Responses of Cyanobacteria to Low Level Osmotic Stress: Implications for the Use of Buffers

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The effects of low level osmotic stress on the growth and physiology of three cyanobacteria, Anacystis nidulans PCC 6301, Anabaena cylindrica PCC 7122 and Anabaena variabilis ATCC 29413, were investigated. No significant differences in the rates or patterns of growth were found in any strain when subjected to salinity stresses up to 100 mM-NaCl. The rates of photosynthetic oxygen evolution and nitrogenase activity were depressed rapidly and temporarily upon the addition of NaCl to the medium, to an extent which was dependent upon the amount of NaCl added. Nitrogenase activity was more sensitive to NaCl than was photosynthesis, and recovery took longer. Internal Na+ concentrations increased transiently upon upshock. This may be responsible for the observed inhibition of photosynthesis and nitrogenase activity, and recovery may be dependent upon the ability of the cell to adjust to sudden increases in internal Na+. These results indicate that metabolic disruption is transient and that, in the long term, growth and metabolic activity remain unaffected.

INTRODUCTION

Standardization of variable parameters such as temperature and pH are important in any physiological study. Growth and metabolism of micro-organisms in closed systems frequently result in pH changes. This requires the addition of a buffering component to the medium to maintain the pH within a given range. These compounds are usually employed at low concentrations within the range 10–50 mM, but all buffers have additional effects which are unrelated to pH stabilization.

The influence of buffers on the physiology of some cyanobacteria has been studied (Bothe et al., 1977; Bridges & Ward, 1976; Ferguson et al., 1980), and recent evidence (Smith et al., 1983) suggested that the effects of some common buffers on the cell structure and physiology of Anabaena cylindrica are due to the elevation in ionic strength that occurs on the addition of buffer, e.g. HEPES/NaOH, to the growth media. Those results contrast with earlier reports, e.g. Batterton & van Baalen (1971), Mackay et al. (1983) and Richardson et al. (1983), which showed that many freshwater cyanobacteria sustain growth in media of elevated ionic strength.

In view of the widespread use and importance of such compounds, we have examined the growth and physiology of three species of cyanobacteria to assess the effects of low level osmotic stress up to 100 mM-NaCl.

METHODS

Strains and growth. Anabaena variabilis ATCC 29413 and Anabaena cylindrica PCC 7122 were maintained in axenic culture in BG-11, medium (Rippka et al., 1979). Anacystis nidulans PCC 6301 was maintained in BG-11 medium, in axenic culture. The medium was supplemented with NaCl (AnalaR grade) at concentrations up to 100 mM to determine the effects of osmotic stress on growth, photosynthesis and nitrogenase activity. Cultures were routinely maintained at 28 °C in 250 ml Erlenmeyer flasks containing 100 ml medium. Illumination was at a constant photon flux density of 45 µmol m⁻² s⁻¹.

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Growth measurements. The cell number and biovolume of *Anacystis nidulans* PCC 6301 were determined using a Coulter counter (model ZB) fitted with a C1000 channelyzer (Coulter Electronics, Luton, UK) linked to a BBC microcomputer, for direct estimation of mean cell volumes. Intracellular volumes were determined for *Anabaena cylindrica* PCC 7122 and *Anabaena variabilis* ATCC 29413 after fragmenting the cyanobacteria in an MSE Soniprep 150 at half maximum amplitude (6 x 10 s bursts with 30 s between each burst) which resulted in more than 90% of the cells being separated as unicells. This technique, resulting from our optimization of sonic treatment for maximum recovery of unicells, gave a constant intracellular volume : chlorophyll a ratio of 0.045 \( \mu \text{g Chl} \mu \text{m}^{-3} \). Protein was determined in cell sonicates by the turbidometric protein assay (Meijbaum-Katzenellenbogen & Dobrzynska, 1959). Chlorophyll a concentrations were determined according to Mackinney (1941). Phycocyanin was measured in cell supernatants 30 min after addition of salt, from the absorbance at 620 nm.

