¹³C Nuclear Magnetic Resonance Study of Biosynthesis of Glucosylglycerol by a Cyanobacterium under Osmotic Stress

By MARK A. MACKAY† AND RAYMOND S. NORTON*

School of Biochemistry, University of New South Wales, Kensington, NSW 2033, Australia

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¹³C nuclear magnetic resonance spectroscopy was used to study the biosynthesis and turnover of *O*-α-D-glucopyranosyl-(1→2)-glycerol (glucosylglycerol) in the marine cyanobacterium *Synechococcus* N100 during osmotic shock. There was a rapid increase in the level of this heteroside following hyperosmotic shock, with most of the solute being synthesized from extracellular carbon via photosynthetic CO₂ fixation. However, changes in the ¹³C labelling patterns during the time course of the response suggested that up to 10% of the newly synthesized material may be derived from intracellular carbon. No increase in glucosylglycerol was observed when cells were subjected to hyperosmotic shock in the dark. These experiments indicated that a good yield of highly ¹³C-enriched glucosylglycerol, which could serve as a source of labelled glucose and glycerol, could be obtained simply and quickly by subjecting this cyanobacterium to hyperosmotic stress in the presence of 90% ¹³C-enriched bicarbonate.

INTRODUCTION

An important means of survival for cells subject to salt stress is the accumulation of internal solutes to maintain osmotic balance with their surroundings. Borowitzka *et al.* (1980) showed, using natural-abundance ¹³C nuclear magnetic resonance (NMR) spectroscopy, that the marine cyanobacterium *Synechococcus* N100 accumulated the heteroside *O*-α-D-glucopyranosyl-(1→2)-glycerol (glucosylglycerol) in response to hyperosmotic stress. Subsequently, it was proposed that marine cyanobacteria could be distinguished by their accumulation of glucosylglycerol as a major osmoregulatory solute and by their related ability to grow in a seawater-based medium containing NaCl at a maximum of 6–11% (w/v) (Mackay *et al.*, 1983). Analysis by ¹³C NMR of the major organic osmoregulatory solutes accumulated by 36 strains of cyanobacteria from a wide range of environmental sources further suggested that a correlation existed between the type of solute accumulated and the maximum salinity tolerated for growth (Mackay *et al.*, 1984). Thus, sucrose and trehalose were the dominant solutes accumulated by strains with the lowest salt tolerance, typically those from freshwater environments (including soil, hot springs, etc.), glucosylglycerol was accumulated by more salt-tolerant strains, typical of a marine environment, and quaternary nitrogen-containing compounds such as glycine-betaine or L-glutamate-betaine, either alone or in combination with sucrose and/or trehalose, were the major organic solutes accumulated by hypersaline strains. Glucosylglycerol accumulation by cyanobacteria under conditions of osmotic stress has also been noted by Erdmann (1983), Richardson *et al.* (1983) and Reed *et al.* (1984).

In this study we have employed ¹³C NMR spectroscopy in conjunction with ¹³C labelling to investigate the biosynthesis and turnover of glucosylglycerol in the marine cyanobacterium *Synechococcus* N100 following an osmotic shock. Numerous studies of metabolism have been carried out in which the fate of ¹³C label(s) has been monitored *in vivo* by ¹³C NMR

† Present address: Department of Clinical Chemistry, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead 2145, Australia.

spectroscopy (Norton, 1980; London, 1982). In the case of osmoregulation this approach has not been utilized previously, although the incorporation of ^{13}C label into *O*- α -D-galactopyranosyl-(1-2)-glycerol (galactosylglycerol) by the red alga *Gigartina corymbifera* (London *et al.*, 1975; Kollman *et al.*, 1979a) and into glucosylglycerol by the cyanobacterium *Agmenellum quadruplicatum* (Kollman *et al.*, 1979b) has been investigated by analysis of ^{13}C NMR spectra of the isolated heterosides.

