14,15}N_2O$	$^{15,15}N_2O$
Boiled 144S fraction+$^{15}NH_2OH$+NO_2^-	3	2	4	3	2
144S fraction+$^{15}NH_2OH$	10	1	8	2	28
144S fraction+$^{15}NH_2OH$+phenazine methosulphate	4	170	8	2	84
144S fraction+$^{15}NH_2OH$+NO_2^-	16	2	45	175	30
144S fraction+$^{15}NH_2OH$+NO_2^-+phenazine methosulphate	20	37	169	195	95

Table 7. *Gaseous products formed during anaerobic oxidation of $^{15}NH_2OH$ by 144S fraction*

Each incubation flask contained: Na_2HPO_4–KH_2PO_4 buffer, pH7.5, 150μmol; $MgCl_2,6H_2O$, 7.5μmol; 144S fraction (6mg of protein) containing 9mg of bovine serum albumin; phenazine methosulphate, 7.2μmol where appropriate; $NaNO_2$, 3.0μmol where appropriate, and $^{15}NH_2OH$, HCl, 3.6μmol in the side arm (total volume of reaction mixture 2.0ml). The centre well contained 0.2ml of 2M-KOH. Conditions for incubation and gas analysis were as described in the Experimental section.

Experiment	Corrected peak heights for NO and N_2O				
	^{14}NO	^{15}NO	$^{14,14}N_2O$	$^{14,15}N_2O$	$^{15,15}N_2O$
Boiled 144S fraction+$^{15}NH_2OH$+NO_2^-	1	1	2	1	1
144S fraction+$^{15}NH_2OH$	3	2	6	1	5
144S fraction+$^{15}NH_2OH$+phenazine methosulphate	0	176	6	1	32
144S fraction+$^{15}NH_2OH$+NO_2^-	0	0	24	86	22
144S fraction+$^{15}NH_2OH$+NO_2^-+phenazine methosulphate	160	150	22	32	36

When nitrite was included in the incubations, nitrite reductase activity was still evident, ^{14}NO and $^{14,15}N_2O$ being the main products. The yield of gas from nitrite was significantly less than the yield from hydroxylamine with the purified enzyme, whereas for 144S fractions there was an equivalence of NO and N_2O production from nitrite and hydroxylamine.

Variation of amounts of gaseous products with pH. The pH of the incubation mixture had a profound effect on the relative quantities of NO and N_2O produced from the two substrates in the presence of the purified enzyme, as shown in Fig. 1. There was an inverse relationship between pH and NO production. A decrease in pH below 7.0 caused an increase in ^{14}NO derived from nitrite. At pH values below 7.0, ^{15}NO derived from $^{15}NH_2OH$ decreased sharply. Changes in N_2O production were more complicated for, although the amount of $^{15,15}N_2O$ increased with pH, the amount of $^{14,15}N_2O$ remained constant. The amount of nitrite-derived $^{14,14}N_2O$ also increased with pH and may reflect a pH-dependent change in the ratio of products of the nitrite reductase system.

Variation of gaseous products with substrate concentration. With the purified enzyme at pH7.0 and a constant concentration of $^{15}NH_2OH$ (0.9mM), the nitrite concentration was varied between 1.5 and 50mM in the incubation mixture. The amount of ^{14}NO derived from nitrite increased with an increase in nitrite concentration and flattened out when the nitrite concentration was above 10mM. The production of $^{14,14}N_2O$ remained constant but the amount of mixed-label N_2O increased. No ^{14}NO was produced when $^{15}NH_2OH$ was omitted.

Table 8. *Gaseous products formed during anaerobic oxidation of $^{15}NH_2OH$ by 144S fraction with different electron acceptors in the presence and absence of nitrite*

Each incubation flask contained the same components as for Table 7, except for electron acceptors which were: phenazine methosulphate or ferricyanide, 7.2μmol; dichlorophenol-indophenol or Methylene Blue, 3.6μmol. The concentration of phenazine methosulphate provided twice as many oxidizing equivalents as did the other electron acceptors in this experiment. Conditions for incubation and gas analysis were as described in the Experimental section.

