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Sodium transport in filamentous nitrogen fixing cyanobacteria

SHREE KUMAR APTE and JOSEPH THOMAS

Biology and Agriculture Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085

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Abstract. Two filamentous, nitrogen fixing cyanobacteria were examined for their salt tolerance and sodium (Na^+) transport. *Anabaena torulosa*, a saline form, grew efficiently and fixed nitrogen even at 150 mM salt (NaCl) concentration while, *Anabaena* L-31, a fresh water cyanobacterium, failed to grow beyond 35 mM NaCl. *Anabaena torulosa* showed a rapidly saturating kinetics of Na^+ transport with a high affinity for Na^+ (K_m, 0.3 mM). *Anabaena* L-31 had a much lower affinity for Na^+ (K_m, 2.8 mM) than *Anabaena torulosa* and the pattern of uptake was somewhat different. Both *Anabaena* spp. exhibited an active Na^+ extrusion which seems to be mediated by a Na^+–K^+ ATPase and aided by oxidative phosphorylation. *Anabaena* L-31 was found to retain much more intracellular Na^+ than *Anabaena torulosa.* The results suggest that the saline form tolerates high Na^+ concentrations by curtailing its influx and also by an efficient Na^+ extrusion, although these alone may not entirely account for its success in saline environment.

Keywords. Nitrogen fixation; cyanobacteria; sodium transport; salt tolerance.

Introduction

Sodium is the predominant soluble cation in most saline soils and waters. Although cyanobacteria are known to require Na^+ for growth (Allen and Arnon, 1955; Kratz and Myers, 1955) and nitrogen-fixation (Apte and Thomas, 1980) the physiological processes involved in their salt-tolerance are not known. Ion transport in cyanobacteria is a generally neglected phenomenon, and Na^+ transport in particular has been studied only in the unicellular cyanobacterium *Anacystis nidulans.* In this organism, Na^+ transport has been shown to be regulated by an active extrusion of the cation by a proton antiport system (Dewar and Barber, 1973; Paschinger, 1977), a mechanism characteristic of certain bacteria (Rothstein, 1972) and sensitive to energy transfer inhibitors (Paschinger, 1977). In an earlier report (Apte and Thomas, 1974) we have shown the ability of a fresh water cyanobacterium *Anabaena* L-31 and a saline form *Anabaena torulosa* to absorb Na^+. In this paper, we describe the details of the kinetics of Na^+ transport in these cyanobacteria as revealed by the use of radiotracer $^{22}Na^+$. The results suggest that the Na^+ transport properties of cyanobacteria from saline habitats are better suited for their survival in such environments.

Abbreviations used: DCMU, 3-(3, 4-Dichlorophenyl) -1, 1-dimethylurea; CCP, carbonyl cyanide, *m*-chlorophenyl hydrazone; DNP, 2, 4-dinitrophenol; DCCD, N, N' -dicyclohexylcarbodiimide; BBOT, 2, 5-(5-ditetrabutyl-2-benzoxazolyl)-thiophene; ATPase, Adenosine triphosphatase.

Materials and methods

Microorganisms

Two filamentous, N_2-fixing cyanobacteria *Anabaena torulosa* (Apte and Thomas, 1980) and *Anabaena* L-31 (Thomas, 1970), were used in axenic condition. *Anabaena* L-31 was isolated from a paddy field and is a fresh water inhabitant. *A. torulosa* was isolated from a brackish soil and is known to be characteristic of saline waters (Desikachary, 1959).

Culture conditions and growth

Cyanophycean medium (David and Thomas, 1979), diluted five-fold and free of combined nitrogen, was used for the maintenance of all the cultures. Cyanobacteria were grown under constant aeration (2 litre/min) at 5000 lux and 25° C. Salt tolerance of cyanobacteria was examined on agar slants (1% agar, w/v) containing various concentrations of NaCl. Chlorophyll *a* determined as described by Mackinney (1941) was used as index of growth. In long term experiments (6 days) as the cell density increased, the distribution of light became uneven, and chlorophyll *a* concentration could not serve as a satisfactory index of growth. In such experiments, increase in total N, determined by micro-kjeldahl analysis (Steyermark, 1961) was preferred. Nitrogenase activity assays were performed as described previously (David *et al.*, 1980).

