USE OF NITROUS OXIDE AS THE TERMINAL ELECTRON ACCEPTOR DURING GROWTH AND RESPIRATION OF *BRADYRHIZOBIUM JAPONICUM* USDA 143

by

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(ABSTRACT)

Bradyrhizobium japonicum USDA 143 grew chemoorganotrophically when supplied with exogenous nitrous oxide as the terminal electron acceptor, or as the alternate terminal electron acceptor to nitrate under anoxic conditions. Cell growth and dissimilatory N_2O reduction were significantly inhibited by acetylene when either N_2O or N_2O plus nitrate served as terminal electron acceptor(s). Reduction of nitrous oxide accounted for 20% of the energy for cell growth in cultures supplied with nitrate as the terminal electron acceptor. Nitrous oxide was produced stoichiometrically in cultures supplied with nitrate and acetylene and growth was proportionately reduced compared to cultures supplied with an equal amount of nitrate. Exogenous nitrous oxide delayed the reduction of nitrate in cultures supplied with both electron acceptors. The final cell yield and/or growth rate of the cells were reduced when N_2O was $\geq$ 15% of the culture flask headspace. Direct amperometric monitoring of nitrous oxide respiration indicated a specific activity of 0.082 $\pm$ 0.004 μmoles N_2O/min/mg cell-protein. The respiration was inhibited by azide.

A Clark-type electrode with a platinum cathode, and the instrumentation for monitoring hydrogen uptake amperometrically were used to monitor the reduction of N_2O during anaerobic respiration.

First I thank my major professor, Dr. John Neal, whose guidance and calm helped carry me through the rough parts of this project. I also thank the members of my committee, Drs. R. E. Benoit, J-S Chen, and N. R. Krieg, for their formal and informal advice. To these gentlemen, I acknowledge that the education you provided me goes far beyond the contents of this thesis. I also recognize the immeasurable help, understanding, and encouragement provided by the friends, including the technical staff, in the Microbiology section.

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I thank my parents, William and Gloria Tucker, who provided emotional support (even in light of the fact they cannot perceive what they did wrong to encourage me to pursue this profession). I also thank my parents-in-law, Barbara and Richard Harvey, for the understanding and support they provided me and my wife.

Most of all I thank my wife, Karen, without whom this degree would have little meaning. Her encouragement, understanding, patience, and impatience made this degree possible. It is to her that this thesis is lovingly dedicated in recognition of her help and many sacrifices.

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ABSTRACT

Bradyrhizobium japonicum USDA 143 grew chemoorganotrophically when supplied with exogenous nitrous oxide as the terminal electron acceptor, or as the alternate electron acceptor to nitrate under anoxic conditions. Cell growth and dissimilatory N_2O reduction were significantly inhibited by acetylene when either N_2O or N_2O plus nitrate served as terminal electron acceptors(s). Reduction of nitrous oxide accounted for 20% of the energy for cell growth in cultures supplied with nitrate as the terminal electron acceptor. Nitrous oxide was produced stoichiometrically in cultures supplied with nitrate and acetylene and growth as proportionately reduced compared to cultures supplied with an equal amount of nitrate. Exogenous nitrous oxide delayed the reduction of nitrate in cultures supplied with both electron acceptors. The final cell yield and/or growth rate of the cells were reduced when N_2O was $\geq$ 15% of the culture flask headspace. Direct amperometric monitoring of nitrous oxide respiration indicated a specific acitivity of 0.082 $\pm$ 0.004 μmoles N_2O/min/mg cell-protein. The respiration was inhibited by azide.

INTRODUCTION

Denitrification is the dissimilatory reduction of ionic nitrogen oxides to gaseous nitrogen oxides or molecular nitrogen (12). The nitrogen oxides serve as electron acceptors for respiration under anoxic conditions (12). The proposed route for the reduction of the nitrogen oxides is NO_3^- ---> NO_2^- ---> [NO] ---> 1/2 N_2O ---> 1/2 N_2 in bacteria that contain the full complement of enzymes for denitrification (15). Nitrous oxide is the first non-toxic gaseous nitrogen oxide produced, and some denitrifying bacteria lack the ability to synthesize the N_2O reductase that is necessary for further reduction of N_2O to N_2 (12). However, of the N_2O-reducing bacteria that have been studied, N_2O can serve as the sole terminal electron acceptor and support growth (13).

Neal et al. (11) demonstrated that chemolithotrophically grown Bradyrhizobium japonicum USDA 143 can utilize nitrate as a terminal electron acceptor. Although the presence of nitrous oxide reductase was demonstrated, they did not determine if the reduction of N_2O was linked to energy generation (12).

The purpose of this paper is to report that B. japonicum USDA 143 can obtain energy for growth using N_2O as the sole terminal electron acceptor. The reduction of N_2O by actively growing cultures of B. japonicum was determined by gas chromatography. The rate of respiration was determined with polarography. The energy gain for cell growth from res-

piration with N_2O was compared to the energy gain with nitrate or oxygen as the terminal electron acceptors. Acetylene and azide, which are inhibitors of dissimilatory N_2O reduction (1, 14, 17), were used to inhibit growth and respiration, respectively, of B. japonicum using N_2O as the terminal electron acceptor.

MATERIALS AND METHODS

CULTURE PURITY AND MAINTENANCE: The purity of cultures of Bradyrhizobium japonicum USDA 143 was determined by observation of morphology by phase contrast microscopy and Gram stain reaction, and by the ability to use mannitol as a carbon/energy source. The stock culture of B. japonicum from which subcultures were derived for all experiments was tested periodically for its ability to nodulate soybean plants (16).

The bacterial culture was maintained in 500-ml polypropylene centrifuge bottles containing 250 ml of yeast extract-mannitol broth supplemented with KNO_3 (0.5 g/l). The headspace of the culture vessel was flushed with oxygen-free argon for a minimum of five minutes. The vessel was then inoculated and sealed with a screw cap. B. japonicum cells were grown at 26°C for 8 days. The cells were harvested by centrifugation, and suspended in anaerobic defined medium (10). For cell growth experiments, the resuspended cells were transferred anaerobically (5) to 150 ml of anaerobic defined media contained in a 530-ml reagent bottle. The bottle was sealed with a rubber stopper and sub-samples were removed as required by syringe for initial inoculation. For polarography experiments, the resuspended cells were transferred anaerobically (5) to a test tube containing oxygen-free argon and sealed with a serum stopper.

TREATMENT OF GASES: Argon was obtained from a commercial supplier and freed of oxygen by passage over hot copper pellets (6). Reagent grade nitrous oxide (98% pure), pharmaceutical oxygen, and acetylene

were used as purchased. In some experiments, acetylene was also generated by adding calcium carbide to water and trapping the gas under water. All gases were sterilized by passage through cotton filters immediately before use.

CULTURE MEDIA: The defined medium described by Madigan and Gest (10) was modified as follows to contain mg/l of 0.479 mg $CoCl_2 \cdot 6H_2O$, 0.367 mg $NiCl_2 \cdot 6H_2O$ and 820 mg NH_4Cl in place of $(NH_4)_2SO_4$. Where required, 10 g/l of mannitol was added as a carbon/energy source. The medium and 0.67 M phosphate buffer were prepared anaerobically (5) and sterilized separately by autoclaving. To avoid a precipitation in the medium, the phosphate buffer was added by injection with a syringe after the medium had cooled, 4.2 ml were added to 140 ml of defined medium contained in a 530-ml reagent bottle that had been sealed with a rubber stopper. The final pH of the medium, after the addition of the buffer, was 6.8. A vacuum was established in each reagent bottle and the desired volume of each gas was injected. Atmospheric pressure was restored by the addition of oxygen-free argon. When specified, nitrate was added to the defined medium from an anaerobic (5) stock solution containing KNO_3 (10 g/l).

OPTIMUM pH FOR GROWTH: Phosphate buffers (0.67 M) ranging from pH 5.8 to 8.2 were prepared anaerobically (5). Four culture flasks were prepared at each pH with 20% N_2O and 80% argon in the headspace. The pH of the medium was determined by removing 2 ml of medium from each culture flask, pooling the samples, and then determining the pH. Three of

the four culture flasks were incubated statically at 26°C on their sides to increase the gas-liquid interface. Growth was monitored by periodically removing a sample and determining the A_{540}: with the medium from the fourth uninoculated culture flask serving as the blank. The experiment was terminated when no further increase in turbidity occurred. Upon termination of the experiment, the pH of the culture fluid was determined.

OPTIMUM INITIAL NITROUS OXIDE CONCENTRATION: Defined medium (pH 6.8) containing 10 g mannitol/1 was prepared anaerobically as described. The culture flask atmosphere was adjusted to contain 0, 10, 20, 30, 40, or 60% N_2O, followed by the addition of oxygen-free argon to bring the cultures' headspace to atmospheric pressure. The culture flasks were inoculated and incubated on their sides at 26°C. Growth was monitored periodically by removing 3 ml of sample and determining the change in A_{540} on a Bausch and Lomb spectronic 88. Experiments were terminated when no further increase in turbidity occurred. Each treatment was replicated three times.

Since the highest growth rate and final cell density corresponded to the lowest initial concentration of N_2O in the headspaceof the culture vessel, the experiment was repeated using initial concentrations of 0, 5, 10, or 15% N_2O in the headspace of the cultures.

GROWTH WITH NITROUS OXIDE AS A TERMINAL ELECTRON ACCEPTOR: Anaerobic, defined medium (pH 6.8) with 10 g mannitol/1 was prepared as previously described. Culture flasks were prepared with 10% N_2O in the

headspace to ascertain cells could use N_2O as the sole terminal electron acceptor for growth. Controls included cultures with 10% N_2O and 5% acetylene (acetylene inhibits the dissimilatory reduction of N_2O (1, 17), or with only argon in the headspace to ascertain the possible growth of the cells when no terminal electron acceptor was supplied. The culture flask with 10% N_2O and 5% C_2H_2 in the headspace also served as the control to determine the loss of N_2O from the headspace due to sampling. A culture flask with 10% N_2O in the headspace but lacking mannitol as a carbon/energy source was prepared to determine growth due to endogenous respiration. A culture flask with 5% acetylene in the headspace was included to determine if any possible electron acceptor was introduced with the acetylene. To determine the effect of N_2O reduction upon growth of cells respiring with nitrate, culture flasks were prepared with 0.33 g KNO_3/l. Culture flasks containing defined media with 0.33 g KNO_3/l were also prepared with 10% N_2O, or 5% acetylene in the headspace to ascertain the effect of exogenous N_2O, or inhibition of endogenous N_2O reduction, respectively, relative to growth with nitrate as the sole exogenous terminal electron acceptor. The headspace of all the culture flasks were brought up to atmospheric pressure by the addition of oxygen-free argon. Experiments were initiated by inoculation of the culture flask with 5 ml of a B. japonicum cell suspension. A control consisting of 0.33 g KNO_3/l and 10% N_2O in a flask that had been inoculated with 5 ml of a heat-killed cell suspension was included to correct for the loss of N_2O due to sampling from the culture supplied

with nitrate and 10% N_2O. All treatments were prepared in triplicate and incubated statically at 27°C. Periodically 4 ml of culture were removed and growth was monitored by following the changes in the A_{540} and by performing viable plate counts on yeast extract-mannitol agar. Viable plate counts were performed only when there was a noticable change in the turbidity of the culture. The remainder of the samples were frozen for later assay of nitrate concentration. To determine the headspace concentration of N_2O by gas chromatography, 9-ml gas samples were periodically removed from the headspace and stored in 10-ml Vacutainer tubes containing 1 ml of 1 M KOH to remove CO_2.

UTILIZATION OF ELECTRON ACCEPTORS: To determine the rate of utilization of nitrate as a terminal electron acceptor, the frozen culture samples were thawed and immediately analysed for nitrate colorimetrically (3).

The concentration of N_2O in the culture flask atmosphere was determined by injecting 0.5 ml gas samples from the Vacutainer tubes into a Perkin-Elmer 910 gas chromatograph equipped with a column support system (5.5 m x 2.0 mm inside diameter) containing Porapack Q; the thermal conductivity detector was set at 300°C and 175 mA (1, 17). The oven and injector temperatures were 100°C and 110°C, respectively. The carrier gas was helium at 50 ml/min. External N_2O standards were used to calibrate the chromatograph. Samples were corrected for solubility of N_2O by using the Bunsen absorption coefficient (15).