Experiment	Corrected peak heights for NO and N_2O				
	^{14}NO	^{15}NO	$^{14,14}N_2O$	$^{14,15}N_2O$	$^{15,15}N_2O$
144S fraction+$^{15}NH_2OH$+phenazine methosulphate	1	77	2	0	31
144S fraction+$^{15}NH_2OH$+ferricyanide	1	51	3	0	30
144S fraction+$^{15}NH_2OH$+dichlorophenol-indophenol	0	18	2	0	40
144S fraction+$^{15}NH_2OH$+Methylene Blue	0	13	2	0	62
144S fraction+$^{15}NH_2OH$+NO_2^-+phenazine methosulphate	55	57	6	9	4
144S fraction+$^{15}NH_2OH$+NO_2^-+ferricyanide	34	32	7	12	10
144S fraction+$^{15}NH_2OH$+NO_2^-+dichlorophenol-indophenol	19	16	13	18	9
144S fraction+$^{15}NH_2OH$+NO_2^-+Methylene Blue	21	18	21	38	19

Table 9. *Gaseous products formed during anaerobic $^{15}NH_2OH$ oxidation by partially purified enzyme*

Each incubation flask contained: Na_2HPO_4–KH_2PO_4 buffer, pH7.5, 150μmol; partially purified enzyme (80μg of protein); phenazine methosulphate, 7.2μmol where appropriate; $NaNO_2$, 3.0μmol where appropriate, and $^{15}NH_2OH$,HCl, 3.6μmol in the side arm (total volume of reaction mixture 2.0ml). The centre well contained 0.2ml of 2M-KOH. Conditions for incubation and gas analysis were as described in the Experimental section.

Experiment	Corrected peak heights for NO and N_2O				
	^{14}NO	^{15}NO	$^{14,14}N_2O$	$^{14,15}N_2O$	$^{15,15}N_2O$
Boiled enzyme+$^{15}NH_2OH$+NO_2^-+phenazine methosulphate	6	2	1	2	2
Enzyme+$^{15}NH_2OH$	3	3	1	1	1
Enzyme+$^{15}NH_2OH$+phenazine methosulphate	1	310	30	2	76
Enzyme+$^{15}NH_2OH$+NO_2^-	1	1	3	1	1
Enzyme+$^{15}NH_2OH$+NO_2^-+phenazine methosulphate	80	262	30	20	60

When the nitrite concentration was maintained at 10mM (pH7.0) and the concentration of $^{15}NH_2OH$ was varied between 1 and 25mM, there was an initial increase in production of both ^{14}NO and ^{15}NO up to 7.5mM-$^{15}NH_2OH$, at which concentration saturation was reached. Both $^{14,15}N_2O$ and $^{15,15}N_2O$ production increased at a linear rate as the $^{15}NH_2OH$ concentration was increased, but $^{14,14}N_2O$ production remained constant. From results for ^{15}NO production, the apparent K_m for NH_2OH was 1.5mM when nitrite was at saturating concentration. This value is two orders of magnitude lower than the value found by Hooper (1968).

Stoicheiometry of the hydroxylamine oxidase–nitrite reductase system

The stoicheiometric relationship between oxidation of hydroxylamine and reduction of nitrite was determined with the purified enzyme at pH6.0, 7.4 and 8.5. At each pH value the stoicheiometry of hydroxylamine oxidized/mol of nitrite reduced was in

