

## Sodium requirement and metabolism in nitrogen-fixing cyanobacteria

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**Abstract.** Sodium affects the metabolism of eukaryotes and prokaryotes in several ways. This review collates information on the effects of  $\text{Na}^+$  on the metabolism of cyanobacteria with emphasis on the  $\text{N}_2$ -fixing filamentous species.  $\text{Na}^+$  is required for nitrogenase activity in *Anabaena torulosa*, *Anabaena* L-31 and *Plectonema boryanum*. The features of this requirement have been mainly studied in *Anabaena torulosa*. The need for  $\text{Na}^+$  is specific and cannot be replaced by  $\text{K}^+$ ,  $\text{Li}^+$ ,  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . Processes crucial for expression of nitrogenase such as molybdenum uptake, protection of the enzyme from oxygen inactivation and conformational activation of the enzyme are not affected by  $\text{Na}^+$ . Mo-Fe protein and Fe protein, the two components of nitrogenase are synthesized in the absence of  $\text{Na}^+$  but the enzyme complex is catalytically inactive. Photoevolution of  $\text{O}_2$  and  $\text{CO}_2$  fixation, which are severely inhibited in the absence of  $\text{Na}^+$ , are quickly restored by glutamine or glutamate indicating that  $\text{Na}^+$  deprivation affects photosynthesis indirectly due to deficiency in the products of  $\text{N}_2$  fixation.  $\text{Na}^+$  deprivation decreases phosphate uptake, nucleoside phosphate pool and nitrogenase activity. These effects are reversed by the addition of  $\text{Na}^+$  suggesting that a limitation of available ATP caused by reduced phosphate uptake results in loss of nitrogenase activity during  $\text{Na}^+$  starvation.

$\text{Na}^+$  influx in *Anabaena torulosa* and *Anabaena* L-31 is unaffected by low  $\text{K}^+$  concentration, is carrier mediated, follows Michaelis-Menten kinetics and is modulated mainly by membrane potential. Treatments which cause membrane depolarisation and hyperpolarisation inhibit and enhance  $\text{Na}^+$  influx respectively. These cyanobacteria exhibit rapid active efflux of  $\text{Na}^+$ , in a manner different from the  $\text{Na}^+/\text{H}^+$  antiporter mechanism found in *Anacystis nidulans*.

$\text{Na}^+$  requirement in nitrogen metabolism including nitrate assimilation, synthesis of amino acids and proteins, in respiration and oxidative phosphorylation, in transport of sugars and amino acids, cellular distribution of absorbed sodium, physiological basis of salt tolerance and prospects of reclamation of saline soils by cyanobacteria are the other aspects discussed in this review.

**Keywords:** Cyanobacteria; sodium requirement; nitrogen fixation; sodium transport; salt tolerance.

### Introduction

Sodium influences the metabolism of microbes, plants and animals. The food we eat is tasteless without sodium chloride, but apart from this gastronomical role,  $\text{Na}^+$  is a key element in regulating a variety of metabolic processes in animal cells including

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Abbreviations used: CCCP, Carbonylcyanide m-chlorophenyl-hydrazone; DCCD, N,N'-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP, dinitrophenol.

responses which underlie the action potentials of nerve axons (Hodgkin, 1964). There is abundant evidence from mammalian systems that the sodium pump generated by  $\text{Na}^+$ ,  $\text{K}^+$  ATPase, in addition to expelling excess  $\text{Na}^+$ , effects a circulation of  $\text{Na}^+$  and supports transport of inorganic and organic metabolites (Heinz, 1974; Harold, 1982; Kimmich, 1982). Moreover, transmembrane  $\text{Na}^+$  fluxes have been shown to be involved in ATP synthesis (Hallam and Whittam, 1977). ATPases stimulated by  $\text{Na}^+$  and  $\text{K}^+$  have also been demonstrated in plant plasmalemma preparations but their precise functions are not well understood (Harold, 1977). Recent work suggests that electrogenic proton translocating ATPases isolated from plant microsomal membranes are involved in the active transport of various solutes (Poole, 1978; Sze and Churchill, 1983). In addition to proton extruding ATPase(s) vectorial redox reactions facilitate such primary solute transport in bacteria (Harold, 1982; Elferink *et al.*, 1984).

Distinct from the enzyme-linked primary transport involving  $\text{Na}^+$  and related processes, secondary active transport such as the  $\text{Na}^+/\text{H}^+$  antiporter, first predicted by Mitchell (1966), has been since elucidated (Krulwich, 1983; Heinz and Grassel, 1984). The  $\text{Na}^+/\text{H}^+$  antiporter which catalyses translocation of  $\text{Na}^+$  and  $\text{H}^+$  in opposite directions may operate in either direction across the cell membrane. Moreover, movement of  $\text{Na}^+$  ions coupled to another metabolite in the same direction by the symport mechanism also facilitates substrate transport. The  $\text{Na}^+/\text{H}^+$  exchange reactions have been found widely in prokaryotic and eukaryotic membranes. At least some prokaryotes possess a membrane potential-dependent  $\text{Na}^+/\text{H}^+$  antiporter which catalyses  $\text{Na}^+$  extrusion in exchange for  $\text{H}^+$  whereas in other bacteria the antiporter may function electroneutrally at appropriate external pH values. The bacterial  $\text{Na}^+/\text{H}^+$  antiporter thus constitutes a critical component of  $\text{Na}^+$  circulation designed to maintain a diffusion potential of  $\text{Na}^+$  for use by  $\text{Na}^+$ -coupled bioenergetic processes (Krulwich, 1983). The prokaryotic antiporter is also involved in pH homeostasis in the alkaline pH range. In eukaryotes, an electroneutral  $\text{Na}^+/\text{H}^+$  antiporter has been found in a wide variety of cell and tissue types. The normal direction of this antiporter appears to be that of  $\text{Na}^+$  uptake and  $\text{H}^+$  extrusion which is implicated as a component of a complex  $\text{Na}^+$  circulation process. The antiporter also influences, in many experimental systems, internal pH which in turn could affect early events in a variety of differentiating and proliferating systems (Krulwich, 1983).

$\text{Na}^+$  is also involved in other metabolic processes. Many heterotrophic bacteria possess  $\text{Na}^+$ -sensitive decarboxylases. For instance, the oxaloacetate decarboxylase permits growth of *Salmonella typhimurium* and *Aerobacter aerogenes* on citrate only in the presence of  $\text{Na}^+$  (O'Brien and Stern, 1969a, b) and in *Klebsiella aerogenes* this decarboxylase functions as a  $\text{Na}^+$  translocase (Dimroth, 1980). The methylmalonyl-CoA decarboxylase involved in succinate fermentation in *Veillonella alcalescens* is specifically activated by  $\text{Na}^+$  (Hilpert and Dimroth, 1984).

A crucial role of  $\text{Na}^+$  is its contribution in causing salinity. Over 950 million hectares of land area of the earth are salt affected and nearly one-third of the irrigated land or 76.7 million hectares are estimated to be affected by salinity posing a serious threat to ecological balance and to the agricultural economy of mankind (Epstein, 1980). The ways in which plants cope with salt stress are not fully understood, although some aspects of salt tolerance are being exemplified. During salt stress plants as well as algae and bacteria resort to osmotic adjustment by building up high internal concentrations

of inorganic and/or organic solutes (Flowers *et al.*, 1977; Szalay and MacDonald, 1980). In plants, the inorganic ions are sequestered in vacuoles which occupy about 90 % of the mature cell volume. Another mechanism of sodium tolerance, as in animal cells, is the highly selective transport of potassium in plants (Epstein, 1980), algae and bacteria (Szalay and MacDonald, 1980).

From the foregoing it is clear that the influence of sodium on the life processes of various groups of organisms has received considerable emphasis. A striking exception to such major attention is the group of cyanobacteria or blue-green algae, prokaryotes capable of oxygenic photosynthesis which are widely prevalent in conventionally favourable climates of tropical and temperate regions as well as in extremes of heat and cold (Fogg *et al.*, 1973). They are abundant in brackish soils of coastal areas (Carter, 1933) and the Na<sup>+</sup>-rich saline alkali soils (Singh, 1950). Such cyanobacteria have been used in reclamation of the latter type of soils of Northern India with some success (Singh 1961). Many cyanobacteria are agronomically important in tropical rice paddies where they fix atmospheric nitrogen and carbon using solar energy. A major recent development is the unequivocal demonstration that Na<sup>+</sup> is essential for cyanobacterial nitrogenase activity (Apte and Thomas 1980, 1984).

In this article we shall collate the available information on the requirement and metabolism of sodium in cyanobacteria and stress the recent advances in sodium transport and nutrition especially its role in cyanobacterial nitrogen fixation. A great deal of the recent work described here relates to experiments carried out in our laboratory during the last five years. It compliments and extends earlier and current work in other laboratories.

## Sodium requirement for nitrogen assimilation in cyanobacteria

### *General features*

The entire taxonomic group of cyanobacteria, irrespective of their morphological organisation, ecological distribution and nature of carbon and nitrogen nutrition, requires Na<sup>+</sup> for growth. During the last four decades there have been several reports indicating the need for Na<sup>+</sup> especially in nitrate-grown cultures of many cyanobacteria such as the unicellular diazotrophs (incapable of N<sub>2</sub>-supported growth) *Chroococcus* sp. (Emerson and Lewis, 1942), *Microcystis aeruginosa* (McLachlan and Gorham, 1961) and *Anacystis nidulans* (Kratz and Myers, 1955) as well as the diazotrophic (capable of N<sub>2</sub>-supported growth) filamentous cyanobacteria *Anabaena variabilis*, *Nostoc muscorum* (Kratz and Myers, 1955), *A. flos-aquae* (Bostwick *et al.*, 1968) and *A. cylindrica* (Brownell and Nicholas, 1967). Whether Na<sup>+</sup> is needed in the presence of other forms of combined nitrogen has not been examined sufficiently but we have recently found that growth of NH<sub>4</sub><sup>+</sup>-supplemented cultures of two *Anabaena* spp. is not affected by Na<sup>+</sup> starvation (S. K. Apte and Joseph Thomas, unpublished results).

