31P and 13C-NMR Studies of the Phosphorus and Carbon Metabolites in the Halotolerant Alga, *Dunaliella salina*1

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**ABSTRACT**

The intracellular phosphorus and carbon metabolites in the halotolerant alga *Dunaliella salina* adapted to different salinities were monitored in living cells by 31P- and 13C-nuclear magnetic resonance (NMR) spectroscopy. The 13C-NMR studies showed that the composition of the visible intracellular carbon metabolites other than glycero is not significantly affected by the salinity of the growth medium. The T2 relaxation rates of the 13C-glycerol signals in intact cells were enhanced with increasing salinity of the growth medium, in parallel to the expected increase in the intracellular viscosity due to the increase in intracellular glycerol. The 31P-NMR studies showed that cells adapted to the various salinities contained inorganic phosphate, phosphomonoesters, high energy phosphate compounds, and long chain polyphosphates. In addition, cells grown in media containing up to 1 molar NaCl contained triplyphosphates. The triplyphosphate content was also controlled by the availability of inorganic phosphate during cell growth. Phosphate-depleted *D. salina* contained no detectable triplyphosphate signal. Excess phosphate, however, did not result in the appearance of triplyphosphate in 31P-NMR spectra of cells adapted to high (>1.5 molar NaCl) salinities.

Members of the genus *Dunaliella* (division Chlorophyceae, order Volvocales, family Polyplepharidae) are unicellular, motile, green algae, which lack a rigid cell wall. *Dunaliella* has the capacity to tolerate and adapt to a wide range of salt concentrations (0.1–5.5 m NaCl). This is achieved through the ability of the algae to survive the initial osmotic stress, and then adjust their intracellular osmotic content to the new required level, by synthesis or elimination of glycerol (3, 4).

Little is known about the intracellular phosphate compounds in *Dunaliella*, and no characterization of the intracellular phosphate compounds is available. Ginzburg and Ginzburg (9) measured an intracellular inorganic phosphate concentration of 400 mm for 12 species of *Dunaliella*, cultured at 0.5 or 2 m NaCl and 2 mm phosphate. Pick et al. (20) showed that the cells accumulate phosphate in proportion to its concentration in the growth medium. Gimmler and Moller (8) studied the phosphates of *D. parva* cells following osmotic shocks. Following a hyperosmotic shock, they observed a very rapid increase in the endogenous inorganic phosphate level, and in the level of an unidentified phosphate compound, at the expense of the acid-soluble, cellular organic phosphates. A hypoosmotic shock caused a rapid decrease in the intracellular inorganic phosphate level. The ATP to ADP ratio, the phosphorylation potential (ATP/ADP × Pi), and the 3-phosphoglyceric acid to Pi ratio also decreased.

In general, algal intracellular phosphate levels can vary widely, depending on whether the algae are growing under phosphorus-rich or phosphorus-limited conditions (11). Like other microorganisms, algal cells possess the ability to accumulate excess phosphate. This is stored in the form of condensed phosphates, which can be either cyclic (metaphosphates) or linear unbranched chains (polyphosphates). The existence of intracellular polyphosphate granules, whose size (30–500 nm) and number were affected by the growth conditions, was demonstrated in the algae *Chlorella pyrenoidosa* (19) and *Anabaena flos-aquae* (21) by electron microscopic studies combined with x-ray energy dispersive analysis.

In this paper, we report the use of 31P-NMR spectroscopy to characterize the intracellular phosphate metabolites in living *D. salina* cells adapted to different salinities and phosphate concentrations. In vivo 31P-NMR has been used extensively in studies of mammalian cells and organs, including organs in living animals (2, 7 and references therein), and recently also in studies of unicellular photosynthetic organisms (10, 14, 15, 18, 24, 25).

We also report the use of 13C-NMR spectroscopy to study *D. salina* grown in media containing 13C-enriched carbonate and monitored under conditions similar to those used in the 31P studies. 13C-NMR was employed previously in numerous studies of intact cells and organs (1). The glycerol in *D. salina* grown in 2.1 m NaCl was previously monitored by natural abundance 13C-NMR studies (17). 13C-labeled *D. salina* permitted additional characterization of several other intracellular metabolites (5). In the latter study, it was also shown that following a hypoosmotic shock, glycerol is converted to α(1→4)glucan, and the reverse process occurs following a hyperosmotic shock. In this study we monitored, by in vivo 13C-NMR, the intracellular glycerol, metabolites, and membranal components in cells adapted to a wide range of salinities.

