CHEMOLITHOTROPHIC NITRATE DEPENDENT GROWTH OF *Rhizobium japonicum* ON CARBON MONOXIDE AND ITS RELATIONSHIP TO HYDROGENASE ACTIVITY

by

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(ABSTRACT)

Anaerobic chemolithotrophic growth of *Rhizobium japonicum* occurred in the presence of carbon monoxide with nitrate serving as the electron acceptor. Under conditions where the atmospheric concentrations of CO were varied, the cells grew in up to 50% (v/v) CO. Optimum growth was observed in the presence of 20-30% CO. Under these conditions absorbance (660 nm) reached a maximum of 0.33 after 15 days of growth, after which no further increase was noted. Colony forming units increased from 1 X 10^6 cells per ml to a maximum of 2.0 X 10^8 cells per ml of culture medium. Growth in the absence of CO was substantially less indicating that CO was required for growth. The rate of disappearance of NO_3^--nitrogen from the culture medium during growth was correlated with the growth rate.

Hydrogen uptake was measured amperometrically with cells

grown in the presence of CO with nitrate, nitrite, nitrous oxide or oxygen serving as the electron acceptor. Addition of acetylene in the presence of N_2O resulted in a 92.5% inhibition of N_2O-dependent H_2 uptake.

Demonstration of H_2 uptake activity with NO_3^-, NO_2^- or N_2O as the only electron acceptor substantiated the presence of dissimilatory nitrate, nitrite, and nitrous oxide reductase(s). Hydrogenase activity with O_2 as the electron acceptor in CO grown cultures showed the presence of cytochrome components necessary for transferring electrons from H_2 to O_2. Increasing the H_2 concentration above 0.5% (v/v) resulted in repression of growth in CO grown cultures. CO:oxidoreductase, the enzyme responsible for oxidation of CO was demonstrated in anaerobic, CO grown cultures.

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I thank my major professor, Dr. John L. Neal, without whose continued guidance, patience and encouragement this project would not have been possible. He made it an invaluable and an enjoyable learning experience. I thank the members of my committee, Drs. R. E. Benoit, J-S. Chen and N. R. Kreig, for sharing their expertise during the course of this work.

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My sincere appreciation is extended to my husband Dr. Leslie Gunatilaka and my little daughter, Lucky for their continued patience, support and reassurance during many hours of both frustration and triumph. May our long life together provide ample opportunity for repayment.

My parents, Mr. U.B. Ekanayake and late Ms. B.M. Ekanayake are gratefully acknowledged for their many sacrifices, immense support, and unmeasurable love. I lovingly dedicate this thesis to my father and my late mother.

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INTRODUCTION

Carbon monoxide is considered to be one of the most prevalent atmospheric pollutants. However, the turnover of CO in the lower atmosphere is quite rapid and soil is known to be an excellent biological sink for CO due to the presence of different aerobic and anaerobic bacteria which metabolize CO (Colby et al., 1979: Hegeman, 1980). Microbial CO oxidation can occur by several biochemical mechanisms and may be either an adventitious reaction or a reaction yielding a carbon source and energy for cell growth. Utilitarian CO oxidation has been reported in both aerobic (Meyer and Schlegel, 1979) and anaerobic (Uffen, 1976; Uffen, 1983) bacterial species.

Utilitarian CO oxidixers represent a unique group of chemolithotrophs since they can use the electrons derived from the oxidation of CO for growth, assimilate carbon dioxide produced for cellular synthesis, and withstand CO inhibition (Meyer and Schlegel, 1983).

Selected strains of Rhizobium japonicum have been shown to be capable of chemolithotrophic growth in a mineral salts medium using H_2 as the source of energy and CO_2 as the carbon source (Hanus et al., 1979; Lepo et al., 1980). Cow pea rhizobia and some strains of R. japonicum can carry out dissimilation of NO_3^- and NO_2^- to N_2O and nitrogen under chemoorganotrophic

conditions (Zablotowicz et al., 1978; Daniel et al., 1980). More recently, chemolithotrophic, anaerobic dissimilation of NO_3^- by R. japonicum has been demonstrated (Neal et al., 1983a). Subsequent studies (Neal et al., 1983b) have shown that NO_3^-, NO_2^-, and N_2O, as well as O_2, could serve as electron acceptors during H_2 uptake by cell suspensions (Neal et al., 1983b).

Factors that regulate the synthesis of the H_2 uptake system in free living R. japonicum include H_2, low levels of carbon substrates and O_2 (Maier et al., 1978; Simpson et al., 1979). The expression of H_2 oxidizing activity in free living R. japonicum is dependent on maintaining low levels of O_2 in the medium (Maier et al., 1978) for oxygen rapidly represses the synthesis of the H_2 uptake system. Recently, it has been shown that molecular H_2 represses the growth of R. japonicum USDA strain 110 (Lim and Uratsu, 1983).

Preliminary experiments in our laboratory showed that under anaerobic chemolithotrophic growth conditions, in the presence of H_2 and CO_2 and with NO_3^- serving as the electron acceptor, low concentrations of CO were not detrimental to growth of selected strains of R. japonicum. Further experimentation indicated that perhaps CO could serve as a carbon and energy source during chemolithotrophic growth by R. japonicum (Appendix, Fig. 1).

The purpose of this thesis is to report the anaerobic, chemolithotrophic growth of R. japonicum with CO serving as the apparent carbon and energy source with NO_3^- serving as the electron acceptor. We have determined the H_2 uptake activity with different electron acceptors, namely, NO_3^-, NO_2^-, N_2O and O_2. Carbon monoxide:acceptor oxidoreductase, the enzyme responsible for CO oxidation in several aerobic and anaerobic bacteria, has been demonstrated.

MATERIALS AND METHODS

Bacterial strain, culture maintenance and purity. Based upon preliminary experiments with several strains of rhizobia that indicated CO was not toxic to growth under anaerobic conditions, USDA strain 6 was selected for further study. The bacterial strain was inoculated and maintained by periodic transfer in 150 ml of anaerobic (Holdman and Moore, 1972) autotrophic medium (Madigan and Gest, 1979) containing 1 g KNO_3/l in an atmosphere of 20% CO and 80% Ar. The cultures contained in 500 ml serum stoppered reagent bottles were incubated at 26°C on their sides to maximize the exposure of the medium to the gases in the head space. Highest purity CO available commercially was purchased from Industrial Gas Supply, Radford, VA. Prior to use, the gases were cleared of trace amounts of O_2 and CO_2 by passing through a train of flasks containing alkaline pyragallol and 20% potassium hydroxide. This procedure was followed for all subsequent investigations.

Considerable care was taken during all experiments to ensure the absence of culture contaminants. Samples from all liquid cultures were streaked onto yeast-mannitol agar to assay for growth of contaminants. Microscopic examination of suspensions of cultures, Gram stain reaction, growth in yeast mannitol broth and scanty or lack of growth in nutrient and tryptone broth were used

to establish the purity of cultures throughout the investigation. Periodically, subsamples from the culture were inoculated onto soy bean plants grown in nitrogen-free plant nutrient media contained in sterile cellophane bags to confirm retention of nodulating ability and symbiotic efficiency (Maier et al., 1978).

Growth experiments. Cultures of USDA 6 were incubated in anaerobic autotrophic media to an absorbance of 0.2 to 0.3 measured at 660 nm, using a Bausch and Lomb (Spectronic 88) spectrophotometer. The cells were harvested by centrifugation under Ar and resuspended in 50 ml of potassium phosphate-$MgCl_2$ buffer (pH 7.0) sparged with O_2-free Ar. This procedure was repeated three times. Following final centrifugation, the cells were resuspended in 200 ml of anaerobic autotrophic medium minus KNO_3 in 500 ml serum stoppered reagent bottles. The reagent bottle atmosphere was adjusted to contain 20% CO and 80% Ar. The culture flasks were incubated for an additional 48 h at 26°C. To determine the amount of CO required for optimum growth, 5.0 ml suspensions of the above culture were inoculated into serum stoppered reagent bottles containing autotrophic media plus 1g KNO_3/1 which approximated to 1 X 10^6 cells per ml. The reagent bottle atmosphere was adjusted to contain 0, 10, 20, 30, 40, and 50% CO with remaining gas volume occupied by O_2-free Ar. The cultures were incubated for a total of 18 days at 26°C. Growth was monitored by measuring change in absorbance at 660 nm and increase

in viable cell numbers (colony forming units). The number of colony forming units were determined by plating appropriate serial dilutions on yeast extract mannitol agar and incubating the plates for 7 days at 26°C. The effect of varying amounts of H_2 in the culture head space was determined in a similar manner, except that the concentration of CO or CO_2 was maintained at 20% and the concentration of H_2 was varied from 0 to 10%. Growth was monitored by following increase in absorbance at 660 nm. Appropriate controls consisted of medium with and without inclusion of ammonium and without NO_3^- as the electron acceptor. Endogenous growth was determined with NO_3^- and NH_4^+ included in the medium, but without the addition of H_2, CO_2 and/or CO. The culture head space pressure was brought to one atmosphere by addition of O_2-free Ar. All experiments were replicated four times.

