

# Role of Molybdenum in Nitrate Reduction by *Chlorella*<sup>1</sup>

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J. M<sup>a</sup> VEGA, J. HERRERA, P. J. APARICIO, A. PANEQUE, AND M. LOSADA

Departamento de Bioquímica, Facultad de Ciencias y CSIC, Universidad de Sevilla, Spain

## ABSTRACT

Molybdenum is absolutely required for the nitrate-reducing activity of the nicotinamide adenine dinucleotide nitrate reductase complex isolated from *Chlorella fusca*. The whole enzyme nicotinamide adenine dinucleotide nitrate reductase is formed by cells grown in the absence of added molybdate, but only its first activity (nicotinamide adenine dinucleotide diaphorase) is functional. The second activity of the complex, which subsequently participates also in the enzymatic transfer of electrons from nicotinamide adenine dinucleotide to nitrate (F<sub>NH</sub><sub>2</sub>-nitrate reductase), depends on the presence of molybdenum. Neither molybdate nor nitrate is required for nitrate reductase synthesis *de novo*, but ammonia acts as a nutritional repressor of the complete enzyme complex. Under conditions which exclude *de novo* synthesis of nitrate reductase, the addition of molybdate to molybdenum-deficient cells clearly increases the activity level of this enzyme, thus suggesting *in vivo* incorporation of the trace metal into the pre-existing inactive apoenzyme.

Competition studies with tungstate corroborate these conclusions and indicate that the only role played by molybdenum in *Chlorella* is connected with the reduction of nitrate to nitrite. Tungsten seems to act by replacing molybdenum in the nitrate reductase complex, thus rendering inactive the F<sub>NH</sub><sub>2</sub>-nitrate reductase portion of the nicotinamide adenine dinucleotide nitrate reductase complex.

The assimilatory nitrate-reducing system of the green alga *Chlorella* has been thoroughly characterized in recent years (13-16, 21, 23, 24, 28, 29) and has been shown to be similar to that of higher plants (6, 9). The reduction of nitrate to ammonia proceeds in two independent and well defined steps: (a) the reduction of nitrate to nitrite, catalyzed by the flavo-molybdoprotein NADH-nitrate reductase; and (b) the reduction of nitrite to ammonia, catalyzed by the iron protein ferredoxin-nitrite reductase. In the transfer of electrons from NADH to nitrate, catalyzed by the enzyme complex NADH-nitrate reductase, two enzymatic activities which act sequentially can be independently assayed although not physically separated. The first is a FAD<sup>2</sup>-dependent NADH-diaphorase, which can use a variety of oxidized compounds (such as cytochrome c) as electron acceptor; the second is the molybdenum-dependent nitrate reductase proper or terminal nitrate reductase, which can use reduced flavin nucleotides (or viologens) as electron donors, and has therefore been named F<sub>NH</sub><sub>2</sub>-

nitrate reductase. A peculiar characteristic of this second enzyme is that it can be reversibly converted both *in vivo* and *in vitro* into an active or inactive form, ammonia acting as the nutritional promoter of the metabolic inactivation. All the enzymes of the *Chlorella* nitrate-reducing system, including nitrite reductase, are repressed by ammonium ion.

The role of molybdenum as an essential trace element for algae and higher plants in the process of nitrate reduction has been firmly established (6, 8, 9, 17). However, although molybdenum was early identified by Nicholas and Nason (19) as the metal prosthetic group of nitrate reductase from soybean leaves, workers in the field (3, 6, 14) have until recently been unable to find evidence for either its presence or its function as an electron carrier in nitrate reductase preparations purified from a variety of green plants species. Hewitt and coworkers (1, 2, 9) have reported that, in molybdenum-deficient plants grown in the presence of nitrate, molybdenum is required for the synthesis of nitrate reductase and have suggested that it is involved in the induction process and acts not merely as the constituent metal. Heimer *et al.* (7) and Wray and Filner (27) have recently studied the effect of tungstate as a competitive inhibitor of molybdate on nitrate assimilation in higher plant tissues and have described the structural and functional relationships of enzyme activities induced by nitrate in barley. Their results support the idea that the nitrate reductase complex is formed in the presence of tungstate but is functional only with respect to NADH-diaphorase. By adding radioactive <sup>99</sup>Mo (as molybdate) to a culture of *Chlorella* cells at the moment derepression of the enzymes of the nitrate-reducing system was initiated by removal of ammonia from the medium, we have recently been able to demonstrate that the metal becomes incorporated into nitrate reductase and remains associated with it during purification (3). Furthermore, we showed that after a mild heat treatment of the enzyme exogenous molybdate chemically reduced by hydrosulfite can be used as electron donor for the enzymatic reduction of nitrate (3).

