Studies of the Hydroxylamine Metabolism of *Nitrosomonas europaea*. I. Purification of Hydroxylamine Oxidase*

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ABSTRACT: The chemoautotrophic bacterium *Nitrosomonas europaea* derives its energy from the oxidation of ammonia and hydroxylamine. Short-term incubation experiments with intact cells reveal that both substrates are quantitatively oxidized to nitrite. When the structural integrity of the cell is destroyed, all ability to oxidize ammonia is lost, and only 10% of the hydroxylamine-oxidizing specific activity remains. Moreover, not more than 30–40% of the total hydroxylamine metabolized by crude cell-free extracts of *Nitrosomonas* is converted to the expected physiological product, nitrite. Efforts to obtain cell-free ammonia-oxidizing activity have failed. However, very active hydroxylamine oxidation occurs in the presence of added terminal electron acceptor compounds. Again, only a fraction of the hydroxylamine utilized can be accounted for as nitrite. Maximum nitrite formation occurs in the presence of phenazine methosulfate. The enzyme responsible for hydroxylamine oxidation, hydroxylamine oxidase, has been isolated in highly purified form by the sequence of steps: ultracentrifugation → sucrose density gradient centrifugation → Sephadex G-200 chromatography. Although only 30–40% of the total oxidized hydroxylamine is converted to nitrite by crude extracts, this number rises progressively as the enzyme is isolated and reaches 70% for the purified enzyme. The improved stoichiometry is at least in part accounted for by the removal of a soluble cytochrome c_{552} containing material (molecular weight approximately 20,000 g/mole) which has been denoted as fraction F_2. This substance specifically inhibits the formation of nitrite by hydroxylamine oxidase. It has no effect on either the rate or extent of hydroxylamine oxidation. Hydroxylamine oxidase is the initial acceptor of electrons from hydroxylamine. The enzyme is unable to ultimately transfer electrons to molecular oxygen, however, and this function is performed by the terminal oxidase of the cell. Evidently, a marked reduction in the efficiency of sequential electron transfer accompanies cellular lysis. The striking stimulation of hydroxylamine oxidase activity by added terminal electron-acceptor compounds is in keeping with this observation.

The bacterium, *Nitrosomonas europaea*, is a strict autotroph and derives its energy from the aerobic oxidation of ammonia to nitrite according to the over-all equation

$$NH_4^+ + 1.5O_2 \longrightarrow 2H^+ + NO_2^- + H_2O + 66 \text{ kcal} \quad (1)$$

Hofman and Lees (1953), using the inhibitor hydrazine, demonstrated the accumulation of hydroxylamine in the culture media, and thus established that hydroxylamine is an intermediate in ammonia oxidation. The over-all equation for hydroxylamine oxidation has been established to be

$$NH_2OH + O_2 \rightarrow NO_2^- + H_2O + H + 59 \text{ kcal} \quad (2)$$

Cell-free extracts of *Nitrosomonas* are capable of oxidizing hydroxylamine but not ammonia; however, only a small proportion of the hydroxylamine-oxidizing capacity compared to that of intact cells is retained. Although the addition of appropriate electron-acceptor compounds increases this activity, not all the hydroxylamine that is utilized can be accounted for as nitrite (Nicholas and Jones, 1960; Falcone *et al.*, 1963; Anderson, 1964). Molecular oxygen is essential for nitrite formation, and when hydroxylamine is oxidized anaerobically in the presence of artificial electron acceptors, no nitrite is formed (Falcone *et al.*, 1963; Anderson, 1964; Hooper and Nason, 1965; Aleem and Lees, 1963). The oxidation of hydroxylamine to nitrite is complex and involves both the transfer of electrons to an electron acceptor (initially to cytochrome *b* or *c*) (Falcone *et al.*, 1963; Anderson, 1964; Hooper and Nason, 1965; Aleem and Lees, 1963) and the addition of an oxygen atom to nitrogen.

Various laboratories have isolated and partially purified the enzyme responsible for hydroxylamine oxidation, which is here referred to as hydroxylamine oxidase, as originally suggested by Falcone *et al.* (1963). The enzyme has also been referred to as hydroxylamine–cytochrome *c* reductase (Hooper and Nason, 1965; Aleem and Lees, 1963). However, since the enzyme is

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capable of catalyzing the transfer of electrons from hydroxylamine to numerous artificial and natural electron acceptors, hydroxylamine oxidase seems more appropriate. The enzyme is partially sedimented following sonic oscillation of *Nitrosomonas* and subsequent centrifugation at 144,000*g* for 1 or 2 hr, and for this reason, hydroxylamine oxidase has been considered to be particulate in nature (Falcone *et al.*, 1963). Difference spectra of partially purified preparations are interpreted as indicating the presence of cytochromes of the *b* and *c* type, and possibly a flavoprotein (Falcone *et al.*, 1963; Hooper and Nason, 1965; Aleem and Lees, 1963). However, conflicting reports exist as to whether or not the terminal oxidase is also physically associated with the enzyme (Falcone *et al.*, 1963; Hooper and Nason, 1965; Anderson, 1964).

To date, published studies of hydroxylamine metabolism by extracts of *Nitrosomonas* have primarily focused on the ability of hydroxylamine oxidase to reduce added mammalian cytochrome *c* (hydroxylamine–cytochrome *c* reductase) (Falcone *et al.*, 1963; Hooper and Nason, 1965; Aleem and Lees, 1963). On the other hand, it has been uniformly observed that in the presence of mammalian cytochrome *c*, only a small proportion of the total hydroxylamine oxidized by crude cell-free extracts is converted to nitrite, and that during the purification of hydroxylamine oxidase, the ability to form nitrite is progressively lost without a proportional loss in hydroxylamine-oxidizing activity. However, the nitrite producing function of purified hydroxylamine oxidase is at least partially restored when some other electron acceptor (such as phenazine methosulfate or pyocyanine) is substituted for mammalian cytochrome *c* (Nicholas and Jones, 1960).

Since the physiological product of hydroxylamine metabolism is nitrite, in this study a different approach to the study of cell-free hydroxylamine metabolism has been taken. Initially, experiments were performed to determine optimum conditions for nitrite formation by crude cell-free extracts of *Nitrosomonas* and to elucidate the basis for the marked reduction in hydroxylamine-oxidizing activity that occurs when the structural integrity of the cell is destroyed. Various enzyme fractionation procedures were then explored with the view of isolating hydroxylamine oxidase with maximal retention of nitrite formation.

The studies presented below indicate that the reduction in hydroxylamine-oxidizing activity that invariably accompanies lysis of *Nitrosomonas* cells is due to the concomitant physical separation of the terminal oxidase from hydroxylamine oxidase, which are both soluble enzymes. Although the addition of electron-acceptor compounds stimulates hydroxylamine oxidation, the extent to which nitrite is formed strongly depends upon the nature of the acceptor employed. In the presence of phenazine methosulfate, it is possible to isolate highly purified hydroxylamine oxidase with retention of up to 70% of its nitrite-forming ability. The ratio of nitrite formed to hydroxylamine oxidized increases rather than decreases as the enzyme is purified owing to the separation of a substance containing a *c*-type cytochrome which inhibits nitrite formation. The molecular properties of hydroxylamine oxidase are the subject of the second paper of this series (Rees, 1968).

Experimental Procedure

Culture Methods. Pure cultures of *N. europaea* (kindly provided by Dr. E. L. Schmidt, University of Minnesota) were grown at 25° with forced aeration in a completely inorganic medium by the method previously described (Rees and Nason, 1965).

Reagents. Phenazine methosulfate (PMS),[1] horse heart cytochrome *c* (type 3), and bovine pancreas DNase were purchased from Sigma. A stock solution of PMS was prepared as a 5×10^{-3} M aqueous solution and stored frozen in low actinic glass as recommended by Singer and Kearney (1957). Cytochrome *c* was reduced by the hydrogen–palladium asbestos procedure of Smith (1955). Crystalline bovine serum albumin was obtained from the Mann Research Laboratories, and lysozyme (two-times crystallized) from the Worthington Biochemical Corp. Versene (EDTA) and horse spleen ferritin (two-times crystallized) were supplied by Nutritional Biochemical Corp., and hydroxylamine hydrochloride by Mallinckrodt. All other chemicals were reagent grade and were used without further purification.

Chromatographic Materials and Methods. Sephadex G-100 and G-200 and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Inc. The V_0 of the Sephadex columns (defined as the volume containing the peak concentration of the indicator) was determined with either Blue Dextran 2000 or ferritin, and the V_i (defined as the volume at which the indicator is first detected) with NH_4Cl, the ammonia being assayed with Nessler reagent (Hewett and Nicholas, 1964). Whatman cellulose ion-exchange resins were prepared as follows. Diethylaminoethylcellulose (DEAE-cellulose) and carboxymethylcellulose (CM-cellulose) were washed by cycling between HCl and NaOH as recommended by Peterson and Sober (1955). DEAE-cellulose was converted to the phosphate form by washing with 2.0 M K_2HPO_4 and was stored in this solution. CM-cellulose was used in the acid form. Dowex 50W-X8 (200–400 mesh) was washed with boiling water, cycled between 1.0 N HCl and 0.5 N NaOH, and converted to the NH_4^+ form with NH_4Cl. All chromatographic procedures were performed in a 4° cold room.