RELATIVE ENERGY PRODUCTION USING VARIOUS TERMINAL ELECTRON ACCEPTORS: To determine the relative energy production for growth of B. japonicum with nitrate, oxygen, or N_2O as the terminal electron acceptor, defined medium containing 10 g mannitol/l was prepared as described before. At concentrations ranging from 0.14 to 1.38 μmoles, nitrate, O_2, or N_2O was added as the sole terminal electron acceptor. A culture with no terminal electron acceptor was included to determine the initial concentration of cells (i.e., cell-protein/ml). The cultures were diluted with equal volumes of either the nitrate solution or anoxic distilled water. After inoculation, the culture flasks were incubated at 27°C on their sides. Each treatment was prepared in quadruplicate. Growth of one culture at each concentration of each electron acceptor was monitored periodically by removing 3-ml samples and measuring the change in A_{540}. When no further increase in turbidity occurred, the corresponding cultures with NO_3^- or N_2O as terminal electron acceptors were assayed for their NO_3^- or N_2O content, respectively, as described before. If the terminal electron acceptor was undetectable, the cells were harvested by centrifugation, suspended in 3-ml of distilled water, and immediately frozen. After all cultures had reached their stationary phase, the protein content of each culture was determined using the procedure of Lowry et al. (9) after alkaline hydrolysis, with bovine serum albumin (BSA) as the standard. For each electron acceptor employed, the relationship of protein content of the culture to moles of electron acceptor supplied was determined using linear regression.

RATE OF RESPIRATION: To monitor the rate of respiration, a Clark-type electrode was used (Section II). The cuvette was filled with defined medium (pH 6.8) and sparged with oxygen-free argon. Additions were made to the cuvette through a sealed entry hole using a Hamilton gas-tight syringe. Nitrous oxide was injected into the cuvette as a saturated solution prepared by bubbling anaerobic water with N_2O for 2 hours. Fifty µl of the N_2O saturated water was injected into the cuvette and, after the electrode stabilized, 50 or 100 µl of cells were injected into the cuvette. Respiration was recorded to base line and all treatments were repeated at least three times. Using the Bunsen absorption coefficient (15) of N_2O corrected for the barometric pressure, it was calculated that 0.95 µmoles of N_2O was injected into the cuvette. Sodium azide was used to inhibit respiration by injecting 90 µl of a 2% solution into the cuvette. Acetylene, commercially purchased or generated from calcium carbide, was used to prepare saturated solutions of the gas. Protein content of the cells was determined after alkaline hydrolysis using the method of Lowry et al. (9) with BSA as the standard.

RESULTS

OPTIMUM PH FOR GROWTH: The optimum pH for growth ranged between 6.8 to 7.2 with only a slight difference in final cell densities for each pH examined (data not shown). However, at pH 8.2 there was a significant decrease in turbidity relative to the other cultures. Upon termination of the experiment, a change in the initial pH of the culture media could not be detected.

OPTIMUM INITIAL NITROUS OXIDE CONCENTRATION: Varying the initial concentration of N_2O in the culture headspace from 10 to 60% resulted in an inverse relationship of cell density and growth rate to the N_2O concentration in the headspace (Figure 1A). Further experiments using initial concentrations of 5, 10, and 15% N_2O in the headspace showed that the final cell density was proportional to the N_2O concentration (Figure 1B). However, the growth rate decreased with 15% N_2O, with respect to the apparent growth rate with 5 or 10% N_2O (Figure 1B). Therefore, 10% N_2O was chosen as the optimal initial concentration of N_2O.

GROWTH WITH NITROUS OXIDE AS A TERMINAL ELECTRON ACCEPTOR: With N_2O as the sole terminal electron acceptor, turbidity reached a maximum of 0.35 after six days incubation, followed by a decrease in turbidity to 0.26 after 14 days incubation (Figure 2A). Increase in turbidity did not occur in culture flasks lacking N_2O as a terminal electron acceptor nor did it occur in the presence of N_2O + C_2H_2 or C_2H_2 alone. Viable cell numbers increased from 2.7 X 10^7/ml to a maximum of 2.5 X 10^8 cells/ml during the course of the growth experiment (Figure 2B). In con-

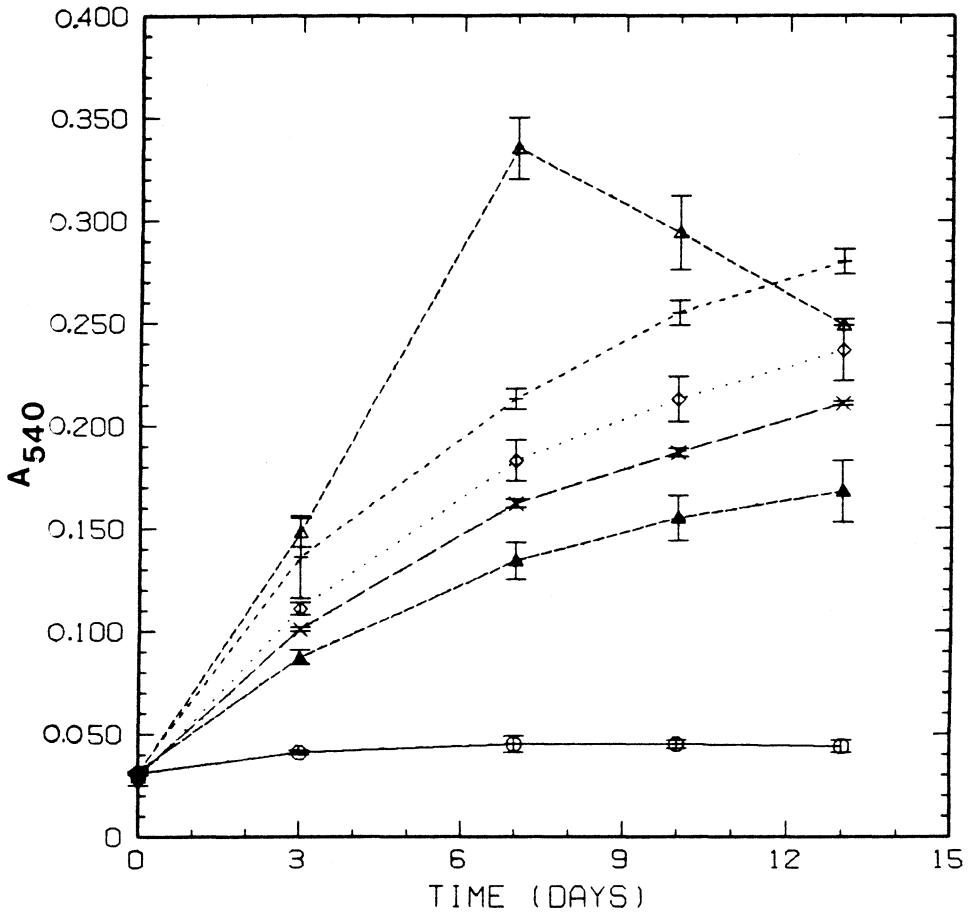

Fig. 1A: The effect of various concentrations of N_2O (0 to 60% of the culture's headspace) upon growth of B. japonicum USDA 143. Symbols: (o) 0%, (△) 10%, (+) 20%, (◇) 30%, (x) 40%, and (▲) 60% N_2O in the headspace of the cultures. Vertical lines are the SEM.

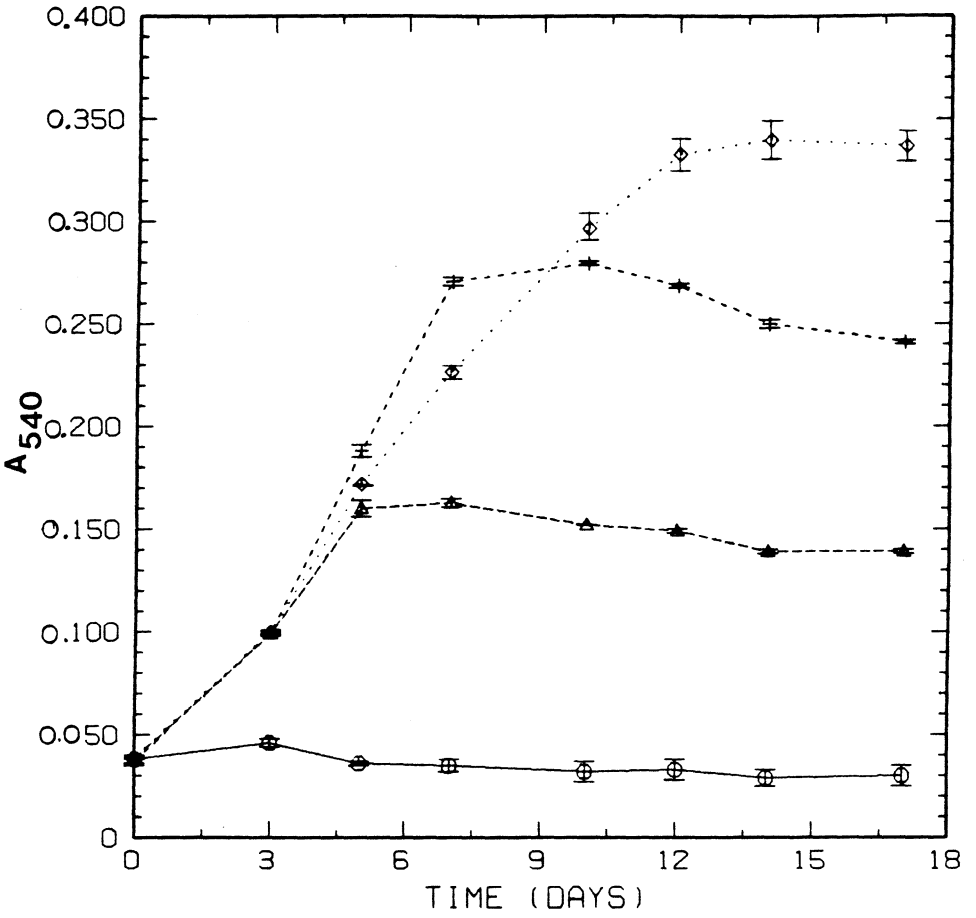

Fig. 1B: The effect of various concentrations of N_2O (0 to 15% of the culture's headspace) upon growth of B. japonicum USDA 143. Symbols: (o) 0%, (△) 5%, (+) 10%, and (◇) 15% N_2O in the headspace of the cultures. Vertical lines are the SEM.

trast, cultures lacking N_2O, or containing N_2O + C_2H_2 or C_2H_2 alone in the culture flask headspace, showed no increase in viable cell numbers. The cell growth experiments indicated that N_2O was serving as a terminal electron acceptor for growth during anaerobic respiration. Cell growth, as measured by the increase in A_{540}, increased significantly when both nitrate and N_2O were supplied as the terminal electron acceptors (Figure 3A) compared to growth with nitrate as the sole terminal electron acceptor. Addition of C_2H_2 to the culture flask atmosphere caused a significantly lower cell density compared to the cell density with nitrate serving as the terminal electron acceptor (Figure 3A). Cell growth, as determined by viable cell count, showed a similar, but less evident trend (Figure 3B). The number of cells increased from 2.7×10^7 to 3.1×10^8 cells/ml after five days incubation with nitrate serving as the only terminal electron acceptor. After seven days incubation, the cell number had decreased to 1.7×10^8 cells/ml. Cell numbers continued to increase up to 10 days of incubation when nitrate and N_2O were supplied as exogenous terminal electron acceptors. Although not conclusive, the data suggest that nitrate and N_2O could be used as the terminal electron acceptor simultaneously. To investigate this possibility, further experiments were conducted to determine the rate of disappearance of nitrate and/or N_2O from the culture flask during cell growth.

UTILIZATION OF ELECTRON ACCEPTORS: Use of N_2O as an electron acceptor in the presence or absence of nitrate is shown in Figure 4A.

