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Lag Phase of Ammonia Oxidation by Resting Cells of *Nitrosomonas europaea*

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Cells of the ammonia-utilizing autotroph *Nitrosomonas europaea* catalyze the aerobic production of nitrite from ammonia with hydroxylamine as a presumed intermediate. Hydroxylamine dehydrogenase from *Nitrosomonas* has been characterized (3) and preliminary characterization of the cell-free conversion of hydroxylamine to nitrite has been described (1), but little is known about the mechanism of ammonia oxidation. In this study, nitrite production and oxygen utilization concomitant with hydroxylamine oxidation were found to be linear with respect to time when catalyzed by a freshly diluted suspension of resting cells of *Nitrosomonas*. With ammonia as a substrate, however, the rate of nitrite production and the rate of oxygen utilization were initially zero and gradually increased to a constant level. This kinetic pattern is referred to as the "lag" phase of ammonia oxidation. The lag was diminished when the cells were preincubated in a dilute suspension or when a small quantity of hydroxylamine was included in the reaction mixture. These observations indicate that ammonia oxidation, in contrast to hydroxylamine oxidation, by *Nitrosomonas* requires a physiological or biochemical cellular state dependent on endogenous metabolism or hydroxylamine oxidation.

Cells of *Nitrosomonas* (culture from E. L. Schmidt, University of Minnesota) were grown in batch culture to a concentration of 67 μg (wet weight) per ml of culture, harvested, and washed by suspension at a concentration of 30 mg of wet cells per ml of 50 mM phosphate solution, *p*H 7.5, as described previously (2). Cells either were washed twice for 20 min and 15 hr, respectively, or were washed twice for 20 min. In either case, significant nitrite did not appear in the supernatant fraction of a third wash. Washed cells were suspended at a concentration of 200 mg of wet cells per ml in 50 mM phosphate solution, *p*H 7.5, and were stored at 4 C in a test tube closed with parafilm. The *p*H of the cell suspension remained at 7.5 during storage.

Oxygen utilization and nitrite formation coupled to hydroxylamine oxidation by a suspension of 3.3 mg (wet weight) of cells/ml were essentially linear with time, whereas oxygen utilization and nitrite formation coupled to ammonia oxidation took place initially with a lag phase of approximately 7 min followed by a high constant rate (Fig. 1). The lag was readily reproducible

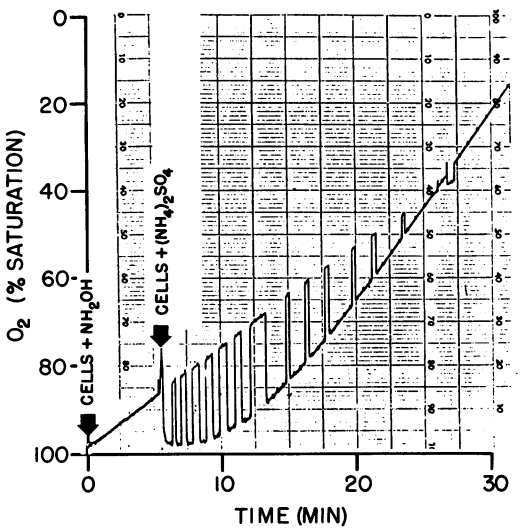

FIG. 1. *Time course of oxygen utilization coupled to ammonia or hydroxylamine oxidation as catalyzed by Nitrosomonas. At the times indicated by the arrows, either (upper tracing) 0.05 ml of a suspension of cells [200 mg (wet weight)/ml] and 0.1 ml of 0.03 M NH_2OH or (lower tracing) 0.05 ml of a suspension of cells and 0.1 ml of 0.1 M $(NH_4)_2SO_4$ were added to 2.85-ml volumes of air-saturated 0.05 M phosphate solution (pH 7.5, 25 C) contained in two separate reaction vessels. The resulting change in oxygen concentration in each vessel was measured polarographically with a separate electrode utilizing a Y.S.I. Oxygen Monitor (Yellow Springs Instruments, Yellow Springs, Ohio) and a Leeds and Northrop 100-mv recorder. The two reactions were carried out simultaneously, recording alternately from one electrode and then the other.*

with separate batches of cells grown and suspended at a concentration of 200 mg/ml, as described in this work, and was observed with unwashed cells, with cells immediately after washing,

or with cells which had been stored for 1 or 2 days. Preliminary results indicated that the lag was more pronounced in cells harvested from cultures near the end of exponential growth or from cultures in the stationary phase of growth. Thus, the biochemical and physiological changes which occurred during a 7-min incubation of a dilute *Nitrosomonas* suspension resulted in activation of ammonia oxidation as contrasted with hydroxylamine oxidation.