Spectral Measurements. All spectral measurements were performed at room temperature with either a Cary Model 14 recording spectrophotometer or a Beckman DU spectrophotometer with 1.0-ml cuvets having a 1-cm light path.

Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed using 7.5% cross-linked cylindrical gels of 5-mm i.d. as described by Davis (1964). Samples were layered on the gels with the aid of sucrose. The current was initially maintained at 1

[1] Abbreviations used: PMS, phenazine methosulfate; TCA, trichloroacetic acid.

ma/tube. When the marker dye (bromophenol blue) had entered the gel, the current was increased to 3 ma/tube. Electrophoresis was performed at pH 9.5 using the Tris–glycine buffer system (0.6 g of Tris, 2.88 g of glycine, and H_2O to 1 l.) (Davis, 1964). Protein was stained by immersing the gel for 1 hr in a solution of 1 g of Amido Schwarz/100 ml of 7.5% acetic acid. The gels were destained electrophoretically (Schwabe, 1966).

Sedimentation Analyses. Sucrose density centrifugation analysis was performed on 5.0- (Spinco SW39 rotor) or 30-ml (Spinco SW25 rotor) sucrose density gradients using a Spinco Model L ultracentrifuge. Sucrose density gradients were prepared at 25° using an apparatus similar to that described by Britton and Roberts (1959). The gradients were stored 4 hr at 4° prior to application of the sample. Gradient fractions were collected by the method of Martin and Ames (1961).

Chemical Assay Methods. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) with a crystalline bovine serum albumin standard. The concentration of mammalian cytochrome *c* was determined by measuring the optical density (λ 550 mμ) of a solution completely reduced with solid $Na_2S_2O_4$, together with the extinction coefficient 27.6×10^{-3} M^{-1} cm^{-1} for reduced cytochrome *c* (Margoliash and Frohwirt, 1959).

Nitrite was assayed colorimetrically in a final volume of 2 ml by the diazo-coupling procedure (Hewett and Nicholas, 1964). Hydroxylamine was determined by the Csaky method in a final volume of 7.5 ml, essentially as described by Anderson (1964).

Enzyme Assay Methods. The enzyme assays described below were conducted at room temperature, and shown to be directly proportional to protein concentration and linear with time for the indicated reaction periods. The rates of corresponding control mixtures containing heated enzyme (5 min at 100°) were also determined and in all cases showed no activity.

Hydroxylamine Oxidase. Hydroxylamine oxidase activity was routinely measured by determining both the extent of nitrite formation and hydroxylamine utilization as a function of time (in the presence or absence of added electron acceptor compounds as indicated) as well as by determining the rate at which hydroxylamine reduced mammalian cytochrome *c*. When PMS was employed, an aliquot of the stock solution was diluted with buffer no more than 6 hr prior to use. Similarly, fresh 0.1 M stock solutions of hydroxylamine (in 0.08 M NaOH) were prepared every 6 hr. The PMS and hydroxylamine solutions were stored at 0° and PMS was protected from light. All assays were performed in 0.1 M sodium glycine buffer at pH 8.8 in the presence of 1×10^{-4} M NH_2OH.

The rate of cytochrome *c* reduction was measured in the presence of 1×10^{-4} M mammalian cytochrome *c* by recording the increase in optical density at 550 mμ as a function of time 30 sec after the addition of hydroxylamine and for at least 3 min thereafter. The quantity of cytochrome *c* reduced was calculated using the difference extinction coefficient, 18.5×10^{-3} M^{-1} cm^{-1}, for mammalian cytochrome *c* (Margoliash and Frohwirt, 1959).

Terminal Oxidase Spectroscopic Assay. As a measure of terminal oxidase activity, the rate of oxidation of reduced mammalian cytochrome *c* was determined in 0.1 M potassium phosphate (pH 7.4) using reduced horse heart cytochrome *c* at a final concentration of 1×10^{-4} M. The decrease in optical density at 550 mμ was recorded as a function of time 30 sec after the start of the reaction and at 30-sec intervals thereafter for at least 5 min. The quantity of cytochrome *c* oxidized was determined using the difference extinction coefficient for cytochrome *c* cited above. Unless otherwise noted, the terminal oxidase activity was assayed spectrophotometrically.

Polarographic Assay. The rate of oxygen uptake was measured with the Clark oxygen electrode (Yellow Springs Instrument Co., Antioch, Ohio), operated at a polarizing voltage of 0.6 v. The electrode was calibrated with air-saturated 0.10 M potassium phosphate (pH 7.4) at room temperature in a 3-ml lucite chamber. For measurement of oxygen uptake, enzyme and buffer were first added to the chamber and the electrode set in place; when temperature equilibration had been reached, the reaction was initiated by injection of 0.1 ml of 0.3 M *p*-phenylenediamine and measured for at least 3 min. (The *p*-phenylenediamine had been previously dissolved in 0.10 M potassium phosphate (pH 7.4) and stored tightly stoppered in the dark at 60°.) Between each assay, the electrode and reaction chamber were rinsed with 0.5 N HCl, distilled H_2O, and buffer.

Preparation of Cell-Free Fractions. Cell lysis was accomplished by osmotic rupture of EDTA–lysozyme-

TABLE I: Comparison of NH_3 Oxidation by Untreated and EDTA–Lysozyme-Treated Cells of *N. europaea*.

Preparation	μmoles of NO_2^-/ml	% Act. Rel to Cells in Buffer
Cells in buffer[a]	4.5	100
Cells in sucrose[b]	3.3	73
Spheroplasts in sucrose	3.3	73

[a] Cells (2 mg wet wt/ml) were serially diluted in 0.05 M potassium phosphate (pH 7.5). A 0.1-ml aliquot of the $^1/_{100}$ dilution was added to 0.8 ml of 0.05 M potassium phosphate (pH 7.5) and the reaction was begun by the addition of 0.1 ml of 0.025 M $(NH_4)_2SO_4$ in buffer. After 15 min at 25°, NO_2^- was determined as described under Experimental Procedure. All reactions were run in duplicate. [b] Cells in buffer and EDTA–lysozyme-treated cells in 0.25 M sucrose were serially diluted in 0.25 M sucrose–0.05 M potassium phosphate (pH 7.5), and 0.1-ml aliquots of the $^1/_{100}$ dilutions were assayed as above but in sucrose–phosphate instead of phosphate buffer alone.

