biochem; ascorbic acid, Baker; GABA, California Corp. for Biochemical Research; glutamic acid, Pfanstiehl; piperidine, Fisher; trimethaphan camphorsulfanate, diazepam, and levorphanol tartrate, Hoffmann-La Roche; pentolinium tartrate, May and Baker; chlorisondamine chloride, Ciba; haloperidol, McNeil Laboratories; naloxone hydrochloride, Endo Laboratories; (+)-nicotine (> 96 percent optically pure), T. Kisaki; *N*-benzyl nornicotine and *N*-benzyl piperidine, L. G. Abood; phenylpropyldiethylamine hydrobromide, R. B. Barlow; and clonidine hydrochloride, Boehringer-Ingelheim. All other drugs were obtained from Sigma.

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Carbon-13 Nuclear Magnetic Resonance Study of Osmoregulation in a Blue-Green Alga

Abstract. *The process of osmoregulation in a unicellular blue-green alga,* Synechococcus *sp., has been studied by natural-abundance carbon-13 nuclear magnetic resonance spectroscopy of intact cells and cell extracts. 2-O-α-D-Glucopyranosylglycerol was identified as the major organic osmoregulatory solute. This demonstrates the presence of a major osmoregulatory solute in a blue-green alga and is also an example of an osmoregulatory role for glucosylglycerol.*

All cells that grow in a solution must "osmoregulate" to prevent net water movement across their plasma membranes and consequent cell damage (*1*). Species of eukaryotic algae (*2–5*) and marine bacteria (*6*) achieve this by accumulating specific, low-molecular-weight, neutral organic solutes in their cytoplasm in direct response to the salt concentration of their growth media. By contrast, prokaryotic algae (blue-green algae or cyanobacteria) have been reported to accumulate only inorganic ions (*7*), particularly KCl (*8*), in response to high-saline environments. Up to now extensive accumulation of organic solutes in the cytoplasm has not been demonstrated in this division (Cyanophyta). Therefore, we have investigated osmoregulation in intact cells of a unicellular blue-green alga with the aid of natural-abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy. This technique is a useful new means of studying the process of osmoregulation in marine and halophilic organisms because it enables the identification and quantitation of all major organic solutes in both living cells and cell extracts (*9–11*). The results reported here show that 2-*O*-α-D-glucopyranosylglycerol is the major organic osmoregulatory solute. This demonstrates the presence in a blue-green alga of a major organic osmoregulatory solute and is an example of an osmoregulatory role for glucosylglycerol.

The unicellular blue-green alga, *Synechococcus* sp. Nägeli (Chroococcales, Cyanophyta) (strain RRIMP N 100), isolated from rock surfaces in the marine intertidal zone in Sydney, has a broad salt tolerance in culture, growing in basal medium (*12*) with no added NaCl (that is, 0.03*M* total salts) and in media with NaCl additions up to 1.69*M* total salts (*13*). Figure 1a shows a natural-abundance ^{13}C NMR spectrum of intact *Synechococcus* cells grown in medium with 1.04*M* salts. Except for a prominent resonance from tris buffer present in the growth medium, the only observable resonances come from the glycoside 2-*O*-α-D-glucopyranosylglycerol. A ^{13}C NMR spectrum of the extracellular supernatant recorded after accumulation (Fig. 1a) was complete showed only the tris resonance, which indicates that (i) all the glucosylglycerol was intracellular and (ii) it did not leak out of the cells during spectral accumulation. Figure 1b shows the spectrum of an aqueous extract of completely broken cells. Quantitative comparison of spectra from intact cell suspensions and cell extracts indicates that essentially all of the glucosylglycerol is visible in the former. Therefore, most of the glycoside must be freely mobile in intact cells.

