Membrane electrogenesis and sodium transport in filamentous nitrogen-fixing cyanobacteria

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Transport of Na⁺ and its relationship with membrane potential (ΔΨm) was examined in Anabaena L-31 (a fresh water cyanobacterium) and Anabaena torulosa (a brackish water cyanobacterium) which require Na⁺ for diazotrophic growth. The data on the effect of N,N'-dicyclohexylcarbodiimide indicated that ΔΨm was generated by electron-proton extrusion predominantly mediated by ATPase(s). In addition, operation of a plasmalemma-bound, non-ATP-requiring, H⁺-pumping terminal oxidase was suggested by the sensitivity of ΔΨm to anaerobiosis, cyanide and azide, all of which inhibit aerobic respiration. The response of ΔΨm to external pH and external Na⁺ or K⁺ concentrations indicated that a diffusion potential of Na⁺ or K⁺ may not contribute significantly to ΔΨm.

Kinetic studies showed that Na⁺ influx was unlikely to be a result of Na⁺/Na⁺ exchange but was a carrier-mediated secondary active transport insensitive to low concentrations (<10 mM) of external K⁺. There was a close correspondence between changes in ΔΨm and Na⁺ influx; all the treatments which caused depolarisation (such as low temperature, dark, cyanide, azide, anaerobiosis, ATPase inhibitors) lowered Na⁺ influx whereas treatments which caused hyperpolarisation (such as 2,4-dinitrophenol, nigericin) enhanced Na⁺ influx. Remarkably low intracellular Na⁺ concentrations were maintained by these cyanobacteria by means of active efflux of the cation.

The basic mechanism of Na⁺ transport in the fresh water and the brackish water cyanobacterium was similar but the latter demonstrated less influx, more efficient efflux, more affinity of carriers for Na⁺ and less accumulation of Na⁺, all attributes favouring salt tolerance.

Sodium ion (Na⁺) is an important requirement for cyanobacterial growth [1, 2] and nitrogen fixation [3–5]. Recently, Na⁺ has been found to be essential for nitrogenase activity although it does not influence synthesis of the enzyme [5]. Na⁺ is one of the predominant cations in most soils and waters, especially in the brackish soils from the coastal areas and saline alkali soils, which are frequently populated by cyanobacteria [6]. The osmotic adaptation under salt stress is achieved in cyanobacteria either by accumulation of K⁺ [7] or by the synthesis of carbohydrates (like glucopyranosylglycerol and sucrose), polyols and amines [8, 9]. However, basic information on other processes which influence salt tolerance such as Na⁺ transport and its regulation is lacking except in the unicellular Anacystis nidulans, which exhibits an active, energy-dependent extrusion of the cation [10, 11]. The mechanisms responsible for Na⁺ influx in cyanobacteria have received only scant attention [10–12], particularly in N₂-fixing forms where investigations on the relationship between

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Abbreviations. BBOT, 2,5-(5-bistetradutyl-2-benzoxazolyl) thiophene; CCCP, carbonylcyanide m-chlorophenylhydrazone; DCCD, N,N'-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; P₅0₅, tetraphenylphosphonium; CM/5, fivefold-diluted cyanophycean medium [17].


Na⁺ transport and salt tolerance have only begun recently [13]. Such information on the mechanisms of salt tolerance in these photosynthetic microbes will be advantageous in studying the responses of higher plant cells to salt stress [14].

This paper examines the transport of Na⁺, especially its influx in two heterocystous, N₂-fixing cyanobacteria, Anabaena L-31 and Anabaena torulosa, which were earlier shown to differ in their ability to resist salt stress [13]. Data obtained by the use of a membrane-specific lipophilic cation and definitive metabolic inhibitors have helped identify the factors influencing Na⁺ transport, particularly influx, and its relationship to the electric potential difference across the plasma membrane in these cyanobacteria.