The present work is intended to clarify the role played by molybdenum in nitrate reduction by *Chlorella*.

## MATERIALS AND METHODS

*Chlorella fusca* Shihira et Kraus (*pyrenoidosa*, strain 211-15 from Pringsheim's culture collection at Göttingen) was grown autotrophically as described previously (29). When ammonia or ammonium nitrate replaced nitrate as the source of nitrogen, the molarity of each compound was also maintained at 8 mM. In the experiments where ammonium ions were involved, the culture media were buffered with 20 mM sodium phosphate, pH 7.5. In the experiments involving the effect of metals, molybdate was omitted from the standard nutrient solution and either sodium molybdate or sodium tungstate was added as indicated. The algae used for inoculation were grown on nitrate media lacking added molybdate in order to produce a low molybdenum inoculum. Growth was determined by meas-

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<sup>2</sup> Abbreviation: FAD: flavin adenine dinucleotide.

uring absorbance changes at 660 nm. Similar results were obtained when growth measurements were verified either by cell counting in a hemocytometer or by dry weight determinations; a slope change in the growth curve (as plotted semilogarithmically) occurred generally after a period of about 50 hr and appeared to be due, at least in the case of nitrate cultures, to depletion of the nitrogen source (unpublished data).

For preparation of the alga crude extracts, the cells were harvested at the logarithmic phase by low speed centrifugation and after washing were ground in the cold with alumina in a mortar. The broken material was extracted with 50 mM tris-HCl, pH 7.5, containing 10  $\mu$ M FAD, and, after centrifugation for 20 min at 20,000g, the supernatant was used for determining the specific activities of the nitrate reductase complex as previously described (15, 28, 29). Enzyme activity units are expressed as  $\mu$ moles of substrate utilized or product formed per minute.

Sucrose density gradient centrifugation of the cell-free extracts was carried out as follows: an aliquot (0.15 ml) of the crude extract was layered on top of the gradient, which was composed of 4.35 ml of a linear 5 to 20% (w/v) sucrose gradient layered over 0.15 ml of 50% (w/v) sucrose. Sucrose was dissolved in 50 mM tris-HCl, pH 7.5, containing 0.2 M NaCl and 10  $\mu$ M FAD. Centrifugation was carried out at 45,000 rpm for 9 hr at 5°C in a Spinco L2-50B ultracentrifuge with SW-50 L rotor. Gradients were fractionated from the bottom of the tube with a Densi-Flow from Buchler Instruments Inc. Fractions of four drops each were collected using a LKB fraction collector and diluted up to 0.7 ml with the solvent used for sucrose solution. Activities of the nitrate reductase complex were then determined in aliquots of each fraction.

## RESULTS

Under our culture conditions, the normal molybdenum supply for *Chlorella* cells is 16 nM, a concentration that is well within the range for optimal growth found by Arnon *et al.* (4) for *Scenedesmus*. In fact, the omission of molybdate from the standard nutrient solution had little effect on the growth of *Chlorella* (presumably owing to contamination of the other components with the trace metal), and no toxic effect was observed when molybdate was added at concentration up to 100  $\mu$ M; 1 mM became clearly inhibitory. It was therefore surprising to find that the activity levels of the nitrate reductase complex were affected markedly by the concentration of molybdate added to the medium (Fig. 1). When no molybdate was added, the activity of the corresponding cell-free extracts was extremely low with respect to both NADH-nitrate reductase and

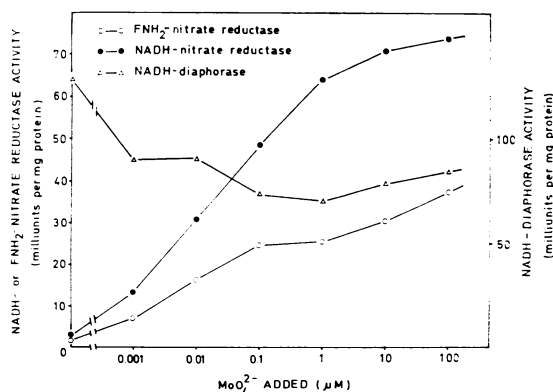


FIG. 1. Effect of molybdate added to culture media on the activity levels of the nitrate reductase complex in *Chlorella* cells grown with nitrate.

