ACETYLENE INHIBITION OF NITROUS OXIDE REDUCTION BY DENITRIFYING BACTERIA

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Summary Acetylene (0.1 atm) caused complete or almost complete inhibition of reduction of N_2O by whole cell suspensions of *Pseudomonas perfectomarinus*, *P. aeruginosa* and *Micrococcus denitrificans*. Acetylene did not inhibit reduction of NO_3^- or NO_2^- by these organisms. In the presence of acetylene there was stoichiometric conversion of NO_3^- or NO_2^- to N_2O with negligible subsequent reduction of the latter. In the absence of acetylene there was no or only transient accumulation of N_2O. The data are consistent with the view that N_2O is an obligatory intermediate in the reduction of NO_2^- to N_2 in all of the three organisms studied.

The pathway by which nitrogenous oxides are reduced to dinitrogen (N_2) by denitrifying bacteria appears to depend on the species. Partial purification of enzyme fractions from *Pseudomonas perfectomarinus* (10), and studies of inhibition of nitrous oxide (N_2O) reductase by azide (N_3^-), cyanide (CN^-) and dinitrophenol (DNP) in *Pseudomonas denitrificans* (8) showed that N_2O was an obligatory intermediate in the reduction of nitrite (NO_2^-) to N_2. However, studies of the effects of inhibitors such as N_3^-, CN^- and DNP on reduction of NO_2^- and N_2O in *Pseudomonas stutzeri* (1), *P. denitrificans* (12) and *Micrococcus denitrificans* (11) suggested that N_2O was not an obligatory intermediate.

Fedorova *et al.* (4), in a study of extra-terrestrial life detection, reported that acetylene (C_2H_2) inhibited the reduction of N_2O during denitrification.

We confirm here that C_2H_2 inhibits reduction of N_2O by three denitrifying bacteria, *P. perfectomarinus*, *P. aeruginosa* and *M. denitrificans*. We report also that during reduction of NO_3^- or NO_2^-, C_2H_2 causes accumulation of N_2O with a stoichiometry which suggests that N_2O is an obligatory intermediate in the reduction of NO_2^- to N_2 in all three species of denitrifier.

MATERIALS and METHODS

Organisms *Pseudomonas perfectomarinus* was obtained from Dr. W.J. Payne, University of Georgia. *Micrococcus denitrificans* (NRC #14029) was obtained from the National Research Council of Canada, Ottawa. *Pseudomonas aeruginosa* was from the Macdonald College culture collection (Mac #67).

Media and Incubation *P. perfectomarinus* was grown aerobically on a rotary shaker for 24 hr at 25° C in the medium of Best and Payne (2) modified as follows: 0.2 M NaCl, 0.05 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$, 0.01 M $NaNO_3$, 0.5% Tryptone and 0.15% Yeast Extract (Difco). Late log phase cells were harvested from 60 ml of growth medium and washed three times by centrifugation (3300 g at 4° C for 15 min) and resuspension in nitrate-free artificial sea water (ASW: NaCl, $MgSO_4$, KCl and $CaCl_2$ as above). Aliquots (0.5 ml) of the cell suspension containing (1.6×10^{11} cells) were transferred to 50-ml sterile Erlenmeyer flasks each containing 10 ml of the above medium without nitrate. After closing with sterile serum stoppers (Suba-Seal, England), the flasks were evacuated and back-filled with sterile helium to one atmosphere three times. When required, 5.0 ml of gas phase was replaced with the same volume of acetylene (C_2H_2) to give a final concentration of 0.1 atm. Also, when desired 0.5 ml N_2O, or 0.4 ml of 100 mM $NaNO_2$ or $NaNO_3$ was added to give a final concentration of 560 µg N per flask. Flasks were incubated at 24° C with occasional shaking.

P. aeruginosa and *M. denitrificans* were grown in Vernon's medium (14) under initially aerobic conditions on a rotary shaker for 24 hr at 32° C. Late log phase cells were harvested and treated similarly to those of *P. perfectomarinus* except that they were washed with 0.85% sodium chloride (NaCl) solution. Aliquots (0.5 ml) of the cell suspension (containing 2.6 and 3.4 $\times 10^{11}$ cells for *P. aeruginosa* and *M. denitrificans*, respectively) were transferred to 50-ml Erlenmeyer flasks each of which contained 0.5% peptone and 0.15% yeast extract in 10 ml of 10 mM sodium phosphate (pH 6.8). Subsequent procedures were as described above.

Analytical Bacteria were counted in a Petroff-Hausser chamber after appropriate dilution of the samples. Protein was estimated (7) with bovine albumin as standard after washing the cells with ASW or 0.85% NaCl.

Nitrate was determined by the Griess reaction (13) and nitrate by the brucine method (5). Samples were kept frozen prior to analysis. Samples for nitrite and nitrate analysis were withdrawn from flasks at various times by hypodermic syringe, and diluted 20 times with distilled water before they were frozen.

Gas chromatographic analysis of N_2O was as previously described (9). Corrections for N_2O solubility and normalization of initial concentration of N_2O were made.

