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Carbon-13 NMR Studies of Salt Shock-Induced Carbohydrate Turnover in the Marine Cyanobacterium *Agmenellum quadruplicatum*[1]

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ABSTRACT

Carbon turnover in response to abrupt changes in salinity, including the mobilization of glycogen for use in osmoregulation was studied with pulse-chase strategies utilizing nuclear magnetic resonance (NMR)-silent and NMR-detectable ^{12}C and ^{13}C isotopes, respectively. Growth of *Agmenellum quadruplicatum* in 30%-enriched ^{13}C bicarbonate provided sufficient NMR-detectability of intracellular organic osmoregulants for these studies. A comparison of NMR spectra of intact cells and their ethanol extracts showed that the intact cell data were suitable for quantitative work, and, when combined with ESR measurements of cell volumes, yielded intracellular glucosylglycerol concentrations without disrupting the cells. NMR pulse-chase experiments were used to show that ^{13}C-enriched glycogen, which had previously been accumulated by the cells under nitrogen-limited growth at low salinities, could be utilized for the synthesis of glucosylglycerol when the cells were abruptly transferred to hypersaline media, but only in the light. It was also shown that the accumulation of glucosylglycerol in the light occurred on a time scale similar to that of cell doubling. Depletion of glucosylglycerol when cells abruptly transferred to lower salinities appeared to be rapid—the intracellular pool of this osmoregulant was decreased 2-fold within 2 hours of hypotonic shock.

Salt-tolerant cyanobacteria synthesize low mol wt organic solutes when grown at supraoptimal NaCl concentrations (7, 11). In general, the major compound(s) synthesized by a cyanobacterium correlates with the maximum salt concentration the strain tolerates (7). Thus, freshwater strains, *i.e.* those whose maximum salinity tolerance is about 0.9 M NaCl or less, synthesize sucrose and trehalose, while marine strains, which range in maximum salt tolerance from about 1.0 to 2.0 M NaCl, synthesize glucosylglycerol. Hypersaline strains, exhibiting maximum tolerances in the range of 2.2 to 3 M NaCl, synthesize the quaternary ammonium compound glycine betaine, sometimes in combination with sucrose or trehalose (7, 12). While identification of the organic solutes which serve as osmoregulants is an important step towards understanding salt tolerance, detailed information on their synthesis, breakdown, and interaction with the total pool of fixed carbon is still lacking.

^{13}C-NMR has proven to be advantageous for identifying organic osmoregulants because it detects all classes of low mol wt organic solutes (7), provides data on their freedom of motion (10), and permits a comparison of intact cells and cell extracts (2). Furthermore, magnetic resonance techniques offer the possibility of noninvasively analyzing molecular transformations *in vivo*. However, studies relying on naturally abundant ^{13}C require large amounts of material, *e.g.* 5 to 10 L of cyanobacterial culture and at least several hours of scanning time (2, 7, 10). These limitations have precluded an analysis of metabolic changes in the carbon pool that occur on shorter time scales.

In the present study, to increase the detectability of intracellular organic solutes, cultures of the marine cyanobacterium *Agmenellum quadruplicatum* (strain PR6) have been provided with bicarbonate enriched with ^{13}C. Such an enrichment strategy was previously exploited (6) for NMR studies of carbohydrates in this marine cyanobacterium, but in that study cells were grown at relatively low salinity and therefore did not accumulate low mol wt organic solutes; hence, prolonged signal acquisition was required for obtaining the NMR spectra. *A. quadruplicatum* grows in a broad range of salinities, from about 0.1 to 1.5 M NaCl (14). This organism synthesizes primarily glucolsylglycerol when grown at high salinity (7), and a more diverse array of organic solutes, including glucose and glycerol derivatives when grown at low salinity (6). Here we have expolited the substantial accumulation of low mol wt carbohydrates that occurs at high growth salinities, together with the strategy of ^{13}C enrichment, to increase the NMR detectability of fixed carbon compounds and to study changes in fixed carbon species in *A. quadruplicatum* subjected to hyper- and hypoosmotic transitions in the dark and in the light.

