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The physical state of osmoregulatory solutes in unicellular algae

A natural-abundance carbon-13 nuclear-magnetic-resonance relaxation study

By Raymond S. NORTON,* Mark A. MacKAY and Lesley J. BOROWITZKA
Roche Research Institute of Marine Pharmacology, Dee Why, N.S.W. 2099, Australia

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Natural-abundance ^{13}C n.m.r. spin-lattice relaxation-time measurements have been carried out on intact cells of the unicellular blue–green alga *Synechococcus* sp. and the unicellular green alga *Dunaliella salina*, with the aim of characterizing the environments of the organic osmoregulatory solutes in these salt-tolerant organisms. In *Synechococcus* sp., all of the major organic osmoregulatory solute, 2-*O*-α-D-glucopyranosylglycerol, is visible in spectra of intact cells. Its rotational motion in the cell is slower by a factor of approx. 2.4 than in aqueous solution, but the molecule is still freely mobile and therefore able to contribute to the osmotic balance. In *D. salina*, only about 60% of the osmoregulatory solute glycerol is visible in spectra of intact cells. The rotational mobility of this observable fraction is approximately half that found in aqueous solution, but the data also indicate that there is a significant concentration of some paramagnetic species in *D. salina* which contributes to the overall spin-lattice relaxation of the glycerol carbon atoms. The non-observable fraction, which must correspond to glycerol molecules that have very broad ^{13}C resonances and that are in slow exchange with bulk glycerol, has not been properly characterized as yet, but may represent glycerol in the chloroplast. The implications of these findings in relation to the physical state of the cytoplasm and the mechanism of osmoregulation in these cells are discussed.

Cells that grow in a solution must 'osmoregulate' to prevent net water movements across their plasma membranes and consequent cell damage (Borowitzka, 1981). Many marine and halophilic species achieve this by accumulating in their cytoplasm low-molecular-weight organic solutes, the concentrations of which respond to changes in the solute concentration of the external medium. In order for these intracellular solutes to function effectively in maintaining the cell iso-osmotic with its surroundings, they should be freely mobile within the cytoplasm. Immobilization by, for example, strong binding to macromolecules in the cell would diminish substantially the contribution of these solutes to the decrease in intracellular water activity. However, measurements of the intracellular concentrations of putative osmoregulatory solutes are invariably carried out after disruption of the cell, a procedure that may dissociate any interactions of these solutes with macromolecules, thereby giving an artificially high value for the concentration of free solute in the intact cell. Furthermore, it has not been established for any organic osmoregulatory solute that the unbound fraction enjoys sufficient mobility to fulfill its postulated role in osmoregulation. In the present paper we show that ^{13}C n.m.r. spectroscopy may be used to characterise the state of osmoregulatory solutes in intact cells of salt-tolerant algae, without the need for prior ^{13}C enrichment.

Recently it has been found that the natural-abundance ^{13}C n.m.r. spectra of intact cells and cell extracts from marine and halophilic organisms are dominated by resonances from low-molecular-weight organic solutes, which, in many cases, play a role in osmoregulation (Norton, 1979; Norton & de Rome, 1980; Borowitzka *et al.*, 1980; Norton, 1980). However, no attempt has been made to quantify the freedom of motion of the osmoregulatory solutes in intact cells. This can be achieved by n.m.r. spectroscopy by measurement of spin-relaxation parameters. In the present study we

Abbreviation used: n.O.e., nuclear Overhauser enhancement.

* Present address and address for reprint requests: School of Biochemistry, University of New South Wales, Kensington, 2033, Australia.

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have measured ^{13}C spin-lattice relaxation times (T_1) and nuclear Overhauser enhancements (n.O.e.) for the major organic osmoregulatory solutes in intact cells of the unicellular blue–green alga *Synechococcus* sp. (a marine isolate) and the unicellular halophilic green alga *Dunaliella salina* (a salt-lake isolate), and have determined if the total pools of these osmoregulatory solutes are visible in spectra of intact cells.

