EFFECT OF TUNGSTEN ON NITRATE AND NITRITE REDUCTASES IN *AZOSPIRILLUM BRASILENSE* SP 7

by

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Cette thèse est dédiée à mes parents

qui m'ont toujours encouragé

ABSTRACT

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Effect of tungsten on nitrate and nitrite reductases in *Azospirillum brasilense* Sp7

Azospirillum brasilense reduced nitrate in W-containing Mo-limited medium, but at lower rates than in W-free medium. However nitrate reduction by *Paracoccus denitrificans* was completely suppressed under the same conditions. Nitrite reductase activity of growing *A. brasilense* was negatively affected by tungstate. Nitrite accumulation was shown to be the result of an inhibitory effect of nitrate on nitrite reductase activity. Both resting whole cell and cell-free extract preformed nitrite reductase activities were equally affected by increasing levels of tungstate. Preformed nitrate reductase activity of the cell-free extract was shown to be more sensitive to increasing concentrations of tungstate than whole cell activity, suggesting that the cytoplasmic membrane served as a protective barrier against tungsten inactivation of nitrate reductase.

Addition of molybdate had no effect on nitrate reductase activities of growing *A. brasilense* cells. However *A. brasilense* cells grown in treated Mo-depleted medium could not reduce nitrate. Disc gel electrophoresis revealed that ^{99}Mo was incorporated in the nitrate reductase of W-free grown

cells, but not of W-grown cells. Aerobically grown *A. brasilense* cells transported ^{99}Mo more efficiently than anaerobically grown cells. Under denitrifying conditions, *A. brasilense* grown in W-free medium steadily accumulated ^{99}Mo for 12 h. On the other hand, *P. denitrificans* grown under the same conditions ceased uptake after 1 h. However both bacteria were incapable of accumulating significant amounts of ^{99}Mo in 10 mM tungstate-containing media.

Nitrous oxide reductase activity could be detected after disc gel electrophoresis of the soluble fraction.

RESUME

M.Sc. Christian Chauret Microbiologie

Effet du tungstène sur la nitrate et la nitrite réductases chez *Azospirillum brasilense* Sp7

Azospirillum brasilense a réduit le nitrate dans des milieux contenant du W et limités en Mo, mais à des taux plus bas que dans des milieux libres de W. Cependant la réduction du nitrate par *Paracoccus denitrificans* fut complètement supprimée sous les mêmes conditions. L'activité nitrite réductase des cellules croissantes d'*A. brasilense* fut affectée négativement par le tungstate. Il a été démontré que l'accumulation du nitrite était le résultat de l'effet inhibiteur du nitrate sur l'activité nitrite réductase. Les activités préformées nitrite réductase des cellules entières au repos de même que des extraits acellulaires furent également affectées par des quantités croissantes de tungstate. Il a été démontré que l'activité préformée nitrate réductase des extraits acellulaires était plus sensible à des quantités croissantes de tungstate que l'activité des cellules entières, suggérant que la membrane cytoplasmique servait de barrière contre l'inactivation par le tungsten de la nitrate réductase.

L'addition de molybdate n'a pas eu d'effet sur les activités nitrate réductase des cellules croissantes d'*A. brasilense*. Cependant, les cellules d'*A. brasilense* croissant dans un milieu traité réduit en Mo ne pouvait pas réduire le nitrate. L'électrophorèse en gel à disque a révélé que le ^{99}Mo était incorporé dans la nitrate réductase des cellules libres de W, mais ne l'était pas dans les cellules croissant en présence de W. Les cellules d'*A. brasilense* croissant en aérobie ont transporté le ^{99}Mo plus efficacement que les cellules croissant en anaérobie. Sous des conditions dénitrifiantes, *A. brasilense* croissant en milieu libre de W a accumulé le ^{99}Mo constamment pour 12 h. A l'opposé, *P. denitrificans* poussant similairement a cessé d'accumuler le ^{99}Mo après 1 h. Cependant les deux bactéries ne furent pas capables d'accumuler des quantités significatives de ^{99}Mo dans les milieux contenant 10 mM de tungstate.

L'activité oxyde nitreux réductase a pu être détectée par l'électrophorèse en gel à disque dans la fraction soluble.

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TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

1. INTRODUCTION

Azospirillum brasilense is a chemoheterotrophic gram-negative spirillum, which fixes N_2 under microaerobic conditions, and is often associated with the rhizosphere of several tropical grasses (Bülow and Döbereiner 1975, Day and Döbereiner 1976). It is also a denitrifier under anaerobic conditions; it reduces nitrate to nitrite, nitrous oxide, and to gaseous N_2 (Neyra *et al.* 1977, Nelson and Knowles 1978).

Dissimilatory nitrate reductase, the first enzyme in denitrification, is a molybdoenzyme and its formation is stimulated by traces of molybdenum (Taniguchi and Itagaki 1960, Lester and DeMoss 1971). Tungstate is known to be an antagonist of nitrate reductase formation (Enoch and Lester 1972, Sperl and DeMoss 1975, Burke *et al.* 1980). In fact, *Escherichia coli* cells grown in tungstate-containing Mo-limited medium produce an inactive nitrate reductase, containing a W-cofactor, which can be activated on addition of molybdate (Sperl and DeMoss 1975, Scott *et al.* 1979, Amy and Rajagopalan 1979). Amy and Rajagopalan (1979) demonstrated that this W-cofactor could complement a nitrate reductase deficient mutant of *Neurospora crassa, nit-1*, to form an intact but inactive nitrate reductase. On the other hand, Lalande (1984) showed that *A. brasilense* cells growing in tungstate-containing (10 and 25 mM) Mo-limited medium were capable of reducing nitrate.

The concentration of molybdenum in soils is generally very low: about 15 μmol/kg mostly in the form of insoluble MoS_2 (Pope *et al.* 1980). Accordingly soil bacteria have developed specific, high affinity systems for processing molybdenum. *Bacillus thuringiensis*, cowpea *Rhizobium*, and *Azospirillum lipoferum* secrete catechol derivative siderophores that mediate the transport of molybdenum (Ketchum and Owen 1975, Patel *et al.* 1988, Saxena *et al.* 1989). *Klebsiella pneumoniae* and *Bradyrhizobium japonicum* each have both high- and low-affinity Mo uptake systems (Imperial *et al.* 1984, Maier *et al.* 1987). The high-affinity systems are expressed under Mo-limited conditions. However no study on the molybdenum metabolism of *A. brasilense* has yet been published.

Nitrite reductase, in *A. brasilense*, is a *cd*-cytochrome (Lalande and Knowles 1987). Nitrite reduction, in *Pseudomonas stutzeri*, is inhibited by nitrate (Kodama *et al.* 1969, Körner and Zumft 1989). However nitrate does not have any effect on nitrite reduction in *Pseudomonas fluorescens* and *Alcaligenes* sp. (Betlach and Tiedje 1981, Juszczak and Domka 1988).

The objectives of this study were (1) to confirm the results of Lalande (1984) on the apparent resistance of *A. brasilense* nitrate reductase activity to tungsten inactivation; (2) to understand the mechanism of this apparent resistance; and (3) to determine if tungsten and nitrate have some effects on nitrite reduction in *A. brasilense*. This study will hopefully lead to a better

understanding of the molybdenum requirement of *A. brasilense*.

2. LITERATURE REVIEW

2.1. *Azospirillum brasilense*

Azospirillum brasilense was found living in the rhizosphere of *Digitaria decumbens* cv *transvala* (Döbereiner and Day 1975, Day and Döbereiner 1976). It is also very abundant in the rhizosphere of several other tropical grasses (van Berkum and Bohlool 1980). *A. brasilense* has been increasingly studied in the past 15 years, especially for its capacity to fix nitrogen under microaerobic conditions (Day and Döbereiner 1976, Okon *et al.* 1977, Nelson and Knowles 1978, Tibelius and Knowles 1983).

A. brasilense can denitrify under anaerobic conditions, using nitrate as an electron acceptor instead of O_2 (Neyra *et al.* 1977). These authors reported that nitrate reductase (see section 2.2.2.) was induced by nitrate and anaerobiosis and required protein synthesis. Nitrite accumulated in the medium, but not in the cells. Lalande and Knowles (1987) reported that growth on nitrate was characterized by a diauxic type of curve: the first log phase was associated with nitrate reduction, whereas the second one corresponded to nitrite reduction. Estimates of apparent K_m values were 14 μM NO_3^- for nitrate reductase, 28 μM NO_2^- for nitrite reductase, and 1.8 μM N_2O for N_2O reductase (Lalande and Knowles 1987). However growth with nitrite and nitrous oxide as the final electron

acceptors had to be induced by small amounts of nitrate. Lalande and Knowles (1987) also demonstrated that nitrite reductase in *A. brasilense* was a *cd*-type cytochrome found only in the soluble fraction of the cells. Nitrite is converted to nitrous oxide in the presence of acetylene, and free nitric oxide is not detected (Zimmer *et al.* 1984). The capacity to denitrify might have both positive and negative effects in a plant-*Azospirillum* association: denitrification results in the loss of fixed nitrogen to a form not readily available to the plant; on the other hand, nitrate utilization might enable *A. brasilense* cells to outcompete other bacteria in the rhizosphere, establish themselves, and then to fix N_2. In fact nitrate-dependent nitrogenase activity has been reported in *A. brasilense* (Bothe *et al.* 1981).

Therefore *A. brasilense* is involved in all the steps of the N cycle with the exception of nitrification. One group of workers (Kundu *et al.* 1987) has reported an incomplete nitrification in *A. brasilense*, but more evidence will be required before classifying this bacterium as a nitrifier.

2.2. MOLYBDOENZYMES

2.2.1. Molybdenum cofactor

Molybdenum is known to be a necessary cofactor (Moco) for several redox reactions: for example nitrogen fixation (Bulen

and Le Comte 1966, Shah *et al.* 1984), nitrate reduction (Taniguchi and Itagaki 1960), xanthine dehydrogenation (Smith *et al.* 1967), aldehyde oxidation (Rajagopalan *et al.* 1962), sulfite oxidation (Cohen *et al.* 1972), and formate dehydrogenation (May *et al.* 1986). On the basis of fluorescence spectra and absorption spectra, Johnson *et al.* (1980) reported that the active cofactor in all molybdoenzymes tested, with the exception of nitrogenase, are most likely composed of molybdenum and a reduced form of a novel pterin. According to these authors, the pterin as well as an unidentified side chain are "undoubtedly involved in metal complexation as well as in the strong noncovalent interactions which anchor the cofactor to the various protein molecules". However, according to L'vov *et al.* (1980), this noncovalent binding is easily broken by changes in pH, heat, and ionic strength. These molybdopterin cofactors are not necessarily identical. The molybdopterin cofactor from the methanogen *Methanobacterium formicium* formate dehydrogenase has unique features. It includes 7-substituted pterins (instead of 6) such as methanopterin (May *et al.* 1986).

2.2.2. NITRATE REDUCTASE

2.2.2.1. TYPES OF NITRATE REDUCTASE

Nitrate is the most oxidized state of fixed nitrogen. Nitrate can be reduced by bacteria in either assimilatory or dissimilatory processes. Nitrate is reduced to nitrite in a two-electron transfer. Assimilatory reduction occurs when nitrate is used as the nitrogen source for growth. The end products of this reaction are NH_4^+ and eventually glutamate, which can be subsequently used for protein synthesis (Payne 1981). Accordingly assimilatory nitrate reductase activity is repressed by NH_4^+. On the other hand, this enzyme is not affected by oxygen, and can function under aerobic conditions. This soluble enzyme is not membrane-bound. Therefore it is not involved at all in energy metabolism. Dissimilatory (or respiratory) nitrate reductase also carries out the reduction of nitrate to nitrite. However this process occurs usually under strict anaerobic conditions (Payne 1981, Knowles 1982). This nitrate reductase is tightly coupled to the electron transport chain, and is, therefore, membrane bound. Nitrate is used as terminal electron acceptor instead of O_2. Accordingly the bacteria possessing this form of nitrate reductase can obtain energy from nitrate respiration.

The membrane-bound nitrate reductases from the denitrifiers *Paracoccus denitrificans* and *Achromobacter denitrificans* are very similar to that from the non-denitrifying *Escherichia coli* (Ferguson 1987). In these bacteria, nitrate reductase basically consists of three types of polypeptides: α (MW 150 kDa), ß (MW 60 kDa), and γ (MW 20 kDa) subunits. The nitrate reductase I of *Klebsiella pneumoniae* was also purified and characterized; and it also consisted of three different subunits, having molecular masses of 117 kDa, 57 kDa, and 52 kDa which are present in a 1:1:2 molar ratio. The holoenzyme of *E. coli* consists, on the other hand, of 2α, 2ß and 4 γ subunits (Ingledew and Poole 1984). MacGregor *et al.* (1974) purified the nitrate reductase of *E. coli* RK 7, and the molecular mass, as measured on an agarose column or in the ultracentrifuge, was 720 kDa and 774 kDa respectively. A *b*-type cytochrome (γ-polypeptide) appears to be involved in the transfer of electrons from ubiquinol of the electron transport chain to the α-polypeptide (Ferguson 1987). The latter subunit comprises the active site, as well as the FeMo cofactor. The active site of the enzyme is at the cytoplasmic side of the inner membrane (Boogerd *et al.* 1983). The role of the ß-polypeptide chain in the oxidation-reduction reaction is unknown, but it seems to be important in membrane attachment of the holoenzyme (Ingledew and Poole 1984).

2.2.2.2. MOLYBDENUM REQUIREMENT AND EFFECT OF TUNGSTEN

Many physiological studies have been performed mostly in the last 20 years, to characterize the role of molybdenum in nitrate reduction. In *E. coli* for example, dissimilatory nitrate reductase formation was stimulated by traces of molybdate (Taniguchi and Itagaki 1960, Lester and DeMoss 1971). Lester and DeMoss (1971) used reagent grade chemicals to prepare their medium, which was not purified (to remove potential Mo contamination) in any particular way. They were surprised, therefore, "to encounter such large effects by the addition of trace levels of molybdate" knowing that molybdenum was a potential contaminant in their medium. Enoch and Lester (1972) studied the role of selenium and molybdenum in the metabolism of *E. coli*. They observed that the addition of high levels of tungsten (1 mM sodium tungstate) increased the molybdate deficiency of their defined medium, as shown by lower nitrate reductase and formate dehydrogenase activities. In fact tungstate caused a greater inhibition of the formation of formate dehydrogenase than nitrate reductase, in nitrate-grown, anaerobic cells. At high levels of tungstate (10^4 molar excess to added molybdate), half the nitrate reductase activity remained whereas formate dehydrogenase activity was totally inhibited. Therefore they concluded that nitrate reductase activity had a greater affinity for molybdenum than formate dehydrogenase. They also discovered that in

"molybdenum-deficient medium" (i.e. no addition of molybdate), 1 mM tungstate completely inhibited nitrate reductase activity. They were the first authors to report that tungstate had an inhibitory effect on the formation of bacterial dissimilatory nitrate reductase. Previously Pinsent (1954) reported that tungsten was a competitive inhibitor of molybdenum utilization in nitrate medium by aerobically cultured coliform bacteria. The same result was then reported in *Aspergillus niger* (Higgins *et al.* 1956). The following year, Takahashi and Nason (1957) found that tungstate was also a competitive inhibitor of molybdenum utilization in *Azotobacter vinelandii*, and that it also inhibited nitrate assimilation and nitrogen fixation. In addition, disc electrophoresis studies seemed to indicate that molybdenum-deficient spinach (*Spinacea oleracea* L.) leaves incorporated radiolabelled tungstate (^{185}W) in nitrate reductase although part of the radiolabelled tungsten dissociated during electrophoresis (Notton and Hewitt 1971). A "tungstoprotein analogue" was formed, but lacking nitrate reductase activities. The same result was obtained with a culture of the alga *Chlorella* (Paneque *et al.* 1972). These workers further demonstrated that when the synthesis of nitrate reductase occurred in the presence of both radioactive tungstate and cold molybdate, the enzyme was then active and less of the labelled tungsten was incorporated than in the absence of molybdate.

As with Spinach plants and *Chlorella* cells, *E. coli* cells cultured in the presence of 1 mM tungstate without added molybdenum synthesized an inactive nitrate reductase (Sperl and DeMoss 1975). They also showed that if the cells were washed and resuspended in W-free medium containing chloramphenicol (to prevent protein synthesis) and 1 mM molybdate, nitrate reductase activity was immediately restored. These workers then showed that ^{99}Mo was associated with the reactivated enzyme. This was done by subjecting the heat-released fractions from membranes to disc electrophoresis. Following electrophoresis, the gel tubes were incubated anaerobically in a tube containing phosphate buffer, nitrate and reduced methylviologen. Nitrate reductase activity was related to the appearance of a colorless band of oxidized methylviologen in the blue background. The gels were sliced, and radioactivity was determined in each slice. Using a different experimental approach, Amy and Rajagopalan (1979) also demonstrated that molybdenum could restore nitrate reductase activity in deficient cells. Tungsten-grown cells of *E. coli* (without addition of molybdenum) produced a W-containing cofactor that could complement a nitrate reductase deficient mutant of *Neurospora crassa, nit-1*, to form an intact but inactive nitrate reductase. The *nit-1* extracts were subjected to sucrose gradient centrifugation, and two peaks of NADPH-cytochrome *c* reductase activity were observed (4.5S and 7.9S). Subramanian and Sorger (1972)

reported that an inactive nitrate reductase from *Neurospora* sedimented at 7.8S. Addition of 1 mM of molybdate to the *nit-1* extracts previously complemented with either a metal-free cofactor or a tungsten-containing cofactor restored nitrate reductase activity which was then detected in the 7.9S region, thus indicating the cofactor was formed without molybdate but required molybdenum for the expression of nitrate reductase activity (Amy and Rajagopalan 1979).

Until 1980, most of the work in this field had been done with *E. coli* nitrate reductase of the respiratory formate-nitrate reductase system. At that time, Burke *et al.* (1980) examined the effects of molybdenum and tungsten upon synthesis of nitrate reductase in *Paracoccus denitrificans*, a true denitrifier. They found that in the absence of added molybdate, there was an 85 % inhibition of the nitrate reductase activity in cells cultured in 1 mM tungstate-containing medium. Their data indicate that traces of contaminating molybdenum only supported about 20 % of the activity observed when the medium was supplemented with 1 μM molybdate. It suggests that addition of molybdenum was required for full expression of the enzymatic activity. They also isolated a mutant strain (M-1) lacking nitrate reductase activity (Calder *et al.* 1980). This mutant synthesized a membrane protein that "had similar electrophoretic mobility to the active nitrate reductase" (Burke *et al.* 1980). Giordano *et al.* (1980), working with *E. coli* K12, observed that traces of

contaminating molybdenum supported only 35 % of the activity of cells grown in the presence of 1 μM molybdate, and the relative rocket area was 28 % that of the control. If 1 mM tungstate was present during growth, the activity was then 8 % that of the control supplemented with 1 μM molybdate, and 23 % that of the control not supplemented with molybdate, and the relative rocket area of the nitrate reductase protein was similar to the area produced by the protein from the cells cultured in 1 μM molybdate (W-free). Scott and DeMoss (1976) obtained the same result using electrophoresis under non-denaturing conditions. These data support the idea that, in W-containing medium, an inactive nitrate reductase is synthesized by bacteria, in which tungsten may somehow stabilize the protein structure. In W-free medium, contaminating molybdenum can support the synthesis of an active enzyme, but it appears that increasing the level of intracellular molybdate does increase the synthesis of nitrate reductase.

In contrast with all these reports, Lalande (1984) reported that growth of *Azospirillum brasilense* Sp 7 in tungstate-containing medium (10 and 25 mM), without addition of molybdate, did not result in nitrate reductase-deficient cells.

2.2.2.3. MOLYBDENUM CONTENT OF NITRATE REDUCTASE

The molybdenum content of the enzyme can be determined by various methods. MacGregor *et al.* (1974) found, by using neutron activation analysis, that the enzyme from *E. coli* K12 contained 3.2 ± 0.5 moles of molybdenum per mole of enzyme. Using the same strain of *E. coli*, Adams and Mortenson (1982) measured, by using the dithiol method (Clark and Axley 1955), that 0.80 mole of Mo was present per mole of enzyme. Atomic absorption gave a similar result: 0.61 mole of Mo per mole of enzyme in *E. coli* (Chaudry and MacGregor 1983). L'vov *et al.* (1980) stated that Mo is weakly bound to the Moco and dissociates easily upon purification.

Mo is likely involved in keeping the appropriate redox state of the active site of nitrate reductase (Stouthamer 1976). Electron paramagnetic resonance was used to show that molybdenum present in the nitrate reductase is mostly in the Mo(V) EPR active oxidation-reduction state. Reduction with dithionite generates the Mo(IV) state which is EPR silent, whereas oxidation with ferricyanide gives Mo(VI) oxidation state (Adams and Mortenson 1982). Consequently the reduction of nitrate may be brought about by the Mo(IV)-Mo(VI) cycle.

2.2.3. NITROGENASE

Nitrogenase is found in free-living bacteria or cyanobacteria , which use N_2 by nonsymbiotic means. It is also found in some bacteria living in close symbiotic associations with higher plants (legume/*Rhizobium* and actinorhizal symbioses) (Dilworth 1974, Brill 1980, Benson 1988). Nitrogenase can reduce N_2, as well as acetylene, cyanide, cyclopropene, and many other small molecules (Hardy *et al.* 1969).