MATERIALS AND METHODS

Organisms and growth conditions

Two filamentous, heterocystous, N₂-fixing cyanobacteria, Anabaena torulosa (a sporulating brackish water form) [15] and Anabaena L-31 (a fresh water form) [16], were isolated in this laboratory and used in axenic condition. Fivefold-diluted cyanophycean medium (CM/5) [17], free of combined nitrogen and containing 1 mM Na⁺ was used for the maintenance and growth of all the cultures. Cyanobacteria were grown photoautotrophically at 25°C under constant illumination (5000 Ix) and aeration (2 I·min⁻¹). Cells were harvested after 5 days in the late-logarithmic phase of growth.
Determination of transmembrane Na⁺ fluxes and intracellular Na⁺ concentration

All the experimental media contained combined nitrogen-free CM/5 (Na⁺, 1 mM) and were buffered to pH 7.0 with 20 mM Hepes/LiOH, unless otherwise specified. Transmembrane Na⁺ fluxes were studied by measuring exchange rates of radiotracer ²²Na⁺ added as carrier-free ²²NaCl. Influx was initiated by the addition of 0.23 µCi ²²NaCl to 1 ml assay mixture containing 20–25 µl packed cells. Na⁺ extrusion was determined in cyanobacterial suspensions which were preequilibrated in experimental media containing ²²NaCl for 24 h under constant aeration and illumination. Such suspensions were centrifuged for 3 min at 1000 x g and the pellet resuspended in identical buffered medium. Assays were terminated by rapid centrifuging 0.2 ml assay mixture at 12 500 g for 1 min. Assays were terminated after 15 min by rapidly centrifuging 0.2 ml assay mixture at 12 500 x g for 1 min in an Eppendorf microcentrifuge. The pellet was washed, dissolved in formic acid, transferred to 10 ml BBOT (0.4%, w/v, in toluene/methanol, 1:1) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Corrections were made for the nonspecific binding of Ph₄P⁺ (< 10%) as described by Harold and Papineau [19]. Values obtained by extrapolation of time course curves of Ph₄P⁺ uptake to t = 0 min were subtracted from equilibrium values of Ph₄P⁺ uptake (i.e. after 15 min). Intracellular radioactivity was also corrected for internal cell volume and membrane potential was calculated using the Nernst equation [20].

Determination of internal cell volume

The intracellular volume was measured as described by Rottenberg [21] using [U⁻³H]H₂O and Mg³⁵SO₄ (instead of a sugar). The average intracellular volume along with standard deviation of eight replicates was 0.426 ± 0.02 and 0.621 ± 0.01 µl · (µl packed cells)⁻¹ of A. torulosa and Anabaena L-31, respectively.

Chemicals, radioisotopes and gases

All the inorganic salts were obtained from British Drug Houses (Bombay, India). ²²NaCl, [U⁻³H]H₂O and Mg³⁵SO₄ were acquired from Amersham International (Amersham, England, UK) and [phenyl⁻¹⁴C]tetraphenylphosphonium
Table 1. Effect of physiological modifications and specific inhibitors on sodium influx and membrane potential of nitrogen-fixing cyanobacteria

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Prior to assay cyanobacterial suspensions were subjected to one of the following pretreatments: pre-incubation in dark for 7 h; anaerobiosis (argon sparging) for 5 h; incubation at 4°C for 1 h; pre-incubation with the inhibitor for 10 min. Assays contained 1 mM Na+ and were terminated after 1 min. Values for Na+ uptake along with standard deviations (five replicates) in controls were 464 ± 10.8 and 842 ± 14.3 cpm · (μl internal cell volume)⁻¹ of A. torulosa and Anabaena L-31 respectively. Membrane potential was determined using [³¹⁴C]Ph₄P⁺ in assays of 15-min duration and the values showed standard deviations up to 4% of the mean value of 4–6 replicates.

The effects of a variety of physiological modifications on Na⁺ influx in Anabaena spp. are shown in Fig.2. The initial linear phase of uptake lasted only 1 min in A. torulosa and 3 min in Anabaena L-31. The latter cyanobacterium showed a 4–5-fold higher influx of the cation compared to the saline form.

Membrane potential was determined using [³¹⁴C]Ph₄P⁺ during 15-min assays. Sodium influx was measured in A. torulosa ( ● ) and Anabaena L-31 ( △ ) in assays of either 1-min ( ○ , △ ) or 3-min ( ● , ▲ ) duration. Na⁺ concentration was 1 mM. Preliminary experiments had shown that Li⁺ did not affect Na⁺ transport, hence the pH was maintained with 20 mM Hepes/LiOH.