**MATERIALS AND METHODS**

**Growth Conditions.** *Dunaliella salina* was grown in batch cultures in media containing NaCl at the indicated concentration, 50 mm NaHCO3, 5 mm KNO3, 5 mm MgSO4, 0.3 mm CaCl2, 0.8 μM ZnCl2, 0.02 μM CoCl2, and 0.2 nm CuCl2 (initial pH, 7.5–8.2). Algal cultures were grown under continuous illumination with white fluorescent lamps (light intensity 3800 lux) at 26°C with slow (80 rpm) continuous shaking within a New Brunswick Psychrometer incubator. Adaptations to different NaCl or phosphate concentrations were made by growing for several days under the desired conditions. Cells for 13C-NMR studies were cultured in the same medium as above, except that it contained 25 mm [13C, 10%] NaHCO3 for the last 2 to 3 division cycles.

**Preparation of Cells for NMR Measurements.** Algae at the logarithmic phase (1–2.5×106 cells/ml) were concentrated by centrifugation at 4°C (2000 g for 10 min). All subsequent oper-
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Measurements. 31P- and 13C-NMR experiments were conducted at 4 ± 1°C and 12 ± 1°C, respectively (unless indicated otherwise). The cells were aerated during the NMR measurements by passing humidified air through silicon tubing immersed in the cell suspension: the tubing allows gas exchange but is impermeable to the medium.

NMR measurements were performed with a Bruker CXP-300 FT NMR spectrometer. 31P-NMR spectra were recorded at 121.5 MHz, using 60° pulses and a repetition time of 1 s, or 90° pulses and a repetition time of 7 s. An external reference containing 1 mM H3PO4, 0.88 mM HCl, and 113 mM PrCl3 as a shift reagent was used in some of the experiments. Assignments of the 31P resonances were established by addition of the commercially available compounds to an extract of the cells. 13C-NMR spectra were recorded at 75.47 MHz, using 45° pulses and a repetition time of 1 s, with broadband proton decoupling (1 W). The intra-cellular glycerol served as an internal chemical shift reference. The assignment of the 13C resonances was based on previous studies (5).

T1 measurements were performed by using the inversion recovery method, applying 180°-90°-90° pulses. Each measurement of T1 of the algal phosphate signals included 9 variable τ values, from 0.01 to 6 s, and a 6 s repetition time. Each measurement of T1 of the glycerol 13C signals included 19 variable τ values, from 0.002 to 10 s, and a 10 s repetition time. T1 values were determined from a nonlinear exponential fit (23) of the magnetization recovery versus the delay (τ) curve.

NOE2 of the glycerol resonances was determined by comparing intensities in spectra recorded with complete 1H decoupling (90° pulse and 10 s repetition time) with those in spectra recorded with 1H gated decoupling, i.e. decoupling applied during the pulse only. To maintain the same temperature (within 2°C) throughout the experiment, 8 scans were accumulated with full decoupling and then 8 scans were accumulated with gated decoupling; this sequence was repeated 8 times.

RESULTS AND DISCUSSION

31P-NMR Studies of the Phosphate Metabolites. The stability of cellular metabolism under the conditions of the NMR experiment was established by observation of the cells’ motility and shape under the microscope and by following the phosphate metabolites. The composition and content of the phosphorus compounds reflect the energy state of the cell, the high energy metabolites being a most sensitive monitor of the cell’s viability.

Figure 1 is a fully relaxed 31P-NMR spectrum of D. salina grown with 1 mM NaCl and 0.2 mM phosphate (standard growth conditions). Under these conditions, the following prominent resonances appear in the 31P NMR spectrum: (a) phosphomonoesters (PME), including glycerol phosphate and sugar phosphates; (b) inorganic phosphate in the growth medium (Pizx); (c) intracellular inorganic phosphate (Piin); (d) an unidentified peak (X); (e) two large signals of tripolyphosphate—one due to the two terminal phosphate groups (PPP) and the other, at a higher field, due to the middle phosphate (PPP); (f) NTP signals due to α- and β-NTP. The γ-NTP and β-NDP overlap the large PPI signal. The resonance denoted α-NDP may also include signals due to nicotinamide adenine dinucleotides and α-NDP; (g) a signal due to the middle phosphate residues of medium size (4–10 residues) polyphosphate chains (P–(P)–P). The signal of the terminal phosphate residues of these polyphosphates also overlaps that of the terminal phosphates of tripolyphosphate.