RESULTS

Fig. 1 shows data from an experiment in which dense cell suspensions of *P. perfectomarinus*, in the absence (Fig. 1A) and in the presence of 0.1 atm of C_2H_2 (Fig. 1B), were supplemented with N_2O, NO_2^- and NO_3^-. Reduction of N_2O proceeded rapidly in the control but was completely inhibited by 0.1 atm C_2H_2. Reduction of NO_2^- occurred more slowly. No N_2O accumulated in the control, while in the presence of C_2H_2 there was stoichiometric conversion of NO_2^- to N_2O with no subsequent reduction of the latter. Reduction of NO_3^- to NO_2^- was complete within 8 hr whether C_2H_2 was present or not. Subsequent

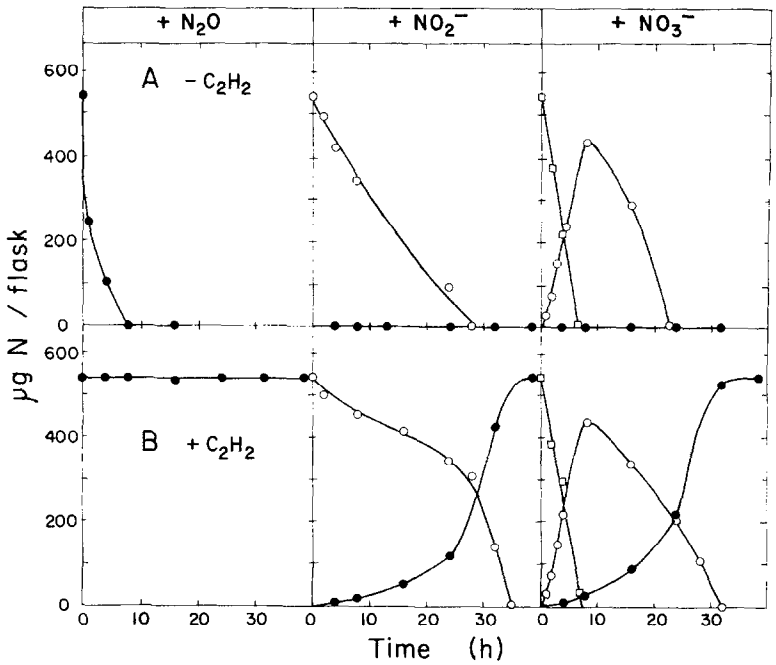

Fig. 1. Reduction of added N_2O, NO_2^- and NO_3^- by dense suspensions of *P. perfectomarinus* incubated in a He atmosphere at 24° C (A) in the absence and (B) in the presence of 0.1 atm pC_2H_2. Symbols are ● N_2O, o NO_2^- and □ NO_3^-.

reduction of the accumulated NO_2^- occurred more slowly. In the absence of C_2H_2 no N_2O accumulated, but in the presence of C_2H_2 there was stoichiometric conversion of the added N to N_2O with no further reduction of the latter. Thus the effect of C_2H_2 on N_2O reduction was very pronounced. Acetylene caused a slight delay in the reduction of NO_2^- but did not affect the rate of reduction of NO_3^-.

Figs. 2 and 3 show the results of similar experiments using *P. aeruginosa* and *M. denitrificans*. The patterns of reduction of the nitrogenous oxides in these two organisms were very similar and C_2H_2 caused complete inhibition of N_2O reduction in *P. aeruginosa* and almost complete inhibition in *M. denitrificans*. These organisms differed from *P. perfectomarinus* in showing transient accumulation of N_2O in the absence of C_2H_2. Compared to *P. perfectomarinus* they showed rates of reduction greater than could be accounted for by the

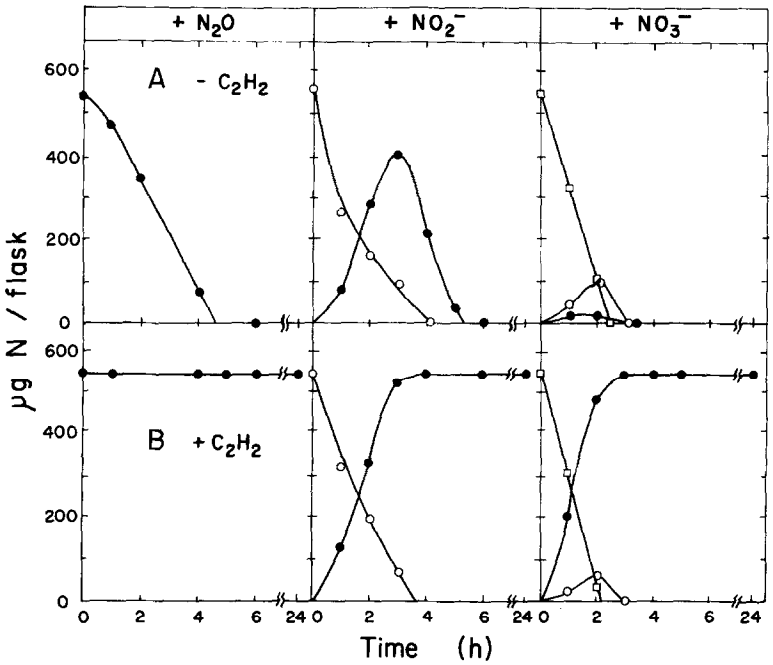

Fig. 2. Reduction of added N_2O, NO_2^- and NO_3^- by dense suspensions of *P. aeruginosa*. Otherwise, the same as for Fig. 1.

