Molybdenum as a Micronutrient for *Nitrobacter*

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ABSTRACT

FINSTEIN, M. S. (University of California, Davis), AND C. C. DELWICHE. Molybdenum as a micronutrient for *Nitrobacter*. J. Bacteriol. **89**:123-128. 1965.—The requirement of *Nitrobacter* for molybdenum was investigated by use of a medium purified by chemical means, as well as by depletion by the prior growth of this autotrophic bacterium. During 5 days of growth, added molybdenum induced an approximately 11-fold increase in both nitrite utilization and cell-mass development. In short-term studies, maximal response was obtained with concentrations as low as 10^{-9} M. No other metal tested substituted for molybdenum. It is estimated that a minimum of 2,000 atoms of molybdenum is required for the synthesis of one *Nitrobacter* cell. Molybdenum did not directly influence the thermodynamic efficiency of *Nitrobacter*. The efficiency of molybdenum-deficient cultures and of cultures supplied with the micronutrient declined equally during the early stage of development, despite large differences in the amount of nitrite consumed. After the utilization of about 100 μ moles of nitrite per ml, cultures supplied with molybdenum became relatively less efficient. A procedure is described whereby large masses of *Nitrobacter* cells can be obtained with relative ease.

The difficulty of obtaining large amounts of *Nitrobacter* has impeded the biochemical investigation of this chemoautotrophic nitrite-oxidizing bacterium. One major result of a considerable effort to improve culture conditions was the demonstration that particulate matter is not needed for growth (Aleem and Alexander, 1958). Later, Gould and Lees (1960) showed that *Nitrobacter* can be grown in continuous culture. Another approach to the improvement of yields has been the use of dialysis techniques to remove metabolic products from the culture medium, on the premise that the accumulation of nitrate interferes with growth (Gould and Lees, 1960; Boon and Laudelout, 1962). Despite these developments, however, the difficulty of growing cells in quantity continues to be a serious obstacle in the study of *Nitrobacter*.

Nutritional studies have indicated that the growth requirements of *Nitrobacter* include calcium (Kingma-Boltjes, 1935), iron, phosphorus, and magnesium (Aleem and Alexander, 1960). Zavarzin (1957) was the first to note that molybdenum enhances the development of freshly inoculated cultures, as measured by nitrite oxidation. Subsequently, he reported that the effect varied with the pH of the medium. Within a

range of 7.7 to 8.3, added molybdenum approximately doubled substrate consumption but at pH 8.8 molybdenum suppressed growth (Zavarzin, 1958). Aleem (1959) also noted a slight, but variable, stimulation with molybdenum. To the present time, this element has not generally been included in *Nitrobacter* media.

The present report concerns the influence of molybdenum on the growth of *Nitrobacter* and the essentiality of this micronutrient. The investigation led to a study of *Nitrobacter* efficiency, results of which are also reported.

MATERIALS AND METHODS

A *Nitrobacter* culture, originally derived from local garden soil, was tested for purity by placing loopfuls on the following solid media: nutrient agar; 0.3% yeast extract plus 1.5% agar; nutrient agar plus 0.3% yeast extract (NY agar); NY agar plus 1% beef liver; NY agar plus 1% malt extract; NY agar plus 1% beef heart; NY agar plus 1% hemoglobin; Tryptone Glucose Extract Agar; Veal Infusion Agar; Potato Dextrose Agar; orange serum agar #77; soil extract agar; and old *Nitrobacter* culture (NO_2^- exhausted) plus 1.5% agar (NB agar). When available, commercial dehydrated products were used. All commercial materials were Difco products except the orange serum agar, which is manufactured by Sunkist Growers, Inc., Los Angeles, Calif. Since no colonies developed during 1 week of incubation on these media, the culture was considered to be pure. In every case, purity was again checked at

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time of harvest by prolonged incubation on organic media. Incubations throughout this investigation were carried out at 28 to 30 C. During the course of the investigation, the maintenance of purity was routinely monitored by plating cultures that had achieved substantial growth onto NY and NB agar and incubating for as long as 3 weeks. No colonies developed, indicating that culture purity was maintained.