METHODS

Apparatus. Electromagnetically stirred cultures were grown in three 1 litre quickfit Buchner filter flasks with side arms fitted with 60 ml syringes. The flasks, stoppered with plain dreschel bottle heads from which most of the stem had been removed, were interconnected, outlet to inlet, by heavy-wall silastic tubing. CO_2 -free air, generated by bubbling air through 5 M-NaOH, was blown slowly over the surface of the medium. To stop air leaks into the vessels, a positive pressure was maintained by submersing the gas outlet under distilled water.

Medium. *Synechococcus* N100 from the Australian Collection of Marine Microorganisms, Townsville, Australia, was grown in low-salt medium which contained the following (l^{-1}): NaCl, 14.61 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10.0 g; glycylglycine, 6.6 g; NaNO_3 , 1.5 g; KCl, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g; K_2HPO_4 , 0.03 g; citric acid, 6 mg; ammonium ferric citrate, 6 mg; disodium calcium EDTA, 1 mg; vitamin B_{12} , 20 μg ; A-5 trace elements, 1 ml (Stanier *et al.*, 1971); and 3 M-NaOH, 6.6 ml, to adjust the pH. To ensure that administered carbon of known $^{12}\text{C}/^{13}\text{C}$ ratio was the only source of exogenous carbon, medium was always freshly prepared in autoclaved Millipore Super-Q water. Medium was degassed under vacuum at a temperature above 50°C , then filter sterilized. Solid bicarbonate, either 90 atom % ^{13}C (Stohler Isotope Chemicals) or natural-abundance, was added during the final stages of vacuum filter sterilization to give complete medium with 5 mM- NaHCO_3 and pH 7.7–7.8 at 36 – 40°C .

Growth. Growth was monitored by measuring the optical density at 800 nm. To ensure uniform growth rates and correct $^{12}\text{C}/^{13}\text{C}$ ratio in cells prior to osmotic shock, inocula were precycled with bicarbonate of the appropriate $^{12}\text{C}/^{13}\text{C}$ ratio. After at least five cell doublings under cool white fluorescent light (Sylvania F96T12/CW/HO) at $30\ \mu\text{E m}^{-2}\text{ s}^{-1}$ and 36 – 38°C , 25 ml of inoculum was transferred to fresh medium in each of three 1 litre flasks and the lighting increased to 60 – $80\ \mu\text{E m}^{-2}\text{ s}^{-1}$. When cultures reached an OD_{800} values of 0.02 – 0.05 , lighting was increased to 890 – $1000\ \mu\text{E m}^{-2}\text{ s}^{-1}$. This raised the temperature of the culture, which in most experiments was held in the range 38 – 40°C , the variation within any experiment being $\pm 1.5^\circ\text{C}$.

Prior to salt shock, *Synechococcus* N100 grew exponentially, with each of the three cultures producing almost identical growth rates and OD_{800} values. Doubling times were in the range 160–200 min. Light microscopy revealed that these cultures were composed of approximately 30% single cells, 30% pairs of cells and 40% chains of four cells, indicating rapid growth. During much of the exponential growth phase, cultures produced significant quantities of small bubbles of oxygen. Exponential growth proceeded until at least OD_{800} 0.30, when bicarbonate started to become limiting. Cultures had utilized all available bicarbonate by OD_{800} 0.40, at which point the medium alkalinity had increased to pH 8.0–8.1 at 39°C . Bicarbonate was the only limiting nutrient at this time and if more was added, growth resumed at the original exponential growth rate.

