Arch. Microbiol. 99, 221–230 (1974)

Nitrogen Metabolism and Ultrastructure in *Anabaena cylindrica*

II. The Effect of Molybdenum and Vanadium

Peter Fay and Laura de Vasconcelos*

Department of Botany and Biochemistry, Westfield College, University of London, London

Received March 18, 1974

Abstract. The structural and functional symptoms of molybdenum deficiency in *Anabaena cylindrica* grown in a medium without combined nitrogen and thus dependent on fixation of elemental nitrogen, resemble those brought about by nitrogen starvation. However, the substantially increased rate of heterocyst differentiation in this culture is not accompanied by a corresponding increase in nitrogenase activity; on the contrary, enzyme activity is severely impaired in the absence of molybdenum. When the supply of molybdenum, or of ammonia, is restored, the alga recovers rapidly. Vanadium exerts an inhibitory effect upon nitrogen-fixing ability of the alga, and its presence in the molybdenum-deficient culture results in the amplification of the symptoms of molybdenum deficiency.

Key words: Anabaena cylindrica — Molybdenum — Vanadium — Nitrogenase — Ultrastructure — Storage Products — Heterocyst Frequency.

Among the micro-nutrients essential for the growth of blue-green algae, molybdenum is distinguished by being the constituent of two enzyme systems involved in the assimilation of inorganic nitrogen, nitrate reductase and nitrogenase (Nicholas and Nason, 1954; Bulen and Le Comte, 1966). Primary productivity studies in several oligotrophic lakes of North America and New Zealand have shown that molybdenum can be a limiting factor for the growth of blue-green algae, and that molybdenum may have a marked effect on the size of the zooplankton population as well as on fish yield (Goldman, 1964, 1966).

Fogg (1949) noticed that shortage of molybdenum increased heterocyst production in *Anabaena cylindrica* when grown in a medium free from a source of combined nitrogen. The significance of this observation was not fully appreciated until the suggestion of the involvement of heterocysts in nitrogen fixation was put forward (Fay *et al.*, 1968). Molybdenum deficiency was shown to result in greater accumulation of sugars and decreased production of amino acids in a *Nostoc* species incubated in the absence of combined nitrogen (Arnon, 1958).

* Present address: Department of Microbiology, University of Lourenço Marques, Moçambique.

Ever since Bortels in 1933 found that vanadium can substitute for molybdenum in nitrogen fixation by *Azotobacter* species, contradictory observations were reported on the requirement for vanadium. Holm-Hansen (1954) and Allen (1956) were unable to find conclusive evidence of a requirement for vanadium in nitrogen fixation by blue-green algae, and later Holm-Hansen (1968) attributed the vanadium effect to small amounts of molybdenum contaminating the vanadium salts used. However, more recently it has been shown that vanadium can partially substitute for molybdenum in the nitrogenase of *A. vinelandii*, and that the specific activity of the vanadium-containing enzyme system is only about 10% of that of the pure molybdenum-nitrogenase (McKenna *et al.*, 1970; Burns *et al.*, 1971; Benemann *et al.*, 1972).

In a previous communication (De Vasconcelos and Fay, 1974) we reported on the effect of nitrogen starvation. In the present study we have examined the effects of molybdenum and of vanadium on the morphology, ultrastructure and function of *Anabaena cylindrica*.

Materials and Methods

Culture methods for the growth of *A. cylindrica* Lemm. and methods of preparation for examination with the light and electron microscope as well as for pigment determination and nitrogenase assay were described in the previous paper (De Vasconcelos and Fay, 1974).

Molybdenum- and vanadium-deficient media were prepared by omitting sodium molybdate and ammonium vanadate from the standard growth medium, and by purifying all macro-nutrient according to the method of Nicholas and Fielding (1950). The purified medium contained less than 0.5 $\mu g \cdot l^{-1}$ molybdenum and about 0.2 $\mu g \cdot l^{-1}$ vanadium.