Analysis of low molecular weight carbohydrates by gas-liquid chromatography. Carbohydrate accumulation profiles were obtained for cells exposed to salinities in the range 20–100 mM-NaCl. Low molecular weight carbohydrates were assayed following freeze-drying of samples pelleted together with an internal standard of 0-1 mg mannitol. Samples were then extracted in 2 ml dimethyl sulphoxide (DMSO) and trimethylsilyl derivatives were prepared by the addition of 0.5 ml hexamethyldisilazane and 0.25 ml trimethylchlorosilane. Samples were analysed using a Varian 3700 gas-liquid chromatograph fitted with a flame ionization detector and a column of 2% methyl phenyl silicone gum (SE52) on a diatomite support (Halligan & Drew, 1971). Chromatograms were recorded and quantified using a Hewlett Packard HP3930A integrator.

Photosynthetic activity. Photosynthetic rates were determined using a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, UK) at 30 °C and a saturating, but not inhibitory, photon flux density of 500 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) incident at the outermost surface of the oxygen electrode chamber. Cells were exposed to different salinities by the addition of NaCl to a final concentration in the range 20–100 mM as required, following the addition of NaHCO\(_3\) to a final concentration of 4 mM (to maintain an adequate supply of carbon for photosynthesis).

Nitrogenase activity. Acetylene reduction was measured as described by Stewart et al. (1967), using a GowMac series 752 instrument fitted with a flame ionization detector (GowMac Instrument Co., Shannon, Ireland). Samples (1 ml) of culture were placed in 7 ml Bijou bottles containing 10% (v/v) \( \text{C}_2\text{H}_2 \) in air and incubated for 30 min at a photon flux density of 45 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) at 30 °C. Cells were exposed to different salinities by the addition of NaCl to a final concentration of 20–100 mM as required.

Cellular Na\(^+\) concentrations. Determinations were made by \( ^{24}\text{Na}^+ \) tracer techniques. The isotope was provided by the Scottish Universities Research and Reactor Centre, East Kilbride, UK, as \( ^{24}\text{NaHCO}_3 \) (analytical grade). Cells of *Anacystis nidulans* PCC 6301 were harvested during late exponential growth, centrifuged at 3000 \( \times \) g for 15 min, and resuspended in BG-11 medium containing a small amount of radioactive tracer of specific activity 100 kBq mmol\(^{-1}\). Cells were pre-incubated for 18 h to equilibrate before the addition of NaCl at either 100 mM or 454 mM containing \( ^{24}\text{Na}^+ \) at the same specific activity (see Reed et al., 1984b). Replicate 1 ml aliquots were taken at each sample time and filtered through Whatman cellulose nitrate filters of 0.45 \( \mu \text{m} \) pore size, and rinsed with medium of appropriate salinity for 20 s to remove all extracellular \( ^{24}\text{Na}^+ \). After washing, cells and filters were transferred to vials to which 5 ml scintillation fluid (Packard scintillation fluid 299) was added. Control experiments were done to estimate carry-over in cell walls and filters with DMSO permeabilized cells. Residual \( ^{24}\text{Na}^+ \) in these samples was <10% of that in viable cells. Samples were counted using a Packard 300 CD liquid scintillation spectrophotometer with fully automatic quench correction.

RESULTS

Long-term effects of low level NaCl stress on growth and metabolic activity

Figs 1(a), 1(b) and 1(c) show the growth of batch cultures of *Anacystis nidulans* PCC 6301, *Anabaena cylindrica* PCC 7122 and *Anabaena variabilis* ATCC 29413 in BG-11/BG-11\(_i\) medium containing NaCl at either 0, 20, 50 or 100 mM, respectively. All the organisms grew under these conditions with no significant differences in the lengths of the lag or exponential growth phases between the different NaCl treatments for each organism, or in the rates of growth when compared to the control (0 mM-NaCl).

The photosynthetic and nitrogenase activities of the cultures after 7 d growth in each medium are given in Table 1. The rates of photosynthetic oxygen evolution for cells grown in each treatment were not significantly different to those of the control cells, with *Anacystis nidulans* PCC 6301 showing the highest photosynthetic activity in each medium. The two filamentous isolates (*Anabaena cylindrica* PCC 7122 and *Anabaena variabilis* ATCC 29413) showed similar rates of photosynthetic oxygen evolution and acetylene reduction with no marked effects due to NaCl addition in all treatments. Analysis of low molecular weight carbohydrates revealed that...
Cyanobacterial responses to low salt stress

![Graph](image)

Fig. 1. Growth of (a) *Anacystis nidulans* PCC 6301, (b) *Anabaena cylindrica* PCC 7122 and (c) *Anabaena variabilis* ATCC 29413 in NaCl at: ○, 0 mM; ●, 20 mM; □, 50 mM; ■, 100 mM. The results are the mean of three replicates.