Salt shock. To induce hyperosmotic shock, 200 ml of fresh medium, in which the NaCl concentration was increased to 2.75 M, was added to 800 ml of culture. This produced an increase in NaCl concentration of 0.5 M in the medium. For dark hyperosmotic shock, vessels were wrapped in foil, covered in black plastic and preincubated for 15 min prior to salt shock without added bicarbonate. For all other experiments the 200 ml hypersaline solution contained 25 mM- NaHCO_3 of known $^{12}\text{C}/^{13}\text{C}$ ratio. Zero-time samples were always withdrawn before hyperosmotic shock. Samples of 100 ml from each vessel were combined and cooled, without freezing, using liquid N_2 (usually taking 3–4 min). Sample times were recorded when samples had been cooled to about 5°C . Samples were maintained at 4°C during the following operations: cells were centrifuged at 20800 g for 15 min; cell pellets were frozen and thawed twice, and disrupted by two passages through a French press at 100–124 MPa; the cell walls were removed by centrifugation at 48000 g for 30 min, and the supernatants and pellets were freeze-dried.

^{13}C NMR spectroscopy. The dried supernatants were resuspended in 2 ml D_2O ($\geq 99.75\%$ D, Australian Atomic Energy Commission). ^{13}C NMR spectra were recorded at 25.05 MHz on a Jeol FX-100Q spectrometer operating in the pulsed Fourier transform mode with noise-modulated, on-resonance proton decoupling. Spinning sample tubes of 10 mm outside diameter were used. Typical spectral acquisition parameters were 5000 Hz spectral width, 8192 time-domain addresses, 16 μs (approx. 60°) radio-frequency pulses, 1.42 s pulse recycle time and 1000–32000 scans (depending on the solute concentration and relative abundance of ^{13}C in the cell extract). Sample temperatures were in the range 24 – 28°C depending on salt concentration. Spectra were processed with 1.0 Hz exponential broadening. Glucosylglycerol was identified by comparison of chemical shifts with published values (Norton *et al.*, 1982). Chemical shifts are expressed in p.p.m. downfield from tetramethylsilane.

Quantification of ^{13}C enrichment. To relate measured peak intensities to molar concentrations it was necessary either to record spectra under conditions where the interval between pulses was sufficient to ensure complete relaxation of the carbons under study or to employ acquisition parameters which, while not necessarily permitting complete relaxation between pulses, had a constant effect on intensities throughout the series of experiments, such that relative intensities could be measured accurately. The spin-lattice relaxation times of carbons in natural-abundance glucosylglycerol are in the range 0.4–0.8 s (Norton *et al.*, 1982), so to ensure complete relaxation between 90° radiofrequency pulses, a recycle time of 3 s would have to be used. In the present experiments about 60° radiofrequency pulses and a recycle time of 1.42 s were used. Comparison of these spectra with those recorded using longer recycle times (up to 8 s) showed that there were no significant perturbations of peak intensities. These comparisons also confirmed that glucosylglycerol was stable under the measurement conditions.

All of the carbons in natural isotopic abundance glucosylglycerol have at least one directly-bonded hydrogen, so their relaxation was dominated by ^{13}C – ^1H dipolar interactions (Norton *et al.*, 1982). They therefore displayed complete nuclear Overhauser enhancement (NOE) values of 2.99 under conditions of complete proton decoupling, as employed here. ^{13}C – ^{13}C dipolar relaxation due to adjacent ^{13}C isotopes can be ignored for carbons with directly-attached hydrogens, even at 90% enrichment (Moreland & Carroll, 1974).

Peak intensities were measured by cutting and weighing (to 0.01 mg) spectra recorded on paper. Contributions of the isotopomers leading to splitting of the C-2' resonance were determined as in Kollman *et al.* (1979a) with the following exception: when the total weight of the upfield and downfield peaks of the triplet of the C-2' resonance was greater than the weight of the central peak, the contribution of the triplet was assumed to be equal to the weight of the central peak plus upfield and downfield peaks and the contribution of the singlet nil.