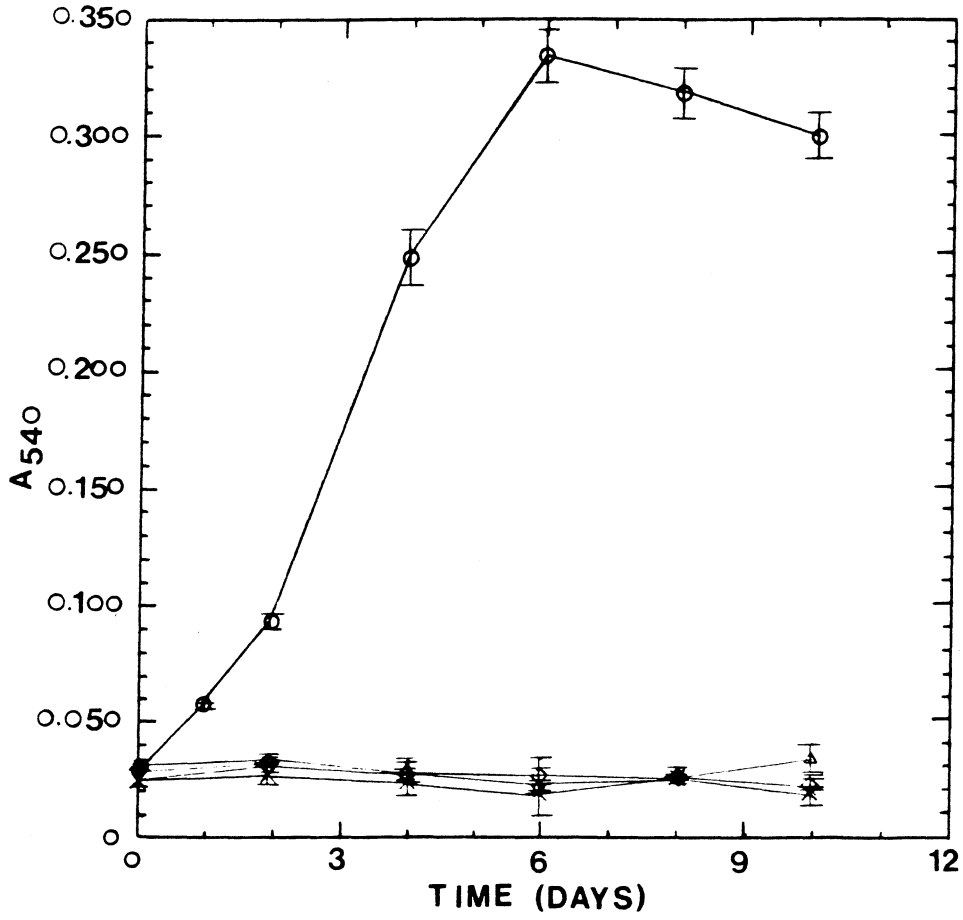

Fig. 2A: Growth of B. japonicum USDA 143 with 10% N_2O as the terminal electron acceptor (o) as compared to growth without N_2O present (i.e. 100% argon in the head-space) (△), or N_2O reduction inhibited by the presence of 5% acetylene (◇). Further controls include lack of growth in the presence of 10% N_2O with no carbon/energy source present (+), and lack of growth with 5% acetylene in the headspace of the cultures (x). Vertical lines are the SEM.

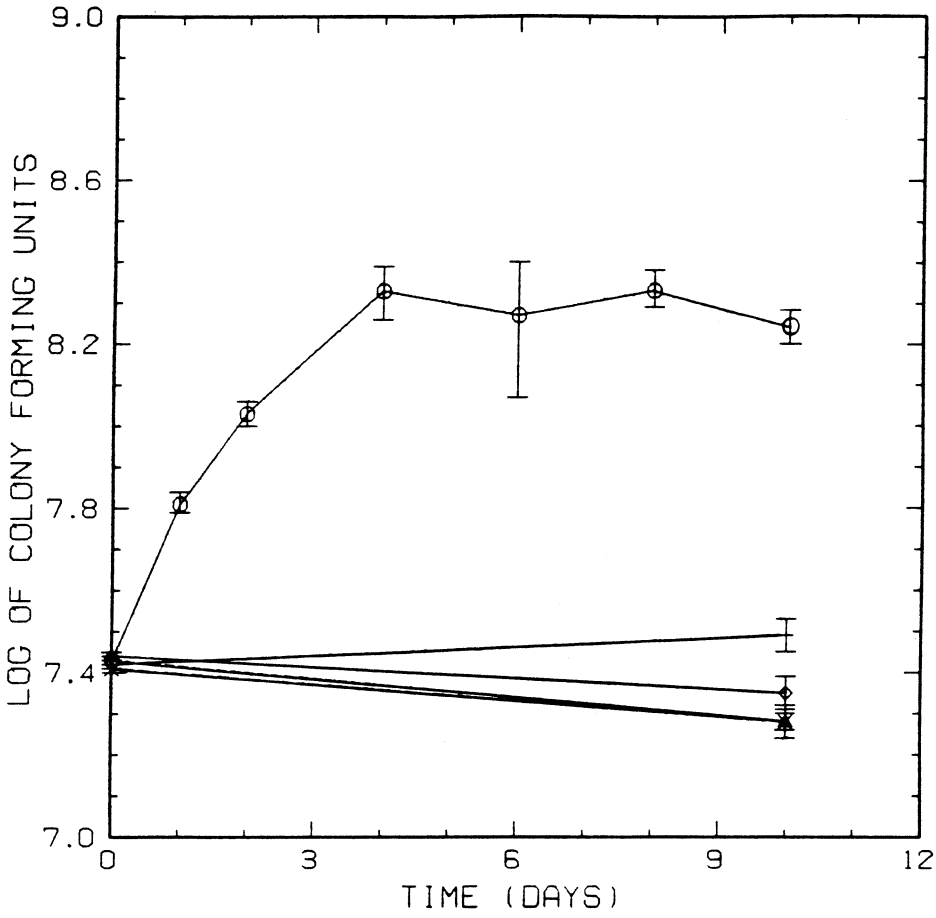

Fig. 2B: Growth of B. japonicum USDA 143 with 10% N_2O as the terminal electron acceptor (o) as compared to growth without N_2O present (i.e. 100% argon in the head-space) (△), or N_2O reduction inhibited by the presence of 5% acetylene (◇). Further controls include lack of growth in the presence of 10% N_2O with no carbon/energy source present (+), and lack of growth with 5% acetylene in the headspace of the cultures (x). Vertical lines are the SEM.

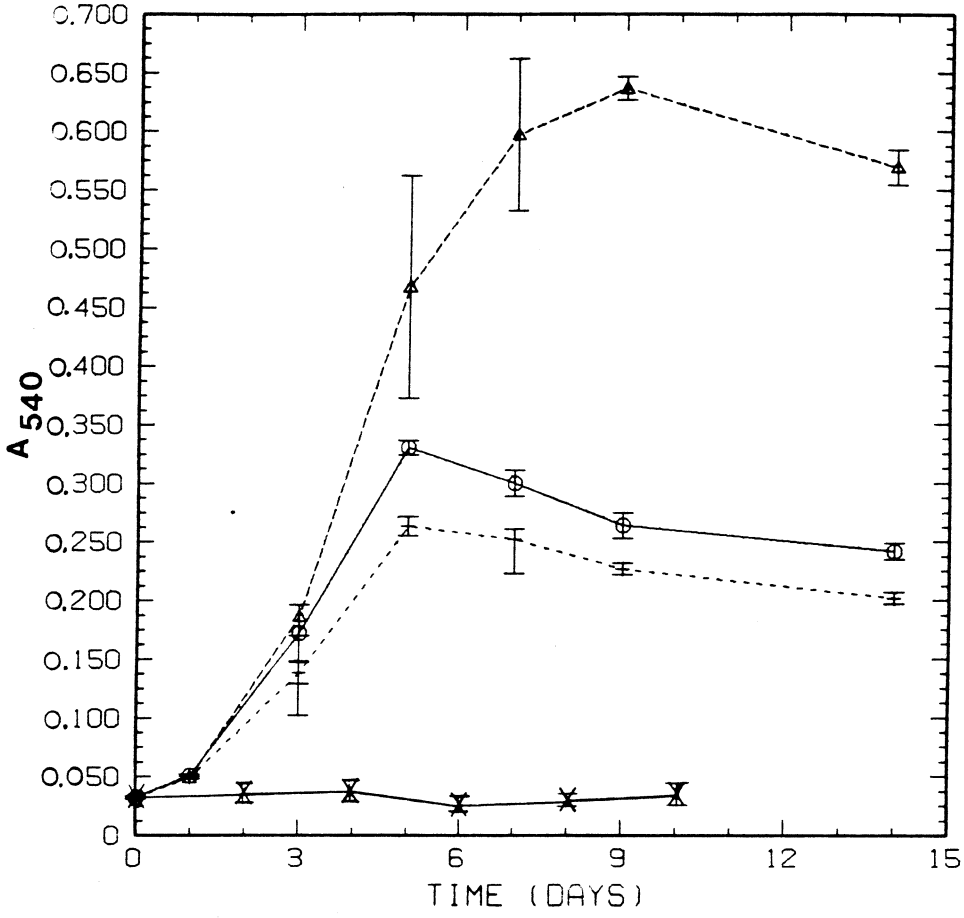

Fig. 3A: Growth of B. japonicum USDA 143 with 0.033% nitrate as the terminal electron acceptor (o) as compared to growth with 0.033% nitrate using 5% C_2H_2 to inhibit endogenous N_2O reduction (+), growth with 0.033% nitrate and 10% N_2O as the supplied exogenous terminal electron acceptors (▲), and lack of growth with no terminal electron acceptors (i.e. 100% argon in the headspace of the culture flask) (x). Vertical lines are the SEM.

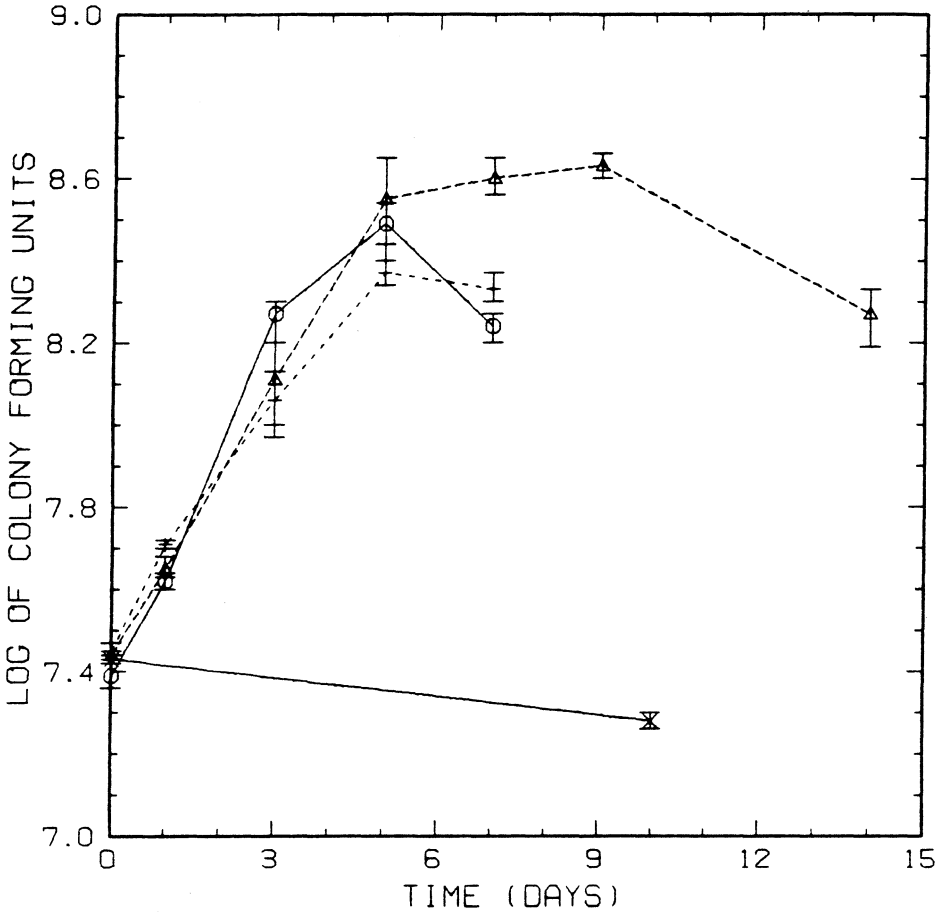

Fig. 3B: Growth of B. japonicum USDA 143 with 0.033% nitrate as the terminal electron acceptor (o) as compared to growth with 0.033% nitrate using 5% C_2H_2 to inhibit endogenous N_2O reduction (+), growth with 0.033% nitrate and 10% N_2O as the supplied exogenous terminal electron acceptors (▵), and lack of growth with no terminal electron acceptors (i.e. 100% argon in the headspace of the cultureflask) (x). Vertical lines are the SEM.