Table 1. Rates of photosynthetic oxygen evolution and acetylene reduction from salt-adapted cultures of *Anacystis nidulans* PCC 6301, *Anabaena cylindrica* PCC 7122 and *Anabaena variabilis* ATCC 29413

<table>
<thead>
<tr>
<th>Organism</th>
<th>NaCl concn (mM)</th>
<th>Oxygen evolution [µmol O₂ h⁻¹ (µg Chl a)⁻¹]</th>
<th>Acetylene reduction [nmol C₂H₂ h⁻¹ (µg Chl a)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td><em>Anacystis nidulans</em></td>
<td>380</td>
<td>388</td>
<td>395</td>
</tr>
<tr>
<td><em>Anabaena cylindrica</em></td>
<td>191</td>
<td>209</td>
<td>209</td>
</tr>
<tr>
<td><em>Anabaena variabilis</em></td>
<td>184</td>
<td>181</td>
<td>191</td>
</tr>
</tbody>
</table>

Table 2. Analysis of low molecular weight carbohydrates

The amounts of internal osmoticum (sucrose) in *Anacystis nidulans* PCC 6301, *Anabaena cylindrica* PCC 7122 and *Anabaena variabilis* ATCC 29413 were measured after 14 d incubation. Results are the mean of five determinations.

<table>
<thead>
<tr>
<th>Organism</th>
<th>NaCl concn (mM)</th>
<th>Internal sucrose concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td><em>Anacystis nidulans</em></td>
<td>19-1</td>
<td>33-2</td>
</tr>
<tr>
<td><em>Anabaena cylindrica</em></td>
<td>13-1</td>
<td>19-2</td>
</tr>
<tr>
<td><em>Anabaena variabilis</em></td>
<td>23-0</td>
<td>41-5</td>
</tr>
</tbody>
</table>

Sucrose was the major soluble carbohydrate accumulated by all three organisms in response to salt stress. Sucrose was present in higher amounts than in the control in all cases and the amount varied according to the different degree of osmotic adjustment required (Table 2), with the maximum amount being present in 100 mM-NaCl.
Fig. 2. Effects of adding 100 mM NaCl on (a) photosynthesis and (b) nitrogenase activity in: △, *Anacystis nidulans* PCC 6301; ○, *Anabaena cylindrica* PCC 7122; □, *Anabaena variabilis* ATCC 29413. The results are the mean of three replicates.

**Transient effects of low level NaCl stress on metabolic activity**

A comparison of the cellular responses to the addition of NaCl, at concentrations up to 100 mM, showed that the rates of photosynthetic oxygen evolution and acetylene reduction were depressed rapidly upon the addition of NaCl to an extent which was dependent upon the amount of NaCl added. Nitrogenase activity was depressed to a greater extent than photosynthetic rate. Time courses for cells transferred to 100 mM NaCl showed a recovery to at least 90% of the initial rate of photosynthesis within 50 min, while nitrogenase activity required approximately 2 h to return to 90% of the initial rate (Fig. 2a, b). No adverse effects of increased ionic strength on cell structure and filament breakage or consequent phycocyanin release were observed in any experimental treatment.

Cells of *Anacystis nidulans* PCC 6301 transferred to 100 mM NaCl allowed rapid entry of Na\(^+\) from the medium (Fig. 3a). Thus 2 min after transfer the internal Na\(^+\) concentration was 10 mM, compared to 1.5 mM initially, declining to 3 mM within 60 min. Data showing the effects of transfer to a high salt medium containing 454 mM NaCl (the concentration of NaCl in seawater) reflect a similar trend (Fig. 3a), with an initial rapid accumulation, and resultant high internal Na\(^+\) concentration of 290 mM, which declined after 60 min to a level approaching 10 mM.
When cells of *Anacystis nidulans* PCC 6301 were treated with NaCl at concentrations above 100 mM, a direct relationship was observed between the internal Na\(^+\) concentration (at 2 min) and the external NaCl concentration (Fig. 3b). At lower external NaCl concentrations, internal Na\(^+\) accumulation after 2 min was dramatically decreased (see also Fig. 3a).