Analytical procedures. The amount of protein in the cell suspension, after prior hydrolysis with sodium hydroxide, was determined colorimetrically (Lowry et al., 1951) with bovine serum albumin (Sigma Chemicals) as the standard.

Sub samples of the liquid culture removed for absorbance measurements were frozen for colorimetric analysis of NO_3^- (Cataldo et al., 1975), NO_2^- (Hanson et al., 1981) and NH_4^+ ions (Hanson et al., 1981).

Enzyme assays. Carbon monoxide:acceptor oxidoreductase

activity was determined by a whole cell assay procedure using methyl viologen as an artificial electron acceptor (Uffen, 1983; Wakim and Uffen, 1983; Uffen, 1976). Cells grown in anaerobic, autotrophic medium with 20% CO for 16 days were harvested by centrifugation under Ar gas in screw capped centrifuge bottles. The cells were resuspended in 10 ml of anaerobic 100 mM N-tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid buffer (pH 8.0) containing 0.15 mM dithiothreitol. This whole cell suspension was frozen until analyses. Prior to enzyme analysis, the cell suspension was gently thawed, and sparged with O_2-free N_2. The reaction mixture (3 ml) containing 2.0 mM MV and 0.15 mM dithiothreitol in 0.1 M 2(N-cyclohexylamino)-ethanesulfonic acid buffer (pH 9.0) was transferred to serum stoppered reaction cuvettes that had been evacuated and gassed out with O_2-free N_2 five successive times. The reaction mixture was sparged with O_2-free CO for 5 minutes. The cuvettes were shaken vigorously and reduced with dithionite (2-6 µl of a 10 mM solution) until a light blue color developed. The reaction mixture was equilibrated at 25°C for ten min. The reaction was started by adding the whole cell preparation (100 µl). The contents in the cuvette were mixed and the CO:MV oxidoreductase activity was measured spectrophotometrically at a wavelength of 578 nm using a Bausch and Lomb (Spectronic 88) spectrophotometer. In all assay systems the observed rates were linear for at least 20 sec. and

proportional to the amount of cell suspension added. Carbon monoxide oxidation activity was calculated from the extinction coefficient of MV ($E_{578\ nm}$ = 9.7 mM^{-1} cm^{-1}), according to the equation in which two moles of MV are reduced per mole of CO oxidized (Wakim and Uffen, 1983). One unit of enzyme activity was defined as the amount of enzyme catalyzing the transformation of 1 μ mole of CO per min. Specific activities are expressed as I.U./mg protein. All chemicals used were obtained from Sigma Chemical Company.

Ribulose bisphosphate carboxylase activity was estimated by a whole cell assay as described by Lepo et al., (1980), and Tabita et al., (1978). Cultures grown in anaerobic autotrophic medium with 20% CO for 16 days were harvested by centrifugation, washed and resuspended in 50 mM Tris chloride buffer (pH 8.0). A portion of this suspension was autoclaved for 15 min to be used as the dead cell control.

To each volume of cell suspension, one half volume of toluene was added, gently mixed for 3 min and allowed to stand in ice for 10 min. The toluene layer and the bubbles were carefully removed with a pasteur pipette. The toluene treated cells (50 μl) were pre-incubated for 5 min at 30°C in a mixture of 100 μl of 160 mM Tris hydrochloride buffer (pH 8.0) with 50 mM magnesium chloride and 50 μl of 100 mM sodium bicarbonate. One ml of the $NaHCO_3$ contained 20 μ moles of $NaH^{14}CO_3$ (0.1 μCi/μmole) (ICN

Pharmaceuticals Inc.). The reaction was initiated by adding 50 µl of a 1.6 mM tetrasodium D-ribulose-1,5-diphosphoric acid (RUBP) (Sigma Chemical Co.) in 2-(N-morpholino) ethane sulfonic acid (MES) buffer (pH 8.0). After 0, 10 and 15 min. the reaction was stopped by adding 4N hydrochloric acid. All assays were carried out in serum stoppered scintillation vials. The unreacted $^{14}CO_2$ was allowed to dissipate overnight. The reaction mixture was evaporated to dryness at 80°C, resuspended in 100 µl of water and then added to 2 ml of Scintiverse scintillation fluid. Radioactivity of the acid stable products was measured using Beckmann LS-3150T liquid scintillation counter. One unit of enzyme activity catalyses the RUBP dependent fixation of 1 µmole of CO_2 per min. and specific activity is expressed in units per mg protein.

Hydrogen uptake rates were measured amperometrically (Wang et al., 1980; McCrae et al., 1978) using a Clark-type electrode having a 1.5 ml cuvette. The cuvette was sparged with O_2-free N_2 to maintain anaerobic conditions and a 1.5 ml aliquot of an anaerobic cell suspension was added. Hydrogen was added as 100 µl of H_2 saturated anaerobic, autotrophic medium. The electrode was allowed to equilibrate several minutes before H_2 uptake was initiated by the addition of 100 µl of either O_2, NO_3^-, NO_2^- or N_2O containing medium. Nitrous oxide was added as a saturated medium. Acetylene has been shown to specifically inhibit the reduction of N_2O to N_2 (Yoshinari et al., 1976;

Balderston et al., 1976). The effect of C_2H_2 on the rate of H_2 uptake with O_2 and N_2O was measured by addition of 100 µl of a C_2H_2 saturated medium prior to the addition of each electron acceptor. The specific activity of the uptake hydrogenase for each electron acceptor was expressed as µmoles of H_2 utilized per min. per mg of protein.

RESULTS

Anaerobic chemolithotrophic growth with CO. The effect of varying concentrations of CO upon anaerobic growth of R. japonicum is shown in Fig. 1A and Fig. 1B. Under the experimental conditions employed, the amount of growth was related to the concentration of CO in the culture atmosphere. Optimum growth, as judged by the total increase in absorbance, was obtained when the percentage of CO in the headspace was adjusted to 20 and 30%, respectively (Fig. 1A). Correspondingly, colony forming units increased from about 1 X 10^6 cells per ml to 2 X 10^8 cells per ml with the concentration of CO adjusted to 20% in the culture atmosphere (Fig. 1B). Increasing the concentration of CO in the culture atmosphere to 30% resulted in a slight, but significant reduction in the maximum number of colony forming units (1 X 10^8 per ml). Adjustment of the amount of CO in the culture atmosphere to below 20% and above 30% decreased the total amount of cell growth during the incubation period of 18 days. Growth in the absence of CO, but in the presence of NO_3^- as an external electron acceptor, did occur, but was significantly less than in the presence of CO. Growth in the absence of CO was attributed to endogenous respiration. Periodic microscopic examination of the cells in the cultures used as inoculum for these experiments showed an extensive accumulation polyhydroxybutyrate

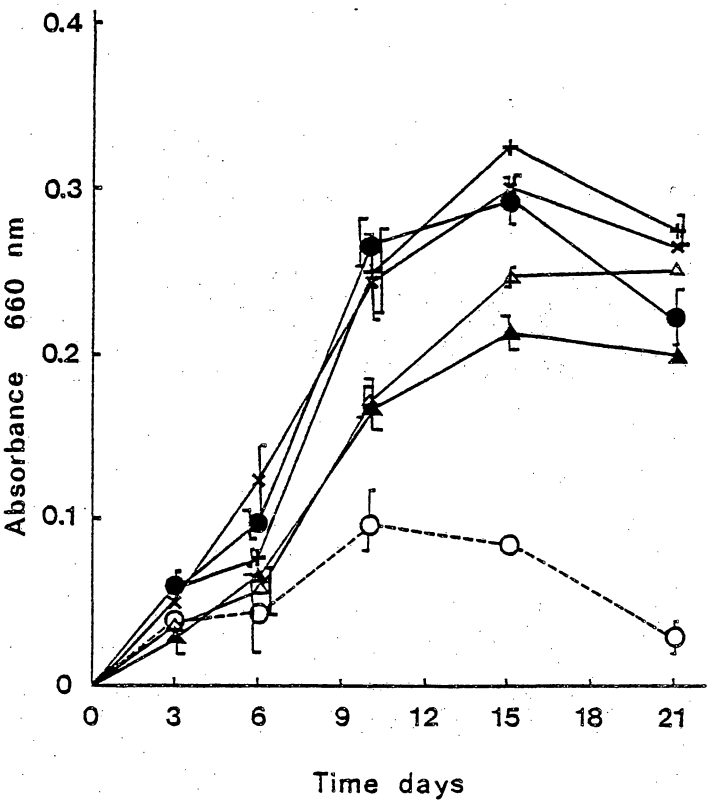

Fig. 1. (A) Change in absorbance (660 nm) of cultures of R. japonicum, strain USDA 6 during anaerobic, chemolithotrophic growth with different concentrations of CO in the head space, (O), 0% CO; (●), 10% CO; (**X**), 20% CO; (**+**), 30% CO; (**Δ**), 40% CO; and (**▲**), 50% CO. The additional gas in the head space was Ar. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

