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In vivo NMR studies of the alga *Dunaliella salina* embedded in beads

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To facilitate in vivo NMR studies of microorganisms under stable environmental conditions a technique was developed in which algae are embedded in Ca-alginate beads and continuously perfused during the measurements. *Dunaliella salina* was shown to grow, multiply and recover from osmotic shocks while embedded within the beads. Using this technique the detailed kinetics of glycerol metabolism of *Dunaliella salina* following an osmotic shock, were investigated.

Osmotic shock; NMR; Ca-alginate bead; (*Dunaliella salina*)

1. INTRODUCTION

A major restriction in employing NMR techniques to study the metabolism of algae and bacteria [1–5], has been the difficulty to maintain organisms under stable environmental conditions during the NMR measurements. Relatively high concentrations of cells (from 10^9 to 10^{12} cells/ml) must be used and therefore the availability of nutrients, the pH of the medium, the concentration of oxygen, etc., are strongly modulated with time. To overcome this drawback, several techniques were previously described [6–9]. Herewith, we describe a method based on embedding algae in Ca-alginate beads [10,11] adapted to NMR studies. Constant perfusion of the algae inside the beads permitted control of the environmental conditions. In a previous study, Ca-alginate beads were used for immobilizing the large *Catharanthus roseus* plant cells [12]. ^{31}P NMR spectra of these cells exhibited similar features when perfused in suspension and in beads [13,14]. Here we demonstrate that the unicellular algae *Dunaliella salina* can adapt and grow in the Ca-alginate beads and show a normal ability to recover from hypoosmotic and hyperosmotic shocks. ^{13}C and ^{31}P NMR studies of *Dunaliella* embedded in the beads demonstrate the potential applications of such systems for characterizing metabolic events by magnetic resonance techniques.

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2. MATERIALS AND METHODS

Dunaliella salina were grown in media containing 1 M NaCl, as previously described [15]. For experiments designed to follow a hypertonic shock in the dark, the algae were grown for 2 days in a low nitrate medium containing 0.5 mM instead of 5 mM KNO_3; it was shown that under these conditions a high level of starch was accumulated [15]. For ^{13}C measurements, cells were cultured as described above except that 24 h before harvesting the algae were transferred to a medium containing 25 mM of 10% ^{13}C-labeled $NaHCO_3$.

Preparation of beads: cells were concentrated to 100–250 times their normal growth concentration, and mixed with a 3% Na-alginate solution at a 1:1 volume ratio. The suspension was extruded dropwise with the aid of a peristaltic pump (flow rate: 2 ml/min), through a 1 ml syringe fitted with a 27G needle, into a solution containing 1 M NaCl and 100 mM $CaCl_2$. During the extrusion, the syringe was attached to a vortex-mixer whose speed controlled the size of the beads. The formed beads were maintained for 20 min in the high $CaCl_2$ solution and then transferred to the normal growth medium (containing 0.3 mM $CaCl_2$).

O_2 evolution of *Dunaliella* embedded in beads was measured with an oxygen electrode [17]. For determination of chlorophyll, the beads were dissolved in methanol and assayed

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according to Arnon [16]. Intracellular glycerol was determined as described by Ben-Amotz and Avron [18] following its release from the beads by incubation at 70°C for 15 min.

Dunaliella cells embedded in beads were fixed, for electron microscopy, as described by Ben Amotz et al. [15]. Micrographs were obtained with a Philips 515 electron microscope at 80 kV.

NMR measurements were carried out with about 2 ml of beads, packed in a 10 mm NMR tube and constantly perfused with fresh medium at a rate of 3–4 ml per min. ^{13}C NMR spectra were recorded at 26°C, with a Bruker CXP-300 NMR spectrometer, at 75.47 MHz, using 45° pulses, a repetition time of 1 s and a broad band proton decoupling (about 1 W). ^{31}P NMR spectra were recorded at 4°C and 20°C with a Bruker AM-500 NMR spectrometer, at 202.46 MHz, using 60° pulses and a repetition time of 1 s.