Materials and methods

Chemicals

Glycerol and urea were obtained from Ajax Chemicals, Sydney, N.S.W., Australia, as analytical-grade reagents, and ^2H_2O (>99.7% ^2H) from Merck, Sharp and Dohme, Montreal, Canada. Floridoside (2-*O*-α-D-galactopyranosylglycerol) was isolated from the red alga *Delisea fimbriata* by extraction with methanol, partition of the extract between ethyl acetate and water, chromatography of the water-soluble fraction on a column of mixed-bed ion-exchange resin (Amberlite MB 3; Ajax Chemicals), and crystallization from methanol. The m.p. and $[\alpha]_D$ were in good agreement with literature values (Charlson *et al.*, 1956). 2-*O*-α-D-Glucopyranosylglycerol was isolated from the *Synechococcus* isolate described below by extraction of disrupted cells with methanol, chromatography of the water-soluble component on Amberlite MB 3 resin, then high-pressure liquid chromatography on a semi-preparative scale column containing C18 reverse-phase silica (Whatman Partisil-10 M9/ODS-2). This afforded more-than-95% pure glucosylglycerol $[\alpha]_D + 126°$ ($c = 1\,g \cdot dl^{-1}$ in water) literature value + 121° (Charlson *et al.*, 1956).

Growth of algae

D. salina (strain RRIMP N25), isolated from Lake MacLeod in Western Australia, was grown in the medium described by Johnson *et al.* (1968) without $VOCl_2$ (vanadyl dichloride) but containing NaCl at $125\,g \cdot litre^{-1}$ and Tris buffer at $0.6\,g \cdot litre^{-1}$, adjusted to pH 8.0. *Synechococcus* sp. (strain RRIMP N100) was grown in the medium of Pintner & Provasoli (1958) containing $60\,g \cdot litre^{-1}$ NaCl at pH 7.4. Both algae were grown at 22–23°C in 20-litre batch cultures, with aeration, stirring and illumination at $70\,\mu E \cdot s^{-1} \cdot m^{-2}$ by cool-white fluorescent lights. Cells were harvested at late exponential phase by centrifugation. Samples for n.m.r. analysis were resuspended in a medium essentially identical with that on which the cells were grown, except for the presence of 50% (v/v) ^2H_2O.

^{13}C n.m.r. spectroscopic methods

^{13}C n.m.r. spectra were obtained at 15.04 MHz on a Jeol FX-60 spectrometer essentially as described previously (Norton, 1979; Borowitzka *et al.*, 1980). Spin-lattice relaxation times (T_1) were determined by use of a $(180°\text{-}\tau\text{-}90°\text{-}t)_n$ pulse sequence. The delay time, t, between each 90° pulse and the following 180° pulse was more than 3.5 times the longest T_1 being measured in all cases. T_1 values were determined from a least-squares fit of the exponential-magnetization-recovery curve M_τ versus τ, where M_τ is the initial value of the magnetization after a 90° pulse at time τ after the 180° pulse. Only values of $\tau \lesssim T_1$ were employed in calculating the T_1 values reported here. Estimated accuracy of T_1 values is 5–10% for model compounds and 15–20% for intact cells. Integrated intensities were measured digitally from spectra of model compounds and by cutting and weighing peaks in spectra of intact cells. In all cases fully-relaxed spectra recorded with 90° pulses were analysed. The n.O.e. values were obtained by comparing intensities in spectra recorded with complete ^1H decoupling with those in spectra recorded with ^1H decoupling gated on only during data acquisition and a delay of $10 \times T_1$ between pulses (Opella *et al.*, 1976). Estimated accuracy of n.O.e. values is ±0.3 for spectra of model compounds, ±0.5 for intact cells.

Spectra of intact cells

Spectra were accumulated in the dark at temperatures in the range 29–34°C. Samples were open to the air, but were not bubbled with any gas.

The *Synechococcus* sp. has a cell wall that renders it fairly resistant to damage, so the sample tubes (10 mm outer diameter) could be spun. Under these conditions the cells slowly settled in the n.m.r. tube, giving rise to an increase in the signal intensity over the first 4–5 h, after which there was no further change. For experiments requiring long spectral accumulation times (e.g. T_1 and n.O.e. measurements) the cells were allowed to settle before the start of the experiment, whereas for short runs the cells were resuspended every 20 min or so. These cells showed no leakage of glucosylglycerol or phycobilin pigment after periods of 24 h or more in the probe.

D. salina is a much more fragile organism, with a very reduced cell wall, and, under the conditions employed here, could not sustain sample spinning without considerable cell damage and consequent leakage of intracellular constituents. However, because the cells are motile, settling did not occur. Cells retained their motility and characteristic pear shape for longer periods if they were removed from the probe every 15–20 min and shaken gently to aerate the suspension. Bubbling with air caused unacceptable cell damage. Even under optimal conditions, suspensions of *D. salina* were kept in the probe for only about 4 h, in order to avoid any significant leakage of glycerol into the medium.