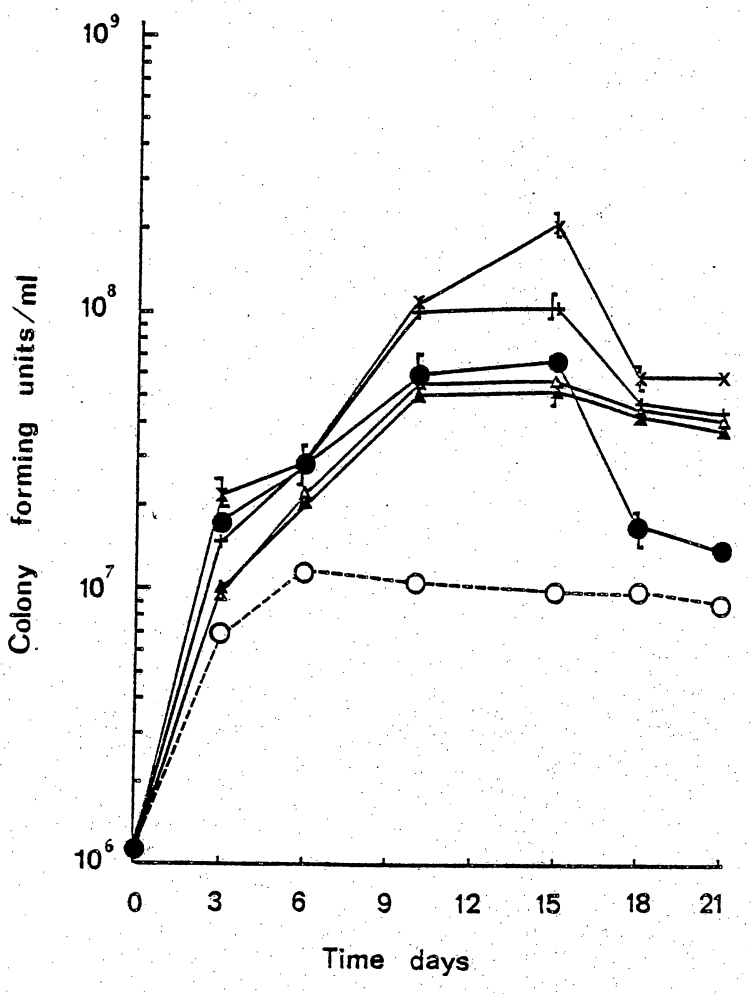

Fig. 1. (B) Change in colony forming units per ml of cultures of R. japonicum, strain USDA 6 during anaerobic, chemolithotrophic growth with different concentrations of CO in the head space, (○), 0% CO; (●), 10% CO; (×), 20% CO;(+), 30% CO;(Δ), 40% CO; and (▲), 50% CO. The additional gas in the head space was Ar. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

often associated with rhizobial cells. The results of these growth experiments indicate that CO is required for growth and that the percentage of CO in the culture headspace for optimum growth was about 20%.

The disappearance of NO_3^- from the culture medium and the transitory occurrence of NO_2^- is shown in Fig. 2A and Fig. 2B. In the presence of CO, anaerobic chemolithotrophic growth was accompanied by a decrease of NO_3^- from 80 μg/ml to less than detectable limits after 18 days incubation (Fig. 2A). Nitrite accumulated in the culture medium during the early stages of cell growth (Fig. 2B), but was transitory and reached a level of less than 1 μg/ml in all treatments by the end of the experimental growth period. In the absence of CO, a slight decrease in NO_3^- occurred, coupled with the transitory occurrence of NO_2^-, supporting the conclusion that in the absence of CO, endogenous carbon sustained minimal growth. Periodic analysis of the concentration of NH_4^+ (Appendix, Table 1) in the culture medium showed that a sufficient amount remained throughout the growth period to repress the synthesis of assimilatory nitrate and nitrite reductases. A significant increase in growth of R. japonicum was found to occur in the presence of CO that was accompanied by a substantial loss of NO_3^- from the medium. The results of this experimentation strongly suggests that anaerobic chemolithotrophic growth occurred at the expense of CO, with

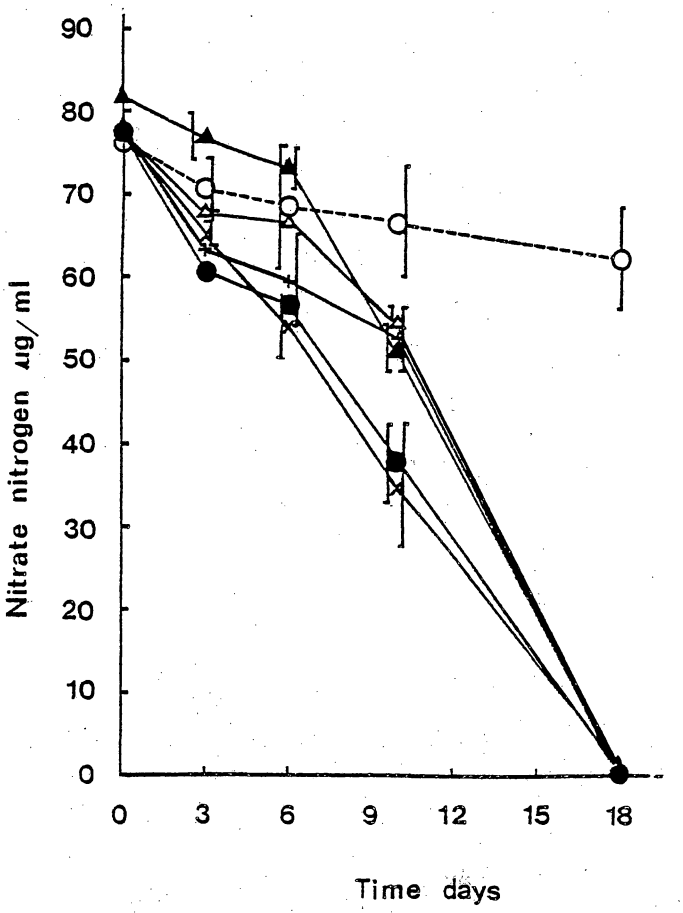

Fig. 2. (A) Change in concentration of NO_3^- of cultures of R. japonicum, strain USDA 6, during anaerobic, chemolithotrophic growth with different concentrations of CO in the head space, (O), 0% CO; (●), 10% CO; (x), 20% CO;(+), 30% CO;(Δ), 40% CO; and (▲), 50% CO. The additional gas in the head space was Ar. The initial concentration of NO_3^- in the medium was 0.5 g/l of KNO_3. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

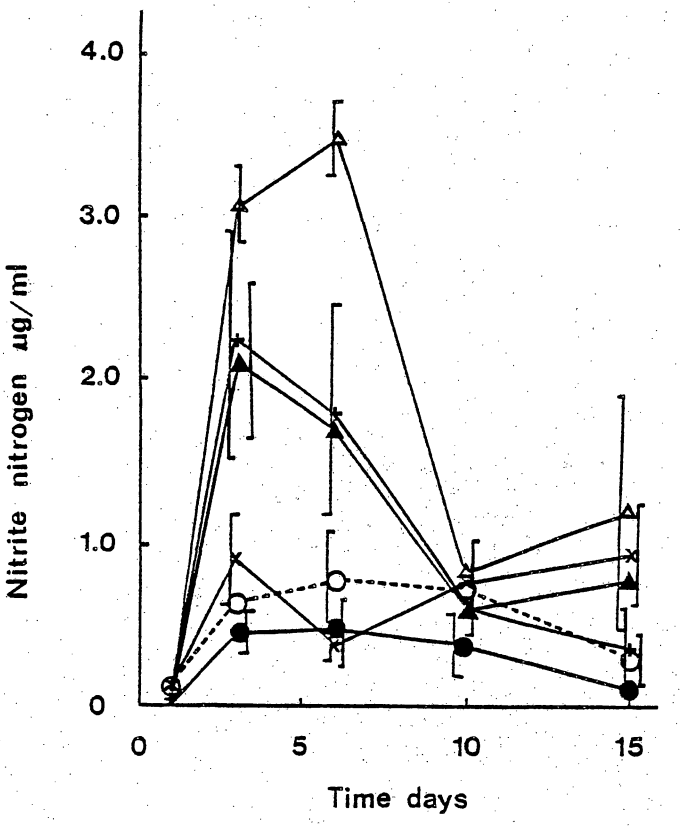

Fig. 2. (B) Change in concentration of NO_2^- of cultures of R. japonicum, strain USDA 6, during anaerobic, chemolithotrophic growth with different concentrations of CO in the head space, (○), 0% CO; (●), 10% CO; (x), 20% CO; (+), 30% CO; (Δ), 40% CO and (▲), 50% CO. The additional gas in the head space was Ar. The initial concentration of NO_3^- in the medium was 0.5 g/l of KNO_3. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

NO_3^- serving as the electron acceptor.