This change could occur in the absence of added ammonia. Stimulation of ammonia oxidation and elimination of the lag phase resulted when a 1:60 dilution of the 200 mg per ml of cell suspension in phosphate buffer was incubated for 7 min in the absence of substrate (Fig. 2). Endogenous oxygen utilization and nitrite formation occurred during the period of incubation. Apparently, a process which can be coupled to the endogenous metabolism of *Nitrosomonas* and which is inhibited in a concentrated cell suspension is required to generate a cofactor of ammonia oxidation, to remove a specific inhibitor of ammonia oxidation, or to increase the rate of ammonia uptake by the cell.

Incubation of cells of *Nitrosomonas* with 10^{-5} M NH_2OH resulted in an increased rate of formation

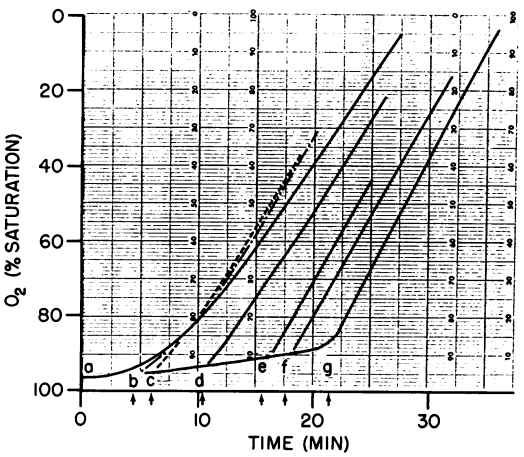

FIG. 2. *Effect of delayed addition of substrate on the kinetics of oxygen utilization coupled to ammonia oxidation by Nitrosomonas. Oxygen utilization was assayed as indicated in Fig. 1. Whereas cells and phosphate solution were mixed at T = 0, $(NH_4)_2SO_4$ was added to seven separate reaction vessels (a to g) at T = 0 (vessel a) or at the times indicated by the arrows (vessels b to g). The figure was made by superimposing and tracing the chart records of six separate experiments having made the oxygen concentrations equal at T = 0. Previous to $(NH_4)_2SO_4$ addition, the time course of oxygen utilization was the same in each vessel. Lines b and c are dotted for clarity.*

TABLE 1. *Effect of 10^{-5} M NH_2OH on the kinetics of nitrite synthesis from ammonia by Nitrosomonas cells*

Reaction mixture[a]	Amt of nitrite produced during various time intervals (min)				
	0–2.5	2.5–5	5–10	10–15	15–20
A. Cells	2.3[b]	1.2	6.5	13	20
B. Cells + NH_2OH	9.3	12.7	23	25	24
C. Cells + $(NH_4)_2SO_4$	5.8	21.2	103	140	180
D. Sum of A + B + C	17	37	136	170	230
E. Cells + NH_2OH + $(NH_4)_2SO_4$	35	85	180	200	200

[a] To start the reaction, a 0.05-ml volume of *Nitrosomonas* cells [200 mg (wet weight)/ml] was added to a test tube containing 9.9 ml of 1 mM $(NH_4)_2SO_4$ or 10^{-5} M NH_2OH in 0.05 M phosphate, *p*H 7.5, at 24 C, and the contents were quickly mixed by inversion. Aeration and mixing during the course of the reaction were accomplished by rotation of the test tube at 20 to 30 rev/min while held as nearly horizontal as possible. The nitrite content was determined by taking 1.0-ml samples from the incubation mixture at the indicated times as described previously (3).

[b] These values are expressed as nanomoles.

of nitrite as compared with the endogenous rate (Table 1). Furthermore, the presence of 10^{-5} M NH_2OH in addition to 1 mM $(NH_4)_2SO_4$ resulted in a stimulation of nitrite synthesis as compared with the sum of nitrite synthesized (i) in the endogenous control, (ii) in the presence of hydroxylamine, and (iii) in the presence of ammonium sulfate alone. The stimulation of nitrite synthesis was greatest during the first 10 min of the reaction and resulted in partial elimination of the lag phase of ammonia oxidation. In *Nitrosomonas*, the oxidation of hydroxylamine is probably coupled to adenosine triphosphate synthesis and the generation of reduced pyridine nucleotide. The present results suggest that one of those compounds is required, directly or indirectly for activation of the ammonia oxidation process.

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