

Effect of Sodium on Nitrogen Fixation in *Anabaena torulosa* and *Plectonema boryanum*

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The cyanobacterium *Anabaena torulosa* required sodium and molybdenum for nitrogenase activity and diazotrophic growth. Addition of sodium or molybdenum to cultures deficient in either element restored nitrogenase activity. Heterocyst differentiation was unaffected by sodium but molybdenum deficiency enhanced differentiation. The non-heterocystous cyanobacterium *Plectonema boryanum* 594 also required sodium for nitrogenase activity but synthesized presumptive nitrogenase component I and II proteins during sodium deficiency. The results show that in cyanobacteria nitrogenase is synthesized even in the absence of sodium but functions only in its presence.

INTRODUCTION

Sodium is an important nutritional requirement for cyanobacteria (Allen & Arnon, 1955; Kratz & Myers, 1955; Batterton & Van Baalen, 1971). The amount needed is very low but the requirement is specific (see Wolk, 1973) and cannot be substituted by potassium, lithium or caesium. The biochemical basis of sodium nutrition is not clear. Sodium deficiency has been shown to increase the assimilation of nitrate in *Anabaena cylindrica* whereas the incorporation of $^{15}\text{N}_2$ decreases (Brownell & Nicholas, 1967). Recently sodium has been found to be essential for nitrogenase activity in cyanobacteria (Apte & Thomas, 1980, 1983) although its mode of action remains to be established.

Molybdenum forms part of the iron–molybdenum cofactor (Shah & Brill, 1977; Eady *et al.*, 1980), a constituent of the MoFe protein or component I of nitrogenase (Eady & Postgate, 1974), and there is evidence that it is the putative substrate binding site in functional nitrogenase (Nagatani & Brill, 1974; Thorneley *et al.*, 1980). Molybdenum has also been implicated in the regulation of nitrogenase synthesis, although its precise role has been a matter of controversy (Brill *et al.*, 1974; Kennedy & Postgate, 1977). Thus molybdenum is essential for the synthesis of both component I and II in *Clostridium pasteurianum* (Cardenas & Mortenson, 1975) and of component I in *Azotobacter vinelandii* (Nagatani & Brill, 1974), while in *Klebsiella pneumoniae* (Kahn *et al.*, 1982), *Anabaena cylindrica* (Hallenbeck & Benemann, 1980) and *Plectonema boryanum* (Nagatani & Haselkorn, 1978) nitrogenase synthesis is independent of molybdenum.

We have examined the sodium requirement of *Anabaena torulosa*, a heterocystous and aerobic diazotroph, and of *Plectonema boryanum*, a non-heterocystous and microaerophilic diazotroph. In order to gain information on the mode of action of sodium, we compared the results with the molybdenum requirement in these organisms.

METHODS

Organisms and culture conditions. *Anabaena torulosa* was isolated in this laboratory by Fernandes & Thomas (1982). *Plectonema boryanum* 594 was a gift from Dr R. Haselkorn, University of Chicago, Illinois, USA. Starter cultures were prepared using five-fold diluted cyanophycean medium, CM/5 (David & Thomas, 1979) devoid of molybdenum and sodium and modified by adding either 3 mM-NH₄Cl and 5 mM-HEPES, pH 7.0, for *A. torulosa*,

or 10 mM-Ca(NO₃)₂·4H₂O, pH 7.0, for *P. boryanum*. The cyanobacteria were grown photoautotrophically at 25 °C under continuous illumination (5000 lx) and aeration (2 l min⁻¹). Chlorophyll *a* determinations were used to measure growth (Mackinney, 1941).

Induction and assay of nitrogenase. Cultures from the exponential growth phase in ammonium- or nitrate-supplemented media were harvested on Whatman GF/C fibre glass filters, aseptically washed and reinoculated into CM/5 medium free of combined nitrogen for induction of nitrogenase. CM/5 medium, which was suitably modified to avoid the inclusion of molybdenum or sodium or both, served as the basal medium for all the experiments. For *A. torulosa* nitrogenase was induced under aerobic conditions and for *P. boryanum* under microaerophilic conditions obtained by continuous sparging with argon. Nitrogenase activity was assayed as described previously (David *et al.*, 1980) except that for *P. boryanum* all the operations including assays were done under an argon gas phase.