Results

1. The Effect of Molybdenum

The algal material for these studies was grown at 2500 lux light intensity and 25° C temperature under steady-state conditions in a continuous culture apparatus (Fay and Kulasooriya, 1973), in the complete medium. At the beginning of each experiment, the alga was washed free of molybdenum, transferred into the molybdenum-deficient medium and incubated under otherwise similar conditions during the experimental period. Samples were withdrawn regularly, and assayed for pigment content and nitrogenase activity, and examined with the light and electron microscope. After the alga had been incubated for 24 days in the molybdenum-deficient medium, ammonia was added to the culture (in the form of 10^{-3} M diammonium hydrogen phosphate), or alternatively, after 7 and 16 days of molybdenum starvation, larger portions of the culture suspension were separated and supplemented with molybdenum (0.1 mg l^{-1}, in the form of sodium molybdate). This was in order to test the ability of the alga to recover from symptoms of molybdenum deficiency and to study the process of recovery.

Growth in the molybdenum-deficient medium led to striking changes in the appearance of the alga, becoming orange-green to pale yellow in colour with time. Results of pigment analyses (Fig. 1 a) showed the changes to be associated with a rapid decrease (by more than 80% after 48 h)

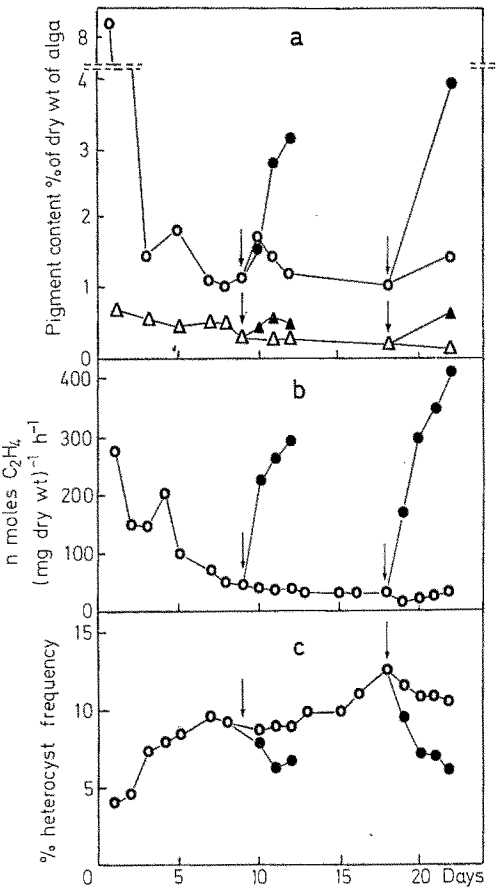

Fig. 1 a—c. Effect of molybdenum deficiency (open circles, open triangles) and of subsequent supply of molybdenum (dark circles, dark triangles) on a pigment (△——▲ chlorophyll; ○——● phycocyanin) content, b nitrogenase activity, and c heterocyst production of *Anabaena cylindrica*. Arrows indicate times of transfer into medium containing molybdenum

in the amount of phycocyanin and a fall (by about 50%) in the chlorophyll content. Examination under the light microscope revealed that molybdenum deficiency greatly enhanced the production of heterocysts, raising heterocyst frequency from about 4% to 12% (Fig. 1 c).

The effects of molybdenum deficiency on pigmentation and heterocyst abundance resemble the characteristic symptoms of nitrogen starvation described in the previous paper (De Vasconcelos and Fay, 1974). However, while increased heterocyst frequency was accompanied by increased nitrogen-fixing activity when the alga was deprived of a source of nitrogen, this was not the case when molybdenum was eliminated from the culture medium. On the contrary, nitrogenase activity dimin-

