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Studies of osmoregulation in salt adaptation of cyanobacteria with ESR spin-probe techniques

(spin labeling/cell volume/blue-green algae/salt tolerance)

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ABSTRACT **Sucrose is accumulated in response to NaCl-induced stress in the cyanobacterium *Synechococcus* 6311. Internal cell volume was measured by ESR spectra with 2,2,6,6-tetramethyl-4-oxopiperidinoxy free radical (TEMPONE) as a spin probe in order to calculate sucrose concentrations inside the cell. This method is rapid and reliable and provides an unambiguous measurement of absolute volumes in different osmotic environments. Because the osmolar concentration of sucrose does not counterbalance the osmolar concentrations of ions in the growth medium, we suggest that sucrose accumulation is one of the mechanisms involved in the process of adaptation to salt of *Synechococcus* 6311. The accumulation of sucrose in non-N_2-fixing cyanobacteria such as *Synechococcus* 6311 and in N_2-fixing cyanobacteria such as *Nostoc muscorum* suggests a common mechanism of osmoregulation of fresh water cyanobacteria in response to increasing NaCl concentrations in the growth medium.**

It has been shown that several mechanisms are involved in cyanobacterial salt adaptation. Reports on salt-water species have demonstrated that glucopyranosylglycol accumulates in *Synechococcus* sp. Nageli (1) and that inorganic ions such as K^+ accumulate in *Aphanotheca halophytica* (2).

The fresh water cyanobacterium *Nostoc muscorum* also has shown a certain degree of salt adaptability. As a response to increasing salt concentrations, photosynthetic activity was enhanced, drastic changes in the intracellular organization of the thylakoidal assembly were observed (3), and sucrose was accumulated (4).

Synechococcus 6311 was chosen for the present study for two reasons: (*i*) in this unicellular organism, which lacks organelles and whose thylakoids contribute negligible volumes, the accurate measurement of cell volumes permits the calculation of cytoplasmic concentrations of solutes; and (*ii*) in this non-N_2-fixing organism, the effects of increases in NaCl concentrations in the growth medium can be studied. This is not possible in the case of N_2-fixing organisms because of the high sensitivity of the N_2-fixing system to salt (3, 5).

It has been observed that when *Synechococcus* 6311 cells are grown in a continuous culture system in salt-containing medium, photosynthetic activity is enhanced and soluble sugars accumulate (unpublished data). In this work, we attempt to describe the mechanism of osmoregulation of *Synechococcus* 6311 cells during salt adaptation, to identify the osmoregulants involved, and to apply ESR spin probes to the determination of osmotic volume of the cell.

MATERIALS AND METHODS

Culture. *Synechococcus* 6311 was obtained from E. Padan, Hebrew University of Jerusalem, Israel. The culture was grown in Kratz and Myers "C" medium (6). Each culture was originated from stock cells grown in Petri dishes with growth medium containing 1.5% agar. Two-week-old cultures were transferred to liquid medium, stirred on a magnetic stirrer, flushed with 1% CO_2/99% air, and illuminated with cool white fluorescent light (I = 4 W/m^2 at 30°C).

Analytical Methods. Culture density was determined at 550 nm by using a Bausch and Lomb Spectronic 20 spectrophotometer. Chlorophyll content was determined at 663 nm in 80% acetone extract as described by MacKinney (7). The number of cells was determined with a hemacytometer chamber (Clay Adams). Soluble sugar content was determined by the method of Dubois *et al.* (8). The cells were harvested by centrifugation (7,000 × *g* for 10 min), washed twice with isoosmotic NaCl solutions and sonicated for 45 sec in a Branson Sonifier cell disruptor, model W-140. Cell sonicate was treated with phenol/sulfuric acid reagent and calibrated against glucose standard. Soluble sugars were identified by thin-layer chromatography as described (3). Sucrose and glucose in cells were determined enzymatically. Cells were centrifuged (7,000 × *g* for 10 min), the pellet was resuspended in 5 ml of distilled H_2O, and the mixture was sonicated for 1 min. The cell sonicate was centrifuged (85,000 × *g* for 1 hr), and the supernatant was treated with glucose oxidase (Sigma; 20 units/ml) in an O_2-electrode (Clark type, Yellow Springs Instrument) in the absence of peroxidase. Glucose was determined by the amount of O_2 consumption and calibrated against a glucose standard. For sucrose determinations, the supernatant was incubated with invertase (Sigma, 200 units/ml) for 1 hr at 37°C, and the glucose was determined as described above. (No endogenous invertase activity was detected in these assay conditions.) The sucrose concentration was determined by subtracting the concentration of free glucose from that of glucose after inversion.