Nitrogenase is a two-component metalloenzyme. The first component (dinitrogenase) is a MoFe protein, which consists of an $\alpha 2 \beta 2$ heterotetramer (Brill 1980). It has a molecular mass of approximately 240 kDa. The nitrogenase cofactor contains a Mo:Fe:S ratio of 1:8:6, with two Mo per molecule of nitrogenase, and it is the nitrogenase redox centre. The second component (dinitrogenase reductase) is an Fe protein. It is composed of an $\alpha 2$ homodimer with a single Fe_4S_4 cluster, with a molecular mass between 55 and 67 kDa depending upon the source of the protein. Two binding sites for MgATP are found in dinitrogenase reductase. For nitrogen fixation to occur, there has to be an electron flow from the Fe protein to the MoFe protein, coupled with MgATP hydrolysis (Dilworth 1974).

The Mo requirement for nitrogen fixation was discovered a long time ago (Bortels 1930). The role molybdenum plays in the activity and the synthesis of nitrogenase has been studied by

utilizing its analogue, tungsten. As stated in section 2.2.2.2., tungsten was found to be an inhibitor of nitrogen fixation in *A. vinelandii* (Takahashi and Nason 1957). ^{185}W was specifically associated with the FeMo protein component of nitrogenase (Benemann *et al.* 1973). This W-containing protein fraction did not reduce acetylene, and did not evolve hydrogen. According to inorganic chemistry, no chemical differences in the linear cubane Mo-Fe-S and W-Fe-S clusters can explain why W is not used in nitrogenase (Averil 1983). In addition, *Klebsiella pneumoniae* did not grow, under nitrogen-fixing conditions, when molybdenum was omitted from the medium, and nitrogenase specific activity was negligible (2 % that of the control supplemented with molybdate) (Brill *et al.* 1974). Adding tungstate to molybdate starved cells decreased even more the nitrogenase specific activity. Therefore tungsten seemed to have a specific antagonistic effect on nitrogen fixation.

In some experiments, however, the antagonistic effect of tungsten on nitrogen fixation was less obvious. Harper and Nicholas (1978) showed that increasing concentrations of tungstate (100-400 μM) had an inhibitory effect on the nitrate reductase activity of soybeans, but stimulated acetylene reduction activity associated with the plants. The tungstate diminished plant growth, regardless of the N source; but the effect was more pronounced when the plants were grown on nitrate. In the latter case, more carbon was available for

nodule growth and activity because the plants could not grow on nitrate. Nodulation of the nitrate-grown plants exceeded that of urea-grown plants at the highest level of tungsten, because urea metabolism is not inhibited by tungsten. This study suggests that the C:N ratio controls nodulation. In an earlier study, Davies and Stockdill (1956), in a field experiment, found that addition of molybdenum, or of tungsten (without molybdenum), stimulated pasture growth. Vanadium, however, had no such effect. These workers postulated that tungsten could assume the role of molybdenum (although less effectively) in nitrogen fixation by *Rhizobium*. Therefore these two studies suggest that tungstate may have positive effects on nitrogen fixation under certain conditions.

Hallenbeck and Benemann (1980) reported that, under molybdenum depletion conditions, the nitrogenase component I apoenzyme of *Anabaena cylindrica* was synthesized, and nitrogenase activity could be stimulated by addition of molybdenum without protein synthesis. In contrast, when *K. pneumoniae* was cultured in molybdenum-depleted medium, nitrogenase component I was not detected antigenically (Brill *et al*. 1974). In *C. pasteurianum*, Cardenas and Mortenson (1975) reported that tungsten affected the synthesis of both nitrogenase components, and that addition of high levels of molybdenum, without protein synthesis, did not activate nitrogenase activity. Therefore either Mo could not be incorporated into a possible preformed apoenzyme, or the

apoenzyme itself was not synthesized. In such a case, molybdenum would be essential for both nitrogenase activity and synthesis.

2.2.4. ALTERNATIVE NITROGENASES

The possibility of nitrogen fixation occurring in the absence of molybdenum or in the presence of other transition metals has intrigued scientists for many years. Several reports indicate that the inhibition of growth, under nitrogen-fixing conditions, produced by molybdenum depletion could be reversed by the addition of vanadium to the medium (Burns *et al.* 1971, Nagatoni and Brill 1974). The substitution of vanadium for molybdenum is logical since the two metals can perform oxidation-reduction reactions at low redox potentials (Cammack 1986).

Bishop *et al.* (1980) hypothesized that an alternative nitrogenase existed in *Azotobacter vinelandii*. They obtained, in N-free medium lacking molybdenum, at least four new proteins that appeared to be repressed by NH_4^+. A few years later, a new nitrogenase was isolated and characterized from *A. vinelandii* (Hales *et al.* 1986). This new nitrogenase consisted of two proteins. The first one was very similar to the conventional Fe protein, whereas the second protein (Av1) was shown to contain V and Fe (instead of Mo and Fe) with a V:Fe ratio of about 1:13.

An alternative nitrogen-fixation system has also been demonstrated in *Azotobacter chroococcum* (Robson *et al.* 1986). These authors showed that nitrogenase activity was proportional to added $VOSO_4$ between 1 and 10 nM. In a second experiment, they inoculated Mo-starved V-starved *A. chroococcum* cells into medium containing 10 nM $VOSO_4$. Nitrogenase activity, expressed as production of H_2, was rapidly activated in the first 30 min. This period was followed by a slower increase in nitrogenase activity. Growth was observed after 6 h. Vanadium was required for diazotrophic growth of the cells (Robson *et al.* 1986).

The alternative nitrogenase is strongly repressed by molybdenum (Bishop *et al.* 1988). Mo-nitrogenase is more stable, and catalyses less wasteful side-reactions (Cammack 1986). V-nitrogenase catalyses the reduction of acetylene not only to ethylene, but also to ethane (Dilworth *et al.* 1987). This reaction could be used in the screening of other bacteria for the presence of a V-nitrogenase, however it has been shown that Mo-nitrogenase can produce ehtane under some conditions, so there is a problem with using this test for screening (Dr. R. W. Miller; personal communication).*Clostridium pasteurianum* (Dilworth *et al.* 1987) and *Anabaena variabilis* (Kentemich *et al.* 1988) cultured on vanadium reduced acetylene partly to ethylene and partly (2-3 %) to ethane. The ethylene formation activity of *A. variabilis* was about one-third that of the molybdenum grown cells, which did not produce any detectable

ethane. Finally Kentemich *et al.* (1988) stated that vanadium did not alter the morphology of the heterocysts of *A. variabilis*. Eady (1990) reported that in *A. vinelandii* and *A. chroococcum* the extracted FeVco has very similar chemical, biological, and spectroscopic properties to FeMoco.

A third nitrogenase has been isolated in *Azotobacter vinelandii* (Chisnell *et al.* 1988). This enzyme contains neither molybdenum nor vanadium, but only iron (Fe-nitrogenase). Nitrogen was fixed by *A. vinelandii* cells lacking the genes *nifHDK* and cultured in Mo-free and V-free medium. These workers observed that the pattern of substrate reduction efficiency was $H^+ > N_2 > C_2H_2$. The Fe-nitrogenase was repressed by molybdenum or vanadium, as the V-nitrogenase was repressed by molybdenum. So the Fe-nitrogenase is less efficient, and wastes more hydrogen than the Mo-nitrogenase (Cammack 1988). Recent studies now show that there may be an Fe-nitrogenase in other bacteria. Bothe *et al.* (1990) stated that *Anabaena variabilis* cells can grow, under nitrogen-fixing conditions, with neither V nor Mo in the medium, suggesting the presence of a third nitrogenase. Fallik *et al.* (1990) presented hybridization data showing evidence that *Azotobacter paspali* possibly contains all three nitrogenases.

Cammack (1988) stated that these results discredit molybdenum and vanadium as essential to nitrogenase activity. Iron may indeed be the cofactor that binds nitrogen in the process of nitrogen fixation.

2.3. MOLYBDENUM UPTAKE AND TRANSPORT

Molybdenum is an essential trace element for most microorganisms in soils. However the concentration of molybdenum in soils is generally very low: about 15 μmol/kg mostly in the form of insoluble MoS_2 (Pope *et al.* 1980). To counteract this problem, some organisms have developed specific, high affinity systems for processing molybdenum.

The first step is to bind Mo from the environment. It has been demonstrated in the recent years that some bacteria requiring molybdenum can synthesize chelators capable of scavenging traces of Mo. This was first observed in *Bacillus thuringiensis* (Ketchum and Owens 1975). Patel *et al.* (1988) reported that an entire catechol-derivative siderophore molecule (2,3-dihydroxybenzoic acid) was involved in the transport of molybdenum in cowpea *Rhizobium*. They indicated that tungsten could not dissociate the molybdenum-siderophore conjugate. *Azospirillum lipoferum* secreted a related compound (3,5-dihydroxybenzoic acid) under both molybdenum-limited as well as molybdenum-supplemented conditions (Saxena *et al.* 1989). These workers stated that molybdenum was coordinated with 3,5-DHBA, which enhanced molybdenum uptake. It is known that *A. brasilense* cells also secrete, under Fe-deficient conditions, a siderophore (2,3-DHBA), which mediates the transport of Fe (Bacchawat and Ghosh 1987). However there has been no report on the occurrence of a siderophore-mediated

transport of molybdenum in *A. brasilense*.

The root-nodulating bacterium *Bradyrhizobium japonicum* has high- and low-affinity Mo uptake systems under nitrogen-fixing conditions (Maier *et al.* 1987). The high-affinity system is present under Mo-depletion conditions. These systems are also present in *K. pneumoniae* (Imperial *et al.* 1984). *B. japonicum* was cultured, under nitrogen-fixing conditions, in a medium containing traces of Mo. The supernatant was then taken, sterilized, and added to some Mo metabolism deficient mutants (strains JH-90, JH-119, JH-14, and JH-143). The mutants JH-90 and JH-119, which both have only a low-affinity Mo uptake system, were then capable of fixing N_2; suggesting extracellular Mo-binding factors were in the supernatant, and that these mutants were incapable of secreting t..ese chelators. However adding sterilized supernatant had no effect on nitrogenase activities of mutants JH-14 and JH-143, which have both high- and low-affinity Mo uptake systems. Therefore these strains do not fix nitrogen because they are inefficient in some intracellular Mo processes (Maier *et al.* 1987).

Under Mo-limited conditions, the molybdate transport ability of the cells could eventually influence the capacity to fix nitrogen, so Maier and Graham (1988) studied molybdate transport by the *B. japonicum* USDA136 strain. In a previous study, this strain was found to be the best able (out of 20 strains studied) to bind molybdate (Graham and Maier 1987). Molybdate was transported by a high affinity system: the

apparent K_m was 1 x 10^{-7} M, and the V_{max} was about 5 nmol.g^{-1} of dry cells.min^{-1}. Maier and Graham (1988) also observed that tungstate was a competitive inhibitor of molybdenum uptake with a K_i of 3.4 x 10^{-8} M.

The transport of ^{99}Mo into *Clostridium pasteurianum* cells, under nitrogen-fixing conditions, was energy dependent; it required sucrose and this process was inhibited by glycolysis inhibitors (Elliott and Mortenson 1975). Molybdate uptake in *C. pasteurianum* proceeded with an apparent K_m of 4.8 x 10^{-5} M and a V_{max} of 55 nmol·g^{-1} of dry cells·min^{-1}. In addition they noticed that tungstate competitively inhibited molybdate uptake, with an apparent K_i of 2.4 x 10^{-5} M, suggesting that W was transported by the molybdenum uptake system. Following binding to cell membrane, Mo is then processed intracellularly.

Hinton and Mortenson (1985) showed, by using anaerobic gel electrophoresis (N_2-fixing conditions) and autoradiography to locate the ^{99}Mo zones, that *C. pasteurianum* incorporated Mo in the following sequence: firstly in a low molecular weight species, then in a Mo binding-storage protein and a 30 kDa molybdoprotein. Because they observed an initial rapid rate of Mo accumulation, they suspected either that Mo was being incorporated into a high concentration of preformed "demolybdoproteins", or that Mo-binding proteins were produced under Mo-starvation conditions, and that when molybdenum is later made available to the cell, it binds very tightly to

these proteins and cannot be easily exchanged (Elliott and Mortenson 1975). Hinton and Mortenson (1985) also detected Mo incorporation into the MoFe protein 1 h after the beginning of Mo uptake. *Klebsiella pneumoniae* also accumulated Mo under nitrogen-fixing conditions, and Mo accumulation was repressed by O_2 and NH_4^+ (Pienkos and Brill 1981). *K. pneumoniae* cells metabolized Mo to non-exchangeable form by means of specific *nif* products. *K. pneumoniae* cells could maintain an internal concentration of molybdenum 25-fold higher than what is required for nitrogenase. On the other hand, *Azotobacter vinelandii* accumulated Mo constitutively, and this process was not repressed by O_2 or NH_4^+ (Pienkos and Brill 1981). *A. vinelandii* secretes a blue white siderochrome constitutively: N,N'-bis (2,3 dihydroxybenzoyl)-L-lysine. This chelator appeared to coordinate molybdate, and was hyperproduced under Mo-limited nitrogen-fixing conditions (Page and Tigerstrom 1982). In addition, these authors reported that this siderochrome could also bind to VO_3^-, WO_4^{2-}, Fe^{2+}, and Fe^{3+}. *A. vinelandii* stored very high levels of Mo, much higher levels than in *K. pneumoniae*, even when Mo was not required for growth. The molybdenum accumulated was bound mainly to a Mo storage protein which can then furnish Mo for component I synthesis when Mo is depleted in the environment (Pienkos and Brill 1981). It was later demonstrated that this Mo storage protein could also bind W constitutively (Hales and Case 1987). However this bacterium had a higher affinity for Mo

despite the overwhelming amount of W in the growth medium. Pienkos and Brill (1981) also found that the Mo storage protein is a tetramer of two pairs of different subunits binding at least 15 atoms of Mo per tetramer. This is more efficient than *C. pasteurianum* storage protein, which is a monomer binding 6 atoms of Mo per molecule (Mortenson and Thorneley 1979). Pienkos and Brill (1981) hypothesized that *A. vinelandii* possibly accumulates Mo constitutively because it "evolved in habitats with large fluxes in the concentration of Mo". It is obviously more advantageous to store high levels of Mo so that it would be available in times of scarcity.

2.4. MOLYBDENUM METABOLISM MUTANTS

2.4.1. *Escherichia coli chlD* MUTANTS

The study of mutants with lesions in Mo transport or Mo metabolism should help in understanding how Mo is processed and inserted in the Mo cofactor. *E. coli* mutants that are selected for chlorate resistance in the absence of O_2 (*chl*) lost the formate-nitrate reductase system. The *chlA*, *-B*, *-D*, *-E*, and *-G* mutants are pleiotropic: they lack nitrate reductase activities as well as other molybdoenzyme activities, but they are capable of synthesizing the nitrate reductase apoenzyme (Stewart and MacGregor 1982). Consequently these loci are involved in the synthesis of molybdenum

cofactors (molybdopterin cofactors). These workers postulated that the products of the *chlA* and *E* loci are associated with the biosynthesis of the pterin. Five years later, Johnson and Rajagopalan (1987) isolated two molybdopterin-deficient mutants of *E. coli* K12, and demonstrated that "there are two biochemically distinguishable complementation groups at both *chlA* (*chlA* and *chlM*) and *chlE* (*chlE* and *chlN*), and that all four complementation groups were involved in the biosynthesis of the molybdopterin". Miller and Amy (1983) pointed out that the products of the *chlB*, *D*, and *G* loci are required for the processing of molybdenum. This was demonstrated by the finding that 1 mM molybdate suppressed the *chlD* and *chlG* mutant phenotypes. High concentrations of molybdate possibly circumvented the role of these gene products.

More recently, Giordano *et al.* (1984) established that 10 mM tungstate inactivated three molybdoenzymes in *E. coli* K12; nitrate reductase, formate benzyl-viologen oxidoreductase and trimethylamine-N-oxide reductase. These three enzymes are normally synthesized and induced under different conditions. Mixing soluble fractions of *chlA* and *chlB* mutants, under appropriate conditions, led to the formation of all three active enzymes in both particulate and soluble forms. This process is termed "complementation" (Azoulay *et al.* 1969). The *chlB* product (association factor) was thought to be implicated in the modification of the molybdenum cofactor in nitrate reductase during synthesis and assembly of the subunits of the

enzyme (MacGregor 1975). The *chlA* product (Protein PA) might be required for the insertion of molybdenum into the molybdenum cofactor to establish its active configuration (Giordano *et al.* 1982). These two purified proteins have the ability to activate, by complementation, all three enzymes. The fractions of the *chlB* mutants grown with 10 mM tungstate could not reconstitute the enzymes, when mixed with fractions of *chlA* mutants grown without tungstate. Nevertheless if 1 mM molybdate was added to the *chlA* mutant fraction, the recovery in all three enzymatic activities was noticed (Giordano *et al.* 1984). On the other hand, the fractions of the *chlB* mutants grown in W-free medium could reconstitute the three enzymes, when mixed with fractions of *chlA* mutants cultured with 10 mM tungstate. They concluded that tungstate inhibited the expression of the *chlA* gene product. However, the following year they demonstrated, using rocket immunoelectrophoresis analysis, that cells growing in 10 mM tungstate synthesized an inactive protein PA to the same level as during growth in the absence of tungstate. Using column chromatography, they also revealed that inactive and active protein PA are of approximately the same molecular weight (Giordano *et al.* 1985). Because increasing levels of molybdate did not have any significant effect either on the amount of protein PA present or on its activity, Giordano *et al.* (1985) stated that protein PA probably only required the level of molybdenum contamination normally found in growth medium.

Johann and Hinton (1987) reported that a *chlD* mutant accumulated Mo in the periplasmic space. The *chlD* cells appeared to concentrate molybdenum to a level at least 20-fold higher than the level in the growth medium (Scott and Amy 1989). Johann and Hinton (1987) postulated that *chlD* is an operon involved in the active transport of Mo (transport operon). Two open reading frames (ORFs) were revealed from DNA sequence analysis of a fragment that complemented an *E. coli chlD* mutant. One ORF, responsible for complementing the *chlD* mutant, showed regions of homology with nucleotide binding proteins and hydrophilic inner membrane proteins. The other ORF showed a sequence coding for a hydrophobic inner membrane protein, which was perhaps a binding protein involved in transport systems.

Miller *et al.* (1987) constructed some *E. coli* strains carrying fusions of the *lac* genes to the *chlD* promoter by using Mu d1 (Ap *lac*) bacteriophage. Transcription from the *chlD* promoter was reflected by ß-galactosidase activity. They then observed that low concentrations of molybdenum, less than 100 nM, with nitrate in the mediur induced the expression of the *chlD* gene (ß-galactosidase activity). In this way *E. coli* cells can generate sufficient levels of molybdenum to supply the needs of the cells. On the other hand, in the presence of high levels of molybdenum, the *chlD* gene expression was lowered greatly, even though nitrate reductase activity was increased. These workers postulated that under such

conditions, molybdenum might be supplied (for incorporation into the cofactor) in a nonenzymatic way. This experiment demonstrated that the *chlD* gene expression was regulated by metal availability. In addition Miller *et al.* (1987) constructed *chlD-lac* fusions plus an additional mutation in a *chl* or *nar* gene. The *nar* genes are generally involved in nitrate reductase induction. In this way they found that the *chlB*, *narC*, *narI*, and *narL* genes had an effect on the expression of the *chlD* gene. They finally concluded that "molybdenum utilization, Mo cofactor metabolism, and nitrate reductase induction are linked" (Miller *et al.* 1987).

It was reported that molybdenum, as well as tungsten, enhanced the nitrate induction of *narC* in *chlD* mutants; indicating that a molybdenum compound acted as a coinducer. Similarly, addition of molybdenum (or tungsten) was also required for the repression of the *frd* genes (encoding for fumarate reductase) by nitrate in *chlD* mutant, suggesting the same molybdenum compound could also become a corepressor (Pascal *et al.* 1982, Iuchi and Lin 1987). Iuchi and Lin 1987 came to the conclusion that the coeffector was not the Mo effector, but either molybdate itself or a compound such as Mo-X (Ugalde *et al.* 1985). Therefore this system does not allow nitrate reductase synthesis under Mo-starvation conditions. At the same time, the dual repression of *frd* genes by nitrate and molybdate inhibits the synthesis of fumarate reductase as long as nitrate reductase synthesis can result in

an active form.

2.4.2. Moco AND FeMoco BIOSYNTHESIS PATHWAYS

Maier *et al.* (1987) stated that more molybdenum is required for nitrogen fixation than for nitrate reduction, probably because nitrate reductase has a greater catalytic efficiency. Accordingly bacteria capable of producing both FeMoco and Moco must somehow regulate the pathways of utilization of molybdenum for both biosyntheses. Ugalde *et al.* (1985) studied these interactions in *K. pneumoniae*. The *mol* mutants of *K. pneumoniae* required a high concentration of molybdate to exhibit the same level of activity expressed by the wild type. These authors found that the substitution of cystine for sulfate as the sulfur source in the medium reduced the molybdenum requirements of these *mol* mutants to the levels required by the wild type strain. However the new sulfur source did not affect the molybdenum requirement of the wild type for maximal nitrogenase activity, and indeed higher nitrogenase activities (acetylene reduction) were also observed with cystine as the sulfur source. On the other hand, the molybdenum requirement of *K. pneumoniae mol* mutants (and *E. coli chlD* mutants) for nitrate reductase activity was increased by at least 100-fold. Thus Ugalde *et al.* (1985) then hypothesized that cystine in these *mol* mutants either allowed the formation of FeMoco in the presence of low levels of

molybdate, or that sulfate interfered with the utilization of molybdate. Molybdenum and sulfur enter the cell by two independent mechanisms (Ugalde *et al.* 1985). Molybdate could be transformed by the *mol* product into the intermediate Mo-X. If the concentrations of molybdate are high, molybdenum would then be apparently incorporated non-enzymatically. Mo-X could then either be incorporated into a pteridine to produce an active Moco for nitrate reductase, or it could react with cystine to produce the first intermediate (Mo-Y) for the FeMoco pathway. This process is, thus, enhanced by high intracellular concentrations of either Mo-X or cystine (as well as the *nifQ* product). In summary, high levels of cystine, in the absence of a *mol* (or *chlD* in *E. coli*) product, would scavenge Mo-X, and likely reduce the rate of synthesis of Moco.