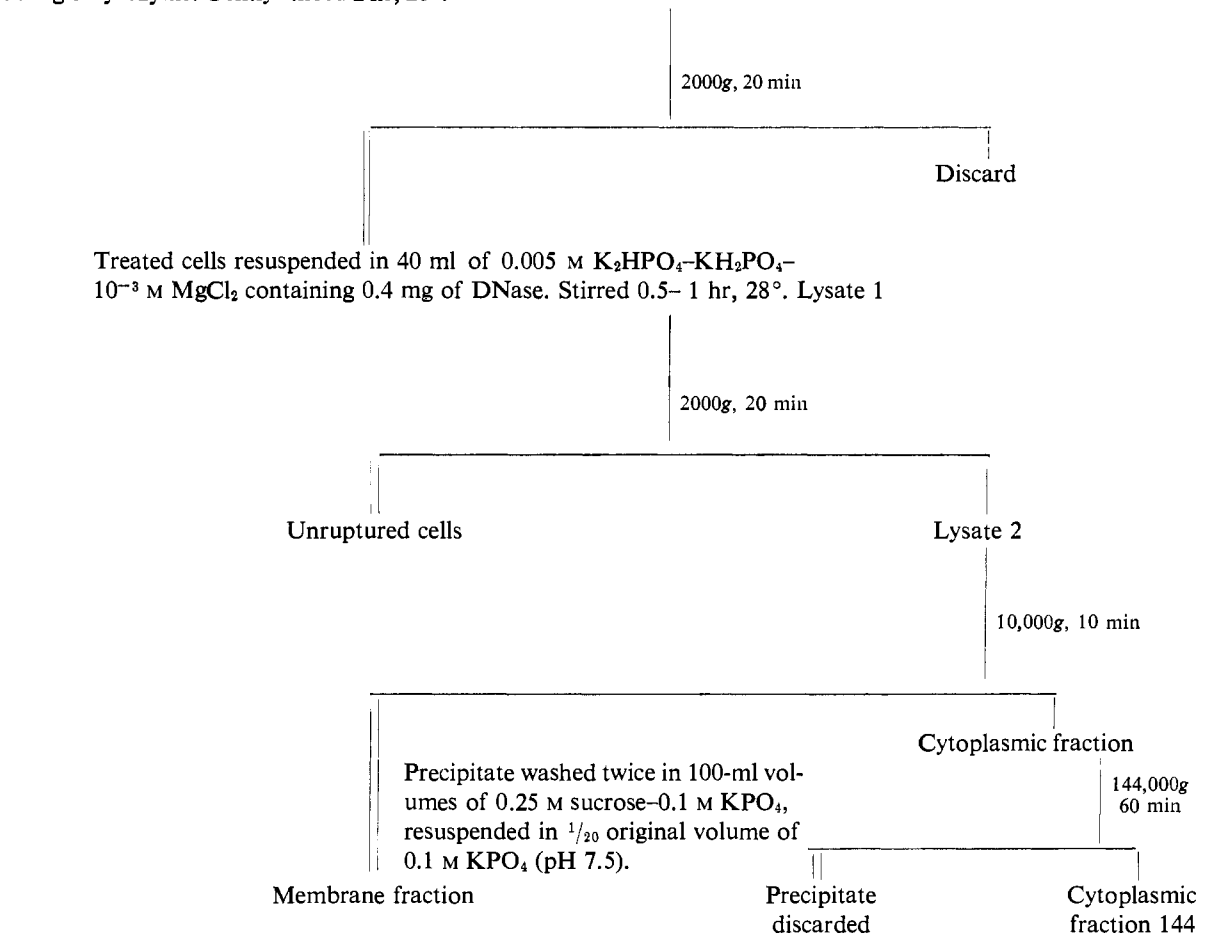

FIGURE 1: The preparation of cell-free fractions of *N. europaea.*

treated cultures. For reasons which will be discussed, this method is preferred over other commonly employed cell disruption techniques. The details of this method have been described previously (Rees and Nason, 1965) and are summarized in Figure 1, which also outlines the preparation of the various fractions which were studied.

Results

Effect of Cell Rupture. As is usual when it is desired to purify an enzyme, studies were initially performed to determine the method of cell rupture leading to the highest recovery of the enzyme in soluble form. In a previous communication (Rees and Nason, 1965), it was shown that osmotic rupture of *Nitrosomonas* cells treated with EDTA–lysozyme by the method outlined in Figure 1 results in quantitative recovery of the terminal oxidase in the supernatant solution following centrifugation of the lysate at 144,000*g* for 1 hr (cytoplasmic fraction 144). Under these conditions, hydroxylamine oxidase is also recovered in the 144,000*g* supernatant solution (see Table II). On the other hand, in agreement with the results of others (Falcone *et al.*, 1963; Anderson, 1964; Hooper and Nason, 1965), following lysis of the cell by exposure to either sonic oscillations or high pressure, approximately 50% of the terminal oxidase and hydroxylamine oxidase activities are removed from solution by the high-speed centrifugation procedure (see Rees and Nason (1965) for a discussion of this point).

To date, all workers have reported that lysis of the *Nitrosomonas* cell results not only in a total loss of ammonia oxidation, but also in the loss of most of the hydroxylamine oxidase activity. The present study represents no exception. Nevertheless, since the enzymes responsible for the initial oxidation of ammonia are thought to lie in the vicinity of the peripheral membranes of the organism (Aleem and Lees, 1963), it was of interest to compare the ammonia-oxidizing activity of untreated and EDTA–lysozyme-treated cells. For this study, 0.25 M sucrose was included in both treated and untreated cultures, since in the absence of sucrose the EDTA–lysozyme treatment results in cell lysis. Table I reveals that although the presence of 0.25 M sucrose has

TABLE II: Distribution of Terminal Oxidase, Hydroxylamine Oxidase, and Protein in Cell-Free Preparations of *N. europaea*.

Fraction[a]	Protein (%)	Terminal Oxidase (%)	Hydroxylamine Oxidase (%)
Lysate 1	100	100	100
Lysate 2	100	100	90
Membrane fraction	31	0.5	8
Cytoplasmic fraction	53	100	91
144,000*g*, 2 hr supernatant solution (cytoplasmic fraction 144)		100	85

[a] Fractions were prepared as described in Figure 1. All precipitates were resuspended in 0.05 M potassium phosphate (pH 7.8). Hydroxylamine oxidase, terminal oxidase (as measured both spectrophotometrically in the presence of reduced cytochrome *c* and polarographically in the presence of 0.03 M *p*-phenylenediamine), and protein were determined as described under Experimental Procedure. All values are expressed relative to lysate 1.

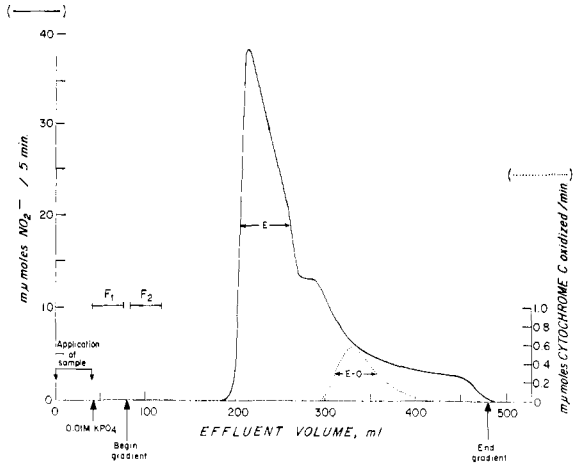

FIGURE 2: Chromatography of cytoplasmic fraction 144 on DEAE-cellulose. Cytoplasmic fraction 144 (43 ml) (0.8 mg of protein/ml) was applied to a 2.5 × 15 cm pressure-packed column of DEAE-cellulose phosphate. Addition of the sample was followed successively by 32 ml of 0.01 M potassium phosphate (pH 7.8) flowing at a rate of approximately 0.4 ml/min. Three milliliter fractions were collected. Two red bands lacking enzymatic activity eluted early, one breaking through prior to completion of addition of the sample (F_1), and another appearing shortly after the gradient was begun (F_2). The absorption spectrum of an aliquot of each of the effluent fractions in which the two colored bands were distributed was recorded from 650 to 380 mμ with water serving as the reference solution. Solid sodium dithionite was added to the sample cuvet, and the absorption spectrum was repeated. Both fractions contained cytochrome c_{552}. The height of the cytochrome c_{552} peak following reduction was determined by subtracting $OD_{575\ m\mu}$ from $OD_{552\ m\mu}$, and these values were used to determine the elution curve of each of the cytochrome c_{552} containing fractions. Separate 0.01-ml aliquots of each effluent tube were examined for hydroxylamine oxidase (NO_2^- production in the presence of PMS) and the terminal oxidase activity as described in Experimental Procedure. Appropriate effluent fractions were pooled to give fractions F_1 and F_2, hydroxylamine oxidase (fraction E), and hydroxylamine oxidase–cytochrome oxidase (fraction E-0).

a small inhibitory effect on ammonia oxidation, the untreated and treated preparations exhibit the same level of activity. On the other hand, osmotic rupture of the treated cells by transfer to a hypotonic environment resulted not only in the complete loss of the ability to oxidize ammonia, but also in the loss of 90% of the hydroxylamine-oxidizing activity (see, for example, Table III).

Thus far, studies have failed to define conditions which restore ammonia-oxidizing ability to cell lysates. However, as reported by others (Nicholas and Jones, 1960; Falcone *et al.*, 1963; Anderson, 1964), stimulation of hydroxylamine oxidation occurred when the lysates were treated with various electron acceptor compounds. With none of the electron acceptors tested, however, was it possible to recover more than a small proportion of the total hydroxylamine oxidized as nitrite. In this regard and in agreement with Nicholas and Jones (1960), PMS gave the highest recovery of nitrite. Concentrations of PMS greater than 10^{-4} M were inhibitory, and the reaction proceeded maximally in the presence of 5×10^{-5} M PMS and at a pH of 8.8.