3. RESULTS AND DISCUSSION

The ability of *Dunaliella* to grow and divide while immobilized in calcium alginate beads was investigated by measuring the chlorophyll, glycerol and rate of photosynthesis per unit volume of beads. When the cell concentration inside the beads was low (1×10^6 cells/ml beads) the algae divided at about half the rate observed under optimal suspension conditions (3–4 divisions in 24 h). At initial cell concentrations of 0.5×10^8 cells/ml beads and 2.5×10^8 cells/ml beads the rate of growth decreased to about a third of that value. This is probably due to self shading caused by the high cell concentration. Cell growth in the highly concentrated beads reached a plateau after 2 days as indicated by the change in both the Chl/ml beads and the glycerol/ml beads (fig.1). The initially more concentrated beads reached a higher plateau than the initially less concentrated beads (fig.1). After the cells were entrapped in the beads they seemed to undergo an adaptation period of about 1 day, during which the photosynthetic rate of O_2 evolution decreased to about one half (fig.1). Following the adaptation period, the rate of photosynthesis in the beads corresponded to about 50% of that in cells grown in suspension at optimal conditions, and remained constant for at least two days (fig.1). *Dunaliella* cells embedded in the beads were able to recover from a hypotonic shock from 1 M to 0.5 M NaCl as indicated by the decrease in glycerol concentration following the shock (fig.1). Thus the results show that *Dunaliella* can divide, photosynthesize and osmoregulate while trapped in the beads.

We also attempted to entrap *Dunaliella* by the previously described agar thread entrapping technique [7,9] and entrapping is possible, but it seems as if the heat treatment required in this procedure adversely affected these algae, and the NMR spectra obtained were significantly different from those of intact cells. As is seen here this was not the case with the bead entrapment. Furthermore, incubation of the algae entrapped within threads was not nearly as successful as the bead entrapped algae.

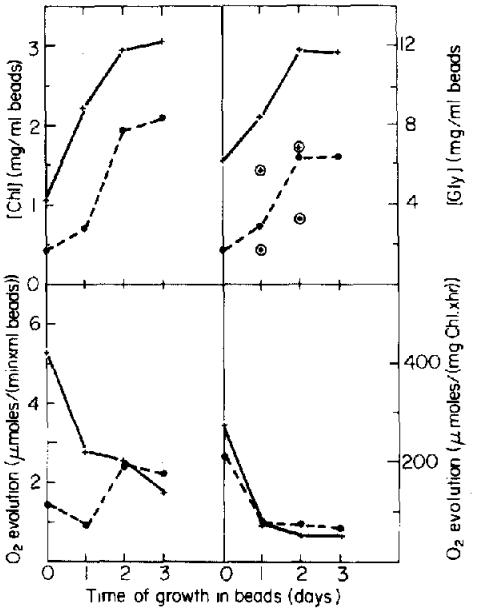

Fig.1. Chlorophyll content, glycerol content and O_2 evolution of *Dunaliella salina* grown in alginate beads. The full lines and broken lines represent beads with an initial density of 2.5×10^8 cells and 0.5×10^8 cells per ml beads, respectively. The encircled + and • symbols represent the glycerol content 2 h after the algae trapped in beads, at initial densities of 2.5×10^8 cells/ml beads and 0.5×10^8 cells/ml beads, respectively, were exposed to a hypotonic shock from 1 M to 0.5 M NaCl.

Fig.2 shows an electronmicrograph of *Dunaliella* grown in calcium alginate beads. The cells are present in clusters, each cluster presumably originating from a single cell, indicating proliferation inside the beads. The cells are rich in thylakoids, resembling cells grown in suspension under low light conditions [19]. The general shape of the algae is somewhat distorted from its ellipsoidal shape in suspension, probably due to the effect of the surrounding alginate polymer.