MATERIALS AND METHODS

Organism and Culture Conditions. Stock cultures of *Agmenellum quadruplicatum* (PR6, ATCC No. 27264, PCC No. 7002) cells, also designated as *Synechococcus sp.*, (kindly provided by Dr. Van Baalen, Institute of Marine Science, University of Texas, Port Arkansas, TX) were grown in batch cultures on ASP-2 medium (14), aerated with 1% CO_2 in air, stirred, and illuminated by cool-white fluorescent light. Cultures for ^{13}C NMR analysis were grown in 250 ml Erlenmeyer flasks containing 100 ml of ASP medium, buffered with 50 mM Tricine (pH 8.4) and 10 mM sodium bicarbonate enriched with 30% ^{13}C at 32 to 34°C. To promote glycogen accumulation, some cells were grown at one-tenth of the nitrate concentrations of the ASP-2 medium, where indicated.

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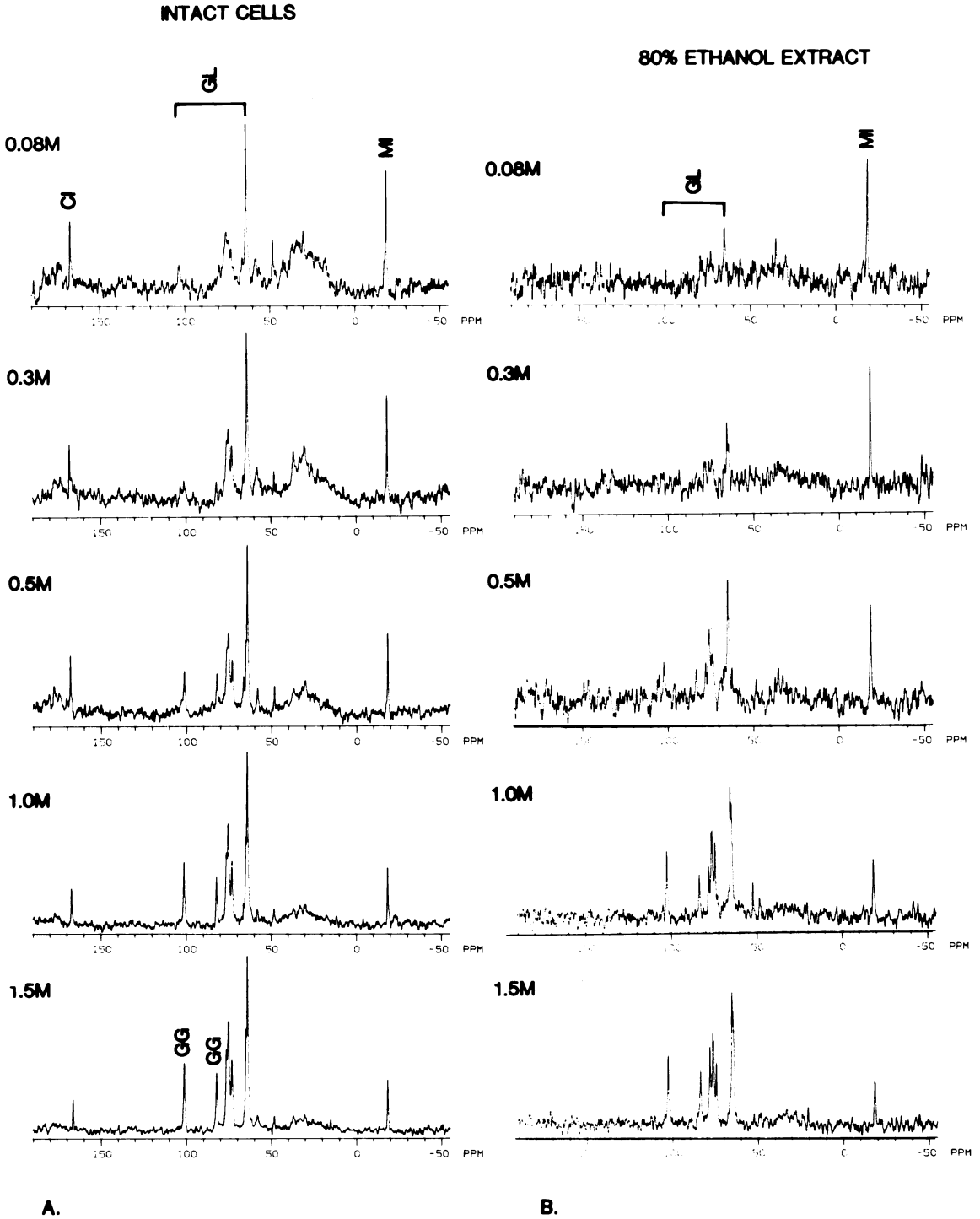

FIG. 1. ^{13}C NMR spectra of *A. quadruplicatum* grown at different salinities. A, Intact cells; B, their 80% ethanol extracts. Chl contents of the 4 ml NMR samples were 0.84, 0.84, 0.76, 0.76, and 0.52 mg for cells grown in 0.08, 0.3, 0.5, 1.0, and 1.5 M NaCl, respectively. CI refers to bicarbonate, GL to glycogen, GG to portions of the glucosylglycerol spectrum that are distinct from glycogen features, and MI refers to methyl iodide.