RESULTS

Both Anabaena spp. possessed a true Na⁺ uptake system (Fig.1). Intracellular Na⁺ concentration had no significant effect on the rates of Na⁺ influx. However, in view of the inhibition of diazotrophic growth under Na⁺ deficiency all other experiments were performed using cultures grown in media containing 1 mM NaCl.

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cultures to anaerobiosis by sparging argon for 5 h (Table 1) or 18 h (Fig.2), which inhibited respiratory electron transport and oxidative phosphorylation, diminished Na⁺ uptake and also decreased their membrane potential. These effects were observed even on replacement of argon by N₂ (data not shown). A. torulosa showed initial stimulation of Na⁺ influx (Table 1; Fig.2) upon argon sparging but accumulated 40% less Na⁺ in 60 min than the aerobic cultures (Fig.2). Incubation of cultures at 4°C in light severely inhibited Na⁺ influx and caused a marked decrease in membrane potential in both Anabaena spp. Moreover, in Anabaena L-31 such treatment completely abolished the normal saturation kinetics of Na⁺ influx resulting in a linear influx with time.

DCMU, an inhibitor of photosynthetic electron transport, stimulated Na⁺ influx and hyperpolarised the membrane at a concentration (1 μM) which completely inhibited photoevolution of O₂ but a higher concentration (10 μM) was inhibitory (Table 1). Cyanide and azide lowered Na⁺ influx and membrane potential in both cyanobacteria at concentrations (10 mM) which inhibited respiratory O₂ consumption. 2,4-Dinitrophenol, an uncoupler of oxidative phosphorylation, enhanced initial rates of Na⁺ influx and membrane potential (Table 1) but such enhancement was short-lived and time-course experiments showed that Na⁺ uptake by dinitrophenol-treated cells was comparable with untreated controls (data not included).

External pH influenced Na⁺ influx and membrane potential (Fig.3). In A. torulosa Na⁺ influx, which was maximal at pH 6.0, decreased upon increasing the external pH to 8.0. Anabaena L-31 showed a similar effect except that the maximal Na⁺ influx was observed at pH 6.5. In both Anabaena spp. transition from pH 6.0 to pH 7.0 resulted in

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Anabaena torulosa</th>
<th>Anabaena L-31</th>
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<tbody>
<tr>
<td></td>
<td>Na⁺ influx</td>
<td>Na⁺ influx</td>
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<tr>
<td></td>
<td>membrane potential</td>
<td>membrane potential</td>
</tr>
<tr>
<td>1. Control</td>
<td>100 ± 2.2</td>
<td>100 ± 2.2</td>
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<tr>
<td>2. Dark</td>
<td>74 ± 2.2</td>
<td>79 ± 2.2</td>
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<tr>
<td>3. DCMU:</td>
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<tr>
<td>1.0 μM</td>
<td>135 ± 2.0</td>
<td>125 ± 2.0</td>
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<tr>
<td>10.0 μM</td>
<td>66 ± 2.1</td>
<td>78 ± 2.1</td>
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<td>4. KCN:</td>
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<tr>
<td>10.0 mM</td>
<td>64 ± 2.0</td>
<td>65 ± 2.0</td>
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<td>5. NaNO₃:</td>
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<tr>
<td>10.0 mM</td>
<td>63 ± 2.0</td>
<td>65 ± 2.0</td>
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<td>6. Anaerobiosis:</td>
<td>150 ± 2.0</td>
<td>71 ± 2.0</td>
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<td>7. Dinitrophenol:</td>
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<tr>
<td>1.0 μM</td>
<td>162 ± 2.0</td>
<td>131 ± 2.0</td>
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<tr>
<td>10.0 μM</td>
<td>297 ± 2.1</td>
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<tr>
<td>100.0 μM</td>
<td>130 ± 2.0</td>
<td>108 ± 2.0</td>
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<tr>
<td>8. Low temperature (4°C)</td>
<td>24 ± 1.3</td>
<td>21 ± 1.3</td>
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Fig. 4. Effect of (a) CCCP and (b) DCCD on the membrane potential and sodium influx in nitrogen-fixing Anabaena torulosa. Culture suspensions (0.5 ml) were treated with the inhibitor for 10 min prior to the addition of radioactivity. Duration of the assay for measurement of Na\(^+\) influx was 1 min (○) or 3 min (●) and of membrane potential (▵) was 15 min. Na\(^+\) concentration was 1 mM. Na\(^+\) influx in control along with standard deviations (five replicates) was 467 ± 10.5 cpm (μl internal cell volume\(^{-1}\)) in both a and b.

