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Oxidation of Ammonia by Spheroplasts of *Nitrosomonas europaea*

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The ammonia-oxidizing activity of *Nitrosomonas europaea* spheroplasts was restored by addition of hydroxylamine or by preincubation with Mg^{2+}.

Nitrosomonas europaea oxidizes ammonia to nitrite through hydroxylamine. Although cell-free systems oxidizing hydroxylamine have been characterized, all efforts have been unsuccessful in obtaining such systems for ammonia oxidation. Recent reports (3, 4) suggest that ammonia oxidation by this organism involves an oxygenase. If ammonia to hydroxylamine is catalyzed by an oxygenase, the reaction is very likely to be a mixed-function type, requiring a reducing equivalent for the reduction of the remaining half of the oxygen molecule. This reducing equivalent can be supplied only during the oxidation of hydroxylamine to nitrite, since this organism derives all of the energy and reducing power for growth from the oxidation of ammonia to nitrite. If this assumption is correct, the oxidation of ammonia to hydroxylamine has to be coupled somehow to the oxidation of hydroxylamine to nitrite, which explains the difficulty in obtaining such a system in a cell-free state.

We prepared spheroplasts of *Nitrosomonas* cells and showed that hydroxylamine can restore the ammonia-oxidizing activity of inactive spheroplasts. While this work was in progress, the effect of hydroxylamine in diminishing the lag observed during ammonia oxidation by whole cells of *Nitrosomonas* was reported (2).

A culture of *Nitrosomonas* (Schmidt strain) was kindly supplied by A. B. Hooper, University of Minnesota. The cells were grown with forced aeration in 15-liter batches at 28 C by using ATCC medium no. 221. The *Nitrosomonas* cells were harvested at the stationary phase, washed three times in 0.1 M potassium phosphate buffer (*p*H 7.5), and stored in the same buffer (10 mg of wet cells/ml). The spheroplasts were prepared by the method of Rees and Nason (3), except that the concentrations of lysozyme and ethylenediaminetetraacetic acid (EDTA) were doubled. The spheroplasts were washed twice in 0.25 M sucrose-0.1 M potassium phosphate (*p*H 7.5) buffer and were suspended in the same buffer. Nitrite was determined by the method of Bratton and Marshall (1).

The *Nitrosomonas* spheroplasts thus prepared were free from whole cells when examined under an electron microscope. In Warburg experiments, these spheroplasts were not able to oxidize ammonia (Table 1, B) but regained the oxidizing ability in the presence of hydroxylamine (D). Nitrite could not replace hydroxylamine in this recovery process (E) but did not inhibit the process when present (F). Hydroxylamine was oxidized rapidly (C) and nearly completely within 0.5 hr, but the rate of oxygen uptake in flask D was fairly constant during the 4-hr incubation period. No intact cells were found after incubation. Although cytochrome *c* was included routinely in the reaction mixtures, its omission had no effect on the results.

This effect of hydroxylamine was confirmed by using a Gilson Oxygraph (Clark Oxygen Electrode; Fig. 1). There was some endogenous oxygen uptake by spheroplasts which was not increased by the addition of ammonia (curve a). Hydroxylamine was rapidly oxidized (curve b), but, after the completion of hydroxylamine oxidation, the rate of oxygen uptake returned to the original level. In the presence of ammonia, however, the rate was increased as a result of ammonia oxidation (curve c). This phenomenon was reproducible, and the rate of ammonia oxidation increased successively after the successive additions of hydroxylamine.

Since EDTA, which was used in the preparation of spheroplasts, has been reported to remove the outer cell wall layer of structural protein of marine ammonia-oxidizing bacteria (5), presumably because of chelation of divalent metals, the effect of Mg^{2+} was tested on the spheroplasts. Although 1.5 μmoles of Mg^{2+} had no effect when added during the reaction, the spheroplasts preincubated with Mg^{2+} before dilution with the sucrose-phosphate buffer regained the ability to oxidize ammonia (curve d).

The oxidation of ammonia in the experiment (curve d) was confirmed by nitrite determination, although the recovery of nitrite was less than that accounted for from the oxygen consumption, unlike the nearly stoichiometric recovery obtained in experiment D in Table 1. This extra consumption of oxygen must have been due to increased endogenous metabolism by ammonia addition. This recovery effect by Mg^{2+} was duplicated by Ca^{2+} and Mn^{2+} at the same concentration. A preliminary electron microscopic study indicated that the Mg^{2+} treatment increased the number of membranous folds and invaginations inside spheroplasts.

These studies confirm and extend the report by Hooper (2) of the effect of hydroxylamine in ammonia oxidation and suggest a possible structural requirement of cell membranes for the oxidation of ammonia.

TABLE 1. *Effect of hydroxylamine on the oxidation of ammonia by Nitrosomonas spheroplasts*

Reaction system[a]	Oxygen utilized (μmole)	Nitrite found (μmole)	Nitrite formed from ammonia (μmole)
A. Spheroplast	1.0	0.25	
B. Spheroplast + $(NH_4)_2SO_4$	1.4	0.42	0.17
C. Spheroplast + NH_2OH	3.0	2.7	
D. Spheroplast + $(NH_4)_2SO_4$ + NH_2OH	7.9	5.0	2.3
E. Spheroplast + $(NH_4)_2SO_4$ + $NaNO_2$	1.5	1.3	0.05
F. Spheroplast + $(NH_4)_2SO_4$ + NH_2OH + $NaNO_2$	8.5	6.7	3.0

[a] The reaction was carried out in Warburg vessels at 30 C for 4 hr. The reaction mixture, in a total volume of 2.5 ml, contained spheroplasts (from 25 mg of wet cells) and 0.3 μmole of cytochrome *c* (equine heart) in 0.25 M sucrose-0.1 M potassium phosphate (*p*H 7.5) buffer. Additions: $(NH_4)_2SO_4$, 5 μmoles; $NH_2OH \cdot HCl$, 2 μmoles; $NaNO_2$, 1 μmole.

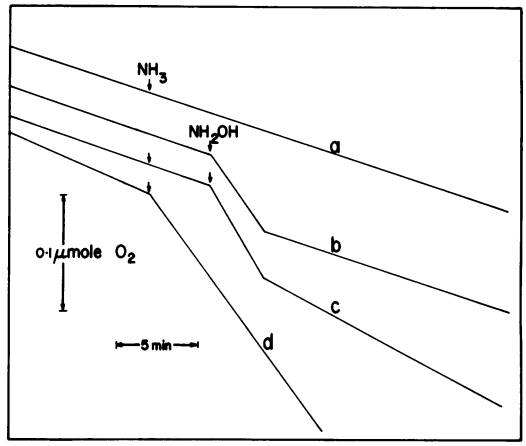

FIG. 1. *Time course of oxygen utilization by Nitrosomonas spheroplasts. At the times indicated by arrows, either 2.5 μmoles of $(NH_4)_2SO_4$ or 0.05 μmole of $NH_2OH \cdot HCl$ was added. The reaction mixture contained 0.1 ml of spheroplasts (derived from 10 mg of wet Nitrosomonas cells), 0.025 ml of 3.3 mM cytochrome c (equine heart), and 1.375 ml of 0.25 M sucrose-0.1 M potassium phosphate (pH 7.5) buffer (20 C). In experiment d, 0.1 ml of spheroplasts was preincubated with $MgSO_4$ (0.015 ml of 0.1 M) at 20 C for 5 min before the initiation of the experiment.*

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