Biochemical Assays. A glycogen-enriched fraction was prepared according to Ernst *et al.* (5). Cells were boiled in 30% w/v KOH for 2 h and the insoluble material was removed by centrifugation. The KOH soluble material was adjusted to 60% ethanol and maintained on ice for 2 h and then centrifuged to yield a pellet that was analyzed either by NMR or chemical means for undigested glycogen. The ethanol extracts, which contained low mol wt carbohydrates, were also analyzed chemically and by NMR.

Low mol wt organic solutes were extracted from cells by incubating them overnight with 80% ethanol at room temperature (11). Insoluble material was removed by centrifugation (2000*g* for 30 min). The ethanol was removed at reduced pressure and the residue was dissolved in water for chemical and NMR analyses.

Carbohydrate concentrations were estimated by the phenol-sulfuric acid test relative to standard solutions of glycogen or glucose (4). Low mol wt carbohydrates were separated on a

Biogel P2 column (2 × 200 cm) eluted with water. The Chl concentration was determined in methanol extracts using the equations of Mackinney (8).

NMR and ESR[3] Experiments. The harvested cells were concentrated by centrifugation (5000*g* for 10 min) and resuspended in a final volume of 3.6 ml in the growth medium; 0.4 ml of D_2O was added to provide a lock signal. The concentrated cells were placed in a 1 cm diameter NMR tube containing two 1 mm diameter capillaries with degassed methyl iodide as a line height standard. The Fourier transform, proton-decoupled ^{13}C NMR spectra were obtained on the UCB-180 instrument, Chemistry Department, Berkeley, using a line broadening of 20 Hz and 1 s intervals between pulses. NMR data were usually obtained within a few hours of harvesting cells, or, in some cases, with material that had been frozen immediately after harvesting the cells. The effect of the ^{13}C enrichment of the quality of the spectra was tested (results not shown), and it was found that 30% enrichment provided adequate signal-to-noise ratios without undue distortion from carbon-carbon spin interactions; therefore, this enrichment percentage was routinely used. Concentrated cells were treated with 1 mM of a freely permeable nitroxide spin probe, Tempone (2,2,6,6-tetramethyl piperidone-*N*-oxyl) and 90 mM of a membrane-impermeable paramagnetic quenching agent, Mn-EDTA, to elicit the ESR signal of the intracellular spin probe, which was used to determine the cell volume (9). Volume determinations with a series of quencher concentrations showed that 90 mM of tetramethyl ammonium Mn-EDTA did not osmotically compress the volumes; hence, no corrections for osmotic effects were made.

RESULTS

Effect of ^{13}C Enrichment on Detectability of Organic Solutes by NMR. Spectra of intact cells of *Agmenellum quadruplicatum*, grown in media containing a range of NaCl concentrations and 10 mM $NaHCO_3$ enriched with 30% ^{13}C bicarbonate, are shown in Figure 1A. The spectra represent 800 scans of cells containing about 0.8 mg of Chl per sample. The spectra of cells grown in high-salt are comparable to previously published spectra obtained with natural abundance ^{13}C which required at least 10,000 scans and more densely packed cells (2, 7). The peak positions at the highest growth salinities correspond to the resonances of glucosylglycerol (6). Spectra of 80% ethanol extracts (Fig. 1B) are similar to those of intact cells and are dominated by the resonances of glucosylglycerol. At the lower growth salinities, the intact cells exhibit a substantial signal at 66 ppm that is not evident in the ethanol extract. The signal at −18 ppm represents the methyl iodide line height standard.

Effect of Extracellular NaCl Concentration on the Pattern of NMR Detectable Solutes. The effect of increasing growth salinities on the NMR spectra of *A. quadruplicatum* is also shown in Figure 1. At the two highest salt concentrations, the glucosylglycerol spectrum is clearly apparent. At lower salt concentrations the signal-to-noise ratio is reduced, but the prominent spectral feature at about 66 ppm suggests that at least one new molecular species is appearing at lower growth salinities.