Cell Volume. Cell volume was determined with ESR spectra. Samples for ESR experiments contained (final volume, 40 μl) 1 mM TEMPONE (2,2,6,6-tetramethyl-4-oxopiperidinooxy free radical), 20 mM sodium ferricyanide, 75 mM Na_2Mn-EDTA (the osmotic strength of this combination of paramagnetic agents measured 300 mosM/kg with a vapor pressure osmometer Wescor model S100 B), and cells suspended at a final chlorophyll concentration of 400–500 μg/ml in media described in the figure legends. The presence of ferricyanide ensured that if TEMPONE molecules were to become chemically reduced within cells, they would subsequently be reoxidized in the extracellular environment. The samples were placed into 100-μl sealed glass capillaries, and spectra were recorded at room temperature in a Varian model E-109E spectrometer. The measurements were conducted in the dark at 10 mW and a modulation amplitude of 0.4 gauss, with a time constant of 0.064 sec and a scan time of 8 min.

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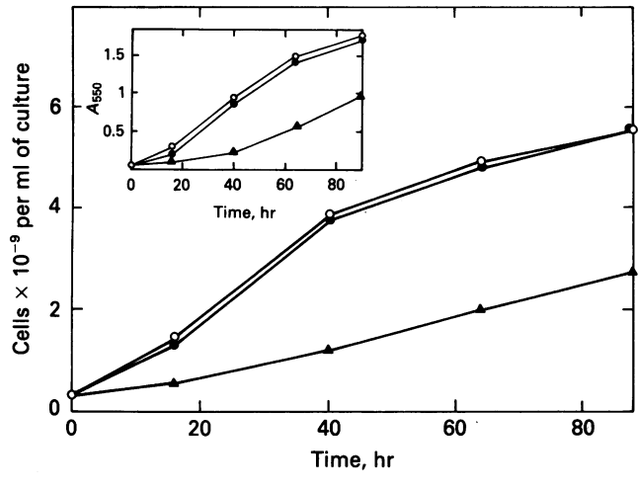

FIG. 1. Comparative growth of *Synechococcus* 6311 cells in the absence (○) and presence of 0.3 M (●) and 0.6 M NaCl (▲). Cultures were innoculated with identical densities and setup in 2-liter batch cultures; samples were withdrawn and analyzed. Each point represents the average of three experiments.

RESULTS

Batch cultures of *Synechococcus* 6311 exposed to a growth medium containing 0.3 and 0.6 M NaCl were analyzed regularly during their log phase of growth for changes in growth and cell composition. Cell growth was not affected in cells growing in the presence of 0.3 M NaCl, whereas growth was partially inhibited in the presence of 0.6 M NaCl; however after 40 hr the cells regained a growth rate similar to that of the control cells (Fig. 1). The rates of chlorophyll, carotenoids, and protein synthesis in cells grown in the presence of 0.3 M NaCl were somewhat lower than those in control cells and were even lower in cells grown in the presence of 0.6 M NaCl (results not shown).

It has been suggested that sugar accumulation during salt stress plays an essential role in osmotic regulation in cyanobacterium *Nostoc muscorum* cells (4). Separation of soluble sugars in *Synechococcus* 6311 cells by thin-layer chromatography showed that sucrose is the major soluble sugar accumulated in salt-grown cells (Fig. 2).

A comparison of the soluble sugar content of cells grown in the presence and absence of 0.3 M and 0.6 M NaCl is shown in Fig. 3. The soluble sugar content increased markedly in cells grown in the presence of 0.3 M and 0.6 M NaCl. In the case of cells grown in 0.6 M NaCl, soluble sugar content also was enhanced, although total cell biomass remained constant during the lag phase (the first 16 hr). The salt-grown cells accumulated sucrose at a constant rate after the first 40 hr of growth.