Very little is known about the requirement of Na<sup>+</sup> for N<sub>2</sub>-fixing cultures of cyanobacteria except for certain *Anabaena* spp.. Thus Allen and Arnon (1955) and Brownell and Nicholas (1967) found that *A. cylindrica* specifically required Na<sup>+</sup> during growth in the absence of combined nitrogen, while Apte and Thomas (1980, 1984) reported an absolute growth requirement of Na<sup>+</sup> for N<sub>2</sub>-fixing cultures of *A. torulosa*

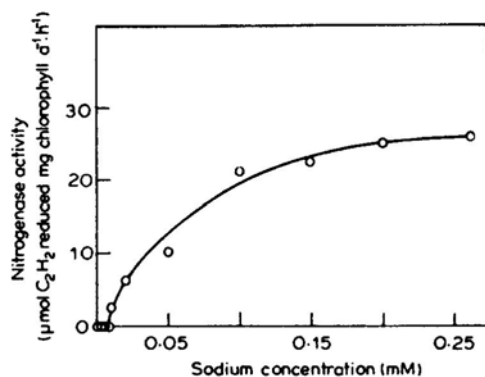
(a brackish water species) and *Anabaena* L-31 (a fresh water species). The  $\text{Na}^+$  requirement is highly specific and the cation cannot be replaced by  $\text{K}^+$  (Allen and Arnon, 1955; Kratz and Myers, 1955; Bostwick *et al.*, 1968; Apte and Thomas, 1984)  $\text{Rb}^+$ ,  $\text{Cs}^+$  (Allen and Arnon, 1955)  $\text{Li}^+$ ,  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  (Apte and Thomas, 1984). The last authors also found that all the salts of  $\text{Na}^+$  tested supported growth irrespective of the accompanying anion.

The reported concentrations of  $\text{Na}^+$  required by cyanobacteria are quite low.  $\text{Na}^+$  requirement saturated at 1 ppm ( $\approx 17 \mu\text{M}$ ) NaCl in fresh water cyanobacteria and at 100 ppm (1.7 mM) in marine forms (Batterton and Van Baalen, 1971). Allen and Arnon (1955) reported  $\text{Na}^+$  requirement in the range of 1.5 ppm (17–85  $\mu\text{M}$ ) while Kratz and Myers (1955) found a much higher requirement (680  $\mu\text{M}$ ). Recently Apte and Thomas (1984) demonstrated that about 20–25  $\mu\text{M}$  was the minimum level of  $\text{Na}^+$  required for detectable  $\text{N}_2$ -supported growth of the fresh water cyanobacterium *Anabaena* L-31 and the brackish water species *A. torulosa*. The concentrations required for optimum growth of the fresh water and the saline species have been found to be 0.5 mM and 20 mM respectively (S. K. Apte and Joseph Thomas, unpublished results). These concentrations are at least 10–20 times higher than those required for some of the known essential micronutrients of cyanobacteria (Wolk, 1973). The amount of  $\text{Na}^+$  needed has sometimes been found to vary with the nature of nitrogen nutrition. Thus, Brownell and Nicholas (1967) observed a much higher requirement (400  $\mu\text{M}$ ) for *A. cylindrica* during growth on nitrate as compared to only 4  $\mu\text{M}$  during growth on  $\text{N}_2$ . On the other hand we have noticed a much lower requirement ( $< 15 \mu\text{M}$ ) for *A. torulosa* during growth on nitrate compared to the amount (20 mM) required for optimum diazotrophic growth (S. K. Apte and Joseph Thomas, unpublished results).

In contrast to the requirement for low quantities of  $\text{Na}^+$  reported above, there are cases of halophilic cyanobacteria which need considerably high concentrations of NaCl (see section on salt tolerance). However, this requirement is for osmoregulation and not nutritional.

#### *Sodium requirement for nitrogen fixation*

In diazotrophically grown *A. cylindrica*  $\text{Na}^+$  deficiency was found to decrease the incorporation of  $^{15}\text{N}_2$  but increase the incorporation of  $^{15}\text{NH}_4$  or [ $^{14}\text{C}$ ] -glutamate into protein (Brownell and Nicholas, 1967). Apte and Thomas (1980) clearly demonstrated the dependence of cyanobacterial nitrogenase activity on the presence of  $\text{Na}^+$ . In two heterocystous diazotrophs, *A. torulosa* and *Anabaena* L-31, it was shown that  $\text{Na}^+$  deficiency resulted in significant loss of nitrogenase activity. Readdition of  $\text{Na}^+$  to  $\text{Na}^+$ -deficient cultures quickly enhanced nitrogenase activity, in a much shorter time (2 h) than that required ( $\approx 20$  h) for the induction of nitrogenase synthesis. Moreover, the activity increased very rapidly and was fully restored 5–6 h after addition of  $\text{Na}^+$ . The minimum level of  $\text{Na}^+$  required for restoration of nitrogenase activity was calculated to be 25  $\mu\text{M}$  in *A. torulosa* (figure 1). These data suggested a role for  $\text{Na}^+$  in the activation of presynthesised enzyme rather than in its *de novo* synthesis. Attempts to demonstrate this by preventing fresh synthesis with chloramphenicol were however unsuccessful, since chloramphenicol was found to rapidly inhibit nitrogenase activity (Apte and Thomas, 1980).



**Figure 1.** Determination of minimum level of external  $\text{Na}^+$  required for *in vivo* nitrogenase activity in *Anabaena torulosa*. Cyanobacterium was grown, in five-fold diluted combined nitrogen-free cyanophycean medium (CM/5; David and Thomas, 1979) devoid of  $\text{Na}^+$ , for 30 h. Different concentrations of  $\text{Na}^+$  were then added to separate aliquots of this culture and incubated under normal growth conditions. Nitrogenase activity ( $\text{C}_2\text{H}_2$  reduction) was determined 5 h after addition of  $\text{Na}^+$ , by the procedure of David *et al.* (1980).

A requirement of  $\text{Na}^+$  for nitrogenase activity has also been shown subsequently for the nonheterocystous cyanobacterium *Plectonema boryanum*-594 (Apte and Thomas 1984) which fixes  $\text{N}_2$  only under microaerophilic conditions. Comparisons were made between the electrophoretograms of sodium dodecyl sulphate (SDS) extracts of proteins obtained from  $\text{N}_2$ -fixing cultures with those repressed in the synthesis of nitrogenase. Using the approach along with the calibration of molecular weights, presumptive nitrogenase proteins having molecular weight of 63 K (MoFe protein) and 33 K (Fe protein) were detected in both  $\text{Na}^+$ -deficient as well as  $\text{Na}^+$ -supplemented *P. boryanum* (Apte and Thomas, 1984).

Recently we have obtained conclusive evidence for the non-requirement of  $\text{Na}^+$  for nitrogenase synthesis in *A. torulosa* using the approach described by Peterson and Wolk (1978). *A. torulosa* iron-containing proteins were radiolabelled with  $^{59}\text{Fe}$ . Soluble proteins were then extracted and electrophoresed on Polyacrylamide gels under non-denaturing anaerobic conditions and the distribution of radioactivity was determined. In cultures without combined nitrogen and thus derepressed for nitrogenase, six  $^{59}\text{Fe}$  peaks were detected which corresponded with distinct protein bands. Of these, two iron-containing proteins absent in grown repressed cultures and in extracts exposed to  $\text{O}_2$ , were identified as nitrogenase proteins. Both the proteins were present, in comparable amounts on unit protein basis, in derepressed cultures grown in the absence as well as in the presence of  $\text{Na}^+$  (table 1). It is thus clear that cyanobacterial nitrogenase proteins are synthesised even in the absence of  $\text{Na}^+$  but seem to function only in its presence. The cation thus appears to be essential for the expression of nitrogenase activity.

#### *Sodium, molybdenum and cyanobacterial nitrogenase*

A comparative analysis of  $\text{Na}^+$  and molybdenum requirements for nitrogen fixation has helped in obtaining more information on the role of  $\text{Na}^+$  (Apte and Thomas, 1984).

**Table 1.** Effect of sodium on the synthesis of nitrogenase proteins in *Anabaena torulosa*.

| Culture conditions   | Distribution of radioactivity<br>(dpm mg total protein <sup>-1</sup> ) |            |
|--|--|------------|
|  | MoFe protein   | Fe protein |
| Combined nitrogen-free CM/5*,<br>devoid of Na <sup>+</sup> | 60250  | 30100      |
| Combined nitrogen-free CM/5*,<br>with 1 mM Na <sup>+</sup> | 66117  | 32604      |
| CM/5* supplemented with 3 mN<br>NH <sub>4</sub> Cl.        | 1429   | 0          |

Cyanobacterium was grown in culture media supplemented with radioactive <sup>59</sup>FeCl<sub>3</sub> (1 μCi. ml<sup>-1</sup>) for 30 h. Total proteins were extracted and electrophoresed on Polyacrylamide vertical slab gels under non-denaturing, anaerobic conditions as described by Peterson and Wolk (1978). Gels were cut into 1 mm slices which were analysed for radioactivity by a NaI-crystal scintillation γ-spectrometer. Nitrogenase proteins were identified from among six <sup>59</sup>Fe-labelled iron proteins by: (i) incorporation of <sup>59</sup>Fe in them, (ii) their absence in NH<sub>4</sub><sup>+</sup>-grown cultures, (iii) their disappearance upon exposure to oxygen, (iv) their apparent molecular weights, and (v) rate of migration towards anode.

\* Five-fold diluted cyanophycean medium (David and Thomas, 1979).