Measurement of molybdenum and sodium uptake. Uptake was examined by using the radiotracers ⁹⁹Mo and ²²Na. Exponential phase cultures of *A. torulosa* were harvested, washed and inoculated in CM/5 medium without molybdenum and sodium and were grown either in the presence or absence of ammonium for 24 h. These molybdenum- and sodium-deficient cultures were harvested, resuspended in the fresh medium and used in uptake experiments. The assay mixture for sodium uptake contained 0.5 mM-sodium and ²²NaCl to give 0.25 μCi (9.25 kBq) ml⁻¹. Molybdenum uptake was measured in a mixture of [⁹⁹Mo]ammonium molybdate (4 μCi ml⁻¹) and MoO₃ to give 50 μM-molybdenum. The final volume of the assays was 2.5 ml. After the desired period of assay 2 ml samples were filtered on Whatman GF/C fibre glass filters. Residues were washed three times with solutions of unlabelled NaCl or MoO₃ (at equimolar concentrations to those of sodium and molybdenum in the assay mixture) and then three times with distilled water. The entire washing procedure took less than 1 min. The filter papers were dried and counted in 10 ml BBOT (0.4%, w/v) in a toluene/methanol mixture (1:1), using a Beckman LS-100 C liquid scintillation counter.

Preparation of cell-free extracts and SDS-PAGE. Cyanobacterial suspensions were concentrated to 50 μg chlorophyll *a* ml⁻¹, sonicated (2 min ml⁻¹) in a MSE-Soniprobe, and centrifuged at 1000 *g* for 10 min. The supernatant was precipitated with cold 10% (w/v) TCA and the pellet was washed twice with 70% (v/v) ethanol and once with ether. After drying, the pellet was solubilized in 0.025 M-Tris/HCl buffer, pH 8.3, containing 2% (w/v) SDS and 5% (w/v) β-mercaptoethanol, by incubating in a boiling water bath for 5 min. A 22 to 15% gradient slab gel was prepared with 25 ml of each of the following two solutions: 14.55% (w/v) acrylamide, 0.45% (w/v) bis-acrylamide, and 0.8 mg ammonium persulphate ml⁻¹; and 21.34% acrylamide, 0.66% bis-acrylamide and 0.4 mg ammonium persulphate ml⁻¹. Both the solutions were prepared in 0.1 M-Tris/HCl, pH 8.3, containing 0.1% SDS. The running gel was overlaid with a stacking gel containing 2.5% acrylamide, 0.625% bis-acrylamide and 0.1% SDS in 0.1 M-Tris/HCl, pH 6.8. The solubilized preparations were loaded on to the stacking gel and electrophoresed for 8 h at 20 mA with 0.025 M-Tris/glycine, pH 8.3, containing 0.1% SDS as a running buffer. Gels were fixed, stained with Coomassie Brilliant Blue R and photographed.

Chemicals, radioisotopes and gases. All the inorganic salts were from Sarabhai M. Chemicals, Baroda, India, or BDH, except for Ca(NO₃)₂·4H₂O which was from Merck. ²²NaCl was from Amersham and [⁹⁹Mo]ammonium molybdate from Isotope Division, Bhabha Atomic Research Centre, Trombay, India. Molecular weight standards for electrophoresis were obtained from Pharmacia. All the other chemicals were supplied by Sigma. Argon and acetylene gases were obtained from Indian Oxygen, Bombay, India, and standard ethylene from Matheson Gas Products, NJ, USA.

