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Membrane electrogenesis and sodium transport in filamentous nitrogen-fixing cyanobacteria

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Transport of Na^+ and its relationship with membrane potential ($\Delta\psi_m$) 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 $\Delta\psi_m$ was generated by electrogenic 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 $\Delta\psi_m$ to anaerobiosis, cyanide and azide, all of which inhibit aerobic respiration. The response of $\Delta\psi_m$ to external pH and external Na^+ or K^+ concentrations indicated that a diffusion potential of Na^+ or K^+ may not contribute significantly to $\Delta\psi m$.

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 $\Delta\psi_m$ 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 N_2-fixing forms where investigations on the relationship between 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, N_2-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, N_2-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 lx) and aeration (2 l · min^{-1}). Cells were harvested after 5 days in the late-logarithmic phase of growth.

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Abbreviations. BBOT, 2,5-(5-bistetrabutyl-2-benzoxazolyl) thiophene; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; Ph_4P^+, tetraphenylphosphonium; CM/5, fivefold-diluted cyanophycean medium [17].

Enzyme (IUB Recommendations 1984). ATPase (EC 3.6.1.34).

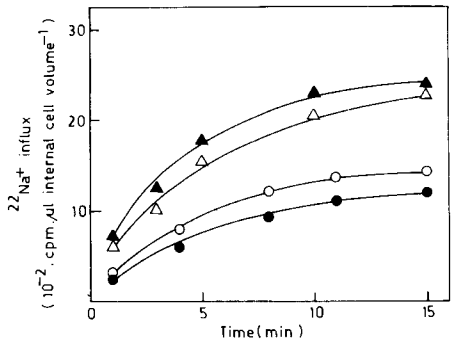

Fig. 1. *Sodium uptake by nitrogen-fixing* Anabaena torulosa (○, ●) *and* Anabaena *L-31* (△, ▲). Cyanobacteria were grown for 24 h in media without sodium (○, △) or supplemented with 1 mM NaCl (●, ▲). Filaments were harvested, washed once with nitrogen-free, Na^+-free CM/5 containing 20 mM Hepes/LiOH at pH 7.0 and resuspended in identical buffered medium. Assays were initiated by the addition of 0.1 μCi carrier-free $^{22}NaCl \cdot ml^{-1}$. Residual Na^+ contamination in Na^+-free media was < 15 μM as revealed by atomic absorption spectrophotometry

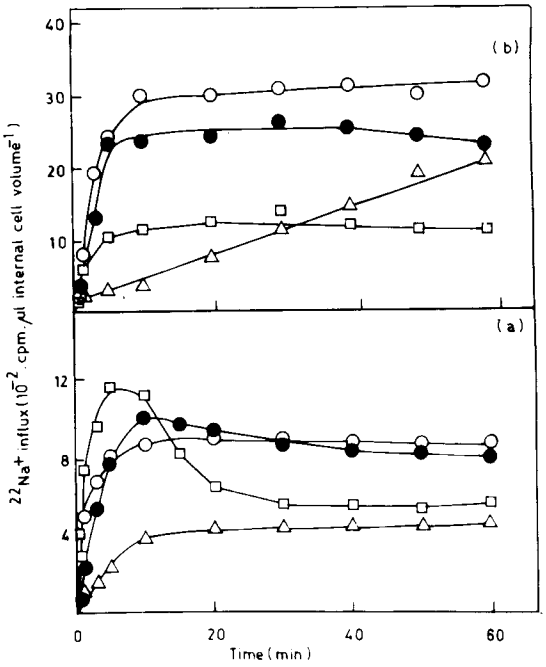

Fig. 2. *Effect of certain physiological modifications on sodium uptake by nitrogen-fixing cultures of (a)* Anabaena torulosa *and (b)* Anabaena *L-31.* Assay conditions were as follows: (○) control, 5000 lx, 25 °C, air; (●) dark preincubation for 7 h and during assays, 25 °C, air; (□) anaerobic preincubation by sparging with argon for 18 h and during assays, 5000 lx, 25 °C; (△) incubation at 4 °C for 60 min prior to and during assays, 5000 lx, air. All the assays contained 1 mM Na^+ and were initiated by the addition of 0.23 μCi $^{22}NaCl$ (carrier-free) · (ml assay mixture)$^{-1}$