Nitrate, nitrite, nitrous oxide, and oxygen-dependent uptake -hydrogenase. The specific activities of uptake-hydrogenase of R. japonicum grown chemolithotrophically with 20% CO in the culture headspace in the presence of NO_3^- are shown in Table 1. Hydrogen uptake occurred following the addition of NO_3^-, NO_2^- or N_2O indicating the presence of dissimilatory nitrate reductase, nitrite reductase, nitrous oxide reductase, and presumably, nitric oxide reductase. Addition of Ar saturated medium to the cell suspension did not result in H_2 uptake. By addition of O_2-saturated medium to a cell suspension of the same density, a substantially greater rate of H_2 uptake occurred, indicating that terminal cytochromes necessary to allow O_2 to serve as an electron acceptor are synthesized by the cells during growth with CO.

Acetylene has been shown to block the reduction of N_2O to N_2 gas (Yoshinari et al.,1976; Balderston et al., 1976). Addition of C_2H_2 to the cell suspension followed by introduction of N_2O caused an almost complete cessation of H_2 uptake (Table 1), indicating a stoppage of electron flow from H_2 via the electron transport chain to N_2O.

Carbon monoxide MV oxidoreductase and ribulose bisphosphate carboxylase. Carbon monoxide oxidoreductase apparently is the enzyme responsible for the catalysis of conversion of CO to CO_2.

Table. 1. Hydrogen uptake by R. japonicum, strain USDA 6, grown under 20% CO with nitrate for 21 days. Hydrogen uptake was measured amperometrically.

Electron acceptor	Acetylene µl	Specific activity nanomoles/min/mg protein
None	0	0 *
O_2	0	40.93 ± 7.34
NO_3	0	11.76 ± 0.46
NO_2	0	15.42 ± 1.33
N_2O	0	27.93 ± 1.86
O_2	50	41.88
N_2O	50	0.45

* ± S.E.M.

Concentrations of electron acceptors used were as follows; O_2, 100 µl of a O_2-saturated medium; KNO_3, 100 µl of a solution containing 0.0872 g/l; KNO_2, 100 µl of a solution containing 0.0892 g/l; N_2O, 9.7 µl of a N_2O saturated medium and 90.3 µl of a Ar saturated medium.

Whole cells, grown for a period of 16 days under a culture atmosphere of 20% CO were found to synthesize CO:MV oxidoreductase as measured spectrophotometrically with MV as the electron acceptor. Specific activity, based upon the equation that two moles of MV are reduced per mole of CO oxidized, was found to be 3.87 I.U. per mg of protein. Whether the enzyme responsible for oxidation of CO is a dehydrogenase or a true oxidoreductase was not determined.

In chemolithotrophic bacteria, the carbon requirement for cell synthesis is satisfied by the fixation of CO_2. Carbon dioxide is apparently fixed via the Calvin cycle, of which ribulose 1,5 bisphosphate (RuBP) carboxylase is one of the key enzymes. Whole cells, grown anaerobically for 16 days chemolithotrophically with 20% CO were assayed for the presence of RuBP carboxylase. Under the described experimental conditions, RuBP carboxylase could not be detected.

Influence of H_2 concentration on growth with CO and CO_2

Based upon experimental results showing that an active uptake hydrogenase was synthesized during cell growth with 20% CO, experiments were conducted to determine if varying amounts of H_2 in the culture headspace affected the cell growth. The results of these experiments are shown in Fig. 3A. Addition of H_2 to the culture atmosphere in excess of 0.5% decreased cell growth substantially when compared to growth only with CO. Although

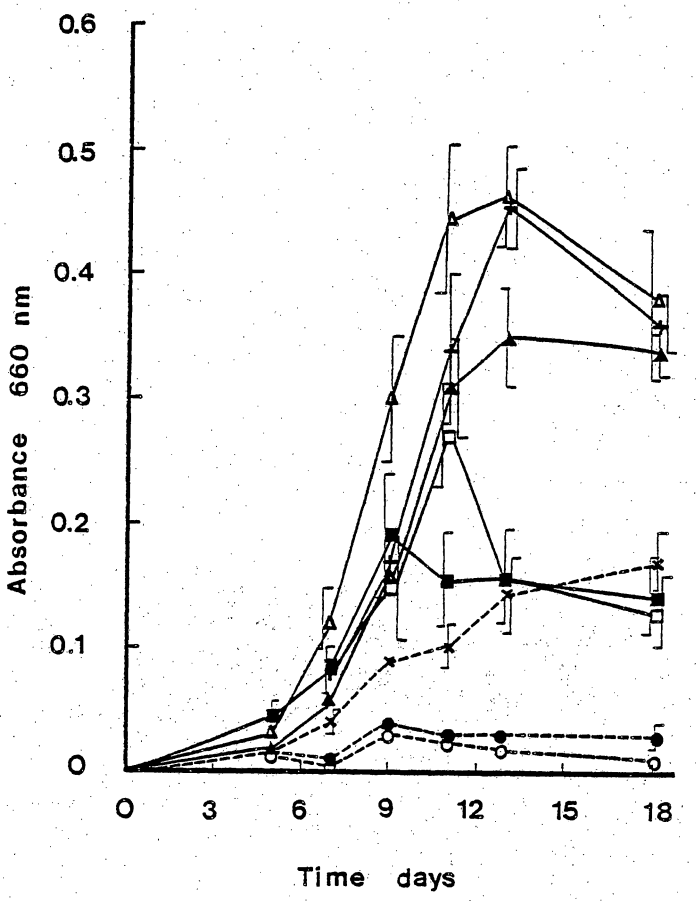

Fig. 3. (A) Change in absorbance (660 nm) of cultures of R. japonicum, strain USDA 6, during anaerobic, chemolithotrophic growth with 20% CO and different concentrations of H_2 in the head space. Symbols: (X), 0% H_2 and 0% CO; (+), 0% H_2 and 20% CO; (Δ), 0.5% H_2 and 20% CO; (▲), 1.0% H_2 and 20% CO; (□), 5.0% H_2 and 20% CO; (■), 10% H_2 and 20% CO. All above treatments had 1 g/l KNO_3 and $(NH_4)_2SO_4$, respectively. Symbols: (○) 0 g/l KNO_3 and 1 g/l $(NH_4)_2SO_4$; (●), 0 g/l KNO_3 and 0 g/l $(NH_4)_2SO_4$. The above treatments had no CO or H_2. The additional gas in the head space was Ar. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