A typical result which is obtained upon addition of PMS to lysates is presented in Table III, in which the rates of hydroxylamine utilization and nitrite formation by intact cells and lysate both in the presence and absence of PMS are compared. (For this study, cells were ruptured by exposure to high pressure rather than by chemical treatment. Thus, the only difference between the intact cell and the lysate is a physical change in the structural organization of the cellular components. To further avoid ambiguity of interpretation, the cell debris consequent to lysis was not removed prior to assay.) Table III illustrates that, as expected, the rates of hydroxylamine utilization and nitrite formation are equivalent in intact cells. However, following lysis, only 9.3 and 3.1% of the hydroxylamine-utilizing and nitrite-forming activities, respectively, remained. Thus, not only does

TABLE III: The Effect of Cell Rupture of *N. europaea* upon Hydroxylamine Oxidase Activity in the Presence and in the Absence of PMS.[a]

Preparation	Total Recov of Enzyme Act. (μmoles/min)		Sp Act.	
	NH_2OH Disappearance	NO_2 Formation	NH_2OH Disappearance	NO_2 Formation
Intact cells	5.0	5.3	32.7	34.7
Intact cells plus 5×10^{-5} M PMS	7.5	7.2	49.0	47.0
Lysate	0.45	0.02	3.7	1.47
Lysate plus 5×10^{-5} M PMS	97.5	31.3	750	240

[a] Freshly harvested cells were thoroughly washed in 0.1 M potassium phosphate (pH 7.5) at 4°, collected by centrifugation at 2000*g* for 20 min, and resuspended in the same buffer to a final volume of 15 mg of protein/ml. A dilution of cells in 0.1 M sodium glycine buffer (pH 8.8) was determined that completely utilized 1×10^{-4} M NH_2OH in 120 min at 25°. The rates of utilization of NH_2OH and production of NO_2^- were determined in the absence and presence of 5×10^{-5} M PMS. The reaction mixture was aerated by gentle swirling, and 0.5-ml aliquots were removed at intervals. NH_2OH was determined on 0.3 ml (final volume, 7.5 ml) and NO_2^- was determined on 0.2 ml (final volume, 2 ml) (see Experimental Procedure). All reactions were run in triplicate. Samples containing heat-treated cells (5 min at 100°) as well as endogenous controls were included to correct for nonenzymatic losses of hydroxylamine and production of nitrite. A portion of the cell suspension was treated by passage through a french press (20,000 psi, three times, 4°), and the resulting lysate assayed identically as above without prior centrifugation. To determine protein concentration, samples were treated with 10% TCA (0°, 10 min), and the precipitate was resuspended in 0.1 M NaOH–2% Na_2CO_3 and analyzed as described in Experimental Procedures.

ysis result in a marked diminution of hydroxylamine oxidase activity, but of the total quantity of substrate oxidized, only one-third is recovered as nitrite. Table III also reveals that a striking increase in the rate of hydroxylamine utilization as well as in the rate of nitrite formation occurs in the presence of 5×10^{-5} M PMS. It is important to note, however, that although treatment with PMS results in a dramatic increase in the reaction rates, it does not alter the stoichiometry of the reaction, either in the presence or absence of PMS, only one-third of the total substrate which was utilized appeared as nitrite.

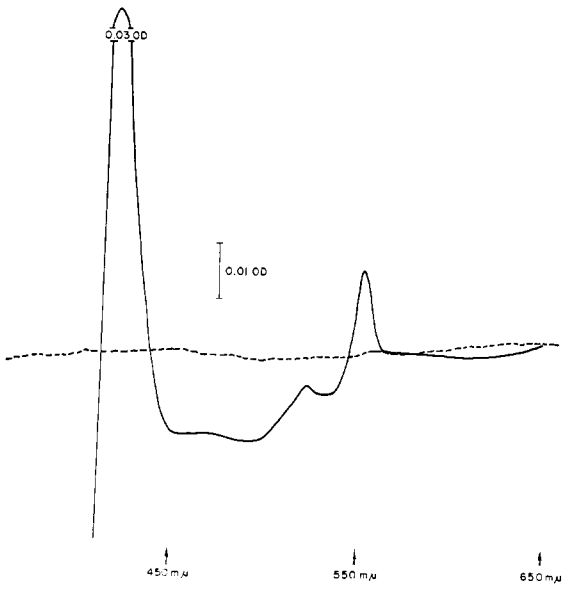

FIGURE 3: Spectral characteristics of fraction F_2 obtained by chromatography of cytoplasmic fractions 144 on a DEAE-cellulose column. Protein concentration: 0.4 mg/ml; (– – –) base line; (—) sodium dithionite difference spectrum.

The recovery of 33% of the total hydroxylamine oxidized by lysates as nitrite is not an invariable result. On occasion, and for unexplained reasons, as little as 5–10% of the substrate is accounted for as nitrite. For most lysates, however, this number is between 30 and 40%. The altered stoichiometry is not explained by the accumulation of a stable precursor of nitrite, for despite the complete disappearance of hydroxylamine, no further production of nitrite occurs with time. Later, evidence will be presented which indicates that the incomplete recovery of nitrite is due at least in part to the interaction of a soluble *Nitrosomonas* cytochrome with the hydroxylamine oxidase system. This material causes hydroxylamine to be oxidized to products other than nitrite.

These studies indicate that the optimum starting material to use in purifying hydroxylamine oxidase is the supernatant solution following brief ultracentrifugation (144,000*g*, 1 hr) of the lysate, and that it is necessary to include PMS in the reaction mixture to obtain maximum nitrite formation. Experiments pertaining to the purification of hydroxylamine oxidase will now be described. During the course of these studies, a fraction containing a *c*-type cytochrome was discovered which has the un-

TABLE IV: Effect of Various DEAE-cellulose Fractions of Cytoplasmic Fraction 144 on Hydroxylamine Oxidase Activity (NH_2OH Utilization and NO_2^- Production) of Fraction E.[a]

Fraction	Protein (mg/ml) in Reaction Mixture	No Added Electron Acceptor		5×10^{-5} M PMS	
		NH_2OH Utilized (mμ-moles/ml)	% Converted to NO_2^-	NH_2OH Utilized (mμmoles/ml)	% Converted to NO_2^-
Lysate 1	0.15	27	23	77.5	15
Cytoplasmic fraction 144	0.08	20	23	77.5	15
E	0.009	0	0	77.5	49
E-0	0.006	0	0	76.5	42
F_1 + E	0.018 + 0.009	0	0	89	38
F_2 + E	0.015 + 0.009	0	0	84.6	13
E-0 + E	0.006 + 0.009	0	0	89	38
F_1	0.018	0	0	1	0
F_2	0.015	0	0	0	0

[a] Cytoplasmic fraction 144 was chromatographed on DEAE-cellulose and appropriate effluent fractions and eluates were pooled as described in Figure 3. Hydroxylamine oxidase activity both in the absence and presence of 5×10^{-5} M PMS was determined in duplicate after a 45-min reaction time by hydroxylamine utilization and nitrite formation as described in Experimental Procedure.

usual property of inhibiting nitrite formation without a concomitant inhibition of hydroxylamine oxidation.

Purification of Hydroxylamine Oxidase. As noted above, both hydroxylamine oxidase and the terminal oxidase are recovered in the soluble cytoplasmic fraction 144 under the conditions here employed. Various lines of evidence have been presented which favor the view that hydroxylamine oxidase and the terminal oxidase are either present in the same macromolecular complex (Falcone *et al.*, 1963) or that they are physically separate enzymes (Anderson, 1964; Hooper and Nason, 1965). It was of interest, therefore, to determine the distribution of the two activities when cytoplasmic fraction 144 was subjected to various enzyme fractionation procedures.

Behavior of Cytoplasmic Fraction 144 of DEAE-cellulose. As shown in Figure 2, the addition of cytoplasmic fraction 144 to a DEAE-cellulose column followed by a linear gradient elution procedure yielded (a) two separate rapidly moving red bands (designated as fractions F_1 and F_2) containing cytochrome c_{552} (see Figure 3) but lacking enzymatic activity, (b) a hydroxylamine oxidase fraction (designated as fraction E) which eluted with a sharp front but considerable trailing, and (c) a terminal oxidase fraction (fraction E-0) which also contained a small amount of hydroxylamine oxidase activity.

The fact that in the reaction catalyzed by the above hydroxylamine oxidase eluate (fraction E) approximately 50% of the hydroxylamine utilized appeared as nitrite, in contrast to the considerably lower nitrite yield of 15% by the original cytoplasmic fraction prior to DEAE-cellulose chromatography (see Table IV), prompted an examination of hydroxylamine oxidase activity in various combinations of the eluates from the DEAE-cellulose column. The results summarized in Table IV demonstrate that the addition of the cytochrome c_{552} containing fraction F_2 to the hydroxylamine oxidase eluate (fraction E) causes a decrease in the nitrite yield from 49% to about 13%. On the other hand, hydroxylamine utilization by fraction E is not impaired as a result of addition of fraction F_2. The adverse effect of fraction F_2 on hydroxylamine oxidase is destroyed by heat (100°, 5 min) and is not lost upon dialysis against 0.1 M potassium phosphate (pH 7.8) for 12 hr at 4°. After treatment of fraction F_2 with saturated ammonium sulfate, its inhibitory activity may be quantitatively recovered from the precipitate so obtained. By contrast, fraction F_1, whose spectral properties are virtually identical with those of fraction F_2 (Figure 3), has no effect on hydroxylamine oxidase activity.