Large-scale cultivation was carried out in 20-liter Pyrex carboys containing 18 liters of a medium made with reagent or national formulary grade salts inoculated with culture (approximately 1%, v/v) from shaken flasks. Medium A consisted of: KNO_2 , 0.3 g; K_2HPO_4 , 0.5 g; KH_2PO_4 , 0.5 g; NaCl , 0.2 g; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 25 μg (where added); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg; KHCO_3 , 1.5 g; and distilled water, 1 liter. The first five ingredients were autoclaved together, as were the calcium and magnesium salts. The last two salts were sterilized together by passage through a Millipore filter (pore size, 0.45 μ). Sterile air was introduced into the medium from a glass tube 8 mm in diameter. At the first development of turbidity, daily sampling by means of a sampling tube and the determination of the nitrite level by the method of Rider and Mellon (1946) were begun. Increments of NaNO_2 were added in amounts needed to prevent the culture from becoming depleted of substrate during the subsequent 24 hr.

One experiment was carried out by use of a medium made with purified salts (medium B). Phosphate, chloride, and sulfate were subjected to copper sulfide coprecipitation (Hewitt and Hallas, 1951), HCl was purified by redistillation, and NaOH was purified by coprecipitation techniques with $\text{Mg}(\text{OH})_2$ (Munns and Johnson, 1960). These were used in the acidification and reneutralization procedures. Iron and carbonate ion were purified by recrystallization, the latter at room temperature by addition of twice-distilled ethyl alcohol. Medium B consisted of: NaNO_2 , 0.25 g; KH_2PO_4 , 0.20 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg; KHCO_3 , 1.5 g; and glass-distilled water, 1 liter. A low phosphorus content was chosen, because phosphates may contain appreciable amounts of contaminating molybdenum (Nicholas, 1952). The first and second pairs of salts were autoclaved separately, and the last two ingredients were sterilized together by filtration. The ingredients were combined aseptically to give 300 ml of medium in 1-liter Erlenmeyer flasks, and were then inoculated with molybdenum-deficient culture. Aeration was provided by shaking on a reciprocating shaker.

During the experiment with medium B, nitrite was estimated semiquantitatively at daily intervals with a loopful of culture obtained with an acid-washed platinum loop fitted to a glass handle. If it appeared that the substrate might become depleted during the subsequent 24 hr, a measured increment of NaNO_2 was added to the culture.

At the termination of the experiment, residual nitrite was determined; this value was subtracted from the cumulative amount of substrate originally in the medium and the added increments to give the total nitrite consumed during the incubation.

Glassware prerinsed with 1% NaOH followed by treatment with ethylenediaminetetraacetic acid and HNO_3 (Munns and Johnson, 1960) was used in most of the experiments. When necessary, pipettes were sterilized in glass tubes to avoid contamination from metal cannisters.

The molybdenum content of *Nitrobacter* was determined by use of a minimum of 190 mg of cells (oven-dry weight) which had been harvested with a Sharples centrifuge and washed with dilute HCl . The procedure of Johnson and Arkley (1954) was used for the assay.

To determine cell weight, sufficient culture was passed through tared Millipore filters to collect 5 to 10 mg of cells (weighed after drying over CaCl_2). The error introduced by the chemical precipitate and inoculum was estimated from a large volume of medium which had received boiled inoculum. The weight of the extraneous particulate matter so collected, which usually amounted to a few tenths of a milligram per liter, was subtracted from the experimental values. To calculate thermodynamic efficiency, it was assumed that 50% of the cell dry weight consisted of carbon.

To calculate thermodynamic efficiency, the equation of Baas-Becking and Parks (1927) was used. Overall efficiency was calculated by use of the values for cumulative cell weight and nitrite oxidized at the time of sampling. Interval efficiency was calculated by using the value for the increment of cell weight during the preceding 24 hr and the corresponding quantity of nitrite consumed.

RESULTS

Requirement of molybdenum for growth. During the cultivation of *Nitrobacter* on a large scale, the rate of substrate utilization frequently became drastically retarded after the consumption of about 15 μmoles of nitrite per ml of medium. To determine whether some micronutrient deficiency was responsible for slow growth, the following salts were added, at approximately 10^{-5} M concentrations, to separate cultures: ZnSO_4 , CuCl_2 , NaVO_3 , BaCl_2 , $\text{K}_2\text{Al}_2(\text{SO}_4)_4$, $\text{K}_2\text{Cr}_2\text{O}_7$, $\text{Ni}(\text{NO}_3)_2$, TiO_3 , $\text{Co}(\text{NO}_3)_2$, NaWO_4 , and Na_2MoO_4 . The cobalt and tungsten salts slightly inhibited substrate oxidation as judged qualitatively; except for molybdenum, the other amendments had no discernible effect. Addition of sodium molybdate resulted in a striking acceleration in both the rate of nitrite utilization and increase in turbidity, indicating that the culture had depleted the medium of this micronutrient.