RESULTS

Anabaena torulosa showed complete dependence on sodium for growth under nitrogen-fixing conditions. After 5 d the chlorophyll *a* content of the sodium-deficient and sodium-supplemented cultures was found to be 2.1 and 17.9 μg ml⁻¹ respectively. Such a requirement was not seen when the medium was supplemented with combined nitrogen either in the form of nitrate or ammonium. The requirement was specific for sodium; it could not be replaced by other cations including K⁺, Li⁺, Ca²⁺ or Mg²⁺ (Table 1). All the sodium salts tested supported growth of the cyanobacterium irrespective of the accompanying anion. The requirement was very low since a sodium concentration above 22 μM supported good growth of the cyanobacterium (Table 2). This value comprised approximately 15 μM-sodium detected as contaminant in sodium-deficient medium plus the added 8.0 μM-NaCl.

The sodium requirement for growth, heterocyst differentiation and nitrogenase activity of *A. torulosa* was compared with its requirement for molybdenum – a known constituent of MoFe protein of nitrogenase. Response of the cyanobacterium to sodium starvation was very rapid but molybdenum-starved cultures could be obtained only after several passages in molybdenum-free

Table 1. *Specificity of sodium requirement for growth of nitrogen-fixing Anabaena torulosa*

Various salts were added to sodium-deficient medium to increase the concentration of cation by 1.0 mM. The initial chlorophyll *a* content of the cultures at the time of inoculation was $3.43 \mu\text{g ml}^{-1}$ and the final chlorophyll *a* content was determined 5 d after inoculation. No growth occurred when KCl, K_2HPO_4 , LiOH (pH adjusted to 7.0 with 0.05 M-HCl), MgSO_4 or CaCl_2 were added.

Salt added	Growth (increase in chl. <i>a</i> , $\mu\text{g ml}^{-1}$)
None	0.0
NaCl	14.7
Na_2SO_4	12.8
Na_2HPO_4	14.5

Table 2. *Effect of sodium on the growth of nitrogen-fixing Anabaena torulosa*

The initial chlorophyll *a* content of the cultures at the time of inoculation was $3.43 \mu\text{g ml}^{-1}$. Growth was measured, as the increase over this initial value, 5 d after inoculation. Sodium was added as NaCl and represents the concentration above background sodium contamination, which was $15 \mu\text{M}$ (as determined in putatively sodium-free medium).

Sodium concn (μM)	Growth (increase in chl. <i>a</i> , $\mu\text{g ml}^{-1}$)
0	0.0
5	0.1
8	4.6
10	8.5
20	6.9
50	10.6
100	11.7
250	14.8
500	15.1
1000	15.4

Table 3. *Effect of sodium and molybdenum on growth, heterocyst differentiation and nitrogenase activity of Anabaena torulosa*

Basal medium was supplemented with MoO_3 to give $50 \mu\text{M}$ -molybdenum or with NaCl to give 0.5 mM-sodium. Measurements were made 30 h after transfer to nitrogen-free media.

Culture conditions ...	{ - Mo - Na	+ Mo - Na	- Mo + Na	+ Mo + Na
Growth (increase in chl. <i>a</i> , $\mu\text{g ml}^{-1}$)	1.1	2.0	2.8	6.2
Heterocysts (% of total cells)	18.8	10.3	16.0	10.1
Nitrogenase activity [$\mu\text{mol C}_2\text{H}_2$ reduced (mg chl. <i>a</i>) $^{-1}$ h $^{-1}$]	1.5	7.3	11.5	52.5

medium. Under both molybdenum and sodium deficiency, growth and nitrogenase activity were inhibited (Table 3). Sodium had no effect on heterocyst differentiation but molybdenum deficiency resulted in a higher percentage of heterocysts. Good growth and nitrogenase activity were observed only when both molybdenum and sodium were present (Table 3).