Experiments designed to determine what fraction of the total intracellular pool of solute was observed in the spectra were carried out by preparing a suspension of intact cells as described above. Some material was removed for whole-cell n.m.r. and the remainder disrupted, *D. salina* at 4°C by three bursts of 3kJ in a Braun sonicator, and *Synechococcus* sp. by two freeze–thaw cycles and passage through a French pressure cell twice at 124MPa and 4°C. Whole-cell material after n.m.r. was centrifuged at 4000 *g* for 10min, the pellet wet weight was recorded and the supernatant assayed by ^{13}C n.m.r. for osmoregulator leakage. Disrupted cell material was assayed both unaltered and after denaturing the protein present by addition of solid urea to a final concentration of 8M. Urea was chosen in preference to 6M-guanidinium chloride because the high electrolyte concentration in the latter significantly reduced the signal intensity (see below), whereas urea had no effect.

Effect of salt

It is well known that electrolytes can have deleterious effects on ^{13}C n.m.r. spectra recorded with full ^{1}H decoupling, although it is widely accepted that the problems are minimal at low resonance frequencies. At the high salt concentrations (up to 2M-NaCl) employed here, significant effects are observed even at 15.04MHz. For a 10% solution of dioxan in $^{2}H_2O$, the 90° pulse width for ^{13}C increases from 16μs in the absence of NaCl to 19μs in 1M- and 20μs in 2M-NaCl. Corresponding sample temperatures are 30, 32 and 35°C. The most dramatic effect, however, is on signal intensity, which, for a 10% (v/v) solution of dioxan, decreases to 70% of the value in $^{2}H_2O$ on addition of 0.25M-NaCl, then to 61% in 1.0M-NaCl, and 59% in 2.0M-NaCl. This decrease is not accompanied by any loss of n.O.e. or effective proton decoupling power [$H_2 \simeq 0.2$mT (2G)] or by an increase in linewidth. These intensity losses would have to be taken into consideration in experiments where the total salt concentration was changed significantly, but did not create problems in the present study, in which total salt concentrations in growth media were in the range 1–2M.

Results

Intact cells

Figs. 1(*a*) and 1(*b*) show natural-abundance ^{13}C n.m.r. spectra of suspensions of intact cells of *D.*

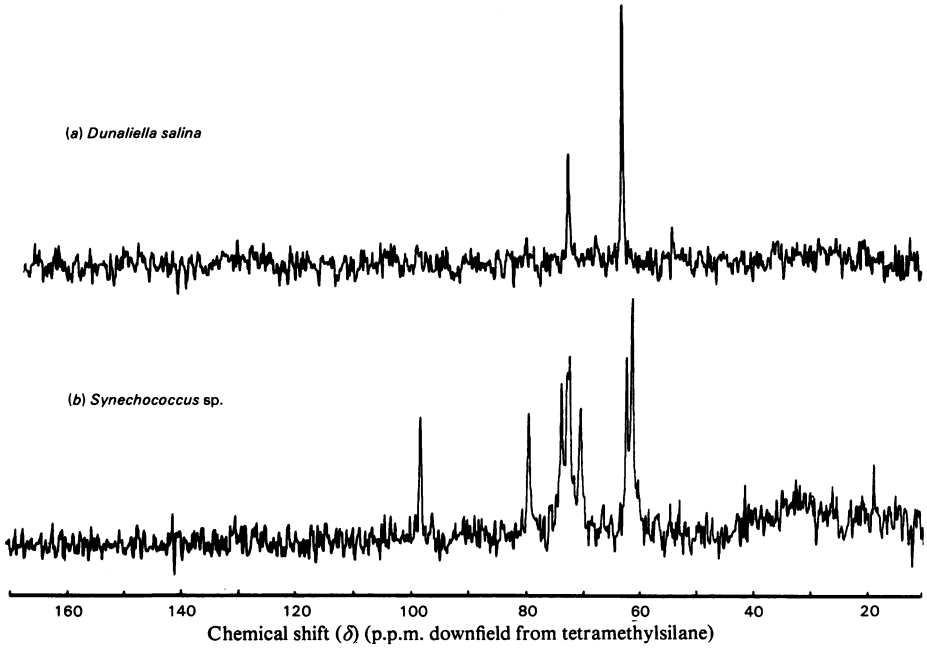

Fig. 1. *Natural-abundance ^{13}C n.m.r. spectra of intact algal cells recorded at 15.04MHz under conditions that ensure complete relaxation of the relevant ^{13}C nuclei between pulses*

(*a*) *Dunaliella salina* cells (0.16g/ml) in ^{2}H-labelled medium containing 12.5% NaCl (1000 scans, 90° pulses, recycle time 4.5s, exponential broadening 1.46Hz). (*b*) *Synechococcus* sp. cells (0.15g/ml) in ^{2}H-labelled medium containing 6% NaCl (8400 scans, 90° pulses, recycle time 1.5s, exponential broadening 1.60Hz). Vertical gain is half that of (*a*).

salina and *Synechococcus* sp. respectively. In each spectrum only one major solute is observed, glycerol in *D. salina* (Borowitzka & Brown, 1974), and 2-*O*-α-D-glucosylglycerol in *Synechococcus* sp. (Borowitzka *et al.*, 1980). Spectra of the extracellular supernatants recorded after completion of the spectra in Fig. 1 showed that the solutes observed are intracellular, and that there is no significant leakage from the cells during data accumulation.