The main features of the ^{13}C NMR spectrum of *Dunaliella* in beads (fig.3), are very similar to those

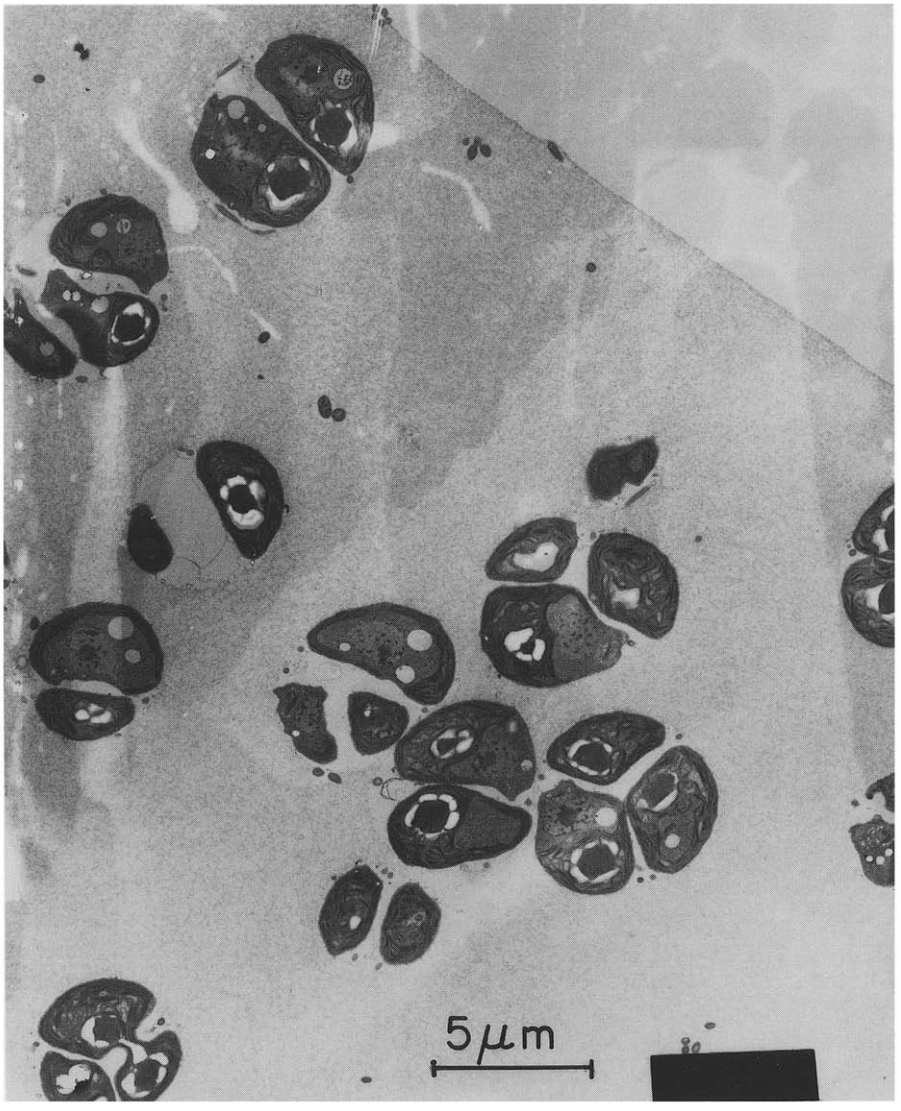

Fig.2. Electronmicrograph of *Dunaliella salina* grown for one day in Ca-alginate beads. Experimental conditions were described under section 2.

in spectra recorded with algae in suspension [5,20]. The spectrum includes signals due to membranal components (methyl, methylene and methene carbons of fatty acid chains in lipids) as well as soluble metabolites such as glycerol, lactic acid, alanine and glutamic acid. ^{31}P NMR spectra of embedded algae exhibited signals due to phosphomonoesters, internal and external P_i and nucleoside triphosphates (not shown). The intensity ratio of the nucleoside triphosphates to internal P_i in the spectra of *Dunaliella* in beads (about 1:5) was similar to that observed previously in spectra of algae in suspension in the dark, at 4°C [20], indicating that the energy state of embedded cells is similar to that of cells maintained in suspension.