Molybdenum forms a part of the iron molybdenum cofactor (FeMoCo) (Shah and Brill, 1977; Eady *et al.*, 1980), a constituent of the MoFe protein or component I of nitrogenase (Eady and Postgate, 1974) and is generally considered to be the substrate-binding active site of nitrogenase (Nagatani and Brill, 1974; Smith, 1977; Thorneley *et al.*, 1980). Molybdenum has also been implicated in the regulation of nitrogenase synthesis (Brill *et al.*, 1974; Kennedy and Postgate, 1977). This regulatory role has been a subject of controversy. Thus molybdenum is reported to be essential for the synthesis of both components I and II in *Clostridium pasteurianum* (Cardenas and Mortenson, 1975) while in *Klebsiella pneumoniae* (Kahn *et al.*, 1982) and *Azotobacter* (Nagatani and Brill, 1974; Pienkos *et al.*, 1981) inactive component I and active component II are synthesised in the absence of molybdenum. Although molybdenum is essential for cyanobacterial N<sub>2</sub> fixation (Wolfe, 1954 a, b; Taha and Elrefai, 1962; Fay and de Vasconcelos, 1974) it is not required for the synthesis of nitrogenase protein in *P. boryanum* (Nagatani and Haselkorn, 1978) and *A. cylindrica* (Hallenbeck and Benemann, 1980).

Many responses of N<sub>2</sub>-fixing cyanobacteria to Na<sup>+</sup> deficiency (Apte and Thomas, 1980, 1984) are similar to that observed during molybdenum deficiency (Wolfe, 1954a; Fay and de Vasconcelos, 1974; Apte and Thomas, 1984). Nitrogenase proteins are synthesised under both Na<sup>+</sup> (Apte and Thomas, 1984; and unpublished results) as well as molybdenum starvation (Nagatani and Haselkorn, 1978). Absence of either of the elements, however, results in loss of nitrogenase activity and restricts diazotrophic growth. In *A. torulosa*, addition of Na<sup>+</sup> as well as molybdenum to cultures deficient in the respective cation restores nitrogenase activity. The kinetics of the restoration

process, comprising a 2–3 h lag, subsequent to rapid increase and full restoration by 6 h is quite comparable for both  $\text{Na}^+$  and molybdenum (Apte and Thomas, 1984). In the nonheterocystous *P. boryanum*, enhancement of nitrogenase activity following addition of  $\text{Na}^+$  (Apte and Thomas, 1984) or molybdenum (Nagatani and Haselkorn, 1978) occurs much faster, thus indicating that in *Anabaena* a possible lag occurs in the transport of these ions into heterocysts.

Uptake of both molybdate (Wolfe 1954a, b) and  $\text{Na}^+$  (S. K. Apte and Joseph Thomas, unpublished results) is much higher under  $\text{N}_2$ -fixing conditions than in  $\text{NH}_4^+$ -grown cultures where synthesis of both nitrogenase and nitrate reductase is repressed. Nitrate-grown cultures also show a drastically reduced uptake of  $\text{Na}^+$  but uptake of molybdenum is substantial, being 50 % of  $\text{N}_2$ -fixing cultures (S. K. Apte and Joseph Thomas, unpublished results). A molybdenum co-factor is a constituent of nitrate reductase, and a substantial uptake of molybdenum is probably needed to maintain nitrate reductase activity.

Molybdenum-deficient cultures of cyanobacteria differ from  $\text{Na}^+$ -deficient cultures in one important respect. While the absence of molybdenum enhances heterocyst differentiation (Fay and de Vasconcelos, 1974) it is unaffected by  $\text{Na}^+$  deficiency (Apte and Thomas, 1980). In cyanobacteria the heterocyst pattern is thought to be governed by a specific nitrogenous inhibitor, produced by preexisting heterocysts, which diffuses through the vegetative cells establishing decreasing concentration gradient away from heterocysts. New heterocysts arise by differentiation of those vegetative cells where the concentration of this inhibitor falls below a critical level (Fogg, 1949; Wolk, 1967; Wilcox *et al.*, 1973; Reddy and Talpasayi, 1974). Molybdenum deficiency results in nitrogen starvation apparently leading to a fall in the level of the nitrogenous heterocyst inhibitor. We find that  $\text{Na}^+$  deficiency also causes nitrogen starvation (Apte and Thomas, 1983a). The postulated inhibitor appears to be still present in  $\text{Na}^+$ -deficient cultures in quantities adequate to regulate the heterocyst pattern.

#### *Possible mechanism of nitrogenase activation by sodium*

Evidence for and against the possible ways by which the presence of  $\text{Na}^+$  may facilitate nitrogenase activity is considered below.

*Effect on heterocysts:* Since heterocysts are the sole sites of aerobic (Peterson and Burris, 1976; Thomas *et al.*, 1977; Peterson and Wolk, 1978) and possibly of even anaerobic (Murry *et al.*, 1984) nitrogen fixation in heterocystous cyanobacteria, a change in number or quality of heterocysts is bound to affect nitrogenase activity. However, as mentioned earlier,  $\text{Na}^+$  deficiency does not influence heterocyst differentiation (Apte and Thomas, 1980), nor are the heterocysts 'leaky' (structural or biochemical lesions) to  $\text{O}_2$  since even under anaerobiosis nitrogenase activity depends on  $\text{Na}^+$  (Apte and Thomas, 1982, 1984). Finally, the requirement is also observed in the nonheterocystous microaerophilic diazotroph, *Plectonema boryanum* (Apte and Thomas, 1984).

*Transport of molybdenum:*  $\text{Na}^+$  has been found to influence the transport of certain cations (Dewar and Barber, 1973), anions like phosphate (Kodama and Taniguchi, 1977) amino acids (Lanyi *et al.*, 1976; MacDonald *et al.*, 1977) and sugars (Stock and

Roseman, 1971) in bacteria. In view of the close resemblance of the effects of  $\text{Na}^+$  and molybdenum deficiency it was considered possible that  $\text{Na}^+$  deprivation might adversely affect molybdate transport. However, we have shown recently that in *A. torulosa* uptake of  $\text{Na}^+$  and molybdate is independent of the presence of the other ion both under  $\text{N}_2$ - and  $\text{NH}_4^+$ -supported growth.  $\text{Na}^+$ , even at concentrations 50 times higher than those of molybdenum, did not affect molybdate uptake (Apte and Thomas, 1984).

*Protection of nitrogenase from oxygen:* As mentioned earlier,  $\text{Na}^+$  dependence of cyanobacterial nitrogenase is also observed under anaerobic conditions (Apte and Thomas, 1984). Prolonged anaerobiosis enhances nitrogenase activity of  $\text{Na}^+$ -supplemented *A. torulosa* cultures by over threefold but does not affect the low activity of  $\text{Na}^+$ -deficient cultures. The dependence of nitrogenase activity of  $\text{Na}^+$  is thus not consequent to a role of the cation in protecting nitrogenase from oxygen.

*Effect on membrane potential and supply of reductant:* Nitrogenase needs a constant supply of reductant (electrons) for its activity. A possible route of electron transfer to cyanobacterial nitrogenase is from NADPH generated by oxidative pentose phosphate pathway via ferredoxin: NADP<sup>+</sup> oxidoreductase (Apte *et al.*, 1978; Lockau *et al.*, 1978). Such an electron transfer, considered to be thermodynamically unfavourable may become favourable under appropriate membrane potential *in vivo* (Haaker *et al.*, 1980). The importance of membrane potential in regulating nitrogenase activity has been demonstrated in several diazotrophs like *Azotobacter vinelandii* (Laane *et al.*, 1980), *Rhodospseudomonas capsulata* (Haaker *et al.*, 1982), *Rhizobium leguminosarum* bacteroids (Laane *et al.*, 1979) and *Anabaena variabilis* (Hawkesford *et al.*, 1981). In all these diazotrophs hyperpolarisation increases while depolarisation decreases nitrogenase activity. Paradoxically, in *A. torulosa*, while there is little or no nitrogenase activity during  $\text{Na}^+$  deficiency, a remarkable hyperpolarisation of membrane potential occurs, which is not reversed immediately by re-addition of  $\text{Na}^+$  (figure 2). A similar hyperpolarisation induced by the ionophore nigericin, however, enhances nitrogenase activity in  $\text{Na}^+$ -supplemented cultures (S. K. Apte and Joseph Thomas, unpublished results). Clearly, therefore, the effect of  $\text{Na}^+$  on nitrogenase activity is independent of its effect on membrane potential.

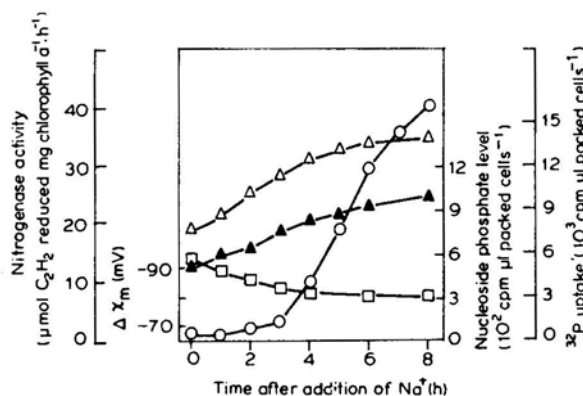
Possible influence of  $\text{Na}^+$  on generation and supply of electrons has not been examined so far. It will be interesting to see whether an externally supplied reductant like  $\text{Na}^+$ -free dithionite or more preferably a physiological source like glucose-6-phosphate stimulates nitrogenase activity in isolated heterocysts or cell-free extracts from  $\text{Na}^+$ -deficient cyanobacteria.