To determine the effect of different levels of

molybdenum on *Nitrobacter* development, 50-ml portions from an 18-liter culture (medium A) devoid of added micronutrient were dispensed into 250-ml Erlenmeyer flasks. Prior to subdivision, the culture was in the stationary phase; the rate of nitrite utilization had been constant at about 2 μ moles per ml per day, and a total of approximately 30 μ moles of NO_2^- per ml had been consumed. The extent of nitrite consumption and the increase in turbidity was determined after 35 hr of exposure to different levels of molybdenum (Fig. 1). To give the net change, turbidity determined at the time of subdivision was subtracted from the reading at the end of the 35 hr. Measured either by nitrite consumption or by development of turbidity, a stimulatory effect of the micronutrient is evident; a concentration of molybdenum as low as 10^{-9} M elicited the maximal response. At higher concentrations, molybdenum was slightly inhibitory, but even a 10^{-2} M concentration proved relatively innocuous. Thus, essentially the same response, both in nitrite oxidation and cell development, was obtained over a concentration range of 10^{-4} to 10^{-9} M.

The experiment reported in Fig. 1 measured the effect of molybdenum during a relatively short incubation period. It was expected that prolonged incubation, with a resultant heightened demand on the micronutrient supply, would more clearly demonstrate the requirement for molybdenum. This possibility was explored by use of chemically purified medium B with incubation over an extended period of time (Table 1). Among various experiments, the length of the apparent lag phase varied from 2 to 4 days; the values reported in Table 1 are those for the duration of incubation after the onset of active nitrification ("days of active growth"). Considering only the data obtained after 5 days of active nitrification, nitrite disappearance was 10.6 times greater with an adequate molybdenum supply than without added micronutrient. Similarly, cell yield was increased 10.9-fold by the addition of molybdenum. Clearly, an adequate supply of molybdenum is needed for the development of *Nitrobacter*.

After 5 days of active growth, the magnitude of the response observed was essentially the same whether measured by amount of substrate utilized or cell yield. Therefore, thermodynamic efficiency, expressed as the ratio of cell weight produced per unit of nitrite consumed, is the same with or without supplemental molybdenum. By supplying molybdenum, a greater mass of cells was obtained, but at the expense of a proportionately greater amount of substrate. Apparently, the relative abundance of the micronutrient has no direct effect on thermodynamic efficiency.

FIG. 1. Effect of molybdenum on cell division and nitrite utilization. Curves show average values obtained for duplicate treatments after 35 hr of incubation in presence of molybdenum concentrations shown.

TABLE 1. Effect of molybdenum on *Nitrobacter* proliferation*

Expt no.	Treatment	Days of active growth	NO ₂ ⁻ consumed		Dry wt of cells		Efficiency†	
			Amt	S _x	Amt	S _x	Per cent	S _x
1	No added Mo	5	14.7	1.7	5.7	1.1	11.4	1.1
	Plus Mo	5	156	3.1	62	2.4	11.2	0.30
2	No added Mo	8	19.0	1.6	5.6	1.1	8.7	1.9
	Plus Mo	8	244	12	56.6	3.1	6.4	0.8

* Figures given are the average of four replications of each treatment. Per cent efficiency is calculated on the basis of thermodynamic values used by Baas-Becking and Parks (1927) and assuming cells contained 50% carbon on a dry weight basis. S_x = standard error.

† Per cent efficiency =

$$\frac{\mu\text{moles carbon in cells}}{\mu\text{moles nitrite oxidized}} \times \frac{118}{17.5} \times 100.$$

After 8 days, the response to molybdenum measured by nitrite disappearance was slightly greater than the response measured by cell weight, perhaps resulting from the dissolution of older cells. The influence of extended incubation on efficiency will be considered more fully later.