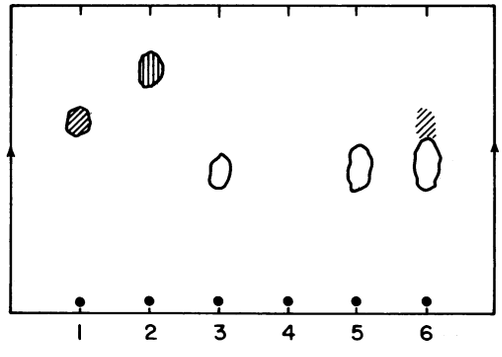

FIG. 2. Analysis of soluble sugars in *Synechococcus* 6311 cells grown in the absence and presence of 0.3 M NaCl and 0.6 M NaCl. Spots: 1, glucose; 2, fructose; 3, sucrose; 4, control; 5, 0.3 M NaCl; 6, 0.6 M NaCl.

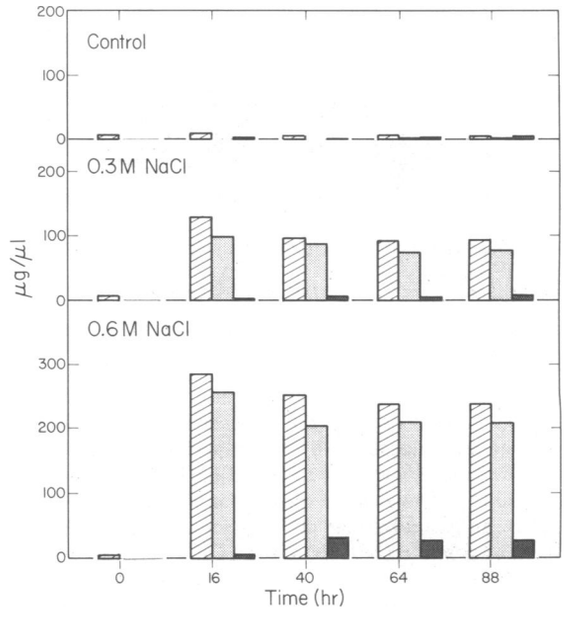

FIG. 3. Comparative soluble sugar content of *Synechococcus* 6311 cells grown in the absence and presence of 0.3 M NaCl and 0.6 M NaCl. Samples were withdrawn from the cultures used in Fig. 1 and analyzed. Each bar represents the average of three experiments. ▨, Soluble sugar; □, sucrose; ■, glucose.

In order to calculate the soluble sugar concentration in cells, the internal volume of the cell was measured by ESR spectra. Previous work has demonstrated the accuracy of this method, which appears to be the simplest and most rapid method currently available for such determinations (ref. 9; unpublished data).

Fig. 4 shows the measurement of the internal volume of a *Synechococcus* 6311 cell suspension. The ESR signal of TEMPONE in the absence of an quenching agent had a line height that was directly related to the total concentration of TEMPONE in the sample (curve a). Upon addition of impermeable broadening agents such as $Na_3Fe(CN)_6$ and $Na_2MnEDTA$, the

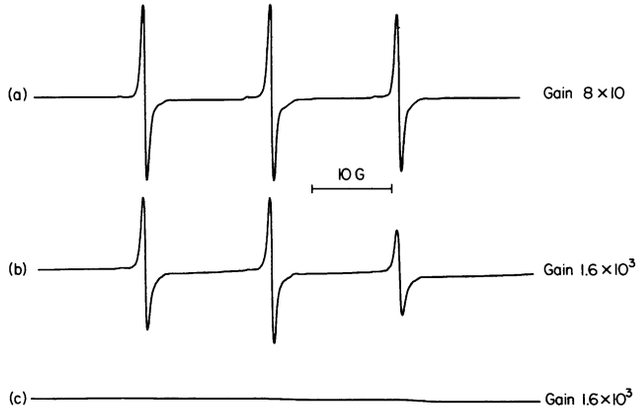

FIG. 4. ESR signal of TEMPONE on *Synechococcus* 6311 cells. Conditions were as described in *Methods*. Curves: a, ESR signal of TEMPONE in cell suspension in the absence of quenching agents; b, ESR signal of TEMPONE in cell suspension in the presence of 20 mM $Na_3Fe(CN)_6$ and 75 mM NaMnEDTA (The quenching agents broaden the external spin-probe signal, and the remaining unbroadened spin signal is directly proportional to the cell volume.); and c, broadened ESR signal of TEMPONE in the absence of cells and in the presence of 20 mM $Na_3Fe(CN)_6$ and 75 mM NaMnEDTA.