2.5. DENITRIFYING ENZYMES

2.5.1. GENERAL OVERVIEW

Denitrification is the process by which nitrate (NO_3^-) and nitrite (NO_2^-) are reduced to nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen gas (N_2). Denitrification usually proceeds anaerobically: the N oxides function as alternative electron acceptors, replacing oxygen. Therefore in flooded anaerobic soils, fixed nitrogen can be lost to the atmosphere.

Several aspects of denitrification have been reviewed in the last decade: ecology (Knowles 1982); enzymology (Hochstein and Tomlinson 1988); evolution (Betlach 1982); and genetics (Ingraham 1985).

Nitrate reductase catalyzes the first step in denitrification (see section 2.2.2). The second step is catalyzed by nitrite reductase.

2.5.2. NITRITE REDUCTASE

According to Payne (1981), nitrite reduction is the reaction that characterizes denitrification. This reaction distinguishes denitrification from ammonia assimilation. Two different types of dissimilatory nitrite reductase are known: one is a metalloprotein containing copper; and the second one is a heme protein formed with *c*- and *d*-type cytochromes (Hochstein and Tomlinson 1988). Since *A. brasilense* possesses a *cd*-cytochrome nitrite reductase (Lalande and Knowles 1987), only this type of protein will be discussed here.

The *cd*-type protein is composed of two identical subunits, each of which contains a *c*- and *d*-type cytochrome (Huynh *et al.* 1982). It has a molecular mass of approximately 120 kDa (Newton 1969, Sawhney and Nicholas 1978).

Mancinelli *et al.* (1986) purified nitrite reductase from both the membrane and cytoplasmic fractions from *Paracoccus halodenitrificans*. Osmotic lysis of the spheroplasts

demonstrated that little if any activity was in the periplasm, and the nitrite reductase appeared to be firmly bound to the membrane. By using reduced phenazine methosulfate as the artificial electron donor, they observed that the membrane nitrite reductase produced mainly N_2O, whereas the cytoplasmic nitrite reductase evolved mostly NO. If methylviologen was the electron donor, then NO and NH_4^+ were respectively produced. These authors concluded that the products of nitrite reduction were determined by the location of the enzyme, and by the redox potential of the electron donor. However there is still controversy about the product of this reaction. Payne (1981) proposed that the conversion of NO_2^- to N_2O required two enzymes; one reducing NO_2^- to NO, and a second one reducing NO to N_2O. NO would be a free intermediate in this model. Another model suggested that NO_2^- is reduced directly to N_2O by a single enzyme, and NO is not evolved as a free intermediate (Garber and Hollocher 1982). The reduction of NO_2^- would occur before the N-N bond formation. A third way has been proposed in which NO_2^- is also reduced directly to N_2O by a single enzyme. However the N-N bond would be formed prior to any reduction (Averill and Tiedje 1982).

Mancinelli *et al.* (1986) also detected nitrite reductase activity in *P. halodenitrificans* by subjecting the various purified enzymes to disc electrophoresis under non-denaturating conditions. The technique they used was very similar to that utilized to localize nitrate reductase

activity (Sperl and DeMoss 1975; see section 2.2.2.2.). Nitrite reductase could also oxidize methylviologen, and the appearance of an oxidized band was related to a positive activity. The electrophoretic behavior of both nitrite reductases isolated from the membrane and the cytoplasmic fractions of *P. halodenitrificans* suggested they were identical.

The enzyme from *Thiobacillus denitrificans* was also found to be membrane bound (Sawhney and Nicholas 1977). On the other hand, Ferguson (1987) stated that the nitrite reductases from *P. aeruginosa* and *P. denitrificans* "are located in the periplasm and can be released from the cells as water-soluble proteins upon disruption of the cell wall". Nitrite reductase in *P. stutzeri* was also most likely located in the periplasmic space (Coyne *et al.* 1989). Since nitrite reductase is involved in oxidative phosphorylation, these proteins are probably associated with the cytoplasmic membrane, but they may be easily solubilized upon cell disruption (Knowles 1982).

As stated above (section 2.2.2.1.), the active site of nitrate reductase is exposed at the cytoplasmic surface of the membrane. Boogerd *et al.* (1983) postulated that a nitrate/nitrite antiporter facilitated the uptake of nitrate against an opposing membrane potential. This antiporter would deliver nitrite to the nitrite reductase, which is likely located in the periplasm (or on the periplasmic side of the membrane).

Nitrite reductase activity might be controlled by nitrate. Kodama *et al.* (1969), working with *P. stutzeri*, showed that nitrite reduction was initiated only after exhaustion of nitrate in the medium. This suggests that nitrate inhibited nitrite reductase activity. These workers postulated that two mechanisms could explain this: either nitrate represses the formation of the nitrite reducing system to some extent, or nitrate competes as an electron acceptor. The latter assumption would require that nitrate is preferentially utilized as an electron acceptor even in the presence of high levels of nitrite. Payne and Riley (1969), working with *Pseudomonas perfectomarinus*, demonstrated that all the enzymes required for reduction of nitrite to N_2 were present, but not active until nitrate was completely reduced. Körner and Zumft (1989) also reported an instant inhibition of nitrite reduction in whole cells of *P. stutzeri* on addition of nitrate, which suggested "an indirect action on nitrite reductase or a mechanistic site of action before the enzymatic reduction step". Lalande and Knowles (1987) postulated that the long lag in the nitrite reduction of *A. brasilense* observed in the presence of high levels of nitrate might be due to an effect on the synthesis or activity of nitrite reductase.

On the other hand, Betlach and Tiedje (1981) reported that nitrate did not directly inhibit nitrite reduction in *Alcaligenes* sp. or in *Pseudomonas fluorescens*. They stated

that nitrite accumulation was dependent on the relative rates of nitrate and nitrite reduction. In addition, nitrite reduction by a *Pseudomonas* strain was shown not to be influenced by presence of nitrate (Juszczak and Domka 1988).

2.5.3. NITRIC OXIDE REDUCTASE

Nitric oxide (NO) reductase is the least characterized enzyme in denitrification (Zumft *et al.* 1979, Ferguson 1987, Hochstein and Tomlinson 1988). In the past, there were doubts about the chemical reactivity of nitric oxide as well as its presence as a stable intermediate in denitrification. Today, however, several studies have demonstrated the occurrence of nitric oxide reductase in some denitrifying bacteria. For example, Betlach and Tiedje (1981) observed that an *Alcaligenes* species, *Pseudomonas fluorescens*, and a *Flavobacterium* isolate "all produced small amounts of nitric oxide during denitrification in a pattern suggesting that nitric oxide was also under kinetic control similar to that of nitrite and nitrous oxide." Garber *et al.* (1982) first demonstrated that exogenous nitric oxide was energy yielding in *P. denitrificans*.

NO reductase is an important enzyme in the global N cycle because it catalyzes the formation of N-N bonds, "and thus, in essence, reverses dinitrogen fixation" (Heiss *et al.* 1989). Urata and Satoh (1985) showed that the NO reductase from a

Rhodopseudomonas sphaeroides strain co-purified with the cytochrome bc_1 complex, and was thus membrane bound. A similar finding was also reported in *Pseudomonas stutzeri* (Heiss *et al*. 1989). These workers demonstrated that NO reductase was membrane bound, and that it stoichiometrically transformed NO to N_2O in an ascorbate-phenazine methosulfate-dependent reaction.

2.5.4. NITROUS OXIDE REDUCTASE

N_2O reductase is the last enzyme in denitrification. It can reduce N_2O to N_2 coupled to ATP formation, and can utilize methylviologen as the artificial electron donor (Kristjansson and Hollocher 1980). This enzyme is usually purified as a copper protein (Snyder and Hollocher 1987). In *P. stutzeri* and *P. fluorescens*, it has a molecular mass of about 120 kDa (Matsubara and Zumft 1982). Nitrous oxide reductase does not contain Mo or Fe (Kristjansson and Hollocher 1980). These authors observed that this protein was extremely labile at room temperature, and very sensitive to O_2. The partially purified enzyme from *P. denitrificans* was also reported to be very labile, and to lose half its activity in about 30 min (Kristjansson and Hollocher 1981).

Pseudomonas perfectomarina cells grown anaerobically in dithiozine-treated medium supplemented with either nitrate or nitrite lacked nitrous oxide reductase activity. When these

cells were then incubated with copper and rifampin, they reduced nitrous oxide (Matsubara *et al.* 1982). This suggested that a copper-deficient apoenzyme was synthesized during growth in copper depleted medium.

The properties of various nitrous oxide reductases differ, although these variations might reflect differences in methodology, especially whether or not purification is carried out entirely under anaerobic conditions (Hochstein and Tomlinson 1988). The proteins from *P. perfectomarina* and *Alcaligenes* sp. both have a molecular mass of 120 kDa, and are composed of two identical subunits (Coyle *et al.* 1987; Matsubara and Sano 1985). They contain 8 and 5 copper atoms per mole of enzyme, respectively. Nitrous oxide reductase from *P. denitrificans* is a cytoplasmic enzyme having a molecular mass of 144 kDa, and containing 8 copper atoms per mole (Snyder and Hollocher 1987). Recently nitrous oxide reductase from *Achromobacter cycloclastes* was purified to homogeneity under aerobic conditions by various methods (Hulse and Averill 1990). This enzyme has a molecular mass of 72 kDa, and contains 3.8 copper atoms per molecule.

Nitrous oxide reductase activity is inhibited by acetylene (Balderston *et al.* 1976, Yoshinari and Knowles 1976); so accumulation of N_2O can be correlated to denitrification rates in general, or more specifically to nitrite reductase activity in pure cultures.

3. MATERIALS AND METHODS

3.1. BACTERIAL STRAINS AND GROWTH MEDIA

3.1.1. *Azospirillum brasilense*

Stock cultures of the bacteria used in this work were kept in 10 % glycerol at -80° C. *Azospirillum brasilense* Sp7 (ATCC 29145) was cultured in a malate-containing medium (Tibelius and Knowles 1983). The medium contained, per liter of deionized water: 1.0 g malic acid (neutralized with NaOH before addition to the medium); 3.0 g K_2HPO_4; 2.0 g KH_2PO_4; 0.1 g NaCl; 0.006 g Sequestrene (13 % iron); 0.1 g $MnSO_4.H_2O$; 1.0 g NH_4Cl. The pH of the medium was adjusted to 6.80 prior to autoclaving. To avoid precipitation, 0.2 g $MgSO_4.7H_2O$ and 0.02 g $CaCl_2.2H_2O$ were autoclaved separately and added to the cooled medium. This medium was not supplemented with sodium molybdate, $Na_2MoO_4.2H_2O$ (Mo-limited medium) unless otherwise stated, and sodium tungstate (99.8 % $Na_2WO_4.2H_2O$, 0.0006 % Mo) was added to the batch culture when stated. Atomic absorption analyses (see section 3.5.) showed that 9 out of 10 batches of medium sampled at random had an average Mo concentration of less than 10 ppb. For denitrification studies, this medium was supplemented with various amounts of $NaNO_3$ as specified in the text. For nitrogen fixation studies, N sources were omitted and if necessary the medium was made semi-solid by adding

0.2 % (w/v) Noble agar. Purity was checked on nutrient agar plates.

3.1.2. *Paracoccus denitrificans*

Paracoccus denitrificans (ATCC 19367) was used as a control in some experiments. It was cultured in a modified GGS medium (Burke *et al.* 1980) containing, per liter of deionized water: 3.0 g KH_2PO_4; 7.0 g $K_2HPO_4.3H_2O$; 0.0075 g $MnSO_4$; 0.006 g Sequestrene (13 % Fe); 3.38 g sodium glutamate; 2.50 g sodium succinate; and various amounts of $NaNO_3$ as specified in the text. The pH of the medium was adjusted to 7.0 prior to autoclaving. To prevent precipitation, 0.2 g $MgSO_4.7H_2O$ and 0.053 g $CaCl_2.2H_2O$ were autoclaved separately and added to the cooled medium.

3.1.3. *Azotobacter vinelandii*

Azotobacter vinelandii (MAC 617, culture collection, Department of Microbiology, Macdonald College) was used as a biological method to deplete traces of molybdenum in the medium used for *A. brasilense* growth (Imperial *et al.* 1984). *A. vinelandii* was cultured in a Mo-limited modified Burk medium (Strandberg and Wilson 1968). The medium contained, per liter of deionized water: 0.2 g KH_2PO_4; 0.8 g K_2HPO_4; 0.001 g Sequestrene (13 % Fe); 20 g sucrose; 1.9668 g ammonium

acetate (25 mM). The pH was adjusted to 7.0 prior to autoclaving. To prevent precipitation, 0.2 g $MgSO_4.7H_2O$ and 0.045 g $CaCl_2.2H_2O$ were autoclaved separately and added to the cooled medium. *A. vinelandii* cells were grown in this Mo-limited medium until they reached mid-log phase. The cells were then harvested by centrifugation (10000 x g, 4° C, 10 min), washed once in the medium to be treated, and resuspended to one-half of the original culture density in the medium to be treated. This suspension was incubated 20 min at 25° C. It was then centrifuged (10000 x g, 4° C, 20 min) and filtered (0.45-μm pore size). The treated medium was stored at 4° C, and plated out for sterility check.

3.2. PHYSIOLOGICAL STUDIES WITH *A. brasilense* AND *P. denitrificans*

3.2.1. INOCULUM PREPARATION

For *A. brasilense*, a stock culture was used to inoculate 10 mL of tungstate free (W-free) Mo-limited medium in a 50-mL Erlenmeyer flask. A loopful was spread on a nutrient agar plate for purity check. It was grown aerobically on a gyratory shaker (250 rpm) at 30° C for 12 h. This culture was then transferred to 100 mL of W-free medium in a 250-mL flask and similarly incubated for 12 h (late log phase; approximately 200 μg protein^{-1}.mL^{-1}). The same procedure was used for *P.*

denitrificans, with the exception that the times of incubation were 10 h, and cells in the late log phase reached approximately 350 μg protein.mL^{-1}.

3.2.2. BATCH CULTURES

3.2.2.1. SPARGING FLASKS (800-mL CULTURE)

A culture, as prepared in section 3.2.1., was harvested by centrifugation (10000 x g, 4°C, 10 min) (Sorvall RC-5B, Du Pont Instruments), washed twice in 67 mM Sorensen's phosphate buffer (pH 7.0) (Sober 1968), and the final pellet was resuspended in 40 mL of the appropriate growth medium. This was used to inoculate (5 % v/v) a 1-L Erlenmeyer flask containing 760 mL of growth medium (W-free or W-containing medium as specified) supplemented with 0.25 mM $NaNO_3$. This batch culture was incubated at 30° C, stirred by a teflon-coated magnetic bar, and sparged at 450 $mL.min^{-1}$ with N_2 (Union Carbide Canada Ltd, Toronto, Ontario), at least 15 min prior to inoculation, to maintain anaerobiosis. Liquid samples (3 mL) were aseptically withdrawn by syringe, at appropriate times, from a septum located at the bottom of the flask. One mL was used to measure optical density, and the remainder was frozen for nitrate, nitrite and protein analyses. Gas samples were withdrawn from a Suba-seal (William Freeman Company, Barnsley, England) connected to the gas outlet tubing.

3.2.2.2. SPARGING FLASKS (5-L CULTURE)

A culture (100 mL), as prepared in section 3.2.1., was transferred into 900 mL of W-free medium, and incubated aerobically at 30° C for 12 h. These cells were harvested by centrifugation (10000 x g, 4° C, 10 min), washed once in 67 mM Sorensen's buffer (pH 7.0), and resuspended in 50 mL of the appropriate medium. This was used to inoculate (1 % v/v) 4950 mL of medium supplemented with 10.0 mM $NaNO_3$. In some experiments, the medium contained 1 μM Na_2MoO_4 and 7 μCi $^{99}Mo.(nmol\ Mo)^{-1}$. The medium was dispensed in a 12-L carboy fitted with a rubber stopper. The medium was sparged at 450 $mL.min^{-1}$ with N_2, and stirred by a teflon-coated magnetic bar. The incubation was at 30° C in a Puffer-Hubbard incubator (Grand Haven, Michigan). Gas samples were withdrawn from a Suba-seal connected to the gas outlet tubing.

3.2.2.3. VACUUM FLASKS (100-mL CULTURE)

A culture (100 mL), as prepared in section 3.2.1., was harvested by centrifugation (10000 x g, 4° C, 10 min), washed once in 67 mM Sorensen's buffer, and resuspended in 25 mL of W-free medium. Ten mL was used to inoculate (10 % v/v) 90 mL of culture medium (supplemented with 1.00-1.25 mM $NaNO_3$) contained in a 500 mL Erlenmeyer vacuum flask sealed with a rubber stopper, evacuated and backfilled with N_2 prior to

inoculation. Each flask was incubated on a gyratory shaker (250 rpm) at 30° C. Liquid samples (3 mL) were aseptically withdrawn by syringe, at appropriate times, from a septum located at the bottom of the flask. Optical density was measured, and the remainder was frozen for nitrate, nitrite and protein analyses. Gas samples were withdrawn aseptically from a septum located at the top of the flask.

For all the anaerobic treatments, the oxygen levels were monitored regularly using a Fisher-Hamilton gas partitioner to ensure anaerobiosis was maintained throughout any experiment.

3.2.2.4. AEROBIC CULTURES FOR GROWTH CURVES

A resuspended culture, as prepared in section 3.2.2.1., was used to inoculate (5 % v/v) 47.5 mL of growth media contained (in 125-mL Erlenmeyer flasks) supplemented with various concentrations of tungstate. A control flask was set up with 25 mM NaCl to monitor the effect of the salt on aerobic growth. The flasks were incubated aerobically on a gyratory shaker (250 rpm) at 30° C, and 0.5 mL samples were aseptically withdrawn at appropriate times for determination of optical densities.

3.2.3. NITROGEN FIXATION ASSAYS

A culture (100 mL), as prepared in section 3.2.1., was harvested by centrifugation (10000 x g, 4° C, 10 min) and washed three times in 67 mM Sorensen's buffer (pH 7.0). The cells were then resuspended in 5.0 mL of N-free W-free Mo-limited medium, and 0.125 mL was used to inoculate (1.4 % v/v) 8.375 mL of semi-solid medium kept at 55° C in a water bath. The medium was contained in 14-mL serum bottles. Different treatments consisted of adding various combinations of 830 μM Na_2MoO_4 and 10 mM Na_2WO_4. The flasks were plugged with cotton, incubated 36 h at 30° C without shaking. The cotton plugs were then removed and replaced by sterile rubber stoppers with metal crimps. Flaming was avoided at this step in order to prevent contamination with C_2H_4. Acetylene was added to the gas space (5 % v/v), and the flasks were incubated statically at 30° C for another 24 h. Gas samples were then analysed for C_2H_4 and C_2H_2 using a Perkin-Elmer Gas Chromatograph (Model 3920) equipped with a flame ionization detector (Nelson and Knowles 1978).

3.2.4. BATCH CULTURE HARVESTING AND PREPARATION OF THE VARIOUS FRACTIONS.

3.2.4.1. CELL-FREE EXTRACT FROM 800-mL CULTURE

The batch culture was set up as in section 3.2.2.1. This was used mainly for producing whole cells and cell-free-extracts for nitrate reductase and nitrite reductase assays. At appropriate times, the cell culture was siphoned under anaerobic conditions into centrifuge bottles, and the cells were harvested by centrifugation (10000 x g, 4° C, 10 min). The cells were maintained in conditions as anaerobic as possible by flushing flasks and tubes with N_2 during transfers. Cells were washed three times in 67 mM Sorensen's buffer, and resuspended in 5 or 10 mL of Sorensen's buffer plus 0.1 mL of 100 mM phenylmethylsulfonyl-fluoride (PMSF) to inhibit protease activity. These cells were broken under anaerobic conditions using a sonicator (model W-220F, Ultrasonics, inc. Plainview, NY, U.S.A. 11803). The cell suspension was subjected to five bursts of 5 min each (130 watts), separated by cooling periods of 5 min. This suspension was centrifuged (10000 x g, 4° C, 10 min) to precipitate unbroken cells.

3.2.4.2. CELL-FREE EXTRACT FROM 5-L CULTURE

The cells were harvested as in section 3.2.4.1. However the cells were resuspended in 40 mL (4 x 10 mL) of 67 mM Sorensen's buffer plus 0.4 mL PMSF. Sonication proceeded as explained above by sonicating 10-mL sample at a time.

3.2.4.3. PREPARATION OF SOLUBILIZED MEMBRANES

Conditions were kept as anaerobic as possible by flushing everything with N_2. Cell-free extract from a 5-L culture (section 3.2.4.2.) was ultracentrifuged (180000 x g, 4° C, 60 min) (model L8M, Beckman). The supernatant (soluble fraction) was kept on ice (or at -80° C) for further assays (NO_2^- reductase, protein), and the precipitated membranes were resuspended in 6 mL of 67 mM Sorensen's buffer. The membranes were homogenized, using a tissue homogenizer, under a stream of N_2. To solubilize the membranes of *A. brasilense*; 0.2 % deoxycholate, 1 % Triton X-100, 10 % sucrose, and 10 mM ethylenediaminetetraacetic acid (all final concentrations) were added to the suspension of homogenized membranes to make up a final volume of 8 mL (modified from Schink and Schlegel 1979). This suspension was incubated at room temperature for 1 h. The suspension was then ultracentrifuged (105000 x g, 4° C, 60 min), and the supernatant (solubilized membranes) was kept for assays (NO_3^- reductase, NO_2^- reductase, protein). The

membranes of *P. denitrificans* were solubilized by adding 40 mM Tris-HCl buffer pH 7.5 (final concentration), deoxycholate and KCl (respectively 1 % and 0.01 M final concentration) to make up a volume of 8 mL (Calder *et al.* 1980). This suspension was incubated on ice for 30 min, and ultracentrifuged as above. For cultures grown in ^{99}Mo, aliquots (0.1 mL) from each fraction were taken and transferred into scintillation fluid, and counted using the ^{32}P channel of a Beckman LS 7500 Liquid Scintillation Spectrometer. Data were adjusted to take into account the 66-h half-life of ^{99}Mo.