Estimation of Intracellular Concentrations of Glucosylglycerol. The spectra in Figure 1 were used to estimate the intracellular concentrations of glucosylglycerol. An 80% ethanol extract of cells grown at 1.5 M NaCl was analyzed by column chromatography and no mono- or disaccharides were detected. The extract was quantitated chemically and used to calibrate an NMR spectrum obtained for the same sample. This calibration point was used to analyze the cellular content of glucosylglycerol of the remaining NMR spectra and, in conjunction with ESR volume measurements of the cells, and was used to determine the intracellular concentrations of glucosylglycerol at the higher growth salinities. Table I summarizes the results of these determinations.

Table I. *Intracellular Glucosylglycerol Content*

Cell volumes were determined with ESR methods (9). NMR determinations were based on average line heights, with reference to the concentration of the ethanol extract of cells grown in 1.5 M NaCl, which had been determined chemically after demonstrating it consisted of a single carbohydrate species.

NaCl in growth medium	Glucosylglycerol Content		
	NMR determination-cells	NMR determination-ethanol extracts	Chemical determination-ethanol extracts
	M		
0.08	ND[a]	ND	0.12
0.3	ND	ND	0.24
0.5	0.42	ND	0.32
1.0	0.60	0.81	0.72
1.5	1.18	1.28	1.20

[a] Not determined.

Time Course of Glucosylglycerol Accumulation. The high sensitivity of carbohydrate analysis in the cells enriched with ^{13}C-bicarbonate permitted the time course of osmoregulant accumulation following salt upshock (sudden increase in salt concentration) to be determined. Figure 2 demonstrates the NMR spectra of equivalent samples, transferred at time 0 from a low salt medium (0.3 M NaCl) to a high salt medium (1 M NaCl), containing the enriched bicarbonate. The samples were harvested at the times indicated, stored at −20°C, and thawed at a later time for NMR analysis. As shown in Figure 2a, the accumulation of glucosylglycerol, from inorganic carbon fixed subsequent to the salinity shock, becomes apparent between 4 and 12 h (Fig. 2b) and does not increase substantially afterwards in the presence of high salt (Fig. 2, c and d). The bicarbonate peak at 158 ppm decreases with time; there is a concominant increase in the glucosylglycerol spectra, which ceases after 12 h. To rule out that depletion of the bicarbonate, as reflected in the disappearance of the 158 ppm NMR feature, caused the plateau in the size of the NMR spectra, experiments with replenishment of the enriched bicarbonate were performed (not shown) and showed no significant increases of glucosylglycerol after about 12 h. This implies that the maximum glucosylglycerol accumulation occurred on the order of 12 h of growth.

Visualization of Intracellular Glycogen. Glycogen can be observed in the cells by NMR (Fig. 3). The broadness of the lines makes observation more difficult but discernible signals are seen when the cells are grown under conditions that promote glycogen accumulation (low salinity, dark-to-light transition). These experiments confirm that glycogen, which had been shown to be present by chemical assays, can be detected, by NMR. Comparison of these spectra with the glucosylglycerol spectra shown in Figure 1 reveals pronounced differences, particularly at 101 ppm, between the monomeric and polymeric carbohydrate species. These data suggest that both glycogen and low mol wt solutes can be discerned when both are present simultaneously. However, because of their relatively poor detectability, glycogen NMR signals can be neglected for quantitating glucosylglycerol cell contents, except for those conditions of low salinity growth that lead to high glycogen and low glucosylglycerol accumulation.

Organic Solute Conversion Subsequent to a Salinity Decrease in the Light. The noninvasive nature of the NMR analysis of cell carbohydrates made it possible to use the same cells that had been analyzed in Figure 1 to examine the effect of hyposaline shock on the status of the osmoregulants in *A. quadruplicatum*. Cells from samples grown in 1.5, 1.0, and 0.3 M NaCl were

[3] Abbreviation: ESR, electron spin resonance.