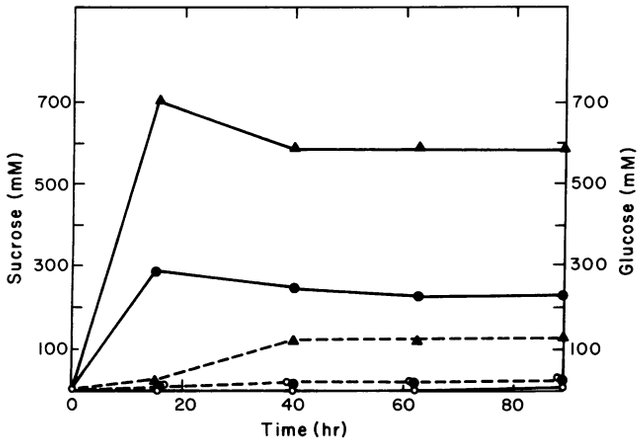

FIG. 5. Comparative sucrose (——) and glucose (---) concentrations in *Synechococcus* 6311 cells grown in the absence (○) and presence of 0.3 M (●) and 0.6 M NaCl (▲). Each point represents the average of three experiments.

signal outside the cells was broadened. Therefore, the resulting spectrum line (curve b) was due to the spin probe located within the cell. In the absence of cells, mixing the probe with the quenching agents resulted in a broadened spectrum (curve c). The internal volume of the cells was calculated by relating the internal signal to the total signal.

The internal concentration of sucrose and glucose in *Synechococcus* 6311 cells grown in the presence and absence of 0.3 M and 0.6 M NaCl also was calculated by using the volume determinations (Fig. 5). Cells grown in the presence of 0.3 M and 0.6 M NaCl synthesized sucrose in response to the increasing external NaCl concentrations, whereas no sucrose was detectable in control cells. In cells grown in the presence of 0.6 M NaCl, the sucrose concentration increased up to 700 mM after 16 hr of exposure to salt and reached a constant level of 580 mM after 40 hr. The glucose concentration in these cells increased after 16 hr and reached a constant level of 140 mM after 40 hr of exposure to salt. Cells grown in the presence of 0.3 M NaCl reached a constant concentration of 235 mM sucrose after 60 hr of growth.

The changes in cell volume of control and salt-adapted cells in response to increasing concentrations of a nonpermeant sugar,

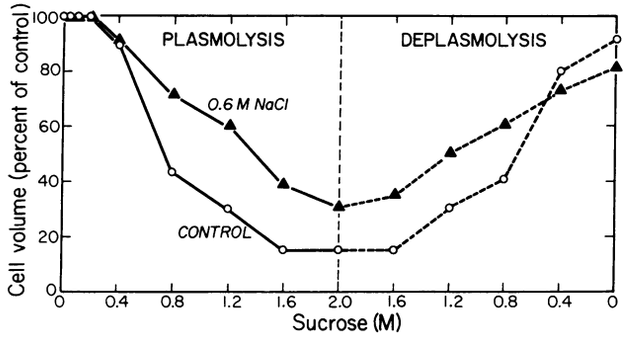

FIG. 6. Changes in cell volume of *Synechococcus* 6311 cells grown in the presence (▲) and absence (○) of 0.6 M NaCl. The cells were harvested after 96 hr of growth. (*Left*) The harvested cells were incubated with different sucrose concentrations at 30°C in the light for 20 min, and the cell volume was measured. (*Right*) Cells that were incubated for 20 min at 30°C in the light in 2 M sucrose solutions were centrifuged, washed, and resuspended in decreasing sucrose solutions, which were incubated for 30 min at 30°C in the light, and the cell volume was measured. Each point represents the average of three experiments.