3.2.5. ^{99}Mo UPTAKE EXPERIMENTS

3.2.5.1. ^{99}Mo TRANSPORT

Cells were cultured aerobically in 500 mL of W-free Mo-limited medium and harvested aerobically, unless otherwise stated, washed twice in Sorensen's buffer, and resuspended in 32 mM K-PO_4 buffer (pH 7.0) in such a way that the $O.D._{430}$ in the assay was approximately 1.0. Assays were done in 30- and 60-mL serum bottles containing 2.5 and 10 mL of buffer, respectively. The assay buffer contained 10 μM Na_2MoO_4 and 5.82 μCi $^{99}Mo.(nmol\ Mo)^{-1}$. At appropriate times, triplicates (0.3 mL) were removed and rapidly filtered (HA type filters, pore size, 0.45 μm; Millipore Corporation). The filtering unit was a 10-sample filtering manifold. After

rapidly filtering the samples, the cells on the filter were washed three times with 3 mL of 32 mM K-PO_4 buffer (pH 7.0) containing 1.0 mM Na_2MoO_4. Background counts correcting for non-specific binding of molybdate to heat-killed cells were subtracted from all sample counts. The filters were then placed in 5 mL of scintillation fluid, and counted as in section 3.2.4.3.

3.2.5.2. ^{99}Mo ACCUMULATION UNDER GROWING CONDITIONS

The set-up in section 3.2.2.3. was used to monitor ^{99}Mo accumulation under denitrifying conditions over 12 h, with the exception that the inoculum was grown in treated medium. The medium contained 1 μM Na_2MoO_4 and 5.80 μCi ^{99}Mo.(nmol Mo)$^{-1}$. Samples (0.3 mL) were withdrawn by syringe, and rapidly filtered and counted as in section 3.2.5.1. Background counts correcting for ^{99}Mo retention by filters without cells were subtracted from all the sample values.

3.3. ASSAYS

3.3.1. NITRATE REDUCTASE ASSAY

The nitrate reductase assay was modified from the procedure used by MacGregor (1978). The assays were done in 14-mL vials, which were sealed with silicone-reinforced butyl

rubber stoppers and aluminum crimps. The assay mixture (2 mL) contained 0.2 mL of 20 mM methyl viologen; 10 μL of 10 mM Cleland's reagent; and various volumes of Na_2WO_4 solution pH 7.0 (prepared in 67 mM Sorensen's buffer) and of 67 mM W-free Sorensen's buffer pH 7.0 were added to produced the desired concentration of tungstate. The vials were evacuated and backfilled with He using a manifold. The reaction was started by adding 0.2 mL of a solution (kept anaerobically) of $NaHCO_3$ (0.8 %) and $Na_2S_2O_4$ (0.8 %) as the reducing agent, 0.1 mL of $NaNO_3$ (10 mM final concentration), and 0.2 mL of either whole cell suspension, cell-free extract suspension, or membrane preparation. The flasks were incubated on a gyratory water-bath shaker (250 rpm) at 30° C. After 10 min, the flasks were opened and vortexed so that methyl viologen became oxidized. One half of the assay mixture was immediately centrifuged to remove the cells and analyzed for nitrite, whereas the remaining solution was frozen until it was assayed for protein.

3.3.2. NITRITE REDUCTASE ASSAY

The assay mixture for the nitrite reductase assay was the same as for the nitrate reduction assay with the exception that sodium nitrate was replaced by 10 mM $NaNO_2$. In addition, acetylene (10 %) was added to the gas phase to block N_2O reduction (Yoshinari *et al.* 1977). The acetylene utilized was

scrubbed by passing it through two concentrated H_2SO_4 traps in order to remove contaminants (CH_3COCH_3, H_2S, NH_3, PH_3, and SO_2) that could damage the detector (Tough and Crush 1979) . A sample (C.2 mL) from the gas phase was removed using a He-flushed syringe and injected into a gas chromatograph (Model 3920 Perkin Elmer) equipped with a ^{63}Ni electron capture detector operated at 290° C and 3.0 mA standing current for determination of N_2O concentration (Chan and Knowles 1979).

3.4. POLYACRYLAMIDE GEL ELECTROPHORESIS

Native 7.5 % polyacrylamide tube gels (pH 8.9) were prepared essentially by the prccedure of Davis (1964). The loading mixture contained the following: 1.0 mL of protein (soluble fraction or solubilized membranes as prepared in section 3.2.4.3.); 0.375 mL of 40 % glycerol; and 0.125 mL of 0.012 % bromophenol blue as the tracking dye. A stacking gel (2.5 % polyacrylamide) was put on top of the separating gel and 0.3 mL of the mixture was layered on the stacking gel. The gels were subjected to electrophoresis at 2 mA per gel for 150 min. After electrophoresis was completed, the gels were removed anaerobically by extruding with water, and were introduced into test tubes containing 15 mL of 6.67 mM methyl viologen in 67 mM Sorensen's buffer (pH 7.0). This mixture was continually sparged with N_2 to maintain anaerobiosis. Excess dithionite (20 mg) was then added to reduce the methyl

viologen. The tubes were sealed with a Suba-seal, and the mixture was allowed to equilibrate for 5 min. Nitrate reductase, nitrite reductase, and nitrous oxide reductase activities on the gels were determined by adding anaerobically, by means of a He-flushed syringe 25 mM $NaNO_3$, 25 mM $NaNO_2$, and 10 % (v/v) N_2O, respectively (all final concentrations) (modified from Sperl and DeMoss 1975). The tubes were gently shaken for 15 min. The observation of a colorless band of oxidized methyl viologen in the blue background of the gels was indicative of activity. In ^{99}Mo incorporation experiments, the position of the band was marked with a needle, and the gel was sliced in 1.5 mm slices. The slices were dissolved in 30 % H_2O_2 at 65° C for 5 h, and counted for radioactivity as in section 3.2.4.3.

Some gels were stained for proteins by placing them into 20 mL of 12.5 % trichloroacetic acid for 5 min, then adding 1 mL of 0.25 % Coomassie Brilliant Blue G250. After 15 min, the gels were transferred in 5 % acetic acid for 12 h (Diezel *et al.* 1972).

3.5. ANALYSES

Protein content was assayed according to the modified Lowry method (Tibelius and Knowles 1983) using sodium citrate instead of sodium tartrate, and using bovine serum albumin as the standard. When tungstate precipitates were formed, 1 M

sodium hydroxide (final concentration) was added first to dissolve the acid precipitates. Following this, the normal steps were carried out.

Nitrate and nitrite were determined according to Griess-Ilosvay, using sulfanilamide reagent with and without hydrazine copper reduction. The azo-dye was formed and estimated colorimetrically with an autoanalyzer (Chemlab Instruments, Hornchurch, England). The instrument was calibrated with $NaNO_3$ and $NaNO_2$ (Fisher grade reagents), and with Na_2WO_4 for tungstate-containing samples.

Optical densities were obtained using a DU-70 Spectrophotometer (Beckman). The pH values were recorded using a Fisher Accumet pH Meter Model 230.

Measurement of traces of contaminating molybdenum in the medium was attempted using a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer equipped with a graphite furnace. The purging gas was argon. $A_{313.5}$ was measured by using integrated peak area. The samples were prepared by adding 0.2 % HNO_3 to extract Mo. Samples (20 μL) were then injected by means of an autosampler. They were first dried at 120° C for 50 s, charred at 1300° C for 20 s to remove organic carbon, and atomized at 2700° C for 7 s. The standard utilized was an atomic absorption molybdenum standard (Fisher Scientific Company) containing 1000 ppm ± 1 % of molybdenum metal in dilute nitric acid. The detection limit was established at 10 ppb.

3.6. MATERIALS

Carrier-free $Na_2{}^{99}MoO_4$, at a concentration of approximately 4 $Ci.mL^{-1}$, was obtained from Nordion International Inc., Kanata, Ontario. Na_2WO_4 was purchased from Fisher Scientific Company, Fair Lawn, NJ. Sequestrene was bought from CIBA-GEIGY Corp., Greensboro, NC. Deoxycholate, Triton X-100, Tris-HCl buffer and Cytoscint scintillation fluid were all obtained from ICN Biochemicals, Cleveland, OH. Methyl viologen and EDTA were purchased from Sigma Chemical Company, St-Louis, MO. Acrylamide was obtained from Bio-Rad Laboratories, Richmond, CA.

All the glassware was washed in 2N HCl and rinsed with deionized water to remove trace metals.

4.RESULTS

4.1. EFFECT OF TUNGSTEN ON AEROBIC GROWTH

Cells of *Azospirillum brasilense* Sp 7 were grown aerobically in the presence of increasing concentrations of sodium tungstate (Fig. 1). Flasks containing 25 mM NaCl were also used. It was found that 25 mM NaCl had no measurable effect on growth over a period of 24 h (Fig. 1). In fact, the generation times in both controls (with and without 25 mM NaCl) were identical (Table 1). On the other hand, Figure 1 shows that increasing levels of tungstate, from 1 to 25 mM, resulted in a decrease in growth rate, although there was very little effect of 1 mM tungstate. The generation times obtained with very high concentrations of tungstate were about twice those of the controls (Table 1).

4.2. EFFECT OF TUNGSTEN ON ANAEROBIC GROWTH, NITRATF AND NITRITE REDUCTION

A. brasilense cells were grown anaerobically in Mo-limited medium as described in section 3.2.2.1. Growth was minimal because N-oxides were present in small concentrations (Fig. 2). In the absence of tungstate, nitrate was completely reduced to nitrite in 4 h. The same result was obtained if the Mo-limited medium was supplemented with 830 μM molybdate

Fig. 1. Aerobic growth of *Azospirillum brasilense* with 18.7 mM NH_4Cl in the presence of various concentrations of tungstate, as described in section 3.2.2.4. Concentrations of tungstate were: 0 mM (O); 0 mM plus 25 mM NaCl (■); 1 mM (●); 5 mM (△); 10 mM (▲); and 25 mM (□). The curves are regressions of the order 3.

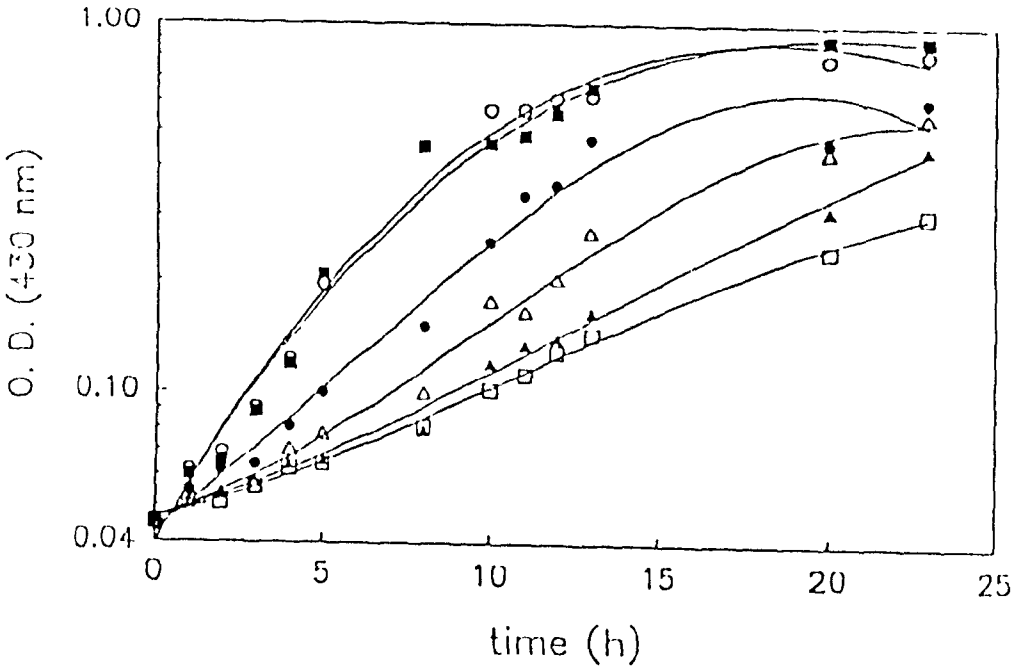
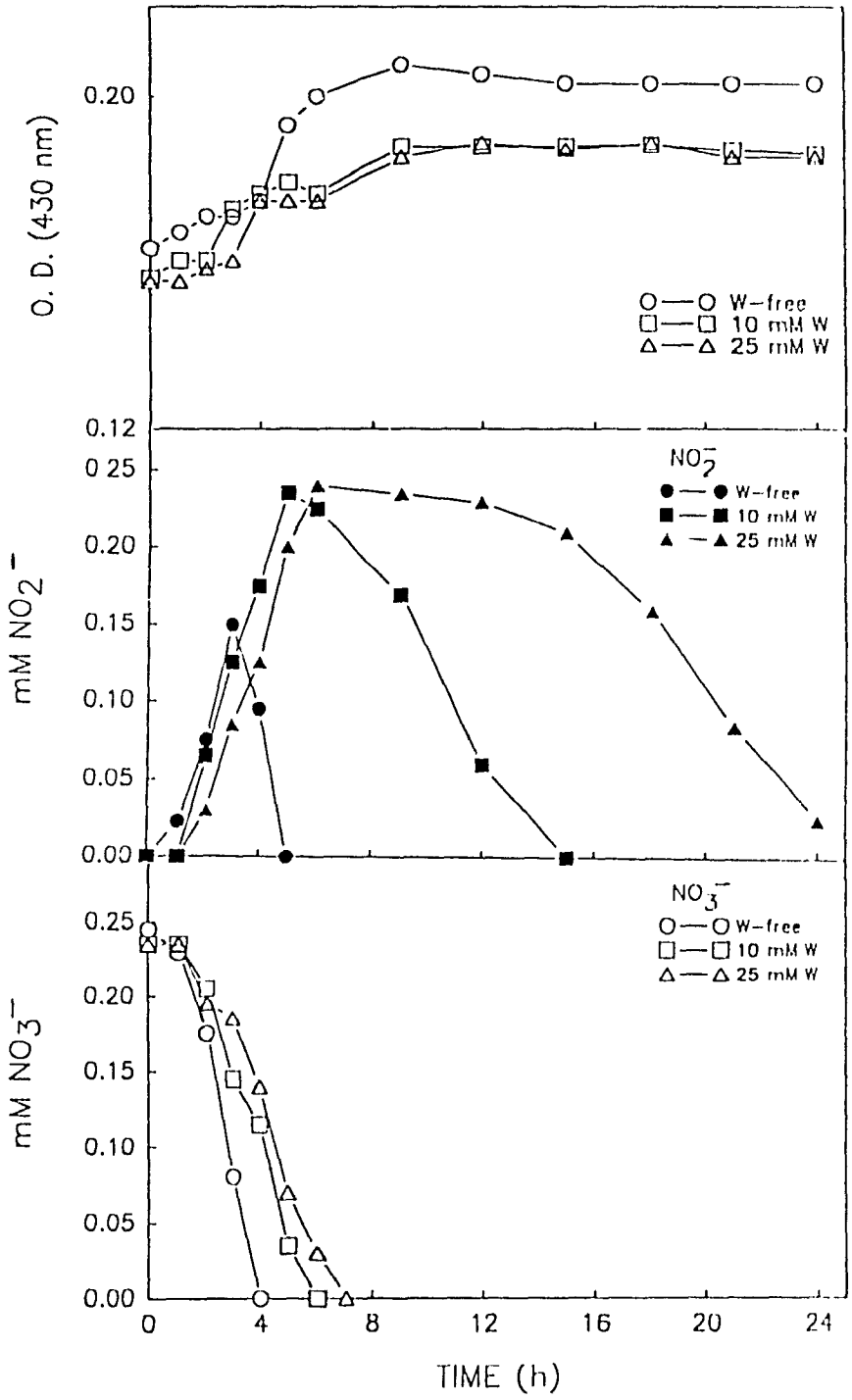

1.00
0.10
0.04
O. D. (430 nm)
0
5
10
15
20
25
time (h)

Table 1. Generation times of *Azospirillum brasilense* Sp 7 grown aerobically in the presence of various concentrations of tungstate, as described in section 3.2.2.4. The data were calculated from the interval 5-10 h.

mM tungstate	Generation Time (h)
0	3.57
0 (+ 25 mM NaCl)	3.57
1	3.84
5	5.05
10	6.73
25	8.42

Fig. 2. Anaerobic growth of *Azospirillum brasilense* in Mo-limited batch cultures supplemented with 0.25 mM $NaNO_3$ and zero, 10 or 25 mM tungstate. Cells were grown aerobically, harvested during late-log phase, washed and resuspended (7.5 μg protein.mL^{-1}) at time zero in 800 mL of medium sparged with N_2 at 450 $mL.min^{-1}$


O. D. (430 nm)
0.20
0.12
O—O W-free
□—□ 10 mM W
△—△ 25 mM W
mM NO2−
0.25
0.20
0.15
0.10
0.05
0.00
NO2−
●—● W-free
■—■ 10 mM W
▲—▲ 25 mM W
mM NO3−
0.25
0.20
0.15
0.10
0.05
0.00
NO3−
O—O W-free
□—□ 10 mM W
△—△ 25 mM W
0
4
8
12
16
20
24
TIME (h)

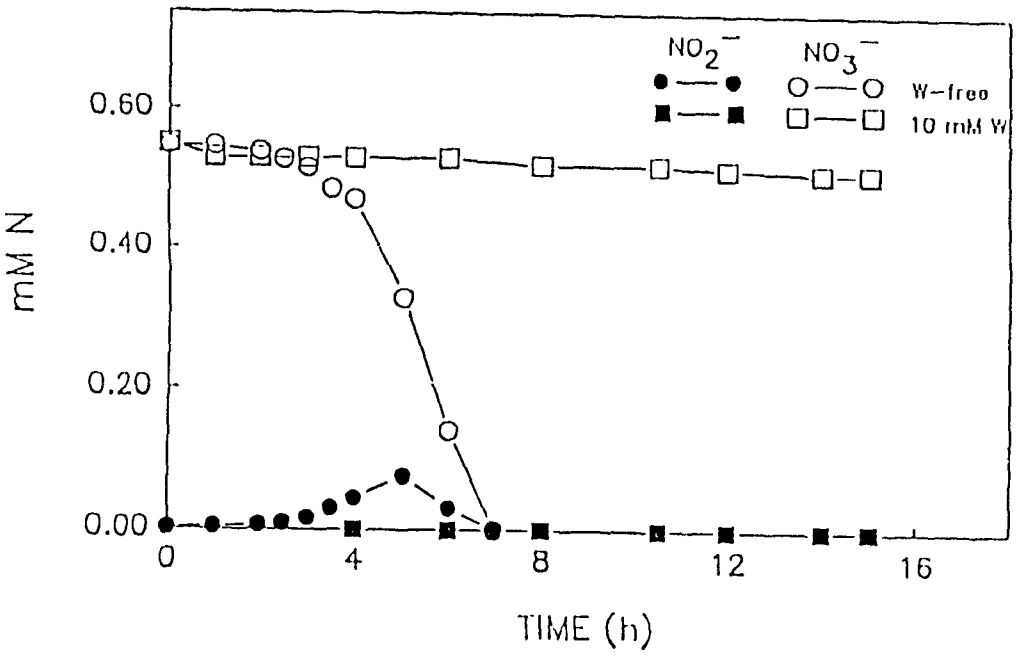
(data not shown). About 0.15 mM nitrite was accumulated from 0.25 mM nitrate in W-free medium. In tungstate-containing media, anaerobic growth was reduced (Fig. 2). Nitrate was completely reduced, but at lower rates than in W-free medium. The nitrate reductase specific activities, estimated from these curves, even though lower than the activities of the cells grown in W-free medium, were still relatively high considering that 10 and 25 mM tungstate were present (Table 2). In tungstate-containing media, nitrite accumulated to a greater extent, and the onset of nitrite reduction was delayed, compared to W-free medium (Fig. 2). Nitrite reduction in W-free medium started after 3 h of incubation, whereas in the presence of 10 mM tungstate, it started after 5 h (Fig. 2), and the activity was about one-third that estimated in W-free medium (Table 2). In the medium supplemented with 25 mM tungstate, nitrite reduction started after 6 h (Fig. 2), proceeded very slowly for the next 9 h, and then resumed at a rate close to that in 10 mM tungstate (Table 2).

Paracoccus denitrificans was also grown anaerobically in the presence and absence of 10 mM tungstate as a control (Fig. 3). The experimental protocol was identical to that utilized for *A. brasilense*. Nitrate reduction in *P. denitrificans* was inhibited by 10 mM tungstate. Nitrite accumulation in the control (Fig. 3) was much less than was observed in *A. brasilense* (Fig. 2).

