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# A critical examination of the role of de novo protein synthesis in the osmotic adaptation of the halotolerant alga *Dunaliella*

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The role of protein synthesis during the osmotic adaptation of *Dunaliella salina* was examined utilizing protein synthesis inhibitors. 3–4 h of preincubation with cycloheximide or chloramphenicol were required to observe full inhibition of protein synthesis. Algae treated with cycloheximide, chloramphenicol or both reagents prior to a hyper- or hypo-osmotic shock showed no significant difference in their osmotic response when compared to untreated controls. Thus, protein synthesis is not essential for the osmotic adaptation response. Cell division was halted following a hyper-osmotic shock, but proliferation was reinitiated already during the late phase of the osmotic response and exponential growth was resumed soon after completion of the osmotic response.

Protein synthesis; Chloramphenicol; Cycloheximide; Halotolerance; Glycerol synthesis; (*Dunaliella*)

## 1. INTRODUCTION

*Dunaliella salina*, like other members of the genus *Dunaliella*, are extremely halotolerant, unicellular, green and motile algae. *Dunaliella* is unique in the absence of a rigid, polysaccharide cell wall; the cells are enclosed only within a thin plasma membrane and consequently, are able to change rapidly their volume and shape in response to external osmotic changes. *Dunaliella* is capable of growth in media of widely different osmotic pressures (or water activities), generated by dissolved salt, and furthermore, can rapidly adjust to hypo- or hyper-osmotic changes. The osmoregulatory response of *Dunaliella* functions by varying the intracellular concentration of primarily a single compatible solute, glycerol. The intracellular concentration of glycerol is directly proportional to the external salt concentration reaching, at high salt concentrations, over 50% of the cellular content. In responding to hypo- or hyper-osmotic changes in the medium, the cells first react as osmometers, swelling or shrinking, soon followed by rapid synthesis or elimination of glycerol. Full osmotic adaptation is marked by reassumption of the original cell volume due to the accumulation of an osmotically compatible content of glycerol [1].

A basic question regarding the osmotic responses of *Dunaliella* is to what extent do they require activation of gene expression, as would be if the biochemical processes underlying these responses were regulated at the level of enzyme biosynthesis. In a previous attempt to address this question [2], adaptation to a hyperosmotic shock of *D. viridis* was studied under conditions meant to inhibit de novo transcription or translation. The cells were first starved for nitrogen and then transferred to a high-salt, nitrogen-free medium containing inhibitors of transcription or of cytoplasmic or organellar protein synthesis. The accumulation of glycerol in the treated cells was then compared to that in the untreated controls. The absence of any significant effect of the inhibitors led the authors to conclude that de novo

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protein synthesis was not required for osmotic regulation in *Dunaliella*.

Several considerations warranted a reexamination of this conclusion. First, the study included only a hyperosmotic, but not a hypoosmotic shock. Recent evidence indicates that adaptations to these two types of shock may involve different biochemical pathways (Oren-Shamir, M. and Avron, M., unpublished). Second, the report did not include a demonstration that the various treatments indeed brought about a complete cessation of RNA or protein synthesis.

In the present study we investigated two aspects of the osmotic regulation in *D. salina*. First, the effect of protein synthesis inhibitors on both hypo- and hyper-osmotic shock responses was examined under conditions which were demonstrated to completely stop both cytoplasmic and chloroplast protein synthesis. Second, we addressed ourselves to the question whether osmotic adaptation following shock was sufficient for the resumption of cell proliferation. That is, whether once completing their osmotic adaptation, the cells resume proliferation immediately, or require an additional lag time before cell division can start.

## 2. MATERIALS AND METHODS

### 2.1. *Strain and growth conditions*

The source of the *Dunaliella salina* strain used in this study and the growth medium were essentially as described [3]. The NaCl concentration of the media was maintained at 1 M unless otherwise stated. Algal cultures (50 ml) were grown in 250 ml Erlenmayer flasks, in a New Brunswick psycotherm incubator under continuous light (white fluorescent lamps, 3800 lux) at 26°C, with continuous shaking at 100 rpm.