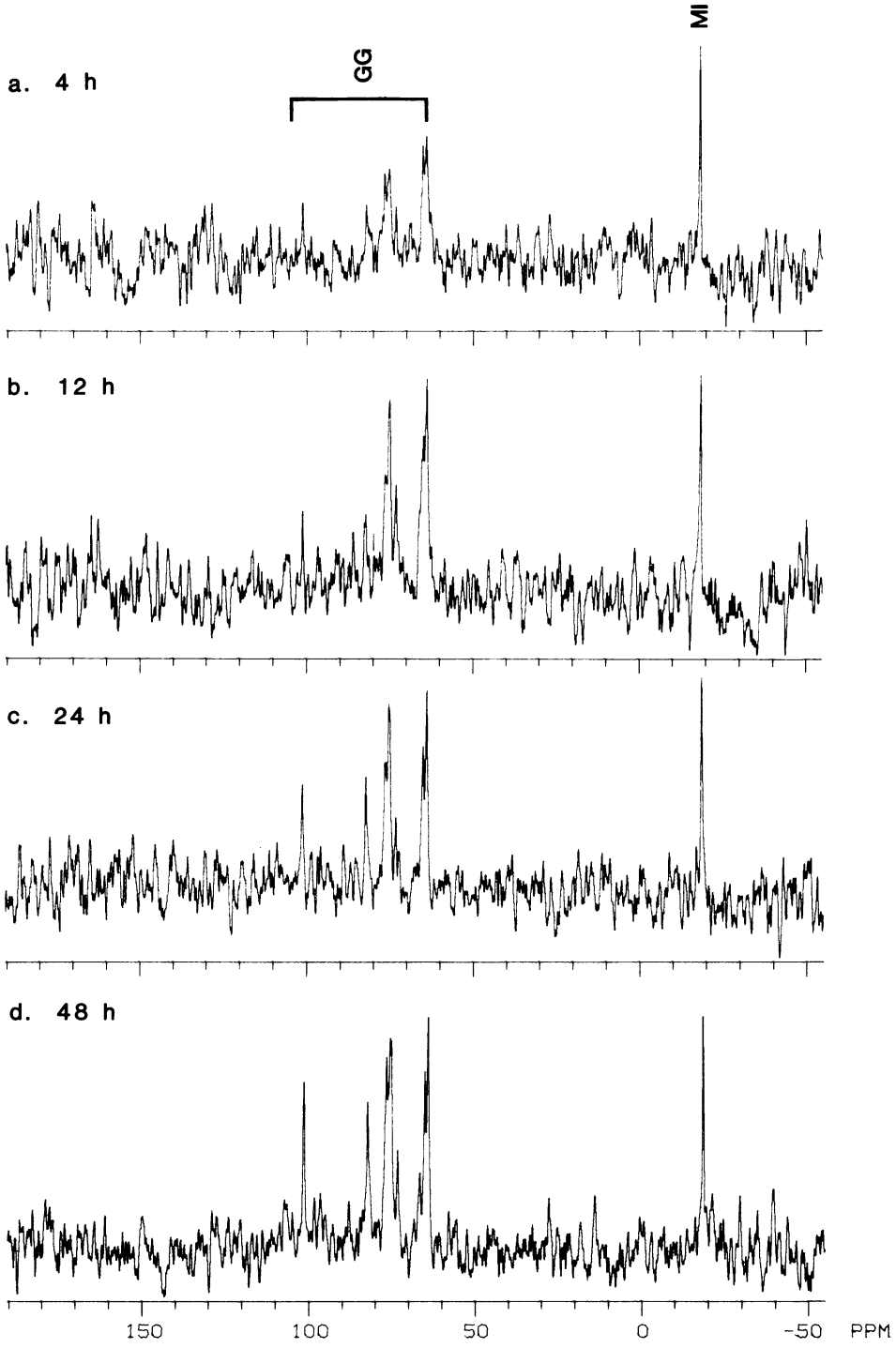

FIG. 2. Time course of the increase in ^{13}C NMR signals. *A. quadruplicatum* were upshocked from 0.3 to 1.0 M NaCl at zero time to a ^{13}C-enriched bicarbonate medium, grown in the light and harvested at the indicated times. Symbols on the spectra are defined in the legend to Figure 1.

resuspended in growth medium containing 0.3 M NaCl, and were maintained in the light for 18 h, harvested, and reanalyzed. These cells exhibited NMR spectra identical to those of cells grown at 0.3 M NaCl, *i.e.* they exhibited spectra characteristic of intracellular glycogen (*cf.* Fig. 3). A preliminary experiment of the time course of the glucosylglycerol disappearance resulting from the salinity decrease showed that approximately half of the compound was lost within 2 h of the hyposaline shock. This high rate of glucosylglycerol disappearance continued until its NMR spectrum could no longer be detected, 8 h after the hyposaline transition (spectra not shown).