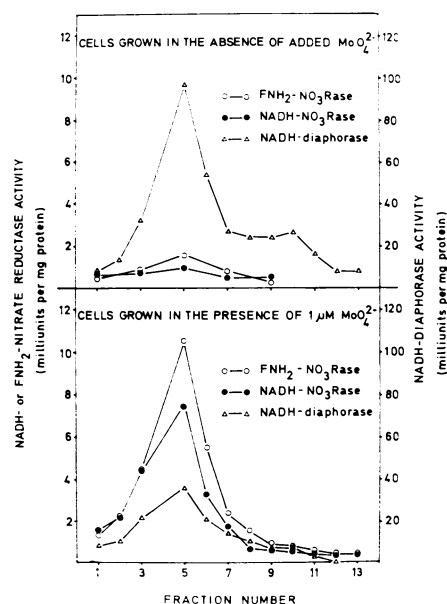


FIG. 2. Analysis by sucrose density gradient centrifugation of extracts from *Chlorella* cells grown with nitrate in the absence and in the presence of added molybdate with respect to the distribution of activities of the NADH-nitrate reductase complex. Fraction 1 corresponds to the gradient bottom.

FNH<sub>2</sub>-nitrate reductase; it was, however, high (even above average value) with respect to NADH-diaphorase. The addition of increasing amounts of molybdate, up to 100  $\mu$ M, gradually increased the levels of NADH- and FNH<sub>2</sub>-nitrate reductase activities up to 20-fold. By contrast, the level of NADH-diaphorase activity decreased somewhat initially but then remained more or less high and constant until the end. It remains an interesting fact that the levels of nitrate reductase activity may rise so high in response to the molybdenum supply, considering that the requirements for optimal growth are much lower. According to the results presented below, it seems that the complete enzyme complex is apparently synthesized in greater amounts than those needed for the fulfillment of its essential role in nitrate reduction.

When the extracts prepared from *Chlorella* cells grown both in the absence of added molybdate and in the presence of 1  $\mu$ M molybdate were simultaneously submitted to sucrose density gradient centrifugation analysis, it could be ascertained that the complete nitrate reductase complex was formed even in the absence of the trace metal, although, under these conditions, it was only active with respect to NADH-diaphorase (Fig. 2). Thus, molybdenum seems to be required as a functional constituent of its second moiety, nitrate reductase proper, rather than for the synthesis of the complete enzyme complex or of part of it.

We have previously shown (13, 15) that in *Chlorella* all the activities of the nitrate reductase complex are fully repressed by the addition of ammonia to the culture medium, their cellular levels decreasing to negligible values after a treatment of 12 hr. It was, therefore, interesting to investigate if the *de novo* synthesis of the enzyme (promoted by its derepression, as a consequence of the removal of ammonia) was independent of molybdenum as an inducer. The experiment described in Figure 3 clearly demonstrates that when ammonium-repressed cells (which were tested to lack all the pertinent activities) were subjected (by removing ammonia and adding nitrate) to derepression for a period of 4 hr in the presence of different concentrations of added molybdate, the synthesis of the

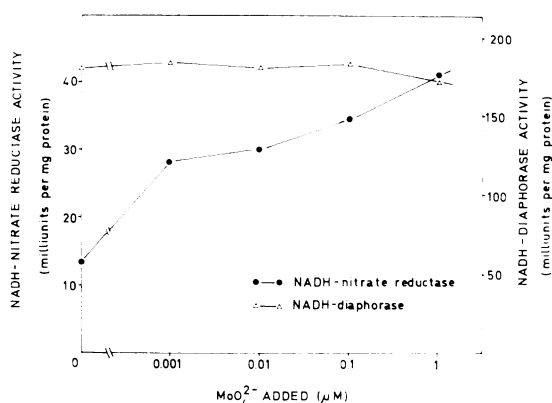


FIG. 3. Effect of molybdate added to culture media on *de novo* synthesis and activity levels of the nitrate reductase complex in *Chlorella* cells. Cells grown on ammonia, which did not contain any of the activities of the nitrate reductase complex, were subjected to derepression for a period of 4 hr by removing ammonia and adding nitrate in the presence of the indicated concentrations of molybdate added.