### 2.2. *Determination of cell number, volume and glycerol content*

Cell number and volume were determined with a model ZM Coulter counter, and glycerol as in [4].

### 2.3. *Assay of protein synthesis*

Culture aliquots of 1 ml, containing about $1 \times 10^6$ cells, were twice spun (2000 rpm, 5 min) and resuspended in a sulfate-free medium (5 mM $MgCl_2$ replaced the 5 mM $MgSO_4$ present in the complete medium). The cells were incubated for 3 h and 0.5 mCi $Na_2{}^{35}SO_4$ (New England Nuclear, spec. act. ~500 mCi/mmol) was then added. After various incubation times aliquots of 100 $\mu$l were removed, and the cells washed by two cycles of centrifugation and resuspension in 100 $\mu$l cold medium. 1 vol. of 2% deoxycholate in 0.1 M NaOH and 1 ml of 5% trichloroacetic acid were added to each sample followed by centrifugation (4000 rpm, 5 min). The pellets were dissolved in 50 $\mu$l of 0.5 M NaOH. Aliquots of 10–20 $\mu$l of each sample were added to 10 ml of a scintillation solution and counted.

### 2.4. *Gel electrophoresis*

Aliquots of 0.5 ml, $5 \times 10^5$ cells, of $^{35}S$-labeled cultures were centrifuged and resuspended in 20–30 $\mu$l Laemmli gel electrophoresis sample buffer, applied to 7.5–15% polyacrylamide gradient-SDS gels, and electrophoresed as described [5]. The gel was dried and autoradiographed.

## 3. RESULTS

### 3.1. *Establishing conditions for effective inhibition of protein synthesis*

Cycloheximide and chloramphenicol were used to inhibit cytoplasmic and chloroplast protein synthesis, respectively. To establish the effective concentrations of the drugs, they were added individually or in combination to cultures of *D. salina* and the effect on cell growth and protein synthesis was tested after various times of incubation. The results (fig.1) indicated that cycloheximide (CYH) at 1 $\mu$g/ml was sufficient to stop cell

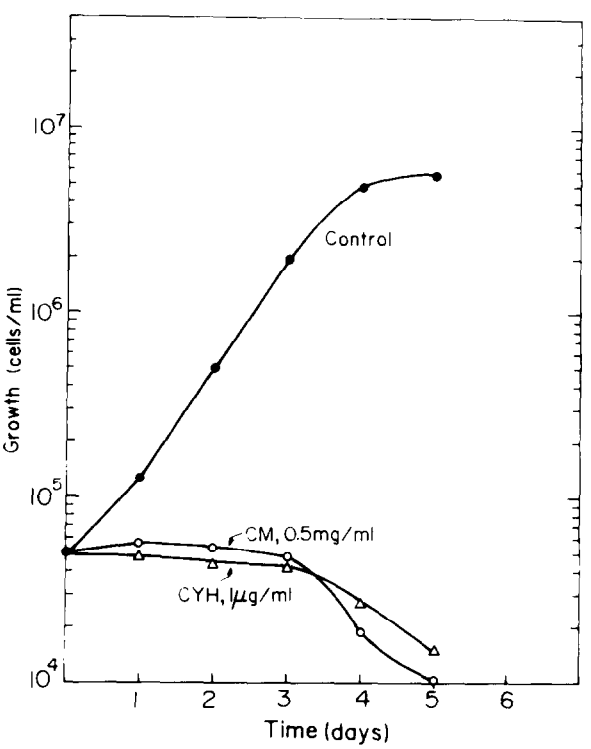


Fig.1. Effect of protein synthesis inhibitors on algal growth. *D. salina* was grown to $5–6 \times 10^6$ cells/ml, as described in section 2. Cells were diluted with fresh media to the indicated density and cycloheximide (CYH) and chloramphenicol (CM) were added to the final concentrations indicated.

division. The algae were, however, refractive to chloramphenicol (CM) at 12.5 $\mu$g/ml, the concentration previously used to inhibit protein synthesis during osmotic recovery [2]. A concentration of 500 $\mu$g/ml chloramphenicol was required to inhibit fully cell division. Relatively high chloramphenicol concentrations (100–1000 $\mu$g/ml) are generally employed to inhibit fully growth and chloroplast protein synthesis in many algae [6–10].