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 $^{22}Na^+$ added as carrier-free $^{22}NaCl$. Influx was initiated by the addition of 0.23 μCi $^{22}NaCl$ 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 $^{22}NaCl$ for 24 h under constant aeration and illumination. Such suspensions were centrifuged for 3 min at 1000 × *g* and the pellet resuspended (20 – 25 μl packed cells · ml^{-1}) in identical medium, without radiotracer, to induce efflux. All the assays were normally carried out at 5000 lx light intensity and under aeration (0.2 l · min^{-1}) at 25 °C. All assays were terminated by filtering 0.1 ml suspension on Whatman GF/C filter paper circles (Whatman Ltd, England, UK) and washing first with 50 ml experimental medium (minus radiotracer) and then with 50 ml glass-distilled water. The entire washing procedure took less than a minute per sample and completely removed the diffusible and easily exchangeable (extracellular) fraction of Na^+ (nearly 90% of radioactivity initially bound to the cells). Filter paper circles were dried, transferred to vials containing 10 ml BBOT (0.4%, w/v, in toluene/methanol, 1:1) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Flux rates, determined from assays of 1-min or 3-min duration (i.e. during the initial linear phase), were expressed as cpm · (μl internal cell volume)$^{-1}$.

Intracellular Na^+ concentration was determined by radioisotopic equilibration in medium supplemented with $^{22}NaCl$, for 24 h. Extracellular $^{22}Na^+$ was removed by the washing procedure described above and intracellular activity was corrected for internal cell volume.

Determination of transmembrane electrical potential, $\Delta\psi_m$

Cells were suspended in combined nitrogen-free CM/5, buffered to pH 7.0 with 20 mM Hepes/LiOH, and incubated with 5 μM [*phenyl*-^{14}C]tetraphenylphosphonium (Ph_4P^+, specific activity 19.2 Ci · mol^{-1}) [18] and 20 μM tetraphenylboron which facilitated complete equilibration within 10 min. Assays were terminated after 15 min by rapidly centrifuging 0.2 ml assay mixture at 12 500 × *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 Ph_4P^+ (< 10%) as described by Harold and Papineau [19]. Values obtained by extrapolation of time course curves of Ph_4P^+ uptake to $t = 0$ min were subtracted from equilibrium values of Ph_4P^+ 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-^{3}H]H_2O and $Mg^{35}SO_4$ (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). $^{22}NaCl$, [U-^{3}H]H_2O and $Mg^{35}SO_4$ were acquired from Amersham International (Amersham, England, UK) and [*phenyl*-^{14}C]tetraphenylphosphonium

Table 1. *Effect of physiological modifications and specific inhibitors on sodium influx and membrane potential of nitrogen-fixing cyanobacteria*
Prior to assay cyanobacterial suspensions were subjected to one of the following pretreatments: preincubation in dark for 7 h; anaerobiosis (argon sparging) for 5 h; incubation at 4°C for 1 h; preincubation 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 $[^{14}C]Ph_4P^+$ in assays of 15-min duration and the values showed standard deviations up to 4% of the mean value of 4–6 replicates

Treatment	*Anabaena torulosa*		*Anabaena* L-31	
	Na^+ influx	membrane potential	Na^+ influx	membrane potential
	% control	mV	% control	mV
1. Control	100	−66.2	100	−64.2
2. Dark	74	−62.2	79	−57.6
3. DCMU:				
1.0 μM	135	−70.6	125	−68.4
10.0 μM	66	−62.1	78	−59.0
4. KCN:				
10.0 mM	64	−56.2	65	−53.3
5. NaN_3:				
10.0 mM	63	−58.0	65	−54.7
6. Anacrobiosis	150	−56.3	71	−51.2
7. Dinitrophenol:				
1.0 μM	162	−72.7	131	−68.8
10.0 μM	297	−71.5	162	−66.2
100.0 μM	130	−64.3	108	−63.7
8. Low temperature (4°C)	24	−51.3	21	−33.6

bromide from New England Nuclear (Boston, USA). Nigericin was a generous gift from Prof. W. D. P. Stewart (University of Dundee, UK). All other biochemicals were supplied by Sigma Chemical Co. (St Louis, Missouri, USA). Argon and nitrogen were purchased from Indian Oxygen Ltd (Bombay, India).