RESULTS AND DISCUSSION

Fig. 1 shows ^{13}C NMR spectra of cell-free extracts of *Synechococcus* N100 grown on 90% ^{13}C -enriched $\text{H}^{13}\text{CO}_3^-$ (Fig. 1a) and following hyperosmotic shock in the presence of 90% $\text{H}^{13}\text{CO}_3^-$ (Fig. 1b). In each case the spectrum was dominated by resonances from glucosylglycerol, the level of which increased dramatically following salt shock. The resonances from C-1 of the glucose moiety and C-2' of the glycerol moiety were well resolved, but those of C-2 to C-5 of the glucose moiety overlapped with one another, as did those of C-1' and C-3' of the glycerol and C-6 of the glucose. Thus, while average levels of ^{13}C enrichment could be determined for the latter two groups of carbons, detailed analyses were possible only for C-1 and C-2'. No accumulation of intermediates in the biosynthesis of glucosylglycerol was observed in the spectra.

Expanded views of the C-1 and C-2' resonances from Fig. 1b are shown in Fig. 2. The degree of enrichment at C-2 of the glucose was determined from the relative intensities of the doublet and singlet components of the C-1 resonance, while that at C-1' and C-3' of the glycerol was indicated by the doublet and triplet components of the C-2' resonance. The triplet was in fact a doublet of doublets (Kollman *et al.*, 1979a) in which the two central peaks were not resolved. The degrees of enrichment at C-1 and C-2' were determined from the total intensities of each set of resonances.

Fig. 3 shows the timecourses of glucosylglycerol accumulation following hyperosmotic shock of *Synechococcus* N100 in the light and in the dark. In the light the parallel increase in intensities of the C-1 and C-2' resonances indicated uniform random labelling of the newly-synthesized glucosylglycerol. The rapid and linear increase in glucosylglycerol was maintained for about 70 min. After about 100 min the glucosylglycerol content had increased approximately 4.8-fold, equivalent to the increase expected on the basis of a previous natural-abundance ^{13}C NMR study (Borowitzka *et al.*, 1980). OD_{800} measurements indicated that exponential growth had resumed by about 110 min and the subsequent increase seen in glucosylglycerol content was attributable to increasing cell mass. Osmotic equilibrium was achieved well within one generation time.

As this experiment was done in the presence of 90% $\text{H}^{13}\text{CO}_3^-$, on cells grown in 90% $\text{H}^{13}\text{CO}_3^-$, the level of enrichment was high throughout. C-2 of the glucose moiety showed 90% enrichment at zero time, and remained at 89–91% during the time course, while C-1' and C-3' of the glycerol showed 85% initially, increasing to 90% by 90–100 min.

Fig. 3 also shows the results obtained when cells were salt shocked in the dark without bicarbonate. The level of glucosylglycerol and the level of ^{13}C enrichment (86–89%) remained constant for at least 200 min, while the OD_{800} of the culture after 240 min was the same as

Fig. 1. ^{13}C NMR spectra of cell-free extracts of *Synechococcus* N100 grown on 90% enriched $\text{H}^{13}\text{CO}_3^-$ at a total NaCl concentration of 0.25 M (a), and 171 min after hyperosmotic shock (final NaCl concentration 0.75 M) in the presence of 90% $\text{H}^{13}\text{CO}_3^-$ (b). The spectrum in (a) was recorded with 4000 scans, that in (b) with 2000 scans.

Fig. 3

Fig. 4

Fig. 3. Time courses of the intensities of the C-1 (○, ●) and C-2' (△, ▲) resonances of glucosylglycerol following hyperosmotic shock in the presence of 90% enriched $\text{H}^{13}\text{CO}_3^-$ in the light (○, △) or the dark (●, ▲).