In the course of these experiments we observed, as described in [9,11], that under illumination, chloramphenicol is photochemically converted into a product which is highly toxic to algal growth. To avoid such photochemical conversion during the course of the experiments, light was passed through an orange filter which eliminated wavelengths below 600 nm. Under these conditions, chloramphenicol solutions showed neither spectral changes or increased toxicity.

The effect of the drugs on protein synthesis was assessed by adding [$^{35}$S]sulfate to the cultures and measuring the trichloroacetic acid insoluble radioactivity. In contrast to their 'immediate' effect on growth (fig.1), the inhibitors affected protein synthesis in short-term experiments only gradually (fig.2). During the first hour of incuba-

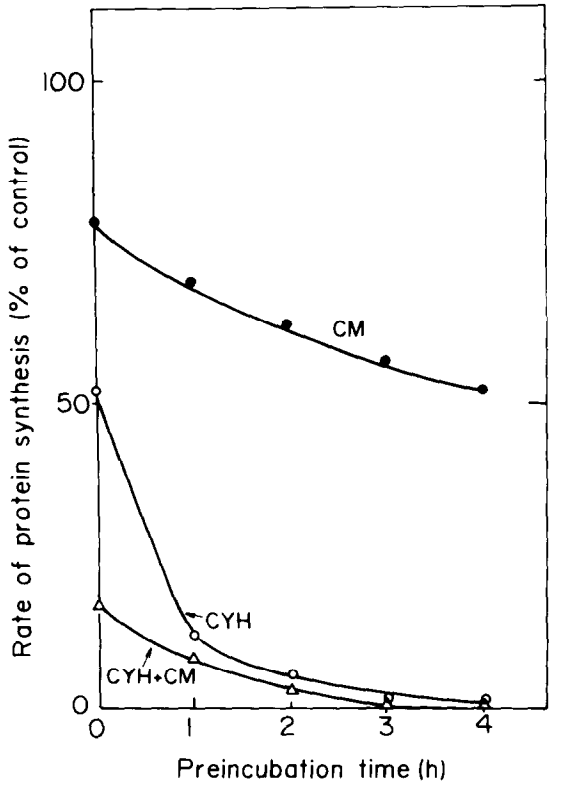


Fig.2. Effect of preincubation with inhibitors on algal protein synthesis. Algae were grown in a sulfate-free medium as described in section 2, followed by addition of $Na_2{}^{35}SO_4$ and protein synthesis inhibitors at the concentrations indicated in fig.1. Trichloroacetic acid-insoluble $^{35}$S was sampled as described in the text. Results are presented as the ratio (%) of the hourly increase in $^{35}$S incorporation in the presence of inhibitors relative to a control without inhibitors.