Fig. 5. Effect of (a) CCCP and (b) DCCD on the membrane potential and sodium influx in nitrogen-fixing Anabaena L-31. Na\(^+\) influx in control along with standard deviations (five replicates) was 829 ± 12.9 cpm (μl internal cell volume\(^{-1}\)) in both a and b. Other details as described in legend to Fig. 4.

Addition of CCCP, a phosphorylation uncoupler, or DCCD nearly abolished the Δψ\(_m\) and severely inhibited Na\(^+\) influx in both cyanobacteria (Figs 4 and 5). DCCD, at 10–100 μM concentration, is known to inhibit H\(^+\)-translocating ATPases including a purified Synechococcus 6713 ATPase [23], although at concentrations above 0.5 mM it can influence other proteins also. The parallel effects of DCCD and CCCP on Δψ\(_m\) and Na\(^+\) influx, and lack of inhibition of these processes by another uncoupler dinitrophenol (Table 1) suggest that the observed effects of DCCD and CCCP are more likely to be due to the inhibition of H\(^+\) extrusion and not to a limitation of available ATP.

Effect of nigericin, an ionophore known to collapse the pH gradient, was examined at pH 6.0. At this pH the transmembrane ΔpH is greatest, the intracellular pH in cyanobacteria being 7.5 [22] and the ionophore has been shown to convert a large ΔpH into Δψ\(_m\) at this pH in certain diazotrophs, including cyanobacteria [24, 25]. When used at concentrations below 1 pg ml\(^{-1}\), nigericin led to a significant enhancement of Na\(^+\) influx and this was accompanied by considerable hyperpolarisation of Δψ\(_m\), in both Anabaena spp. (Fig. 6).

K\(^+\) inhibited Na\(^+\) influx and caused depolarisation of Δψ\(_m\) only at very high external concentration in both cyanobacteria (Table 2). Low concentrations (1–5 mM) of K\(^+\), which strongly affect Na\(^+\) transport in bacteria, algae and higher plants, were ineffective. The inhibition constants (K\(_i\)) for K\(^+\), calculated from Dixon plots, were found to be 28 mM for Anabaena L-31 and 49 mM for A. torulosa.

It was shown earlier that Na\(^+\) influx in these Anabaena spp. was carrier-mediated, followed Michaelis-Menten kinetics and that the affinity (K\(_m\)) for Na\(^+\) differed in the saline (0.3 mM) and fresh-water form (2.8 mM) by nearly 10-fold [13] in apparent accordance with their metabolic requirement for Na\(^+\). Table 3 shows that when exposed to increasing
Table 2. Effect of potassium on sodium influx and membrane potential of nitrogen-fixing cyanobacteria

External concentration of Na⁺ was kept constant at 1 mM in assays of 1 min duration. Values of Na⁺ influx along with standard deviations (five replicates) in controls (i.e. 1 mM K⁺) were 488 ± 6.4 and 829 ± 14.2 cpm · (μl internal cell volume)⁻¹ in A. torulosa and Anabaena L-31 respectively. Other details were as described in Table 1. n.d., not determined.

Table 3. Effect of external sodium concentration on the intracellular sodium levels and membrane potential in nitrogen-fixing cyanobacteria

Intracellular Na⁺ concentrations were determined by radioisotopic equilibration in 22NaCl for 24 h. The values of ΔψNa⁺ and equilibration potential of Na⁺ (ΔψNa⁺) were calculated from the Nernst equation [20]. The data showed standard deviations from 2.5% up to 4.2% of the mean values, with 4 - 6 replicates. n.d. = not determined.