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.

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.

Preincubation of cyanobacterial cultures in the dark completely inhibited photosynthesis in less than 15 min and N_2 fixation in 3 h (data not shown). Cultures incubated in the dark for 7 h showed diminished initial rates of Na^+ influx and partial depolarisation of the membrane (Table 1). Subjecting

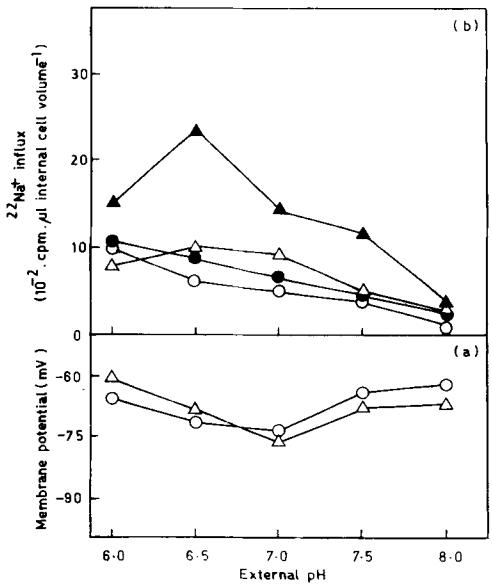

Fig. 3. *Effect of external pH on (a) membrane potential and (b) sodium influx of nitrogen-fixing cyanobacteria.* Prior to assay cyanobacterial suspensions were pre-incubated in buffer of the appropriate pH for 30 min. Membrane potential of *Anabaena torulosa* (○) and *Anabaena* L-31 (△) was determined using $[^{14}C]Ph_4P^+$ 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

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 N_2 (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 O_2 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 O_2 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

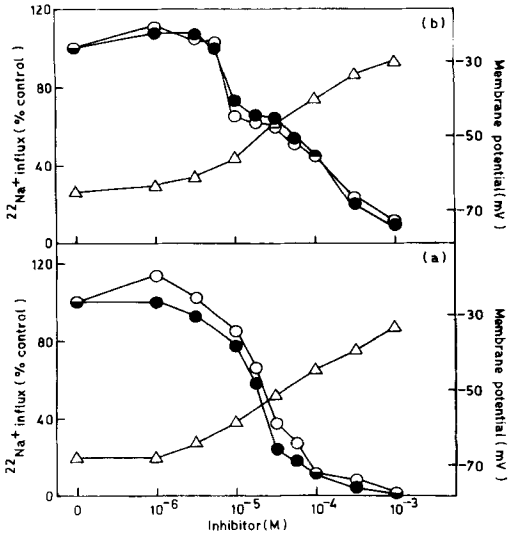

Fig. 4. *Effect of (a) CCCP and (b) DCCD on the membrane potential and sodium influx in nitrogen-fixing* Anabaena torulosa. Culture suspensions (0.5 ml) were treated with the inhibitor for 10 min prior to the addition of radioactivity. Duration of the assay for measurement of Na^+ influx was 1 min (○) 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

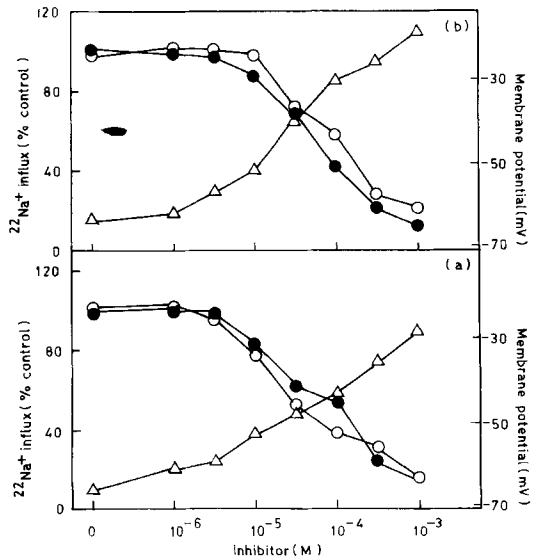

Fig. 5. *Effect of (a) CCCP and (b) DCCD on the membrane potential and sodium influx in nitrogen-fixing* Anabaena *L-31*. Na^+ influx in control along with standard deviations (five replicates) was 829 ± 12.9 cpm · (µl internal cell volume)$^{-1}$ in both a and b. Other details as described in legend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

hyperpolarisation of membrane, indicating that an electrogenic H^+ extrusion was responsible for the generation of membrane potential. A further shift to pH 8.0 however caused depolarisation, probably on account of a significant drop in ΔpH, the intracellular pH in heterocystous cyanobacteria being maintained around 7.5 [22]. The reduced ΔpH probably curtailed H^+ extrusion thereby causing depolarisation.