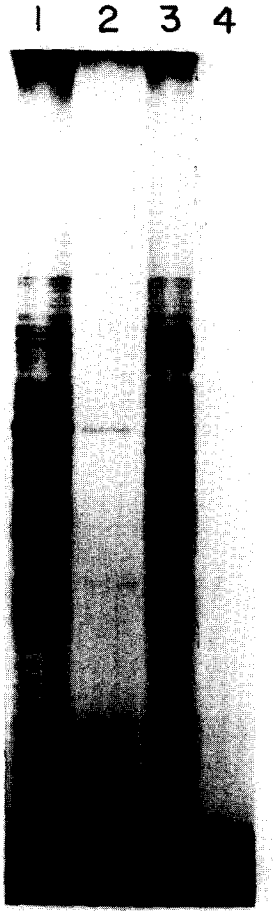


Fig.3. Autoradiograph of polyacrylamide gel electrophoresis of $^{35}$S-labeled algal extracts. Algal cells were preincubated in sulfate-free medium for 1 h, followed by addition of the inhibitors to the concentrations indicated in fig.1, and further preincubation for 2 h. $Na_2{}^{35}SO_4$ was then added, and following 1 h of incubation the cells were collected by centrifugation and the proteins separated by polyacrylamide-SDS gel electrophoresis as described in section 2. Lanes: (1) control, (2) cycloheximide, (3) chloramphenicol, (4) cycloheximide + chloramphenicol.

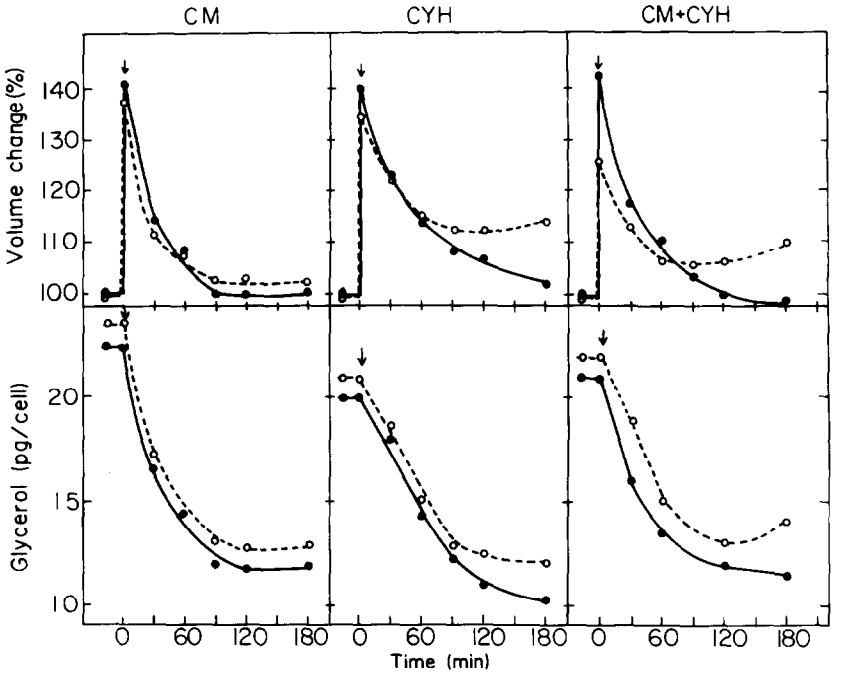


Fig.4. Effect of protein synthesis inhibitors on changes in volume and glycerol content following an osmotic down-shock. Algal cells (approx. $1 \times 10^6$ cells/ml) were preincubated with or without the indicated inhibitors at the concentrations indicated in fig.1, for 4 h. The cultures were centrifuged and cells resuspended in media containing 0.5 M NaCl. Samples were removed at the indicated times for determination of cell volume and glycerol content as described in section 2. Arrows indicate samples that were removed within 1 min following the osmotic shock. Data shown are the average of four separate experiments. (●——●) Control, (○---○) with inhibitor(s).