31P-NMR studies of the freshwater unicellular green algae Chlorella (14, 18, 24), Cosmarium (6), and Scenedesmus (22) and of the marine flagellate Heterosigma akashiwo (26) have revealed the presence of intracellular NTP, sugar phosphates, and polyphosphate chains. Unlike in Dunaliella, however, no signals due to tripolyphosphate were observed in intact cells of any of them. In Scenedesmus (22) and Chlorella (24), an unidentified resonance was observed, with a chemical shift similar to the resonance designated X (Figs. 1 and 2). Further studies are necessary for identification of this signal, as well as for detailed assignment of the sugar phosphate resonances.

T1 values of the identified intracellular phosphate metabolites ranged between 50 ms (for tripolyphosphate) and 500 ms (for Pi). The unassigned signal X had a T1 of approximately 1 s. Thus, except for X, there were no saturation effects in the 31P spectra recorded with 60° pulses and 1 s repetition time. Under these conditions, a spectrum with a good signal to noise ratio could be obtained within 30 min (Figs. 2–4).

The concentration of the phosphates, determined from 31P NMR spectra of cells maintained at 4°C, remained stable for at least 6 h. At 20°C, minor changes were observed during the first 2 to 3 h of measurements. Upon prolonged (overnight) incubation at 35°C without air flow, large changes were observed; the spectrum became dominated by very intense, broad peaks of Pi and medium size polyphosphate chains (see Figs. 4, B and D, and 3C), and the total integrated intensity increased several-fold. These changes reflect the presence of high concentrations of NMR-invisible phosphates in the living cells, probably stored as insoluble, long-chain polyphosphate deposits that are hydrolyzed into shorter chains and free inorganic phosphate.

Effects of Salinity and Phosphate Availability on the Intracellular Phosphate Metabolites. Examination of cells adapted to different NaCl concentrations revealed that cells grown at 1 mM NaCl or lower (0.25 m, 0.5 m) exhibited a spectrum similar to that shown in Figure 2A. At higher salinities (1.5, 2, 4 mM NaCl), however, the amount of tripolyphosphate decreased to a nondetectable level (Figs. 2B and 3A). The tripolyphosphates were not found in cells cultured at high salinities even when excess

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2 Abbreviations: NOE, nuclear Overhauser enhancement; NTP, nucleoside triphosphate; NDP, nucleotide diphosphate.
phosphate was available (Fig. 3B). However, these cells did have long-chain polyphosphates, as is apparent from the polyphosphate signal that appeared upon overnight incubation without aeration at 35°C (Fig. 3C).

Cells grown with excess phosphate (1 mM) throughout the salinity range showed the same spectral features as cells cultured at 0.2 mM phosphate (e.g. Fig. 3, A and B) for cells cultured at 4 mM NaCl. When cells were cultured under phosphate-limited conditions (0.03 mM), the triplyphosphate signals disappeared, and the intracellular Pi-to-ATP ratio increased (Fig. 4A versus Fig. 4C). The resulting 31P NMR spectrum was reminiscent of spectra obtained for cells cultured at high salt. However, in contrast to the cells adapted to high salinities, upon overnight unauerated incubation of the phosphate-depleted cells at 35°C, only a relatively small signal of inorganic phosphate appeared, and no signals due to polyphosphates (Fig. 4B versus Fig. 4D). This suggests that the content of stored polyphosphates in these cells was very low.

These results suggest that the presence of triplyphosphate in the cells is controlled by both the phosphate availability (as are the long-chain polyphosphates) and the salinity of the growth medium. The significance of this finding is not yet clear.

The accumulation of excess phosphate and its storage in the form of high mol wt, condensed, cyclic metaphosphates or linear polyphosphates by algal cells is a well known phenomenon. It was shown (11) that in algae (and in other microorganisms) synthesis of polyphosphates occurs when the energy requiring reactions are saturated or in relative excess due, for example, to lack of CO2. The general conclusion from numerous studies on the interrelations between phosphorus metabolism and photosynthesis is that in algae polyphosphates are synthesized in the light at the expense of ATP generated by photophosphorylation reactions. They are thought to function not as an energy reserve but as a phosphorus reserve which enables the cells to grow under phosphorus depletion. Such a role was also proposed for polyphosphates of bacteria and yeast (13). It has been suggested (16) that in algae short (2–7 residues) oligopolyphosphates are first synthesized and then condensed to high mol wt phosphates (200–300 residues) and mostly converted to metaphosphates. Of the latter, trimetaphosphates regulate inorganic orthophosphate uptake via inositol phosphates; therefore, the ATP-ADP-Pi system, the condensed phosphates, and the inositol phosphates are closely related. In the 31P-NMR spectrum, the signals due to the phosphate groups of the inositol phosphates would be in the area of the inorganic phosphates and sugar phosphates (2–4 ppm) which we cannot yet resolve.