The ^{13}C chemical shifts of glycerol and glucosylglycerol in intact cells are identical within experimental error with those measured for these solutes in aqueous solution. The linewidths are considerably broader, but because of the difficulty in measuring accurate natural linewidths, we have used spin-lattice relaxation-time measurements to characterize the motional freedom of these molecules in intact cells. T_1 and n.O.e. values for intact cells of *D. salina* and the *Synechococcus* are given in Table 1.

In order to determine if there are tightly bound fractions of glycerol or glucosylglycerol which are not observed in spectra of intact cells, the intensities of resonances observed in spectra of intact cells have been compared with those observed after disruption of the cells. Spectra for this purpose were recorded without n.O.e. by using gated ^1H decoupling. For *D. salina*, suspensions of broken cells still containing the debris give glycerol CH and CH_2 resonances with 112 and 110% respectively of the intensities in spectra of intact cells, indicating that very little glycerol is immobilized in the intact cells by interactions that are disrupted simply by cell breakage. Addition of 8 M-urea increases the intensities of these peaks to 222 and 164% respectively of their values in intact cells. Thus it appears that only about 60% of the glycerol in *D. salina* cells contributes to the spectrum under the conditions of our experiments.

For glucosylglycerol in *Synechococcus* the total peak intensity in spectra of intact cells is identical with that observed with both disrupted cells and disrupted cells to which 7 or 8 M-urea has been added.

Table 1. *^{13}C n.m.r. spin-lattice relaxtion data for osmoregulatory solutes in intact cells*

Measurements were made using solution and spectral-accumulation conditions similar to those described in the caption to Fig. 1. The n.O.e. and T_1 values are means of four separate determinations for *D. salina*, and three for *Synechococcus* sp. T_1 values are in s. T_1^{CH} and T_1^{other} are obtained from eqns. (1) and (2) as described below. ^{13}C resonance assignments for glucosylglycerol are discussed in the text. Because of incomplete peak resolution in spectra of *Synechococcus* cells, only an average T_1 is reported for resonances from C-6 and C-1′ (or C-3′), and average n.O.e. values for the four resonances from C-2 to C-5 and the three from C-1′, C-3′ and C-6. T_1^{CH} values for *Synechococcus* were calculated by using the average n.O.e. of 2.6.

	n.O.e.	T_1	T_1^{CH}	T_1^{other}
D. salina				
CH resonance	2.2	0.9	1.5	2.3
CH_2 resonance	2.1	0.7	1.3	1.6
Synechococcus sp.				
C-1	3.1	0.18	0.22	
C-2	2.6	0.19	0.24	
C-3		0.19	0.24	
C-4		0.21	0.26	
C-5		0.21	0.26	
C-6	2.5	(0.17)	(0.21)	
C-1′ or C-3′	2.5	0.19	0.24	
C-2′	2.3	0.31	0.39	
C-3′ or C-1′	2.5	(0.17)	(0.21)	

Model compounds

Corresponding data for glycerol and glucosylglycerol in aqueous solution have been measured at solute concentrations comparable with the intracellular concentrations. The concentration of glycerol in intact *D. salina* grown in medium containing 2.1 M added NaCl has not been determined, but glycerol concentrations at various NaCl concentrations are known for two closely related species, *D. tertiolecta* and *D. viridis* (Borowitzka, 1974), from which it can be estimated that the internal glycerol concentration in *D. salina* would be approx. 2 M. Relaxation measurements were made over a range of glycerol concentrations to assess the influence of an error in this estimate. Furthermore, separate sets of data were acquired in ^2H_2O and in ^2H_2O containing 2.1 M-NaCl, because of the likelihood that the cytoplasm of *D. salina* contains some inorganic salts to make up the difference between the intracellular glycerol concentration (about 2 molal, equals 2 osmolal) and the external salt concentration (2.1 M, corresponding to 4.2 osmolal). The data are summarized in Table 2.