*Conformational activation:* Attempts have been made to ascertain whether  $\text{Na}^+$  deficiency directly or indirectly renders nitrogenase into inactive conformation and whether this can be reversed by preincubation under acetylene (Apte and Thomas, 1984). Preincubation under  $\text{C}_2\text{H}_2$  enhances *in vivo* nitrogenase activity of cyanobacteria (David *et al.*, 1978) by inducing conformational changes such that the affinity for several substrates is altered (Apte *et al.*, 1978). In purified nitrogenase proteins from *Klebsiella pneumoniae* (Thorneley and Eady, 1977) such treatment enhances electron flow through nitrogenase resulting in more and more reduced forms of enzyme and



enhancement of activities. On incubation with  $C_2H_2$ ,  $Na^+$ -supplemented *A. torulosa* shows over two-fold enhancement of  $C_2H_2$  reducing activity while the low activity of  $Na^+$ -deficient cultures is not affected (Apte and Thomas, 1984). Thus, a conformational activation by  $Na^+$ , if at all present, is different from that caused by exposure to  $C_2H_2$ . Possible direct effect(s) of  $Na^+$  on the enzyme molecule itself are not considered here in the absence of such data.

*Supply of ATP:*  $N_2$  reduction is a highly energy intensive process and nitrogenase consumes nearly 15 mol of ATP per mol of  $N_2$  reduced (Postgate, 1982).  $Na^+$  deficiency has been found to significantly decrease the uptake and utilisation of phosphate and thereby reduce the size of nucleotide pools. Restoration of nitrogenase activity following addition of  $Na^+$  to cultures deficient in the cation is preceded by significant enhancement of  $P_i$  uptake and of nucleotide levels (figure 2; and see also section on phosphate uptake and utilisation).



**Figure 2.** Effect of sodium on membrane potential ( $\square$ ), uptake of phosphate ( $\Delta$ ), nucleotide phosphate pool ( $\blacktriangle$ ) and nitrogenase activity ( $\circ$ ) of  $Na^+$ -deficient *Anabaena torulosa*. Membrane potential ( $\Delta\chi_m$ ) was determined by using lipophilic cation [ $^{14}C$ ]-tetraphenylphosphonium as described by Haaker *et al.* (1982). Uptake of  $^{32}P_i$  and its incorporation into nucleotide phosphates were determined by the procedures described by Sugino and Miyoshi (1964) and Kodama and Taniguchi (1977). Values of membrane potential (mV),  $P_i$  uptake and nucleotide phosphates (cpm.  $\mu\text{l}$  packed cells $^{-1}$ ), and nitrogenase activity ( $\mu\text{mol } C_2H_2$  reduced mg chlorophyll  $a^{-1} \cdot h^{-1}$ ) for corresponding  $Na^+$ -supplemented cultures at 0 h were -65.2, 19400, 1405 and 52.63 respectively.

Of all the possibilities discussed above, a limitation on the supply of available ATP to nitrogenase appears, at present, to be the best available explanation for loss of  $N_2$  fixation in the absence of  $Na^+$ . A role of  $Na^+$  in maintaining supply of ATP to support various energy-linked functions has also been observed in *Pseudomonas stutzeri* (Kodama and Taniguchi, 1976, 1977).

#### *Sodium requirement for nitrate assimilation*

Although a requirement of  $Na^+$  for nitrate-supplemented growth of both adiazotrophic and diazotrophic cyanobacteria seems fairly established the nature of such

requirement is not very clear. In nitrate-supplemented *A. cylindrica*, Brownell and Nicholas (1967) found that  $\text{Na}^+$  deficiency resulted in several fold enhancement of nitrate reductase activity resulting in overproduction of nitrite which accumulated in the medium in toxic concentrations and caused loss of phycocyanin and chlorosis of the cultures. The  $\text{Na}^+$ -deficient cultures showed reduced dry wt and protein content but exhibited enhanced incorporation of  $^{15}\text{NO}_3$ ,  $^{15}\text{NO}_2$ ,  $^{15}\text{NH}_4^+$ , and  $[^{14}\text{C}]$ -glutamate into proteins. Based on results obtained using chloramphenicol, it was concluded (Brownell and Nicholas, 1967) that  $\text{Na}^+$  was essential for the synthesis of a protein which regulated (decreased) nitrate reductase activity. No attempts were, however, made to detect such a regulatory protein in  $\text{Na}^+$ -supplemented cultures. Possible effects of  $\text{Na}^+$  on the uptake of nitrate and nitrite and on nitrite reduction were not examined. It is also not clear from the data why, in spite of enhanced nitrate reduction and incorporation into proteins, the cultures showed symptoms of nitrogen starvation such as decreased protein content and more importantly, loss of phycocyanin.

Nitrate-supplemented,  $\text{Na}^+$ -deficient cultures of a related cyanobacterium, *A. torulosa*, also showed loss of chlorophyll *a* but this was not due to accumulation of nitrite which was negligible (S. K. Apte and Joseph Thomas, unpublished results). As compared to  $\text{Na}^+$ -supplemented cultures,  $\text{Na}^+$ -deficient cultures showed reduced uptake of nitrite as has also been reported in a diatom *Phaeodactylum* (Cresswell and Syrett, 1982). In contrast to the results obtained with *A. cylindrica*, we found that in *A. torulosa* both *in vivo* as well as *in vitro* nitrate reductase activity was decreased by prolonged  $\text{Na}^+$  deficiency. The observed decrease in nitrate reduction (25–30%) was however insufficient to explain the extent of growth inhibition (50 %) of  $\text{Na}^+$ -deficient nitrate-supplemented *A. torulosa* (S. K. Apte and Joseph Thomas, unpublished results).

It appears therefore that a requirement of  $\text{Na}^+$  for nitrate-supported growth of cyanobacteria may occur not only at the step of reduction of nitrate, or uptake and reduction of nitrite, but perhaps also at a later stage in the metabolism. More work is warranted to resolve the nature of  $\text{Na}^+$  requirement in nitrate-grown cyanobacteria.

#### *Synthesis of amino acids and proteins*

$\text{Na}^+$  deficiency results in a significant reduction (50–70%) in the content of total nitrogen and nitrogen based compounds, especially amino acids and proteins, in  $\text{N}_2^-$  fixing *A. torulosa* cultures. In comparison, a similar treatment of nitrate- or  $\text{NH}_4^+$ -supplemented cultures causes only a 15–25% reduction (S. K. Apte and Joseph Thomas, unpublished results). However, the activities of two key enzymes involved in  $\text{NH}_4^+$  assimilation and synthesis of other amino acids, namely glutamine synthetase and aminotransferase, are independent of the presence of  $\text{Na}^+$ . Similarly the rate of amino acid incorporation into proteins is not reduced in  $\text{Na}^+$ -deficient cultures of *A. torulosa*. In fact, the rate of protein synthesis is enhanced under  $\text{Na}^+$  starvation. Thus, the reduced contents of amino acids and proteins result not from limitations on the efficiency of machinery responsible for their synthesis but indirectly from loss of nitrogenase activity and nitrogen starvation.

There is increased incorporation of glutamate into protein but decreased protein content in  $\text{Na}^+$ -deficient, nitrate-supplemented cultures of *A. cylindrica* (Brownell and Nicholas, 1967). The decreased protein content in this case also probably results from

nitrogen starvation caused by loss of nitrate reductase activity (see earlier part of this section). It appears that in cyanobacteria, as also in *E. coli* (Lubin and Ennis, 1964), presence of excess  $\text{Na}^+$  decreases the rate of protein synthesis probably by antagonizing the established stimulatory effect of  $\text{K}^+$  on amino acid incorporation (Harold, 1982).

In addition to the quantitative differences,  $\text{Na}^+$  also seems to determine the quality of proteins synthesised by  $\text{N}_2$ -fixing *A. torulosa* cultures. Thus  $\text{Na}^+$ -deficient cultures lack two proteins characteristically present in  $\text{Na}^+$ -supplemented cultures; the role of these proteins is unknown at present.  $\text{Na}^+$ -deficient cultures also contain reduced amounts of phycocyanin—an established marker of nitrogen status in cyanobacteria (Van Gorkom and Donze, 1971; De Vasconcelos and Fay, 1974) and certain other proteins probably including some of the Calvin cycle enzymes (S. K. Apte and Joseph Thomas, unpublished data).

### Sodium requirement in general metabolism

#### *Photosynthesis*

Under  $\text{N}_2$ -fixing conditions,  $\text{Na}^+$  deficiency causes impairment of photosynthetic functions (Apte and Thomas 1982, 1983a).  $\text{Na}^+$ -deficient *A. torulosa* cultures show loss of photosynthetic pigments and severe inhibition of photoevolution of  $\text{O}_2$  and photofixation of  $\text{CO}_2$ . The content of nitrogenous pigments (chlorophyll *a* and phycocyanin) decreases while that of carotenoids increases. Similar loss of pigments occurs also in  $\text{Na}$ -deficient *A. cylindrica* (Brownell and Nicholas, 1967). Phycocyanin is a major constituent of the oxygen-evolving apparatus of photo-system II but since the phycocyanin/chlorophyll *a* ratio is not affected by  $\text{Na}^+$  deficiency the overall pigment composition of photosystem II probably remains unaltered.  $\text{Na}^+$  deficiency also does not affect methyl viologen-induced and light-dependent oxygen uptake (Apte and Thomas, 1982, 1983a). Since this reaction collectively represents all the major photochemical reactions (photolysis of water, electron flow through the entire 'Z' scheme and electron donation to the terminal electron acceptor), the possibility of impairment of structural/functional integrity of photo-systems by  $\text{Na}$ -deficiency is unlikely.