To obtain improved resolution, cytoplasmic fraction 144 was chromatographed on a large DEAE-cellulose column employing a series of gradients which allowed for significantly better separation of the fractions. This is depicted in Figure 4. During the application of the initial linear gradient of 0.05–0.18 M phosphate, a third red band designated as fraction F_3 was eluted in addition to fractions F_1 and F_2. Like F_1 and F_2, it lacked hydroxylamine oxidase activity, but, unlike F_2, it had no inhibitory effect on nitrite formation by an active hydroxylamine oxidase preparation. Figure 4 reveals that hydroxylamine oxidase eluted as two fractions from the DEAE-cellulose column. The majority of the activity (31%) was recovered with no associated terminal oxidase activity, while a small proportion of hydroxyl-

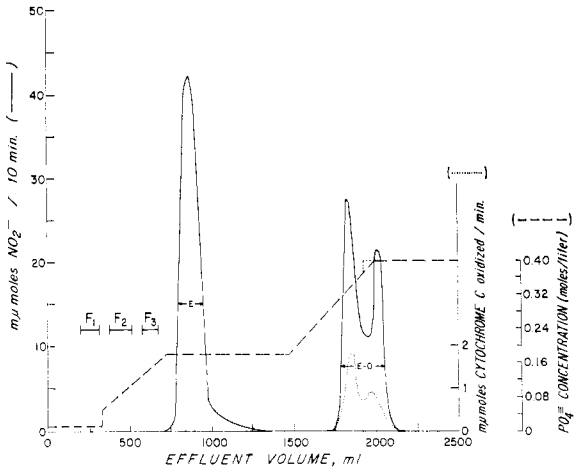

FIGURE 4: Improved chromatography of cytoplasmic fraction 144 on DEAE-cellulose. Cytoplasmic fraction (60 ml) (4.4 mg of protein/ml) was applied to a 5 × 20 cm column of DEAE-cellulose phosphate packed with pressure. The pH of the eluting buffer was maintained at 7.9. The flow rate was approximately 1 ml/min, and 30-ml fractions were collected. The molarity of phosphate varied as follows. Following addition of the sample, 300 ml of 0.01 M phosphate was passed through the column resulting in the elution of fraction F_1. During the subsequent application of a linear gradient of 200 ml of 0.05 M and 200 ml of 0.18 M phosphate fraction F_2 and a third colored protein (fraction F_3) were eluted. As described in the legend for Figure 3, the width of the F_1, F_2, and F_3 elution bands was determined by calculating the $OD_{552m\mu} - OD_{575m\mu}$ of a dithionite-reduced aliquot of each of the effluent tubes in which these fractions were distributed. Following the termination of the first gradient, 0.18 M phosphate buffer was passed through the column resulting in the elution of hydroxylamine oxidase (fraction E). When hydroxylamine oxidase activity could no longer be detected, 200 ml more of the 0.18 M buffer was passed through the column followed by a second linear gradient consisting of 300 ml of 0.18 M and 300 ml of 0.40 M phosphate. During the latter part of this gradient, mixing difficulties developed, and there occured a sudden increase in the molarity of the phosphate entering the column. This is depicted by the (.....) superimposed upon the gradient line in the 1930-ml region of the figure. The terminal oxidase was eluted during this second gradient in association with a small quantity of hydroxylamine oxidase. Separate 0.01 aliquots were used for each enzyme assay.

amine oxidase (4%) eluted at a considerably higher phosphate molarity in association with the terminal oxidase (fraction E-0).

As noted with the previous DEAE-cellulose column, the hydroxylamine oxidase fraction recovered following chromatography, fraction E, converted a considerably greater proportion of the total oxidized hydroxylamine to nitrite than was true for the corresponding cytoplasmic fraction. On measuring the rates of hydroxylamine oxidation and nitrite formation by fraction E both in the presence and in the absence of the F_2 fraction, it was found that fraction F_2 inhibits the rate of nitrite formation without a concomitant decrease in the rate of hydroxylamine oxidation. Moreover, only a small percentage of the total hydroxylamine oxidized is accounted for as nitrite when F_2 is present. For example, in the absence of fraction F_2, the rates of hydroxylamine uptake and nitrite formation by fraction E (0.2 μg of protein/ml) were −0.54 mμmole of NH_2OH/ml min and +0.36 mμmole of NO_2^-, respectively, and 65% of the total oxidized hydroxylamine was accounted for as nitrite. Although the presence of fraction F_2 (14 μg of protein/ml) did not alter the rate of hydroxylamine utilization by fraction E, it markedly inhibited the rate of nitrite formation which fell to +0.10 mμmole/ml min. Moreover, only 17% of the total hydroxylamine utilized by the enzyme appeared as nitrite. In either the presence or absence of F_2, complete oxidation of the substrate (10 mμmoles of NH_2OH/ml) occurred. (These rates were measured identically as described in the legend of Table III.) Thus, fraction F_2 interferes with hydroxylamine oxidation in such a way that products other than nitrite are formed. Further information dealing with the properties of this fraction will be presented below.

Only 35% of the total hydroxylamine oxidase activity was recovered following chromatography of cytoplasmic fraction 144 on DEAE-cellulose; moreover, less than a twofold increase in the specific activity of the enzyme resulted from this fractionation procedure. The addition of fractions E and E-0 to one another did not increase the activity to a greater extent than that expected by summation of the two. Present evidence indicates that this loss in enzymatic activity is a result of denaturation of the enzyme due to the chromatographic procedure (see below).

Gel Filtration Chromatography of Cytoplasmic Fraction 144. Chromatography of cytoplasmic fraction 144 on Sephadex G-100 results in the isolation of two cytochrome-containing fractions (see Figure 5). The first red fraction elutes in the column void volume and represents hydroxylamine oxidase and the terminal oxidase. The second fraction elutes just prior to the salt volume of the column and is identified as fraction F_2 by its inhibitory effect on nitrite formation (see legend of Figure 5 for experimental details) and by its spectral characteristics (see Figure 3). Greater than 100% of the total nitrite-forming activity of cytoplasmic fraction 144 applied to the column is recovered following Sephadex chromatography (130%), as would be expected upon removal of the interfering substance, fraction F_2, from hydroxylamine oxidase. Following chromatography, 70% of the total hydroxylamine oxidized by hydroxylamine oxidase was recovered as nitrite.

When cytoplasmic fraction 144 was chromatographed with Sephadex G-200 rather than G-100, hydroxylamine oxidase and the terminal oxidase partially entered the gel pores, and a clear separation of the hydroxylamine

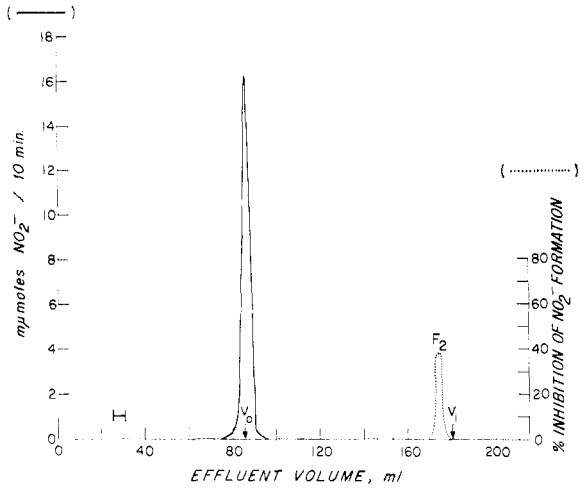

FIGURE 5: Gel filtration of cytoplasmic fraction 144 on Sephadex G-100. Cytoplasmic fraction 144 was concentrated by overnight dialysis at 4° against 3.7 M $(NH_4)_2SO_4$ (pH adjusted to 7.0 with NH_4OH), the precipitate resuspended in 0.15 M phosphate buffer (pH 7.8), and 0.35 ml (15 mg of protein/ml) applied to a 1.7 × 102 cm Sephadex G-100 column. Elution was accomplished using the phosphate buffer at a flow rate of 15 ml/hr. The effluent tubes (2 ml/tube) containing hydroxylamine oxidase activity were pooled (0.1 aliquots were assayed; see Experimental Procedure). A dilution of this pooled material (which in the presence of PMS caused the formation of 3 mμmoles of NO_2^- in 10 min) was used to test for F_2 inhibitory activity (see text) in 0.1-ml aliquots of the effluent tubes. As a control, the effluent tubes indicated by |—| were pooled, and a 0.1-ml aliquot of this control sample was added to the enzyme at the same time that effluent fractions were examined for the presence of inhibitor. Those fractions exhibiting the inhibitory effect were pooled and designated as F_2.