Spin-lattice relaxation parameters for glucosylglycerol were measured at a concentration of 0.45 M, the value determined previously for *Synechococcus* cells grown in media containing 1.03 M added NaCl (Borowitzka *et al.*, 1980). The intracellular concentration of glucosylglycerol does not balance the osmolal concentration of ions in the growth medium (Borowitzka *et al.*, 1980), so data were acquired for glucosylglycerol in ^2H_2O, and in 0.8 M-KCl in ^2H_2O (0.8 M-KCl being the concentration necessary to maintain osmotic balance across the cell membrane if KCl were the only other osmoregulatory solute). These data are given in Table 3, in conjunction with corresponding data on the closely related glycoside floridoside (galactosylglycerol).

Table 2. *^{13}C n.m.r. spin-lattice relaxation times (in s) for glycerol in aqueous solution*

T_1 and n.O.e. values were measured with a spectral width of 1000 Hz as described in the Materials and methods section. Experimental n.O.e. values are not given here because they were all within 0.3 unit of the expected maximum of 3.0, indicating that ^{13}C–^1H dipolar interactions dominate spin-lattice relaxation in all cases. Sample temperatures were 28–29°C in ^2H_2O and 29–33°C in ^2H-labelled growth medium containing 2.1 M-NaCl.

^2H_2O			2.1 M-NaCl in ^2H_2O		
[Glycerol] (mol/litre)	CH resonance	CH_2 resonance	[Glycerol] (mol/litre)	CH resonance	CH_2 resonance
0.30	3.5	2.0	0.30	4.2	2.2
0.99	3.4	1.8_5	1.02	3.5	2.0
2.02	3.1	1.7_5	2.02	3.0	1.7
3.03	2.6	1.5	3.02	2.4	1.4
4.73	1.9	1.1	4.95	1.7	1.0

Table 3. *^{13}C n.m.r. spin-lattice relaxation times (in s) for 0.43 M-glucosylglycerol and 0.45 M-floridoside in aqueous solution*

T_1 and n.O.e. values were measured with a spectral width of 2500 Hz as described in the Materials and methods section. Experimental n.O.e. values are not reported because they were all within 0.3 units of the expected maximum of 3.0, indicating that ^{13}C–^1H dipolar interactions dominate spin-lattice relaxation in all cases. Sample temperatures were 29°C in ^2H_2O and 32°C in ^2H_2O containing 0.8 M-KCl. Chemical shifts in p.p.m. from tetramethylsilane are given in parentheses for ^2H_2O solutions. Resonance assignments are discussed in the text. Peaks from C-2 and C-4 of floridoside are not resolved in 0.8 M-KCl.

	C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′
Glucosylglycerol	(98.9)	(72.7)	(74.1)	(70.8)	(73.1)	(61.8)	(62.7)	(79.9)	(61.6)
^2H_2O	0.62	0.58	0.57	0.61	0.58	0.40	0.47	0.83	0.40
^2H_2O, 0.8 M-KCl	0.58	0.53	0.53	0.53	0.55	0.39	0.50	0.73	0.38
Floridoside	(99.3)	(70.6)	(69.7)	(70.5)	(72.3)	(62.4)	(62.7)	(80.0)	(61.6)
^2H_2O	0.56	0.59	0.57	0.59	0.57	0.42	0.44	0.80	0.49
^2H_2O, 0.8 M-KCl	0.63	0.60	0.59	0.60	0.59	0.40	0.49	0.70	0.51

^{13}C resonance assignments for glucoslyglycerol and floridoside are also given in Table 3. Assignments for glucosylglycerol differ slightly from those tentatively proposed by Kollman *et al.* (1979) from consideration of the ^{13}C n.m.r. spectrum of glucose and glycerol. We have reversed their assignments of C-2 and C-5, based on a comparison of the differences in chemical shifts between α-D-glucopyranose and α-D-galactopyranose with those between glucosylglycerol and galactosylglycerol. The revised assignments yield a closer prediction of the observed shifts for galactosylglycerol, as well as providing an assignment for the spectrum of the latter. However, the close proximity of the resonances from C-2 and C-4 of galactosylglycerol requires that these assignments be regarded as reversible. In both glycosides the assignments for the glycerol methylene carbons C-1′ and C-3′ are interchangeable (Kollman *et al.*, 1979).

Discussion

Theoretical basis for interpretation of relaxation data

A number of interactions can contribute to the relaxation of a ^{13}C nucleus but, for hydrogen-bearing carbons in diamagnetic molecules, spin relaxation usually occurs via ^{13}C–^1H dipole–dipole interactions (Lyerla and Levy, 1974; Wehrli, 1976). The equations that describe the dependence of T_1, linewidth and n.O.e. on τ_R, the correlation time for overall rotational reorientation, are discussed elsewhere (Doddrell *et al.*, 1972; Norton, 1980). In calculating τ_R from T_1, a C–H bond length of 0.11 nm (1.1 Å) will be assumed in the present paper.