Addition of  $\text{Na}^+$  (as  $\text{NaCl}$ ) to  $\text{Na}^+$ -deficient *A. torulosa* cultures restores photoevolution of  $\text{O}_2$  and  $\text{CO}_2$  fixation (Apte and Thomas, 1983a). This effect of  $\text{NaCl}$  is not simulated by several other chlorides indicating that it is a genuine  $\text{Na}^+$  effect and not the 'chloride effect' observed in certain chloroplast preparations (Hind *et al.*, 1969; Izawa *et al.*, 1969). Interestingly,  $\text{NH}_4\text{Cl}$ , glutamine and glutamate completely mimic the  $\text{Na}^+$  effect (table 2). Addition of these compounds to  $\text{Na}^+$ -deficient cultures also restores the photoevolution of  $\text{O}_2$  and  $\text{CO}_2$  fixation; glutamine and glutamate, in fact, are more efficient than  $\text{Na}^+$  (Apte and Thomas, 1983a).

It is well known that physiological photoevolution of  $\text{O}_2$  occurs in the presence of a terminal electron acceptor ( $\text{CO}_2$ ) which is reduced. The extremely poor rates of  $\text{CO}_2$  fixation in  $\text{Na}^+$ -deficient *A. torulosa* thus appear to be responsible for very low rates of  $\text{O}_2$  photoevolution (Apte and Thomas, 1983a). This is supported by the fact that provision of an alternative electron acceptor (like methyl viologen) facilitates normal reaction (table 2). Moreover, the reappearance of  $\text{O}_2$  photoevolution by addition of

**Table 2.** Impairment of photosynthesis by sodium deficiency and its restoration by addition of sodium or products of N<sub>2</sub> fixation in diazotrophically grown *Anabaena torulosa*.

| Culture conditions            | Additions                 | Photosynthetic reactions<br>( $\mu\text{mol. mg chlorophyll } a^{-1} \text{ min.}^{-1}$ ) |  |   |
|-------------------------------|---------------------------|---|--|---|
|                               |                           | Photo-evolution of O <sub>2</sub>   | Methyl viologen-induced O <sub>2</sub> uptake in light | Light-dependent uptake of NaH <sup>14</sup> CO <sub>3</sub> |
| Na <sup>+</sup> -supplemented | Nil                       | 9.86  | 7.15   | 3.51  |
| Na <sup>+</sup> -deficient    | Nil                       | 3.51  | 7.70   | 1.23  |
| Na <sup>+</sup> -deficient    | NaCl (1 mM)               | 8.06  | 7.06   | 2.05  |
| Na <sup>+</sup> -deficient    | KCl (1 mM)                | 3.65  | N.D.   | N.D.  |
| Na <sup>+</sup> -deficient    | NH <sub>4</sub> Cl (3 mM) | 5.24  | 7.38   | N.D.  |
| Na <sup>+</sup> -deficient    | Glutamine (1 mM)          | 8.35  | 6.42   | N.D.  |
| Na <sup>+</sup> -deficient    | Glutamate (1 mM)          | 10.06   | 8.30   | 2.46  |

Thirty h after transfer to Na<sup>+</sup>-free media, cyanobacterium was harvested and resuspended in identical medium. Additions were then made as shown and samples were incubated for 2 h under aeration (21.min<sup>-1</sup>) at 5000 lx and 25°C prior to analysis. Photosynthetic reactions were studied as described by Apte and Thomas (1983a).

N.D. = not determined.

Na<sup>+</sup> or amino acids is accompanied by a concomitant restoration of CO<sub>2</sub> fixation (Apte and Thomas, 1983a). Loss of CO<sub>2</sub> fixation appears to be related to nitrogen starvation which occurs under Na<sup>+</sup> deficiency. Nitrogen starvation decreases protein synthesis and causes degradation of proteins in *Anabaena* (Own by *et al.*, 1979) probably due to activation of certain proteases (Wood and Haselkorn, 1977). Na<sup>+</sup>-deficient cultures also show loss of and decreased content of certain proteins (see section on nitrogen assimilation) and if some of these include Calvin cycle enzymes it may explain the reduced CO<sub>2</sub> fixation under Na<sup>+</sup> deficiency.

To summarise, it can be concluded that Na<sup>+</sup> deficiency influences photosynthesis indirectly through nitrogen starvation: (i) in media supplemented with combined nitrogen (especially NH<sub>4</sub><sup>+</sup>) Na<sup>+</sup>-deficiency does not affect photosynthesis; (ii) Na<sup>+</sup>-deficiency results in loss of nitrogenase activity; (iii) nitrogen starvation is evident from the decreased content of total nitrogen, proteins, amino acids and nitrogenous pigments in Na<sup>+</sup>-deficient cultures; (iv) nitrogen starvation has been found to decrease photosynthetic CO<sub>2</sub> fixation in the cyanobacterium *A. cylindrica* (Lawrie *et al.*, 1976); (v) inhibition of photosynthesis is reversed upon addition of Na<sup>+</sup> which 'switches on' nitrogenase activity; (vi) NH<sub>4</sub><sup>+</sup>, glutamine and glutamate, which are the initial products of N<sub>2</sub> fixation and assimilation in cyanobacteria (Thomas *et al.*, 1975), also rapidly restore photosynthesis in Na<sup>+</sup>-deficient *A. torulosa*. The greater efficiency of amino acids, compared to Na<sup>+</sup>, is probably because they by-pass the energy-consuming nitrogenase reaction.

### Respiration and oxidative phosphorylation

In the unicellular cyanobacterium, *Anacystis nidulans*, high concentrations of  $\text{Na}^+$  have been found to stimulate respiratory  $\text{O}_2$  consumption (Nitschmann and Peschek, 1982; Nitschmann *et al.*, 1982) and anaerobically grown cells were seen to accumulate much more  $\text{Na}^+$  than aerobically maintained cells. These results suggested that  $\text{Na}^+$  extrusion by  $\text{Na}^+ / \text{H}^+$  antiporter is coupled to the respiratory proton gradient and that part of the energy stored in the proton gradient would be dissipated by  $\text{Na}^+$  extrusion decreasing the efficiency of oxidative phosphorylation. A fall in the P/O ratio from 2.9 at low  $\text{Na}^+$  concentration to 0.6 at high concentration (Nitschmann and Peschek, 1982) and decrease of ATP pools in the presence of high  $\text{Na}^+$  (Nitschmann *et al.*, 1982) substantiated the above conclusion. Such deleterious effect of  $\text{Na}^+$  was not found in two facultative chemoheterotrophic diazotrophs *Anabaena variabilis* and *Nostoc* MAC. It has been, therefore, proposed that the high respiratory energy costs of  $\text{Na}^+$  extrusion are responsible for the obligate photoautotrophy of most cyanobacteria.

In  $\text{N}_2$ -fixing *A. torulosa* addition of 10-100 mM NaCl has no effect on respiratory  $\text{O}_2$  consumption (S. K. Apte and Joseph Thomas, unpublished results). This resembles the situation reported in *A. variabilis* and *Nostoc* MAC (Nitschmann *et al.*, 1982). Since all these cyanobacteria are diazotrophs requiring  $\text{Na}^+$ , it is tempting to suggest that the energy expensive  $\text{Na}^+$  extrusion in *A. nidulans* may be associated, in addition to obligate photoautotrophy, with adiazotrophy also. As we shall see later (section on sodium transport)  $\text{Na}^+$  extrusion in certain diazotrophic cyanobacteria does not seem to be mediated by a  $\text{Na}^+/\text{H}^+$  antiporter and this may be a useful device to economise and divert the available energy to a more useful process like  $\text{N}_2$  fixation.

Effect of  $\text{Na}^+$  deficiency on respiration has been examined in *A. torulosa* (Apte and Thomas, 1982) where it increases the dark consumption of  $\text{O}_2$ .  $\text{Na}^+$  deficiency also decreases the uptake of  $P_i$  (figure 2) and together this should result in low P/O ratios and decreased efficiencies of oxidative phosphorylation (S. K. Apte and Joseph Thomas, unpublished results).

### Uptake and utilisation of phosphate

$\text{Na}^+$  deficiency curtails the uptake and utilisation of phosphate in *A. torulosa* grown under  $\text{N}_2$ -fixing conditions and results in a marked depletion of nucleotide pool (mostly nucleotide triphosphates). Addition of  $\text{Na}^+$  to  $\text{Na}^+$ -deficient *A. torulosa* cultures quickly enhances both the uptake of phosphate and nucleotide levels (figure 2). Similar effects of  $\text{Na}^+$  on phosphate uptake and ATP synthesis occur in *Pseudomonas stutzeri* (Kodama and Taniguchi, 1976, 1977). In *A. torulosa* the restoration of these processes precedes that of nitrogenase activity. A detailed investigation of influence of  $\text{Na}^+$  on phosphate uptake systems in  $\text{N}_2$ -fixing cyanobacteria is necessary to identify the nature of regulation of  $P_i$  transport by  $\text{Na}^+$ . It also needs to be determined whether regulation of  $P_i$  uptake by  $\text{Na}^+$  is characteristic of diazotrophic growth of cyanobacteria (which depends on  $\text{Na}^+$ ) alone or also occurs during adiazotrophic growth.

### Transport of sugars and amino acids

Certain halophilic (Lanyi *et al.*, 1976) and alkalophilic (Koyama *et al.*, 1976) bacteria couple  $\text{Na}^+$  gradient with symport/antiport of sugars and amino acids. A requirement

of  $\text{Na}^+$  for transport of glucose and glutamate is also observed in other bacteria, e.g., *Escherichia coli* (Frank and Hopkins, 1969; Halpern *et al.*, 1973), *Salmonella typhimurium* (Stock and Roseman, 1971), *Pseudomonas stutzeri* (Kodama and Taniguchi, 1977). In a recent investigation (Apte, 1984) we found that absence of  $\text{Na}^+$  did not drastically affect the uptake of ( $^{14}\text{C}$ ) glutamate and ( $^{14}\text{C}$ ) glucose in  $\text{N}_2$ -fixing cultures of *A. torulosa*. However, low concentrations (1–5 mM) of  $\text{Na}^+$  stimulated uptake of these solutes while higher concentrations inhibited glutamate uptake probably by lowering the membrane potential. Similar effect of  $\text{Na}^+$  on glutamate transport has also been reported in *A. nidulans* (Lee-Kaden and Simonis, 1982).