The ability of *Dunaliella* to recover from an osmotic shock by synthesizing or eliminating glycerol was monitored using algae in beads perfused in the NMR tube. Modulation of glycerol content after hypotonic and hypertonic shocks was measured in vivo by following the intensity (I) of the ^{13}C signals of glycerol, thus yielding detailed

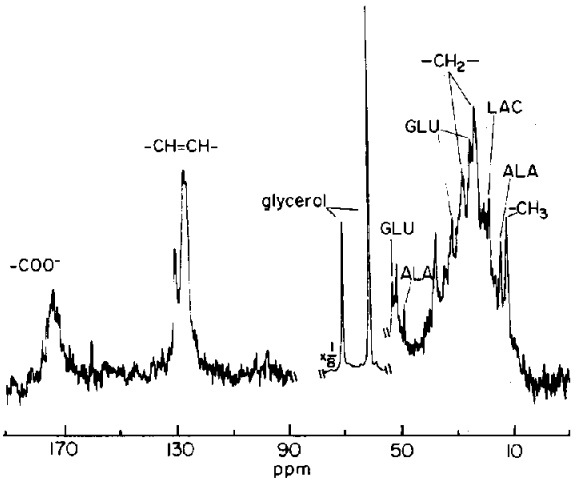

Fig.3. ^{13}C NMR spectrum of *Dunaliella salina* trapped in Ca-alginate beads. The sample contained 2 ml of beads at a concentration of about 5×10^8 cells/ml beads, grown and perfused with media containing 1 M NaCl. 4200 scans were accumulated as described in section 2. LAC, lactate; GLU, glutamate; ALA, alanine; $-CH_3$, $-CH_2$, $-CH=CH-$, lipid methyl, methylene and methene carbons, respectively; -COO-, carboxylate resonance. The chemical shift was referenced to tetramethylsilane (TMS) using the glycerol signals as internal reference (62.6, 73.1 ppm).