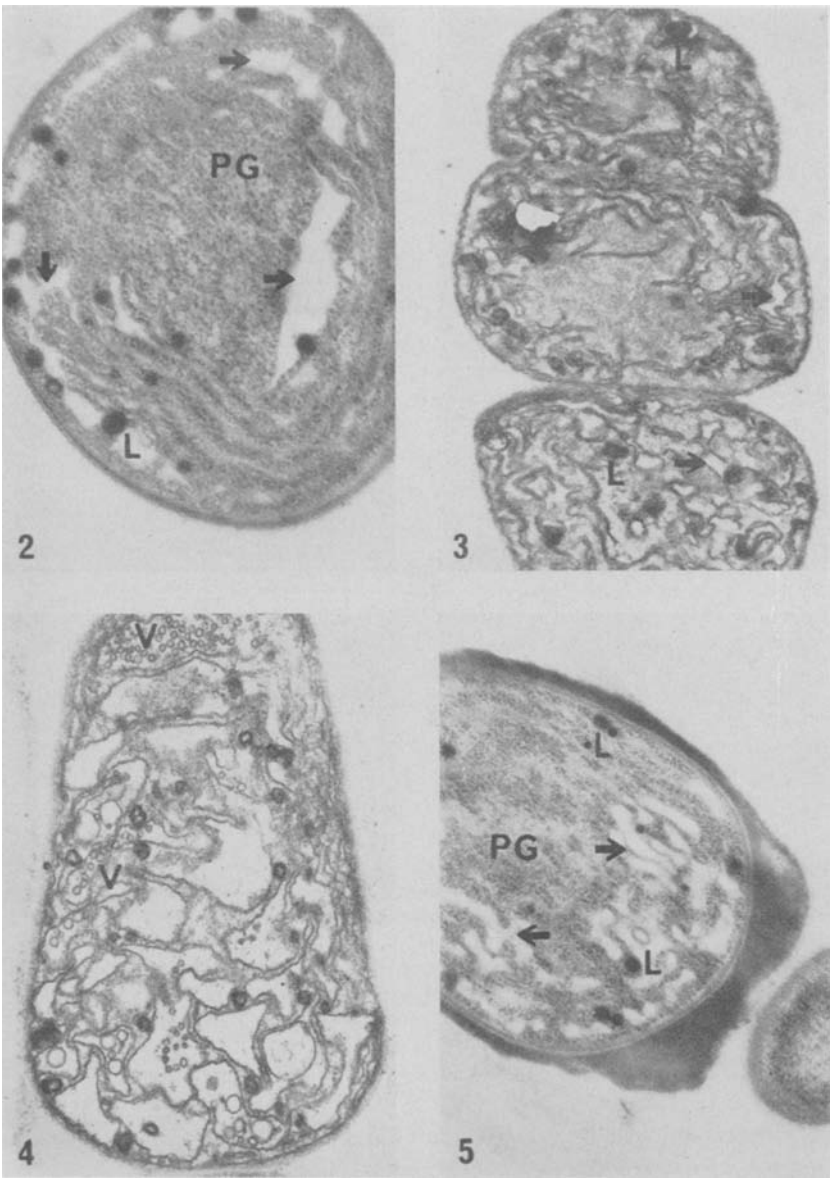

Fig. 2. Electron micrograph showing a vegetative cell from the molybdenum-deficient culture. Note the absence of cyanophycin granules and polyhedral bodies, accumulation of lipid droplets (*L*), abundant deposition of polyglucan granules (*PG*) and initial stages of thylakoid vesiculation (arrows). Fixed with glutaraldehyde-osmium. Magn. ×22850

ished rapidly under these conditions, though it could be maintained at an extremely low level for a relatively long period (Fig. 1 b).

Molybdenum deficiency greatly affected the cellular organization of *A. cylindrica*, especially the ultrastructure of vegetative cells. Cyanophycin granules and polyhedral bodies disappeared while polyglucan granules were deposited densely throughout the cytoplasm and large lipid droplets were formed (Fig. 2). This change in the storage patterns of cells was followed by an equally dramatic change in the ultrastructure of the photosynthetic apparatus which became extensively vesiculated due to the separation of the usually closely adhered thylakoid membranes (Figs. 2 and 3). Prolonged molybdenum deficiency resulted in a drastic reduction of the cytoplasmic granular substance, including ribosomes, and in a massive breakdown of the thylakoid system. The latter was accompanied by a characteristic assembly of small vesicles within the intrathylakoidal space (Fig. 4).

The effect of molybdenum deficiency on the ultrastructure of heterocysts was less well defined and uniform, and appeared to vary according to the damage suffered by the cell in the vegetative stage before its differentiation into a heterocyst. Hence, some heterocysts displayed a more or less normal ultrastructure while others appeared to have inherited the organizational changes from the undifferentiated vegetative cell (Fig. 5).