### **Sodium transport and salt tolerance of cyanobacteria**

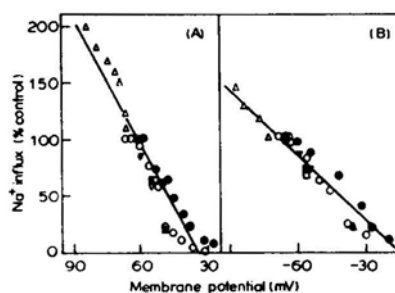
#### *Sodium transport*

Studies on ion transport, in general, have received only scant attention in cyanobacteria. In view of the salt tolerance and the requirement of  $\text{Na}^+$  for growth exhibited by cyanobacteria, studies on the transport of  $\text{Na}^+$  are essential prerequisites for understanding sodium metabolism of these microbes. Such studies are also considered advantageous for understanding the mechanism of salt tolerance of higher plants (Szalay and MacDonald, 1980). In spite of this  $\text{Na}^+$  transport has been examined in detail only in the unicellular diazotroph *A. nidulans* (Dewar and Barber, 1973; Paschinger, 1977; Nitschmann *et al.*, 1982) and in filamentous  $\text{N}_2$ -fixing species of *Anabaena* (Apte and Thomas, 1983b).

The influx of  $\text{Na}^+$  occurs by passive diffusion in *A. nidulans* (Dewar and Barber, 1973; Paschinger, 1977). In two *Anabaena* spp. (Apte and Thomas, 1983b))  $\text{Na}^+$  influx has been shown to be carrier-mediated and follows Michaelis-Menten kinetics. The affinity ( $K_m$ ) of the carrier for  $\text{Na}^+$  is different in the fresh water cyanobacterium *Anabaena* L-31 (2.8 mM) from that of the brackish water species *A. torulosa* (0.3 mM), probably in accordance with their metabolic requirement of  $\text{Na}^+$ . The modulation of  $\text{Na}^+$  influx in *Anabaena* spp. by concanavalin A, known for its ability to bind and reorient certain sugar moieties, suggests that the  $\text{Na}^+$  carrier responsible for influx may be a glycoprotein (Apte and Thomas, 1983b).

$\text{Na}^+$  influx in *Anabaena* spp. has been found to be mediated by a secondary active transport which occurs in response to the proton-motive force generated by the primary active extrusion of protons (S. K. Apte and Joseph Thomas, unpublished results). Of the two components that constitute the proton electrochemical potential gradient, namely a pH gradient and membrane potential,  $\text{Na}^+$  influx is more closely linked with the membrane potential. Nigericin, an ionophore known for its ability to collapse the pH gradient and to convert it into membrane potential at pH 6.0 (Haaker *et al.*, 1980; Hawkesford *et al.*, 1981), causes significant hyperpolarisation of membrane potential in *Anabaena* spp. This is accompanied by concomitant increase in  $\text{Na}^+$  influx, thus clearly establishing the regulation of  $\text{Na}^+$  influx by membrane potential (S. K. Apte and Joseph Thomas, unpublished results). In conformity with this, all treatments which cause depolarisation of membrane such as low temperature, dark incubation, inhibition of respiration by cyanide, azide and anaerobiosis, and ATPase inhibitors [e.g., carbonyl-cyanide m-chlorophenyl-hydrazone (CCCP) and N,N'-dicyclohexyl-

carbodiimide (DCCD)] inhibit  $\text{Na}^+$  influx (figure 3). Similarly, hyperpolarisation caused by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 2,4-dinitrophenol (DNP) or nigericin is accompanied by enhanced  $\text{Na}^+$  influx (S. K. Apte and Joseph Thomas, unpublished results). The transition from an external pH of 6.0 to 8.0 severely inhibits  $\text{Na}^+$  influx in *Anabaena* spp. possibly due to reduced composite proton-motive force at higher pH as has been observed in *A. variabilis* (Reed *et al.*, 1980).



**Figure 3.** Regulation of sodium influx by membrane potential in nitrogen-fixing cyanobacteria: (A) *Anabaena torulosa* (B) *Anabaena* L-31. Data were pooled from experiments using CCCP, (O); DCCD, (●) nigericin, (Δ) low temperature (4°C), (▲); anaerobiosis (argon)(▼); dark incubation, (▼); cyanide, (□); and azide, (■).

$\text{Na}^+$  influx in *Anabaena* spp has been found to be insensitive to low concentrations (1-5 mM) of external  $\text{K}^+$  (S. K. Apte and Joseph Thomas, unpublished results). Higher concentrations of  $\text{K}^+$  (10-100 mM) do inhibit  $\text{Na}^+$  influx but the calculated  $K_i$  values (25-50 mM) are far beyond the known levels of  $\text{K}^+$  in various eco-systems and therefore are probably of no eco-physiological significance. High selectivity for  $\text{K}^+$  and the ability to discriminate against  $\text{Na}^+$  is a general feature of cation transport in bacteria (Harold and Papineau, 1972; Rothstein, 1972), algae (Schaedle and Jacobson, 1967; Shieh and Barber, 1971) and higher plants (Rains, 1972; Flowers *et al.*, 1977; Epstein, 1980). The unicellular cyanobacterium *A. nidulans* also shows selective uptake of  $\text{K}^+$  against  $\text{Na}^+$  (Dewar and Barber, 1973). As against these, *Anabaena* spp. appear to transport  $\text{Na}^+$  and  $\text{K}^+$  independently. Indeed, in *A. variabilis*  $\text{K}^+$  transport occurs by  $\text{K}^+/\text{K}^+$  exchange diffusion which does not catalyse  $\text{Na}^+/\text{K}^+$  exchange (Reed *et al.*, 1981). The relationship between lack of selectivity between  $\text{Na}^+$  and  $\text{K}^+$  and the requirement of  $\text{Na}^+$  in *Anabaena* spp., if any, remains to be established. Increasing external concentrations of both  $\text{Na}^+$  or  $\text{K}^+$  does not significantly affect the membrane potential although some depolarisation occurs above 60 mM (S. K. Apte and Joseph Thomas, unpublished results). It is unlikely, therefore, that membrane potential may be constituted of a significant diffusion potential of  $\text{Na}^+$  or  $\text{K}^+$  ions in cyanobacteria.

Both *A. nidulans* (Paschinger, 1977) and *Anabaena* spp. (Apte and Thomas, 1983b) maintain a low internal  $\text{Na}^+$  concentration brought about by an active extrusion of the cation. The plasmalemma of *A. nidulans* is equipped with a DCCD-sensitive,  $\text{H}^+$ -translocating ATPase (Paschinger, 1977) and an  $aa_3$  type of  $\text{H}^+$ -extruding terminal cytochrome oxidase (Nitschmann and Peschek, 1982; Nitschmann *et al.*, 1982). The resulting proton gradient is coupled with a  $\text{Na}^+/\text{H}^+$  antiporter which brings about an

active extrusion of  $\text{Na}^+$ . When subjected to anaerobiosis or incubated with DCCD, therefore, *A. nidulans* shows net accumulation of  $\text{Na}^+$  (Paschinger, 1977; Nitschmann and Peschek, 1982). In *A. variabilis* proton efflux appears to be mediated by ATPase rather than by respiratory electron transport (Scherer *et al.*, 1984). We have obtained evidence recently that two *Anabaena* spp. generate proton gradients by means of extrusion of  $\text{H}^+$  carried out by both DCCD-sensitive ATPase(s) and respiratory oxidase(s) located in the plasmalemma. These cyanobacteria also exhibit rapid, active efflux of  $\text{Na}^+$  but this is not mediated by  $\text{Na}^+/\text{H}^+$  antiporter since DCCD treatment or anaerobiosis does not cause net  $\text{Na}^+$  accumulation (Apte, 1984). The identical sensitivity of both influx/efflux to *trans* concentrations of  $\text{Na}^+$ , to low temperatures ( $4^\circ\text{C}$ ) and to combined nitrogen suggests that the same carrier may facilitate both inward and outward  $\text{Na}^+$  fluxes.

Recently we have also identified an interesting regulation of  $\text{Na}^+$  transport by combined nitrogen in two *Anabaena* species. (S. K. Apte and Joseph Thomas, unpublished results). When grown in the presence of nitrate or  $\text{NH}_4^+$ , *Anabaena* L-31 and *A. torulosa* showed depolarisation of membrane potential and inhibition of  $\text{Na}^+$  influx compared to cultures grown in the absence of combined nitrogen. This was due to a direct effect of combined nitrogen on membrane potential and  $\text{Na}^+$  influx since addition of nitrate or  $\text{NH}_4^+$  to  $\text{N}_2$ -fixing cultures, only 2 min prior to assay, also produced identical effects. Addition of nitrate or  $\text{NH}_4^+$  *per se* also caused extrusion of  $\text{Na}^+$  preabsorbed by  $\text{N}_2$ -fixing cultures of *Anabaena* spp. Together, the effect of combined nitrogen on influx as well as efflux processes allowed very little  $\text{Na}^+$  to accumulate in the cells. Even at 100 mM external NaCl concentration, nitrate and  $\text{NH}_4^+$  decreased the intracellular  $\text{Na}^+$  levels by 68 % and 50 % in *Anabaena* L-31 and by 45 and 40 % in *A. torulosa*. Thus, *Anabaena* spp. seem to have evolved an attractive mechanism mediated by combined nitrogen to curtail the entry and accumulation of  $\text{Na}^+$ , an ion not required in large quantity under nitrogen-supplemented conditions. The precise mechanism of this regulation awaits analysis.