Addition of molybdenum or sodium to *A. torulosa* cultures starved of the respective cation enhanced nitrogenase activity (Fig. 1). A lag of 2 h (for sodium) and 4 h (for molybdenum)

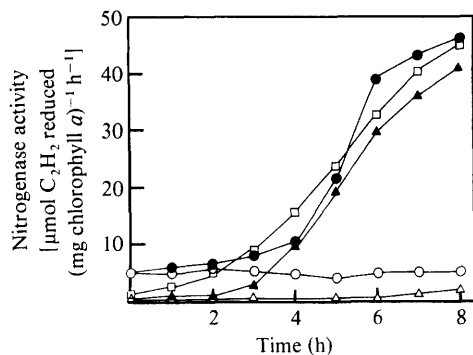


Fig. 1

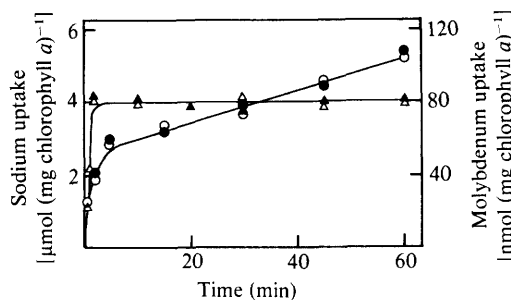


Fig. 2

Fig. 1. Requirement of molybdenum and sodium for nitrogenase activity of *Anabaena torulosa*, grown for 24 h under molybdenum deficiency (○) or sodium deficiency (△). Cultures starved of the respective cation were supplemented at 0 h to give 50 µM-molybdenum (●) or 0.5 mM-sodium (▲). A control culture (□) containing both molybdenum and sodium was included for comparison.

Fig. 2. Uptake of molybdenum and sodium by *Anabaena torulosa*. Uptake of molybdenum without (○) or with (●) 0.5 mM-sodium. Uptake of sodium without (△) or with (▲) 50 µM-molybdenum. Details of the radioactive assays are given in Methods.

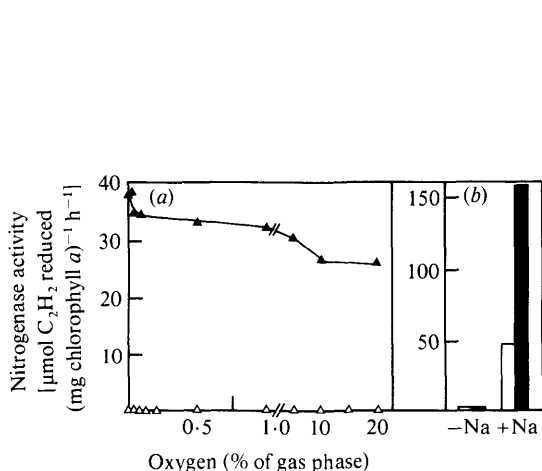


Fig. 3

Fig. 3. Effect of anaerobiosis on nitrogenase activity in *Anabaena torulosa*. (a) Aerobically grown cyanobacterial suspensions from sodium-deficient (△) and sodium-supplemented (▲) cultures were assayed for acetylene reduction under an argon gas phase containing various concentrations (% gas phase, v/v) of oxygen. (b) Aerobically grown cultures were subjected to anaerobiosis by continuous sparging with argon for 90 min, prior to assay, and then incubated for 30 min under 0.1 atm acetylene in argon. The open and closed bars show the nitrogenase activity of aerated and argon-sparged cultures respectively.

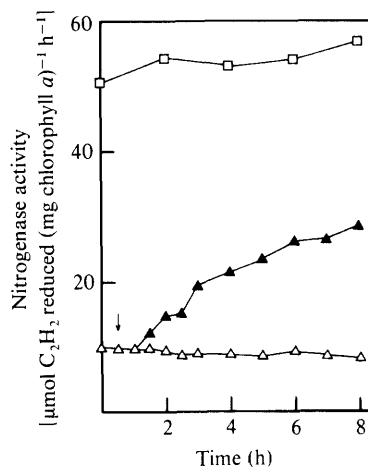


Fig. 4

Fig. 4. Effect of sodium on nitrogenase activity in *Plectonema boryanum* 594. Nitrogenase was induced in nitrogen-free medium with (□) or without (△) 1 mM-sodium under microaerophilic conditions. Sodium (1 mM) was added to sodium-deficient cultures (▲) at the time indicated by the arrow.

preceded such increased nitrogenase activity, which was fully restored by 5 to 6 h following addition of either cation. The duration of this lag could be reduced to 1 h for sodium and about 2 h for molybdenum if more dense cultures of *A. torulosa* (33 µg chlorophyll *a* ml⁻¹) were used.