Table 4. Effect of external sodium concentration on the intracellular sodium levels and membrane potential in nitrogen-fixing cyanobacteria

Introduction to the paper: The effects of sodium on the membrane potential and Na⁺ influx in nitrogen-fixing cyanobacteria were studied. Sodium influx was measured in controls (1 mM K⁺) and in the presence of 1-60 mM Na⁺ (0.1 - 60 mM). The results showed that Na⁺ influx was sensitive to incubation at 4°C and its rate was influenced by the external concentration of Na⁺. The extrusion appeared to be more efficient and also less sensitive to low temperature in A. torulosa than in Anabaena L-31. In the latter case normal kinetics of efflux, just as that of influx (see Fig. 2), was altered by low temperature.

Discussion: The transport of Na⁺ has been probed for the first time in filamentous heterocystous, N₂-fixing cyanobacteria. Na⁺ influx, which has been studied in some detail, was dependent on the proton-motive force of cells. Outwardly directed electrogenic proton pumps in cyanobacteria [22, 26, 27] have been earlier shown to generate the proton electrochemical potential gradient (ΔμH⁺) consisting of two components, namely a pH gradient (ΔpH) and membrane potential (Δψm), in accordance with the chemiosmotic theory of Mitchell [28]. The respiratory and photosynthetic electron transport chains are generally believed to be located on the thylakoids in cyanobacteria [29] and presumably can not generate a proton gradient. Recently a plasmalemma-bound respiratory aα₃ type of cytochrome oxidase has been implicated as a non-ATP-requiring proton pump in cyanobacteria [12] although the evidence is not unequivocal. From the present study of two N₂-fixing Anabaena spp. it appears that both the proton-translocating classical ATPase as well as a respiratory H⁺-translocating oxidase located in the plasma membrane are involved in the membrane electrogenesis. Thus, the additional of an ATPase inhibitor like DCCD and a phosphorylation uncoupler like CCCP (Figs 4 and 5) or of inhibitors of respiratory oxidases like cyanide, azide and anaerobiosis (Fig. 2, Table 1) all result in depolarisation of the membrane. The observed energisation (Table 1) in the presence of dinitrophenol probably resulted from the reported stimulation of electron transport in cyanobacteria [30, 31], by this.

Fig. 7. Sodium extrusion by nitrogen-fixing cultures of (a) Anabaena torulosa and (b) Anabaena L-31. Efflux was initiated by resuspending cells prelabelled with 22NaCl in experimental medium containing either 1 mM Na⁺ (△A) or 0.2 mM Na⁺ (△B). One set was incubated at 4°C (△B). Initial radioactivity (i.e. 100%) in the cells along with standard deviations in five replicates was 1375 ± 18.1 and 1536 ± 22.6 cpm · (μl internal cell volume)⁻¹ in A. torulosa and Anabaena L-31 respectively.

The evidence for existence of Na⁺ extruding pump(s) in N₂-fixing Anabaena spp. is presented in Fig. 7. The efflux was sensitive to incubation at 4°C and its rate was influenced by the external concentration of Na⁺. The extrusion appeared to be more efficient and also less sensitive to low temperature in A. torulosa than in Anabaena L-31. In the latter case normal kinetics of efflux, just as that of influx (see Fig. 2), was altered by low temperature.

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uncoupler. The lack of response to dark treatment suggests that reduced ATP pools [32] do not seriously affect membrane electrogenesis. Of the two components of ΔΨm, which promote Na+ influx in the N₂-fixing Anabaena spp., Na+ influx is more closely linked with the ΔΨm, DCCD and CCCP non-selectively collapse both ΔpH and ΔΨm. But the significant hyperpolarisation of ΔΨm and concomitant enhancement of Na+ influx observed in the presence of nigericin (Fig. 6) clearly establish the regulation of Na+ uptake by membrane potential in these cyanobacteria. In conformity with this, all treatments which cause depolarisation (4°C, KCN, NaN3, argon, DCCD, CCCP) or hyperpolarisation (DMCU, dinitorphenol, nigericin) also, respectively, lower or enhance Na+ uptake (Figs 2–5; Table 1). The incomplete correspondence between Na+ influx and ΔΨm during pH transition from 6.0 to 8.0, may appear to be at variance with the above generalisation.