The compound was identified princi-

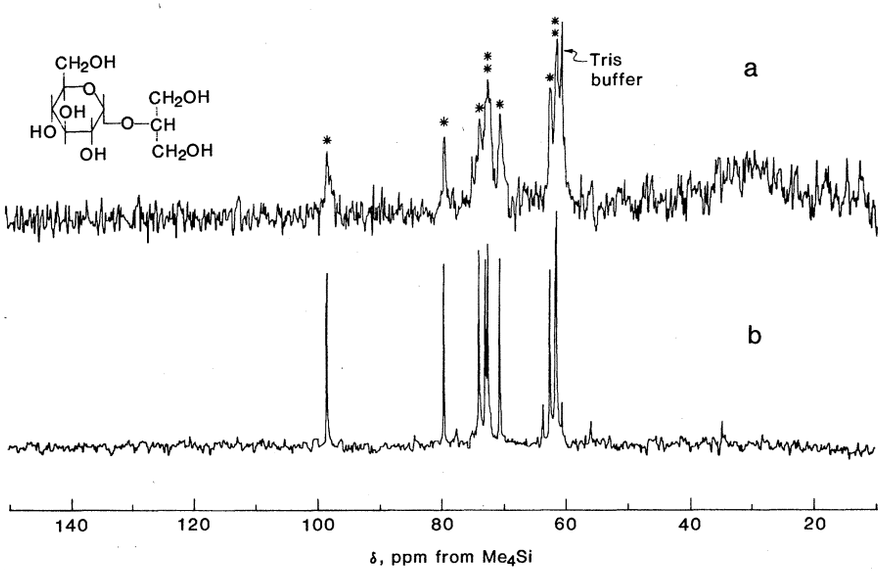

Fig. 1. Natural abundance ^{13}C NMR spectra of (a) intact *Synechococcus* cells (0.13 g, wet weight, per milliliter) grown in medium with 1.04*M* total salts and harvested in the late exponential phase (culture details are given in the legend of Fig. 2) and (b) an aqueous extract of cells (0.4 g, wet weight, per milliliter) grown under the same conditions as in (a). Cells were broken by three freeze-thaw treatments followed by French pressing twice at 18,000 pounds per square inch, the debris was removed by centrifugation, and the resulting supernatant was diluted with D_2O (to 12 percent by volume) to provide a lock signal. Spectra were obtained on a JEOL spectrometer (model FX-60) operating in the pulsed Fourier transform mode at 15.04 MHz and incorporating a 4000-Hz band-pass crystal filter. Sample tubes, 10 mm in outside diameter, were used. For (a) 19,000 scans with a recycle time of 1.0 second (total time, 5.3 hours) were accumulated in 4096 time-domain addresses, a further 4096 addresses with zero value were added before Fourier transformation, and exponential broadening was 1.46 Hz. Sweep width was 2500 Hz, 90° radio-frequency pulses (pulse width, 18 μsec) were employed, and the sample tube was spun at 10 to 15 Hz. Conditions for (b) were as for (a) except that 12,000 scans with a recycle time of 4.0 seconds (total time, 13.3 hours) were accumulated in 8192 time-domain addresses, and exponential broadening was 1.0 Hz. Temperature of the supernatants varied between 26° and 31°C and *p*H between 5.5 and 6.5, depending on composition. The 90° pulse width varied by only 1 to 2 μsec over the range of salt concentrations examined. The resonance at 60.7 parts per million (ppm) arises from the three methylene carbons of tris buffer in the medium. The corresponding quaternary carbon resonance (at 62.7 ppm) is partially saturated under our accumulation conditions. Repeated washing of cells prior to breakage reduced the height of these peaks in extracts below the level indicated in (b). Me_4Si is tetramethylsilane.

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pally by the exact agreement of the ^{13}C chemical shifts observed in the cells and cell extracts with those reported recently for 2-*O*-α-D-glucopyranosylglycerol (*14*). This was confirmed by isolating the compound from an aqueous extract of *Synechococcus* cells by precipitation of protein with 5 percent trichloroacetic acid, followed by desalting on a column of mixed bed ion exchange resin (Amberlite MB 3). This afforded a sample consisting of glucosylglycerol contaminated with about 10 percent free glycerol. The corrected $[\alpha]_D$ was +125° (water), which compared favorably with that reported (*15*) for 2-*O*-α-D-glucopyranosylglycerol ($[\alpha]_D$ +121°).

The concentration of glucosylglycerol in water extracts of cells was determined by ^{13}C NMR spectroscopy with use of the solution and spectral accumulation conditions given in the legend to Fig. 1b. Quantitation was effected by adding known amounts of recrystallized floridoside (2-*O*-α-D-galactopyranosylglycerol) (*16*) to the water extracts and measuring the average height of the resolved single-carbon resonances (*17*).

Figure 2 shows the linear increase in amount of glucosylglycerol per cell mass when the cells are grown in total salt concentrations ranging from 0.03*M* to 1.20*M*. All other free organic solutes are below the detection limit of about 0.5 μmole of solute per milligram of cell protein. At higher total salt concentrations, between 1.20*M* and 1.69*M*, this linear relation between intracellular glucosylglycerol and extracellular salt concentration ceases. These effects are mirrored in the growth parameters (*13*), which indicate increasing cell stress above 1.20*M* total salts.