Carbohydrate Conversion following Upshock in the Light and in the Dark. The conversion of ^{13}C-enriched glycogen in the light and in the dark was studied in pulse-chase experiments by transferring cells from ^{13}C-enriched low-salinity medium to a high-salinity medium containing nonenriched bicarbonate (Fig. 4). Cells containing high glycogen exhibited a substantial conversion of glycogen to glucosylgylcerol in the light. On the other hand, a low to high salt transition in the dark did not lead to any measurable conversion of glycogen to glucosyl glycerol although a significant glycogen depletion was apparent in the NMR spectra. The latter is evident from the decreased intensity of the broad glycogen spectrum relative to the methyl iodide standard. Evidence for respiratory activity of the cells in the dark is provided by the increase in the bicarbonate ^{13}C NMR feature at 158 ppm, which increases at the expense of the glycogen signal.

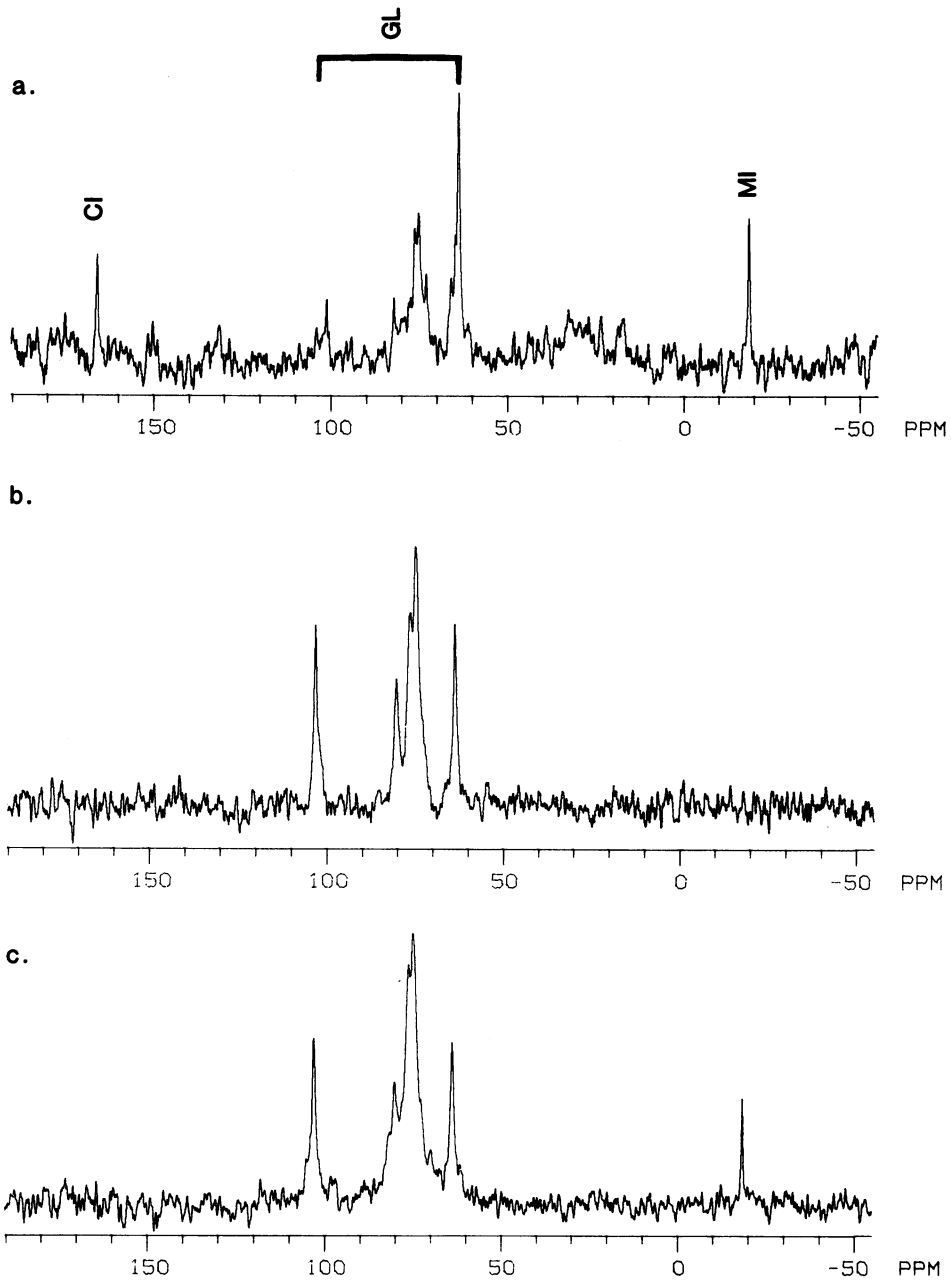

FIG. 3. Glycogen ^{13}C NMR signals in solution and in cells. a, Intact cells grown in 0.3 M NaCl; b, extract of cells shown in (a); c, natural abundance spectra or purified glycogen from mussel (0.2 g/ml). Symbols on the spectra are defined in the legend to Figure 1.

