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Effects of Vanadium and Tungsten on the Nitrogen-fixing Cyanobacterium *Nostoc linckia*

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Abstract. The effects of vanadium and tungsten on growth of the heterocystous cyanobacterium *Nostoc linckia* (ROTH.) BORN. and the activity of its nitrogenase and nitrate reductase were studied. While vanadate stimulated the growth of molybdate-limited cells and also their acetylene-reducing ability, it did not stimulate nitrate reductase activity. Tungstate inhibited growth in molybdate-limited cells and also their acetylene reducing and nitrate reductase activities. Acetylene reduction response of cells grown in a tungstate-containing medium indicated certain difference between the modes of action of vanadium and molybdenum. Tungstate caused a slight increase in heterocyst frequency both in nitrogen-fixing and nitrate-grown material. Cyanophycin granules and polyhedral bodies disappeared in molybdate-deficient cells in which thick deposition of polyglucan-like granules occurred throughout the breakdown of cytoplasm and the thylakoids.

Organisms which fix nitrogen require molybdenum (NAGATANI and BRILL 1974, PEINKOSS *et al.* 1981). That molybdenum is a prosthetic group of nitrogenase (component I) is well documented (PEINKOSS *et al.* 1981). Many of the enzymatic processes of nitrogen fixation take place at a site on component I, showing the requirement of molybdenum for these reactions.

Tungsten inhibits nitrogen-dependent growth by interfering with the utilization of molybdenum (TAKAHASHI and NASON 1957, BULEN 1961). Although the synthesis of some unknown, inactive tungsto-protein, when an organism is grown on tungsten instead of molybdenum, has been reported in higher plants (NOTTON and HEWITT 1971) as well as bacteria (BENEMANN *et al.* 1973, PEINKOSS *et al.* 1981), the question of whether tungsten competitively inhibits molybdenum, remains to be settled.

BURNS *et al.* (1976) suggested that vanadium can substitute for molybdenum in the conventional dinitrogenase of *Azotobacter vinelandii* and *A. chroococcum*. Very few studies of this kind have been made in cyanobacteria; FAY and DEVASCONCELOS (1974) and DE VASCONCELOS and FAY (1974) studied some aspects in *Anabaena cylindrica*. The present paper deals with the effect of molybdenum depletion on the vegetative cells of *Nostoc*

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linckia and the effects of sodium tungstate and sodium vanadate on the activity of two molybdenum-containing enzymes (nitrogenase and nitrate reductase) of this organism.

MATERIAL AND METHODS

Nostoc linckia (ROTH.) BORN. was grown axenically in the medium of ALLEN and ARNON (1955) adjusted to pH 7.5 after autoclaving. The medium either lacked combined nitrogen or was supplemented with 5 mM KNO_3, and was buffered with 4 mM of Tris (hydroxymethyl) aminomethane/HCl. Cultures were grown on 14/10 h light/dark cycle at 26 ± 2 °C and 2000 ± ± 200 lx. They were shaken by hand thrice daily. The organism forms heterocysts in a regularly spaced pattern (about 5.6%) within 36 h after transfer into nitrogen-free medium. To obtain heterocyst-free filaments for differentiation study, it was grown in medium containing KNO_3. Its growth was estimated by measuring culture absorbance at 663 nm in a Bausch and Lomb Spectronic 20 colorimeter. Heterocyst frequency was estimated as a percentage of the total cells by counting at least 12 filaments (about 1000 cells).

Nitrate reductase and acetylene reducing activities were determined as described earlier in KUMAR *et al.* (1983) using methods of CAMM and STEIN (1974) and STEWART *et al.* (1968).