Table 2. Nitrate and nitrite reductases specific activities, calculated from data of growing batch cultures of *Azospirillum brasilense* in Fig. 2, in the presence or absence of tungstate.

mM tungstate	time interval (h)	nmol N.mg protein^{-1}.min^{-1}
Nitrate reduction		
0	0-4	85
10	0-6	54
25	0-7	47
Nitrite reduction		
0	3-5	104
10	5-15	33
25	6-24	17
25	15-24	29

Fig. 3. Effect of 10 mM tungstate on nitrate reduction and nitrite accumulation in *Paracoccus denitrificans* cells. Cells were grown aerobically, harvested during late-log phase, washed and resuspended (57 μg protein.mL^{-1}) at time zero in 800 mL of medium sparged with N_2 at 450 $mL.min^{-1}$.


NO_2^-
NO_3^-
W-free
10 mM W
0.60
0.40
0.20
0.00
mM N
0
4
8
12
16
TIME (h)

4.3. EFFECT OF GROWTH WITH TUNGSTATE ON SPECIFIC ACTIVITIES

The next step was to test the effect of growth with tungstate on enzymatic activities in the presence of artificial electron donors. The cells were grown anaerobically in 800 mL batch cultures for 4 and 5 h respectively, harvested and resuspended in Sorensen's buffer. Nitrate reductase activity was tested in a short-term assay (10 min), and production of nitrite was monitored. Nitrite production was linear, in the presence of 10 mM $NaNO_3$, for at least 10 min (Fig. 4). Nitrate reductase specific activities of the cells grown in W-free medium were five-fold higher than those of cells grown in 10 mM tungstate (Table 3). The specific activities of the cell-free extracts from the cells cultured in W-free and 25 mM tungstate medium were respectively one-third and one-fifth those of the whole cells and again there was a marked effect of tungstate (Table 3).

Nitrite reductase specific activity was assayed with methylviologen as the electron donor, and N_2O accumulation, in the presence of the nitrous oxide reductase inhibitor C_2H_2, was shown to be linear for about 5 min (Fig. 5). Nitrite reductase specific activity of the cells grown in W-free medium were about 4-fold higher than that of the cells grown in 10 mM tungstate (Table 4), suggesting tungstate had an inhibitory effect on nitrite reductase activity. As observed with nitrate reductase activities, nitrite reductase

Fig. 4. Time course assay of nitrate reductase activity in whole cells of *Azospirillum brasilense* (280 μg protein.mL^{-1}). The assay was done with 10 mM $NaNO_3$ and methyl viologen as described in section 3.3.1.

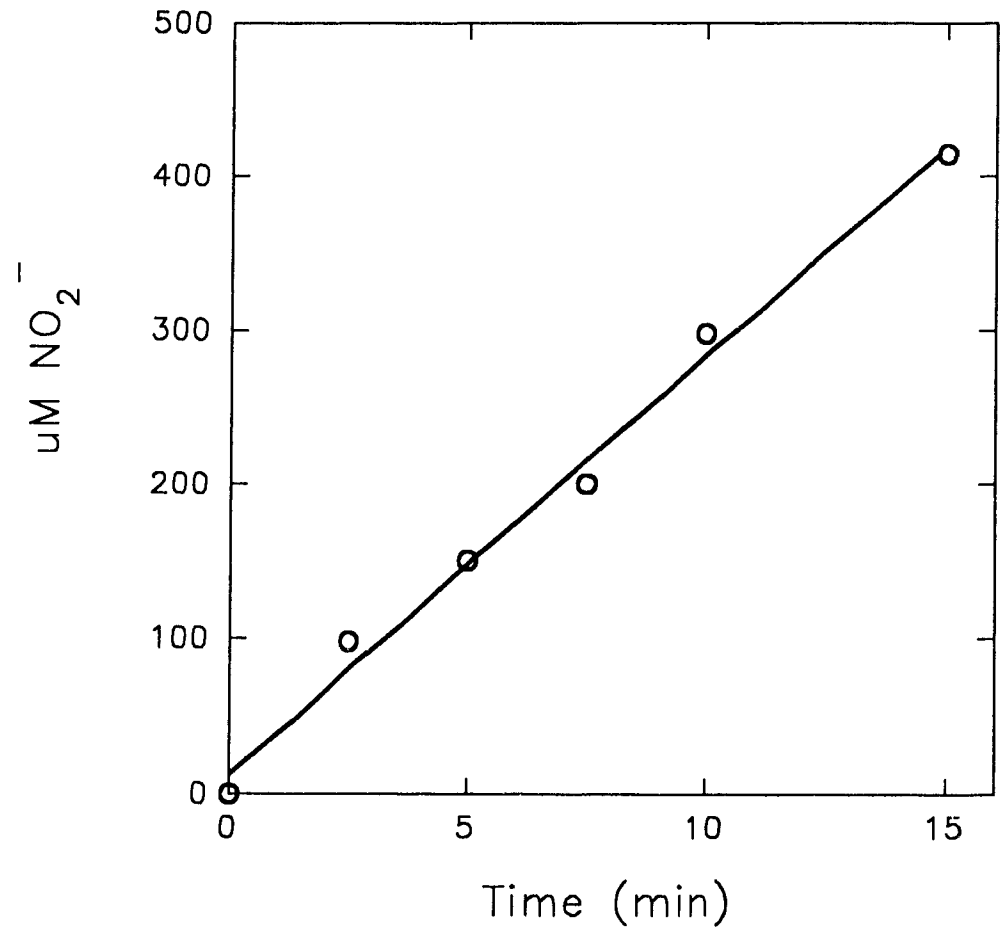

500
400
300
200
100
0
uM NO_2^-
0
5
10
15
Time (min)

Table 3. Specific activities of nitrate reductase in whole cells and cell-free extracts of *Azospirillum brasilense* grown anaerobically in the presence of 0, 10, or 25 mM tungstate for 4 h. Cells were harvested, washed and resuspended (approximately 250 μg protein.mL^{-1}) in W-free buffer. The assay was done as described in section 3.3.1.

mM tungstate	nmol NO_2^-.mg $protein^{-1}.min^{-1}$
Whole cells	
0	158.0 ± 5.1[1]
10	28.6 ± 1.7
25	13.9 ± 0.4
Cell-free extracts	
0	53.0 ± 4.6
10	N.D.[2]
25	3.0 ± 0.3

[1] Data are means ± SEM of triplicates.
[2] Not determined

Fig. 5. Time course assay of nitrite reductase activity in whole cells of *Azospirillum brasilense* (255 μg protein.mL^{-1}). The assay was done with 10 mM $NaNO_2$, methyl viologen, and either 0 or 10 % (v/v) C_2H_2, as described in section 3.3.2. Data represent total flask N_2O calculated per mL of suspension.

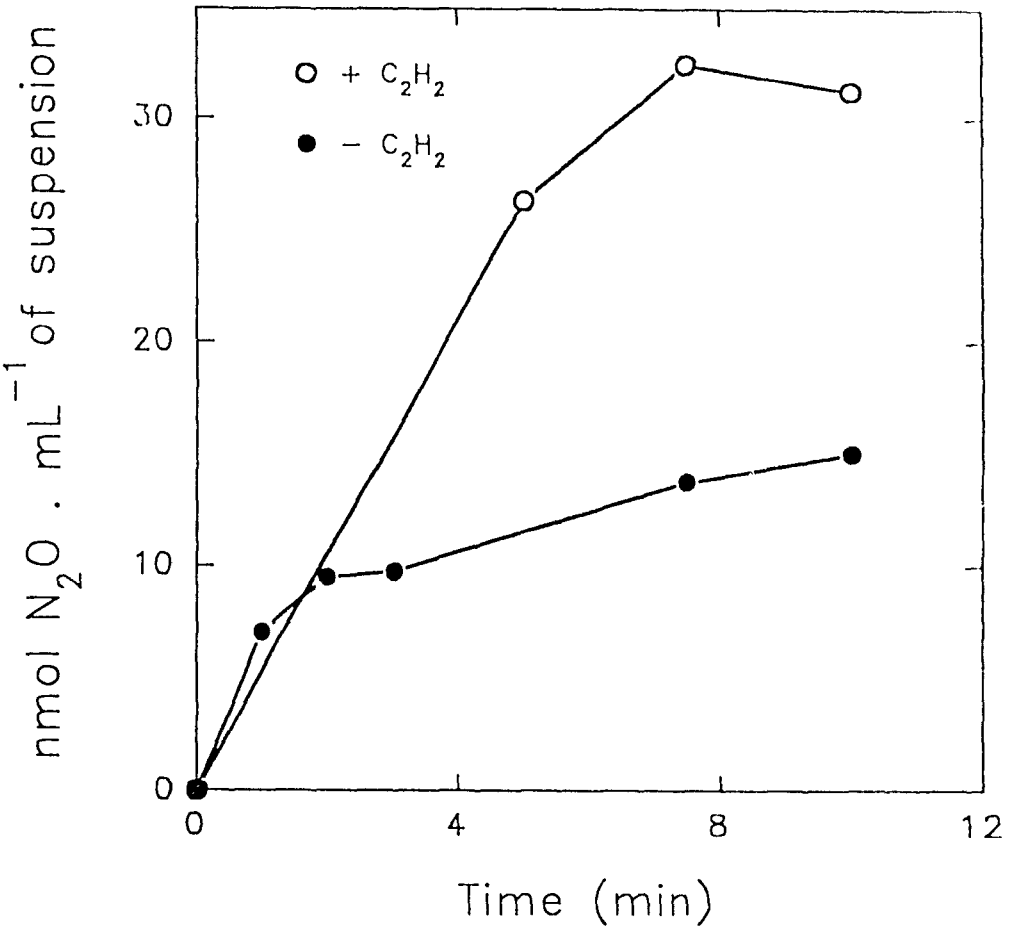

+ C_2H_2
– C_2H_2
nmol N_2O . mL^{-1} of suspension
30
20
10
0
0
4
8
12
Time (min)

Table 4. Specific activities of nitrite reductase in whole cells and cell-free extracts of *Azospirillum brasilense* grown anaerobically in the presence of either 0 or 10 mM tungstate for 5 h. Cells were harvested, washed and resuspended (approximately 250 μg protein.mL^{-1}) in W-free buffer. The assay was done as described in section 3.3.2.

mM tungstate	nmol N_2O.mg $protein^{-1}.min^{-1}$
Whole cells	
0	98.8 ± 7.3 [1]
10	26.5 ± 3.8
Cell-free extracts	
0	66.0 ± 12.2
10	7.2 ± 0.2

[1] Data are means ± SEM of triplicates.

activities of the cell-free extracts were lower than those of the whole cells (Table 4).

4.4. EFFECT OF NITRATE ON NITRITE REDUCTION

Some experiments were then carried out to explain the delay observed in the onset of nitrite reduction in tungsten-containing medium. Figure 6 shows that even if the medium was initially supplemented with 0.25 mM nitrite, nitrite reduction did not start before most of the nitrate had been reduced. This delay in the onset of nitrite reduction might have been caused by an inhibitory effect of nitrate on nitrite reduction. Figure 7 shows that cells growing in W-free medium had completely reduced nitrate after 3.5 h, and were then reducing nitrite at a rate similar to that of nitrate reduction. At this time, 0.25 mM nitrate was added to the growth medium. The instant effect of the addition of nitrate was the formation of a second peak of nitrite accumulation (Fig. 7). After 5 h, when nitrate concentration in the medium was again very low, nitrite reduction resumed at about the same rate as observed initially.

In order to verify if only nitrite reductase activity, and not synthesis, was affected by nitrate, the cells were grown in 800 mL of medium supplemented with 0.25 mM nitrate (Fig. 8). Parts of the cell suspension were removed after 2, 4, and 6 h. The cells were washed, resuspended in Sorensen's

Fig. 6. Nitrate and nitrite reduction in *Azospirillum brasilense* cells grown anaerobically in W-free medium containing 0.25 mM $NaNO_3$ and 0.25 mM $NaNO_2$. Cells were grown aerobically, harvested during late-log phase, washed and resuspended (45 μg protein.mL^{-1}) at time zero in 800 mL of medium sparged with N_2 at 450 mL.min^{-1}.

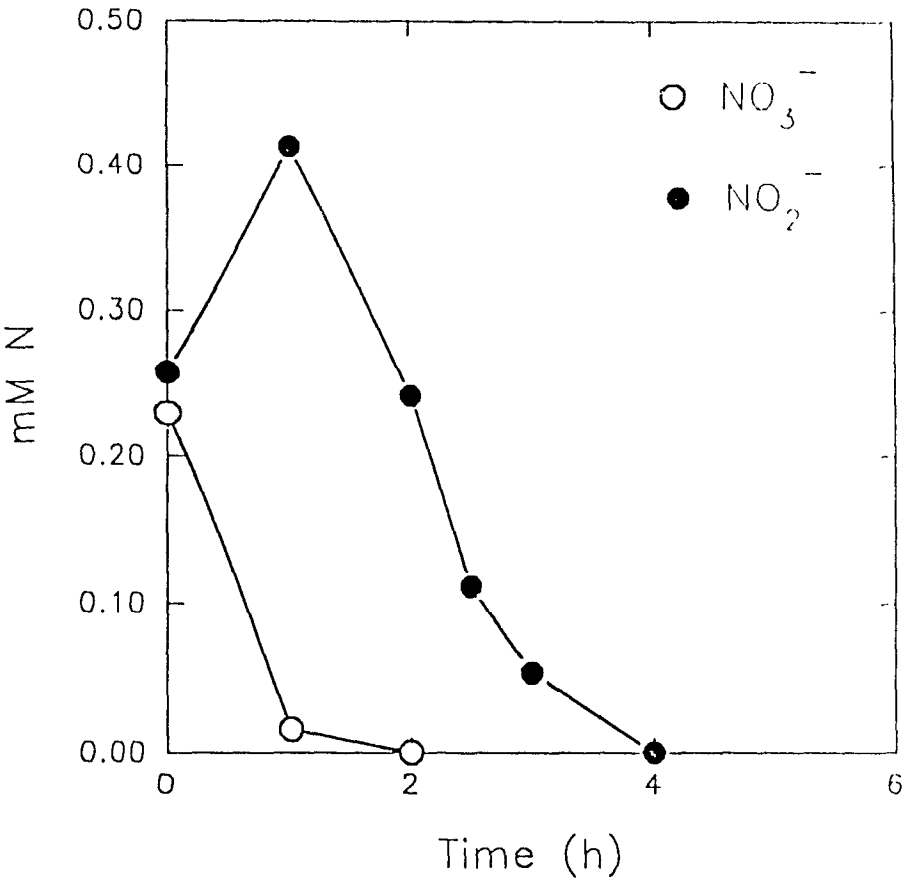

0.50
0.40
0.30
0.20
0.10
0.00
mM N
0
2
4
6
Time (h)
NO3−
NO2−

Fig. 7. Transformation of nitrate and nitrite by *Azospirillum brasilense* in anaerobic W-free medium containing 0.25 mM $NaNO_3$. Cells were grown aerobically, harvested during late-log phase, washed and resuspended (6.4 μg protein.mL^{-1}) in 800 mL of medium sparged with N_2 at 450 mL.min^{-1}. The arrow indicates the time at which fresh $NaNO_3$ was added to the medium to demonstrate the inhibitory effect of nitrate on nitrite reduction.

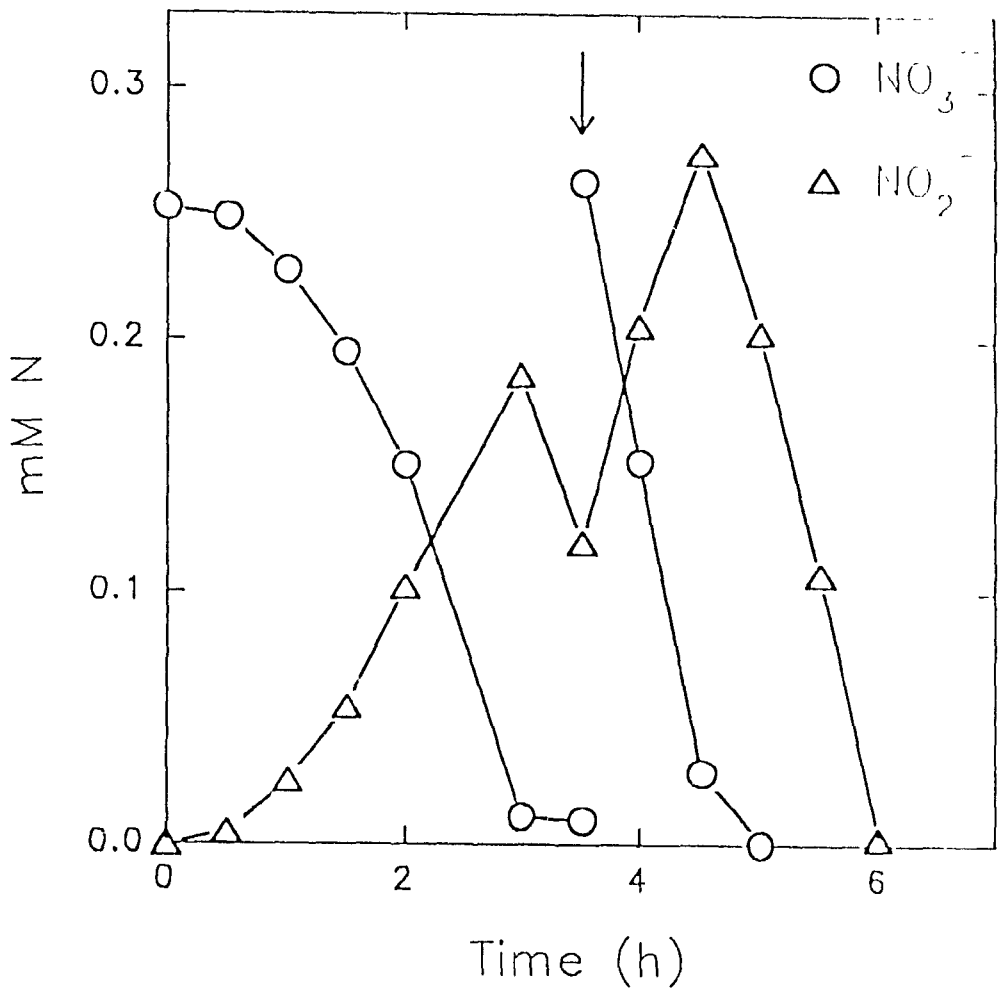
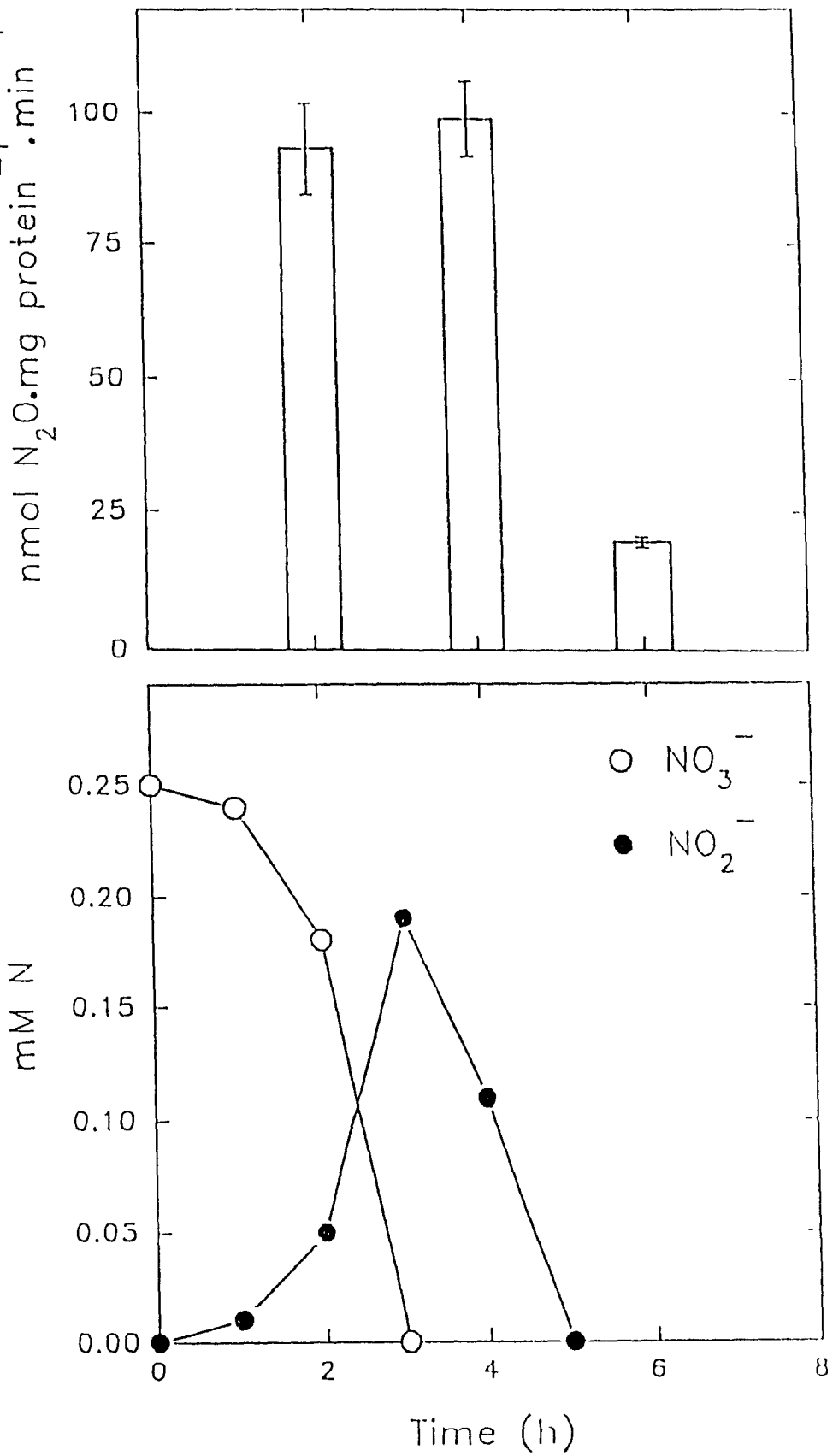

0.3
0.2
0.1
0.0
mM N
0
2
4
6
Time (h)
NO3-
NO2-

Fig. 8. Nitrite reductase specific activities of *Azospirillum brasilense* washed cells measured during three different growth phases. Cells were grown aerobically, harvested during late-log phase, washed and resuspended (7.0 μg protein.mL^{-1}) in 800 mL of medium sparged with N_2 at 450 mL.min^{-1}. At each sampling time, 200 mL of cell suspension was harvested anaerobically, washed, resuspended (approximately 250 μg protein.mL^{-1}) in Sorensen's buffer, and assayed for nitrite reductase activity as described in section 3.3.2. Error bars are ± SEM of triplicates.