Because the extensive proliferation of cells resulting upon the addition of molybdenum could have induced deficiencies of other micronutrients, a mixture of salts was tested for possible growth-promoting influence on cultures adequately supplied with molybdenum. The mixture consisted of CuSO_4 , MnCl_2 , ZnSO_4 , and $\text{Na}_2\text{B}_4\text{O}_7$, each supplied at a final concentration of 2×10^{-6} M. During 8 days of active growth, essentially equal quantities of nitrite were utilized with or without the salt mixture.

FIG. 2. Comparative overall efficiency of *Nitrobacter* grown in the absence of added molybdenum (curve A) and in the presence of 10^{-6} M molybdenum (curve B). Numbers in parentheses give cumulative quantities of nitrite consumed at point shown (micromoles per milliliter).

Molybdenum content of *Nitrobacter*. Cells harvested from two 18-liter cultures (medium A) were assayed for their molybdenum content. The results indicate that rapid growth in the presence of added molybdenum was accompanied by the incorporation of relatively large amounts of the micronutrient. Although growth was severely restricted in the absence of added micronutrient, the cells which developed contained appreciable molybdenum. Apparently, the developing cells scavenged contaminating traces of molybdenum present in the medium. It is estimated that the cells effected approximately a 1 million-fold concentration of the micronutrient from the medium. These deprived bacteria each contained about 2,000 atoms of molybdenum, which may be considered the minimal requirement for the synthesis of one *Nitrobacter* cell. However, during the extended incubation necessitated by the shortage of the micronutrient (30 days for the culture with no added molybdenum), some molybdenum may have been lost from the cells. For this reason, 2,000 atoms per cell may be an underestimate of the true requirement. By way of comparison, it has been estimated that an *Azotobacter* cell contains, at a minimum, 1,000 to 10,000 atoms of molybdenum or vanadium (Burk and Horner, 1935).

Thermodynamic efficiency. In Table 1 it was shown that, after 5 days of active growth, efficiency with or without added molybdenum was essentially the same despite a 10.6-fold difference in the amount of nitrite consumed. The accumulation of nitrate in the medium due to the oxidation of nitrite appears to have had little influence on efficiency for at least the first 8 days of active growth. This observation is at variance with the

supposition of Meyerhof (1916) and others that efficiency is inversely related to nitrate concentration. Although it has been suggested by some investigators that nitrate must be removed from the medium to achieve unrestricted growth (Gould and Lees, 1960; Boon and Laudelout, 1962), the data in the present report indicate that a relatively high concentration of nitrate is not detrimental. To ascertain the influence of extended incubation and the resulting nitrate accumulation on *Nitrobacter* efficiency, further investigation was undertaken (see Fig. 2).

Two carboys of medium, one of which was supplemented with molybdenum, were inoculated. After a lag of 3 days, nitrification commenced simultaneously in both vessels, and thereafter volumes of culture were withdrawn daily for the estimation of overall efficiency. During the first 4 days of active growth, efficiency dropped from about 21 to 13%, regardless of whether 8 or 83 μmoles of NO_2^- per ml had been consumed, indicating that the initial decline was independent of the concentration of nitrate or other metabolic products. With restricted growth (minus molybdenum), the decline in efficiency tended to level off, and after 8 days the value was about 12%. With adequate molybdenum, however, efficiency declined further and reached 6% at the termination of the incubation. The relative decline of efficiency of the molybdenum-sufficient culture during the latter part of the incubation may have been induced by the accumulation of large amounts of nitrate as a result of extensive growth, or possibly was due to a small but significant change in hydrogen ion concentration. The measured pH did not change during the period of the experiment from an initial value of 8. However, nitrous acid has a pK_a of 4×10^{-4} , and nitric acid is completely dissociated. Although the medium used is highly buffered, this production of nitrate will be reflected in a pH shift.

Data from a separate trial, with a single molybdenum-sufficient culture, were used to calculate both overall and interval efficiencies. As with the plus-molybdenum culture of Fig. 2, efficiency appeared to decline in two phases, although evidence for this is not unequivocal. In general, the second decline occurred after the oxidation of about 120 μmoles of NO_2^- per ml, although the exact value varied somewhat among the different experiments. Interval efficiency was lower than overall efficiency; the former value more closely represents efficiency at a given time. In the last 2 days, the interval efficiency became negative. This is a consequence of a decrease in cell weight per unit volume of medium, despite the continued oxidation of nitrite. Apparently, con-

siderable cell dissolution occurs in the aftermath of intensive growth. The conclusion that the accumulation of metabolic products, presumably nitrate, eventually arrests development of the culture and that the decline in apparent efficiency in old cultures is a reflection of this arrested development appears inescapable. The early decline in efficiency discussed above is unexplained.