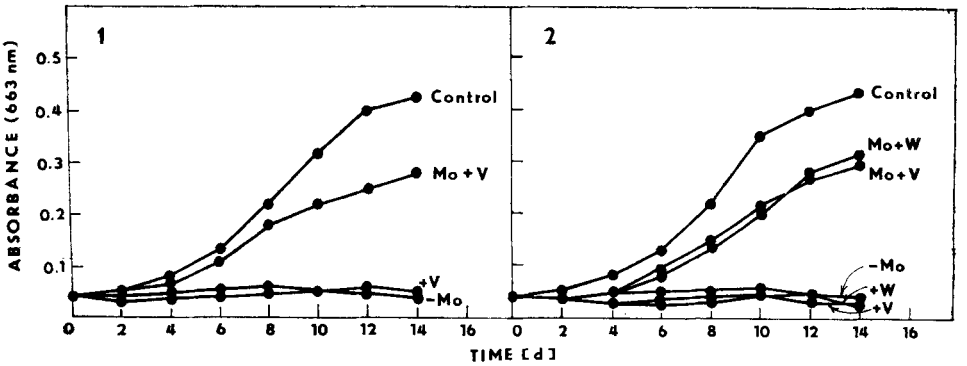
For transmission electron microscopy, cells cultured for 3 d in molybdate--deficient culture medium (from which Mo was omitted) or in normal Mo-containing medium (for controls), were fixed for 4 h with 4% glutaraldehyde buffered with 0.1 M phosphate at pH 7.4, post-fixed with 1% osmium tetroxide, dehydrated with acetone and embedded in Spurr resin. Ultrathin sections were examined with a Hitachi H-700 electron microscope.

To study the effects of vanadium and tungsten, molybdate-grown cells were washed with double-distilled water (glass stills) and incubated 72 h in Allen and Arnon medium lacking both combined nitrogen and molybdate. Cultures used for these experiments were grown in Corning glassware thoroughly cleaned with chromic-sulphuric acid solutions, washed with running

TABLE 1

Effect of molybdenum deficiency on culture absorbance values at 663 and 615 nm. All values are means of five replicates ± standard deviation

Absorbance [nm]	Time [h]	Mo-depleted	Control (Mo-grown)
663	24	0.08 ± 0.03	0.10 ± 0.04
	36	0.07 ± 0.03	0.10 ± 0.03
	48	0.06 ± 0.02	0.12 ± 0.04
	60	0.03 ± 0.01	0.14 ± 0.05
	72	0.03 ± 0.01	0.17 ± 0.05
615	24	0.06 ± 0.02	0.08 ± 0.02
	36	0.06 ± 0.02	0.085 ± 0.03
	48	0.045 ± 0.01	0.11 ± 0.04
	60	0.04 ± 0.02	0.12 ± 0.04
	72	0.03 ± 0.02	0.14 ± 0.05



1. Effect of sodium molybdate (50 μg ml^{-1}) addition on growth in sodium vanadate toxicated (50 μg ml^{-1}) cells under nitrogen fixing conditions.
2. Effect of vanadate and molybdate (5 μg ml^{-1} each) on growth in tungstate (5 μg ml^{-1}) toxicated cells under nitrogen fixing conditions. Mo + W and Mo + V concentrations are 5 μg ml^{-1} each

tap water and then with three rinses of glass-distilled water. All chemicals used in medium preparation were of AnalaR grade (British Drug Houses) or equivalent highest purity available. No other special steps were taken to avoid traces of metal impurities. Sodium vanadate, sodium tungstate and sodium molybdate were purchased from British Drug Houses Ltd. Hydrogen and nitrogen gases were from Indian Oxygen Ltd. (Bombay); acetylene was from Indian Air Gases Co., Mughalsarai (India), and ethylene was from Matheson Gas Company, Lyndhurst (U.S.A.).

RESULTS

When grown in molybdate-limited medium, the organism became pale yellow within about 72 h. The colour change was associated with a rapid decrease (up to about 80% in 72 h) in A_{663} and A_{615} values (Table 1). This culture was used to study the effects of vanadate and tungstate.