Loss of N_2O followed the same trend when N_2O served as the terminal electron acceptor with or without nitrate. A decrease in the concentration of nitrate in the cultures supplied with nitrate as the sole terminal electron acceptor was detectable after 24 hours of incubation (Figure 4B). This indicates nitrate was used as a terminal electron acceptor. In contrast, nitrate was not used as a terminal electron acceptor in the presence of N_2O until three or more days of incubation. Cultures with nitrate as the sole exogenous electron acceptor, but supplied with acetylene, showed the same rate of decrease in the amount of nitrate as cultures without C_2H_2. The amount of N_2O produced by cultures supplied with nitrate and C_2H_2 was stoichiometric relative to the amount of nitrate added to the culture media. In culture flask containing both nitrate and N_2O as the exogenous terminal electron acceptors, dissimilatory reduction of nitrate was delayed in favor of N_2O reduction (Figure 5). Apparently, as the concentration of N_2O is reduced by the dissimilatory reduction to N_2, nitrate and N_2O are concurrently reduced, both serving as terminal electron acceptors during anaerobic respiration.

CELL YIELD: When N_2O, NO_3^-, or O_2 was supplied as the terminal electron acceptor, the cell yield was 950, 2550, and 2600 mg cell-protein/μmole of acceptor, respectively. The coefficients of correlation were 0.93 0.97, and 0.96, respectively. On a mole per mole basis of electron acceptor, NO_3^- and O_2 produced the same relative cell-protein yield. However, N_2O yielded 2.67 fold less cell-protein relative to cultures supplied with an equimolar concentration of nitrate or oxygen.

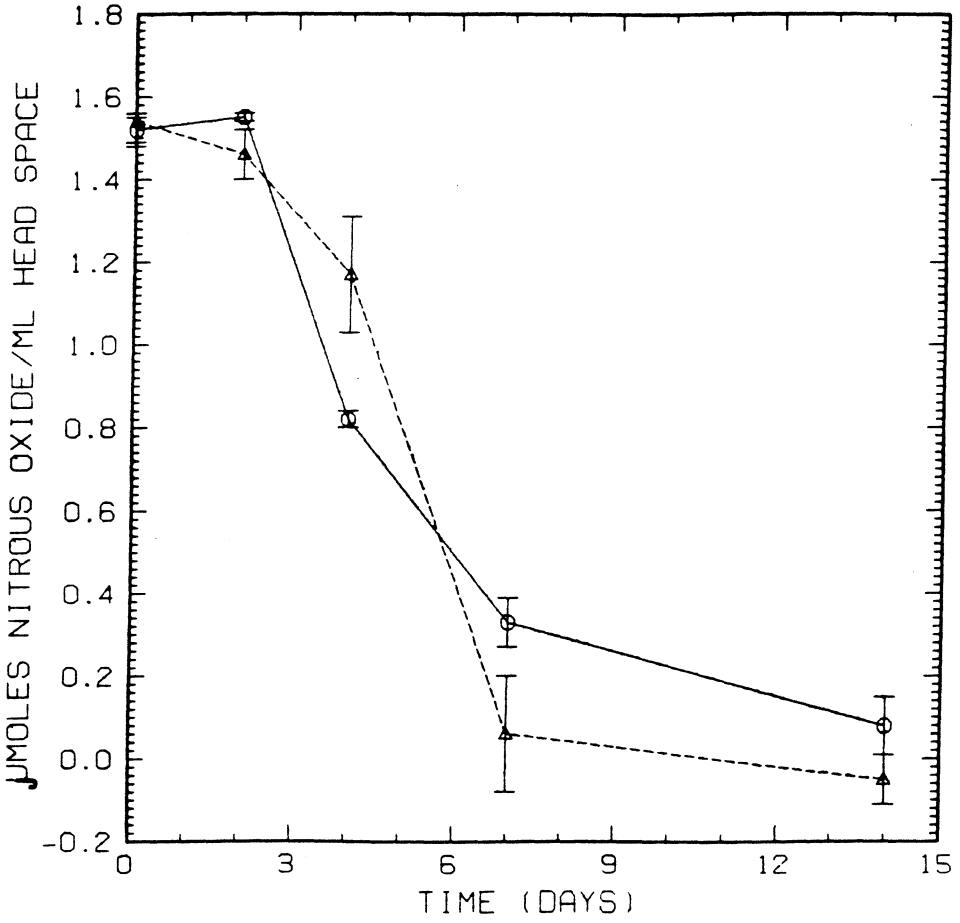

Fig. 4A: Utilization of N_2O by B. japonicum with N_2O, (o), or N_2O and nitrate (Δ) supplied as the terminal electron acceptor(s). Vertical lines are the SEM.

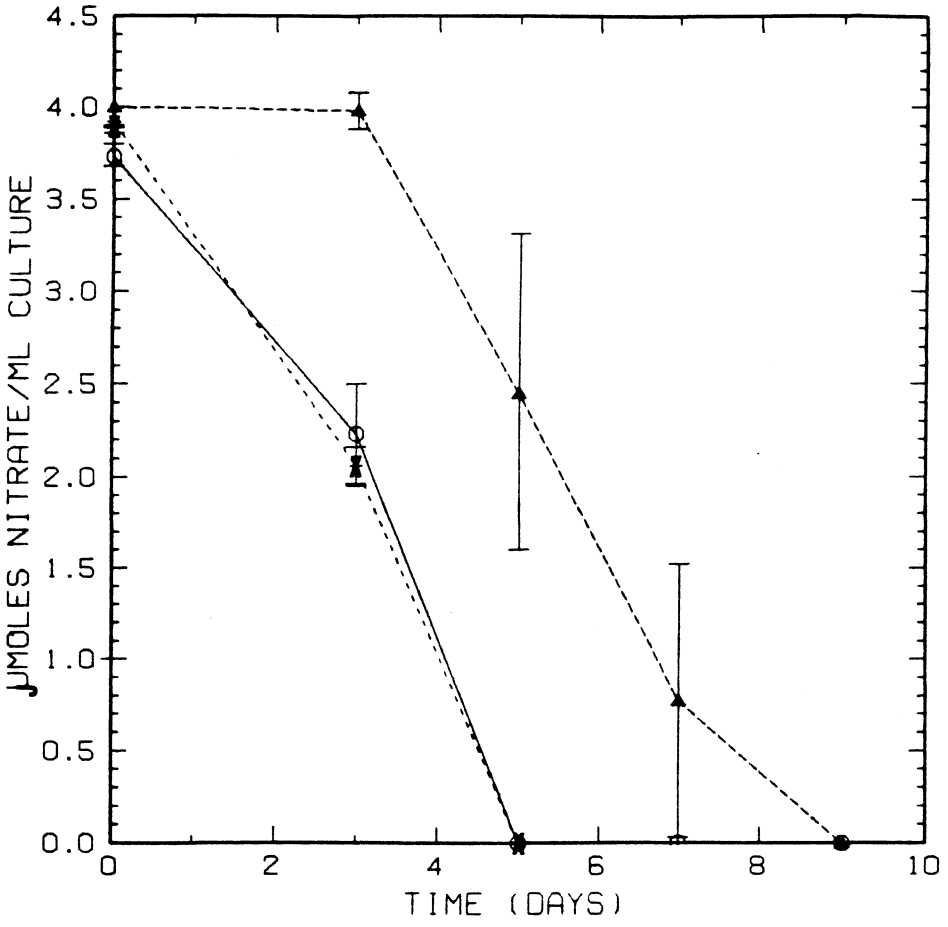

Fig. 4B: Utilization of nitrate by B. japonicum with nitrate (o), nitrate when endogenous N_2O respiration is inhibited by acetylene (x), or nitrate and 10% N_2O (▲) as the terminal electron acceptor(s). Vertical lines are the SEM.

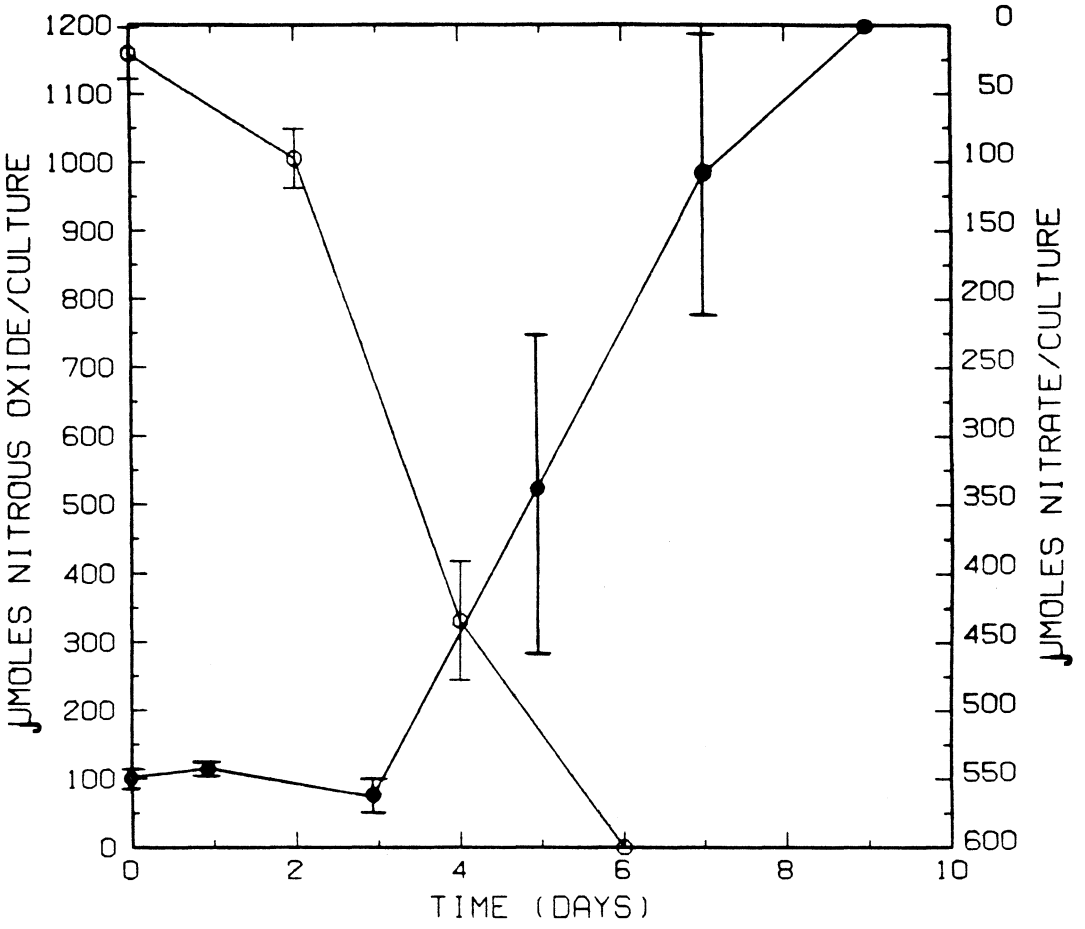

Fig. 5: Reduction of N_2O (o) or nitrate (•) by cultures of B. japonicum USDA 143 supplied with N_2O and nitrate as the terminal electron acceptors. Vertical lines are the SEM.

RATE OF RESPIRATION: The rate of respiration was constant per mg of cell-protein when either 50 or 100 µl of cells were injected into the cuvette (Figure 6). The specific activity for reduction of 528 µmolar N_2O by B. japonicum was found to be 0.082 ± 0.004 µmoles N_2O/min/mg protein. The reduction of N_2O was apparent immediately after the injection of the cells and was linear until base line was reached. Injection of N_2O without the addition of cells resulted in less than 2% deflection after a period of 30 minutes. Injection of cells into the cuvette without N_2O caused no response. Azide, an inhibitor of N_2O reductase (14), inhibited respiration with N_2O.