Addition of CCCP, a phosphorylation uncoupler, or DCCD nearly abolished the $\Delta\psi_m$ and severely inhibited Na^+ influx in both cyanobacteria (Figs 4 and 5). DCCD, at 10–

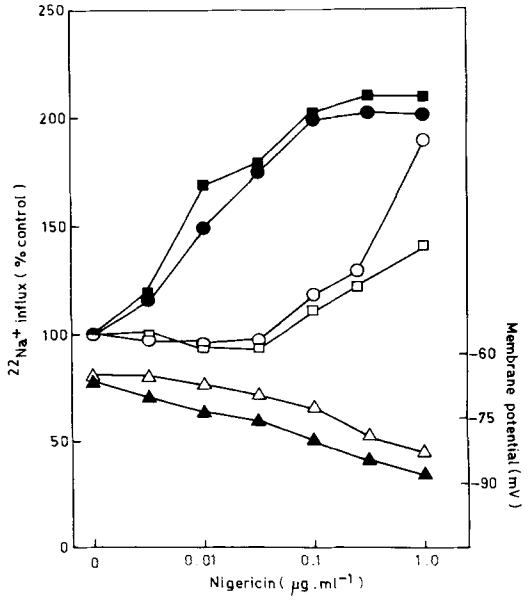

Fig. 6. *Effect of nigericin on the membrane potential and sodium influx of nitrogen-fixing* Anabaena torulosa *(●, ■, ▲) and* Anabaena *L-31 (○, □, △)*. Prior to assay cyanobacterial suspensions were preincubated at pH 6.0 for 30 min followed by incubation in nigericin for 10 min. Duration of the assay was 1 min (○, ●) or 3 min (□, ■) for measurement of Na^+ influx and 15 min for determination of membrane potential (△, ▲). Na^+ concentration was kept at 1 mM. Na^+ influx along with standard deviations (five replicates) in the absence of nigericin was 470 ± 10.7 and 816 ± 13.8 cpm · (µl internal cell volume)$^{-1}$ in *A. torulosa* and *Anabaena* L-31, respectively

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 $\Delta\psi_m$ 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 $\Delta\psi_m$ at this pH in certain diazotrophs, including cyanobacteria [24, 25]. When used at concentrations below 1 µg · ml^{-1}, nigericin led to a significant enhancement of Na^+ influx and this was accompanied by considerable hyperpolarisation of $\Delta\psi_m$ in both *Anabaena* spp. (Fig. 6).

K^+ inhibited Na^+ influx and caused depolarisation of $\Delta\psi_m$ 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

External K^+	*A. torulosa*		*Anabaena* L-31	
	Na^+ influx	membrane potential	Na^+ influx	membrane potential
mM	% control	mV	% control	mV
1	100	−66.7	100	−64.4
2	100	n.d.	100	n.d.
5	100	n.d.	100	n.d.
10	85	−65.9	79	−62.2
20	71	n.d.	59	n.d.
40	63	n.d.	45	n.d.
60	57	−63.8	38	−61.7
80	53	n.d.	35	n.d.
100	49	−58.6	33	−54.4

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 $^{22}NaCl$ for 24 h. The values of $\Delta\psi_m$ and equilibration potential of Na^+ ($\Delta\psi_{Na^+}$) 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