such as sucrose, were measured by ESR spectra as shown in Fig. 6. Sucrose was added to water solutions of quencher [20 mM $Na_3Fe(CN)_6$ and 75 mM $Na_2MnEDTA$]. At sucrose concentrations up to 0.4 M, no significant change in cell volume was observed. At higher concentrations, two different plasmolysis curves were obtained. Control cells underwent a 50% reduction in volume with the addition of 0.7 M sucrose. Salt-adapted cells underwent a 50% reduction in volume when the external sucrose concentration was increased up to 1.35 M. Lowering the external sucrose concentrations after prior incubation in 2 M sucrose resulted in an increased cytoplasmic volume (deplasmolysis): control cells recovered 90% of their initial volume, whereas salt-adapted cells recovered 80% of their initial volume.

DISCUSSION

Cyanobacteria are considered to be the evolutionary link between the heterotrophic prokaryotes (bacteria) and the eukaryotic photoautotrophs (algae and plants). Halobacteria acquired mechanisms for ionic regulation that generate large gradients of Na^+ and K^+ ions across the cytoplasmic membrane and mechanisms for enzymatic adaptation to salt (10). Some higher plants and algae have mechanisms for salt tolerance that include (*i*) contractile vacuoles and (*ii*) osmotic regulation by the accumulation of organic solutes: sugars, amino acids, and polyols (11, 12). Therefore, cyanobacteria may use a combination of mechanisms for salt tolerance like those found in halobacteria and halotolerant higher plants and algae.

Previous research on marine cyanobacteria demonstrated the accumulation of glycerol derivatives in *Synechococcus* sp. Nageli (1) and K^+ ions in *Aphanotheca halophytica* (2). The fresh water cyanobacterium *Nostoc muscorum* was found to show a certain degree of adaptation to saline medium and to accumulate sucrose as an osmotic response to increasing salt concentrations in the growth medium (4). Photosynthetic activity is enhanced and sugars are accumulated in *Synechococcus* 6311 cells growing in salt-containing medium (unpublished data). Such activity produces the larger amounts of energy that are required for the osmoregulatory biosynthesis of sugars.

The enhancement of photosynthetic activity leads to a higher soluble sugar content in the form of sucrose. The fact that both N_2-fixing cyanobacteria (such as *Nostoc muscorum*) and non-N_2-fixing cyanobacteria (such as *Synechococcus* 6311) accumulate the same soluble sugar in response to salinity suggests a common pattern of osmoregulation in fresh-water cyanobacteria. The osmolar concentration of accumulated soluble sugar (sucrose and, to a lesser extent, glucose) does not counterbalance the osmolar concentration of ions in the growth medium. Other soluble sugars and polyols were not found to accumulate in *Synechococcus* 6311 cells (as compared with control cells) in response to the NaCl present in the growth medium (results not shown). These observations suggest (*i*) that soluble sugar accumulation in the form of sucrose is one of the mechanisms involved in the osmoregulatory response of fresh-water cyanobacteria to increasing salinity in the growth medium but (*ii*) that other as yet unidentified solutes must be involved also.

In an attempt to show the osmoregulatory role of sucrose in salt-adapted cells, changes in cell volume were measured when osmotic shock was induced by sucrose (which is nonpermeable). Two different plasmolysis/deplasmolysis curves were obtained; control cell volume was reduced by 50% at an external sucrose concentration of 0.7 M, while salt-adapted cell volume decreased 50% at 1.35 M. These results indicate different osmotic strengths of the cytoplasmic milieus of the cells and, thus, of the osmoregulatory role of the accumulated sucrose. In the deplasmolysis process, control cells recovered 90% of their

original volume, whereas the salt-adapted cells recovered only 80%. The reasons for this difference are not clearly understood and require further investigation.

ESR methods of volume measurements offer a new and rapid way to determine cell volumes in whole-cell suspensions. Because of rapid probe equilibration across membranes, volume measurements with TEMPONE can be made accurately and rapidly. With the use of quenching agents such as MnEDTA, which exhibits more quenching at a given concentration than the commonly used agent ferricyanide (9), we can achieve quenching of extracellular signals without substantial reduction in cell volume due to quencher-induced osmotic shrinkage. This new method should greatly facilitate determinations of intracellular solute concentrations in future experiments and, hence, accelerate progress in our understanding of osmoregulation.

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