The maximum value of the n.O.e., 2.99, is observed for carbon atoms relaxed entirely by ^{13}C–^1H dipolar interactions in small, mobile molecules. The n.O.e. decreases to 1.153 in slowly moving macromolecules (Norton, 1980), even when the ^{13}C-^1H dipolar mechanism dominates relaxation. As reductions in n.O.e. can also occur due to relaxation by other mechanisms (Abragam, 1961; Lyerla & Levy, 1974), it is important to be able to identify these instances. Usually linewidth measurements provide a means of achieving this, as this parameter is a monotonic function of τ_R.

If relaxation mechanisms other than the ^{13}C–^1H dipolar one are significant, the contribution of ^{13}C–^1H dipolar interactions to spin-lattice relaxation (T_1^{CH}) may be obtained from:

$$T_1^{CH} = \frac{1.99}{(n.O.e. - 1)} \times T_1 \quad (1)$$

and the contribution of other mechanisms (T_1^{other}) from:

$$1/T_1^{other} = 1/T_1 - 1/T_1^{CH} \quad (2)$$

provided overall motion is rapid ($\tau_R \lesssim 10^{-10}$s at 15.04 MHz).

Glucosylglycerol in Synechococcus sp.

All the glucosylglycerol present in intact *Synechococcus* cells grown in medium containing 1.03 M added NaCl is visible in natural-abundance ^{13}C n.m.r. spectra recorded at about 30°C. The average n.O.e. value for the glucosylglycerol resonances is 2.6. This value is lower than the theoretical maximum of 2.99 by 0.4 unit, close to the limit of our estimated experimental error. However, we shall proceed on the assumption that this represents a real deviation from the maximum.

A reduced n.O.e. may result from slow overall motion in the case of ^{13}C–^1H dipolar relaxation, or from contributions to relaxation by other mechanisms. If the ^{13}C nuclei of glucosylglycerol were relaxed entirely by ^{13}C–^1H dipolar interactions, and if overall motion were isotropic, the average n.O.e. value (Table 1) would require a τ_R of about 1.5 ns, corresponding to NT_1 (N is the number of directly bonded hydrogen atoms) of about 0.04 s and a natural linewidth of 8 Hz. Clearly this model does not fit the data, as the NT_1 values are in the range 0.2–0.3 s (Table 1) and the natural linewidths, although difficult to estimate because of broadening due to sample heterogeneity, are <5 Hz. Nor can the data be fitted by postulating that the glucosylglycerol is exchanging rapidly (Dwek, 1973) between 'free' and 'bound' states, in the latter of which the n.O.e. is 1.153. The observed linewidths place an upper limit of about 3% on the fraction of 'bound' solute, which would be too small to significantly reduce the observed n.O.e. Finally, the CH and CH_2 resonances of glucosylglycerol and floridoside exhibit the maximum n.O.e. in ^2H_2O and in ^2H_2O containing 0.8 M added NaCl, as shown in Table 3. Therefore it appears that the reduced n.O.e. for intracellular glucosylglycerol is due to non-^{13}C–^1H dipolar contributions. Considering the model compound data once again, we may eliminate all alternative relaxation mechanisms except that arising from paramagnetic agents in the intact cell. The following discussion analyses the ^{13}C–^1H dipolar contribution to relaxation. The paramagnetic contributions will be considered in the next section.

The T_1^{CH} values of the methine carbon atoms of the glucose moiety, C-1 to C-5, are shorter than that of C-2′of the glycerol moiety, and the T_1^{CH} values of the methylene carbon atoms are longer than half the average methine carbon T_1^{CH} values (Table 1). Therefore, glucosylglycerol in the intact cells does not behave as an isotropic rigid rotor. To facilitate interpretation of this data, it is useful to consider first the behaviour of this molecule in aqueous solution. In both glucosylglycerol and floridoside the five hexosyl methine carbon atoms have T_1 values identical within experimental error (Table 3). As all carbon atoms in both molecules exhibit maximal n.O.e., this result indicates that the hexose moieties undergo nearly isotropic motion in solution. The calculated τ_R values are 0.083 and 0.090 ns for glucosylglycerol in ^2H_2O and 0.8 M-KCl in ^2H_2O respectively, and 0.085 and 0.082 ns for floridoside. In each case the hexosyl methylene carbon atom C-6 has a T_1 longer than half that of the methine carbon atoms, due to internal rotation about the C-5–C-6 bond with a correlation time shorter than 0.08–0.09 ns. Furthermore, the significantly longer T_1 values observed for C-2′ of the glycerol moieties indicate that there is fast internal motion about one or both bonds of the glycosidic linkage. Finally, the glycerol methylene carbon atoms may also be rotating rapidly around the carbon–carbon bonds. Nevertheless, the main conclusions are that the hexosyl moieties of glucosylglycerol and floridoside undergo approximately isotropic rotational reorientation in aqueous solution, whereas the hexosyl methylene carbon atom and the glycerol moiety experience fairly rapid internal motion. The presence of KCl has no substantial effect on the rotational motions, the slight effects observed being within the combined experimental errors.