Cyano-bacterium	External Na^+	Intracel-lular Na^+	$\Delta\psi_{Na^+}$	$\Delta\psi_m$
	mM		mV	
Anabaena torulosa	1	0.047	+79.7	−66.7
	10	0.44	+81.4	−66.4
	20	n.d.	n.d.	−65.3
	40	n.d.	n.d.	−65.6
	60	1.86	+90.5	−63.0
	80	n.d.	n.d.	−61.8
	100	3.17	+89.9	−59.2
Anabaena L-31	1	0.09	+62.8	−64.1
	10	0.78	+66.5	−64.1
	20	n.d.	n.d.	−64.1
	40	n.d.	n.d.	−63.5
	60	3.28	+75.7	−61.4
	80	n.d.	n.d.	−58.9
	100	4.91	+78.5	−54.7

salinity, both cyanobacteria maintained remarkably lower internal concentrations of Na^+ compared to its external concentration. The $\Delta\psi_m$ was largely unaffected by 1–60 mM external NaCl in both *Anabaena* spp. although some depolarisation occurred above 60 mM NaCl. It is unlikely therefore that $\Delta\psi_m$ may be constituted of a significant Na^+ (or K^+, see Table 2) diffusion potential in these cyanobacteria. The values of equilibrium potential of Na^+ (i.e. $\Delta\psi_{Na^+}$) calculated from Nernst equation (Table 3), clearly suggest the occurrence of active Na^+ efflux mechanisms working against the concentration gradient in these cyanobacteria.

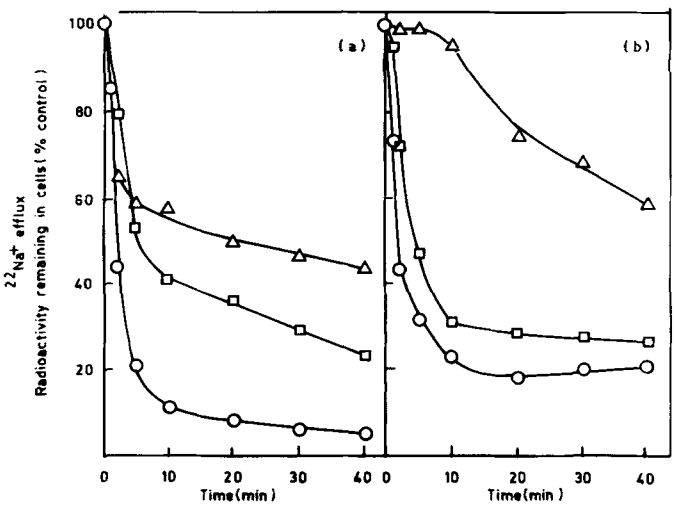

Fig. 7. *Sodium extrusion by nitrogen-fixing cultures of (a)* Anabaena torulosa *and (b)* Anabaena *L-31.* Efflux was initiated by resuspending cells prelabelled with $^{22}NaCl$ in experimental medium containing either 1 mM Na^+ (○, △) or 0.2 mM Na^+ (□). One set was incubated at 4°C (△). Initial radioactivity (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 N_2-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.

DISCUSSION

The transport of Na^+ has been probed for the first time in filamentous heterocystous, N_2-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 ($\Delta\tilde{\mu}_{H^+}$) consisting of two components, namely a pH gradient (ΔpH) and membrane potential ($\Delta\psi_m$), in accordance with the chemi-osmotic 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 aa_3 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 N_2-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 addition 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

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 $\Delta\tilde{\mu}_{H^+}$ which promote Na^+ influx in the N_2-fixing *Anabaena* spp., Na^+ influx is more closely linked with the $\Delta\psi_m$. DCCD and CCCP non-selectively collapse both ΔpH and $\Delta\psi_m$. But the significant hyperpolarisation of $\Delta\psi_m$ 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, NaN_3, argon, DCCD, CCCP) or hyperpolarisation (DCMU, dinitrophenol, nigericin) also, respectively, lower or enhance Na^+ uptake (Figs 2–5; Table 1). The incomplete correspondence between Na^+ influx and $\Delta\psi_m$ 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 $\Delta\tilde{\mu}_{H^+}$ from -125 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 transitions, to be coupled to the composite $\Delta\tilde{\mu}_{H^+}$ rather than to the $\Delta\psi_m$ alone.

The observed values of $\Delta\psi_m$ in N_2-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 $\Delta\psi_{Na^+}$ (Table 3) therefore clearly support the operation of Na^+ extrusion pump(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 N_2-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 N_2-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 K_i 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.

N_2-fixing *Anabaena* spp. also differ from the unicellular adiazotroph *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*, N_2 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 obligate 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.

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