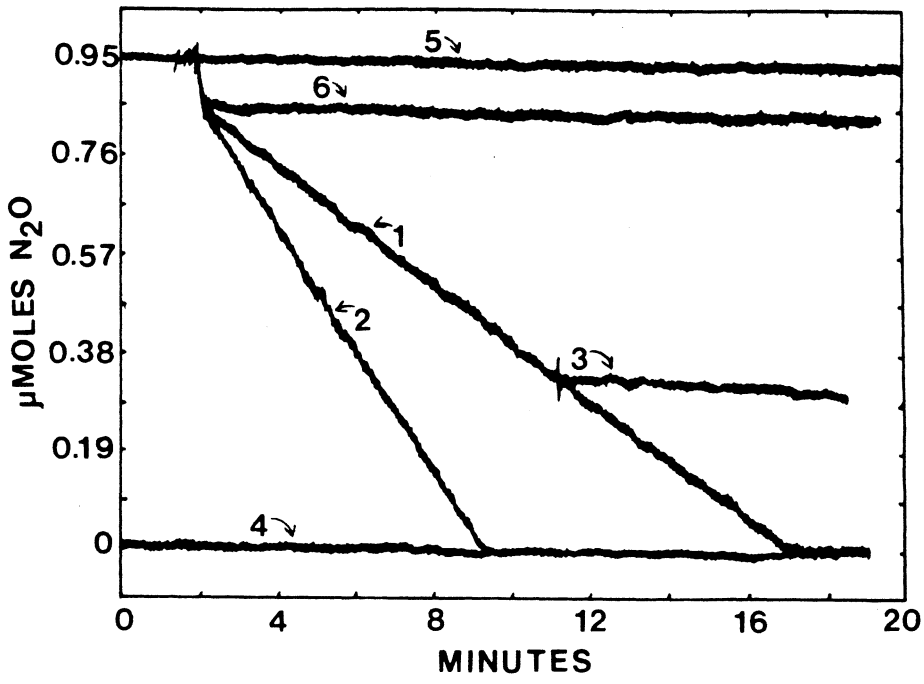

Fig. 6: Representative tracings of amperometric measurements of respiration using 0.69 (1) or 1.38 (2) mg cell-protein of B. japonicum with N_2O as the terminal electron acceptor. Included is inhibition of respiration with 0.1% azide (3). The controls without cells include the electrodes response to anoxic defined media saturated with argon (4), anoxic defined media containing 0.95 µmoles of N_2O (5), followed by injection of 100 µl of defined media saturated argon (6).

DISCUSSION

In many of the reported experiments where N_2O served as the terminal electron acceptor, the concentration of N_2O in the culture headspace was not greater than 10% (v/v) (e.g., 8). Bazylinsli et al. (2) reported that strains of Pseudomonas aeruginosa using N_2O as the terminal electron acceptor showed decreased cell yields when N_2O in the headspace of their cultures exceeded 15%. Their results indicated that reduced cell yield was not because of direct inhibition by N_2O, the failure or collapse of the proton pump forming the proton motive force, or nutritional limitation. N_2O was a free intermediate during denitrification by P. aeruginosa. The results of this study indicates apparent inhibition by N_2O of growth rate and cell yield when using N_2O as the terminal electron acceptor at concentrations $\geq$ 15% in the headspace of culture flask (Figures 1A and B). When cultures were supplied with nitrate + 5% C_2H_2 nitrous oxide was produced stoichiometrically relative to the amount of nitrate supplied. Therefore, nitrous oxide was an intermediate during denitrification by B. japonicum USDA 143.

As has been reported for bacteria using N_2O as the terminal electron acceptor (e.g., 2), cultures of B. japonicum cells using N_2O as the sole terminal electron acceptor showed a continually decreasing growth rate with time (Figure 2B). The concentration of N_2O in the culture medium decreases due to its reduction to N_2 (Figure 4A), and thus, N_2O becomes increasingly limiting as a terminal electron acceptor and limits the rate of growth. As far as we are aware, this is the first report of

B. japonicum USDA 143 obtaining energy for growth by using N_2O as a terminal electron acceptor during anaerobic respiration, either independent of, or in conjunction with nitrate respiration. The data presented show a significant increase in culture turbidity and cell numbers when N_2O was the sole terminal electron acceptor (Figures 2A and B), or when N_2O was an alternate electron acceptor to nitrate (Figures 3A and B). That energy was derived for growth from N_2O respiration was further supported by the reduction in culture turbidity and cell numbers when acetylene was used to inhibit N_2O reduction in the presence of nitrate (Figures 3A and B). These observations are in agreement with previous studies in which N_2O was used as the terminal electron acceptor during growth (e.g., 2). In contrast, El Hassan (El Hassan, Gadalla A. 1982. The Use of Nitrogen Oxides by Cowpea Rhizobia. Master of Science Thesis. University of California. Riverside, California) reported that the addition of acetylene to culture flasks containing nitrate caused a significant increase in cell yields and apparent growth rate of a cowpea rhizobial strain that was capable of substaining growth with nitrate, nitrite, and nitrous oxide as terminal electron acceptors. He proposed that the increase in cell yield and growth rate was possibly due to an increase in electron flux primarily because of a lower amount of electron acceptor caused by the removal of N_2O from the total electron acceptor pool. The results of this study show that the removal of N_2O from the pool of terminal electron acceptors by the presence of acetylene during nitrate respiration causes a reduction in cell yield, pre-

sumably by reducing the availability of a terminal electron acceptor for oxidative phosphorylation. If an electron acceptor is used by a cell for oxidative phosphorylation, then the exclusion of that electron acceptor should not provide extra energy for cell growth; however, it would reduce the cell yield, provided the electron acceptor is not present in inhibitory concentrations. Although El Hassan did not demonstrate that the amount of N_2O produced during respiration with nitrate was inhibitory to the cell growth, the experimental data presented in this paper indicate that the amount of N_2O evolved from nitrate had no effect. The rates of growth (Figures 3A and B) and nitrate reduction (Figure 4B) were the same in cultures provided with nitrate or nitrate + 5% C_2H_2.

The observation that N_2O is preferentially reduced before nitrate by cells of B. japonicum (Figure 9) is contradictory to early work (7) indicating that the ionic nitrogen oxides are reduced before the gaseous nitrogen oxides, and that the gaseous nitrogen oxides do not affect the reduction of the ionic nitrogen oxides. The data (Figure 5) showing that N_2O was reduced before nitrate, thus causing a delay in the reduction of nitrate, may be explained by the K_s values for the nitrate and nitrous oxide reductases of a cowpea rhizobia studied by El Hassan (4) (270 and 41 uM, respectively). In our growth studies where both nitrate and N_2O were included as exogenous terminal electron acceptors, the amount of N_2O was approximately twice that of the nitrate (1150 and 550 µmoles, respectively). Since the K_s values indicate N_2O-reductase can

out compete nitrate-reductase for electrons from the electron transport system, the reduction of N_2O would be favored over the reduction of nitrate. Subsequently, as the N_2O was reduced during respiration, the reduction of nitrate would become more favorable. The data presented show the concurrent reduction of nitrate and N_2O following the initial reduction of N_2O (Figure 5).

The reduction of N_2O, with or without nitrate as an alternate electron acceptor (Figure 4A), was associated with an increase in cell density and number (Figures 2A and B). This further supports the conclusion that N_2O reduction during anaerobic respiration supplies sufficient energy for cell growth by B. japonicum USDA 143.

The reduction of a mole of nitrate produces a 1/2 mole of N_2O; therefore, N_2O reduction provides 20% of the energy for growth during the reduction of nitrate to nitrogen. These relative energy yields fit the oxidative phosphorylation scheme suggested by Koike and Hattori (8) who proposed that the total energy yield is equal for the reduction of nitrate to nitrite, nitrite to N_2O, and N_2O to N_2. However, not all strains of B. japonicum are able to obtain energy for growth from the reduction of nitrite (12).

The specific activity of hydrogenase for chemolithotrophically grown B. japonicum USDA 143 was reported to be 0.039 μmoles H_2/min/mg cell-protein with N_2O as the terminal electron acceptor (11). Assuming that the oxidation of one mole of hydrogen, linked with the electron transport chain for the subsequent reduction of N_2O, occurred with the

subsequent reduction of one mole of N_2O, the specific rate of respiration for chemoorganotrophically grown cells (0.082 μmoles N_2O/min/mg cell-protein) would be twice that reported by Neal et al. (11). A higher rate of growth for cells grown chemoorganotrophically may account for this greater rate of respiration.

Denitrification in rhizobia yields energy, enabling anaerobic growth and possibly enhanced survival under anaerobic conditions (12). It is postulated that rhizobial denitrification contributes significantly to the loss of nitrogen from soils (12). Therefore, when preparing inocula for soybean plants, it may be advisable not to use rhizobia capable of denitrification. On the other hand the ability of rhizobia to denitrify may contribute to their observed persistence in soils (12). Though many slow growing rhizobia have been shown to denitrify, I am not aware of any published reports that these bacteria can obtain energy for growth by reducing N_2O to N_2 (12). The results of this study shows that 20% of the energy for growth during the dissimilatory reduction of nitrate is derived from the reduction of N_2O to N_2 by B. japonicum USDA 143 cells. This may provide a competitive advantage in soil over rhizobia not capable of using N_2O as a terminal electron acceptor for oxidative phosphorylation. However, if survivability of rhizobia in soil is deemed a desired trait, then perhaps strains that most efficiently dissimilate nitrate should be selected. A strain that can use N_2O for energy production presumably would be more desirable, with respect to persistence in soil, than a strain that cannot.

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AMPEROMETRIC METHOD FOR MONITORING ANAEROBIC RESPIRATION USING NITROUS OXIDE AS THE TERMINAL ELECTRON ACCEPTOR

(ABSTRACT)

A Clark-type electrode with a platinum cathode, and the instrumentation for monitoring hydrogen uptake amperometrically were used to monitor the reduction of nitrous oxide during anaerobic respiration.

INTRODUCTION

Directly monitoring the reduction of nitrous oxide as a step in denitrification previously required the use of gas chromatography. It has recently been reported that Clark-type electrodes, with Ag/AgCl anodes and gold (1) or silver (2) cathodes, may be used to directly monitor N_2O utilization. This report presents the use of a Clark-type oxygen electrode with a platinum cathode and a Ag/AgCl anode to monitor respiration by Paracoccus denitrificans with N_2O as the terminal electron acceptor. This system may be used to monitor N_2O reduction during respiration or nitrogen fixation (15), and may be adjusted to monitor hydrogen uptake during chemolitotrophic growth of bacteria (10) or during nitrogen fixation (5).

MATERIALS AND METHODS

CULTURE PURITY AND MAINTENANCE: The purity of cultures of Paracoccus denitrificans ATCC # 17741 was determined by observation of morphology by phase contrast microscopy, Gram stain reaction, and the ability to use mannitol as a carbon/energy source.

The bacterial cultures were maintained in 500-ml polypropylene centrifuge bottles containing 250 ml of yeast extract-mannitol broth supplemented with 0.5 g KNO_3/l. The headspace of the culture vessel was flushed with oxygen-free argon for a minimum of five minutes, the media were then inoculated and sealed with a screw cap. P. denitrificans cells used for the experimentation were grown at 26°C for 5 days, harvested by centrifugation, suspended in anaerobic defined media (6, 9) and transferred anaerobically to a test tube containing oxygen-free argon and sealed with a serum stopper.

TREATMENT OF GASES: Argon was obtained from a commercial supplier and freed of oxygen by passage over hot copper pellets (7). Reagent grade nitrous oxide (98% pure), pharmaceutical oxygen, and acetylene were used as purchased. In some experiments, acetylene was also generated by adding calcium carbide to water and trapping the gas under water. All gases were sterilized by passage through cotton filters immediately before use.

CULTURE MEDIA: The defined medium described by Madigan and Gest (9) was modified as follows to contain mg/l of 0.479 mg $CoCl_2 \cdot 6H_2O$,

0.367 mg $NiCl_2 \cdot 6H_2O$ and 820 mg NH_4Cl in place of $(NH_4)_2SO_4$. The medium and 0.67 M phosphate buffer were prepared anaerobically (6) and sterilized separately by autoclaving. To avoid a precipitation in the medium, after the medium had cooled, 4.2 ml of the phosphate buffer were added with a syringe to 140 ml of defined medium contained in a 530-ml reagent bottle that had been sealed with a rubber stopper. After the addition of the buffer, the final pH of the medium was 6.8.