Fig. 4. Time courses of the degrees of ^{13}C enrichment in C-2 of the glucose moiety (○) and C-1' and C-3' of the glycerol moiety (△) of glucosylglycerol, as detected by the splitting patterns of C-1 and C-2', respectively, when cells grown on natural-abundance bicarbonate were subjected to hyperosmotic shock in the presence of 90% enriched $\text{H}^{13}\text{CO}_3^-$.

immediately after salt shock. These results reflect primarily the lack of photosynthetic carbon fixation, but it is noteworthy that in the dark ATP production is slowed to about 10% of its value in the light (Bottomley & Stewart, 1976). This could slow the incorporation of intracellular carbon into glucosylglycerol during salt shock, with the expected 25–45% contribution (see below) being reduced to 2.5–4.5%, which would be barely detectable. Despite their failure to synthesize glucosylglycerol in response to hyperosmotic shock in the dark, the cells seemed to maintain their integrity.

The above results suggested that photosynthesis was necessary to enable *Synechococcus* N100 to respond to a hyperosmotic shock by producing glucosylglycerol. They did not establish unequivocally that the major source of carbon for this solute was new photosynthate. In order to demonstrate this, cells which had been grown on natural-abundance bicarbonate were subjected to hyperosmotic shock in the presence of 90% $\text{H}^{13}\text{CO}_3^-$ (Fig. 4). The rate of increase in the level of ^{13}C -labelled glucosylglycerol in this case was about 92% of that observed in the corresponding experiment on cells grown on enriched bicarbonate (cf. Fig. 3). Within 30 min, the level of ^{13}C enrichment, as measured by splitting of the resonances of C-1 of the glucose moiety and C-2' of the glycerol moiety, had reached 90%, where it remained for the rest of the experiment (Fig. 4). However, 15 min after the hyperosmotic shock the level of enrichment of C-2 of the glucose moiety was significantly lower than that of C-1' and C-3' of the glycerol moiety.

When the complementary experiment was done, that is cells grown on 90% enriched $\text{H}^{13}\text{CO}_3^-$ were salt shocked in the presence of natural-abundance bicarbonate, a significant increase in the intensities of all resonances of glucosylglycerol was observed during the first hour, following which there was a slight decline. The magnitude of the increase was greater, however, than would be predicted if all of the new glucosylglycerol were synthesized from extracellular carbon.

Fig. 2. Expanded views from Fig. 1(b) of the resonances from C-1 and C-2' of glucosylglycerol in an extract from cells 171 min after hyperosmotic shock in the presence of 90% enriched $\text{H}^{13}\text{CO}_3^-$.

Fig. 5. Changes in the relative proportions of the various isotopomers at C-2 of the glucose moiety and C-1' and C-3' of the glycerol moiety of glucosylglycerol when cells grown on 90% enriched $\text{H}^{13}\text{CO}_3^-$ were salt shocked in the presence of natural-abundance bicarbonate. ●, Proportion of labelled C-2; □, proportion of $^{13}\text{C}-1'-^{13}\text{C}-2'-^{13}\text{C}-3'$; △, proportion of $^{13}\text{C}-1'-^{13}\text{C}-2'-^{12}\text{C}-3'$ plus $^{12}\text{C}-1'-^{13}\text{C}-2'-^{13}\text{C}-3'$; ○, proportion of $^{12}\text{C}-1'-^{12}\text{C}-2'-^{12}\text{C}-3'$.

If the overall concentration of glucosylglycerol increased approximately 4.8-fold (see above), and if all of the newly synthesized material contained 1.1% ^{13}C , equivalent to the natural-abundance level of the bicarbonate in the medium, then the resonance intensities would have increased by only about 5%. The observed increases were in the range 30–50%, the greatest increase being observed for C-2' of the glycerol moiety. Thus, contributions from sources other than extracellular carbon must have accounted for 25–45% of the increase in resonance intensity, equivalent to 6–11% of the additional glucosylglycerol required to balance the hyperosmotic stress.

Thus, in both experiments where the isotopic composition of the bicarbonate was changed upon salt shock, there was evidence of a contribution to newly synthesized glucosylglycerol of up to 10% from intracellular carbon. A possible source of this carbon may be CO_2 released by increased respiration in the light, due to addition of bicarbonate, and immediately refixed. Scherer & Böger (1982) have shown that in cyanobacteria CO_2 is evolved by respiration in the light and immediately refixed without liberation.