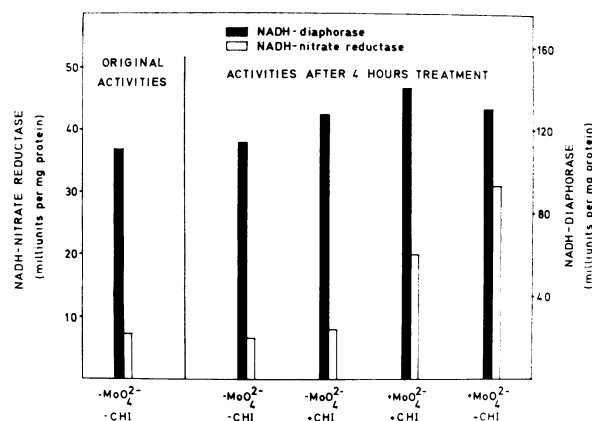


FIG. 4. *In vivo* response to added molybdate ( $100 \mu\text{M}$ ) of molybdenum-deficient *Chlorella* with respect to the activities of the nitrate reductase complex in the absence and in the presence of cycloheximide ( $10 \mu\text{g/ml}$ ).

NADH-nitrate reductase complex (as measured by the level of NADH-diaphorase activity) reached top values independently of the molybdate concentration in the medium. However, as in the experiment mentioned above, only when molybdate was supplied did the enzyme for nitrate reduction become functional. Sucrose density gradient centrifugation analysis of extracts prepared from cells derepressed both in the absence of added molybdate and in the presence of  $1 \mu\text{M}$  molybdate corroborated also the conclusion that the nitrate reductase complex does not require molybdenum as an inducer for its formation, but depends on the trace element for being active as nitrate reductase.

Since molybdenum appeared to be required only for nitrate reductase activity and not for the synthesis of the enzyme complex, we tried to incorporate the trace metal into the preformed apoenzyme, which we already knew to be present in deficient cells and to be inactive with respect to nitrate reductase. As seen in Figure 4, the success of this experiment depended largely on rigid controls. We used, as starting material, cells which had been previously grown in the absence of added molybdate and therefore exhibited high levels of NADH-diaphorase activity and low levels of both NADH-nitrate reductase and

FNH<sub>2</sub>-nitrate reductase activities. When, in the presence of cycloheximide, which inhibits the synthesis of the nitrate reductase complex (13, 15), molybdate was added to the metal-deficient cells for a period of 4 hr, it could be noted that the level of NADH-diaphorase activity remained constant, whereas the level of nitrate reductase increased markedly, thus indicating *in vivo* incorporation of molybdenum into the pre-existing nitrate reductase inactive apoenzyme, under conditions which exclude its *de novo* synthesis. When, in addition, the synthesis of the enzyme was allowed to proceed by omitting cycloheximide from the medium, the increase in nitrate reductase activity was even more pronounced.

Figure 5 shows that nitrate is not essential as an inducer for the *de novo* synthesis of the nitrate reductase complex in *Chlorella*. When ammonia acted as absolute repressor of the enzyme either in the absence or in the presence of nitrate, all the relevant activities were insignificant under both sets of experimental conditions. When ammonia was removed from the culture media, the formation of the enzyme did not depend on nitrate, although its addition to the medium promoted more vigorous synthesis. With respect to the influence of the nitrate concentration in the media (in the range between 4 and 32 mM) on the cellular levels of activity of the nitrate reductase complex, no significant changes have been observed (unpublished data).