A Clark-type oxygen electrode (YSI # 5331) with a platinum cathode and Ag/AgCl anode was used to detect N_2O. The electrolyte solution was 1 M KOH and 0.1 M KCl (2). The polarizing potential verses the reference of Ag/AgCl was adjusted by using two 1.5-volt alkaline batteries connected in a manner so that the first battery provided a "bucking current" for the second battery (5). The bucking current produced a current equal in magnitude but opposite in sign to the residual current flowing through the probe when no N_2O was present (5). A Keithly 480 picoammeter set on 1 μA mode was used to display the results, and the results were recorded using a Fisher Recordall series 5000 set at the 0.1 volt scale. The electrode was inserted via a side port into a 1.8-ml jacketed cuvette as described by Wang (13). The cuvette temperature was controlled by circulating water from a 30°C water bath through the jacket of the cuvette. The cuvette was filled with the defined medium (pH 6.8), then sparged with O_2-free argon. The contents of the cuvette were mixed by vigorously stirring with a magnetic stirrer. The picoammeter was set to zero using its zero control knob and the bucking cur-

rent from the electrode's polarizer (5). The cuvette was sealed using a ground glass stopper with a narrow entry port to hamper gas diffusion (13). The entry port of the stopper was sealed with a glass rod. Additions were made to the cuvette through the stopper's entry port using a Hamilton gas-tight syringe.

To determine the optimum polarizing potential for N_2O detection, a volt meter was connected to the electrode's jack (negative to the anode and positive to the cathode). The polarizing potential was varied, and the corresponding μA recorded. This procedure was repeated after the cuvette was sparged with air, and again repeated after the cuvette was sparged with 98% pure N_2O.

To determine the linearity of the electrodes response to N_2O, a polarizing potential of -0.95 V was used. The medium in the cuvette was bubbled with anoxic argon, then sealed. Distilled water prepared anaerobically (6) was saturated with N_2O by bubbling with 98% pure N_2O for two hours at room temperature. Five or nine μl of this N_2O-saturated solution were injected into the cuvette. The electrode was allowed to stabilize after each injection and another 5- or 9-μl injection was made. This was repeated until the readings went off-scale on the recorder.

The cuvette was prepared as described before with argon. Fifty μl of N_2O-saturated water was injected into the cuvette. After the electrode stabilized, 50 or 100 μl of cells were injected into the cuvette. Respiration was recorded to base line and all treatments were repeated

at least three times. Using the Bunsen absorption coefficient (12) of N_2O, corrected for the barometric pressure, it was determined that 0.95 μmoles of N_2O was injected into the cuvette. The protein content of the samples were determined after alkaline hydrolysis using the method of Lowry et al. (8) with bovine serum albumin as the standard.

INHIBITION OF RESPIRATION: To verify that the electrode was monitoring dissimilatory N_2O reduction, cells were harvested by centrifugation and incubated at 4°C for three days with 15% N_2O in the headspace. Ten mg antimycin A (AA), an inhibitor of dissimilatory N_2O reduction (4), were dissolved in 30 ml of a 67% ethanolic solution. Forty-seven mg of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), a low molecular weight redox mediator that counteracts AA inhibition (4), were dissolved in 100 ml water and the solution adjusted to pH 7 immediately before use. These solutions were sparged with oxygen-free argon for 10 minutes, then sealed with a serum stopper. Nitrous oxide was injected into the cuvette from a saturated solution, and the readings were allowed to stabilize. Cells of P. denitrificans were injected into the cuvette and the respiration was monitored long enough to determine the rate. Then 100 μl of AA was injected into the cuvette, and the rate of respiration monitored. Then 100 μl of TMPD was injected into the cuvette, and respiration was monitored.

RESULTS AND DISCUSSION

The sensitivity of the electrode could be adjusted by altering the polarizing potential. The polarizing potential for optimum N_2O detection was ranged from -0.93 to -1.02 V (Figure 1). In this range, the electrode's response to argon and oxygen was constant. Although there was no plateau region for N_2O detection in this range of potentials, the potential remained constant using the apparatus described above. It was later found that sensitivity could be increased two-fold by the following procedure: the cuvette was prepared with argon and sealed. The electrode potential was adjusted to -1.40 V for 5 minutes, then reversed 5 times. The potential was reduced to -0.95 V and allowed to stabilize. The contents of the cuvette were then sparged with N_2O for 20 to 30 seconds, and then with argon. After the electrode stabilized, the recorder was set to zero. The electrode was about 20 times more sensitive to O_2 than to N_2O, but this was overcome by excluding O_2 from the cuvette. The electrode response was linear from 0.042 to at least 1.9 μmoles N_2O.

By using this apparatus, the N_2O-reducing activity of P. denitrificans was found to be 0.156 ± 0.012 μmoles N_2O min^{-1} mg cell-protein^{-1}. The rate of respiration was constant per mg of cell-protein if 50 or 100 μl of cells were injected into the cuvette. The reduction of N_2O was apparent immediately after the addition of cells and was linear until the base line was reached (Figure 2). This indicates the

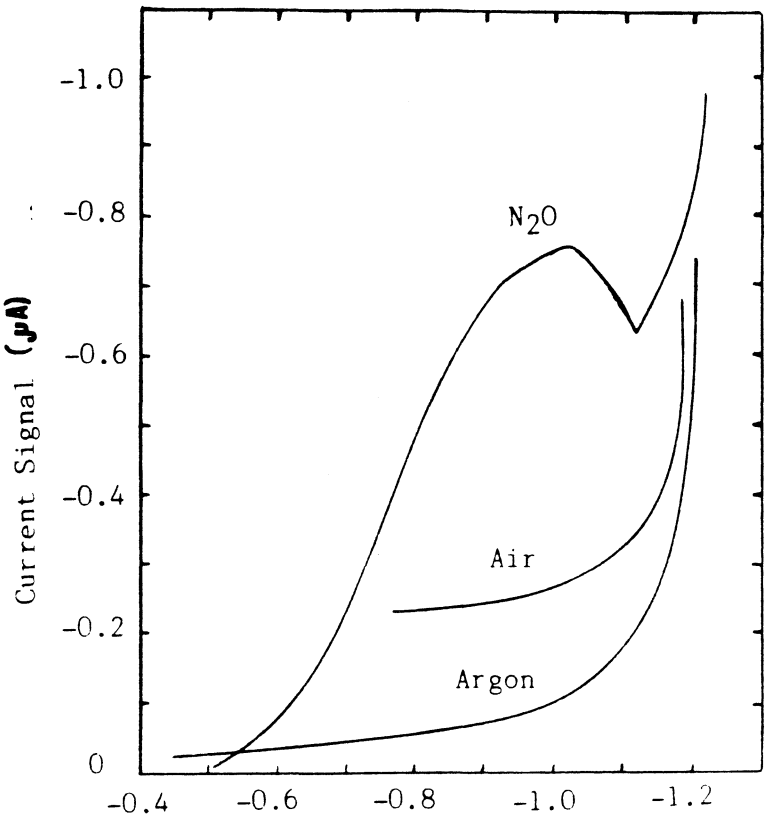

Fig. 1: The current resulting from the application of various polarizing potentials to the Clark-type electrode, in the presence of argon, air, or N_2O saturated solutions.

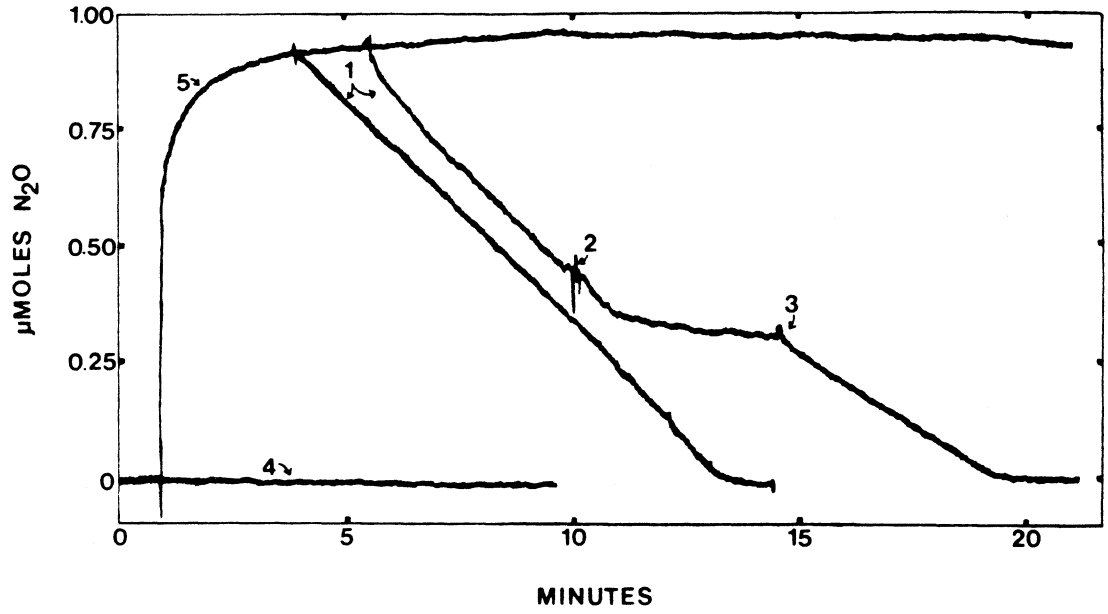

Fig. 2: Representative tracings of amperometric measurements of respiration by P. denitrificans (0.62 mg cell-protein) (1) using N_2O as the terminal electron acceptor, and the effect upon respiration due to the injection of 33 µg antimycin A (2) followed by injection of 4.47 µg of TMPD (3). Also shown is the electrode's response to anoxic defined media saturated with argon (4), and the electrode's response after injection of 0.95 µmoles of N_2O as N_2O saturated distilled water (5).

electrode response was rapid enough to monitor respiration with N_2O. Injection of N_2O without the addition of cells resulted in less than a 2% deflection after a period of 30 minutes. Injection of cells into the cuvette without N_2O caused no response. The rate of respiration was inhibited up to 88% by the addition of AA. The addition of TMPD restored respiration to its pre-AA rate, indicating the electrode was actually monitoring N_2O reduction.

Acetylene, a specific inhibitor of N_2O reductase (3, 14) could not be used to inhibit N_2O reduction because it was detected by the electrode. This held true for commercially purchased acetylene or acetylene generated with calcium carbide and water.

Based upon the Bunsen absorption coefficient, O_2 is 19 times less soluble than N_2O in water at 25°C. A volume of distilled water saturated with O_2 caused about the same % deflection as an equal volume of N_2O-saturated distilled water. Using these same saturated solutions, respiration with N_2O was about 3 times faster than respiration with O_2, on a mole/mole basis. AA will inhibit respiration with N_2O but not O_2 in P. denitrificans (11). These facts preclude the occurance of artefacts with this procedure through contamination of the system by O_2.

Zimmer et al. (15) proposed the following reaction to be occurring at the cathode of their gold cathode, Ag/AgCl anode electrode during N_2O detection:

$$2e^- + N_2O + H_2O \text{ ----> } 2OH^- + N_2$$

This was not verified experimentally. Alefounder et al. (2) suggested the lower solubility of N_2 compared to N_2O may cause gas bubbles to form between the Teflon membrane and the electrode, causing problems of gas diffusion. In use, they reported this did not cause problems. However, I noted that after prolonged use of the electrode with the same membrane, the recordings started to have a saw-toothed appearance, indicating a diffusion problem. Gas could be seen trapped beneath the membrane, and it is believed this gas was N_2; however, this was never tested. The membrane was changed and resensitized to solve the problem.

A Clark-type electrode with a platinum cathode, commonly used for monitoring hydrogen uptake, may also be used to monitor respiration with N_2O. The disadvantages of this system include a high sensitivity to O_2, which may be overcome by excluding O_2, and sensitivity to acetylene. The advantage is the system may be used to directly monitor the reduction of N_2O.