#### *Cellular distribution of absorbed sodium and its incorporation into biomolecules*

Using the radiotracer  $^{22}\text{Na}^+$ , over 90% of the total cell-bound  $\text{Na}^+$  of  $\text{N}_2$ -fixing cultures of *A. torulosa* was found to exist extracellularly in a freely exchangeable state; most of it was held by the extracellular mucopolysaccharide sheath of the cyanobacterium (S. K. Apte and Joseph Thomas, unpublished results). Nearly all of the intracellular  $\text{Na}^+$  (> 96 %) occurred as free  $\text{Na}^+$  in an osmotically active state. There was no evidence for binding or incorporation of  $\text{Na}^+$  into any of the biochemical fractions examined, especially proteins or carbohydrates, in *A. torulosa* grown on  $\text{N}_2$ , nitrate or  $\text{NH}_4^+$ . In *Anabaena*  $\text{Na}^+$  is, therefore, not a constituent of a storage product like the  $\text{Na}^+$ -mannoglycerate in certain red algae (Craigie, 1974). Also, no  $\text{Na}^+$ -binding or -storing protein, similar to molybdenum storage protein of certain diazotrophs (Pienkos and Brill, 1981), could be autoradiographically detected in *A. torulosa* both under repressed as well as induced conditions of nitrogenase synthesis. Thus, unlike molybdenum,  $\text{Na}^+$  appears to regulate nitrogenase activity and cyanobacterial metabolism as a cation, *per se*.



*Physiological basis of salt tolerance*

Many cyanobacteria exhibit considerable tolerance to salt (NaCl) and occurrence of marine, salt lake-inhabiting or brackish water forms is by no means rare (Desikachary, 1959; Fogg, 1973). There are also well documented cases of halophilic cyanobacteria, such as *Microcoleus chthonoplastes* (20-25% NaCl; Van Baalen, 1962), *Spirulina subsalsa* (> 3 M NaCl; Fogg, 1973), *Aphanothece halophytica* (> 3 M NaCl; Yopp *et al.*, 1978) and *Calothrix scopulorum* (5% NaCl; Stewart, 1964; Tel-Or, 1980a) and euryhaline cyanobacteria, capable of growth in freshwater and in varying degrees of salinity (Richardson *et al.*, 1983; Reed and Stewart, 1983). The physiological basis of their salt tolerance has been studied to some extent but remains not fully understood. Following types of mechanisms have been reported:

(i) Accumulation of  $K^+$  occurs in response to increasing external salt concentration and helps in osmoregulation in *Aphanothece halophytica* (Miller *et al.*, 1976; Yopp *et al.*, 1978). Although this organism also accumulates carbohydrates and amino acids as osmotica these are considered secondary events triggered by high intracellular  $K^+$  concentration.

(ii) Exclusion of  $Na^+$  and maintenance of low intracellular  $Na^+$  concentrations appear to be responsible for the salt tolerance of  $N_2$ -fixing cultures of *A. torulosa*, a brackish water species (Apte and Thomas, 1983b). As compared to the fresh water species *Anabaena* L-31, *A. torulosa* shows much reduced influx of  $Na^+$ , much higher affinity of carrier for  $Na^+$  which prevents excess influx at high  $Na^+$  concentrations, and more efficient efflux of  $Na^+$  resulting in lower internal  $Na^+$  concentrations. As mentioned earlier, presence of nitrate or  $NH_4^+$  severely curtailed influx and stimulated efflux of  $Na^+$  in both these *Anabaena* spp. As a consequence, salt tolerance of the fresh water and brackish water species was comparable and was 3-5 fold greater than that of corresponding  $N_2$ -fixing cultures (table 3).

**Table 3.** Comparative halotolerance of a brackish water and a freshwater species of *Anabaena* grown on different nitrogen sources.

| Cyanobacterium           | Nitrogen source during growth | Growth ( $\mu g$ chlorophyll <i>a</i> ) on NaCl (mM) |               |              |              |              |              |
|--------------------------|-------------------------------|--|---------------|--------------|--------------|--------------|--------------|
|                          |                               | 1  | 35            | 85           | 125          | 170          | 250          |
| <i>Anabaena torulosa</i> | $N_2$                         | 7.5<br>(7.3)   | 12.6<br>(9.1) | 6.2<br>(9.2) | 4.4<br>(8.1) | 2.3<br>(5.4) | 2.1<br>(3.6) |
|                          | $(NO_3)^{2-}$ (10 mM)         | 5.9  | 6.6           | 6.8          | 6.3          | 6.0          | 5.3          |
|                          | $NH_4^+$ (3 mM)               | 9.4  | 12.1          | 14.1         | 12.6         | 8.5          | 5.9          |
| <i>Anabaena</i> L-31     | $N_2$                         | 7.9<br>(4.6)   | 3.6<br>(1.0)  | 2.1<br>(0.3) | 0.6<br>(0.1) | 0.3<br>(0.0) | 0.2<br>(0.0) |
|                          | $(NO_3)^{2-}$ (10 mM)         | 6.1  | 5.9           | 5.5          | 5.5          | 5.2          | 4.8          |
|                          | $NH_4^+$ (3 mM)               | 10.3   | 13.2          | 9.3          | 8.0          | 7.8          | 7.5          |

*Anabaena torulosa* (a brackish water species) and *Anabaena* L-31 (a freshwater species) were grown on solid media (1 % agar in CM/5) supplemented with or without combined nitrogen under continuous illumination (5000 lx). Representative data obtained (six replicates each) on fifth day after inoculation are presented. Chlorophyll *a* content was determined after Mackinney (1941). Values in parantheses show the nitrogenase activities in  $\mu mol C_2H_2$  reduced.  $mg$  chlorophyll  $a^{-1}.h^{-1}$ .

(iii) Synthesis of internal osmotica in the form of carbohydrates, polyols, amino acids and quaternary amines facilitates osmoregulation in cyanobacteria. Two to four fold enhanced synthesis of carbohydrates, polyols and amino acids has been reported in *A. halophytica* (Yopp *et al.*, 1978). The main low molecular weight carbohydrate in *Rivularia atra* incubated in 100 % sea water is the disaccharide trehalose (Reed and Stewart, 1983). It has been suggested that marine forms synthesise a unique compound glucosylglycerol (Mackay *et al.*, 1983) for osmoregulation while sucrose is characteristic of fresh water forms (Blumwald *et al.*, 1983). Richardson *et al.* (1983) however showed that *Synechocystis* PCC 6803 isolated from fresh waters also synthesised glucosylglycerol. In a recent study Reed *et al.* (1984) examined 70 cyanobacterial strains for the presence of these compounds and showed that although there is a trend for accumulation of glucosylglycerol in marine species and sucrose in fresh water species, no absolute link exists between a genus or an ecological type on the one hand and the chemical nature of carbohydrate accumulated on the other.

In *Anabaena* spp. we have observed an increased nitrogen demand during salt stress. Under  $N_2$ -fixing condition this is exemplified by the salt-tolerant *A. torulosa* which responds to salinity (upto 85 mM NaCl) by enhanced nitrogenase activity while the salt-sensitive *Anabaena* L-31 fails to respond in this manner. The enhanced salt tolerance of these species in the presence of combined nitrogen (table 3) is also in agreement with this. Thus, nitrogen supplementation appears to subsidise the increased nitrogen demand and hence result in increased tolerance to salt. Such increased demand is probably aimed at synthesis of nitrogenous osmoregulatory compounds like amino acids (Measures, 1975) and quaternary ammonium compounds (Shkedy-Vinkler and Avi-Dor, 1975; Galinsky and Truper, 1982). Indeed, the role of glycinebetaine and other quaternary ammonium compounds in osmoregulation has been demonstrated in the halophilic *Synechocystis* DUN52 (Mohammad *et al.*, 1983).

(iv) Cyanobacterial metabolism adapts to salt stress and certain metabolic features likely to be important in salt tolerance have been identified. Although, high NaCl concentrations inhibit photosynthesis and decrease the level of pigments, fatty acids and hydrocarbons in cyanobacteria (Batterton and Van Baalen, 1971), in a comparative study of four cyanobacteria Tel-Or (1980a,b) found that photosynthesis (photoevolution of  $O_2$ ) was more resistant to salt stress than  $N_2$  fixation. The sensitivity of nitrogenase activity was found to be consequent to the very high sensitivity of ferredoxin-NADP<sup>+</sup>-oxidoreductase to salt. The activity of this enzyme, which has been shown to mediate electron flow to cyanobacterial nitrogenase (Apte *et al.*, 1978; Lockau *et al.*, 1978) had a positive correlation with the degree of salt tolerance of different cyanobacteria. The activities of glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and photosystem I-mediated electron flow, all of which support nitrogenase activity, showed less sensitivity to salt while glutamine synthetase was almost insensitive (Tel-Or, 1980a,b). Among *Anabaena* spp., the salt-tolerant *A. torulosa* shows greater resistance (50% inhibition at 180 mM NaCl) of nitrogenase activity to salt than the salt-sensitive (50 % inhibition at 25 mM NaCl) *Anabaena* L-31 (Apte and Thomas, 1983b). In *A. torulosa* the NaCl concentration responsible for 50 % inhibition of growth (115 mM) and  $N_2$  fixation (> 180 mM) differ widely. Thus the growth inhibition in cyanobacteria under salt stress does not appear to be a direct consequence of inhibition of  $N_2$  fixation (S. K. Apte and Joseph Thomas, unpublished results).