Uptake of sodium

Sodium uptake was determined by using the radiotracer ^{22}Na. Logarithmic phase cultures were harvested, washed and inoculated in N-free cyanophycean medium without Na^+. After incubation for 24 h, to induce Na^+ deficiency, filaments were filtered, washed and resuspended in cyanophycean medium (without Na^+) and used in experiments. Test suspensions, unless stated otherwise, contained $^{22}NaCl$ (0.25 µCi/ml) mixed with non-radioactive NaCl to give a final concentration of 0.5 mM or 10.0 mM Na^+. Assays involving incubation for more than 5 min were performed under aeration (2 litre/min) at 5000 lux and 25°C. Short term incubations were carried out in 5 ml vials held on a rotary wheel (11 r.p.m.), light intensity and temperature remaining the same as above. At the end of a desired period, 2 ml suspension was filtered on a Whatman GF/C paper circle (Whatman Ltd., UK) and the residue was washed with 100 ml of equimolar unlabelled NaCl to completely remove the diffusible and easily exchangeable fraction of Na^+ (nearly 90% of the radioactivity initially bound to cells) followed by washing with distilled water. Filter paper discs with samples were dried, transferred to scintillation vials containing 10.0 ml 2,5-(ditetrabutyl-2-benzoxazyolyl)-thiophene (BBOT) (0.4% w/v in toluene: methanol, 1:1) and counted in a Beckman LS–100 C liquid scintillation spectrometer (Beckman Instruments Inc., California, USA). The kinetic data were evaluated by standard linear regression analysis for calculating kinetic constants.

Chemicals

All inorganic salts were obtained from Sarabhai M. Chemicals, Baroda, or British Drug Houses, Bombay. $^{22}NaCl$ was acquired from Radiochemical Centre,

Amersham, UK and bacto-agar from Difco Laboratories, Michigan, USA. 3-(3,4- Dichlorophenyl)-1, 1-dimethylurea (DCMU), 2,4-dinitrophenol (DNP), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), N,N'-dicyclohexyl carbodiimide (DCCD), Ouabain and BBOT were supplied by Sigma Chemicals Co., St. Louis, Missouri, USA. Concanavalin A was obtained from Calbiochem, San Diego, California, USA. Acetylene was supplied by Indian Oxygen Ltd., Bombay, and pure ethylene from Matheson Gas Products, New Jersey, USA.

Results

Salt-tolerance

Figure 1 shows the growth and nitrogenase activity of cyanobacteria in the presence of different concentrations of NaCl. *Anabaena torulosa was* fairly salt-tolerant growing even at 150 mM NaCl. The salt concentration responsible for 50% inhibition was found to be 115 mM for growth and 150 mM for nitrogenase activity. Significantly at 2–35 mM NaCl concentrations, *A. torulosa* showed better growth and

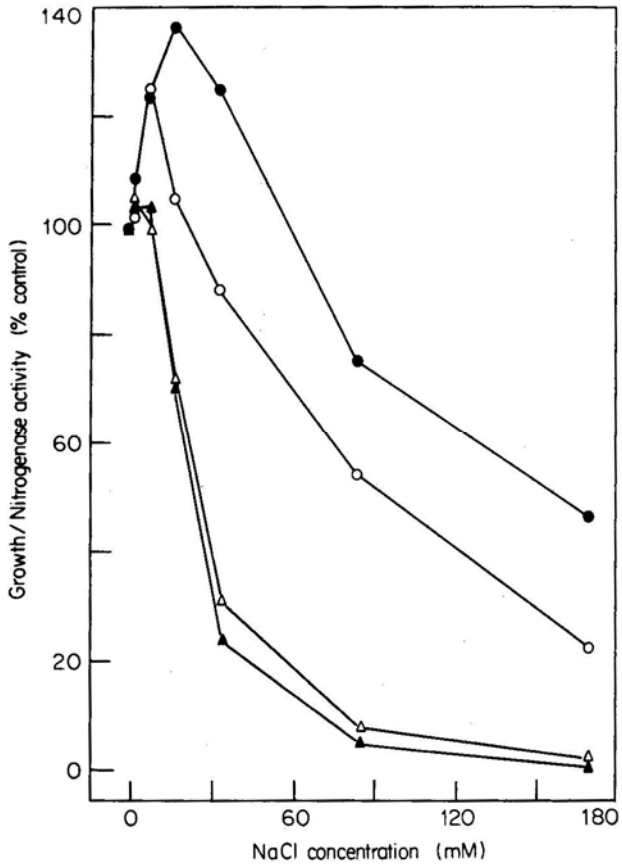

Figure 1. Salt tolerance of nitrogen fixing cyanobacteria: *Anabaena torulosa* (O, ●); *Anabaena* L-31 (Δ, Δ). Growth (chlorophyll *a*) (open symbols) and nitrogenase activity (C_2 H_2 reduction) (closed symbols) were measured 5 days after inoculation. See text for details.