Changes in the relative proportions of the various isotopomers at C-2 of the glucose moiety and C-1' and C-3' of the glycerol moiety when cells grown on 90% enriched $\text{H}^{13}\text{CO}_3^-$ were salt shocked in the presence of natural-abundance bicarbonate are shown in Fig. 5. C-2 was 90% enriched at zero time, and this declined to 82% during the response. Initially C-1' and C-3' were 86–87% enriched, corresponding to relative proportions of 0.76 for the isotopomer containing $^{13}\text{C}-1'$ and $^{13}\text{C}-3'$, 0.24 for that containing $^{13}\text{C}-1'$ or $^{13}\text{C}-3'$ and zero for that containing $^{12}\text{C}-1'$ and $^{12}\text{C}-3'$. During the first 30 min the relative contribution of the first isotopomer decreased and those of the second and third increased. For the next 70–80 min no further change was observed in the relative contribution of the doubly-labelled glycerol, but there was a slower decline in that of the triply-labelled glycerol and a corresponding increase in that of the singly-labelled glycerol. These changes are consistent with significant turnover of ^{12}C (from photosynthate) and exchange with ^{13}C (from intracellular carbon) in the glycerol moiety of glucosylglycerol during the period of adjustment to salt shock. After 100 min, when exponential growth resumed, there was no evidence of further net turnover in the glycerol moiety.

In order to determine whether glucosylglycerol turnover was significant in the absence of osmotic stress, cells which had been grown on natural-abundance bicarbonate were switched to 90% enriched bicarbonate without any change in the total salt concentration. During the 2 h period for which spectra were examined, no significant labelling of glucosylglycerol was observed, but there was significant labelling of a polysaccharide, the ^{13}C chemical shifts of which were consistent with those of glycogen. This indicates that under these conditions glucosylglycerol was either not turning over at all, or was turning over in exchange with an endogenous pool of carbon which was not diluted significantly with exogenous ^{13}C during the 2 h period of this experiment. During this experiment the OD_{800} increased by approximately 25%. The absence of ^{13}C label in the newly synthesized glucosylglycerol associated with this increase in cell mass requires further investigation.

In summary, we have shown, using ^{13}C NMR spectroscopy, that *Synechococcus* N100 responds to hyperosmotic shock by accumulating glucosylglycerol. Most of this solute was synthesized from extracellular carbon via photosynthetic fixation, but the changes detected in the ^{13}C labelling patterns during the time course of the response suggest that up to 10% of the newly-synthesized material may be derived from intracellular carbon, presumably polysaccharide.

Previously, Kollman *et al.* (1979*a, b*) have employed a marine red alga *Gigartina corymbifera* to label galactosylglycerol, from which ^{13}C -labelled galactose and glycerol could be produced, and a cyanobacterium *Agmenellum quadruplicatum* to label glucosylglycerol. The results presented in this paper suggest that a useful alternative means of achieving a high degree of ^{13}C labelling using *Synechococcus* N100 would be to accumulate cell mass by growth on natural-abundance bicarbonate, then subject the cells to hyperosmotic stress in the presence of $\text{H}^{13}\text{CO}_3^-$ in the light. Most of the glucosylglycerol synthesized during the osmotic response would be derived from the labelled bicarbonate, with very little coming from intracellular carbon. This procedure offers the advantage that cells could be grown on a natural-abundance carbon source, which is cheap and requires no precautions such as the exclusion of atmospheric CO_2 . Labelling could then be achieved quickly (within 2 h) and with highly efficient incorporation of ^{13}C label. If uniform 90% ^{13}C enrichment were required, it would be desirable to also grow the cells on ^{13}C -labelled bicarbonate, but even then, the use of a hyperosmotic shock offers a useful way of achieving rapid synthesis of a significant quantity of labelled material.

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