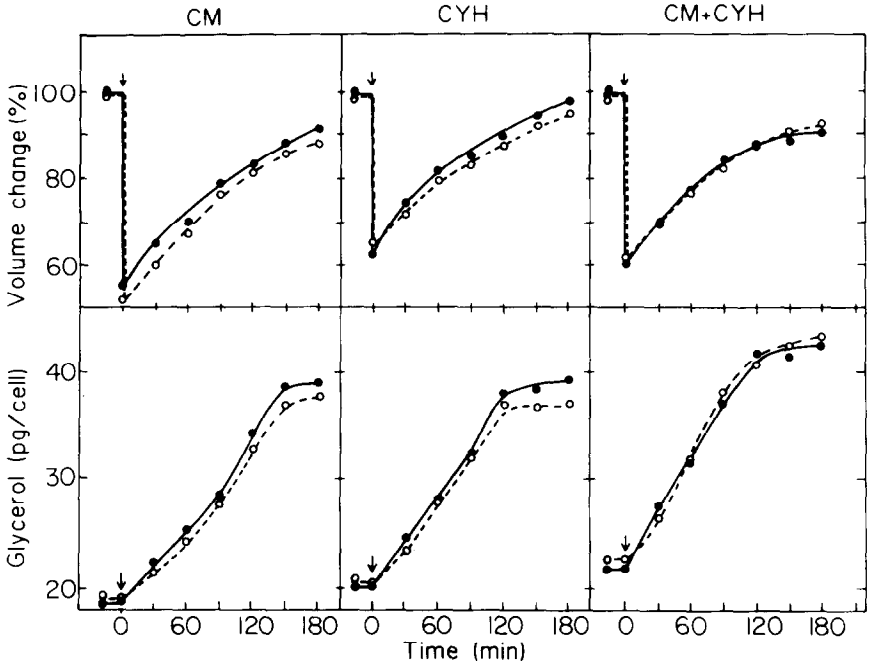


Fig.5. Effect of protein synthesis inhibitors on changes in cell volume and glycerol content following osmotic upshock. Details as in fig.4, except that the cells were resuspended in media containing 2 M NaCl.

tion, cycloheximide and chloramphenicol, at the concentrations employed, inhibited the incorporation of $^{35}S$ into trichloroacetic acid insoluble products by about 50 and 30%, respectively. Even when combined, the drugs still allowed a considerable residual incorporation. However, following a 3 h preincubation, cycloheximide alone, or the combination of both drugs completely eliminated protein synthesis, while chloramphenicol alone inhibited the synthesis by about 50%. These results indicated that in order to achieve a complete inhibition of protein synthesis during the osmotic adaptation, it was necessary to preincubate the cells with the inhibitors for several hours prior to the osmotic treatment. That protein synthesis was completely inhibited under these conditions was also demonstrated by a gel electrophoretic analysis of cell extracts (fig.3). The autoradiograms show that, aside from traces of probably the chloroplast-encoded large subunit of ribulose-bisphosphate carboxylase there is no evidence for the synthesis of proteins in cells pretreated with both cycloheximide and chloramphenicol for 2 h.

### 3.2. *Effect of protein synthesis inhibition on osmotic adaptation*

To test the effect of the protein synthesis inhibitors on the osmotic responses, *D. salina* cells grown in 1.0 M NaCl were transferred to a medium containing 0.5 M NaCl (downshock) or 2.0 M NaCl (upshock). The inhibitors were added 4 h before the osmotic shock. Following downshock (fig.4), the cells show the typical immediate increase and subsequent slow decrease in cell volume as well as the corresponding slow decrease in the cellular content of glycerol. These responses were practically unaffected when protein synthesis was inhibited by singly added, or combined inhibitors. Following upshock (fig.5) the expected reverse time-dependent changes in volume and glycerol content were observed, and here too the inhibitors of protein synthesis did not significantly affect the kinetics or extent of the responses.

### 3.3. *Osmotic adaptation and cell division*

The kinetics of osmotic adaptation were previously reported [1]. Yet, no detailed analysis was reported so far regarding the effect of the osmotic shock and the subsequent adaptation response on the replication of *Dunaliella* cells. To gain some insight into this question, we determined in parallel the kinetics of the change in cell density, glycerol accumulation and cell volume in cultures of *D. salina* transferred from a medium containing 0.5 M NaCl to media containing either 1.0 or 1.5 M NaCl. As a control, a sample of the original