Addition of either ammonia or molybdenum to the culture suspension has speedily restored the healthy appearance of the alga, but the morphological and physiological changes associated with recovery differed markedly depending on the substance used. Pigmentation was rapidly recovered when ammonia was added, but heterocyst production and nitrogenase synthesis became depressed. Ammonia was readily and apparently excessively assimilated by the molybdenum-deficient cells as indicated by the rapid accumulation of cyanophycin granules in the vegetative cells and the formation of enormous polar "plugs" in the heterocysts (Fig. 6). On restoring the supply of molybdenum, nitrogenase

Fig. 3. Section through vegetative cells from the molybdenum-deficient culture illustrating extensive thylakoid vesiculation (arrows) and accumulation of lipid reserves (*L*). Fixed with glutaraldehyde-permanganate. Magn. ×23500

Fig. 4. Ultrastructure of vegetative cells from a 20 day old molybdenum-deficient culture showing extensive loss of granular cytoplasmic substance, disorganization of thylakoids and assembly of small vesicles (*V*) in the intrathylakoidal space. Fixed with glutaraldehyde-permanganate. Magn. ×23500

Fig. 5. Portion of a heterocyst exhibiting symptoms of molybdenum deficiency [see accumulation of polyglucan granules (*PG*) and lipid droplets (*L*), thylakoid vesiculation (arrows)] similar to those seen in undifferentiated vegetative cells (comp. with Fig. 2). Fixed with glutaraldehyde-osmium. Magn. ×21300