The observed lag might have been due to a lag in the transport of the cations and it is possible that sodium regulated molybdenum transport. Accordingly the uptake of molybdenum and sodium by whole filaments of *A. torulosa* was examined. Sodium uptake was very rapid and

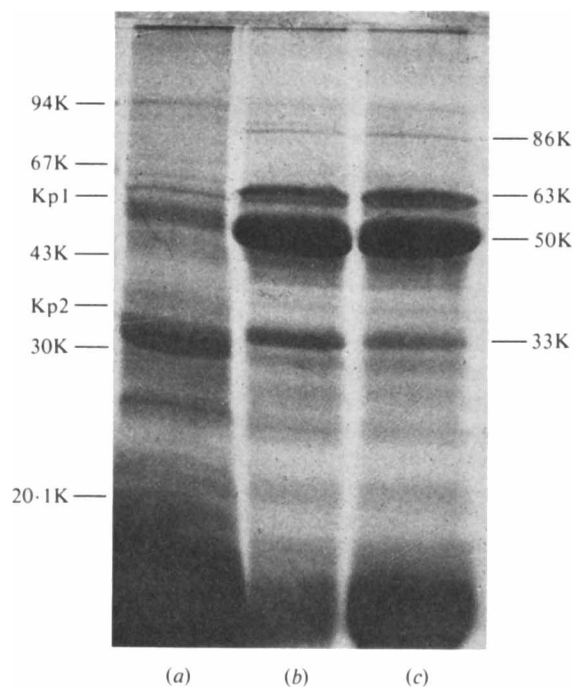


Fig. 5. Electrophoretic pattern of proteins from *Plectonema boryanum* 594. SDS extracts of proteins were electrophoresed on a 22 to 15% (w/v) polyacrylamide gradient slab gel with 0.1% (w/v) SDS. The culture conditions were (a) 10 mM- $\text{Ca}(\text{NO}_3)_2$, aerobic; (b) nitrogen-free, sodium-deficient, argon; and (c) nitrogen-free, sodium-supplemented, argon. The *in vivo* specific activities of cultures a, b and c were 0, 12 and 59 μmol acetylene reduced (mg chlorophyll *a*)⁻¹ h⁻¹, respectively. The amount of protein loaded was (a) 230 μg and (b) and (c) 200 μg . The positions of bands belonging to molecular weight standards and Kp1 and Kp2 as visualized in the same slab gel (tracks not shown) are indicated on the left side. Bands marked on the right side show proteins synthesized under nitrogen-fixing conditions only, of which 63K and 33K bands have been previously identified as *Plectonema* nitrogenase proteins (see text).

saturated within minutes while molybdenum uptake continued slowly for at least 60 min. Uptake of both the cations was independent of the presence or absence of the other cation (Fig. 2). Nitrogen-fixing *A. torulosa* assimilated much more molybdenum than when grown in the presence of ammonium but, in the presence or absence of combined nitrogen, sodium did not affect molybdenum uptake, even at concentrations 50 times higher than those of molybdenum.

Preincubation under acetylene is known to activate nitrogenase both *in vivo* (David & Fay, 1977) and *in vitro* (Thorneley & Eady, 1977) and attempts were therefore made to see if sodium affected this property. In sodium-supplemented cultures of *A. torulosa* this treatment resulted in a 2.2-fold enhancement of acetylene reduction while sodium-deficient cultures showed no such effect.