higher nitrogenase activity than in control experiments without salt. In contrast, similar salt concentrations did not stimulate growth or nitrogenase activity in *Anabaena* L-31 and the cyanobacterium was found to be very sensitive to salt, not growing beyond 35 mM NaCl. Salt concentration of 25 mM caused 50% inhibition of growth and nitrogenase activity in this cyanobacterium.

Kinetics of sodium transport

There was no lag in the uptake of Na^+ which stabilised within a short time in both the cyanobacteria. But the rates and pattern of uptake differed (figures 2, 3). In *A. torulosa* the initial exponential phase of uptake lasted only for 1 min and stabilised quickly at both 0.5 mM and 10.0 mM Na^+ concentrations. At lower concentration of Na^+ (0.5 mM) the cyanobacterium showed higher rates of uptake than

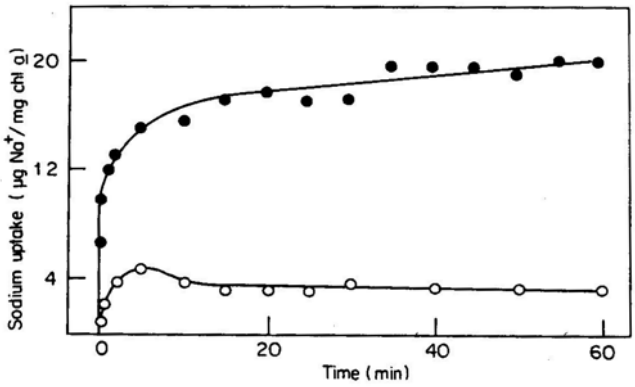

Figure 2. Kinetics of Na^+ uptake by *Anabaena torulosa* at 0.5 mM (O) and 10.0 mM (●) external Na^+ concentrations.

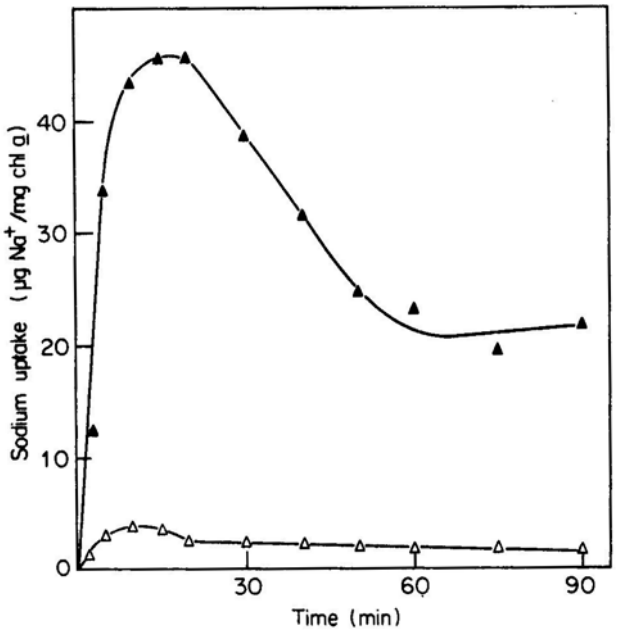

Figure 3. Kinetics of Na^+ uptake by *Anabaena* L-31 at 0.5 mM (Δ) and 10.0 mM (Δ) external Na^+ concentrations.

Anabaena L-31. At higher Na^+ concentration (10 mM) the initial uptake was much more rapid in *Anabaena* L-31 than in *A. torulosa* but was followed by an extrusion of Na^+ till 60 min when it stabilised.