TABLE 2

Percent heterocyst frequencies in Mo-depleted culture media with and without combined nitrogen, supplemented with different concentrations of vanadate or tungstate. Frequency was recorded after 4 days and only mature heterocysts were counted. AA^-, Allen and Arnon's medium lacking combined nitrogen; AA + NO_3, same supplemented with 5 mM KNO_3. ND, not detectable because filaments appeared as 4—7 celled trichomes. Values are means ± standard deviation

Control				Vanadate	Heterocyst frequency [%]		Tungstate	Heterocyst frequency [%]	
+ Mo		− Mo		[μg ml^{-1}]					
AA^-	$AA+NO_3$	AA^-	$AA+NO_3$		AA^-	$AA+NO_3$	[μg ml^{-1}]	AA^-	$AA+NO_3$
5.6 ± 0.4	0	12.3 ± 2.5	12.4±2.3	1	7.6±1.9	12.0±2.4	1	12.8±3.4	12.7±3.6
				5	7.2±1.9	11.8±2.1	5	13.6±3.2	13.8±3.3
				10	6.6±2.1	12.0±2.5	10	14.0±3.3	14.2±3.5
				20	7.8±2.3	12.1±2.2	20	14.8±3.6	14.8±3.4
				50	9.2±2.1	12.4±2.3	50	ND	ND

TABLE 3

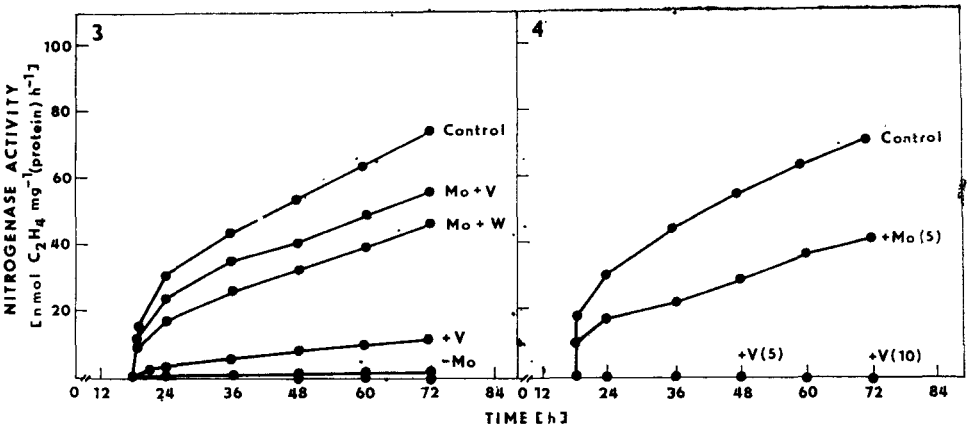
Effects of sodium vanadate and sodium tungstate on nitrate reductase and nitrogenase (acetylene reducing) activities of molybdate-limited cells. For nitrate reductase determination, cultures were supplemented with 10 mM of KNO_3. Activities were determined using procedures mentioned in the text. All values are means of five replicates ± standard deviation

Supplement [μg ml^{-1}]	Nitrate reductase [% of control]	Nitrogenase [% of control]
Nil (normal culture medium)	100	100
Nil (Mo-free culture medium)	6.95 ± 1.5	2.35 ± 0.5
Vanadate, 1	6.9 ± 1.5	15.6 ± 3.2
5	6.6 ± 1.6	15.6 ± 3.5
10	6.65 ± 2.5	23.8 ± 5.4
20	6.5 ± 1.9	15.55 ± 4.7
Tungstate, 1	1.53 ± 0.5	1.05 ± 0.35
5	0.78 ± 0.8	0.80 ± 0.28
10	—	—

Growth stimulation by vanadate in molybdate-limited nitrogen fixing cells was always less than that caused by molybdate addition. There was about 20% growth stimulation in Mo-depleted cells at 10 μg ml^{-1} of vanadate.