The possible role of sodium in protecting functional nitrogenase from oxygen inactivation was tested. This was done in *A. torulosa* in two ways (Fig. 3). Even the short-term anaerobiosis, maintained during the 30 min assay period, caused a 50% increase in nitrogenase activity of sodium-supplemented cultures (Fig. 3a). This indicates that even in heterocysts oxygen protection of nitrogenase does not seem to be perfect. When subjected to prolonged anaerobiosis by sparging for 90 min with argon prior to assay, sodium-supplemented cultures showed a 3.3-fold increase in acetylene reduction (Fig. 3b). The enhancement was probably because of a further strengthening of protection of the enzyme due to oxygen exclusion, as well as stimulation of nitrogenase derepression due to nitrogen starvation. Significantly, in both the experiments nitrogenase activity in sodium-deficient cultures remained unaffected, indicating that sodium was not involved in mediating protection from oxygen.

The filamentous, non-heterocystous cyanobacterium *Plectonema boryanum* also showed a requirement for sodium for nitrogenase activity (Fig. 4). Nitrogenase activity in sodium-supplemented cultures was five times greater than that in sodium-deficient cultures. In the latter cultures addition of sodium enhanced nitrogenase activity. The lag preceding this enhancement was short (30 min) compared to that observed with the heterocystous *A. torulosa* (2 h) and was reduced to less than 30 min if higher density cultures ($35 \mu\text{g}$ chlorophyll *a* ml^{-1}) were used.

The electrophoretic pattern of proteins from sodium-deficient and sodium-supplemented *P. boryanum* induced to synthesize nitrogenase is shown in Fig. 5. We found it convenient to use *P. boryanum* rather than *A. torulosa* for these studies since the former had a much higher nitrogenase content than the latter. Cultures repressed for nitrogenase synthesis, by addition of nitrate to the medium and by aeration, were used as controls (Fig. 5a). Purified *Klebsiella pneumoniae* nitrogenase proteins (Kp1 and Kp2) were also electrophoresed for comparison. Four proteins having molecular weights near 86000 (86K), 63K, 50K and 33K were found to be uniquely synthesized under nitrogen-fixing conditions, irrespective of the presence or absence of sodium (Fig. 5b, c). The protein bands at 63K and 33K are within the known range of molecular weights of nitrogenase proteins, which varies from 51K to 61K for MoFe protein and 30K to 35K for Fe protein in various diazotrophs (Yates, 1980). Similar bands were tentatively assigned earlier to the components of *P. boryanum* nitrogenase by Nagatani & Haselkorn (1978).

DISCUSSION

A specific growth requirement for sodium has been shown for *Anabaena cylindrica* (Allen & Arnon, 1955). We have now demonstrated that *A. torulosa* and *Plectonema boryanum* have a definite and specific requirement for sodium for nitrogenase activity and growth. The amount of sodium required is very low, with 20 to 25 μM being the threshold level required by *A. torulosa*. Growth dependence on sodium is observed only in the absence of combined nitrogen, unlike *A. cylindrica* which has been found to require sodium for normal growth on nitrate (Brownell & Nicholas, 1967). Sodium-deficient *A. cylindrica* showed enhanced nitrate reductase activity but did not grow, probably because of the resulting toxicity of excess nitrite which accumulated in the medium. With *A. torulosa* no such effects were seen and we have been able to maintain it in sodium-deficient media supplemented with nitrate or ammonium for over a year.

The response of *A. torulosa* to sodium starvation is similar to that observed during molybdenum starvation, except that in the latter case heterocyst differentiation is markedly enhanced. Such a stimulation of heterocyst formation during molybdenum deficiency was earlier observed in *A. cylindrica* (Fay & de Vasconcelos, 1974). This suggested that while the molybdenum-deficient cultures did not possess adequate amounts of the postulated nitrogenous inhibitor of heterocyst differentiation (Fogg, 1949; Wolk, 1967), it was present in the sodium-deficient cultures in adequate quantities to regulate heterocyst formation. Since both molybdenum and sodium deficiency eventually resulted in nitrogen starvation due to lack of nitrogenase activity, it appears that nitrogen starvation may not be the sole cause of the absence of such inhibitor.