Long term experiments showed that in both cyanobacteria maximum Na^+ accumulation was attained within first few hours and then a lower level was maintained for a prolonged period of 6 days (figure 4). *Anabaena* L-31 retained 2.7 times more Na^+ than *A. torulosa* at the end of six days, the values being 9.6 µg Na^+/mg N and

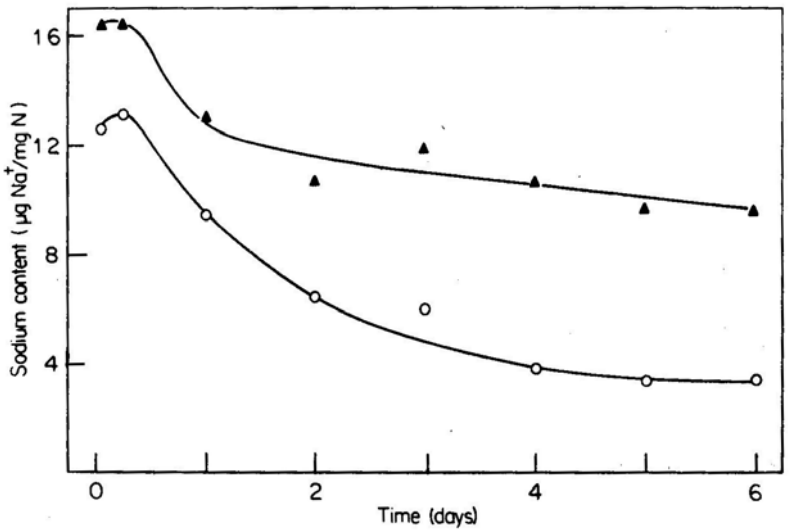

Figure 4. Retention of sodium by *Anabaena torulosa* (O) and *Anabaena* L-31 (Δ) during growth under N_2 fixing conditions. Increase in total N was chosen as the measure of growth in this experiment for reasons specified in the Materials and methods.

3.5 µg Na^+/mg N respectively. Similar trend was observed in the intracellular concentrations of Na^+ in these cyanobacteria. After 18 h of equilibration in the medium containing $^{22}Na^+$ the internal concentrations of Na^+ were estimated on the basis of radioactivity per ml packed cell volume. Values of 97 µM and 0.36 mM for *A. torulosa* and 60 µM and 0.51 mM for *Anabaena* L-31were observed at 1and 10 mM Na^+ in the external medium.

In the presence of increasing external concentrations of Na^+, uptake of Na^+ in both cyanobacteria followed Michaelis-Menten kinetics saturating between 10–20 mM (figures 5, 6). Michaelis constants (K_m) calculated from the double reciprocal plots (figures 5, 6 insects) were 0.3 mM for *A. torulosa* and 2.8 mM for *Anabaena* L-31. These values are in conformity with the observed differences in the rates of Na^+ uptake by these cyanobacteria at different Na^+ concentrations.

Effect of metabolic inhibitors on Na^+ uptake

Light was found to facilitate Na^+ uptake in both cyanobacteria and preincubation in dark resulted in reduced Na^+ uptake (table 1). Addition of inhibitors of photosynthetic electron transport (DCMU) or photophosphorylation (CCCP) was not very effective at concentrations (1 µM) which inhibited photosynthesis (data not shown) but Na^+ uptake was inhibited at higher concentrations (10 µM) (table 1). DNP, an inhibitor of oxidative phosphorylation, enhanced Na^+ uptake in both

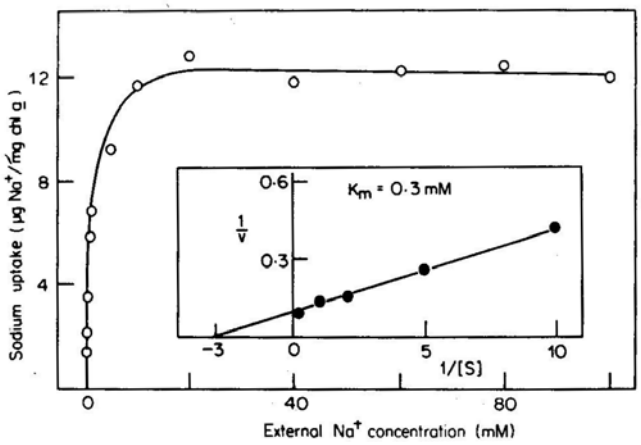

Figure 5. Effect of external Na^+ concentration on Na^+ uptake by *Anabaena torulosa.* Duration of assay was 15 s. No change in cell, size or packed cell volume was noticed at any concentration of Na^+. Inset shows a double reciprocal plot of the data for calculation of Michaelis constant (K_m).