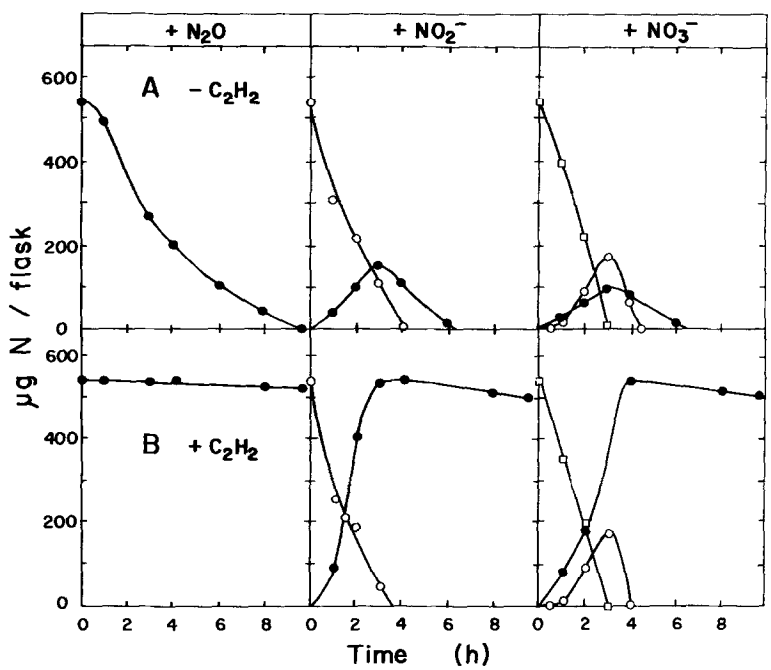

Fig. 3. Reduction of added N_2O, NO_2^- and NO_3^- by dense suspensions of *M. denitrificans*. Otherwise, the same as for Fig. 1.

TABLE 1. Initial and Final Bacterial Protein Concentrations

	Time of incubation (hr)	Initial protein (mg/flask)	Acetylene (0.1 atm)	Final protein (mg/flask) N_2O	NO_2^-	NO_3^-
P. perfectomarinus	40	0.45	-	0.63	0.69	0.93
			+	0.51	0.55	0.67
P. aeruginosa	24	0.70	-	0.77	0.99	1.14
			+	0.74	0.80	1.11
M. denitrificans	12	0.73	-	0.87	1.00	1.30
			+	0.81	0.89	1.11

small differences in cell and protein concentrations.

There was no evidence of reduction of C_2H_2 to ethylene (C_2H_4) in these experiments.

Table 1 shows that only small amounts of growth (measured by initial and final protein concentrations) occurred in the dense suspensions used in the experiments. Growth increments increased in the order $N_2O < NO_2^- < NO_3^-$ and were slightly greater in the absence of C_2H_2 than in its presence.

DISCUSSION

The present data show that C_2H_2, at a concentration of 0.1 atm, causes complete or almost complete inhibition of N_2O reduction without significantly affecting other stages of denitrification. Our unpublished data show that complete inhibition is also shown by 0.01 atm C_2H_2 depending on organisms and experimental conditions. Such growth increments in the order $N_2O < NO_2^- < NO_3^-$ were consistent with the growth and energy yields reported for *P. denitrificans* (6) and with the possible loss of at least one phosphorylation site in the presence of C_2H_2.

Nitrous oxide and C_2H_2 are substrates for and competitive inhibitors of

nitrogenase (3). It is therefore interesting that C_2H_2 should be an inhibitor of N_2O reductase. Unpublished data suggest that the inhibition is non-competitive.

The reported effects of N_3^-, CN^- and DNP on denitrification (1,8,11,12) are explainable in terms of effects on specific cytochrome systems. However, there are no reports of effects of C_2H_2 on such systems.

The fact that C_2H_2 specifically inhibits N_2O reduction and causes stoichiometric accumulation of N_2O during reduction of NO_2^- and NO_3^- suggests that, at least in the organisms studied, N_2O is an obligatory intermediate in the sequence of steps between NO_3^- and N_2. This is consistent with studies of *P. perfectomarinus* (10) but not with studies of *P. stutzeri* (1) and *M. denitrificans* (11).

These results suggest the potential utilization of C_2H_2 inhibition of N_2O reduction in further biochemical studies as well as in the assay of denitrification in complex systems (4). Preliminary experiments (to be published elsewhere) show that the presence of C_2H_2 permits measurements of N_2O accumulation in soils or sediments and may facilitate measurement of the ratio of N_2/N_2O formed during denitrification.

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