### DISCUSSION

The results presented in this paper show that at low concentrations of NaCl (up to 100 mM) the growth of three cyanobacteria was not significantly affected, and they indicate that these strains are physiologically and biochemically adapted to cope with low level osmotic stress. Recent evidence suggests that several mechanisms may be involved in osmotic adjustment. Mackay et al. (1983), Mohammad et al. (1983) and Reed et al. (1984a) have demonstrated the ability of cyanobacteria to accumulate low molecular weight carbohydrates and quaternary ammonium compounds in direct response to osmotic stress. Evidence has also been presented by Miller et al. (1976) to suggest that K\(^+\) is important in the osmotic adjustment of *Aphanathece halophytica*. *Anacystis nidulans* PCC 6301, *Anabaena cylindrica* PCC 7122 and *Anabaena variabilis* ATCC 29413 accumulated sucrose as the major internal osmoticum as a (long-term) response to osmotic stress (Table 2), in agreement with previous results (Reed et al., 1984a; Mackay et al., 1984).

All three organisms showed no appreciable long-term differences in the rates of photosynthetic oxygen evolution or acetylene reduction when grown at different concentrations of NaCl from 0 to 100 mM (Table 1). However, these activities were markedly affected immediately on the addition of NaCl. The cells showed a degree of inhibition which corresponded to the degree of osmotic stress, in agreement with the findings of Smith et al. (1983). However, recovery to within 90\% of the initial rates was rapid (50 min for photosynthesis and 2 h for nitrogenase activity). It would seem that the process of nitrogen fixation is more sensitive to inactivation by salt than is photosynthesis, as previously suggested (Jones & Stewart, 1969; Reed & Stewart, 1983). Recovery may be dependent upon the ability of the cell to adjust to sudden internal Na\(^+\) increases via a Na\(^+\) extrusion mechanism (Dewar & Barber, 1973) since the present study has shown that on upshock, the internal Na\(^+\) concentration of cells of *Anacystis nidulans* PCC 6301 increased rapidly to a level dependent on the external Na\(^+\) concentration. Clearly, even an increase of 7–8 mM would be sufficient to disrupt normal cellular functions by, for example, inducing changes in the membrane potential, affecting pH regulation and enzyme activity, etc. Tel-Or & Harel (1981) have shown that NaCl is a potent inhibitor of photosynthesis, and a rapid Na\(^+\) influx would effect considerable disruption to the photosynthetic system. Rapid Na\(^+\) influx during hyperosmotic shock has also been observed in the unicellular cyanobacterium *Synechococcus* 6311 (Blumwald et al., 1983). It seems likely that the increase in internal Na\(^+\) concentration upon upshock may be responsible for the direct inhibition of photosynthesis and nitrogenase activity observed here since the time courses for recovery of metabolic activity and Na\(^+\) extrusion are rather similar. In conclusion, whilst transient effects of NaCl on metabolic activity were noted which agree with the data of Smith et al. (1983), some contradictions are apparent. In the present study, no filament disruption and consequent phycocyanin release were observed (when measured 30 min after the addition of NaCl at concentrations up to 100 mM), compared to almost 60 \(\mu\)g phycocyanin released (mg dry wt\(^{-1}\)) (measured 5 h after the addition of 50 mM-NaCl) according to Smith et al. (1983). However, the release of phycocyanin observed by Smith et al. (1983) was variable and they did not measure any recovery of metabolic activity. Although the same strain of *Anabaena cylindrica* (ATCC 27899 = PCC 7122; Rippka et al., 1979) was used in both studies, the data of Smith et al. (1983) were obtained from experiments done by resuspending the cells in distilled water to which salt was added to the required concentration, in contrast to the experiments in the present study, which were done in BG-11 culture medium. Recent experiments (Reed et al., 1985) have indicated that K\(^+\) is required in the external medium in order for Na\(^+\) extrusion to occur, and this may explain the contradictions noted above. Our results demonstrate clearly that the effects
of elevated NaCl concentrations up to 100 mM are transient and that, on a long-term basis, the growth of these organisms remains unaffected. The effects described previously (Smith et al., 1983) may not solely be ascribed to elevation in ionic strength with respect to Na⁺.

REFERENCES