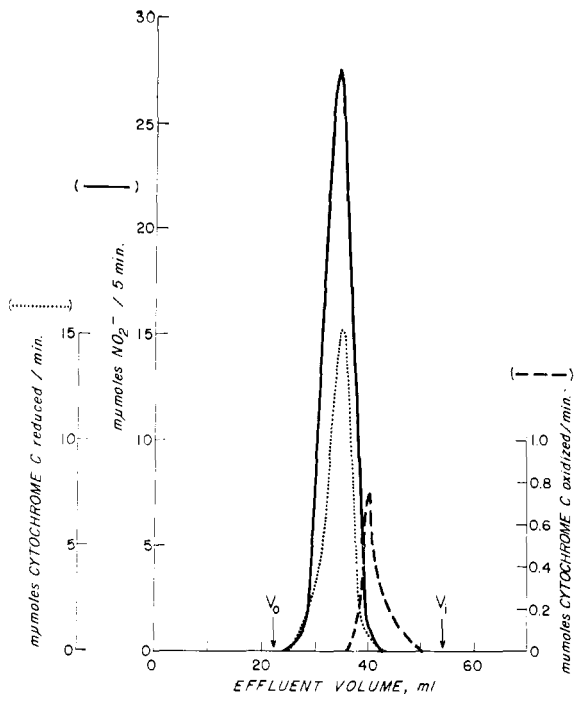

FIGURE 6: Distribution of hydroxylamine oxidase and terminal oxidase on Sephadex G-200. Cytoplasmic fraction 144 was concentrated by overnight dialysis at 4° against 3.4 M $(NH_4)_2SO_4$ containing 1×10^{-3} M EDTA (pH adjusted to 7.0 with NH_4OH). The resulting precipitate was resuspended in 0.1 M phosphate buffer pH 7.3) and 0.35 ml (19 mg of protein/ml) was applied to a 1.3 × 56 cm Sephadex G-200 column (calibrated as described in Experimental Procedure). The 0.1 M phosphate buffer served as eluent, flowing at a rate of 8 ml/hr. Fractions (2 ml) were collected, and 0.01-ml aliquots of each effluent tube were individually assayed for hydroxylamine oxidase activity and terminal oxidase activity as described in Experimental Procedure.

oxidase and terminal oxidase activities resulted as Figure 6 demonstrates. The yield and enzymatic characteristics of the hydroxylamine oxidase fraction recovered from the Sephadex G-200 column were essentially identical with that noted following chromatography on Sephadex G-100.

As shown above, the bulk of the hydroxylamine oxidase activity elutes from DEAE-cellulose prior to the terminal oxidase, and a small proportion of the enzyme (4%) elutes in association with the terminal oxidase (see Figures 2 and 4). However, this evidently represents coelution of two physically unassociated enzymes since hydroxylamine oxidase is well separated from the terminal oxidase following fractionation of cytoplasmic fraction 144 by either Sephadex G-200 column chromatography (Figure 6) or sucrose density gradient centrifugation (see below). These data indicate that the two enzymes are not located within the same macromolecular complex.

Sucrose Density Gradient Centrifugation of Cytoplasmic Fraction 144. Further evidence for the absence of binding between the terminal oxidase and hydroxylamine oxidase is presented in Figure 7. In this study, cytoplasmic fraction 144 was applied to a 30 ml, 3–20% sucrose gradient. Figure 7 reveals that following 30-hr centrifugation at 24,000 rpm, two well-separated red bands are present. The narrow and more distantly migrating band represents hydroxylamine oxidase, while the terminal oxidase is distributed within the diffuse upper band along with the soluble cytochrome c_{552} of the bacterium, which is present in large amounts (see also Falcone *et al.*, 1963).

To determine whether the terminal oxidase and hydroxylamine oxidase activities could be made to migrate together, sucrose density gradient centrifugation was repeated, employing somewhat different conditions. In this study, the usual prolonged exposure to hypotonic conditions was avoided. The preparative procedure outlined in Figure 1 was followed; however, the EDTA–lysozyme-treated cells remained in the hypotonic

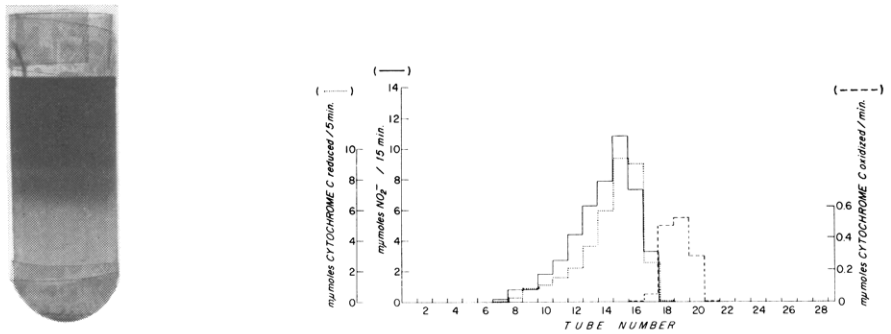

FIGURE 7: Sucrose density gradient centrifugation of cytoplasmic fraction 144. Cytoplasmic fraction 144 was concentrated by overnight dialysis at 4° against 3.4 M $(NH_4)_2SO_4$ (adjusted to pH 7 with NH_4OH) containing 1×10^{-3} M EDTA. The precipitate was resuspended in 0.1 M potassium phosphate (pH 7.4) to a final protein concentration of 14.5 mg/m and dialyzed briefly against a large volume of buffer, and 0.7 ml was applied to a 30 ml, 3–20% linear sucrose gradient (0.1 M potassium phosphate, pH 7.4). Left: the appearance of the gradient after centrifugation for 30 hr at 24,000 rpm, 4°, using a Spinco SW25 rotor. Right: approximately 1-ml fractions were collected and 0.01-ml aliquots were analyzed for hydroxylamine oxidase and terminal oxidase activities as described in Experimental Procedure.

solution only 15 min, after which time the lysate was treated with 0.25 M sucrose (60% of the cells lysed under these conditions). Furthermore, concentration of cytoplasmic fraction 144 was achieved by prolonged ultracentrifugation (22 hr, 144,000*g*) rather than by precipitation with ammonium sulfate. Following resuspension in 0.25 M sucrose, the concentrated material was analyzed on a 10–40% sucrose gradient (5-ml gradient, 30,000 rpm for 22 hr). The relative distribution of the hydroxylamine oxidase and terminal oxidase activities was essentially identical with that shown in Figure 7.

Undoubtedly, within the intact cell, hydroxylamine oxidase and the terminal oxidase are in close approximation. Moreover, since ammonia oxidation is identical by either untreated or EDTA–lysozyme-treated cells (see Table I), this treatment evidently does not disturb the intracellular arrangement of these enzymes. Nevertheless, the above study suggests that the forces, if any, binding them are weak.

In the second of this series (Rees, 1968), it is shown that hydroxylamine oxidase is a cytochrome-containing particle of uniform size which has a molecular weight close to 200,000 g/mole and a sedimentation coefficient (corrected to the viscosity of H_2O at 20°) equal to 10 S. With this information and the data of Figure 7, it is possible to estimate the molecular dimensions of the terminal oxidase. Martin and Ames (1961) established that for many biological materials migrating linearly through a 3–20% sucrose gradient (3–15°), the following empirical relationship holds

$$\frac{r_1}{r_2} = \frac{(s_{20,w})_1}{(s_{20,w})_2} = \frac{M_{w1}}{M_{w2}}$$

where r_1 and r_2 are the distances that compounds with sedimentation coefficients $(s_{20,w})_1$ and $(s_{20,w})_2$ have traveled from the meniscus. Assuming this relationship to be true for hydroxylamine oxidase and the terminal oxidase, an $s_{20,w} = 8$ S and a molecular weight of 128,000 g/mole are calculated for the terminal oxidase.[2]

Sucrose density gradient centrifugation of cytoplasmic fraction 144 not only allows for easy separation of hydroxylamine oxidase from the terminal oxidase (Figure 7), but also a significant purification of the former is achieved in this manner. This is demonstrated in Figure 8A, which shows the typical electrophoretic pattern obtained when the sucrose gradient effluent tubes having the peak hydroxylamine oxidase activity are pooled and examined by polyacrylamide gel electrophoresis. Prior to staining, a single red band is present in the gel, and this band is denoted by the arrow in Figure 8A. It is of interest that it is possible to recover most of the enzyme after completion of electrophoresis. If rather than staining, the cytochrome-containing band is sectioned from the gel and eluted with dilute phosphate buffer, 70% of the total hydroxylamine oxidase activity (assayed both in the presence of PMS and in the presence of mammalian cytochrome *c*) is recovered.

As discussed above, separation of the terminal oxidase

[2] In a previous communication (Rees and Nason, 1965), it was reported that *N. europaea* contains two carbon monoxide combining compounds. One of these was identified as the terminal oxidase, cytochrome *o*, and the other was thought to represent a P-450 compound similar to that which is present in mammalian ribosomes. However, recent unpublished studies performed in collaboration with Dr. David Wilson at the Johnson Research Foundation, University of Pennsylvania, indicate that the latter compound is actually a form of cytochrome *a*. At the temperature of liquid N_2, there are reduced peaks at 430 and 595 mμ which shift to 446 and 599 mμ upon combination with carbon monoxide. Thus the *Nitrosomonas* terminal oxidase consists of both cytochromes *o* and *a*, and it is not yet clear whether one or both are assayed by the methods employed in this study. Thus, some ambiguity exists in defining the molecular weight of the terminal oxidase of this organism. However, the density gradient data indicate that the upper limit of the molecular weight of whichever component is assayed is about 128,000 g/mole.

and hydroxylamine oxidase activities of *Nitrosomonas* results when cytoplasmic fraction 144 is subjected to either column chromatography employing Sephadex G-200 or sucrose density gradient centrifugation. Fraction F_2 is removed from hydroxylamine oxidase by either of these procedures; moreover, a significant purification is achieved with excellent recovery of enzymatic activity. These findings led to the development of the definitive purification procedure for hydroxylamine oxidase presented below.