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CONCLUSIONS

B. japonicum USDA 143 obtained energy for growth using N_2O as the terminal electron acceptor. Exogenous or endogenous N_2O increased cell yield when nitrate was supplied as an exogenous terminal electron acceptor. The reduction of N_2O accounted for 20% of the energy for growth when nitrate was supplied as the sole terminal electron acceptor. It is proposed that high concentrations of N_2O, relative to those of nitrate, caused a delay in the use of nitrate as a terminal electron acceptor. Inhibition of N_2O reduction by azide and acetylene occurred, characteristic of the nitrous oxide reductases found in other denitrifying bacteria.

A Clark-type electrode with a platinum cathode, commonly used for monitoring hydrogen uptake, may also be used to monitor respiration with N_2O. The disadvantages of this system include a high sensitivity to O_2, which may be overcome by excluding O_2, and sensitivity to acetylene. The advantage is the system may be used to directly monitor the reduction of N_2O.

APPENDIX 1

PROTOCOLS

PREPARATION OF YEAST EXTRACT-MANNITOL BROTH

To 1 liter of distilled water, add the following:

Yeast Extract	0.4 g
Mannitol	10.0 g
K_2HPO_4	0.5 g
NaCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.1 g
KNO_3	0.5 g

Adjust pH to 6.8 with 1 N NaOH.

To prepare solid media, omit KNO_3 and add 15 g agar after adjusting the pH.

PREPARATION OF AUTOTROPHIC GROWTH MEDIUM MADIGAN AND GEST (1979)

* = Modifications to the original media of Madigan and Gest

	x 10 stock solution g/l of distilled water
Na_2EDTA	0.2
$FeSO_4 \cdot 7H_2O$	0.12
$MgSO_4 \cdot 7H_2O$	2.0
$CaCl_2 \cdot 2H_2O$	0.75
NaCl	10.0
Trace element solution	10.0 ml
Vitamin stock	10.0 ml
$NiCl_2$ solution *	10.0 ml
$CoCl_2$ solution *	10.0 ml

Stock Solutions

Trace element solution

	g/250 ml distilled water
H_3BO_3	0.700
$MnSO_4$	0.398
$Na_2Mo_4 \cdot 2H_2O$	0.188
$ZnSO_4 \cdot 7H_2O$	0.060
$Cu(NO_3)_2 \cdot 3H_2O$	0.010

Vitamin solution

	g/100 ml distilled water
Thiamine·HCl	0.1
Biotin	0.1

* $CoCl_2$ solution

$CoCl_2 \cdot 6H_2O$ 4.79 g/100 ml distilled water
dilute 1:1000 then add as described above

* $NiCl_2$ solution

$NiCl_2 \cdot 6H_2O$ 3.6735 g/100 ml distilled water
dilute 1:1000 then add as described above.

To prepare medium 200 ml of the x10 stock solution is added to 1800 ml of distilled, deionized water that has been passed through a carbon filter. * 1.63 g NH_4Cl and 20 g Mannitol are added.

* Reduce then sterilize the medium by autoclaving. Then allow to cool to room temperature. Add 3 ml of the anaerobic phosphate buffer (reduced on the same manner as the media). The bottles are then ready for gassing.

Phosphate buffer

	g/30 ml distilled water	/900 ml
KH_2PO_4	1.2	36
K_2HPO_4	1.8	54

Reduce and sterilize in the autoclave.

All water was distilled by reverse osmosis, then deionized and filtered on an ion exchange and carbon filter column.

PREPARATION OF ANAEROBIC MEDIA

Modification of procedure in Holdemon and Moore's Anaerobe Laboratory Manual (1975).

The rheostat for the Hungate apparatus (Hungate, 1969) is set at 90% and the column of copper pellets was allowed to warm. Argon is gently passed through the column for at least 5 minutes. While argon is still entering the column, hydrogen is added to the column to reduce the copper pellets. A condensate should be noticed at the bend of the glass tube that channels gas from the column. This condensate and the copper pellets developing a bright copper color indicate the pellets have been reduced and are able to remove O_2 from gases. Two liters of autotrophic medium is prepared as described before and placed in a 2.8-l Erlenmeyer flask. 200 ml of distilled water is added to this and the flask is sealed with a stopper that has a condenser, a stoppered hole, and a glass tube attached to it. The glass tube extends to 0.5 cm from the bottom of the 2.8-l flask and has anoxic argon flowing through it. Cold tap water is passed through the condenser's jacket at a rate that prevents the condenser from warming during the reduction of the media. The medium is heated with a Bunsen burner until it starts to reflux in the condenser. The medium must be boiled for 30 more minutes. The heat

must be removed periodically to prevent the medium from perking out of the condenser. After the medium has been boiled for 30 minutes the flask is lowered into a pan of ice to expedite cooling, while argon continues to bubble through the medium.

After the medium has cooled to room temperature, the condenser is sealed with a stopper as the 2.8-l flask is unstoppered. A disposable 1 ml pipette connected to a 60 cc syringe by tygon tubing is used to enter the flask. The syringe must be rendered free of O_2 by filling and emptying the syringe at least 5 times with the headspace of the flask containing the medium. The pipette is lowered into the medium and the desired volume is drawn into the syringe. The pipette is lifted out of the medium, but kept in the flask so 0.5 ml of argon may be drawn into the pipette. The pipette is removed and the tip is flamed to remove any liquid that may absorb O_2 and to expand the argon to expel O_2. The medium is then transferred to a 530-ml reagent bottle that has been flushed with anoxic argon from a gas cannula for at least 5 minutes. The flow rate from the cannula should be enough to cause at least a 1-cm depression in a flame when the gas is directed at the flame. Before removing the pipette from the bottle, 0.5 ml argon is drawn into the pipette. The tip of the pipette is heated before it is returned to the 2.8-l flask. When the desired volume of medium has been transferred to the bottle, a rubber stopper is quickly heated over a flame and poised over the mouth of the reagent bottle. The cannula is drawn up to 1 cm from the mouth of the reagent bottle and held there for 5 or 10 seconds.

Then as the cannula is removed, the stopper is inserted and sealed with a twist. The medium is then sterilized by placing the sealed bottles in a press and autoclaving (fast exhaust may be used). When the medium has cooled to room temperature, buffer and other supplements are added with a syringe and needle.

Anoxic buffer, water, or KNO_3 solutions may be prepared by reducing distilled water in the same manner described for the media. Dry reagents are weighed and transferred to the reagent bottles, then flushed with anoxic argon for 10 minutes. If large amounts of dry reagents are added, the reagent bottle is shaken once or twice while flushing with argon. The desired volume of distilled, anoxic water is then transferred to the bottle. The bottles are then sealed, clamped, and autoclaved.

GASSING OF CULTURE FLASK

To draw a vacuum, two side arm flasks are connected together in a series, and then to a faucet aspirator with tygon tubing. The flask closest to the sink contains water to monitor gas flow. Both flasks are wrapped with electrical tape for safety in case of implosion. The side arm of the flask without water is attached to tygon tubing that has a male Leur-lock fitting at the end. A vacuum is drawn and a sterile 18 G 1-inch needle is locked on the Leur-lock. The needle is inserted into the reagent bottle's stopper, previously sterilized with alcohol. While it is being exhausted, the bottle is placed on its side in a plastic cup for safety in case of implosion. The bottle is exhausted for 1 minute

or until gas bubbles are observed in the medium. This produces about a 90% vacuum relative to the headspace of the flask. To terminate the exhaustion process aseptically, the needle is removed from the stopper. The bottle maintains a vacuum well for at least 5 hours. For safety, the bottles are handled as little as possible.

To add the correct volume of the desired gas, a side arm of a flask is sealed with a sterile gas filter sealed with a serum bottle stopper. This flask is fitted with a rubber stopper with two openings. The desired gas is allowed to flow into this flask for 5 minutes while it is unstoppered. Then the flask is sealed with the stopper while continuing to flush the flask with the desired gas entering and exiting through the holes in the stopper. The exhaust is bubbled through water to monitor the flow of the gas. With an appropriate sized syringe, the gas filter is flushed 5 times by inserting a needle into the serum stopper and drawing gas through the filter. The needle is then removed to expel the gas. The syringe is used to withdraw the appropriate volume of gas, which is injected into the vacuumed reagent bottle. The barometric pressure and temperature should be recorded at this time for future calculations.

To restore atmospheric pressure in the flask, anoxic argon is then fed into a split gas-line. One line releases gas under water, while the other line is fitted with a 3 ml syringe barrel (i.e. Leur-lock) filled with sterile cotton (i.e. gas filter). A 22 G, 1-inch needle is attached to the gas filter and inserted into the stopper of the vacuumed

reagent bottle. Gas flow must be maintained at a sufficient rate to prevent water being drawn into the gas-line that is under water, but gas flow cannot be so great as to put more than the atmospheric pressure of gas into the flask.

LOWRY PROTEIN DETERMINATION

The following reagents are to be prepared in advance. If these are sterilized, they may be stored indefinitely.

Reagent A: 10 g of Na_2CO_3 dissolved in 100 ml of 0.5 N NaOH. (i.e. 2 g NaOH in 100 ml. deionized water)

Reagent B: 1 g of $CuSO_4 \cdot 5H_2O$ dissolved in 100 ml of deionized water.

Reagent C: 2 g K,Na tartrate dissolved in 100 ml of deionized water.

Standard protein solution: This must be prepared the day of use. 30 mg of bovine serum albumin (BSA, Sigma) is dissolved in 100 ml of deionized water (final volume). To 9 ml of this solution, add 3 ml of 4 N NaOH.

Procedure:

Prepare triplicates of the following standards, except for the 0 BSA, which is the blank. Number these 1-28, then treat as described below.

Tube #	ml BSA	ml d'H_2O	[Protein] ug/ml
1	0	1	0
2-4	0.1	0.9	30
5-7	0.2	0.8	60
8-10	0.3	0.7	90
11-13	0.4	0.6	120
14-16	0.5	0.5	150
17-19	0.6	0.4	180
20-22	0.7	0.3	210
23-25	0.8	0.2	240
26-28	1.0	0	300

Cells are centrifuged at 10,000 rpms (RCF = 5000 x g) for 20 minutes in the large rotor and suspended to a known volume of deionized wa-

ter. Three ml of the cell suspension is mixed with 1 ml of 4.0 N NaOH and is incubated in an 80°C water bath for 30 minutes, with glass marbles on the mouth of the test tubes. Cool to room temperature and suspend and precipitates.

Prepare in triplicate as described below.

Tube #	ml cell digest	ml d'H_2O
29-31	0.05	0.95
32-34	0.1	0.9
35-37	0.2	0.8
38-40	0.3	0.7

Treat standards and samples as described below:

Mix throughly 50 ml of reagent A with 2.5 ml of reagent B and 2.5 ml of reagent C in an Erlenmeyer flask. Add 1.0 ml of this to each of the tubes and mix. Let stand for 15 minutes at room temperature.

Fifteen ml of 2 N Folin-phenol solution is mixed with 150 ml of deionized water. After the 15 minute wait, 3.0 ml of this is added to each tube and the contents of the tube are <u>mixed immediately</u> after the addition.

The samples are allowed to incubate for 45 minutes at room temperature. Then the absorbance is read at 660 nm on the spectronic 88 (Bausch and Lomb). The color is stable for 45 to 60 minutes after the incubation period is over. This reaction is time dependent, so the intervals between the addition of Folin-phenol to the samples and stan-

dards should correspond to the intervals between the reading of the samples.

Salicylic Acid Method of Nitrate Determination

From the procedure of Cataldo et. al. (1975)

Reagents:
1) Sa-H_2SO_4: 5% (w/v) salicylic acid in conc. sulfuric acid.
2) 2 N sodium hydroxide.

Standards:

Standards contain 1-60 ug NO_3^--N
(0, 10, 20, 30, 40, 50, and 60 ug NO_3^--N/20 ml H_2O)

Procedure:

1) Add 0.2 ml aliquots to 50-ml Erlenmyer flasks.
2) Mix throughly with 0.8 ml Sa-H_2SO_4.
3) Let stand for 20 minutes at room temperature.
4) Add 19 ml of NaOH slowly to raise pH above 12.
5) Cool samples to room temperature.
6) Measure the absorbance at 410 nm.
7) Color development is rapid and stable for at least 48 hours in the light or dark.