13C-NMR studies of the carbon metabolites. 13C-NMR spectra were recorded for cells grown in media containing 0.1, 0.5, 1, 2, 3, and 4 mM NaCl. Typical spectra are shown in figure 5. As previously noted (3, 5), the difference in salinity markedly affected the concentration of glycerol. It may be noted that glycerol content in cells adapted to a low salt concentration (0.1 mM NaCl)

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**Fig. 2.** 31P-NMR spectra of *D. salina* adapted to different salinities. Cells were prepared as described under "Materials and Methods" and maintained at 4°C. Each trace was obtained by accumulating 1800 scans (30 min) (60° pulses, 1 s delay) and processed using line broadening of 15 Hz. 0 ppm refers to 85% H3PO4. A, Cells grown with 1.0 mM NaCl; B, cells grown with 2.0 mM NaCl.

**Fig. 3.** 31P-NMR spectra of *D. salina* grown with 4.0 mM NaCl: effect of phosphate availability. Experimental conditions as described for Figure 2. A, Cells cultured with 0.2 mM phosphate, maintained at 4°C; B, cells cultured with 1.0 mM phosphate, maintained at 4°C; C, the cells of (B), following overnight unincubated incubation at 35°C. Y, an unassigned signal.
was very low, in the range of concentrations of the other common metabolites (Fig. 5A). However, there are no distinct changes in the composition and concentration of the other NMR-visible metabolites. This suggests that although during the adaptation to a new salt concentration the cellular metabolism undergoes large changes (5), once the cells are adapted the metabolic intermediates reach a similar steady state level at all salinities. Some differences appeared in the membranal signals, primarily in the intensity of (CH$_2$)$_n$, that increased at the very high salinities (3 and 4 M NaCl). Further studies are required to characterize these changes.

**Effect of Salinity on $T_1$ and Nuclear Overhauser Enhancement of Glycerol.** The $T_1$ relaxation times of the $^{13}$C-glycerol signals were measured in intact cells adapted to different salinities as well as in model solutions containing different glycerol concentrations (Fig. 6). For glycerol, which is a small molecule that tumbles rapidly, $^{13}$C relaxation occurs mainly via dipole-dipole interactions between the $^{13}$C and the directly bonded protons. The dipolar relaxation rate ($1/T_1$) is directly proportional to the correlation time for the reorientation of the glycerol molecule ($\tau$), according to

$$1/N T_1 = 2.0325 \times 10^{10} \times \tau,$$

where $N$ is the number of directly bonded protons. $\tau$ is proportional to the viscosity of the glycerol environment (12). The increase in $1/N T_1$ with salinity paralleled the increase observed for glycerol solutions, except at the very low salinity, and was in accord with the expected increase in viscosity due to the increased glycerol concentration (Fig. 6). The higher values for the relaxation rates in the cells relative to the glycerol solutions may reflect the higher viscosity in the cells due to the presence of additional soluble metabolites and macromolecules. This is expected to be more pronounced at the low salinity, where the contribution of glycerol to the total viscosity is smaller, explaining the deviation of the $1/N T_1$ curve at this range.

Although the $^{13}$C-$^1$H dipolar interaction dominates glycerol relaxation, other relaxation mechanisms may also prevail, particularly in the cells. A measure for the contribution of the other mechanisms was obtained from the $T_1$s and the NOEs using the same analysis as Norton et al. (17). These calculations have indicated that the contribution of mechanisms other than the dipolar interaction to the $T_1$ relaxation is small (10–20%). This result differs from that of Norton et al. (17), who determined approximately 40% contribution from mechanisms other than dipolar to the $T_1$ relaxation rate. It therefore makes unlikely a
marked paramagnetic effect (17) on the \( T_1 \) relaxation rate of intracellular glycerol. We have also found that the \( T_1 \) relaxation due to the dipolar interaction and due to the other mechanisms was similarly enhanced by increasing salinity of the growth medium (for example, for \( B_{\text{CHOH}} \), \( 1/N T_1(\text{dipolar}) \) increases from 0.7 to 1.1 s\(^{-1}\), and \( 1/N T_1(\text{butter}) \) increases from 0.1 to 0.3 in cells grown in 0.5 and 4.0 m NaCl, respectively). This suggests that the modulation of the \( T_1 \) with salinity is caused primarily by the increased glycerol content of the cell, in line with the results presented in Figure 6.

**LITERATURE CITED**

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