Purification Scheme for Hydroxylamine Oxidase. The purification procedure finally adopted for hydroxylamine oxidase is summarized in Table V. The methods for preparation of lysate II and cytoplasmic fraction 144 are presented in Figure 1, and the details of sucrose density gradient centrifugation and Sephadex G-200 chromatography have been previously described (see above and Figures 7 and 6, respectively). Table V illustrates that the sequence of steps: ultracentrifugation → sucrose density gradient centrifugation → Sephadex G-200 chromatography results in a 60-fold increase in the specific activity of hydroxylamine oxidase with retention of 40% of the total enzymatic activity. (Enzymatic activity is expressed in terms of the rate of oxidation of hydroxylamine rather than by the rate of nitrite formation, since the latter is influenced by the presence of fraction F_2. Thus, although expressing the specific activity of hydroxylamine oxidase as a function of the rate of hydroxylamine oxidation yields a lower final specific activity than is the case when the rate of nitrite formation is employed, it more accurately reflects the true increase in specific activity achieved by the purification procedure. Similarly, a falsely high estimate of the yield of enzymatic activity is obtained if the nitrite-forming activity of hydroxylamine oxidase is used to calculate this number.) The resulting hydroxylamine oxidase preparation possesses a high degree of homogeneity (Figure 8B) and no detectable terminal oxidase activity. Further evidence of the homogeneity of this enzyme preparation is presented in Rees (1968). The data summarized in the final column of Table V reveal that an increased conversion of hydroxylamine to nitrite accompanies purification of hydroxylamine oxidase. With the purified enzyme, 58% of the total oxidized hydroxylamine was accounted for as nitrite. (This number varies, and occasional pure preparations oxidize as much as 70% of the hydroxylamine to nitrite.) However, no nitrite is formed if mammalian cytochrome *c* is substituted for PMS. From the data in Table V, it is estimated that hydroxylamine oxidase comprises 1–2% of the total protein of *Nitrosomonas*. The enzyme is quite stable and may be stored at or below 0° for months without measurable loss of activity. The molecular properties of hydroxylamine oxidase isolated by this procedure are presented in the following paper (Rees, 1968).

Fraction F_2. Finally, the characteristics of fraction F_2, isolated by column chromatography (DEAE-cellulose or Sephadex G-100), have been examined. It was shown above that while fraction F_2 has no demonstrable enzymatic activity alone, it interacts with hydroxylamine oxidase in such a way that nitrite formation is inhibited while hydroxylamine oxidation proceeds unimpaired. Fraction F_2 only exerts its inhibitory effect when hydroxylamine oxidase is actively oxidizing hydroxylamine, that is, when substrate, added electron acceptor, and enzyme are all present. Fraction F_2 is heat labile and nondialyzable and can be precipitated with concentrated ammonium sulfate. The cytochrome associated with this fraction has spectral properties which are characteristic for a cytochrome *c* (α, β, and γ peaks at 552, 523, and 420 mμ, respectively; see Figure 3). Since fraction F_2 elutes just prior to the salt volume of Sephadex G-100 (Figure 6), its molecular weight prob-

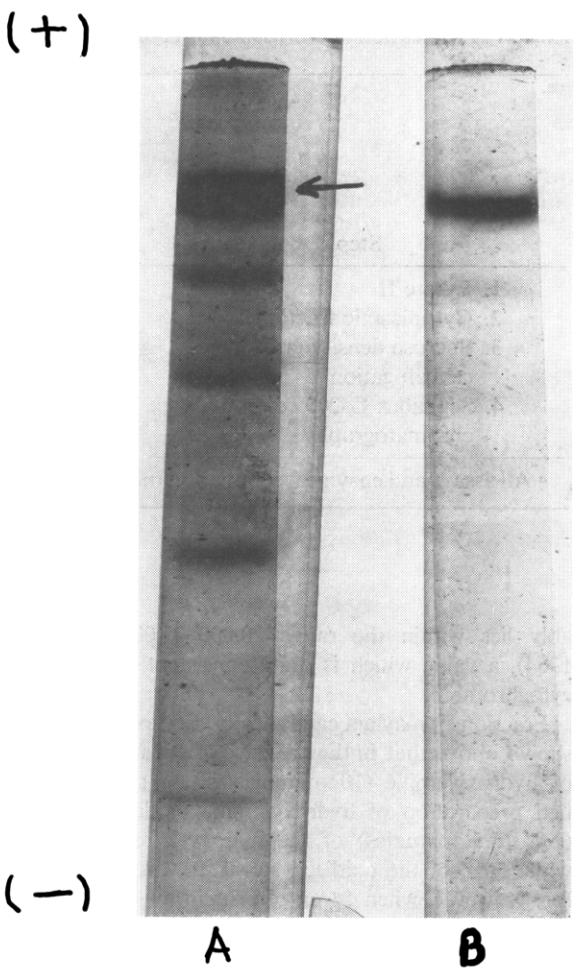

FIGURE 8: Polyacrylamide gel electrophoresis of purified hydroxylamine oxidase. Samples were applied directly to 7.5% gels (5-mm diameter) and electrophoresed for 40 min at 4° (3 ma/tube). Solvent: Tris–glycine buffer, pH 9.5. (A) Hydroxylamine oxidase fraction resulting from sucrose density gradient centrifugation of cytoplasmic fraction 144; 46 μg of protein. The arrow denotes the stained position of the red band that was visible following electrophoresis. (B) Hydroxylamine oxidase fraction resulting from Sephadex G-200 chromatography of the peak sucrose density gradient fraction of the enzyme; 30 μg of protein.

TABLE V: Purification of *Nitrosomonas* Hydroxylamine Oxidase.[a]

Step	Sp Act. (mμmoles of NH_2OH/(ml min mg of protein))	Purification (-fold)	Yield (%)	% Conversion to Nitrite ((mμmoles of NO_2 formed/mμmoles of total NH_2OH oxidized) × 100)
1. Lysate II	10	1	100	29
2. Cytoplasmic fraction 144	17.2	1.7	100	29
3. Sucrose density gradient centrifugation	510	51	60	47
4. Sephadex G-200 column chromatography	600	60	41	58

[a] All assays and enzyme fractionation methods are described under Experimental Procedure and Figure 1.

ably lies within the range 20,000–25,000 (Andrews, 1964), a value which is quite consistent for a soluble cytochrome *c*.

Fraction F_2 may act catalytically. For example, it was shown above that in the presence of substrate amounts of hydroxylamine (10 mμmoles/ml), a partially purified preparation of hydroxylamine oxidase converted 65% (6.5 mμmoles) of the hydroxylamine to nitrite while 35% of the oxidized substrate was unaccounted for. However, when 14 μg of protein/ml of fraction F_2 was added to the reaction mixture, only 17% (1.7 mμmoles/ml) of the hydroxylamine appeared as nitrite, even though 100% (10 mμmoles) was oxidized. Assuming the molecular weight of fraction F_2 to be 20,000 g/mole, then 0.7 mμmole of fraction F_2 was responsible for the disappearance of $6.5 - 1.7 = 4.8$ mμmoles of hydroxylamine. The catalytic interaction of fraction F_2 with hydroxylamine oxidase seems more likely than the alternate explanation, that every one molecule of fraction F_2 reacts with seven molecules of hydroxylamine (or some intermediate of hydroxylamine oxidation).

Although most *c*-type cytochromes are strongly basic (p*K*'s between 10 and 11) this is evidently not true of fraction F_2. At a pH of about 7, this cytochrome does not adsorb to either Dowex 50W in the NH_4^+ form or to CM-cellulose in the H^+ form. Moreover, it binds only weakly to DEAE-cellulose phosphate at a pH of about 8. Thus, in the neutral pH range, the protein seems relatively uncharged.

Discussion

To date, most of our information about the inorganic nitrogen metabolism of *Nitrosomonas* centers about the oxidation of hydroxylamine. In 1960, Nicholas and Jones demonstrated that cell-free extracts prepared by sonic oscillation actively oxidize hydroxylamine in the presence of added electron-acceptor compounds. Of the various electron acceptors they examined, maximum nitrite formation was obtained with PMS and pyocyanine, while methylene blue, benzyl viologen, and mammalian cytochrome *c* were less effective in this regard. These workers noted that although crude extracts are capable of completely oxidizing substrate quantities of hydroxylamine, even under the best conditions only 40–70% of the substrate could be accounted for as nitrite. A 44-fold purification of the enzyme system responsible for hydroxylamine oxidation was achieved by a combination of ammonium sulfate fractionation and DEAE-cellulose column chromatography with an 8% recovery of the total enzymatic activity. (The proportion of the total hydroxylamine oxidized by the purified enzyme which was recovered as nitrite was not examined.)