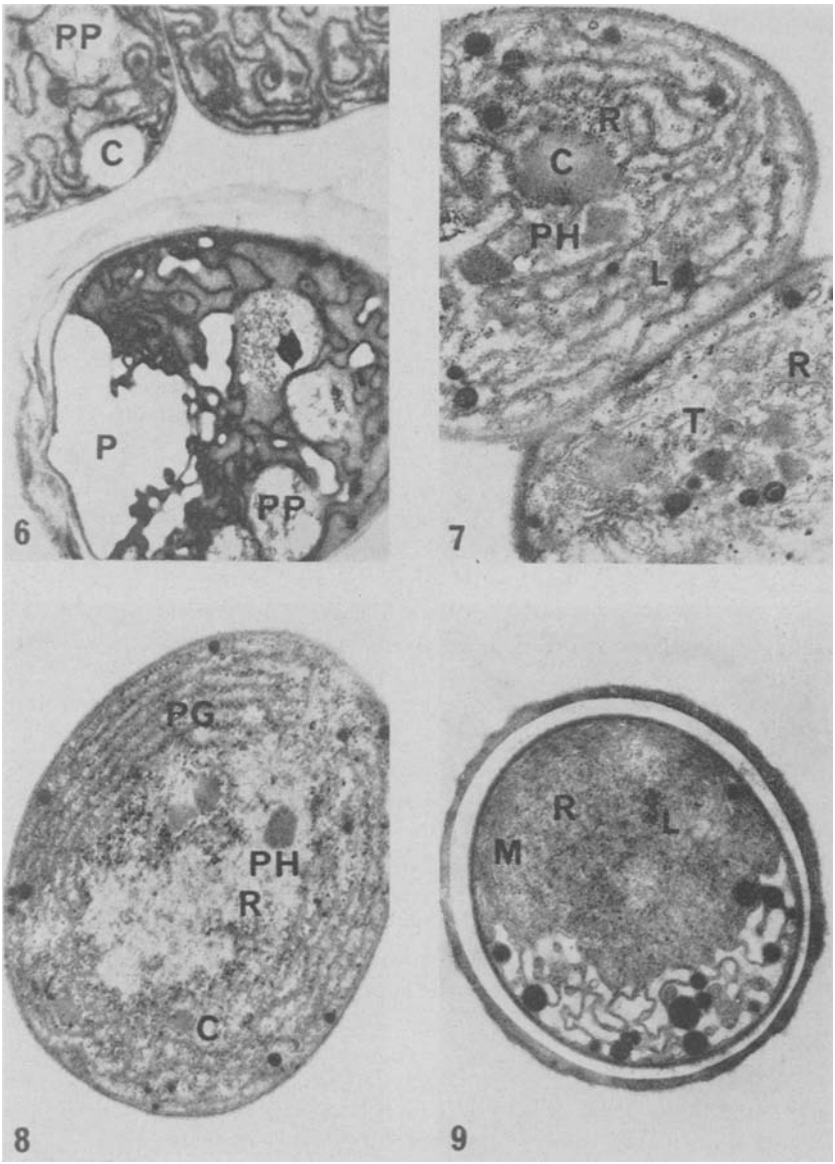

Fig. 6. Section through a heterocyst and vegetative cells one day after the addition of ammonium hydrogen phosphate to the molybdenum-deficient culture. The large electron-transparent space (*P*) near the pore region in the heterocyst indicates the locus of "plug" material removed during the fixation procedure. A similar electron-transparent space (*C*) in one of the vegetative cells is indicative of cyanophycin accumulation. Polyphosphate bodies (*PP*) were deposited in both vegetative cells and heterocysts. Fixed with glutaraldehyde-permanganate. Magn. ×27000

activity and pigment concentrations increased swiftly, and heterocyst differentiation became controlled at the normal level of about 5% of the cell population (Fig. 1 a–c). Many cells regained their normal ultrastructure within 24 h of the addition of molybdenum. The course of their recovery was characterized by the gradual breakdown and disappearance of lipid droplets, re-formation of ribosomes and reorganization of thylakoids (Fig. 7). Cyanophycin granules and polyhedral bodies also formed sparingly (Fig. 8). A great number of (possibly defective) heterocysts were shed soon after molybdenum was added to the deficient culture, but those heterocysts which remained attached to vegetative cells also recovered within 24 h. Fig. 9 illustrates such a heterocyst in the process of recovery: lipid droplets are being dispersed, ribosomes accumulate and new membraneous structures are being formed.

2. The Effect of Vanadium

In these experiments the alga was pre-incubated in a vanadium- and molybdenum-free medium under conditions similar to those described previously. After 7 days when the alga showed clear symptoms of molybdenum deficiency, the culture was divided into four portions which were supplemented with a) both molybdenum and vanadium, b) molybdenum only, c) vanadium only, and d) neither molybdenum nor vanadium, and each incubated for a further period of 7 days.

The results of this experiment are summarized in Table 1. It can be seen that pigment content and nitrogen-fixing activity were higher in cultures containing molybdenum. Heterocyst frequency was lower in the presence than in the absence of molybdenum. Curiously, in all cultures containing vanadium, the rate of heterocyst production was higher while nitrogenase activity and pigment concentration were lower than when it was excluded from the medium. The extremely high frequency of heterocysts (13%) in filaments incubated with vanadium and without molybdenum indicate a severe state of nitrogen starvation.

Fig. 7. Section through vegetative cells fixed one day after supplying molybdenum to a molybdenum-deficient culture. Note thylakoids (*T*) in process of re-organization, lipid droplets dispersing (*L*), ribosomes (*R*), polyhedral bodies (*PH*) and cyanophycin granules re-appearing (*C*). Fixed with glutaraldehyde-osmium. Magn. ×29300

Fig. 8. Section of a vegetative cell completely recovered from symptoms of molybdenum deficiency one day after supplying molybdenum to the molybdenum-deficient culture. *PH* polyhedral body; *C* cyanophycin granule; *R* ribosomes; *PG* polyglucan granules. Fixed with glutaraldehyde-osmium. Magn. ×31300

Fig. 9. Ultrastructure of a heterocyst recovering from symptoms of molybdenum deficiency. Note the presence of ribosomes (*R*), dispersal of lipid droplets (*L*) and new membrane formation (*M*). Fixed with glutaraldehyde-osmium. Magn. × 17750

Table 1. Effect of vanadium on heterocyst production, nitrogenase activity and pigmentation of *Anabaena cylindrica*