DISCUSSION

The methodology introduced here, consisting of NMR determinations of carbohydrate content and ESR cell volume measurements, provides an entirely noninvasive means of quantitating intracellular organic solute concentrations. We have demonstrated that these methods are suitable, in combination with standard cell fractionation and biochemical analysis, for studying carbon turnover processes, including the quantitative and kinetic determination of interconversion of glycogen, glucosylglycerol, and inorganic carbon for these processes. Although the present study did not attempt to optimize signal-to-noise with more prolonged accumulation of the NMR spectra, the utility of these analytical methods has been established. The data presented here show that in the marine cyanobacterium *A. quadruplicatum*, glucosylglycerol can contribute more than 40% of the internal osmolality needed to counterbalance the external salt concentration (Table I).

The comparison of intact cell and ethanol extract ^{13}C-NMR spectra shows similar patterns only at the higher growth salinities. The appearance of an enhanced feature at about 66 ppm in the intact cell spectra relative to the extracts, suggests that an osmotically significant molecular species occurs in the cells at the lower growth salinities. The failure to observe this feature in the ethanol extracts suggests that it may be an ionic species. The large magnitude of the signal, together with the narrow linewidth, which is characteristic of rapidly tumbling molecules, implies a low mol wt organic species. Kollman *et al.* (6), described NMR features of glycerol, glycerate, and glyceric acid in low salinity-grown *A. quadruplicatum*, but none of these molecular species give NMR line positions that can be reconciled with the 66 ppm peak observed in the present study.

The spectra of glycogen in Figure 3 show a substantial difference between intact cells and cell-free glycogen (evident in the larger magnitudes of the peaks at about 83 and 101 ppm in the cell-free material, labeled GG in the figure legends). The same lines are observed to appear in ethanol extracts of the cells (Fig.