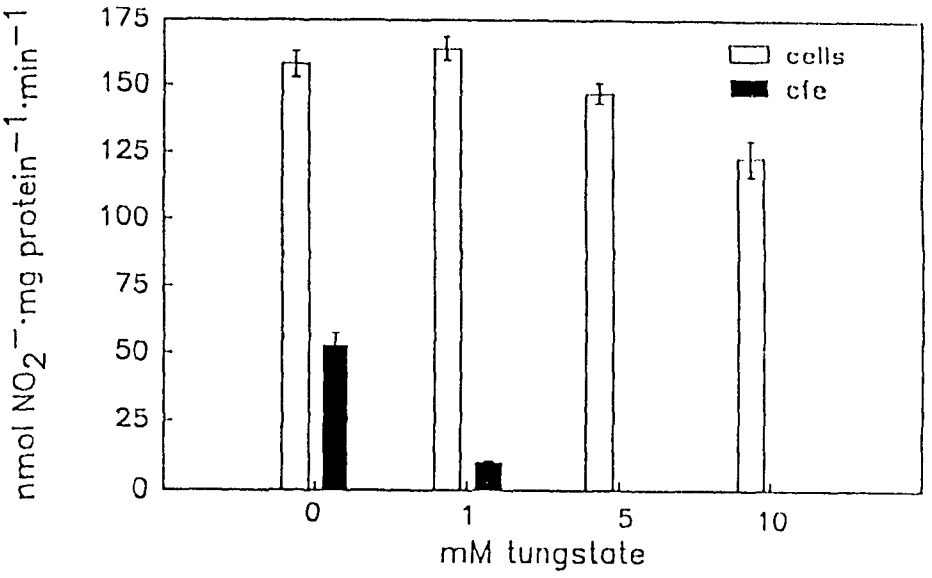

nmol N_2O.mg protein^{-1}.min^{-1}
100
75
50
25
0
NO_3^-
NO_2^-
mM N
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
Time (h)

buffer, and assayed for nitrite reductase activity. Specific activities of the washed cells harvested after 2 and 4 h were not significantly different, even though nitrite in the batch culture was respectively accumulating and being reduced at these sampling times. Therefore removing nitrate stimulated nitrite reduction. Nitrite reductase activity decreased markedly after nitrite had disappeared, suggesting nitrite reductase required nitrite stimulation for maximal activity.

4.5. EFFECT OF TUNGSTATE ON PREFORMED ACTIVITIES

The effect of increasing levels of tungstate on preformed nitrate reductase and nitrite reductase activities was investigated by growing *A. brasilense* cells in 800 mL of W-free medium supplemented with 0.25 mM nitrate for 4 and 5 hours, times at which nitrate and nitrite, respectively, were being actively reduced. The cells were washed and resuspended in Sorensen's buffer. Cell suspensions as well as cell-free extracts were assayed with various concentrations of tungstate using methyl viologen as the electron donor. Increasing levels of tungstate (up to 10 mM) had little effect on nitrate reductase activities of the whole cells (Fig. 9). However 1 mM tungstate resulted in an 80 % inhibition of the cell-free extract activities. On the other hand, preformed nitrite reductase activities of both cell-free extracts and whole cells were equally affected by increasing concentrations of

Fig. 9. The effect of increasing concentrations of sodium tungstate on the specific activities of pre-formed nitrate reductase. *Azospirillum brasilense* cells were grown anaerobically in 800 mL of W-free medium supplemented with 0.25 mM nitrate, harvested after 4 h, washed and resuspended (approximately 275 μg protein.mL^{-1}) in Sorensen's buffer containing the indicated concentration of tungstate. Nitrate reductase assay was done as described in section 3.3.1. Error bars are ± SEM of triplicates.


175
150
125
100
75
50
25
0
nmol NO_2^-·mg protein^{-1}·min^{-1}
cells
cfe
0
1
5
10
mM tungstate

tungstate (Fig. 10).

4.6. EFFECT OF MOLYBDENUM LIMITATION ON NITRATE REDUCTION

The molybdenum contents of some batches of media were analysed by atomic absorption spectrometry. Nine out of ten W-free batches sampled at random contained concentrations lower than 10 ppb, which was the detection limit of the machine.

The effect of various combinations of 830 μM molybdate and 10 mM tungstate on nitrate reduction was studied. A 1-h derepression lag phase was observed (Fig. 11). The curves show that nitrate reduction in W-free medium appeared to be slightly enhanced by addition of molybdate. However nitrate reduction in tungstate-containing medium was not enhanced by addition of molybdate. Parts of the cultures were harvested and nitrate reductase specific activity was assayed with washed cells. It was then observed that the specific activity of cells grown in W-free medium was not significantly increased by molybdate (Table 5). Similarly, the specific activity of W-grown cells was not enhanced by molybdate.

Because media were potentially contaminated with traces of molybdenum (< 10 ppb), it became important to ensure that possible contaminants were removed from the medium. The media were therefore treated with *Azotobacter vinelandii* (section 3.1.3). *A. brasilense* inoculum was grown aerobically in treated medium, and then added to vacuum flasks containing

Fig. 10. The effect of increasing concentrations of sodium tungstate on the specific activities of pre-formed nitrite reductase. *Azospirillum brasilense* cells were grown in 800 mL of W-free medium supplemented with 0.25 mM nitrate, harvested after 5 h, washed and resuspended (approximately 275 μg protein.mL^{-1}) in Sorensen's buffer containing the indicated concentration of tungstate. Nitrite reductase assay was done as described in section 3.3.2. Error bars are ± SEM of triplicates.

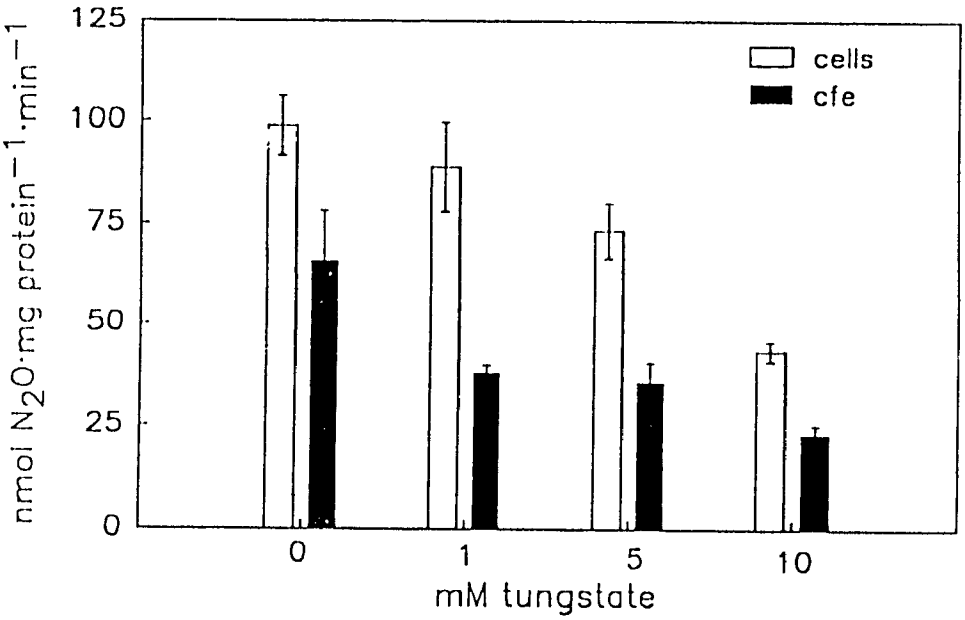

125
100
75
50
25
0
nmol $N_2O \cdot mg\ protein^{-1} \cdot min^{-1}$
cells
cfe
0
1
5
10
mM tungstate

Fig. 11. Effect of the addition of different combinations of molybdate and tungstate in the growth medium on nitrate reduction by *Azospirillum brasilense* cells. Cells were grown aerobically, harvested during late-log phase, washed and inoculated (52 μg protein.mL^{-1}) into vacuum flasks as described in section 3.2.2.3.

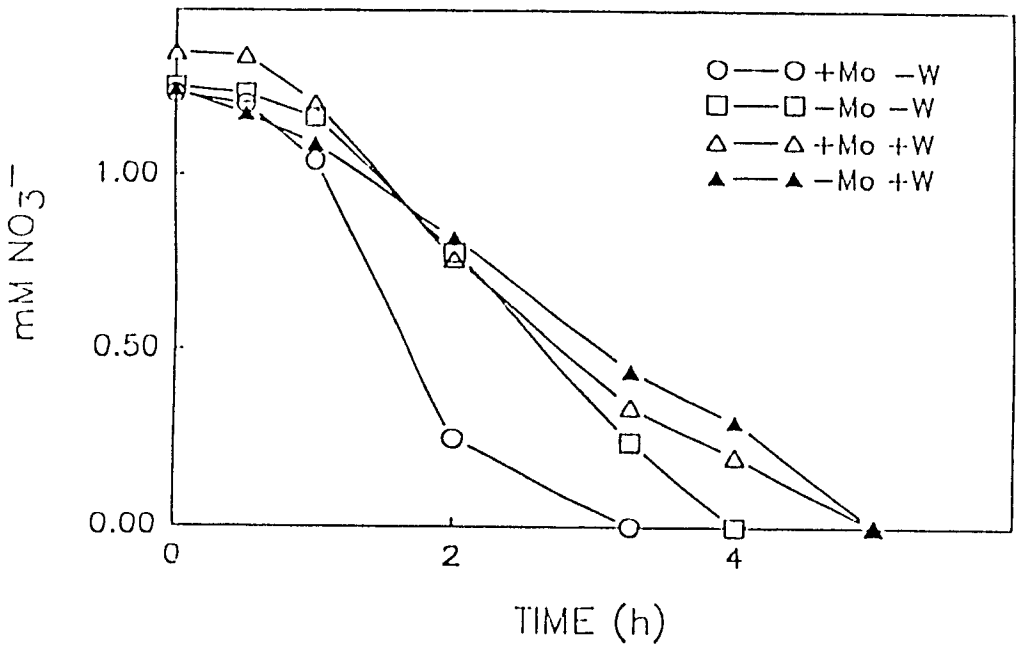

O—O +Mo −W
□—□ −Mo −W
△—△ +Mo +W
▲—▲ −Mo +W
mM NO3−
1.00
0.50
0.00
0
2
4
TIME (h)

Table 5. Nitrate reductase specific activities of cells grown in medium containing various combinations of molybdate and tungstate. Parts of the cultures from Figure 11 were harvested after 4 h, washed and resuspended (approximately 300 μg protein.mL^{-1}) in Sorensen's buffer. The assay was done as described in section 3.3.1.

Growth medium		
Molybdate (μM)	Tungstate (mM)	nmol NO_2^{-1}.mg protein^{-1}.min^{-1}
830	0	126 ± 12[1]
0[2]	0	117 ± 14
830	10	83 ± 7
0	10	82 ± 6

[1] Data are means ± SEM of triplicates

[2] No addition (< 10 ppb Mo)

treated medium, with and without 830 μM molybdate. *A. brasilense* cells grown anaerobically in treated Mo-limited medium could not reduce nitrate, whereas cells supplemented with molybdate reduced nitrate within 3 h (Fig. 12). This indicates that contaminating molybdenum was present in non-treated medium.

4.7. GEL ELECTROPHORESIS AND STAINING ACTIVITY

The location of N oxide reductase activities was determined by growing the cells in 5 L of medium supplemented with 10 mM $NaNO_3$, and sparged with N_2 for 12 h. Cells were then broken and separated into various fractions. Nitrate reductase activity recovered in the solubilized membranes was about 42 % that of the cell-free-extracts, whereas it was approximately 30 % in the soluble fraction (Table 6). Proteins were almost entirely recovered, indicating that solubilization of the membranes was efficient. The solubilized membranes were subjected to disc gel electrophoresis and nitrate reductase was assayed in the gels, as described in section 3.4. The appearance of a white band of oxidized methyl viologen in the blue background was indicative of nitrate reductase activity (Fig. 13). Nitrite reductase and nitrous oxide reductase activities were found in the soluble fraction. Oxidized bands appeared at different positions, depending on the electron acceptor present, indicating that both nitrite and nitrous

Fig. 12. Nitrate reduction by *Azospirillum brasilense* cells grown anaerobically in treated medium supplemented with zero or 830 μM molybdate. Cells were grown aerobically in treated medium, harvested during late-log phase, washed and inoculated (41 μg protein.mL^{-1}) into vacuum flasks as described in section 3.2.2.3.

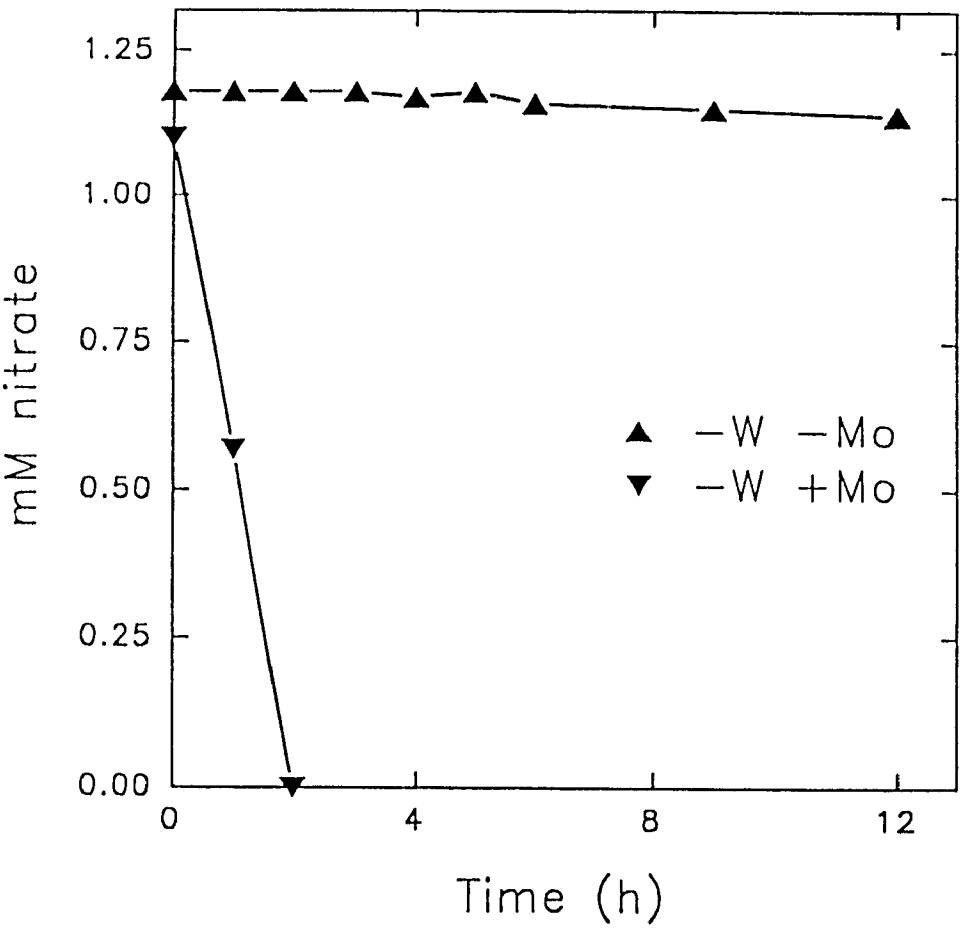
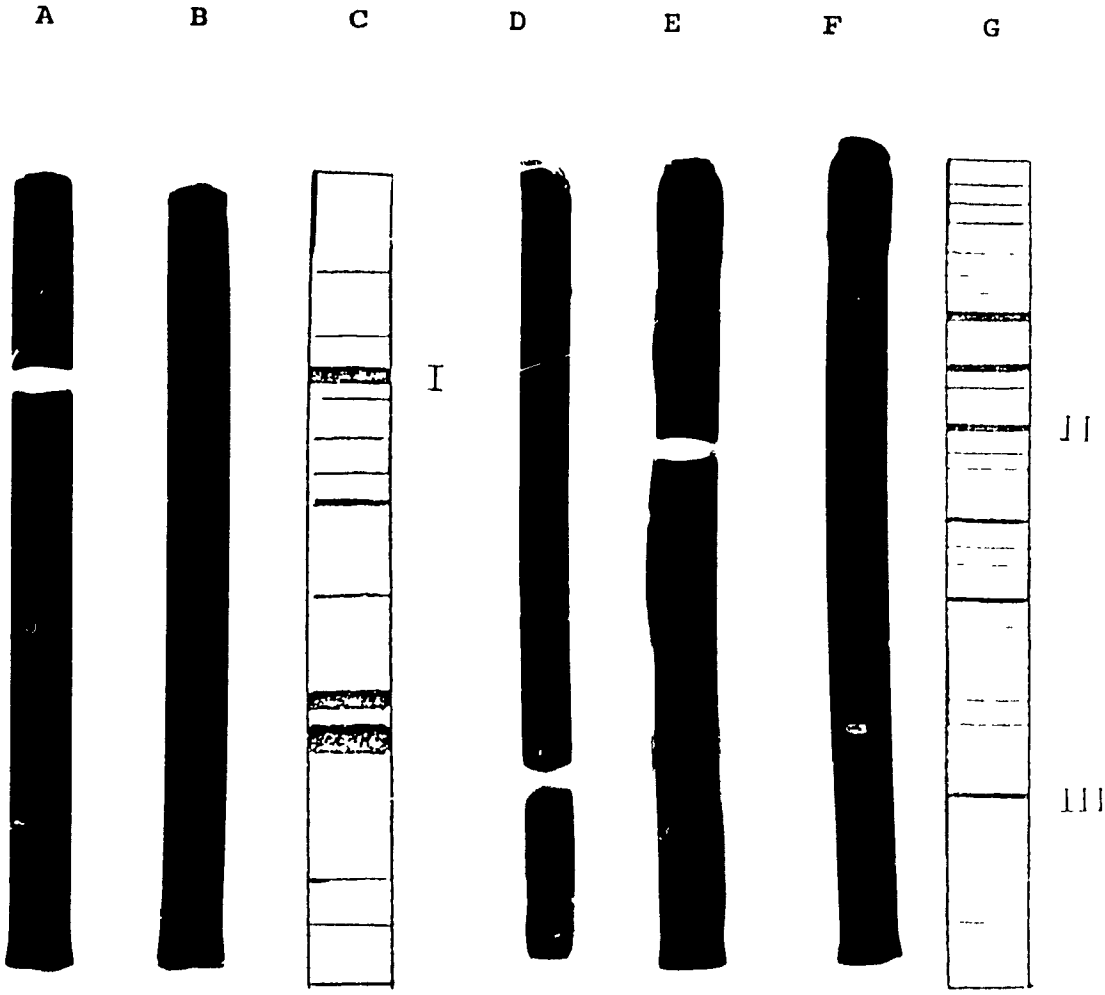

1.25
1.00
0.75
0.50
0.25
0.00
mM nitrate
0
4
8
12
Time (h)
▲ −W −Mo
▼ −W +Mo

Table 6. Location of nitrate reductase activity in *Azospirillum brasilense* cells grown anaerobically in 5 L of W-free medium, as described in section 3.2.2.2. Nitrate reductase assay was done as in section 3.3.1.

Location	Specific activity (nmol NO_2^-.mg protein^{-1}.min^{-1})	Total protein (mg)	Total activity (μmol.min^{-1})
Cell-free extracts	108 ± 4[1]	140	15.1 (100)
Soluble fraction	59 ± 1	79	4.7 (30)
Solubilized membranes	114 ± 7	55	6.3 (42)

[1] Data are means ± SEM of triplicates.

Fig. 13. Polyacrylamide gel electrophoresis of the solubilized membrane fraction and the soluble fraction of *Azospirillum brasilense* grown anaerobically in 5 L of W-free Mo-limited medium for 12 h as described in section 3.2.2.2. Both fractions were subjected to electrophoresis at 2 mA/gel for 150 min, and then assayed for N oxide reductase activities as described in section 3.4. Approximately 1.25 mg of solubilized membranes was applied to each of gels (A), (B),and (C). Gels (A) and (B) were then incubated with and without nitrate, respectively. The white oxidized band in (A) represents nitrate reductase activity. Approximately 0.75 mg of the soluble fraction was applied to each of gels (D), (E), (F) and (G). Gels (D) and (E) were then incubated with nitrite and nitrous oxide respectively, and gel (F) served as a control. The white oxidized bands in (D) and (E) represent nitrite reductase and nitrous oxide reductase activities, respectively. Gels (C) and (G) were stained for protein as described in section 3.4. Bands (I), (II), and (III) correspond to nitrate reductase, nitrous oxide reductase, and nitrite reductase, respectively.


A
B
C
D
E
F
G
I
II
III

oxide reductases were active in the soluble fraction, under these conditions (Fig. 13). Nitrate reductase activity of the soluble fraction was not initially assayed in the gels.