However, it is known that such transitions reduce the total proton-motive force. For example, in Anabaena variabilis a shift in pH has been shown to decrease the ΔΨm, from +25 mV at pH 6.0 to −100 mV at pH 8.0 [22]. Hence, Na+ influx in Anabaena spp. appears, at least during the pH transition, to be coupled to the composite ΔΨm, rather than to the ΔΨm alone.

The observed values of ΔΨm in N₂-fixing A. torulosa (−66.7 mV) and Anabaena L-31 (−64.4 mV) can account for 13-fold and 12-fold accumulation of Na+, respectively, in accordance with secondary active transport. The calculated values of ΔΨm, (Table 3) therefore clearly support the operation of Na+ extrusion pumps(s). Indeed both Anabaena spp. exhibit rapid efflux of Na+ (Fig. 7). The efflux is relatively insensitive to several metabolic inhibitors which influence Na+ influx. Especially notable are dinitrophenol, DCCD and CCCP which have a dramatic effect on influx (Table 1; Figs 4 and 5) but affect efflux only marginally even at 100 μM concentration (data not included). The differential sensitivity of inward and outward Na+ fluxes to these metabolic inhibitors and to trans Na+ concentrations (Figs 1 and 7) clearly distinguishes them as independent processes. An Na+/Na+ exchange does not therefore appear to play a significant role in Na+ uptake by N₂-fixing Anabaena spp.

The relative insensitivity of Na+ influx in Anabaena spp. to low concentrations of K⁺ (Table 2) contrasts with the high selectivity for K⁺ and discrimination against Na⁺ observed in algae [33, 34], higher plants [35, 36] and bacteria [37, 38] including the unicellular cyanobacterium A. nidulans [10]. The present data suggest that in N₂-fixing Anabaena spp. Na⁺ and K⁺ are either transported separately or, if not, at least at low concentrations K⁺ is not a preferred cation over Na+. In A. variabilis K⁺ transport has been recently shown to be mediated by K⁺/K⁺ exchange diffusion and does not catalyse K⁺/Na⁺ exchange [39], which is in agreement with our results. The calculated Kₐ values for inhibition of Na⁺ influx by K⁺ are far higher than the known levels of K⁺ in various ecosystems and therefore are probably of no eco-physiological significance.

N₂-fixing Anabaena spp. also differ from the unicellular diazotroph Anacystis in their regulation of Na⁺ transport. A. nidulans possesses a DCCD-sensitive, proton-translocating ATPase; the resulting H⁺ gradient is utilised for Na⁺ extrusion by an Na⁺/H⁺ antiporter [11]. The lack of net Na⁺ accumulation upon the addition of DCCD suggests that no such mechanism occurs in Anabaena spp. The energy-consuming Na⁺ extrusion reduces the efficiency of oxidative phosphorylation in A. nidulans and this has been correlated with its failure to grow in the dark while in two other facultatively chemoheterotrophic cyanobacteria, A. variabilis and Nostoc MAC, oxidative phosphorylation is much less sensitive to Na⁺ [12]. It is noteworthy that A. torulosa and Anabaena L-31 as also A. variabilis and Nostoc MAC are all, unlike A. nidulans, N₂ fixers. In view of a specific requirement of Na+ for diazotrophy established in cyanobacteria [3–5], it would be interesting to examine whether the differences in the Na⁺ transport characteristics observed between Anacystis and Anabaena spp. are associated with oblige photoautotrophy or diazotrophy or both.

In spite of remarkable differences in their salt tolerance [13] the basic mechanism of Na⁺ transport appears to be identical in A. torulosa and Anabaena L-31. They differ, however, in the rates and magnitudes of influx and efflux, affinity of their carriers for Na⁺ and levels of Na⁺ accumulated. Considerably lower rates of influx, much higher affinity for Na⁺ and more efficient extrusion of the cation indeed strengthen the ability of Na⁺ exclusion in A. torulosa and appear to contribute significantly to its salt tolerance.

The reported measurements of the intracellular volumes of cyanobacteria were performed at the Agriculture and Food Research Council Unit of Nitrogen Fixation, University of Sussex, Brighton. We thank Prof. J. R. Postgate FRS for the facilities provided and Dr Robert Eady for help in these measurements.

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