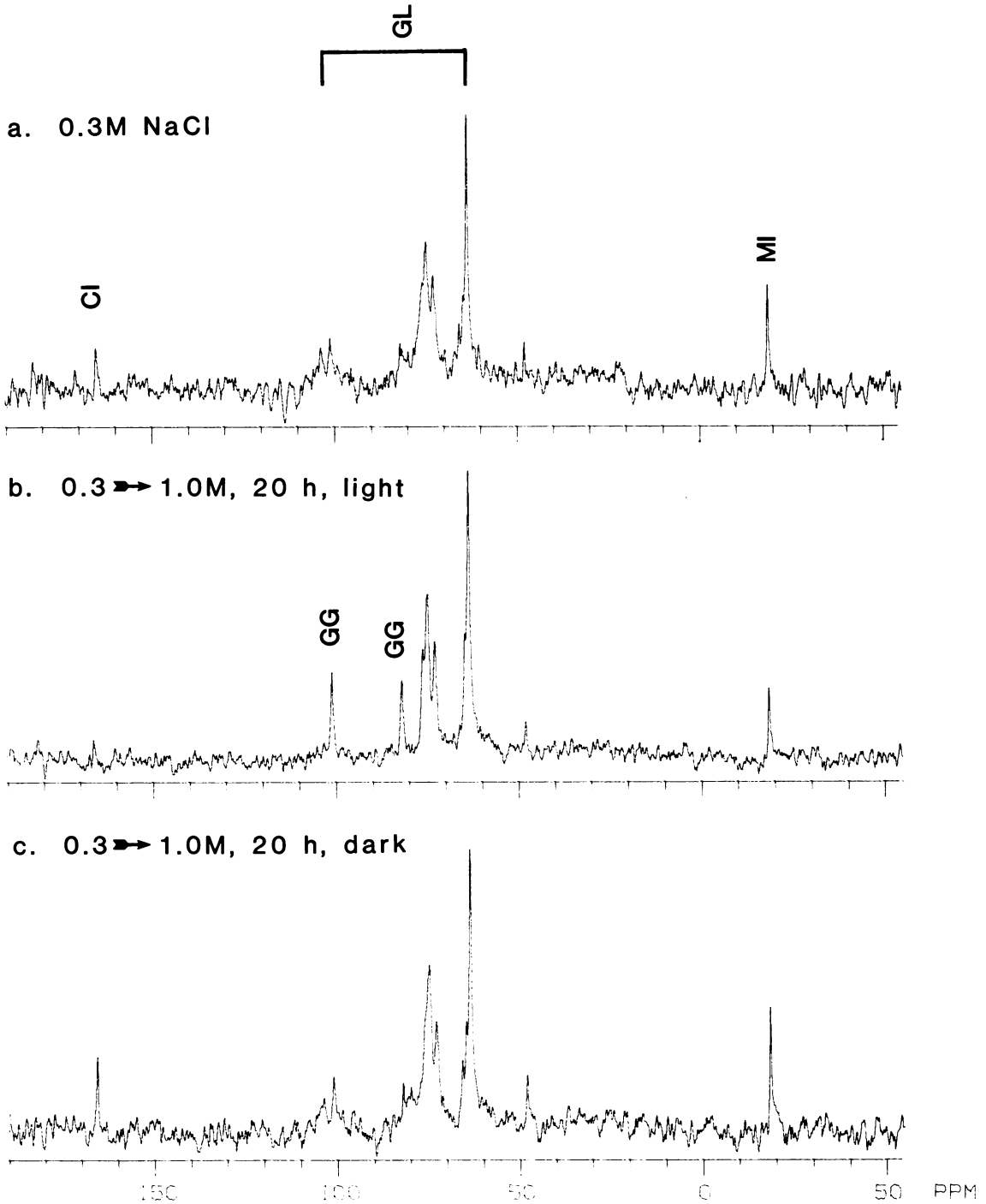

FIG. 4. ^{13}C NMR spectra of cells upshocked from 0.3 to 1.0 M NaCl in the light and in the dark. Cells were grown with enriched ^{13}C and then upshocked in the presence of unenriched bicarbonate. Symbols on the spectra are defined in the legend to Figure 1.

1), supporting the interpretation that these represent glucosylglycerol (which,, unlike glycogen is soluble in 80% ethanol), whereas the other peaks in the range of 66 to 101 ppm are a composite of glycogen and glucosylglycerol.

The time course of glucosylglycerol accumulation in the light after upshock of the cells is comparable to the time course of cell doubling under normal growth conditions. This is consistent with other studies of carbohydrate accumulation (1) and with the observation that the intracellular organic solute pool represents a substantial fraction of the newly fixed carbon. The diversion of fixed carbon to the synthesis of glucosylglycerol can explain, in part, the marked reduction in cell growth rates observed in cells, particularly during the initial period following a salinity increase.

The salinity decrease experiment indicates that glucosylglycerol is depleted while glycogen either is accumulated, or, perhaps, a preexisting glycogen pool is retained in the high salt-grown cells, and that depletion of the accumulated glucosylglycerol is rapid. The most likely fate of the lost carbohydrate material would appear to be incorporation into glycogen, which would conserve the fixed carbon pool. Future studies will seek to quantitate any such carbohydrate conversion, if it occurs. It is also of interest to assess cell viability subsequent to hyposaline shock.

The pulse-chase experiment of Figure 4 demonstrates that glucosylglycerol is produced directly from stored glycogen in the light, since the resuspension of the cells in 10 mM ^{12}C-bicarbonate prior to the hypersaline treatment ensured that essentially all *de-novo* synthesized glucosylglycerol would be NMR silent. The analogous experiment in the dark revealed no conversion of glycogen to glucosylglycerol, although viability of the cells was demonstrated by the appearance of the inorganic carbon NMR signal. Hence, the presence of organic solutes in the salt-treated cells is not mandatory for maintenance of respiratory function.

Previously, it has been observed that cells of another cyanobacterium (*Synechococcus 6311*) take up sufficient salt during hypersaline shock to balance extracellular with intracellular NaCl concentrations. These cells carry out metabolic processes in the light in the presence of substantial intracellular NaCl concentrations but in the absence of high concentrations of intracellular organic solutes. In particular, they are metabolically competent to pump out the internalized NaCl over a period of many hours to reestablish the normal condition of low intracellular sodium (1, 13). Thus, it appears that the presence of organic 'compatible solutes' (3, 15) is not a prerequisite for prolonged cell survival either in the light or in the dark.

These studies and that of Warr *et al.* (16) suggest that osmotic adjustment in cyanobacteria may be accomplished in different ways, depending on nutritional and environmental conditions (light, temperature, salinity). Organic osmoregulants may serve to fulfill both osmotic balance and carbon storage requirements when light is available for photosynthesis. During dark periods, however, these compounds may be required for energy production via respiration, and different osmoregulatory mechanisms, possibly involving a replacement of organic solutes by inorganic ions, may be utilized under these conditions.

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