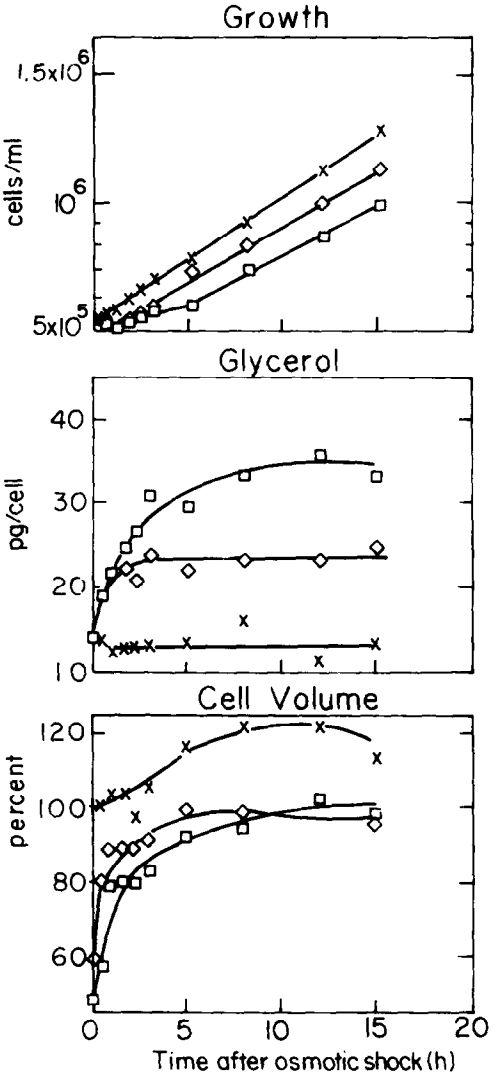


Fig.6. Cell proliferation, glycerol content and cell volume changes following upshock. Algae grown in a medium containing 0.5 M NaCl were diluted with an equal volume of media containing 0.5 M NaCl (×—×), 1.5 M NaCl (◇—◇), or 2.5 M NaCl (□—□). Samples were removed at the indicated times for determination of cell number, glycerol content and cellular volume as described in section 2.

culture was treated identically, but without any change in the NaCl concentration. The results (fig.6) show that the two upshocked cultures accumulate glycerol initially at approximately the same rate, but in the cells transferred to the higher salt concentration glycerol accumulation proceeds for a longer period. Thus, osmotic adaptation was essentially complete after about 2 h in the culture experiencing the lower upshock, but required about 4 h in the culture exposed to the higher upshock.

Cell counts during the adaptation period show lags of similar durations in cell proliferation followed by regular exponential growth. The culture transferred to 1.0 M NaCl resumes a normal growth rate sooner than the culture transferred to 1.5 M salt. In the exponential phase, the growth rate is slightly slower at the higher salt concentration. These results show that hyperosmotic shock causes an immediate halt in cell division but proliferation is reinitiated already during the latter period of glycerol accumulation and exponential growth is resumed not much later than the completion of osmotic adaptation.

## 4. CONCLUDING REMARKS

The present experiments show conclusively that adaptation of *D. salina* to either hypo- or hyperosmotic shock does not depend on de novo protein synthesis. Hence, the dramatic changes in glycerol metabolism induced by the shock cannot be a consequence of regulation at the level of gene expression. The results, however, do not rule out a possible role of post-translational modification in osmotic regulation. Post-translational modifications could be triggered, for example, by alterations in ion fluxes after the initial signal has been transduced.

Another aspect of the adaptation process is the capability of the osmotically adjusted cells to resume cell division. The present measurements indicate that exponential growth starts within a short period of the completion of the osmotic response, with a minor fraction of the cells starting to divide even earlier. Conceivably, the osmotic shock stops the cells at a specific stage of the cell cycle and may therefore synchronize the subsequent growth to some extent. It should be stressed that the accuracy of the measurements is insufficient to conclude whether an additional brief period must elapse before the osmotically balanced cells are able to divide. Therefore, we cannot, at this stage, completely exclude the possibility that in contrast to osmotic adaptation, adaptation to growth in the new medium may involve regulation at the level of gene expression.

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