APPENDIX 2

RESPIRATION OF BACTEROIDS WITH NITROUS OXIDE

Soybean seeds were sterilized, then germinated on folded paper towels in plastic bags (Weaver and Frederick, 1982). When roots were about one inch long, 30 ml of the nutrient solution described by Weaver and Frederick (1982) were added to every plastic bag, then the seeds were inoculated with Bradyrhizobium japonicum USDA 143 in distilled water. The plants were grown in a greenhouse for 25 days with a photoperiod of 16 hours. The nodules were removed and the bacteroids were liberated aerobically using the procedure outlined by Farnden and Robertson (1980). Bacteroids were suspended in anaerobic (Holdemon and Moore, 1975) autotrophic medium (Madagan and Gest, 1979) with 10 mM nitrate and 10 g mannitol/l, and stored in 10 ml Vacutainer tubes. Five percent acetylene was placed in the headspace of the culture to ascertain if nitrogen fixation was occuring by testing for reduction of acetylene to ethylene (e.g. Turner and Gibson, 1980). These cells were stored at 4°C for 60 hours. The cells were then washed in autotrophic medium to remove nitrate, then harvested by centrifugation. Respiration was monitored using a Clark-type electrode (Section II).

Bacteroids were also injected into 10 ml Vacutainer tubes containing 3 ml of anaerobic autotrophic medium with 10 g mannitol/l. Electron acceptors were 15% N_2O or 7 mM nitrate. Controls included uninoculated medium to correct for the loss of N_2O due to sampling, and an inoculated culture with nitrate and 5% acetylene. The cultures were incubated at

room temperature for 24 hours. Gas samples were taken at the time of inoculation and 24 hours after inoculation, then stored in 2-ml Vacutainer tubes with 1 ml of 1 M KOH. These gas samples were tested for N_2O content using the parameters described in the text; the carrier gas was reduced to 30 ml/min. The cell concentration was 1 mg cell protein per 3 ml of culture as determined by the method of Lowry et al. (1951) using BSA as the standard.

RESULTS and DISCUSSION

After initial isolation, bacteroids were shown to be viable by monitoring aerobic respiration with the Clark-type electrode. However, no N_2O reduction was noted, indicating neither the nitrous oxide reductase nor the nitrogenase-complex were active (Payne, 1981; Turner and Gibson, 1980; respectively). Under phase contrast microscopy, the isolated bacteroid suspension was dense with bacteroids, contained no motile cells, and contained few eucaryotic cells or starch granules. After incubation with nitrate, cells still respired with O_2, and apparently respired with N_2O. However, the respiration with N_2O only stopped at the base line once. Respiration continued at least three times beyond the base line, relative to the initial deflection due to the addition of N_2O. With O_2 as the terminal electron acceptor, respiration consistently terminated at the base line. The addition of AA did inhibit the apparent respiration with N_2O, but the addition of TMPD did not restore the rate of respiration to its pre-AA rate. Even after inhibition with AA, apparent respiration continued to be monitored beyond

base line. Respiration with N_2O by B. japonicum USDA 143 previously exposed to nitrate prceeded at a linear rate to a base line that corresponded to 0 μmoles N_2O, indicating the electrode was working properly. There is no apparent explanation for the results observed with bacteroid respiration monitored with the electrode.

The attempt to monitor respiration with N_2O in the 10 ml Vacutainer tubes was also unsuccessful because the reduction of N_2O was not distinguishable from the loss of N_2O due to sampling.

From this experiment, it cannot be concluded that bacteroids respire with N_2O. It was believed the bacteroids affected the electrode in a manner that was not determined.

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APPENDIX 3

CARBON MONOXIDE OXIDATION EXPERIMENTS

Cultures of Rhizobium japonicum USDA 6 were stored on yeast extract-mannitol agar slants. Gunatilaka originally reported that this strain could oxidize CO as a carbon and energy source (Gunatilaka, Malkanthi K. 1983. Chemolithotrophic Nitrate Dependent Growth of Rhizobium japonicum on Carbon Monoxide and Its Relationship to Hydrogenase Activity. Master of Science Thesis. Virginia Polytechnic Institute and State University; Blacksburg, Virginia). I had transferred these cultures back to anaerobic, chemolithotrophic conditions with 20% carbon monoxide as the sole carbon and energy source. Initially I had apparent growth; however, the cultures could not be transferred beyond the third subculture. I also found that these cultures did not reduce the nitrate in the medium, which is contradictory to Gunatilaks's work. Because the conditioned state of the cultures that Gunatilaka used may have been lost, I attempted to condition the culture again. I first grew the cultures on 10% H_2 and 5% CO_2 with nitrate in the medium to verify that the cultures were capable of denitrification. These denitrifying, chemolithotrophic cultures were then cultured under various concentrations of CO (10, 20, and 30%) with nitrate in the media. Experiments with CO_2 sparking and/or microaerophilic conditions were not encouraging. Since R. japonicum produces copious poly-β-hydroxybutyrate, demonstration of nitrate reduction was of limited value on the first subculture. Again, I had many false starts based on increased turbidity, only

to have the culture fail to grow after the third subculture. At this point I started the selection process on cultures of R. japonicum USDA 6 from various sources. Based upon the work on other carbon monoxide-oxidizing bacteria, cultures were repeatedly regassed with 20% CO. Early results were encouraging, because nitrate and nitrite was reduced by the first subculture of the cultures; however, the second subculture did not grow. Selection was attempted by growing cultures of R. japonicum under 10% H_2, 5% CO_2, and various concentrations (5, 10, and 20%) of CO, but was not successful. Anaerobic continuous culture was attempted for 45 days with 5% CO_2, 10% CO and 85% Ar flowing over the stirred culture at 110 ml/min. While nitrite was detected on day seventeen of the culture, it was never detected again. Nitrate was always present in the culture and waste flask. This attempt to isolate the CO-oxidizer was also unsuccessful, and the experiment was abandoned.

Procedure

A previously published method for detecting CO in solution using the difference in spectral properties of reduced hemoglobin and carboxy-hemoglobin was practiced with success.

Alcaligenes eutrophus

Since preliminary work in this lab had indicated that Alcaligenes eutrophus was capable of utilizing CO as the sole carbon and energy source, I cultivated A. eutrophus ATCC 17699 under chemolithotrophic conditions with nitrate in the medium and under 20% CO. These cultures grew even after the sixth subculture.

The A. eutrophus cultures grown on CO still contained nitrite even though anaerobic, chemolithotrophic cultures under 10% H_2 and 5% CO_2 reduced nitrate beyond nitrite. Since nitrite interferes with the hemoglobin assay for CO, it was important to obtain cultures of A. eutrophus that did not contain nitrite. Regassing the cultures to increase growth yields and cause further reduction of the nitrite were not successful. A. eutrophus cultures were cultured under 10% H_2 and 5% CO_2 with 20% nitrous oxide as the terminal electron acceptor. Growth was inhibited by acetylene, indicating nitrous oxide reductase was present. I did not obtain a culture grown on CO that could use nitrous oxide as the terminal electron acceptor. This was not surprising since the nitrous oxide reductase of Paracoccus denitrificans is known to be inhibited by CO.

APPENDIX 4

Data

Table 1A: Data for figure 1A, $A_{540} \times 10^3$).

day	% N_2O					
	0	10	20	30	40	60
0	31 ± 1	31 ± 1	32 ± 0	28 ± 2	30 ± 0	30 ± 0
3	41 ± 1	148 ± 7	136 ± 20	111 ± 3	101 ± 1	87 ± 3
7	45 ± 4	335 ± 15	213 ± 5	183 ± 10	162 ± 2	134 ± 9
10	45 ± 2	294 ± 18	255 ± 6	213 ± 11	187 ± 2	155 ± 11
13	44 ± 3	249 ± 2	280 ± 6	237 ± 15	211 ± 1	168 ± 15

Table 1B: Data for figure 1B, $A_{540} \times 10^3$.

day	% N_2O			
	0	5	10	15
0	38 ± 2	38 ± 0	37 ± 1	39 ± 1
3	46 ± 2	99 ± 1	100 ± 1	100 ± 1
5	36 ± 1	160 ± 4	188 ± 3	172 ± 0
7	35 ± 3	163 ± 2	271 ± 2	227 ± 4
10	32 ± 5	152 ± 0	280 ± 1	297 ± 8
12	33 ± 5	149 ± 1	269 ± 1	333 ± 8
14	29 ± 4	139 ± 1	250 ± 1	340 ± 14
17	30 ± 5	139 ± 1	241 ± 1	337 ± 12

Table 2B: Data for figure 2B, $A_{540} \times 10^3$.

day	Treatment				
	N_2O	Argon	N_2O -Mannitol	N_2O C_2H_2	C_2H_2
0	31 ± 1	26 ± 3	32 ± 1	31 ± 1	26 ± 3
1	57 ± 1				
2	93 ± 1	33 ± 2	37 ± 1	33 ± 4	28 ± 4
4	248 ± 12	30 ± 3	29 ± 1	30 ± 2	25 ± 6
6	334 ± 11	24 ± 1	25 ± 0	27 ± 2	22 ± 10
8	318 ± 11	26 ± 1	28 ± 0	28 ± 1	28 ± 3
10	299 ± 12	34 ± 7	22 ± 1	23 ± 1	20 ± 6

Table 2B: Data for figure 2B, CFU x 10^5.

day	Treatment				
	N_2O	Argon	N_2O -Mannitol	N_2O C_2H_2	C_2H_2
0	271 ± 11	267 ± 6	264 ± 5	276 ± 8	258 ± 12
1	653 ± 38				
2	1080 ± 70				
4	2150 ± 320				
6	1850 ± 680				
8	2160 ± 220				
10	1660 ± 210	192 ± 9	309 ± 27	224 ± 20	192 ± 18

Table 3A: Data for figure 3A, A_{540} x 10^3.

day	Treatment			
	Nitrate	Nitrate N_2O	Nitrate C_2H_2	Argon
0	32 ± 0	33 ± 1	32 ± 1	26 ± 3
1	51 ± 1	49 ± 1	49 ± 1	
2				33 ± 2
3	172 ± 24	186 ± 16	138 ± 9	
4				30 ± 3
5	330 ± 6	467 ± 95	263 ± 8	
6				24 ± 1
7	300 ± 11	597 ± 65	242 ± 19	
8				26 ± 1
9		637 ± 10		
10				34 ± 7
14		569 ± 15		

Table 3B: Data for figure 3B, CFU x 10^5.

day	Treatment			
	Nitrate	Nitrate N_2O	Nitrate C_2H_2	Argon
0	247 ± 20	274 ± 8	280 ± 17	267 ± 6
1	416 ± 16	451 ± 29	513 ± 17	
3	1870 ± 270	1290 ± 290	1140 ± 208	
5	3080 ± 360	3520 ± 990	2330 ± 162	
7	1720 ± 139	4020 ± 405	2160 ± 170	
9		4260 ± 266		
10				192 ± 9
14		1850 ± 300		

Table 4A: Data for figure 4A, μmoles N_2O/ml of culture headspace.

day	Treatment	
	N_2O	N_2O Nitate
0	1.52 ± 0.04	1.54 ± 0.01
2	1.55 ± 0.01	1.46 ± 0.06
4	0.82 ± 0.02	1.17 ± 0.14
7	0.33 ± 0.06	0.06 ± 0.14
14	0.08 ± 0.07	-0.05 ± 0.06

Table 4B: Data for figure 4B, μmoles nitrate/ml of culture media.

day	Treatment		
	Nitrate	Nitrate C_2H_2	Nitrate N_2O
0	3.79 ± 0.05	3.96 ± 0.10	3.95 ± 0.03
3	2.26 ± 0.28	4.05 ± 0.10	2.10 ± 0.10
5	0 ± 0	2.49 ± 0.85	0 ± 0
7		0.79 ± 0.75	
9		0 ± 0	

Table 5: Data for figure 5, μmole of electron acceptor/culture in cultures supplied with nitrate and N_2O as terminal electron acceptors.

day	Electron Acceptor	
	Nitrate	N_2O
0	550 ± 4	1175 ± 24
1	545 ± 5	
2		1018 ± 47
3	561 ± 14	
4		337 ± 82
5	345 ± 120	
6		0 ± 0
7	109 ± 105	
9	0 ± 0	

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