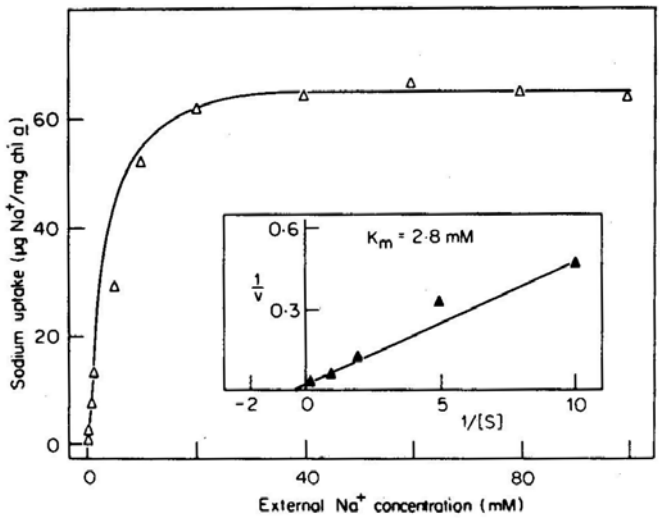

Figure 6. Effect of external Na^+ concentration on Na^+ uptake by *Anabaena* L-31. Duration of assay was 2 min. No change in cell size or packed cell volume was noticed at any concentration of Na^+. Insert shows a double reciprocal plot of the data for calculation of Michaelis constant (K_m).

cyanobacteria. Significant enhancement of Na^+ uptake was noted in the presence of ouabain, a specific inhibitor of Na^+ –K^+ ATPase, while DCCD, an inhibitor of classical ATPase, was not effective or marginally inhibited the uptake (table 1) at concentrations which were adequate to inhibit ATPase (Paschinger, 1977). Concanavalin A showed a differential effect on Na^+ uptake in these cyanobacteria. While Na^+ uptake was enhanced in *A. torulosa* by treatment with concanavalin A, in *Anabaena* L-31 it was inhibited. Gramicidin stimulated Na^+ uptake both in light as well as in dark (table 1).

Table 1. Effect of various inhibitors* on Na^+ uptake by N^2-fixing cyanobacteria.

Inhibitor (concentration μM)	Radioactivity/ml (% control)	
	Anabaena torulosa	*Anabaena* L-31
Control	100	100
Dark	74	79
DCMU		
(10)	140	125
(10)	61	78
CCCP		
(1)	100	99
(10)	98	65
(100)	70	45
DNP		
(10)	162	131
(100)	297	162
DCCD		
(50)	100	92
(200)	90	79
Ouabain		
(10)	135	109
(100)	192	159
Concanavalin A		
(50 μg/ml)	132	100
(200 μg/ml)	186	65
(400 μg/ml)	120	52
Gramicidin (light)		
(5 μg/ml)	130	139
(20 μg/ml)	172	225
Gramicidin (dark)		
(20 μg/ml)	122	143

* Prior to assay cyanobacterial suspensions were pretreated with the inhibitor for 15 min. Dark treatment (No. 2 and 10) consisted of preincubation of aerated cultures in dark for 5 h followed by assays also performed in the dark. The assays were initiated at time zero by addition of 0.25 μCi of $^{22}Na^+$ and stable Na^+ at a final concentration of 10.0 mM in 1.0 ml final volume. Duration of assay was 15 s for *A. torulosa* and 2 min for *Anabaena* L-31, when Na^+ uptake was in the linear phase.

Discussion

Na^+, an important nutritional requirement in cyanobacteria (Allen and Arnon, 1955; Kratz and Myers, 1955) has recently been shown to be essential for nitrogenase activity in *Anabaena* L-31 and *A. torulosa* (Apte and Thomas, 1980). In the present study these cyanobacteria have been found to differ considerably in their ability to resist salt-stress (figure 1). *A. torulosa* exhibits good growth and nitrogenase activity at salt concentrations well beyond the normal tolerance limit of many microbes and crop plants. On the other hand *Anabaena* L-31, a fresh water form, fails to grow beyond 35 mM NaCl. The difference in salt-tolerance seems to be related to the Na^+ transport pattern of these cyanobacteria. In *A. torulosa* Na^+ uptake

saturates quickly (figure 2), follows a Michaelis-Menten kinetics and shows a high affinity for Na^+ (figure 5). *Anabaena* L-31 also shows a Michaelis-Menten type of rapid uptake (figure 3) but it has a much lower affinity for Na^+ (figure 6) than *A. torulosa*. The difference in K_m is probably in accordance with the metabolic requirement of Na^+ in a brackish and a fresh water form. Concentrations of Na^+ required for optimum growth are 0.5 mM for *Anabaena* L-31 (Apte and Thomas, unpublished results) and nearly 10 mM for *A. torulosa* (figure 1).