Treatment	% heterocyst frequency	Nitrogenase activity nmoles C_2H_4 produced (mg dry wt)$^{-1}$ h^{-1}	Pigment content % dry wt	
			phycocyanin	chlorophyll
+ Mo/+ V	4.7	53.7	7.0	0.3
+ Mo/− V	4.3	85.2	10.8	0.6
− Mo/+ V	13.0	20.7	0.8	0.2
− Mo/− V	10.8	29.3	2.1	0.2

Discussion

Molybdenum deficiency in *A. cylindrica* results in changes which partly resemble those observed during nitrogen starvation (De Vasconcelos and Fay, 1974). The alga displays a remarkable ability of endurance under these conditions, remaining capable of a rapid recovery when either ammonia or molybdenum is re-supplied to the culture medium. Considering the involvement of heterocysts in nitrogen fixation by blue-green algae (Stewart *et al.*, 1969; Wolk and Wojciuch, 1971) it may appear paradoxical that an increase in the relative number of heterocysts should be concomitant with a fall (by 75—90%) of nitrogenase activity, compared with the control culture to which molybdenum was supplied at a concentration of 0.1 p.p.m. However, since molybdenum is an integral part of the nitrogenase complex, its absence in the medium will no doubt prevent the production of an active nitrogenase. The extremely rapid increase in nitrogenase activity and the high speed of recovery, when molybdenum is added to the deficient alga, suggest that the alga may continue to synthesize the apoenzyme in the absence of molybdenum at the expense of reserve and (under the existing conditions) less essential proteins.

The results are compatible with the existence of a metabolic control mechanism which regulates the rates of carbon and nitrogen metabolism in *A. cylindrica* (Fogg and Than-Thun, 1960; Cobb and Myers, 1964; Kulasooriya *et al.*, 1972). Continued photosynthesis under nitrogen-starved conditions gives rise to cells which first utilize nitrogenous reserves (biliproteins, cyanophycin granules, polyhedral bodies, ribosomes) and later accumulate starch (polyglucan granules) and fat (lipid droplets). Heterocyst differentiation and nitrogenase synthesis is enhanced during nitrogen starvation. When elemental nitrogen becomes available and subsequent nitrogen fixation gradually restores the normal C : N ratio in the alga, the rate of heterocyst production and enzyme synthesis declines correspondingly. However, when the synthesis of an active nitrogenase is prevented, as it is in the absence of molybdenum, and heterocysts are unable to provide fixed nitrogen to the vegetative

cells, the alga responds by producing more heterocysts. Essentially, this is a similar response to that which has been recorded for symbiotically grown leguminous and non-leguminous plants. There, too, expansion of the nodule tissue occurs when plants are short of nitrogen and the nodule bacteria are unable to synthesize nitrogenase in the absence of molybdenum (Anderson and Spencer, 1950; Hewitt and Bond, 1961).

Addition of vanadium to the growth medium results in the amplification of symptoms characteristic of molybdenum deficiency. It is conceivable that assimilation of vanadium and partial substitution of vanadium for molybdenum in the nitrogenase complex of *A. cylindrica* will result in a less efficient enzyme action, in a similar way as has been established with *Azotobacter* species (McKenna *et al.*, 1970; Burns *et al.*, 1971; Benemann *et al.*, 1972). This may possibly account for the "inhibitory" effect by vanadium upon nitrogenase activity of *Anabaena cylindrica*. Hence, it may be advisable to exclude vanadium from culture solutions when cells are grown on elemental nitrogen.

The results call for some caution when estimations of nitrogen-fixing ability in blue-green algae are based on relative heterocyst numbers. In the case of nitrogen starvation induced by molybdenum deficiency, it is clear that high heterocyst frequency does not necessarily indicate high nitrogen-fixing ability. Similarly, it would be wrong to equate low heterocyst number with low nitrogenase activity, as this may either indicate high nitrogen-fixing efficiency or alternatively it may arise from the depression of heterocyst production when combined forms of nitrogen are available.

Acknowledgements. This work was supported by grant GB1 from Westfield College, London. We thank Mrs. Brenda Thake for helpful comments on the manuscript.

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Dr. Peter Fay
Department of Botany
and Biochemistry
Westfield College
London NW3 7ST, England