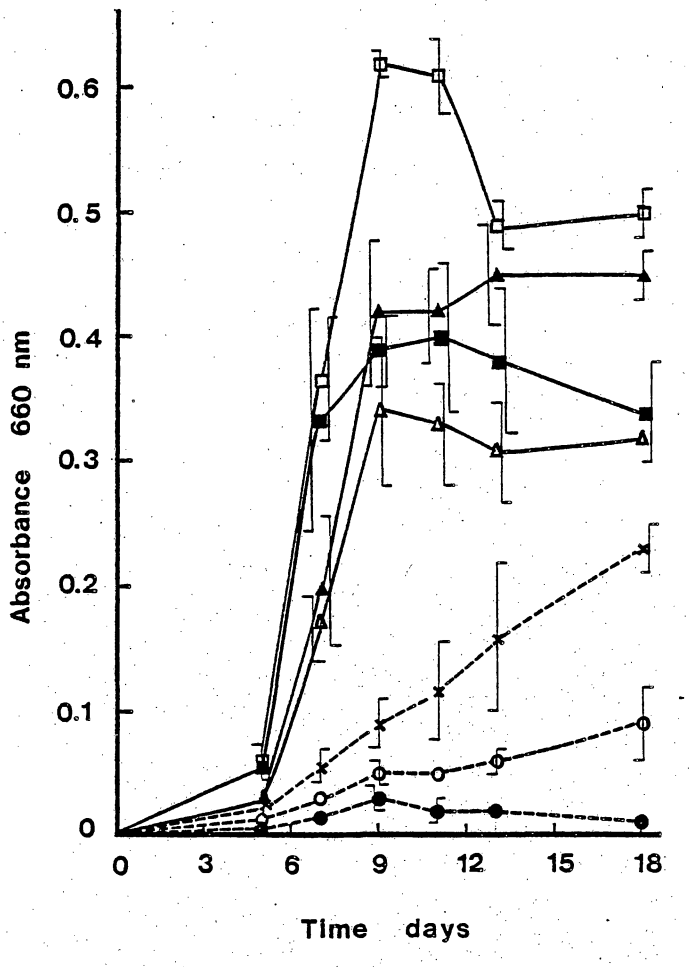

Fig. 3. (B) Change in absorbance (660 nm) of cultures of R. japonicum, strain USDA 6, during anaerobic, chemolithotrophic growth with 20% CO_2 and different concentrations of H_2 in the head space. Symbols: (X), 0% H_2 and 0% CO_2; (Δ), 0.5% H_2 and 20% CO_2; (▲), 1.0% H_2 and 20% CO_2; (□), 5.0% H_2 and 20% CO_2; (■), 10% H_2 and 20% CO_2. All above treatments had 1 g/l KNO_3 and $(NH_4)_2SO_4$, respectively. Symbols: (○) 0 g/l KNO_3 and 1 g/l $(NH_4)_2SO_4$; (●), 0 g/l KNO_3 and 0 g/l $(NH_4)_2SO_4$. The above treatments had no CO_2 or H_2. The additional gas in the head space was Ar. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

incorporation of 0.5% H_2 in the culture atmosphere stimulated the total amount of cell growth, increasing the percentage of H_2 above 0.5% repressed the amount of growth proportionately. Increasing the percentage of H_2 to a level of 10% repressed the amount of cell growth to nearly the level of endogenous respiration. In the absence of an electron acceptor or both an electron acceptor and a source of nitrogen, growth was found to be negligible. In contrast, replacing CO with CO_2 in the culture atmosphere caused a stimulation in cell growth that could be related to the increase in H_2 concentration in the culture atmosphere (Fig. 3B). Optimum growth conditions occurred with the percentage of H_2 in the culture atmosphere adjusted to 5%, followed by rank order of percentage of H_2 of 1.0 %, 10% and 0.5%. As was noted previously, the concentration of NO_3^- in the culture medium decreased in proportion to cell increase as monitored by absorbance (Appendix, Fig. 2A and 3A). Nitrite appeared transiently during the early stages of cell growth, and decreased to a level of less than 1 μg/ml as cell growth progressed (Appendix, Fig. 2B and 3B). Periodic sampling of the culture medium showed that NH_4^+ (Appendix, Table 2A and 2B) remained in sufficiently high concentration during the 18 day incubation period to repress the synthesis of assimilatory nitrate and nitrite reductase. During anaerobic chemolithotrophic growth in the presence of 20% CO, increases in the percentage of H_2 in

the culture headspace above 0.5% repressed cell growth, while in the presence of 20% CO_2, increases in the level of H_2 were accompanied by increased cell growth.

DISCUSSION

Carbon monoxide, one of the most potent and abundant pollutants present in the atmosphere (Swinnerton et al., 1980), is known to be toxic to most organisms. It is toxic to the majority of aerobic microorganisms since CO inhibits cytochrome oxidase activity (Meyer and Schlegel, 1983).

However, a variety of aerobic and anaerobic bacteria have been reported to metabolize CO. Usually, this oxidation activity occurs adventitiously, but a few microorganisms have been reported to use CO for growth (Cypionka et al., 1980; Genthner et al., 1982; Hegeman, 1980; Kiessling and Meyer 1982; Kim and Hegeman 1981a; Lynd et al., 1982; Postgate, 1970; Uffen, 1976; Uffen, 1983).

Insensitivity to CO by these organisms has to be regarded as an exceptional property, (Cypionka and Meyer, 1982) and is attributed to detoxification of CO by the action of CO:acceptor oxidoreductase (Meyer and Schlegel, 1979; Meyer and Schlegel, 1980; Kim and Hegeman, 1981b), increased production of respiratory chain components or induction of CO insensitive terminal oxidases. Recent reports (Cypionka and Meyer, 1983a; Cypionka and Meyer, 1983b) have shown that CO insensitivity of carboxydotrophs does not depend on the presence of CO:acceptor oxidoreductase, but is a result of CO insensitive, alternative terminal oxidases.

Our studies show that R. japonicum, strain USDA 6, is

capable of growth on CO apparently as the sole source of carbon and most probably deriving energy by this oxidation reaction. This study demonstrates that even at a CO concentration of 50% in the head space, significant amounts of growth were obtained. However, optimum growth occurs with 20% CO in the culture atmosphere. The biochemical basis for the depression of growth observed with higher concentrations of CO is not known.

The culture used for this investigation was periodically transferred and maintained in anaerobic, autotrophic growth medium with 20% CO in the head space for 18 months, which strongly suggests that this gas is utilized during synthesis of cellular material.

Chemolithotrophic anaerobic growth with NO_3^- shows that NO_3^- can be used as an electron acceptor. The disappearance of NO_3^- and transitory appearance of NO_2^- provides indirect evidence for the presence of dissimilatory nitrate reductase and nitrite reductase enzymes. Based on colorimetric analysis of the culture medium for NH_4^+, sufficient NH_4^+ was present to repress the synthesis of assimilatory nitrate and nitrite reductases (Tiedje et. al. 1982) during the growth period.

During the initial period of growth, NO_2^- accumulated transiently. This may be due to suppression of nitrite reductase enzyme activity by NO_3^- or due to sequential induction of nitrite reductase by accumulating NO_2^- (Payne and Riley 1969; Payne, 1973).

Bacteria which can grow as chemolithotrophs on CO aerobically or anaerobically and have the ability to denitrify or perform nitrate-nitrite respiration have not been reported to date (Meyer and Schlegel, 1983). Apparently, this may be the first report of a denitrifying CO autotroph.

Carbon monoxide:oxidoreductase activity has been demonstrated in a number of carboxydobacteria (Kim and Hegeman 1981b; Meyer and Schlegel 1980) and organisms which utilize CO anaerobically (Ragsdale et al., 1983a; Ragsdale et al., 1983b; Diekert and Ritter 1982; Diekert and Thauer, 1978; Fuchs et. al. 1974; Kerby and Zeikus, 1983; Lynd et al., 1982; Drake, 1982; Drake et al., 1980; Thauer et al., 1974).

The demonstration of the synthesis of this enzyme by R. japonicum suggests the following reaction:

$$CO + H_2O + \text{Acceptor} \longrightarrow CO_2 + \text{Reduced Acceptor}$$

The activity of this enzyme (3.87 I.U.per mg protein) reported in this study is within the range of values reported, using the same assay system, for other microorganisms (Diekert and Ritter, 1982; Drake et al., 1980; Diekert and Thauer, 1978; Wakim and Uffen, 1983). The demonstration of CO:oxidoreductase activity with an artificial electron acceptor like MV would indicate that the in vivo reaction occurs at a strongly electro-negative potential. In addition, the similarity in the electrode potentials of the in vivo substrate, CO, and the artificial electron

acceptor, MV, would reduce the possibility of measuring non-specific, interfering, oxidoreductase activities. Since this enzyme assay was carried out under strictly anaerobic conditions, with O_2-free CO as the substrate, the possibility of a monooxygenase type reaction can be probably discounted (Meyer and Schlegel 1983; Colby et. al. 1979).

The significance of a CO:oxidoreductase type reaction lies in the fact that it is an exergonic reaction (Colby et al., 1979) as follows: $CO + HOH \longrightarrow CO_2 + H_2$; $G^{\circ\prime} = -20$ KJ' per mole of CO enabling the organism to derive metabolically useful energy.

Various other CO utilizers (Lynd and Zeikus, 1982; Wakim and Uffen, 1983) capable of growth on 100% CO have been shown to carry out oxidation of CO by this metabolic route. Hence, the demonstration of this enzyme in cells grown under chemolithotrophic conditions with CO as the only carbon source shows that CO is oxidized in R. japonicum in a utilitarian manner.

The Calvin cycle constitutes the main pathway of CO_2 assimilation in all eucaryotes and in the vast majority of procaryotic autotrophs. Ribulose 1,5 bisphosphate carboxylase, a key enzyme in Calvin cycle, has been demonstrated in H_2 uptake positive strains of R. japonicum growing under aerobic, chemolithotrophic conditions with H_2 and CO_2 (Lepo et. al., 1980; Simpson et al., 1979; Purohit et. al. 1982) and with formate (Manian and O'Gara 1982).

The inability to demonstrate ribulose 1,5 bisphosphate carboxylase activity in cells grown in CO would indicate that the conditions used for detection of this enzyme were inappropriate for cells grown under the conditions used or the absence of this enzyme.