Figure 6 shows that tungstate very efficiently inhibits the growth of *Chlorella* cells in media with nitrate as the sole source of nitrogen. When, under the experimental conditions described in "Materials and Methods," no molybdate was added to the culture medium, the inhibition of growth was higher than 50% at  $1 \mu\text{M}$  concentration of tungstate and was almost total at concentrations from 10 to  $100 \mu\text{M}$ . The competitive nature of tungstate inhibition with molybdate in nitrate assimilation by this organism was demonstrated, for the addition of molybdate ( $1$  and  $10 \mu\text{M}$ , respectively) to cultures unable to grow after a period of 48 hr, owing to the presence of tungstate ( $10$  and  $100 \mu\text{M}$ , respectively), practically overcame the inhibition of growth. No reversal of inhibition occurred in the corresponding controls to which no molybdate was added.

The effect of tungstate in competition with molybdate on the activities of the nitrate reductase complex in *Chlorella* cells grown with nitrate was investigated next. For this kind of ex-

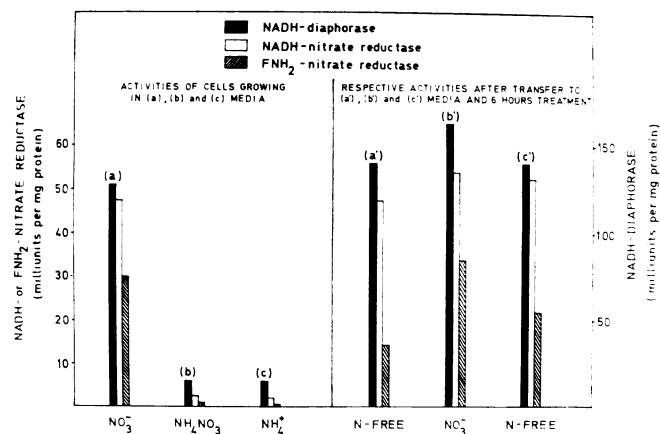


FIG. 5. Repression by ammonia and derepression by its removal of the nitrate reductase complex in *Chlorella*. Left: Activity levels of cells logarithmically growing on media with (a) nitrate, (b) ammonium nitrate, and (c) ammonia. Right: Activity levels of the same cells after collection by centrifugation and transfer to the corresponding (a') N-free, (b') nitrate, and (c') N-free media, where they were kept for 6 hr.

periment, the concentration of molybdate in the media was fixed at 0.1  $\mu\text{M}$ , and the concentration of tungstate was gradually increased from 0 to 100  $\mu\text{M}$ . Since tungstate, as an inhibitor of growth, promoted its effect by competition with molybdate, higher concentrations of tungstate than those employed in the preceding experiment were required to achieve the same degree of inhibition. As shown in Table I, tungstate did not interfere with the synthesis of the nitrate reductase complex (as measured by its NADH-diaphorase activity) and even stimulated it. On the other hand, the levels of NADH-nitrate reductase activity and  $\text{FNH}_2$ -nitrate reductase activity decreased markedly with increasing tungstate concentrations, indicating that this is the only reason why tungstate inhibits the growth of *Chlorella* cells on nitrate media, a suggestion that could be corroborated by the experiment described below.

When ammonia was substituted for nitrate as the sole nitrogen source, the growth of *Chlorella* cells was unaffected by tungstate, even at concentrations as high as 10 or 100  $\mu\text{M}$ , which, under the same conditions including the absence of added molybdate, had been found (see Fig. 6) to inhibit completely the growth of the organism.

The effect of molybdate and tungstate on the levels of the different activities of the nitrate reductase complex in *Chlorella* cells grown with either nitrate nitrogen or ammonium nitrogen is summarized in Table II. Since ammonium is an absolute repressor of the nitrate reductase complex, all the activities of the enzyme were negligible, whatever the case,

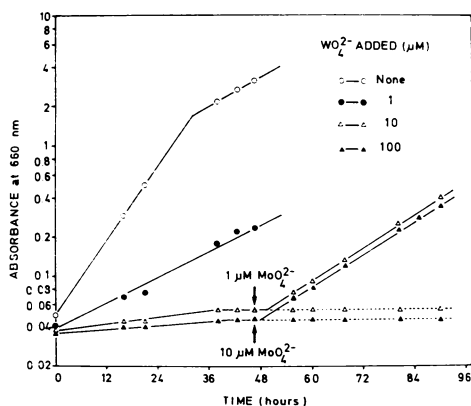


FIG. 6. Effect of tungstate concentration on growth of *Chlorella* on nitrate media lacking added molybdate, and reversal of the inhibition by further addition of molybdate at the time indicated by the arrows.