For glucosylglycerol in intact *Synechococcus* cells the same qualitative description applies, but overall motion is slower. The average T_1^{CH} for the methine carbon atoms of the glucosyl moiety is 0.24 s, corresponding to a τ_R of 0.20 ns. This approx. 2.4-fold increase in τ_R relative to aqueous solution could result from a 2.4-fold greater intracellular viscosity or from a weak association of glucosylglycerol with some other molecule(s) in the cell. We favour the former alternative, but it would be necessary to show that the mobilities of other, unrelated cytoplasmic constituents are reduced to the same extent in whole cells in order to prove this.

Other studies of the internal viscosity of intact cells have employed n.m.r., e.s.r. and fluorescence polarization. In general, internal viscosities have been found to be greater than, or equal to, twice the viscosities of corresponding aqueous solutions (e.g. Neville & Wyssbrod, 1977; Hedrick *et al.*, 1979; Morse *et al.*, 1979). Part of this increase may be due simply to enclosure within a biological membrane (Clement & Gould, 1980). Thus a 2.4-fold increase in the viscosity of the space occupied by glucosylglycerol in *Synechococcus* cells would be comparable with that found in many other cell types. In any

case, the most significant results are first that there is no significant fraction of glucosylglycerol which is 'immobilized' within the cell (such that its ^{13}C resonances are broadened beyond detection), and second that, although the motion of glucosylglycerol in intact cells is slower than in aqueous solution, this molecule is still highly mobile within the cell. We find no evidence for a highly restricted motional state within the cell, as has been implied by studies of water in other algae, including a blue–green alga (Venable *et al.*, 1978).

Glycerol in D. salina

The behaviour of glycerol in this organism is more complex than that of glucosylglycerol in *Synechococcus* sp. in that only about 60% of the total intracellular content of glycerol is observed under our spectral accumulation conditions. We begin by considering the relaxation behaviour of the observable fraction.

Table 1 shows that n.O.e. values for the methine and methylene ^{13}C resonances are quite low. Following the same reasoning as for glucosylglycerol in *Synechococcus* cells, it may be concluded that this result is due to paramagnetic contributions to the relaxation of these carbon atoms. The $^{13}C-^{1}H$ dipolar contributions to relaxation can be calculated from eqn. (1) above. The NT_1^{CH} values for the CH and CH_2 resonances of glycerol in intact cells are 1.5 and 2.6 s respectively (Table 1), indicating that the molecule does not behave as a simple isotropic rigid rotor. Glycerol in aqueous solution behaves similarly, with NT_1 for the methylene carbon atom being greater than that for the methine carbon atom at all concentrations studied, both in the presence and absence of 2.1 M NaCl (Table 2). However, the methine carbon T_1 for 2 M-glycerol in aqueous solution is twice as long as T_1^{CH} for glycerol in *D. salina*. Although glycerol has been the subject of numerous molecular-motion studies by a variety of techniques, including ^{13}C n.m.r. relaxation measurements (Wright *et al.*, 1979), there is no consensus regarding the best model for its motional behaviour. Therefore it seems appropriate to restrict our conclusions to the fact that the average rotational freedom of glycerol in the intact cell is about half that in the corresponding aqueous solution. This probably reflects a higher intracellular viscosity.

Now consider the paramagnetic contribution to relaxation. The data in the second part of Table 2 were obtained in a ^{2}H-labelled medium containing all the constituents present in the growth medium at the appropriate concentrations. This medium was also used for n.m.r. studies of intact cells. The fact that maximum n.O.e. values are found for glycerol in this medium indicates that the concentrations of those trace metals in the medium which could conceivably affect relaxation (Cu^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+}) are too low to be significant. The cells would have to concentrate one or more of them in order to generate the observed results. To establish the concentrations necessary for this to occur, we tested the effects of adding Mn^{2+} and Cu^{2+} on the ^{13}C T_1 values of 2.0 M-glycerol in $^{2}H_2O$, these ions being chosen because they have greater effects on relaxation than on chemical shifts (Dwek, 1973). The results for intracellular glycerol could be approximated by Mn^{2+} and Cu^{2+} concentrations of 1 and 5 mM respectively. These findings do not imply that free Mn^{2+}, free Cu^{2+} or a combination of these are necessarily present in the cytoplasm of *D. salina*. They merely indicate the sort of paramagnetic-ion concentrations that could give rise to the observed effects.