In bacteria, three basic mechanisms for assimilation of C-1 units into C-3 compounds, namely, Calvin cycle, serine pathway and ribulose monophosphate cycle have been established (Colby et al., 1979). Several methylotrophic microorganisms carry out C-1 assimilation by the serine pathway (Bellion and Hersh, 1972; Anthony, 1975) and RUMP pathway (Strom et al., 1974).

The RUMP pathway and the serine pathway have not been investigated in this study. Hence, these possibilities for assimilation of C-1 compounds in autotrophically diverse rhizobia cannot be excluded.

Among the six strains of R. japonicum that possess a H_2 uptake system (Evans et al., 1980) several have been shown to be capable of growing chemolithotrophically using H_2 as the energy source, O_2 or NO_3^- as the electron acceptor and deriving carbon skeletons by CO_2 fixation (Hanus et al., 1979; Lepo et al., 1980; Neal et al., 1983a). The conditions necessary for expression of hydrogenase in free living cultures of rhizobia grown chemolithotrophically have been defined (Maier et al., 1978). Among other factors such as low concentrations of carbon

substrates, decreased O_2 tensions and quantity of combined nitrogen, hydrogenase activity was shown to be dependent upon a preincubation period in the presence of H_2 (Simpson et.al. 1979; Hanus et. al. 1979).

Hydrogenase activity in CO grown cultures have been demonstrated in several aerobic (Cypionka et al.,1980; Meyer and Schlegel 1979; Yagi and Tamiya 1962; Meyer and Schlegel, 1980) and anaerobic (Drake, 1982) CO oxidizing bacteria.

The cultures used for amperometric detection in this study were incubated in the presence of Ar and CO. The demonstration of an active uptake hydrogenase in our studies apparently suggests that this enzyme could be expressed without a preincubation period under H_2. Hydrogenase activity without substrate induction has been demonstrated earlier with a mutant rhizobium (Merberg et al., 1983).

Apparently, all purified hydrogenases are inhibited by CO (Adams and Hall 1979; Badziong et al. 1978; Chen and Blanchard 1978; Schink and Schlegel 1979; Peck et al. 1956; Mortenson and Chen 1974; Van der Werf and Yates 1978) with the exception of the soluble hydrogenase of A. eutrophus (Schneider et al. 1979). Studies with membrane associated hydrogenase of R. japonicum have shown that although CO is accessible to the active site of the purified enzyme, it is inaccessible to the enzyme in the intact membrane of R. japonicum bacteroids (Ruiz argueso et al. 1979).

Similarly, the demonstration of an active hydrogenase in CO grown cultures of R. japonicum provides indirect evidence for its possible insensitivity to inhibition by CO.

The demonstration of H_2 uptake activity in the presence of O_2 as the sole electron acceptor in our study would provide presumptive evidence for the presence of a cytochrome system which is insensitive to CO. Carbon monoxide is known to bind to terminal oxidases and impair respiration and growth of the majority of aerobes (Cypionka and Meyer 1983b). Recently, it was found that carboxydotrophs such as Pseudomonas carboxydovorans (Cypionka and Meyer 1982; Cypionka and Meyer 1983b) and Pseudomonas carboxydohydrogena (Kim and Hegeman 1981a; Kim and Hegeman, 1981b; Kim and Hegeman 1983) have branched electron transport systems with CO insensitive branches. The CO insensitive branch in Pseudomonas carboxydovorans has been further characterised and shown to be composed of a novel, constitutive cytochrome O (cytochrome 563) which functions as an alternate oxidase, enabling this carboxydotroph to withstand CO inhibition (Cypionka and Meyer 1983b).

Studies with R. japonicum bacteroids (O'Brian and Maier 1983) and free living cultures (O'Brian and Maier 1982) have revealed a complex, branched electron transport system having several terminal oxidases. However, the possibility of a non-physiological reduction of O_2 bypassing the CO sensitive

terminal cytochrome oxidases cannot be ruled out at this time.

Acetylene is known to act as a specific inhibitor in the reduction of N_2O during denitrification (Yoshinari and Knowles, 1976; Balderston et al., 1976). The inhibition of uptake hydrogenase activity with C_2H_2 when N_2O is the only available electron acceptor provides further proof for this step in denitrification. Hydrogenase activity with NO_3^-, NO_2^- and N_2O as electron acceptors substantiates the presence of active dissimilatory nitrate reductase, nitrite reductase and nitrous oxide reductase in this study (Neal et al. 1983a, 1983b; Tibelius and Knowles 1983).

The specific activities of hydrogenase with NO_3^-, NO_2^-, N_2O and O_2 as the electron acceptor expressed as nmoles of H_2/min/mg of protein (Table 1) increase in that order.

Because only whole cell preparations were used in this study with no prior investigations on rates of membrane transport of the different electron acceptors, the enzymatic properties such as intrinsic activities, K_m, V_{max}, the cellular location of the different enzymes and cofactors involved, it is not possible to derive further inferences.

Repression of growth by molecular H_2 has been demonstrated in R. japonicum (Lim and Uratsu 1983). However, the effect of H_2 on the activity of CO:MV oxidoreductase is not known. It would be of interest to study the inhibition, if any, of CO:MV

oxidoreductase by H_2.

However, a similar repressive effect of H_2 has been studied in Alcaligenes eutrophus (Gottschalk 1965) when cultures maintained under chemolithotrophic conditions were transferred to organic substrates in the absence of CO_2. This has been shown to be due to the repression of catabolic enzymes involved in degradation of substrates such as fructose, gluconate, acetate and the majority of amino acids. So far, all strains of hydrogen bacteria tested for this property show the H_2 effect with the exception of Paracoccus denitrificans and Aquaspirillum autotrophicum (Bowien and Schlegel 1981). This is called the "Hydrogen Effect" and is interpreted as catabolite repression (Magasanik 1961).

In contrast, a similar experimentation with CO_2 instead of CO shows a significantly better growth even at 5.0% H_2. Repression to the extent of endogenous growth was not observed within the range of H_2 concentrations used.

These observations would agree with a biochemical model where electrons formed due to oxidation of CO and H_2 are sent into the same cytochrome chain. A consequent inhibition of CO oxidation with increasing concentrations of H_2 would result in less growth due to carbon limitation.

It is generally accepted that microbial populations are limited by readily available forms of organic carbon (Alexander,

1961) and that the competitive survival of rhizobia as free living organisms in soil is essential for successful nodulation of uninoculated legumes.

Hence it seems logical to speculate that the metabolic versatility of rhizobia in terms of heterotrophy and chemolithotrophy should aid its ability to successfully compete and survive in the soil. If so, the ability to utilize substrates which are abundant in anoxic soil environments and substrates that are unavailable to the majority of soil microorganisms should enhance an organism's ability to effectively compete in the soil ecosystem.

In addition to these unusual chemolithotrophic features, the ability to carry out denitrification allows the rhizobia to survive and grow under anaerobic conditions (Zablotowicz et al. 1978; Zablotowicz and Focht, 1979; Daniel et al., 1980) as a chemoorganotroph as well as a chemolithotroph (Neal et al., 1983a).

Organisms growing in CO as the sole source of carbon and energy have an unusual mode of life, occupying the boundary between the chemolithotrophs and chemoorganotrophs. They differ from other autotrophs since they can also utilize their carbon substrate for energy . They are different from other heterotrophs because they grow on compounds having no C-C bonds as their sole source of carbon and they must make every C-C bond "de novo".

Hence, the ability of the microorganism dealt with in this

study to use CO for carbon and possibly as a energy source, to utilize H_2 as a energy source, to exist in anaerobic denitrifying conditions using NO_3^- as the electron acceptor, and still to retain the ability to nodulate the soy bean plant is an interesting phenomenon.