The metabolism of hydroxylamine by cell-free extracts of *Nitrosomonas* has since been examined in various laboratories, and the findings that the presence of an added electron acceptor is essential for active hydroxylamine oxidation and that incomplete recovery of nitrite occurs have been repeatedly confirmed (Falcone *et al.*, 1963; Anderson, 1964; Aleem and Lees, 1963; Hooper and Nason, 1965). However, attention has almost uniformly been focused on the ability of hydroxylamine oxidase to catalyze the reduction of added mammalian cytochrome *c* (hydroxylamine–cytochrome *c* reductase). Aleem and Lees (1963) achieved an 85-fold purification of the enzyme with a 35% recovery of mammalian cytochrome *c* reducing activity, and Hooper and Nason (1965) reported at 14-fold purification with a 25% yield. However, little or no nitrite formation accompanied hydroxylamine oxidation by the preparations isolated by these workers.

The present work is essentially an extension of the original observations of Nicholas and Jones (cited above), and it represents the first attempt since their report to isolate an active nitrite-forming system from *Nitrosomonas*. This study confirms the finding that the presence of PMS is essential for maximal nitrite formation. The data of Table II illustrate that following osmotic rupture of EDTA–lysozyme-treated cultures,

both hydroxylamine oxidase and terminal oxidase activities are quantitatively recovered in the 144,000*g*, 2-hr supernatant solution and are, by this definition, soluble enzymes. Furthermore, little or no electron transport activity is found in the various particulate fractions listed in Figure 1, and examination of these fractions by absorption spectroscopy revealed that 95–98% of the hemeproteins of *Nitrosomonas* are distributed in the soluble fraction (see, for example, Figures 2–4 of Rees and Nason, 1965). The enzyme responsible for hydroxylamine oxidation is easily separated from the terminal oxidase of the organism (see above, Figures 2, 4, 6, and 7). This finding, when taken together with the striking stimulation of hydroxylamine oxidation which occurs when electron-acceptor compounds are added to lysates, suggests a mechanism to explain the loss of hydroxylamine-oxidizing activity consequent to cell rupture. Upon rupture of the cell, the terminal oxidase is effectively separated from hydroxylamine oxidase; consequently, the rate of turnover of the latter is slow. The addition of electron acceptors allows the terminal oxidase to be by-passed, and, accordingly, hydroxylamine oxidation is stimulated.

Although only a small fraction of the total hydroxylamine oxidized by crude cell-free extracts is converted to nitrite, the data of Table V reveal that this number increases as hydroxylamine oxidase is purified. This increasing yield of nitrite is at least in part accounted for by the separation of the cytochrome c_{552} containing fraction, which has been designated fraction F_2. Fraction F_2 in some manner specifically interferes with the ability of hydroxylamine oxidase to form nitrite, and it has no effect on either the rate or extent of hydroxylamine oxidation by this enzyme. As discussed above, either alone or in the presence of added electron-acceptor compounds, fraction F_2 does not oxidize hydroxylamine; however, when hydroxylamine oxidase is additionally present, the inhibitory effect of F_2 is readily noted. Moreover, the available evidence indicates that fraction F_2 acts catalytically. This suggests that both fraction F_2 and hydroxylamine oxidase contain components which, when together, catalyze the oxidation of either hydroxylamine or an intermediate of hydroxylamine oxidation to reaction products other than nitrite.[3] Since the presence of fraction F_2 does not accelerate the rate of hydroxylamine oxidation by hydroxylamine oxidase, the substrate of the reconstituted enzyme is more likely to be an intermediate of hydroxylamine oxidation than it is to be hydroxylamine itself.

[3] Although hydroxylamine oxidase is capable of oxidizing hydroxylamine anaerobically if an electron-acceptor compound is present, no nitrite is formed, and Anderson (1964) has shown that under these conditions hydroxylamine is quantitatively converted to the gases, nitrous oxide and nitric oxide. These findings are thought to indicate that during the oxidation of hydroxylamine, a labile intermediate (NOH) is formed which may either be further oxidized to nitrite or may be converted to nitrous and nitric oxide (Anderson, 1964; Falcone *et al.*, 1963). This information suggests that the unidentified products of hydroxylamine oxidation discussed above are these gases.

It is doubtful that the altered stoichiometry of hydroxylamine metabolism which occurs when *Nitrosomonas* cells are lysed can be attributed entirely to the interaction of fraction F_2 with hydroxylamine oxidase, for even when the activity of the electrophoretically homogeneous enzyme is examined, a maximum of 70% of the total substrate oxidized by the enzyme is converted to nitrite. It has been shown that the presence of even a relatively small quantity of the chloride ion (0.1 M) exerts an effect on hydroxylamine oxidation similar to that observed here for fraction F_2 (Rees, 1967). That is, although the rates of hydroxylamine utilization are identical in either the absence or presence of chloride, the presence of 0.1 M chloride causes a significant inhibition of the rate of nitrite formation as well as a decrease in the final quantity of nitrite formed. Thus, it may be that multiple factors (including the nature of the ionic environment and the presence or absence of fraction F_2) are responsible for the altered stoichiometry of hydroxylamine oxidation which is observed when cell-free extracts of *Nitrosomonas* are studied.

The use of an enzyme fractionation procedure which relies on differential adsorption (such as column chromatography on DEAE-cellulose) should be avoided in purifying hydroxylamine oxidase, since the resultant yield of the enzyme is poor and less than a two fold increase in specific activity is achieved. Evidently, denaturation of hydroxylamine oxidase occurs with this procedure. In any event, the employment of adsorption chromatography is unnecessary, for the present study demonstrates that it is possible to isolate hydroxylamine oxidase in pure form relying solely upon centrifugation and molecular sieving techniques.

Acknowledgment

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Studies of the Hydroxylamine Metabolism of *Nitrosomonas europaea*. II. Molecular Properties of the Electron-Transport Particle, Hydroxylamine Oxidase*

Michael K. Rees

ABSTRACT: Hydroxylamine oxidase is the initial acceptor of electrons from hydroxylamine, one of the energy substrates for the chemoautotroph, *Nitrosomonas europaea*.

Hydroxylamine oxidase lacks terminal oxidase activity, and in order to obtain measureable turnover of the enzyme, the presence of an added terminal electron-acceptor compound is essential. In this paper, some of the physical, chemical, and enzymatic features of hydroxylamine oxidase have been studied. Sedimentation velocity experiments indicate that the mass of homogeneous hydroxylamine oxidase is close to 200,000 g/mole and that its sedimentation coefficient is 10 S. Difference spectroscopy studies performed at the temperature of liquid nitrogen demonstrate that the enzyme contains both *b*- and *c*-type cytochromes. Neither flavin nor carbon monoxide combining components are present.

In the preceding paper (Rees, 1968), a procedure was described for isolating highly purified solutions of the hydroxylamine-oxidizing enzyme of *Nitrosomonas europaea*, hydroxylamine oxidase. Information was presented which indicates that the terminal oxidase of the bacterium is not physically associated with hydroxylamine oxidase, and that, as a consequence, an added terminal electron acceptor is essential for turnover of the enzyme. Further, it was shown that the product of hydroxylamine oxidation varies with the electron-acceptor compound employed; for example, although nitrite is not formed in the presence of added mammalian cytochrome *c*, as much as 70% of the oxidized hydroxylamine is accounted for as the expected physiological product, nitrite, when phenazine methosulfate is used. In this paper, some of the physical and chemical characteristics of hydroxylamine oxidase have been studied.

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Experimental Procedure

Reagents. D_2O (at least 99.5%) was obtained from Matheson Coleman and Bell. All chemicals were reagent grade and were prepared as previously described (Rees, 1968). Hydroxylamine oxidase was isolated by the method outlined in Table V of the first paper of this series (Rees, 1968) and was routinely stored at 0°.

Sedimentation Analysis. The Spinco Model E analytical ultracentrifuge was used for sedimentation velocity and sedimentation equilibrium experiments. All studies were performed with the absorption optical system of the instrument together with a split-beam photoelectric scanner and monochromator (Beckman Instruments, Inc.). The optical system was aligned by the method of Schachman *et al.* and the camera lens was focused upon an air–liquid meniscus using off-axis illumination (Schachman *et al.*, 1962). Radially oriented masks (4 × 20 mm) were mounted upon both collimating and condensing lenses to minimize stray light arising from the various optical components. Monochromatic light of wavelength 410 mμ was selected so that very dilute solutions of hydroxylamine oxidase could be examined. The monochromator and photo-