Table I. Effect of Tungstate in Competition with Molybdate on the Enzymatic Activities of the Nitrate-reducing Complex in *Chlorella*

Cells were grown on media with nitrate and the indicated amounts of metals. After 48 hr, growth was measured and cell-free extracts were prepared for estimation of activity levels.

Metals Added		Growth	Specific Activities		
$\text{MoO}_4^{2-}$	$\text{WO}_4^{2-}$		NADH-diaphorase	NADH- $\text{NO}_3$ reductase	$\text{FNH}_2$ - $\text{NO}_3$ reductase
$\mu\text{M}$		$A_{660 \text{ nm}}$	milliunits per mg protein		
0.1	0	1.75	110	46.3	22.2
0.1	1	1.40	130	9.6	5.9
0.1	10	0.54	263	2.1	1.8
0.1	100	0.22	268	0.4	0.6

Table II. Effect of Molybdate and Tungstate in Relation to Nitrogen Source on the Enzymatic Activities of the Nitrate-reducing Complex in *Chlorella*

Cells were grown on media with either nitrate or ammonia and the indicated amounts of metals. After 48 hr, cell-free extracts were prepared for estimation of activity levels.

Nitrogen Source	Metals Added		Specific Activities		
	$\text{MoO}_4^{2-}$	$\text{WO}_4^{2-}$	NADH-diaphorase	NADH- $\text{NO}_3$ reductase	$\text{FNH}_2$ - $\text{NO}_3$ reductase
	$\mu\text{M}$		milliunits per mg protein		
$\text{NO}_3^-$	0	0	250	4.8	2.7
$\text{NO}_3^-$	1	0	176	40.0	15.0
$\text{NH}_4^+$	0	0	0	0.8	0.8
$\text{NH}_4^+$	1	0	0	0.7	0.7
$\text{NH}_4^+$	0	1	0	0.6	0.6
$\text{NH}_4^+$	0	10	0	1.4	0.6
$\text{NH}_4^+$	0	100	0	0.8	0.8

in cells grown at the expense of this form of nitrogen. With nitrate nitrogen, molybdenum behaved as an essential functional component for nitrate reductase activity. Although not shown in the table, it is of interest to mention that cells grown with ammonium nitrogen on media containing tungstate started synthesizing the nitrate reductase complex (until reaching top levels) as soon as ammonia was consumed entirely, but, in this case and by contrast with molybdate, the enzyme was only functional with respect to NADH-diaphorase.

## DISCUSSION

The first evidence that molybdenum is essential for the utilization of nitrate was provided by the work of Steinberg (22), who reported a marked requirement for the trace metal for *Aspergillus niger*, but only when the fungus was grown on media containing nitrate nitrogen, not when the sole source of nitrogen was ammonia. The essentiality of molybdenum for higher plants was demonstrated by Arnon and Stout (5) by producing molybdenum-deficient tomato plants in experiments realized under rigidly controlled conditions. Since this original demonstration, many investigators have shown that molybdenum is indispensable for a variety of microorganisms and plant species (8, 17), including the green algae *Chlorella pyrenoidosa* (12, 26) and *Scenedesmus obliquus* (4, 11). Although it has been clearly shown that higher plants deficient in molybdenum and grown with nitrate show many effects that can be explained by the role of molybdenum in nitrate reduction, there are other effects, also caused by this deficiency, apparently not associated with the source of nitrogen used to grow the plants (8). In *Chlorella* (26) and *Scenedesmus* (4, 11), however, the role of molybdenum was found to be limited to nitrate reduction. The metal was only indispensable for growth when nitrate was the sole source of nitrogen; when a reduced form of combined nitrogen, ammonia or urea, was substituted for nitrate, the requirement for molybdenum was abolished.

The first evidence that molybdenum is associated with nitrate reductase was provided by Nicholas *et al.* (20); they showed that when either *Neurospora crassa* or *A. niger* was grown on highly purified media without added molybdenum a striking reduction in nitrate reductase activity of extracts occurred, and that activity of deficient cultures was restored to normal level 12 hr after molybdenum was added to them. More convincing evidence that molybdenum is indeed a part of the *Neurospora* nitrate reductase was the demonstration by

Nicholas and Nason (18) that dialysis against KCN and GSH deactivated the enzyme, and incubation of the dialyzed enzyme with either  $\text{MoO}_3$  or  $\text{MoO}_4^{2-}$  restored its activity. These authors also showed that a correlation exists between the molybdenum content and the activity of fractions of nitrate reductase preparations. Similar experiments (19) demonstrated that molybdenum is also an active constituent of nitrate reductase from soybean leaves.