In terms of numbers of "osmotically active" or nonbound solute particles, the osmolal concentration of glucosylglycerol in the cell water [determined by the procedure described in (*2*)] does not balance the osmolal concentration of ions in the growth media. There is a linear increase in intracellular glucosylglycerol concentration from 0 in 0.03*M* (0.06 osmolal) total salts to 0.51 osmolal in 1.20*M* (2.40 osmolal) total salts. The intracellular concentration, like the content of glucosylglycerol, diverges from a linear relationship with external salt concentration when that concentration exceeds 1.20*M*.

Although glucosylglycerol is the major free organic solute in the cytoplasm, macromolecules and low-molecular-weight solutes at levels below the ^{13}C NMR detection limit must also contribute to the total solute concentration, with inorganic ions completing the balance between intracellular and extracellular solutions. Up to 1*M* (2 osmolal) intracellular KCl, dependent on extracellular salt concentration, has been reported for another member of the Chroococcales, *Aphanothece halophytica* (*8*). In *Synechococcus* sp. more than 0.9*M* KCl would be required to balance the differential between the intracellular and extracellular solutions. We have shown that activity of the cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, is reduced to 25 percent of maximum in assays incorporating 0.9*M* KCl. If this degree of enzyme inhibition is general, it may account for the depression of growth parameters in media containing 1.20*M* or more total salts (*13*).

The biosynthetic pathway and the molecular basis for regulation of levels of glucosylglycerol in the cytoplasm are not known. In fact, the presence of the compound in another blue-green alga has only recently been reported (*14*). However, the compound is structurally analogous to heterosides found in red algae (Rhodophyta). These are floridoside, found in members of the orders Porphyridiales (*18*), Gigartinales and Bangiales (*3*), and 2-*O*-α-D-mannopyranosylglycerate (digeneaside) in the order Ceramiales (*4*). Osmoregulatory functions have been claimed for the red algal heterosides (*3*) but are still in dispute (*19*). The analogy of the 2-*O*-α-D-sugar-glycerol structures and their proposed osmoregulatory roles are of particular interest because of postulated evolutionary relationships between blue-green algae and red algae based largely on their sharing another group of compounds, the phycobilin photosynthetic accessory pigments.

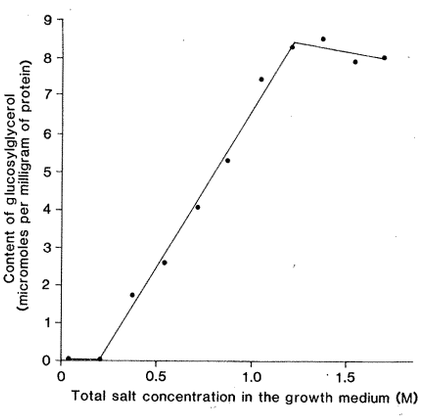

Fig. 2. The increase in glucosylglycerol content of *Synechococcus* cells grown in 20-liter batches in Pintner-Provasoli medium with added NaCl (see text). Cultures were illuminated at 150 μE m^{-2} sec^{-1} with white fluorescent lights, aerated and stirred, and harvested in the late exponential phase. Levels of glucosylglycerol were determined as described in text. Agreement of separate determinations for the same extract was within 5 percent, while duplicate extracts from one culture agreed within 15 percent. Extraction from duplicate cultures was the major source of error, the maximum being 30 percent.

Finally, the technique of natural-abundance ^{13}C NMR is well suited to studies of osmoregulation by organic solutes in prokaryotes, and, with attention to intracellular and tissue compartmentation, to eukaryotes. The advantages over other methods of detecting unknown organic solutes are that the technique is noninvasive and provides information about all classes of free organic solutes present in concentrations above the detection limit. The only potential osmoregulatory solutes not detected are inorganic ions. In the present study it permitted characterization, identification, and quantitation of glucosylglycerol in intact cells and demonstrated that no other free organic compound contributes to any significant extent to osmoregulation in *Synechococcus*.

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13. Growth rate over the range 0.03*M* to 1.20*M* total salts was 0.9 ± 0.2 generations per day, with the exception of a peak at 1.4 generations per day in medium with 0.37*M* salts. At higher NaCl concentrations growth rate decreased linearly from 0.8 generation per day in 1.20*M* total salts to less than 0.2 generation per day in 1.69*M* total salts (the maximum concentration of salts in which growth occurred), culture lag phase increased from 2 to 8 days, and cell yield fell dramatically.
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17. Mass spectrometry confirmed that the ^{12}C:^{13}C ratios in the glucosylglycerol and the floridoside standard were similar. Both compounds are derived, indirectly, from photosynthetic carbon fixation, and, since this process can discriminate against $^{13}CO_2$, this confirmation is necessary to make quantitation based on comparable ^{12}C:^{13}C ratios valid. We thank R. O. Lidgard for these determinations.
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