*Reclamation of saline/alkali/sodic soils by cyanobacteria*

Salt tolerance of cyanobacteria has been exploited in the reclamation of agricultural habitats rendered useless due to excess salt. Singh (1950, 1961) reported that alkali soils of Northern India, variously called reh, kallar or usar, could be reclaimed through naturally occurring population of cyanobacteria, for subsequent cultivation of rice and sugarcane. After substantial growth had occurred during rainy season cyanobacterial mats were ploughed in and left buried in the soil. The annual addition of organic carbon was 35–60 % and that of nitrogen 30–40 % over the initial levels. After the first year the pH of soil fell from 9.5 to 7.6 and there was improvement in tilth, exchangeable calcium (20–33 %) and water holding capacity (40 %). It has been claimed that cyanobacterial incorporation into soil in this manner conserves organic carbon and nitrogen and immobilises  $\text{Na}^+$ , converting the sodium clay into a calcium type (Singh, 1950, 1961). However, as mentioned earlier, in aerated laboratory cultures, almost all of the absorbed  $\text{Na}^+$  remains osmotically active (S. K. Apte and Joseph Thomas, unpublished results). It is therefore likely that  $\text{Na}^+$  is released back into the soil subsequent to the death and decay of cyanobacteria.

In an alternative approach (Thomas, 1978; S. K. Apte and Joseph Thomas, unpublished results), saline 'Kharland' soils from coastal areas of Maharashtra were assessed for beneficial effects on inoculation with the salt tolerant cyanobacterium *A. torulosa*. These soils have much higher salinity levels (electrical conductivity:  $>15 \text{ mmhos.cm}^{-1}$ , or total soluble salts upto 10%) and are rich in sodium (upto 7% NaCl) (Joshi and Kadrekar, 1980). In laboratory experiments *A. torulosa* grew at moderate salinity levels (electrical conductivity:  $8.5 \text{ mmhos.cm}^{-1}$ ) which are likely to occur in inland soils adjoining coastal areas. After five weeks of growth there was considerable enrichment in the nitrogen status and subsequent removal of cyanobacterial mats from soils brought about 12–35 % reduction in soil salinity (S. K. Apte and Joseph Thomas, unpublished results). Such treatment however removed most of the newly fixed nitrogen and carbon and the net gain in nitrogen and carbon content was only marginal ( $\approx 10\%$ ). Thus, if irreversible removal of salt has to be achieved the additional attraction of simultaneous enrichment of nitrogen and carbon status may have to be sacrificed. Moreover, although such a reclamation process appears to be comparable in its efficiency to some of the traditional methods, the ultimate removal of cyanobacterial mats physically appears impractical on a large scale.

The reported success of the approach used by Singh (1950, 1961) and recently by Kaushik and Venkataraman (1982) most probably lies in the apparent ability of cyanobacterial extracellular mucopolysaccharides to 'chelate' considerable amounts of various ions, especially  $\text{Na}^+$  (S. K. Apte and Joseph Thomas, unpublished results). Although such approach has no permanent solution to soil salinity it carries the dual benefits of temporarily immobilising the excess  $\text{Na}^+$  and of partly supplementing the nitrogen requirement of crops. A composite treatment involving both gypsum (to precipitate out  $\text{Na}^+$  salts which can be subsequently leached out by flooding) and cyanobacteria (to entrap and immobilise other salts) has recently been shown to be more effective in reclamation of sodic soils (Kaushik and Venkataraman, 1982). The remarkable ability of cyanobacteria, including fresh water forms, to withstand salt stress in the presence of combined nitrogen (S. K. Apte and Joseph Thomas, unpublished results) also promises to have good prospects in improvement of saline

soils. Possible reclamation of saline/alkali soils employing salt tolerant cyanobacteria along with nitrogenous fertilisers and also in combination with gypsum remains attractive and needs to be critically investigated.

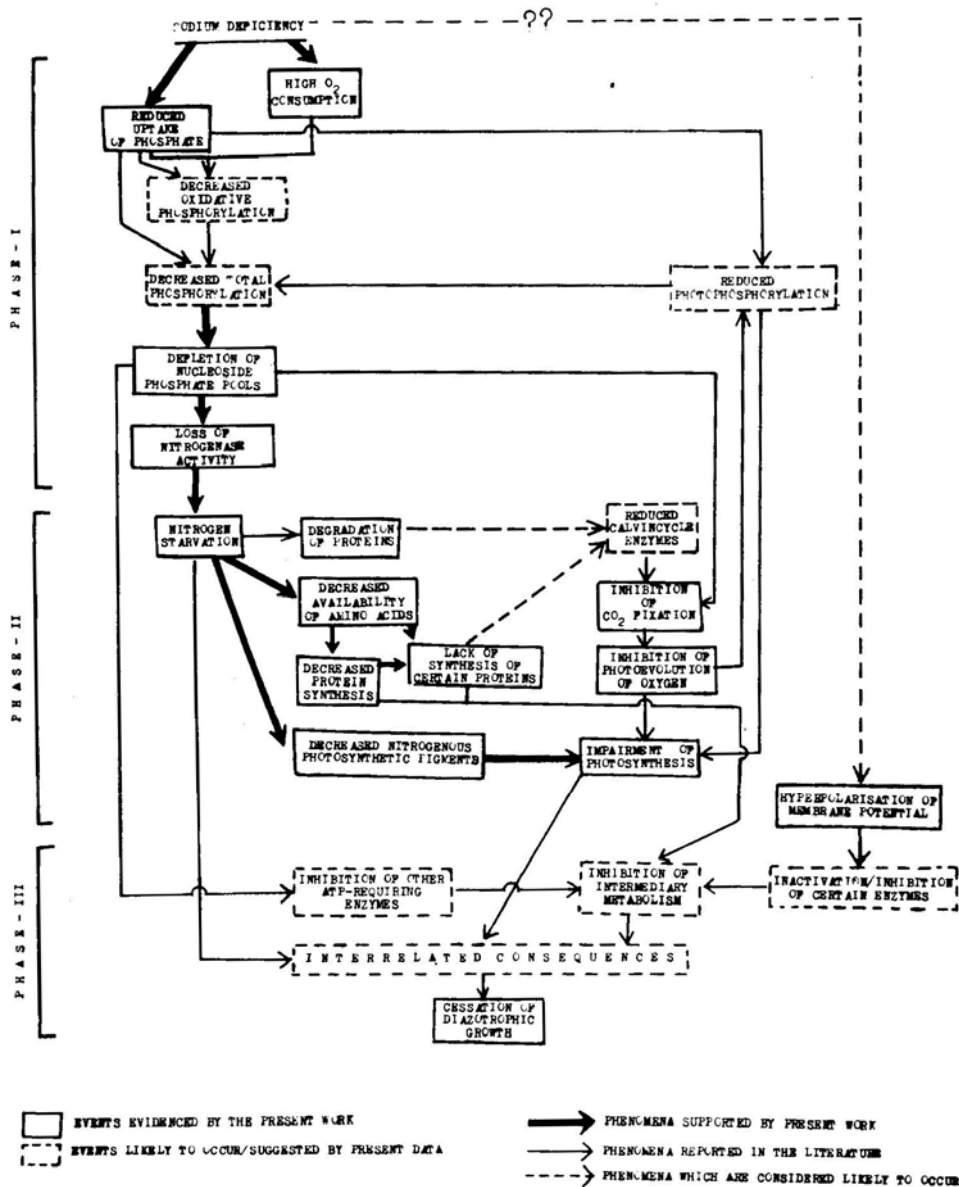


Figure 4. Probable sequence of events that result in retardation of growth of nitrogen-fixing *Anabaena torulosa* under sodium deficiency.

## Conclusions

There is a specific metabolic, requirement of sodium ( $< 100 \mu\text{M}$ ) in cyanobacteria especially under  $\text{N}_2$ -fixing conditions. Although,  $\text{Na}^+$  does not influence several structural and functional features associated with diazotrophic growth like heterocyst differentiation, synthesis of nitrogenase proteins, transport of molybdenum and protection of nitrogenase from oxygen, vital functions like nitrogenase activity, photosynthesis, quality and quantity of proteins, membrane potential and energy status of  $\text{N}_2$ -fixing cells are affected by sodium deficiency. A primary effect of  $\text{Na}^+$  deficiency is inhibition of uptake and utilisation of phosphate leading to depletion of nucleotide phosphate pools. This results in inhibition of  $\text{N}_2$  fixation apparently due to limitation of ATP supply. The sequence of events that possibly results in inhibition of growth of  $\text{N}_2$ -fixing cyanobacteria under sodium deprivation is portrayed in the accompanying model (figure 4). A definite requirement for sodium also exists during nitrate-supported growth but its nature is not yet clear.

Extra cellular mucopolysaccharides chelate significant amounts of sodium. Intracellular sodium exists as a free cation and is not incorporated into any biomolecule, especially proteins. It does not occur in storage products.  $\text{Na}^+$  influx in  $\text{N}_2$ -fixing *Anabaena* spp. is carrier-mediated and is regulated by the proton-motive force, particularly the membrane potential of cells. Low intracellular concentrations are maintained by active efflux. While the nature of this efflux is uncertain in  $\text{N}_2$ -fixing cyanobacteria, in *A. nidulans* it is mediated by  $\text{Na}^+/\text{H}^+$  antiporter and decreases the efficiency of oxidative phosphorylation.

Accumulation of  $\text{K}^+$ , exclusion of  $\text{Na}^+$  and maintenance of low intracellular  $\text{Na}^+$  levels, synthesis of carbohydrates, polyols, amino acids and quaternary amines for osmoregulation and other adaptations of metabolism are principal features associated with and contributing to the salt tolerance in cyanobacteria. The presence of combined nitrogen, which effectively curtails sodium accumulation and also supports the extra nitrogen demand for osmoregulation during salt stress, confers considerable salt tolerance on cyanobacteria. Exploiting the potential of cyanobacteria for reclamation of saline sodic soils needs more serious efforts than those made in the past.

Virtually no information exists on the genetics of cyanobacterial halotolerance. Genetic engineering of these photoautotrophic diazotrophs for enhanced halotolerance and subsequent agricultural exploitation is an attractive area of future research.

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