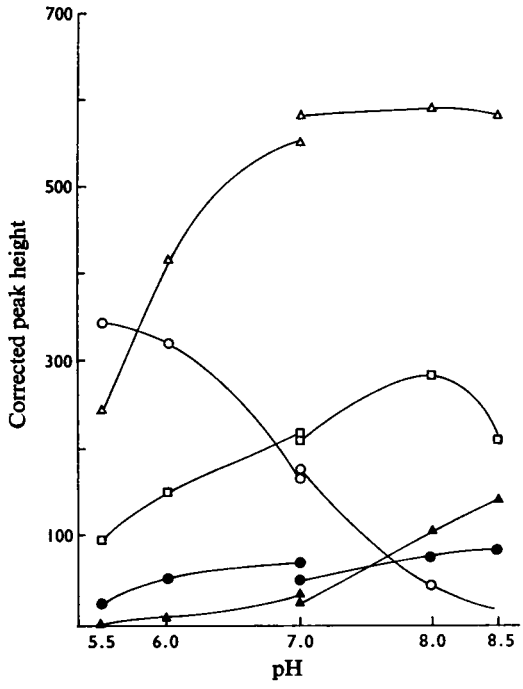


Fig. 1. *Variation of gas production with pH*

The buffers used in these incubations were as follows: pH 5.5–7.0, 0.1 M-citric acid–Na_2HPO_4; pH 7.0–8.5, 0.1 M-Na_2HPO_4–KH_2PO_4. Each incubation mixture contained $^{15}NH_2OH$ (1.8 μmol), $NaNO_2$ (1.5 μmol), phenazine methosulphate (3.6 μmol), and partially purified enzyme (40 μg of protein) in a total volume of 1.4 ml. Evacuation and incubation and analysis of gases was as described in the Experimental section. Identical blank incubations without enzyme were analysed for gaseous products; however, the extent of chemical decomposition of the substrates was insignificant. The pH of the incubation mixtures after gas sampling was unchanged. △, ^{15}NO; ○, ^{14}NO; □, $^{14,14}N_2O$; ▲, $^{15,15}N_2O$; ●, $^{15,14}N_2O$.

the range 1.38–2.24 mol and remained constant during the incubation period.

Discussion

When appropriate techniques are employed with carefully matched Warburg flasks, a uniform system of gas sampling and the application of correction factors to the peak-height measurements, the mass spectrometer provides a very sensitive method for identifying small amounts of ^{15}N-labelled nitrogenous gases unequivocally.

The production of N_2O by washed cells of *Nitrosomonas* during the oxidation of ammonia and hydroxylamine confirmed the report of Yoshida & Alexander (1970). The results reported in the present paper show that the addition of nitrite at concentrations normally attained during batch culture (20 mM) had no inhibitory effect on ammonia oxidation. Mass-spectrometric analysis of the N_2O produced under these conditions, however, showed that $^{15}NO_2^-$ was the main source of this gas and not ammonia. Although the ratio of peak heights for N_2O containing the different isotopic combinations of N did not change during the relatively short incubation periods used in these experiments, they would be expected to alter during prolonged respiration as the oxidation of ammonia increases the intracellular pool of free nitrite.

Washed cells of *Nitrosomonas* also produced N_2O by the reduction of nitrite under anaerobic conditions. This production of N_2O was unaffected by the presence of ammonia, since its oxidation requires molecular oxygen (Rees & Nason, 1966). Ammonia was, however, removed from the medium even when nitrite was present. This loss may have been caused by uptake by the cells with possible conversion into amino acids via glutamate dehydrogenase activity (Wallace & Nicholas, 1969). If ammonia oxidase was specifically inhibited, the aerobic incorporation of $^{15}NH_3$ into cell protein was unaffected (D. J. D. Nicholas, unpublished work). The results with washed cells provided evidence for the existence of a nitrite reductase in this organism, which was active either under anaerobic conditions or aerobically with concomitant oxidation of ammonia to nitrite. Only a few bacterial species have been reported to reduce nitrite under aerobic conditions (Meiklejohn, 1940; Kefauver & Allison, 1957; Skerman *et al.*, 1958). The failure to detect further reduction of N_2O to N_2 gas, together with the very low amounts of ammonia formation during nitrite reduction reported by Wallace & Nicholas (1968) in cell-free extracts of *Nitrosomonas*, suggest that the reductase system may be a mechanism for removing nitrite accumulated intracellularly during ammonia oxidation. Nitrite may also serve as an alternative terminal electron acceptor to oxygen under both anaerobic and aerobic conditions. This faculty may be important in the survival of *Nitrosomonas*, since conditions of temporary anaerobiosis often occur in the soil environment of this micro-organism.