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APPENDIX

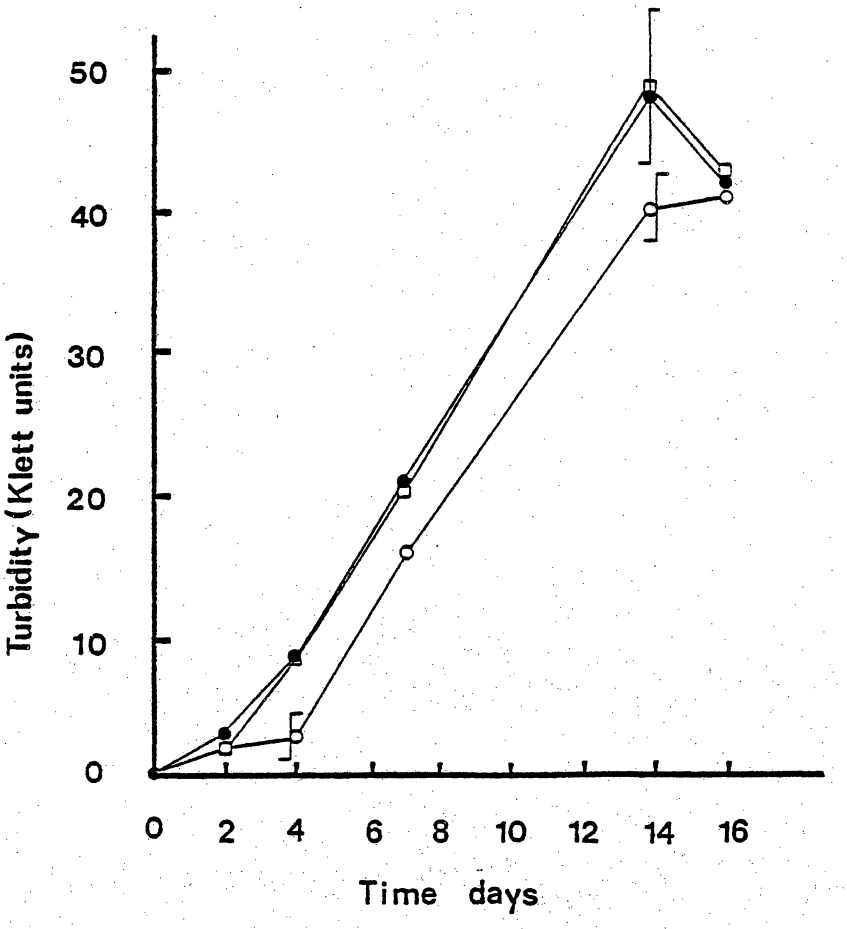

Fig. 1. Change in absorbance (500 nm) of cultures of R. japonicum, strain USDA 6, during chemolithotrophic growth with 20% CO and different concentrations of O_2, (○),0% O_2; (●), 1% O_2; (□), 5% O_2. All treatments had 1 g KNO_3 per 1. The additional gas in the head space was Ar. Vertical lines are standard errors of means . Where not shown the lines are encompassed by symbols.

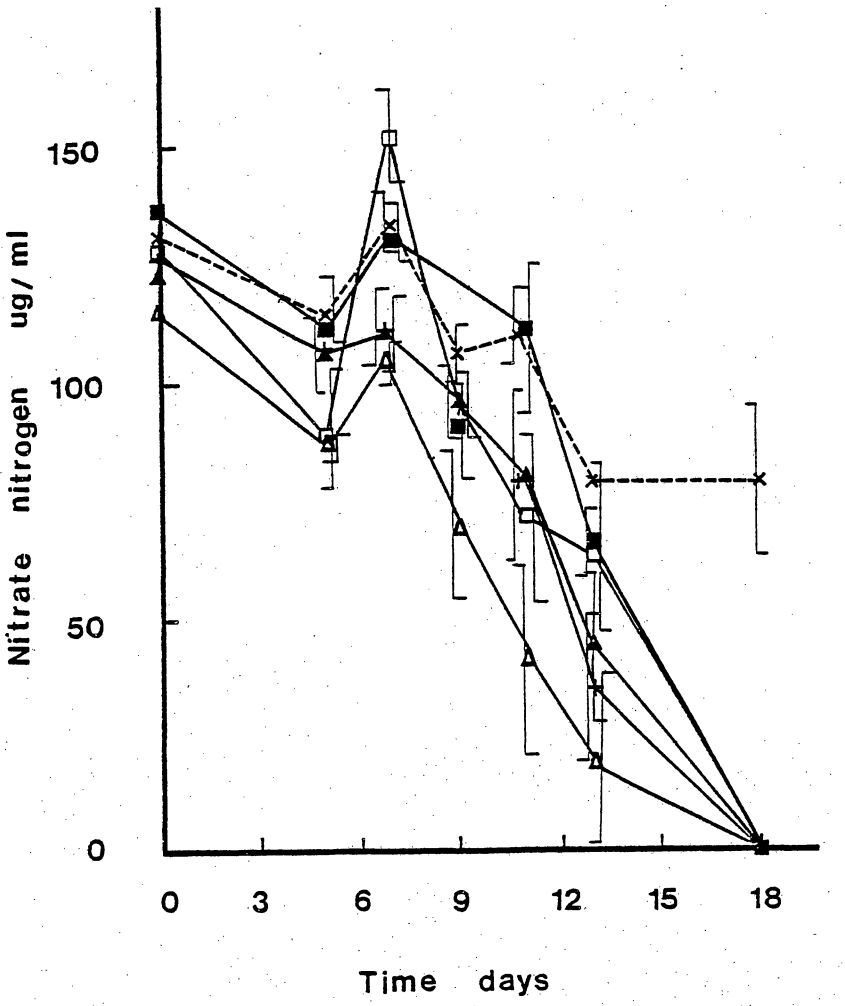

Fig. 2. (A) Change in concentration of NO_3^- of cultures of R. japonicum, strain USDA 6, during anaerobic, growth with 20% CO and different concentrations of H_2 in the head space. Symbols: (X), 0% H_2 and 0% CO; (+), 0% H_2 and 20% CO; (Δ), 0.5% H_2 and 20% CO; (▲), 1.0% H_2 and 20% CO; (□), 5.0% H_2 and 20% CO; (■), 10% H_2 and 20% CO. All above treatment had 1 g/l KNO_3 and $(NH_4)_2SO_4$, respectively. Symbols: (○) 0 g/l KNO_3 and 1 g/l $(NH_4)_2SO_4$; (●), 0 g/l KNO_3 and 0 g/l $(NH_4)_2SO_4$. The above treatments had no CO or H_2. The additional gas in the head space was Ar. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

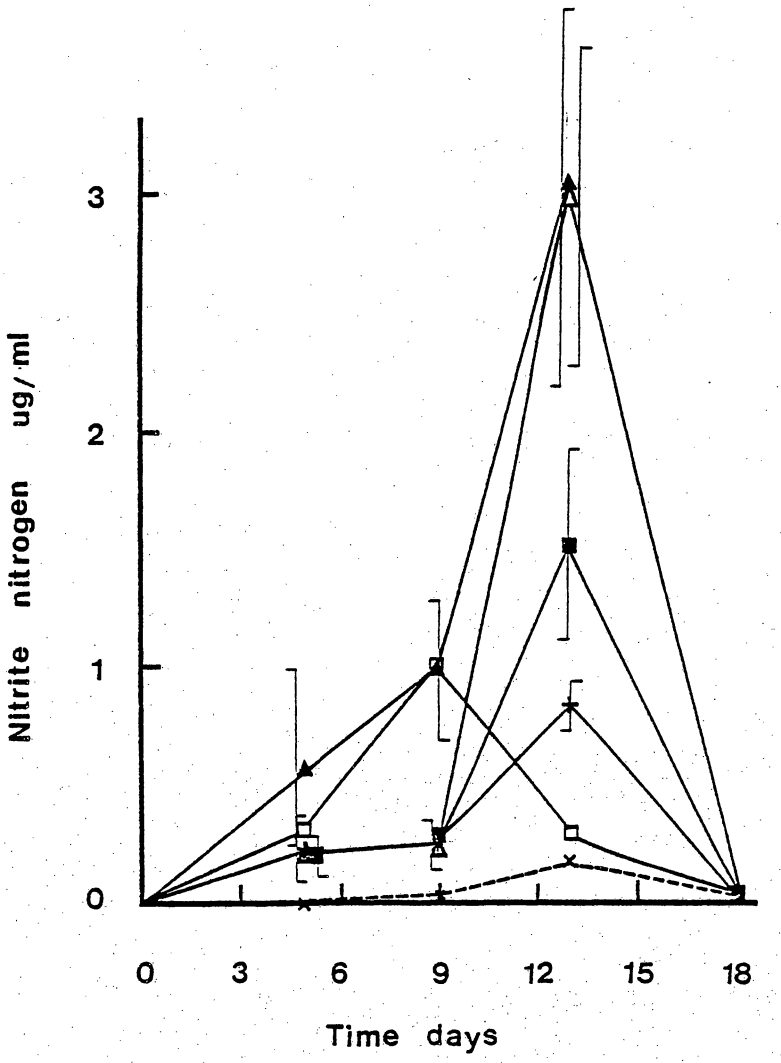

Fig. 2. (B) Change in concentration of NO_2^- of cultures of R. japonicum, strain USDA 6, during anaerobic, chemolithotrophic growth with 20% CO and different concentrations of H_2 in the head space. Symbols: (X), 0% H_2 and 0% CO; (+), 0% H_2 and 20% CO; (Δ), 0.5% H_2 and 20% CO; (▲), 1.0% H_2 and 20% CO; (□), 5.0% H_2 and 20% CO; (■), 10% H_2 and 20% CO. All above treatment had 1 g/l KNO_3 and $(NH_4)_2SO_4$, respectively. Symbols: (○) 0 g/l KNO_3 and 1 g/l $(NH_4)_2SO_4$; (●), 0 g/l KNO_3 and 0 g/l $(NH_4)_2SO_4$. The above treatments had no CO or H_2. The additional gas in the head space was argon. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