Except for the case of soybeans, however, the role of molybdenum in nitrate reduction by green cells as the metal prosthetic group of nitrate reductase could not be considered convincingly demonstrated. As mentioned earlier, previous attempts in different laboratories, including our own, to identify molybdenum as a component of the enzyme or to use reduced molybdate as an electron donor for the enzymatic reduction of nitrate were unsuccessful with preparations from a variety of plants, sometimes highly purified (3, 6, 14). Hewitt and co-workers (1, 2, 8) reported that the introduction of molybdenum by infiltration in excised tissues of deficient higher plants grown with nitrate resulted in the rapid production of nitrate reductase activity. The response to either factor was dependent on the integrity of the cells, as cell-free extracts were unable to respond. Since they further found that the response to molybdenum showed the same sensitivity to antimetabolites of protein synthesis as did induction by nitrate, they concluded that the formation of nitrate reductase depends upon the presence of molybdenum *in vivo*, and the metal is not merely involved in activating pre-existing protein.

Our recent and present results shed new light on the function of molybdenum in nitrate reduction by algae. It was first shown that molybdenum is a component of the nitrate reductase complex from *Chlorella* (3). The experiments reported here have demonstrated that molybdenum is only required for the nitrate reductase activity proper exhibited by the enzyme complex but not for its NADH-diaphorase activity. The same enzyme complex is apparently synthesized in the absence as in the presence of added molybdate, although only the second one is functional for nitrate reduction. They have also shown that the metal can be incorporated *in vivo* into the nitrate reductase apoenzyme pre-existing in deficient cells under conditions which excluded its *de novo* synthesis. Finally, it has been stated that neither molybdate nor nitrate is required for *de novo* synthesis of the nitrate reductase complex, which is completely repressed by ammonia.

Higgins *et al.* (10) reported that tungstate is a competitive inhibitor of molybdate in *A. niger* when nitrate is the sole nitrogen source. There is a similar type of competitive inhibition of tungstate with molybdate in nitrate assimilation (and in  $\text{N}_2$  fixation) by *Azotobacter vinelandii* (25). Afridi and Hewitt (1) tried, with tissues obtained from molybdenum-deficient plants grown with nitrate, the possibility of substitution of tungsten for molybdenum, but did not observe any effect on activity; the element was not antagonistic to molybdenum at the concentration used. More recently, however, Heimer *et al.* (7) and Wray and Filner (27) have examined the effect of tungstate on the formation of active nitrate reductase in suspension cultures of tobacco XD cells and in intact barley shoots. Tungstate prevented the formation of nitrate reductase activity but caused the superinduction of the NADH-cytochrome *c* reductase activity of the enzyme complex; molybdate overcame the effect of tungstate. They concluded that nitrate induces the development of nitrate reductase activity in barley shoots, and tungstate inhibits the formation of this activity.

The results reported in this paper have shown that tungstate is also a competitive inhibitor of molybdate in *Chlorella*. The addition of tungstate to the culture medium inhibited the

growth of the alga when nitrate served as nitrogen source; molybdate completely overcame this inhibition. When ammonia was used as the source of nitrogen, no effect of tungstate was observed on growth, thus corroborating that, in *Chlorella*, the role of molybdenum seems to be limited to nitrate reduction. The effect of tungstate in competition with molybdate could also be observed at the enzymatic level: tungstate interferes only by decreasing the nitrate reductase activity of the nitrate reductase complex, without affecting—or even stimulating—its NADH-diaphorase activity. Preliminary experiments with radioactive  $^{187}\text{W}$  (as tungstate) have indicated that tungsten acts by replacing molybdenum in the nitrate reductase complex and that tungsten can be removed by several treatments which leave the apoenzyme free.

The role of molybdenum in nitrate reduction by *Chlorella* seems to be limited to the reduction of nitrate to nitrite, since the trace metal is neither a component of nitrite reductase (3) nor required for its activity (unpublished data).

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