Nitrogenase activity in sodium- or molybdenum-deficient cultures of *A. torulosa* reappeared after a lag of 2 or 4 h following addition of sodium or molybdenum respectively. This lag, which could be reduced by using dense cultures, was not due to a corresponding lag in the transport of the cations. The kinetic difference in the rates of sodium and molybdenum transport possibly accounted for the difference in the duration of lag following their additions. The observed lag may perhaps be due to barrier(s) to the transport of the cations at the step of their entry into heterocysts. Significantly, in the non-heterocystous *P. boryanum* this lag was much shorter. Transport of molybdenum was independent of the presence of sodium, ruling out a possible symport or antiport of molybdenum with sodium, as has been observed for other cations (Dewar & Barber, 1973), amino acids (Lanyi *et al.*, 1976) and sugars (Stock & Roseman, 1971) in bacteria. Clearly, the sodium requirement of nitrogenase is not related to the effect of sodium on molybdenum transport.

Using anaerobically grown *Anabaena variabilis*, Rippka & Stanier (1978) observed detectable nitrogenase activity only after 20 h. A similar time lag was found in *A. torulosa* (Apte & Thomas, 1980) while in anaerobically grown non-heterocystous *P. boryanum* induction of nitrogenase was observed after 8 to 9 h under our experimental conditions. Induction of nitrogenase in 5 h has been reported by Nagatani & Haselkorn (1978) using high density cultures. In contrast, the enhancement of nitrogenase activity following addition of sodium or molybdenum to deficient cultures of cyanobacteria occurred much faster. Subsequently the activity increased rapidly and was fully restored in *A. torulosa*. These observations favour involvement of molybdenum and sodium in the activation of enzyme pre-synthesized in their absence, rather than in its *de novo* synthesis. Indeed, *P. boryanum* induced to synthesize nitrogenase showed protein bands corresponding to components I and II of nitrogenase, in both the absence and presence of sodium. In this organism, as in *A. cylindrica* the molybdenum independence of nitrogenase synthesis has already been demonstrated (Nagatani & Haselkorn, 1978; Hallenbeck & Benemann, 1980). The close similarity in the requirement of molybdenum and sodium for nitrogenase activity in *A. torulosa* is suggestive of a complementary role for sodium in the enzyme activation. Thus the nitrogenase proteins are synthesized under sodium deficiency but remain catalytically inactive.

Subjecting sodium-deficient *A. torulosa* cultures to anaerobiosis did not promote nitrogenase activity in the absence of sodium and in the anaerobically induced *P. boryanum* also nitrogenase remained inactive under sodium deficiency. This eliminates the possibility that lack of protection from oxygen under sodium deficiency causes nitrogenase to be inactive.

The enhancement of acetylene reduction upon preincubation under acetylene *in vivo* (David & Fay, 1977; David *et al.*, 1978) has been shown to be due to conformational change(s) in the nitrogenase leading to an increase in its affinity towards acetylene (Apte *et al.*, 1978). With purified nitrogenase preparations from *Klebsiella pneumoniae* (Thorneley & Eady, 1977) such treatment resulted in increased electron flow through nitrogenase thereby enhancing its activities. However, the inactive nitrogenase of sodium-deficient cultures did not respond to preincubation under acetylene *in vivo* in our studies.

The nature of 'activation' of nitrogenase by sodium, whether due to direct effects on the enzyme molecule or to indirect effects, remains unresolved. Data provided in this paper indicate that cyanobacterial nitrogenase is synthesized in the absence of sodium, and that sodium is not involved in promoting nitrogenase activity indirectly by influencing molybdenum transport, protecting from oxygen or heterocyst differentiation. We have recently shown (Apte & Thomas, 1983) that impairment of photosynthesis during sodium deficiency is caused by paucity of nitrogenous products due to the absence of a functional nitrogenase. It is possible that sodium may influence membrane potential, which in turn is known to regulate nitrogenase activity, at least in aerobic diazotrophs like *Azotobacter* (Haaker *et al.*, 1980) and *Anabaena variabilis* (Hawkesford *et al.*, 1981). Occurrence of a Na^+/H^+ antiporter is known in cyanobacteria (Paschinger, 1977) and this may be involved in the maintenance of a requisite membrane potential conducive to nitrogenase activity.

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