Table 2 shows the effect of tungstate in slightly increasing the heterocyst frequency in both nitrogen-free and nitrate-containing media, as compared to controls.

Fifty μg vanadate ml^{-1} was toxic to growth in nitrogen-fixing conditions. Vanadate did not stimulate growth in nitrate-grown material. Unlike vanadate. tungstate, even at 5 μg ml^{-1}, inhibited growth in nitrogen-free and nitrate-supplemented media.



3. Effect of vanadate and tungstate on nitrogenase (acetylene reducing) activity. Mo-limited cells were transferred into nitrogen-free medium and supplemented with vanadate and tungstate (10 μg ml^{-1}) each, molybdate and vanadate (10 μg ml^{-1} each), and molybdate + tungstate (10 μg ml^{-1} each), added at 0 time.

4. Effect of molybdate and vanadate addition on nitrogenase (acetylene reducing) activity of tungstate toxicated (10 μg ml^{-1}, 96 h) cells.

There were low, detectable nitrate reductase (about 7%) and nitrogenase (about 3%) activities in molybdate-limited cells. While vanadate supply did not affect the nitrate reductase, it stimulated nitrogenase; in contrast, tungstate inhibited both (Table 3). Figs. 1 and 2 show the reversal of vanadate and tungstate induced growth inhibition by simultaneous addition of molybdate in nitrogen-fixing cells. Tungstate-induced growth inhibition was overcome by adding the same concentration of molybdate alone. Vanadate did not relieve tungstate-caused inhibition at any concentration.

Nitrogenase activity appeared about 19 h after transfer of the organism into medium lacking combined nitrogen (Fig. 3). A low [1.2 nmol C_2H_4 formed mg^{-1} (prot.) h^{-1}] nitrogenase in Mo-limited cells was probably due to residual molybdate contamination. Exogenous addition of molybdate to the cultures restored much of the *in vivo* nitrogenase activity in all Mo-limited, vanadate- and tungstate-supplemented cultures. Conceivably, most of the proteins necessary for nitrogenase activity in these cells might have been synthesized in the absence of Mo.

The activation of inactive nitrogenase in tungstate-containing cultures (Fig. 4) occurred much more slowly. Fig. 4 shows that molybdate alone can overcome the tungstate-induced inhibition of acetylene reduction, but that vanadate can not do so. Densely packed wavy thylakoids were seen in Mo-grown cells (in normal culture medium). Polyhedral bodies, cyanophycin granules and polyphosphate bodies were also seen. No clear trace of thylakoids was seen in Mo-limited cells; this coincided with the fall in A_{663} value in these cells. Cyanophycin granules and polyhedral bodies were not found but small granules resembling polyglucan granules were seen throughout the cytoplasm, and small lipid droplets of osmiophilic nature were also formed. These structural changes in Mo-limited cells were similar to those reported in *Anabaena cylindrica* by FAY and DE VASCONCELOS (1974).

DISCUSSION

The organism seems to be able to withstand the stress of molybdenum depletion, remaining capable of rapid recovery when molybdate is resupplied. Considering that the heterocysts are the main site of nitrogen fixation (FAY *et al.* 1968), it seems paradoxical that an increase in heterocyst frequency should be accompanied by a fall in nitrogenase activity in comparison to controls containing 0.01 μg molybdate ml^{-1}. The molybdate limitation prevents the production of an active nitrogenase, as Mo is an integral part of the nitrogenase complex (PEINKOSS *et al.* 1981). Nitrogen starvation enhances nitrogenase synthesis and heterocyst differentiation. The organism produces a few more heterocysts (though the increase is not significant) when it is not provided with fixed nitrogen. The results of Mo-limitation experiments indicate that high heterocyst frequency need not always be associated with high nitrogenase activity.