In *A. torulosa* and *Anabaena* L-31, Na^+ influx appears to be affected by light mediated metabolism since dark incubation inhibits Na^+ uptake. However this effect is not consequent to the inhibition of photosynthesis since concentrations of DCMU and CCCP required to inhibit Na^+ uptake are several times higher than those required to inhibit photosynthesis (table 1). Gramicidin, known for its stimulatory effect on membrane permeability, stimulates the effect of light and increases Na^+ uptake in dark. Although the absolute quantity of Na^+ taken up is more in light, the per cent enhancement of Na^+ uptake caused by gramicidin in light and dark is comparable. This suggests that light may be involved in maintaining membrane permeability suitable for the transport of this cation and the greater effect of gramicidin in light may therefore be due to additive increase in permeability. In *A. nidulans* also light was found to non-specifically increase the permeability of plasmamembrane (Paschinger, 1977).

Na^+ influx in *A. torulosa* and *Anabaena* L-31 is probably a passive carrier mediated diffusion process while the regulation of Na^+ transport is achieved by an active extrusion of Na^+. The Na^+ extrusion appears to be mediated by an ouabain sensitive $Na^+ – K^+$ ATPase which is distinct from the conventional F_1 F_0 ATPase in being insensitive to DCCD (Paschinger, 1977; Heefner and Harold, 1982). This contrasts with the situation in *A. nidulans* where an active Na^+ efflux is driven by a Na^+ / H^+ antiporter involving a proton translocating ATPase sensitive to DCCD and CCCP (Paschinger, 1977), and resembles that in *Streptococcus faecalis*, where efflux is mediated by a Na^+ stimulated ATPase which is insensitive to several inhibitors of conventional ATPase (Heefner and Harold, 1982). However in *S. faecalis* the ATP driven Na^+ pump requires only ATP but does not require proton motive force since reagents which collapse proton motive force (DCCD, CCCP, valinomycin) are ineffective. In *Anabaena* spp., although DCCD and CCCP are ineffective, Na^+ efflux probably requires a H^+ gradient, since gramicidin which destroys the proton motive force also inhibits efflux (table 1). The "Na^+ pump" of *Anabaena* spp. can therefore be described as a Na^+/H^+ or Na^+/K^+ antiporter similar to Na^+/H^+ antiporter in *A. nidulans* but involving an ouabain sensitive ATPase distinct from the conventional ATPase. The energy source for Na^+ extrusion may be ATP derived from oxidative phosphorylation since DNP inhibits efflux. Both ouabain and DNP cause a more dramatic effect on the efflux in *A. torulosa* indicating that it has a more efficient Na extruding system than *Anabaena* L-31.

Concanavalin A has been shown to inhibit mitochondrial ATPase in yeast (Satav *et al*., 1980) by binding to the F_1 portion which is said to be a glycoprotein. The observed effect of concanavalin A on Na^+ uptake in *A. torulosa* however may not be through its effect on ATPase since DCCD is ineffective. The effect, which is more likely to be due to the ability of concanavalin A to bind certain sugar receptors (polysaccharides/glycoproteins) on plasmamembrane and to reorient them, needs further investigation.

Anabaena L-31 retains much more Na^+ than *A. torulosa* (figure 4) probably in excess of its requirement as shown by the lack of correlation of sodium concentration with growth or nitrogenase activity (figure 1). Apparently this Na^+ accumulation is not a physiological adaptation but arises out of a failure to regulate its intracellular content of Na^+ and results in its lack of survival under salt stress. *A. torulosa* achieves this regulation by curtailing the uptake itself and also by more efficient extrusion of Na^+ than *Anabaena* L-31. Salt-tolerance of *A. torulosa* resembles those of glycophytes rather than halophytes (Flowers *et al.,* 1977). It is known that organic acids, amino acids or carbohydrates accumulate under stress to build-up the osmotic potential (Flowers *et al.,* 1977). We have not studied this aspect but the enhanced nitrogenase activity in *A. torulosa* under salt stress may be aimed at eventual accumulation of amino acids for better osmoregulation. Additionally, the ability of *Anabaena torulosa* to sporulate (Fernandes and Thomas, 1982), a feature with high adaptive value, and to form mats (which probably create microenvironments with decreased salinity) would contribute to the success of this cyanobacterium in saline environment.

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