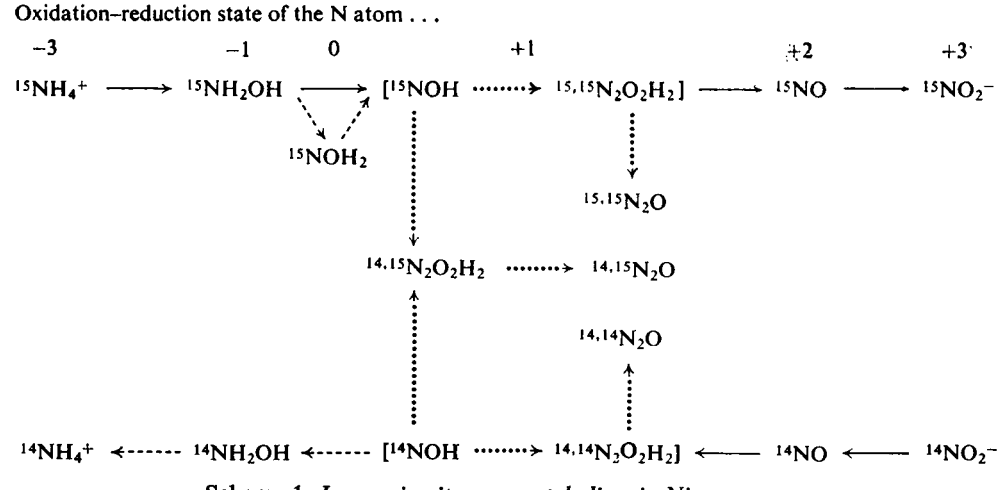
The production of N_2O during the aerobic oxidation of hydroxylamine in cell-free extracts would, in part, account for the poor stoicheiometric yield of nitrite observed by using an added electron acceptor (Nicholas & Jones, 1960; Rees, 1968*a*). As the reaction proceeded, however, the activity of the nitrite reductase would increase because the oxidation of hydroxylamine would provide both substrate and reducing power. The final losses would be

gaseous, i.e., NO and N_2O, produced both during hydroxylamine oxidation and nitrite reduction. The addition of bovine serum albumin to the cell suspension before cell disruption protected hydroxylamine oxidase to the extent that measureable activity was obtained without the use of an electron acceptor. This suggests that a native electron acceptor was present in the extracts. After further enzyme purification, however, this electron acceptor was removed and the addition of phenazine methosulphate was essential for both oxidation of hydroxylamine and reduction of nitrite. This effect of bovine serum albumin may be interpreted as a protection of a physical association between hydroxylamine oxidase and the cytochrome oxidase. Rees (1968*a*) has shown that the cytochrome oxidase can be separated from hydroxylamine oxidase by sucrose-density-gradient centrifugation. In the present work this separation accounts for the observation that the purified enzyme did not oxidize hydroxylamine aerobically in the absence of an electron acceptor. It was also observed that the nitrite reductase activity was decreased in the 75-fold purified hydroxylamine oxidase, suggesting that the former enzyme was a separate protein that could be removed during purification.

The significance of the differential response of gas production to the type of electron acceptor used with hydroxylamine oxidase may be related to the type of dehydrogenation reaction that the enzyme catalyses. Two such sequences may well occur, (i) a two-electron transfer to produce an intermediate in which the N atom has an oxidation–reduction state of +1 (NOH) followed by a one-electron transfer to produce an intermediate in which the N atom has an oxidation–reduction state of +2 (NO), or (ii) a series of three one-electron transfers. As hydroxylamine oxidase is a haemoprotein containing *b*- and *c*-type cytochromes as prosthetic groups, the second sequence of electron transfer might be favoured. In this respect it is the one-electron acceptor, ferricyanide, that reacts most rapidly and accounts for the increased NO values. When nitrite was included, the one-electron acceptor favoured reduction to NO, a one-electron transfer, whereas the two-electron acceptors resulted in the formation of N_2O from nitrite, a two-electron transfer. The presence of nitrite, however, did not alter the efficiency of each type of acceptor for NO or N_2O formation during hydroxylamine oxidation.