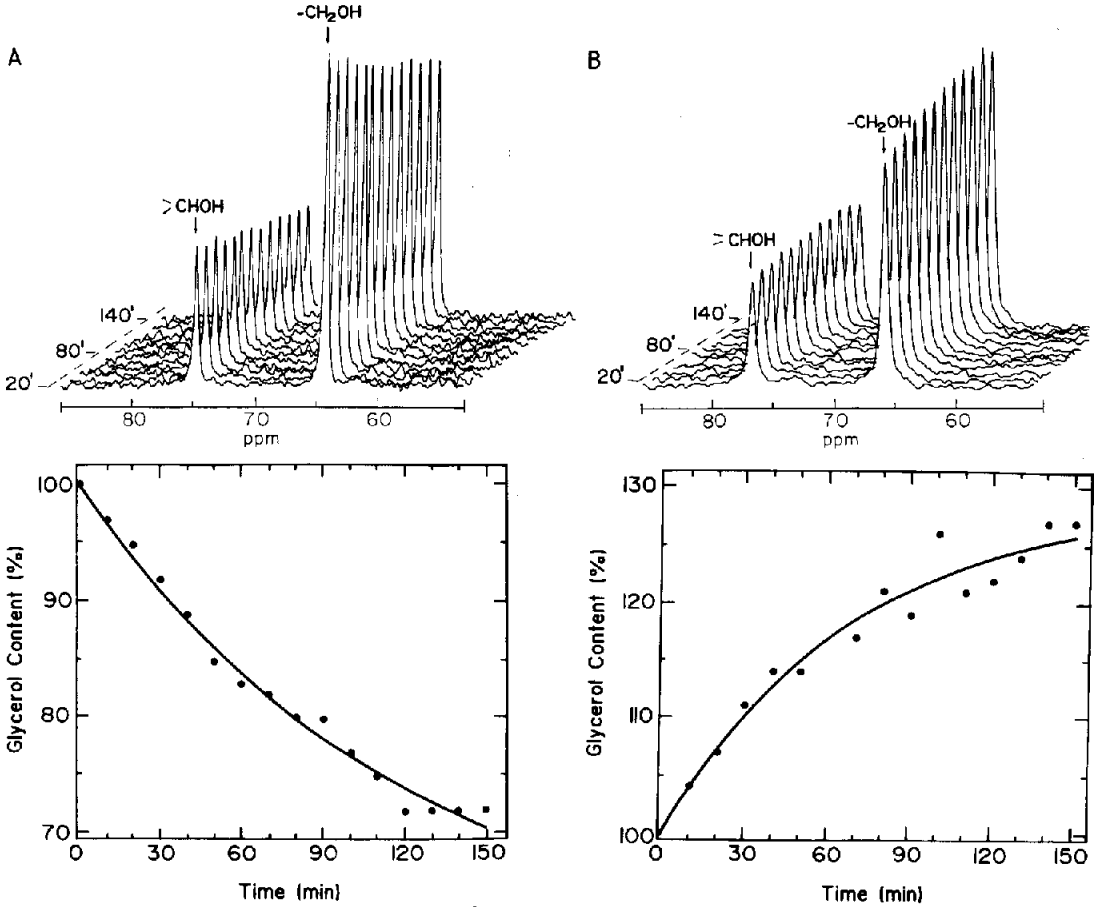

Fig.4. Recovery of *Dunaliella* embedded in beads (5×10^8 cells/ml) from osmotic shocks. (A) Hypotonic shock from 1 M to 0.5 M NaCl and (B) hypertonic shock from 1 M to 2 M NaCl. The beads were initially perfused with medium containing 1 M NaCl at a rate of 3 ml/min at 26°C. For the hypotonic shock, the algae were grown in a usual 1 M NaCl growth medium, while for the hypertonic shock they were grown in a low nitrate medium (see section 2). The osmotic shocks were performed by changing the perfusion medium to a medium containing the desired salt concentration. The upper parts exhibit typical spectra obtained at different times after the shock. The lower traces represent the modulation in glycerol content in terms of the percent from the initial glycerol content (100% ≈15 pgr/cell), obtained as explained in the text. The line through the experimental points was obtained by fitting the data to eqn 1, with the variable parameters indicated in the text.

kinetic data (fig.4). The resulting points were fitted to the exponential equation:

$$I(t) = (I_0 - I_{inf})e^{-at} + I_{inf} \quad (1)$$

where I_0 corresponds to the initial signal intensity, taken as 100%; I_{inf} corresponds to the % intensity (relative to I_0) after adaptation to the shock has been completed; a is a characteristic rate for adaptation. Both I_{inf} and a were variable parameters in the fit. The fit yielded for the hypotonic shock $I_{inf} = (58 \pm 4)\%$ and $a = (0.48 \pm 0.06)\ h^{-1}$ and for the hypertonic shock $I_{inf} = (130 \pm 2)\%$ and $a = (0.60 \pm 0.12)\ h^{-1}$. The final decrease in glycerol concentration due to a hypotonic shock was similar to that observed in algae recovering from the same shock in a suspension (at 2×10^6 cells/ml) [15]. The increase in glycerol concentration following a hypertonic shock was only partial. This may be due to an insufficient amount of starch which serves as the substrate for glycerol synthesis in the dark. Indeed, as determined biochemically, *Dunaliella* trapped in alginate beads in the light recovered completely from a hypertonic shock (not shown).

In conclusion, our technique facilitates time dependent control and regulation of the environmental conditions of algae during NMR measurements. The algae can grow and multiply while embedded in the beads, reaching concentrations that are 200–400 times more concentrated than under normal growth conditions, and still recover from osmotic shocks. This opens a wide range of opportunities for further in vivo NMR research on algae and bacteria.

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