The resonances of glucosylglycerol in intact *Synechococcus* cells also experience a reduction in n.O.e. values owing to interactions with paramagnetic species. T_1^{other} values (calculated from eqn. 2) are in the range 0.9–1.6 s, similar to that found for glycerol in *D. salina* cells (Table 1). Although this similarity may be coincidental, it raises the possibility that these effects have a common origin. Both species may have the ability to sequester heavy-metal ions from their growth media. Alternatively, the effects may be due to manganese from the thylakoid membranes. The thylakoids contain 'tightly' bound and 'loosely' bound pools of manganese (Cheniae, 1970), and the latter may leak into the cytoplasm when the cells are incubated in the dark, as in our n.m.r. experiments.

The glycerol fraction not observed in spectra of intact *D. salina* must represent glycerol that is in slow exchange [Dwek, 1973 (chap. 2)] with free glycerol and is broadened beyond detection under our spectral-accumulation conditions. This would require linewidths of 100 Hz or greater. Broadening of this magnitude could be due to interaction with paramagnetic species and/or to association with slowly tumbling macromolecular structures. Each of these interactions would cause a concomitant loss of n.O.e., which would add to the difficulty of observing this fraction. The finding that the interactions responsible for line broadening are not destroyed simply by disruption of the cell, but require addition of 8 M-urea, implies that these interactions are fairly stable and not dependent on an intact plasma membrane.

If broadening were due solely to association with a macromolecule, the latter would require $\tau_R \geqslant 76$ ns. For comparison, haemoglobin has a τ_R of about 45 ns and bovine serum albumin about 80 ns (Wilbur *et al.*, 1976). However, we believe it more likely that broadening results from a combination of partial immobilization of the glycerol and interaction with paramagnetic species. This raises the possibility that

the non-observable fraction corresponds to glycerol located within the chloroplast. This organelle occupies about half of the cell volume in *D. salina* (M. A. Borowitzka, personal communication), which corresponds quite well with the fact that 40% of the glycerol is not observed. The lifetime of a water molecule inside pea (*Pisum sativum*) thylakoids is >1 ms (Wydrzynski *et al.*, 1978), so the lifetime of a glycerol molecule would be even longer, thereby satisfying the requirement for slow exchange between the observable and non-observable pools. In addition, chloroplasts contain a significant amount of manganese (Cheniae, 1970; Wydrzynski *et al.*, 1978), which may cause the observed line broadening in conjunction with partial immobilization of glycerol within the thylakoids. In spectra of intact *Synechococcus* cells there is no significant fraction (i.e. <10–15%) of glucosylglycerol which is broadened beyond detection, despite the presence of thylakoid membranes in the organism. However, these membranes occupy a smaller fraction of the cell volume than in *D. salina*, and are not organized into a separate organelle (Lang & Whitton, 1973) factors that may alter any effects on the osmoregulatory solute.

Clearly further work is required to characterize the non-observable pool of glycerol in terms of the cause(s) of line broadening and the rate of exchange between glycerol in this pool and the observable glycerol. These questions are particularly relevant to a full understanding of the osmoregulatory process in *D. salina*, for, if the non-observable fraction did represent tightly bound glycerol, the contribution of this fraction to the overall osmotic balance would be diminished. This would raise the possibility that changes in the ratio of 'free' to 'bound' glycerol may constitute a mechanism of short-term osmoregulation in this organism.

In conclusion, our results demonstrate that it is possible to probe the interactions of osmoregulatory solutes in intact unicellular algae by ^{13}C n.m.r. spin-lattice relaxation measurements. These studies can be carried out at the natural abundance ^{13}C level even on a low-magnetic-field-strength spectrometer because of the very high concentrations of these solutes in intact cells. The mobilities of glucosylglycerol in intact *Synechococcus* cells, and of about 60% of the glycerol in intact *D. salina* are only 2–2.4-fold slower than the mobilities of these molecules in free solution. These solutes are therefore able to contribute effectively to the maintenance of osmotic balance across the cell membrane. The cytoplasmic space occupied by these solutes is not highly structured.

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