Although phenazine methosulphate is a two-electron carrier, it is reduced in two one-electron steps with the intermediate formation of phenazine methosulphate-semiquinone (Michaelis & Schubert, 1938). Zaugg (1964) has demonstrated that phenazine methosulphate-semiquinone can exist in aqueous buffered solutions over the pH range used in these experiments. It is therefore possible that phenazine methosulphate acted in this enzyme system as a one-electron acceptor undergoing transfer steps between the semiquinone and either the oxidized or fully reduced form of phenazine methosulphate.

The combined activities of hydroxylamine oxidase and nitrite reductase mediated by the native electron acceptor, or by any one of the added electron acceptors, consistently produced an amount of



Scheme 1. *Inorganic nitrogen metabolism in Nitrosomonas*

The scheme summarizes the labelled intermediates during the oxidation of $^{15}NH_3$ to $^{15}NO_2^-$ and the reduction of nitrite primarily to N_2O but also to ammonia. The proposed mechanism for the formation of mixed-label N_2O is shown. Enzymic reactions ——; possible enzymic reactions – – – –; chemical reactions ····.

$^{14,15}N_2O$ in excess of the amounts of $^{14,14}N_2O$ and $^{15,15}N_2O$. This observation may be explained in terms of a proximity of two active centres, either two active centres of a single enzyme or the active centres of two distinct but associated enzymes. This conclusion is based on the fact that NOH can only exist free in a neutral aqueous environment for extremely short periods before it dimerizes to form hyponitrite. Only if one active site released ^{15}NOH, derived from $^{15}NH_2OH$, and the other active site released ^{14}NOH, derived from nitrite, within the time taken for dimerization could subsequent decomposition lead to the formation of $^{14,15}N_2O$. Stoicheiometry and substrate-saturation results with purified enzyme lend support to the suggestion that the enzymic activities are closely associated.

The present knowledge of intermediates, as found by these experiments, in the metabolism of nitrogen in *Nitrosomonas* is shown in Scheme 1. During the oxidation of hydroxylamine, enzyme-bound intermediates could be at oxidation levels 0, +1 and +2, assuming them to contain a single nitrogen atom. A hypothetical intermediate included in the scheme at oxidation level 0 has not been identified. If the reaction sequence consists of two two-electron transfers, only NOH, as N_2O, would be expected. In the presence of added electron acceptors, with midpoint potentials ranging from +0.011V to +0.350V, intermediate at oxidation level +2 is produced in variable quantities. This intermediate may in fact be NO; however, with 144S fractions containing hydroxylamine oxidase, no added electron acceptor is required and no NO is produced either aerobically or anaerobically. Until it is shown that NO can be enzymically oxidized to nitrite aerobically and not oxidized chemically by reactions (1) and (2), then NO cannot be considered an obligatory intermediate.

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$2NO_2 + H_2O \rightarrow HNO_2 + HNO_3 \quad (2)$$

Anderson (1964) reported a slight stimulation of NO uptake anaerobically by ferricyanide and cytochrome *c* together; however, we were unable to repeat this observation. It appears likely that NO uptake was caused by complex-formation with the iron in the electron acceptors.

The reduction of nitrite by *Nitrosomonas* cells and by 144S fractions without an electron carrier results only in the formation of N_2O. The use of an electron carrier, such as phenazine methosulphate, leads to production of both NO and N_2O. Similar effects have been observed during the reduction of nitrite by using ascorbate with phenazine methosulphate as the electron-donating system in extracts of *Alcaligenes faecalis* (Matsubara & Iwasaki, 1971).

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