DISCUSSION

The stimulatory influence of molybdenum on *Nitrobacter* growth, heretofore reported to be variable and of limited magnitude (Aleem and Nason, 1963), now seems to be firmly established. The magnitude of the response during extended incubations, the linear relationship between growth and added molybdenum in the short-term experiment, and the accumulation of the micro-nutrient by cells growing in deficient medium all indicate that molybdenum is indispensable for *Nitrobacter* growth.

Although the greatest response was achieved with repurified salts, medium made with non-repurified chemicals did not support extensive growth without the addition of molybdenum. Investigators who have attempted the intensive cultivation of *Nitrobacter* have generally obtained restricted growth, as evidenced by the amount of substrate consumed during extended incubations. For example, Meyerhof (1916) reported the oxidation of 106 μ moles of nitrite per ml during a 17-day incubation; Aleem and Alexander (1958), approximately 100 μ moles in 9 days; and Gould and Lees (1960), using tap water in the medium, 140 μ moles in 10 days. These values are in contrast to the oxidation of almost 250 μ moles of nitrite per ml, 10 days after inoculation, with an adequate supply of molybdenum. It appears that *Nitrobacter* has generally been cultivated under molybdenum-deficient conditions, which undoubtedly has contributed to the difficulty of growing this bacterium.

The high efficiencies obtained with young cultures, as well as those reported by Gould and Lees (1960), indicate that the maximal efficiency of *Nitrobacter* is considerably higher than values reported in the early literature (Bass-Becking and Parks, 1927). Meyerhof's (1916) suggestion that efficiency declines rapidly as the culture develops has received experimental support, especially from the rapid decline of the interval efficiency.

Although the accumulation of nitrate may contribute to the decline of efficiency after extensive growth periods, the present investigation indicates that the initial decline of efficiency is independent of the concentration of nitrate. The

data of Gould and Lees (1960), when expressed as efficiencies rather than cell weights, also indicate that the initial decline in efficiency is the same whether or not nitrate is accumulated or removed from the medium.

In the opinion of Aleem and Nason (1963), the accumulation of nitrate does not seriously interfere with culture development, whereas Gould and Lees (1960) contended that the accumulation of nitrate is deleterious to growth. These conflicting views may be reconciled with our observation that nitrate accumulation apparently contributed to the decline in efficiency, but only after a concentration in excess of about 0.1 M was attained.

A routine procedure designed to produce optimal cell yields can be established by use of the data on interval efficiency. When supplied with molybdenum, the most efficient utilization of substrate occurs during the first 4 days of active growth (about 120 μ moles of nitrite oxidized per ml); subsequently, interval efficiency declines rapidly and little additional cell mass is obtained. By harvesting after the utilization of approximately 120 μ moles of nitrite per ml, an 18-liter culture yields about 0.5 g of cells (oven-dry weight).

Zavarzin's (1958) suggestion that molybdenum participates directly in the enzymatic oxidation of nitrite was based, in part, on the observed stimulatory effect of molybdenum on nitrite oxidation by freshly inoculated cultures. The present report shows that substrate consumption and cell yields were both enhanced to the same extent in the presence of adequate molybdenum. The intensification of nitrite oxidation may simply reflect the greater mass of cells that developed, and does not necessarily indicate that molybdenum functions directly in enzymatic nitrite oxidation. There are indications that the role of molybdenum may be less directly connected with the oxidation of nitrite than was earlier supposed. The demonstrated function (Nicholas, Nason and McElroy, 1953) of molybdenum in nitrate reductase suggests, but does not establish, a function in the reverse reaction. Additional studies of this aspect are in progress, and the function of molybdenum in *Nitrobacter* physiology will be considered in detail in a separate communication.

Present information does not establish, nor does it exclude, the existence of identical pathways for the oxidation of nitrite and reduction of nitrate.

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