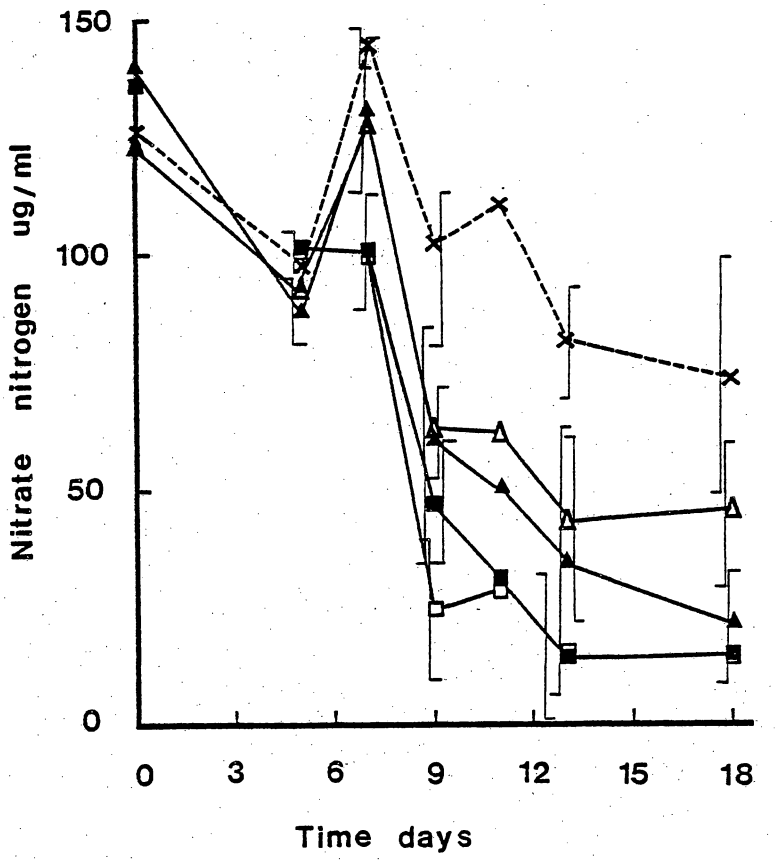

Fig. 3. (A) Change in concentration of NO_3^- of cultures of R. japonicum, strain USDA 6, during anaerobic, chemolithotrophic growth with 20% CO_2 and different concentrations of H_2 in the head space. Symbols: (X), 0% H_2 and 0% CO_2; (Δ), 0.5% H_2 and 20% CO_2; (▲), 1.0% H_2 and 20% CO_2; (□), 5.0% H_2 and 20% CO_2; (■), 10% H_2 and 20% CO_2. All above treatment had 1 g/l KNO_3 and $(NH_4)_2SO_4$, respectively. Symbols: (○) 0 g/l KNO_3 and 1 g/l $(NH_4)_2SO_4$; (●), 0 g/l KNO_3 and 0 g/l $(NH_4)_2SO_4$. The above treatments had no CO_2 or H_2. The additional gas in the head space was Ar. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

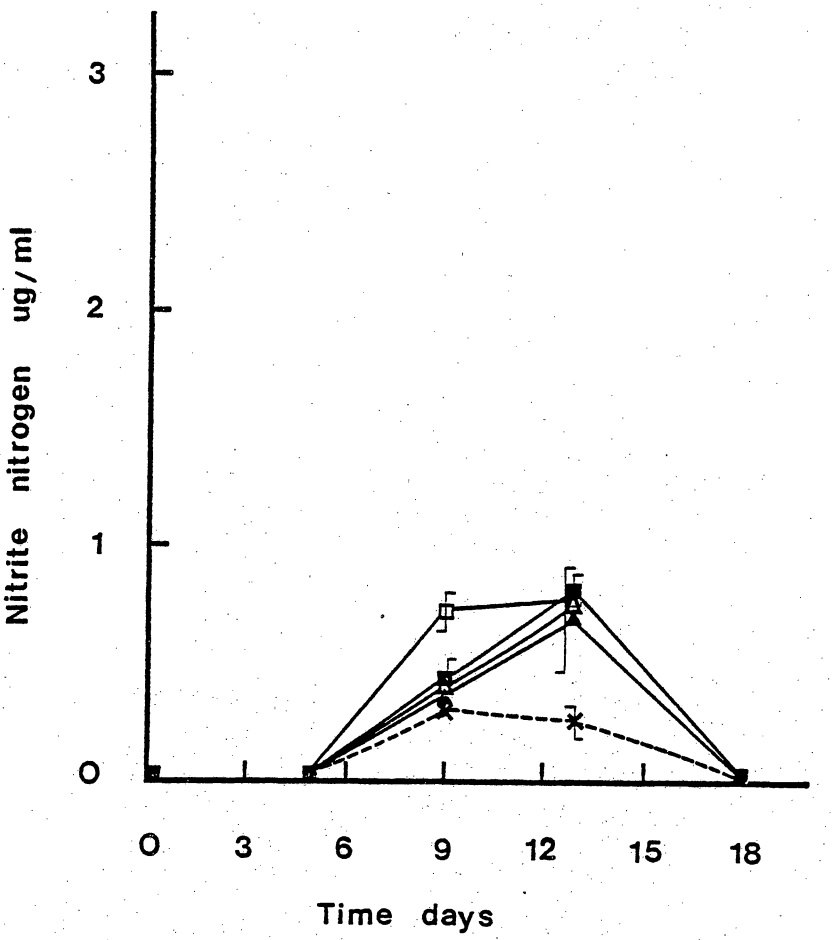

Fig. 3. (B) Change in concentration of NO_2^- of cultures of R. japonicum, strain USDA 6, during anaerobic, chemolithotrophic growth with 20% CO_2 and different concentrations of H_2 in the head space. Symbols: (X), 0% H_2 and 0% CO_2; (Δ), 0.5% H_2 and 20% CO_2; (▲), 1.0% H_2 and 20% CO_2; (□), 5.0% H_2 and 20% CO_2; (■), 10% H_2 and 20% CO_2. All above treatment had 1 g/l KNO_3 and $(NH_4)_2SO_4$, respectively. Symbols: (○) 0 g/l KNO_3 and 1 g/l $(NH_4)_2SO_4$; (●), 0 g/l KNO_3 and 0 g/l $(NH_4)_2SO_4$. The above treatments had no CO_2 or H_2. The additional gas in the head space was argon. Vertical lines are standard errors of means. Where not shown the lines are encompassed by the symbols.

Table. 1. The percentage change in concentration of NH_4^+ in cultures of R. japonicum, strain USDA 6[a].

% CO in the Head Space	% Decrease in NH_4^+ in the Medium at Stationary phase of growth[b]
0	9.18
10	23.13
20	26.00
30	11.84
40	11.95
50	2.71

[a] Cells were grown in anaerobic, autotrophic medium for 15 days on different concentrations of CO in the head space. The additional gas in the head space was Ar.
[b]The initial concentration of NH_4^+ in the medium was 0.5 g/l of $(NH_4)_2SO_4$.

Table. 2. The percentage change in concentration of NH_4^+ in cultures of R. japonicum, strain USDA 6[a].

% Gas in the Head Space		% Decrease in NH_4^+ in the medium at stationary phase of growth[b]
H_2	CO	
0	0	8.10
0	20	3.24
0.5	20	0
1.0	20	0
5.0	20	7.90
10.0	20	16.28
0	0	7.11

[b] The initial concentration of NH_4^+ in the medium was 1 g/l of $(NH_4)_2SO_4$.

[b] Cells were groen in anaerobic, autotrophic medium for 18 days on different concentrations of CO in the head space. The additional gas in the head space was Ar.

Table. 3. The percentage change in concentration of NH_4^+ in cultures of R. japonicum, strain USDA 6[a].

% CO in the Head Space		% Decrease in NH_4^+ in the medium at stationary phase of growth[b]
H_2	CO_2	
0	0	13.63
0	20	22.58
0.5	20	27.27
1.0	20	37.93
5.0	20	40.43
10.0	20	9.76
0	0	9.09

[a] Cells were grown in anaerobic, autotrophic medium for 18 days on different concentrations of CO_2 in the head space. The additional gas in the head space was Ar.
[b] The initial concentration of NH_4^+ in the medium was 1 g/l of $(NH_4)_2SO_4$.

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