P. denitrificans cells were also grown in 5 L of W-free medium supplemented with 10 mM $NaNO_3$, and the cells were separated into various fractions. Specific and total activities were higher than in *A. brasilense* (Table 7). Proteins were entirely recovered, using a different solubilization procedure than for *A. brasilense*. The fractions were subjected to electrophoresis, and staining activity in the gel tubes was positive for both enzymes. Nitrate reductase activity was observed with the solubilized membrane fraction, whereas the oxidized band corresponding to nitrite reductase activity was found with the soluble fraction (Fig. 14).

The R_f values for nitrate reductase and nitrite reductase were determined in both organisms (Table 8). The results suggest that nitrate reductase had the highest molecular mass, whereas nitrite reductase had the lowest.

4.8. ^{99}Mo EXPERIMENTS

4.8.1. ^{99}Mo INCORPORATION

To obtain clear evidence that nitrate reductase in *A. brasilense* was a molybdoenzyme, *A. brasilense* cells were

Table 7. Location of nitrate reductase activity in *Paracoccus denitrificans* cells grown anaerobically in 5 L of W-free medium, as described in section 3.2.2.2. Nitrate reductase assay was done as in section 3.3.1.

Location	Specific activity (nmol NO_2^{-1}.mg protein^{-1}.min^{-1})	Total protein (mg)	Total activity (μmol.min^{-1})
Cell-free extracts	224 ± 28[1]	413	92.5 (100)
Soluble fractions	99 ± 2	333	33.0 (36)
Solubilized membranes	434 ± 23	103	44.8 (48)

[1] Data are means ± SEM of triplicates.

Fig. 14. Polyacrylamide gel electrophoresis of the solubilized membrane fraction and the soluble fraction of *Paracoccus denitrificans* grown anaerobically in 5 L of W-free Mo-limited medium for 12 h as described in section 3.2.2.2. Both fractions were subjected to electrophoresis at 2 mA/gel for 150 min, and then assayed for N oxide reductase activities as described in section 3.4. Approximately 1.20 mg of solubilized membranes was applied to each of gels (A), (B), and (C). Gels (A) and (B) were then incubated with and without nitrate, respectively. The white oxidized band in (A) represents nitrate reductase activity. Approximately 1.75 mg of the soluble fraction was applied to each of gels (D), (E), and (F). Gels (D) and (E) were then incubated with and without nitrite, respectively. The white oxidized band in (D) represents nitrite reductase activity. Gels (C) and (F) were stained for protein as described in section 3.4. Bands (I) and (II) correspond to nitrate reductase and nitrite reductase, respectively.

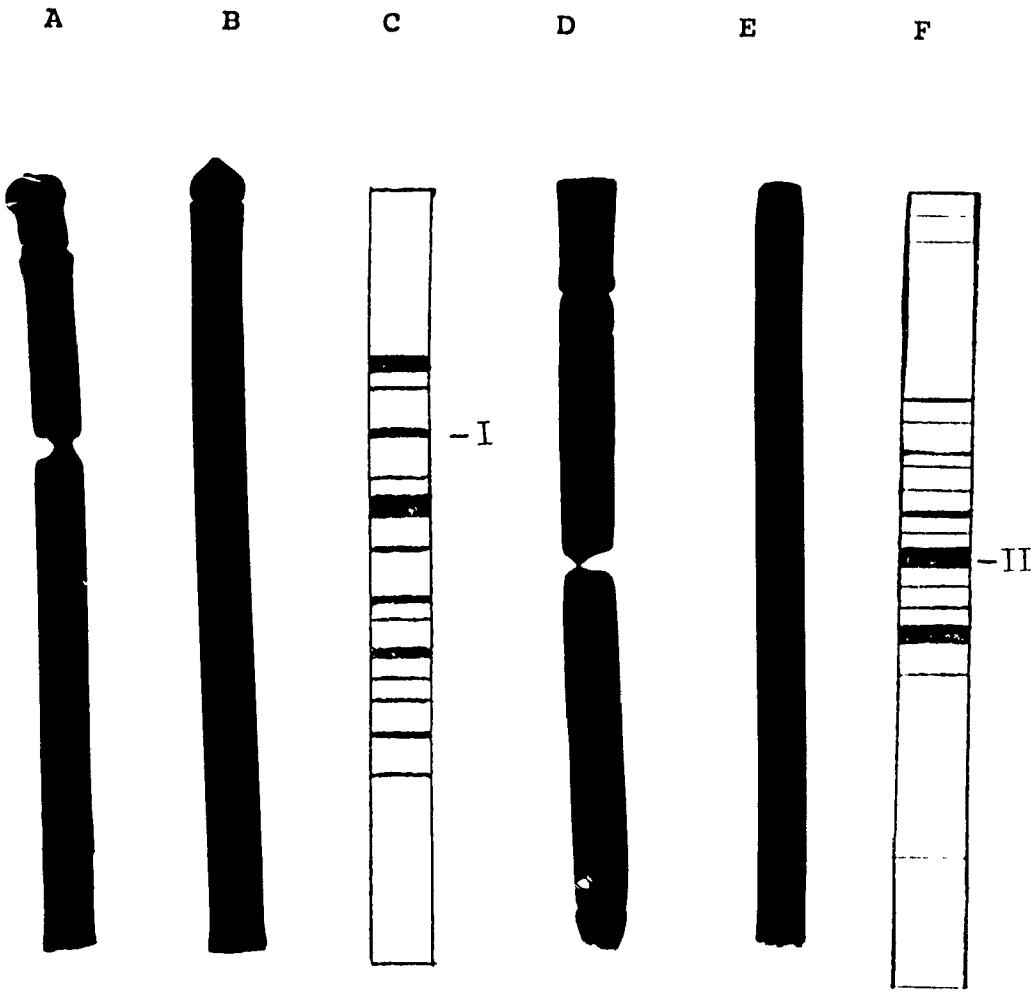

A
B
C
D
E
F
I
II

Table 8. R_f values for each of the bands from Figures 13 and 14.

	R_f values[a]		
Organism/Fraction	NO_3^- red.	NO_2^- red.	N_2O red.
A. brasilense			
Soluble	0.27[b]	0.84	0.36
Sol. Membrane	0.28	N.D.[c]	N.D.
P. denitrificans			
Soluble	N.D.	0.57	N.D.
Sol. Membrane	0.29	N.D.	N.D.

[a] Calculated in relation to the front marker.

[b] Calculated from the experiment presented in Figure 17.

[c] Not determined.

grown anaerobically in 5 L of W-free medium containing 1 μM molybdate and 7 μCi ^{99}Mo.(nmol Mo)$^{-1}$. After 12 h of incubation, the cells were harvested and fractionated. Table 9 shows the fractionation of the cells. All counts were corrected for the decay of the isotope. Membranes contained more than twice as much ^{99}Mo per mg of protein as did the whole cells and the cell-free extracts. Both soluble fraction (0.60 mg) and solubilized membranes (0.20 mg) were subjected to disc gel electrophoresis, and assayed for nitrate reductase. An oxidized band was observed only with the soluble fraction and its location was marked with a needle. This gel tube was sliced in 1.5 mm slices, and radioactivity was counted in each slice. ^{99}Mo incorporation corresponded to the nitrate reductase fraction (Fig. 15a).

In order to determine if tungstate interfered with Mo incorporation into nitrate reductase, *A. brasilense* cells were grown in 5 L of 10 mM tungstate-containing medium supplemented with 1 μM molybdate and 7 μCi ^{99}Mo.(nmol Mo)$^{-1}$. Table 10 shows that the counts obtained with the various fractions of these cells were much lower than those obtained with the cells grown in W-free medium. Counts per mg protein were higher in the cytoplasmic membrane fraction than in any other fractions. Aliquots from both soluble fraction (0.55 mg) and solubilized membranes (0.45 mg) were subjected to disc gel electrophoresis, and assayed for nitrate reductase activity. At first, no oxidized band was observed in any gel. The gel

Table 9. Fractionation of *Azospirillum brasilense* cells grown in 5 L of W-free medium supplemented with 1 μM molybdate and 7 μCi ^{99}Mo.(nmol Mo)$^{-1}$, as described in section 3.2.4.

Fraction	mg protein	10^3 CPM.mg^{-1}	10^5 CPM
Washed cells	122.5	63.8	78.1[1]
Cell-free extracts	70.5	66.4	46.7
Soluble	71.2	55.5	39.5
Cytoplasmic membranes	13.5	154.3	20.8
Solubilized membranes	5.6	144.2	8.1

[1] Data are means of duplicates.

Fig. 15. Incorporation of ^{99}Mo in the soluble fraction of *Azospirillum brasilense* cells grown anaerobically in 5 L of (A) W-free medium and (B) of 10 mM tungstate-containing medium. Medium was supplemented with 10 mM $NaNO_3$ and incubation time was 12 h (section 3.2.2.2). The arrow indicates the fraction where nitrate reductase activity was observed in the gel, as described in section 3.4.

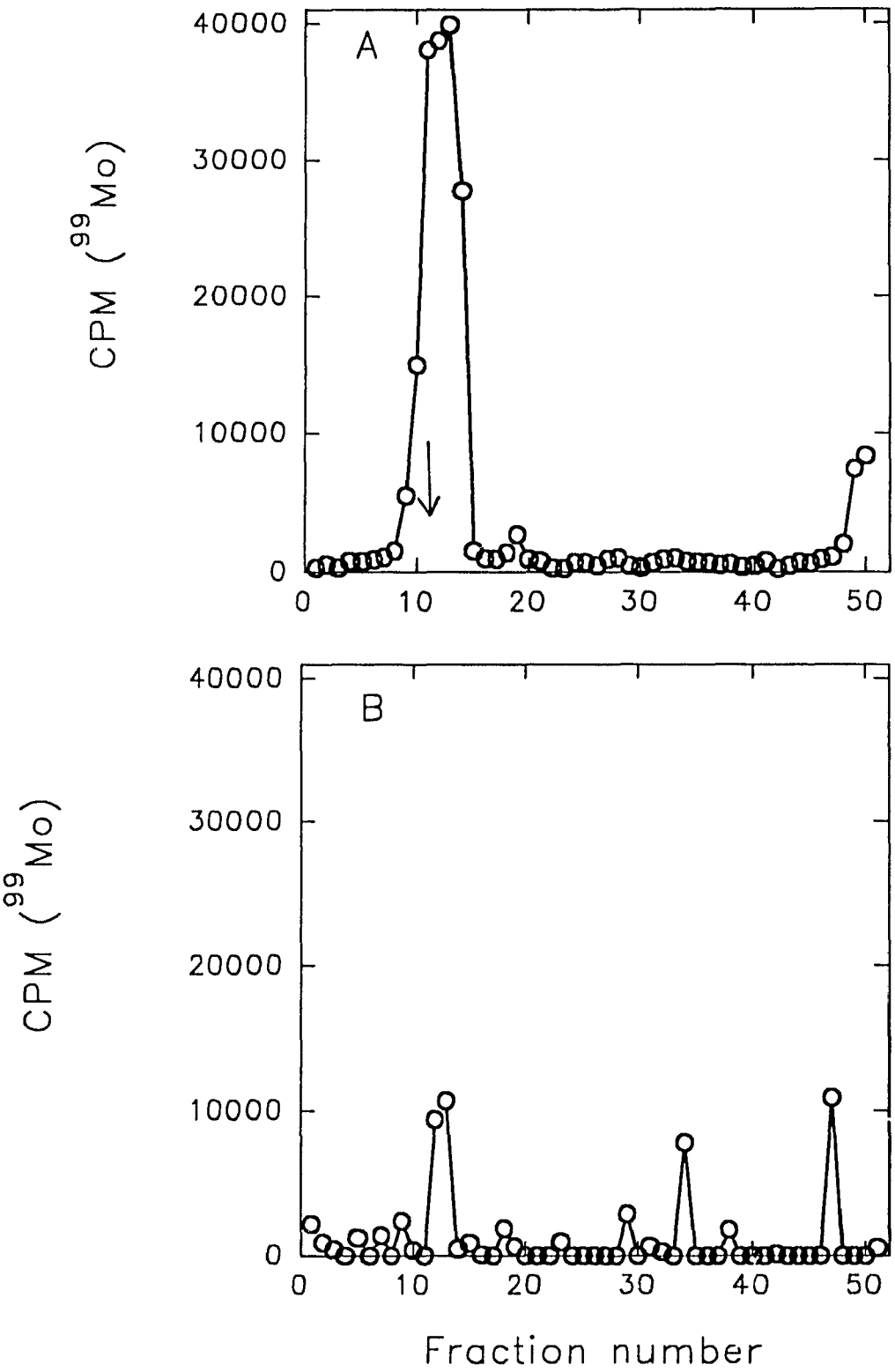

A
B
40000
30000
20000
10000
0
0
10
20
30
40
50
CPM (^{99}Mo)
Fraction number

Table 10. Fractionation of *Azospirillum brasilense* cells grown in 5 L of 10 mM tungstate-containing medium supplemented with 1 μM molybdate and 7 μCi ^{99}Mo.(nmol)$^{-1}$, as described in section 3.2.4.

Fraction	mg protein	10^2 CPM.mg^{-1}	10^3 CPM
Washed cells	120.0	7.4	88.8[1]
Cell-free extracts	83.7	9.7	81.3
Soluble	68.9	9.7	66.8
Cytoplasmic membranes	8.7	50.1	43.6
Solubilized membranes	11.4	26.6	30.3

[1] Data are means of duplicates.

loaded with the soluble fraction was sliced and counted (Fig. 15b). Lower levels of molybdate were found in this gel. In a second electrophoresis run, the gel tubes were incubated in methyl viologen plus nitrate for a longer period (2 h), because it was assumed that the activity of W-grown cells was lower and needed a longer incubation period. In this case, an oxidized band was seen in the solubilized membrane fraction and its location was marked with a needle. However, ^{99}Mo was not incorporated in this region (Fig. 16), suggesting that the activity was supported by molybdenum previously scavenged during aerobic growth of the inoculum in W-free medium.

Protein staining and slicing of a duplicate gel of the one in Figure 15a showed that a major protein band corresponded to a peak of ^{99}Mo incorporation (Fig. 17). This peak was 10-fold smaller than that measured in the staining activity gel, possibly because the protein gel was washed in acetic acid for 12 h. Since the protein gels were not photographed because of radioactivity, only a representative sketch is shown (Fig. 17).

Table 11 is a summary of 4 different experiments in which *A. brasilense* cells were fractionated and assayed for nitrate reductase. Specific activities of the fresh fractions in experiment IV were not determined because of the radioactivity. Nitrate reductase activity was detected in both the soluble fraction and the solubilized membranes in experiments II and III. The soluble fraction was assayed in

Fig. 16. Incorporation of ^{99}Mo in the solubilized membrane fraction of *Azospirillum brasilense* cells grown for 12 h in 5 L of 10 mM tungstate-containing medium supplemented with 10 mM $NaNO_3$ (section 3.2.2.2). The arrow indicates the fraction where nitrate reductase activity was observed in the gel, as described in section 3.4.

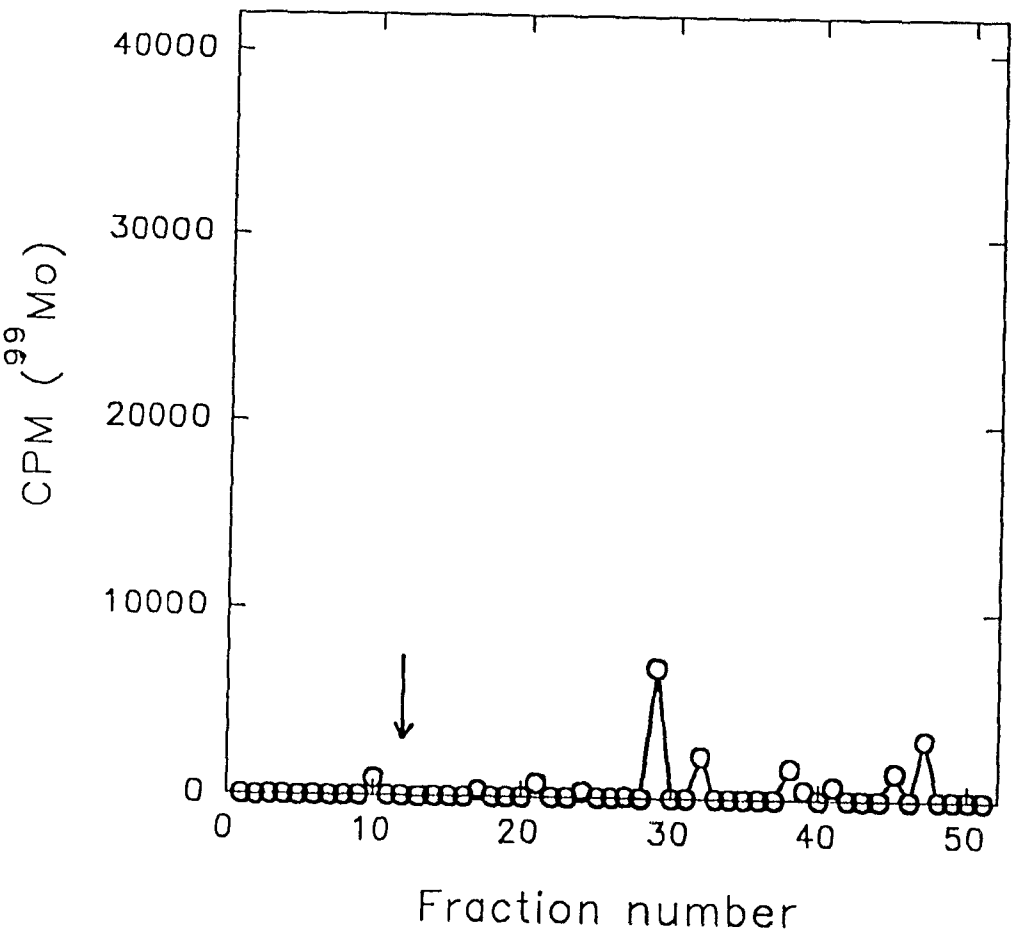

40000
30000
20000
10000
0
CPM (^{99}Mo)
0
10
20
30
40
50
Fraction number

Fig. 17. Incorporation of ^{99}Mo in the soluble fraction of *Azospirillum brasilense* cells grown for 12 h in 5 L of W-free medium supplemented with 10 mM $NaNO_3$ (section 3.2.2.2). The arrows indicate the position of a major protein band observed in the gel tube stained for protein, as described in section 3.4.

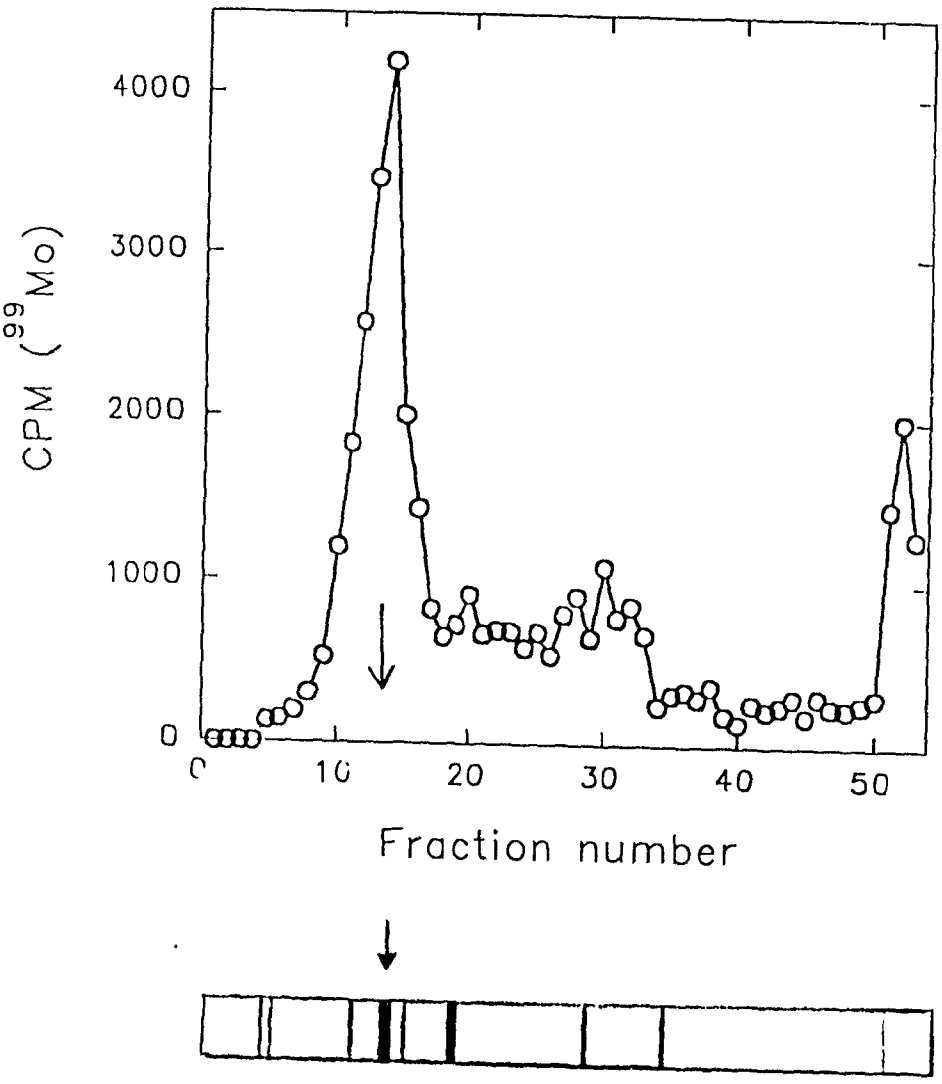

4000
3000
2000
1000
0
CPM (^{99}Mo)
0
10
20
30
40
50
Fraction number

Table 11. Summary of nitrate reductase assays performed in 4 different experiments with *Azospirillum brasilense* cells grown in W-free medium, as described in sections 3.2.2.2. and 3.2.2.3.