NAGATANI and BRILL (1974) and BURNS *et al.* (1976) suggested that vanadium and tungsten can substitute for Mo in the dinitrogenase of *Azotobacter* spp. Though this is plausible, recent work of BISHOP *et al.* (1982) has revealed that the situation is actually much more complex; thus, the relevant proteins appear to be distinct from the conventional dinitrogenase. Further, uptake mechanisms and storage proteins seem to play an important role.

We have not studied any of these points. BISHOP *et al.* (1980) reported the existence of an alternative nitrogen fixation system in *Azotobacter*. If this were also true for *Nostoc*, one might logically expect the wild type strain to grow in Mo-limited medium containing the above metals. In our study, the tungsten inhibition was probably competitive in the presence of Mo, since Mo relieved the tungstate-caused inhibition. But vanadium did not do so. Our tungstate experiments indicate some significant difference between the modes of action of vanadium and molybdenum. The vanadium effect seems to be specific for nitrogen fixation; growth on nitrate, which requires Mo, is not stimulated by vanadium. Vanadium addition did not amplify the symptoms characteristic of Mo-deficiency, but tungstate did so even at fairly low concentrations. Though vanadium may derepress some proteins involved in any alternative nitrogen fixation pathway in *Azotobacter* (BISHOP *et al.* 1982), the same need not necessarily be true for cyanobacteria as well. Indeed, if vanadium played a similar role in cyanobacteria, then these organisms would be expected to grow in Mo-limited, nitrate supplemented, vanadium--containing medium since, because of defective nitrate reductase, vanadium would derepress some proteins needed for the alternative system. What we have actually observed is that addition of vanadium to Mo-limited cells stimulates growth, and that probably vanadium substitutes for Mo in the nitrogenase. This might result in the formation of a partially active vanadium containing nitrogenase. Possibly, this latter enzyme is not sufficiently active to satisfy the nitrogen requirements of the organism, leading to some nitrogen starvation, which, in turn, induces some slight increase in heterocyst frequency. The reason why vanadium does not stimulate growth when the organism is grown on nitrate, is not known. The increase (albeit small) in heterocyst numbers in Mo-limited, tungstate-grown material, unaccompanied by a concomitant increase in nitrogenase activity, probably occurs due to the formation of defective molybdenum-containing enzymes.

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BOOK REVIEW

Sarić, M. R., Loughman, B. C. (ed.): Genetic Aspects of Plant Nutrition (Developments in Plant and Soil Sciences. Vol. 8). — Martinus Nijhoff Publ., The Hague—Boston—Lancaster 1983. 495 pp. Dfl. 195.00; U.S. $ 85.00.

The book contains the contributions to the First International Symposium on Genetic Aspects of Plant Nutrition, organized by the Serbian Academy of Sciences and Arts in Belgrade from August 30 to September 4, 1982. In this volume, 58 papers of participants from 21 countries of Europe, Asia, North and South America and Australia are collected; 21 of these papers were first published in the journal "Plant and Soil", Vol. 72, 1983.

The papers were divided into five logical parts according to symposium sections: I. Cytological and anatomical changes in different genotypes caused by altered nutrient supply, II. Absorption, translocation and accumulation of ions in different genotypes, III. The influence of mineral nutrition on physiological and biochemical processes of genotypes, IV. The influence of mineral nutrition on yield and quality of different genotypes, and V. Genetic investigations concerned with selections of genotypes for a more effective use of mineral elements. Individual contributions are arranged as usual (Summary, Introduction, Material and Methods, Results, Discussion, References). Some of the contributions are reviews. The book contains no indexes and some of the illustrations are of inferior quality; this is excusable by the effort of the publishers to minimize the time interval between the symposium and publication of these proceedings, *i.e.* to bring information as actual as possible.

In the reviewed book the genetic aspects of plant nutrition, a very important problem at the present moment, are submitted in their widest sense and discussed comprehensively. The progress achieved in this field so far has been evaluated and attention directed to the fundamental problems of crop improvement in the future. The volume is of interest to plant physiologists, biochemists, geneticists, agronomists and plant breeders.

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