Fraction	Culture (mL)			
	I (200)	II (5000)	III (5000)	IV[a] (5000)
Soluble				
Total activity[b]	0	164	940	N.D.[d]
Gel[c]	N.D.	N.D.	N.D.	+
Solubilized Membrane				
Total activity	220	255	1260	N.D.
Gel	N.D.	+	+	-

[a] Culture containing 7 μCi.^{99}Mo.(nmol Mo)

[b] Expressed as nmol NO_2^-.min^{-1}.L^{-1}, as in section 3.3.1.

[c] Staining activity in gels performed as in section 3.4.

[d] Not Determined

gels only in experiment IV. The fact that nitrate reductase activity was observed in the soluble fraction, suggest that sonication was partly releasing nitrate reductase from the membrane.

4.8.2. EFFECT OF TUNGSTATE ON ^{99}Mo ACCUMULATION

A. brasilense cells steadily accumulated Mo under denitrifying conditions for at least 12 h, even though nitrate was completely reduced within 2 h (Fig. 18). In 10 mM tungstate-containing medium, *A. brasilense* cells did not accumulate significant amounts of ^{99}Mo, but nitrate was reduced within 3 h. Since the inoculum was grown in medium treated with *A. vinelandii* to remove contaminating molybdenum, this indicates either that the treated medium was not as thoroughly cleaned as that used in Figure 12 and that molybdenum was scavenged aerobically, or that traces of molybdate incorporated under denitrifying conditions (Fig. 18) were enough to support nitrate reduction. *P. denitrificans* cells accumulated Mo during the first hour only, and completely reduced nitrate within 2 h (Fig. 18). In W-containing medium, *P. denitrificans* did not accumulate Mo and did not reduce nitrate significantly.

Fig. 18. Accumulation of Mo and reduction of nitrate by *Azospirillum brasilense* cells (O , ●) and by *Paracoccus denitrificans* cells (Δ , ▲) grown anaerobically in either W-free (open symbols), or 10 mM tungstate-containing (closed symbols) medium supplemented with 1 μM molybdate and 5.8 μCi ^{99}Mo.(nmol Mo)$^{-1}$. *Azospirillum brasilense* and *Paracoccus denitrificans* cells were grown aerobically in treated medium, harvested during late-log phase, washed and inoculated (63 and 101 μg protein.mL^{-1}, respectively) into vacuum flasks as described in section 3.2.5.2. Each point is the mean of duplicates, after subtracting counts obtained with heat-killed cells.

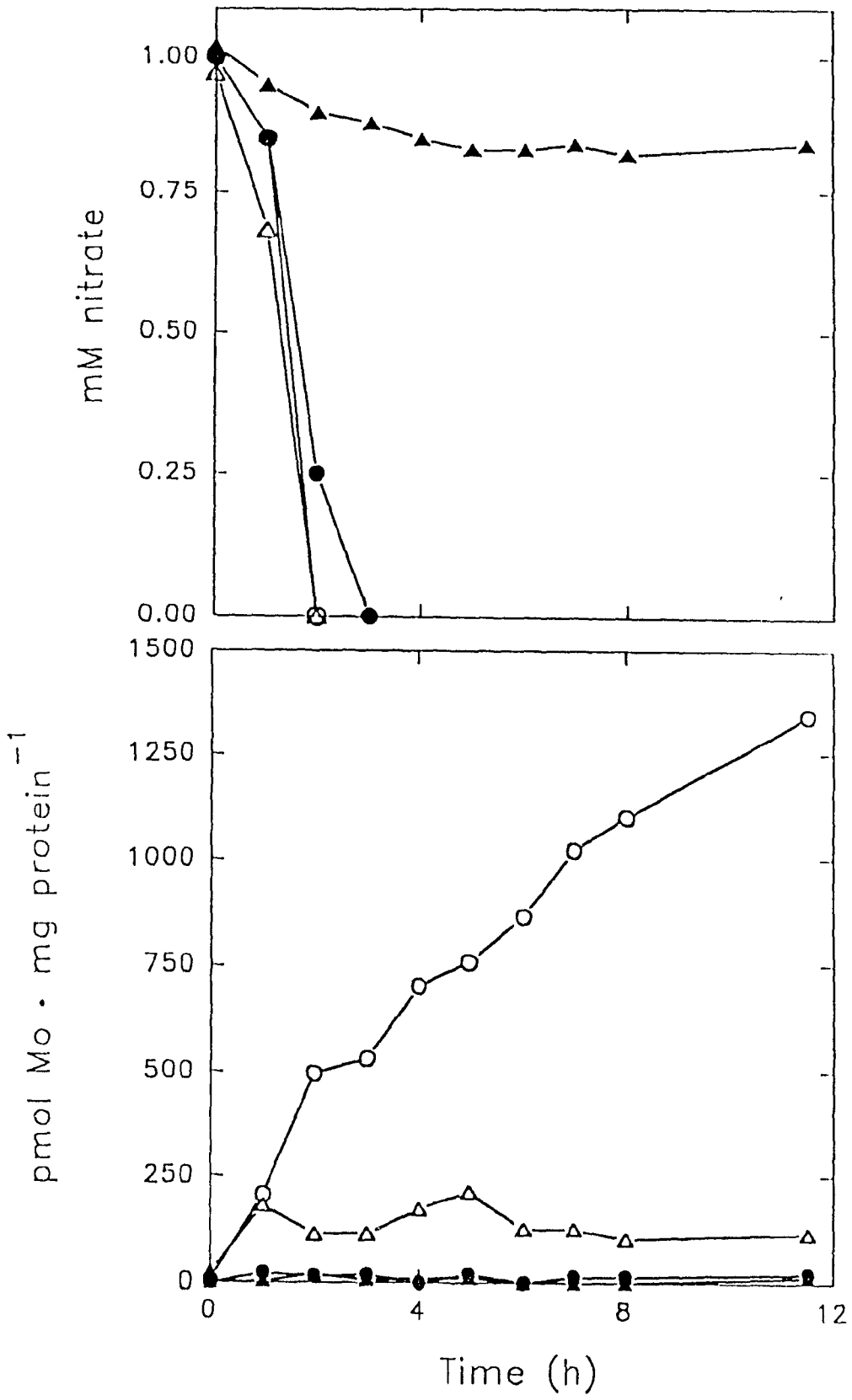

1.00
0.75
0.50
0.25
0.00
mM nitrate
1500
1250
1000
750
500
250
0
pmol Mo • mg protein^{-1}
0
4
8
12
Time (h)

Table 12. ^{99}Mo uptake by *Azospirillum brasilense* cells grown under aerobic and anaerobic conditions, as described in section 3.2.5.1.

Growth conditions	Assay conditions	Sampling time (min)	pmol Mo.mg protein^{-1}
Aerobic	Aerobic	1	390 ± 26[1]
		30	876 ± 108
	Anaerobic	1	88 ± 14
		30	109 ± 10
Anaerobic	Aerobic	1	94 ± 15
		30	134 ± 22
	Anaerobic	1	33 ± 2
		30	105 ± 18

[1] Data are means ± SEM of 2-3 replicates.

Table 13. Effect of different combinations of molybdate and tungstate on growth and nitrogenase specific activity of *Azospirillum brasilense* cells grown in semi-solid N-free medium, as described in section 3.2.3.

Growth medium			
Molybdate (μM)	Tungstate (mM)	μg protein.mL^{-1} [a]	μmol C_2H_4.mg protein^{-1}.day^{-1}
830	0	101.0 ± 5.2[b]	4.8 ± 0.9
0[c]	0	63.9 ± 6.0	6.4 ± 0.5
830	10	43.3 ± 6.8	2.5 ± 0.5
0	10	32.2 ± 1.8	0.09 ± 0.009

[a] Final concentration

[b] Data are means ± SEM of triplicates

[c] no addition (< 10 ppb Mo)

5. **DISCUSSION**

5.1. EFFECT OF TUNGSTATE ON GROWTH

Tungstate was essential for growth of the methanogenic archaeobacterium *Methanocorpusculum parvum* when H_2/CO_2 was the substrate (Zellner *et al.* 1987). Under similar conditions, however, the growth of *Methanobacterium formicicum* was not influenced by 1 mM tungstate (May *et al.* 1988). On the other hand N_2-fixing *Klebsiella pneumoniae* grew slightly in Mo-limited medium, but did not grow at all in 5 mM tungstate-containing Mo-limited medium (Brill *et al.* 1974). In this study, *Azospirillum brasilense* cells grew either aerobically or anaerobically in the presence of tungstate, but the growth rates were decreased by increasing levels of tungstate. Under aerobic conditions, high concentrations of tungstate must interfere somehow with the systems responsible for growth and cell survival. Under denitrifying conditions, the partial inhibitory effect of tungstate on nitrate reductase and nitrite reductase activities (section 5.2.) was reflected by lower growth rates.

5.2. EFFECT OF TUNGSTATE ON NITRATE AND NITRITE REDUCTION

Several studies have shown that tungsten is an inhibitor of the complete formation of the molybdoenzyme nitrate reductase in bacteria grown in Mo-limited media (Enoch and Lester 1972, Sperl and DeMoss 1975, Burke *et al.* 1980). Tungsten is a competitive inhibitor of molybdenum uptake, and high ratios of W to Mo in the growth medium prevent molybdenum utilization by the cells (Pinsent 1954, Takahashi and Nason 1957, Giordano *et al.* 1980). Under these conditions, the bacterial cells synthesize an inactive nitrate reductase, containing a W-cofactor, which can be activated upon the addition of molybdate (Sperl and DeMoss 1975, Amy and Rajagopalan 1979). Tungsten may somehow stabilize an inactive nitrate reductase. However *Azospirillum brasilense* was found capable of reducing nitrate in Mo-limited W-containing medium (Lalande 1984).

Results presented in this thesis confirmed that growing cells of *A. brasilense* can reduce nitrate even in the presence of very high levels of tungstate, but at significantly lower rates than in W-free medium. Nitrate reductase specific activities of resting cells initially grown in tungstate-containing medium were also lower than the activity of resting cells initially grown in W-free Mo-limited medium.

Burke *et al.* (1980) observed an 85 % inhibition of the nitrate reductase activity in *Paracoccus denitrificans* cells cultured in 1 mM tungstate-containing Mo-limited medium. Accordingly *P. denitrificans* was grown in Mo-limited medium, and was used as a control. Inactivation of nitrate reductase in *P. denitrificans* cells by 10 mM tungstate, using the same experimental protocol as for *A. brasilense*, suggested that *A. brasilense* cells were apparently resistant to tungsten inactivation of nitrate reductase.

No effect of tungsten on nitrite reductase activity has ever been published. This study demonstrates that nitrite reductase activity in *A. brasilense* was negatively affected by increasing levels of tungstate in the growth medium. Nitrite reductase, in *A. brasilense*, is a *cd*-cytochrome which contains many cysteine residues bound to the heme group (Bowen 1966). The oxygen atoms in tungstate might be substituted by sulphur producing thiotungstate (Rieck 1967). Accordingly, it seems possible that tungstate might disrupt the SH- bonds present in nitrite reductase by forming a thiotungstate, and this would account for the inhibitory effect of tungsten on nitrite reductase activity.

Nitrite reductase is most likely located at the periplasmic side of the cytoplasmic membrane (Newton 1969, Meijer *et al.* 1979), but nitrate reductase is a membrane-bound enzyme believed to be at the cytoplasmic side of the inner membrane (MacGregor *et al.* 1974, Kristjansson and Hollocher

1980). Preformed nitrite reductase activity, in either whole cells or cell-free extracts, was equally affected by increasing levels of tungstate, suggesting that tungstate was transported to the periplasm where it interacted with nitrite reductase. On the other hand, preformed nitrate reductase activity in whole cells was not significantly affected by 10 mM tungstate, whereas 1 mM tungstate caused an 80 % inhibition of the cell-free extract activities. This suggests that the cytoplasmic membrane, in whole cells, served as a protective barrier against tungsten inactivation of nitrate reductase.

[^{185}W]tungstate incorporation and uptake experiments were planned, but the isotope could not be obtained commercially.

5.3. MOLYBDATE-LIMITED AND SUPPLEMENTED CULTURES

All experiments, up to that point, had been performed in Mo-limited medium. Nitrate reduction, in W-free Mo-limited medium, was not enhanced by addition of molybdate. Since nitrate reductase is a molybdoenzyme, it seemed that *A. brasilense* cells could scavenge traces of contaminating molybdenum in order to support nitrate reduction in Mo-limited medium. Giordano *et al.* (1982), working with *E. coli* K12, observed that traces of contaminating molybdenum supported only 35 % of the activity of cells grown in the presence of 1 μM molybdate. *Azospirillum lipoferum* was found to secrete a catechol derivative siderophore capable of mediating the

transport of molybdenum under both molybdenum-limited and molybdenum-supplemented conditions (Saxena *et al.* 1989). *A. brasilense* cells also secrete a related compound, but it has not been shown to be involved in molybdenum transport (Bacchawat and Ghosh 1987). *Bradyrhizobium japonicum* and *Klebsiella pneumoniae* each have both high- and low-affinity Mo uptake systems under N_2-fixing conditions (Maier *et al.* 1987, Imperial *et al.* 1984). The high-affinity systems are expressed under Mo-limited conditions.

Molybdenum is a likely contaminant of growth media. Atomic absorption revealed that W-free media used in this study contained less than 10 ppb of Mo. Depleting molybdenum from the medium is possible by chromatography on immobilized ethylenediamine, or by filtration through 8-hydroxyquinoline immobilized on glass (Leyden *et al.* 1978, Eskew *et al.* 1984). The method used in this work consisted of inoculating and incubating the growth medium with *Azotobacter vinelandii* cells, which scavenge molybdenum constitutively, for a short period of time (Imperial *et al.* 1984). *A. brasilense* inoculum prepared aerobically in treated medium, and then transferred to vacuum flasks containing treated medium, did not reduce nitrate. This strongly suggested that nitrate reductase in *A. brasilense* was a molybdoenzyme; and that traces of contaminating molybdenum in the medium were enough to support activity.

5.4. ^{99}Mo EXPERIMENTS

5.4.1. ^{99}Mo INCORPORATION

In order to prove that nitrate reductase incorporated molybdenum, staining activity with disc gel electrophoresis was used. This technique, developed by Sperl and DeMoss (1975), is very useful to study metal incorporation in metalloenzymes. Enzymatic activity is seen as a white oxidized band in the blue background of reduced methyl viologen, which is used as an electron donor (Jones and Garland 1977). The gels can be sliced and incorporation of the isotope can be measured in each fraction.

This study demonstrates that nitrate reductase and nitrite reductase activities, in both *A. brasilense* and *P. denitrificans* can be routinely observed by using this technique. Nitrous oxide reductase activity in *A. brasilense* was also observed by this technique. This is, to our knowledge, the first time staining activity in disc gel electrophoresis is reported for nitrous oxide reductase activity. This technique could be used to follow copper incorporation into nitrous oxide reductase.

In this study, incorporation of ^{99}Mo in the nitrate reductase of *A. brasilense* cells grown in W-free medium was shown by disc gel electrophoresis. The slice showing enzyme activity corresponded clearly to ^{99}Mo incorporation. In a

duplicate gel stained for protein, a very large protein band was found at the same position as the oxidized band was seen, and it incorporated ^{99}Mo, indicating that nitrate reductase was a molybdoenzyme, and one of the major proteins present. Activity was observed with the soluble fraction, indicating that sonication partly removed nitrate reductase from the membrane. In preliminary experiments, nitrate reductase activity was detected in both the soluble and the solubilized membrane fractions. In this experiment, solubilization of the cytoplasmic membranes was not very effective, as indicated by the quantity of protein recovered. The low level of proteins in the solubilized membrane fraction might explain why no activity was detected in the gel with this fraction. Another factor was the long time required (4 h) for sonicating the entire cell suspension. It is possible that the cell-free extract, even though kept on ice, could have been more affected in this experiment.

Nitrate reductase activity of cells grown in 10 mM tungstate was also shown by disc gel electrophoresis. It was associated with the solubilized membrane fraction. However no ^{99}Mo incorporation was observed, suggesting that nitrate reductase activity of W-grown cells was not supported by molybdate incorporated during anaerobic growth.

5.4.2. ^{99}Mo TRANSPORT

Data obtained with preliminary transport experiments show that *A. brasilense* cells were much more efficient at taking up Mo aerobically than anaerobically. *K. pneumoniae* cells accumulate Mo under N_2-fixing conditions only, and Mo accumulation is repressed by O_2 and NH_4^+ (Pienkos and Brill 1981). Transport of molybdate by *Clostridium pasteurianum* is possible under N_2-fixing anaerobic conditions only (Elliott and Mortenson 1975). Anaerobically isolated *B. japonicum* bacteroids accumulated twice as much molybdate as did aerobically isolated cells (Maier and Graham 1988). This suggests that the capacity of *A. brasilense* to take up Mo aerobically is an uncommon characteristic among nitrogen fixers.

5.4.3. ^{99}Mo ACCUMULATION

Growing cells were used to study the long-term effect of 10 mM tungstate on ^{99}Mo accumulation under denitrifying conditions. *A. brasilense* cells growing in W-free medium accumulated Mo steadily under anaerobic conditions for at least 12 h even though nitrate was completely reduced within 2 h. On the other hand, *P. denitrificans* cells growing in W-free medium ceased uptake after 1 h. However 10 mM tungstate inhibited ^{99}Mo accumulation in both *A. brasilense* and *P.*

denitrificans cells, even though *A. brasilense* reduced nitrate in tungstate-containing medium. It suggests that nitrate reduction in W-containing medium was not supported by molybdate incorporated during anaerobic growth. This last result agrees with data obtained with dics gel electrophoresis of W-grown cells, in which ^{99}Mo was not incorporated into nitrate reductase which was nevertheless active in the gel.

5.5. EFFECT OF TUNGSTEN AND MOLYBDENUM ON NITROGEN-FIXATION

More molybdenum is required for N_2-fixation than for nitrate reduction possibly because the catalytic efficiency of nitrate reductase is greater (Maier *et al.* 1987). Accordingly, synthesis of the Moco and FeMoco, in *K. pneumoniae* and *E. coli* at least, occurs via two different pathways, which are tightly regulated (Ugalde *et al.* 1985). In *A. brasilense*, preliminary nitrogen fixation experiments revealed that traces of molybdenum supported nitrogen fixation in W-free medium. However nitrogenase activity of cells grown in 10 mM tungstate-containing Mo-limited medium was completelely inhibited. On the other hand, previous results demonstrated that nitrate reductase was active in tungstate-containing Mo-limited medium. It confirmed that, in *A. brasilense*, more molybdenum was needed for nitrogenase activity than for nitrate reductase activity.

5.6. EFFECT OF NITRATE ON NITRITE REDUCTION

Lalande (1984), when they observed that nitrate reductase in *A. brasilense* was not inactivated by 10 and 25 mM tungstate, were trying to inhibit nitrate reduction in order to study the effect of nitrate on nitrite reduction. In our study, it was observed that increasing levels of tungstate in the growth medium correlated with longer delays in the onset of nitrite reduction. In fact, nitrite reduction seemed to start only when nitrate levels in the medium were very low (less that 0.05 mM), whether or not the cells were supplemented with nitrite initially. Nitrate was present for longer periods in tungstate-containing media because nitrate reduction rates were lower than in W-free medium, and it could explain the delay observed in the onset of nitrite reduction.

The immediate effect of adding 0.25 mM nitrate to cells reducing nitrite was the appearance of a second peak of nitrite accumulation. Because both nitrate reductase and nitrite reductase activities were similar, no nitrite accumulation should have been observed if nitrite reductase was still active. Thus, this second peak of nitrite accumulation could only be the result of an inhibitory effect of nitrate on nitrite reduction. So nitrite accumulation was probably not dependent on different rates of reduction, as was the case in *Pseudomonas fluorescens* and *Alcaligenes* sp. (Betlach and Tiedje 1981). Körner and Zumft (1989), working

with *Pseudomonas stutzeri*, also observed the immediate formation of a second peak of nitrite accumulation on addition of 25 mM nitrate to cells reducing nitrite. The immediate inhibition of nitrite reductase activity on addition of nitrate might either be the result of competition between nitrate and nitrite as electron acceptors or an effect of nitrate on nitrite reductase itself.

This study demonstrates that nitrite reductase was synthesized by cells accumulating nitrite, because the specific activity of cells accumulating nitrite, after washing, was equal to the specific activity of cells harvested while actively reducing nitrite. Therefore the effect of nitrate is on nitrite reductase activity only, and probably not on synthesis. Further studies with antibodies raised against nitrite reductase could confirm this assumption.

In conclusion, it is suggested that *A. brasilense* cells can accumulate and store molybdenum constitutively, under both aerobic and anaerobic conditions, in W-free Mo-limited medium. Molybdenum stored under aerobic conditions may then be used for nitrate reductase synthesis and activity in tungstate-containing medium, without being easily exchanged by overwhelming levels of tungstate, since it was shown that tungsten did not appear to be transported through the cytoplasmic membrane where nitrate reductase is probably located.

The Mo-uptake system of *A. brasilense* needs to be studied in greater detail. For example, it would be interesting to determine whether or not Mo transport in *A. brasilense* is mediated by a catechol-derivative siderophore. Purification of nitrate reductase from *A. brasilense* cells grown in W-containing (either Mo-limited or Mo-depleted) medium should be attempted to determine whether or not a W-containing nitrate reductase is synthesized. In addition, further studies on the nitrogenase system would clarify the Mo requirement of this enzyme in *A. brasilense*.

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