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Selection and Characterization of *Dunaliella salina* Mutants Defective in Haloadaptation[1]

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ABSTRACT

A technique for selection of *Dunaliella* mutants defective in their capacity to recover from osmotic shocks has been developed. The selection is based on physical separation of mutants on density gradients. This technique takes advantage of the fact that *Dunaliella* cells, when exposed to osmotic shocks, initially change volume and density due to water gain or loss and subsequently recover their volume and density by readjusting their intracellular glycerol. Eight mutants that do not recover their original density following hyperosmotic shocks have been isolated. The mutants grow similar to wild type cells in 1 molar NaCl, and recover like the wild type from hypotonic shocks but are defective in recovering from hypertonic shocks. A partial characterization of one of the mutants is described.

The unicellular green alga *Dunaliella* has the remarkable capacity to grow and adapt to media ranging in salinity from 50 mM to 5 M NaCl. The major means of osmoregulation of this wall-less alga are by production of intracellular glycerol at concentrations that are proportional to the external NaCl concentration.

The response of *Dunaliella* to changes in the extracellular osmotic pressure occurs in two distinct phases. In the first phase the cells rapidly shrink or swell under hypertonic or hypotonic conditions, respectively. The second phase of adaptation is slower (2–3 h) and involves synthesis or elimination of glycerol. By the end of this period the cells recover their original volume.

Although it is clear that recovery of *Dunaliella* cells from hypertonic shocks involves glycerol production, and a few novel enzymes that seem to be involved in glycerol metabolism have been identified (2, 5), it is still uncertain what triggers glycerol production or elimination in response to osmotic shocks. Changes in Na^+ content (6, 13) pH level (9), phosphate (8), inositol phospholipids (7), and in ultrastructure (11) have been observed following osmotic shocks and were suggested to be involved in triggering glycerol production or elimination.

A valuable approach to study the mechanism of osmoregulation could be characterization of mutants defective in osmoregulation. Towards this aim we have developed a method for the separation of *Dunaliella* mutants defective in their osmotic response. Here, we describe the use of this method for the isolation of several different mutants defective in their response to hyperosmotic shocks and present the partial characterization of one of them.

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MATERIAL AND METHODS

Growth Conditions

Dunaliella salina was obtained from the culture collection of Dr. Thomas (Fisheries Institute, La Jolla, CA). The cells were grown in batch cultures, with periodic dilutions (to be maintained in a logarithmic growth phase).

The growth-medium contained 1 M NaCl, 50 mM $NaHCO_3$, 5mM KNO_3, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.2 mM KH_2PO_4, 0.185 mM H_3BO_3, 7 μM $MnCl_2$, 6 μM Na_2EDTA, 1.5 μM $FeCl_3$, 0.8 μM $ZnCl_2$, 20 nM $CoCl_2$, and 0.2 nM $CuCl_2$; the initial pH was 8.0. Cell suspensions in low-form culture flasks were shaken (80 rpm) in a New Brunswick controlled environment incubator shaker (model G-27) at 26°C. Continuous illumination of 300 to 350 foot-candles was supplied from cool-white fluorescent tubes (Sylvania). Under these conditions the doubling time was 7 to 8 h.

Cell concentration and volume was determined using a Coulter Counter (model F) with a 100 μm orifice.

Mutagenesis

Chemical mutagenesis: 10 mL of 4×10^7 cells/mL were treated for 60 min at room temperature with 0.3 mg of N-methyl-N-nitro-N-nitrosoguanidine. Under these conditions the survival score was 1.5%. The culture was transferred to fresh 1 M NaCl growth media and allowed to recover for a few (4–8) d. UV irradiation: 100 mL containing 10^6 cells/mL were irradiated with a UV lamp (10) at light intensity of 6 erg/cm^2/min. Following irradiation, the cells were kept in the dark for 24 h to avoid DNA photorepair processes.

Osmotic Shocks

Hypotonic shocks were induced by transferring cells grown at 2.5 M NaCl to 1 M NaCl madia. Hypertonic shocks were induced either by transferring cells grown in 1 M NaCl to 2.5 M NaCl or by a twofold dilution with growth media containing 4 M NaCl.

Separation on Density Gradients

To create discontinuous density gradients, 1 mL aliquots of isoosmotic gradient media were layered in 9 mL glass centrifuge tubes. The density of each gradient step was ob-

tained by weighing 1 mL of each layer. The gradients contained 7 layers. A sample of 0.5 mL media containing 5×10^6 to 10^7 cells (at 0°C) were layered on the top of each gradient. For separation of cells after mutagenesis similar gradients of 50 mL were formed, and samples of 5×10^7 cells were loaded on each gradient. The gradients were centrifuged for 30 min, at 3000 rpm at 0°C. The cells, identified as green bands, were carefully removed with a syringe, and their density was determined by weight.

Measurements of Glycerol Content

Glycerol was determined as follows: the cells were washed twice in isotonic media and filtered through miracloth paper. To 200 μL of the cell culture 1 mL of periodate reagent was added (65 mg $NaIO_4$, 10 mL acetic acid, 7.7 g ammonium acetate), and 2.5 mL of acetylacetone reagent (2.5 mL acetylacetone, 247.5 mL isopropanol) was added and mixed. The samples were incubated at 45°C for 20 min. Optical density was determined at 410 nm and compared to calibration standards.

Measurements of Carbohydrate Content

Two mL of 1 N HCl were added to 2×10^7 cells. The mixture was boiled for 20 min, cooled, and centrifuged (5 min, 2000 rpm). Samples of 0.5 mL were mixed with 0.5 mL of 5% phenol; 2.5 mL of H_2SO_4 were added to the samples and the optical density was determined at 488 nm and compared to calibration standards.

ATP Measurements

Osmotic shocks were induced by a twofold dilution with growth media containing 4 M NaCl. Samples of 20 μL were frozen immediately in liquid nitrogen to avoid ATP hydrolysis and to facilitate lysis of the cells. For the ATP measurements the cells were thawed and solubilized with 50 μL detergent (NRB Reagent 3M, Lumac, catalog No. 9225). Luciferase and luciferin were automatically injected into the lysate, and the ATP content was determined from the fluorescence intensity by comparison to ATP standards in a Lumac 3M Bicounter luminometer.

RESULTS

Distribution of *Dunaliella salina* Cells following Hyperosmotic Shocks on Stepwise Sucrose Gradients

Remarkable changes in volume occur in *Dunaliella* cells following hypertonic shocks. Since these volume changes result from water influx or efflux, they should be accompanied also by changes in cell density. As is demonstrated in Figure 1 the density of *D. salina* cells indeed increases immediately following hypertonic shocks (from 1–2.5 M NaCl, Fig. 1B), but cells resume their original density during a recovery period of 2 h (Fig. 1C). It should be noted that the density in samples B and C in Figure 1 are higher than the corresponding layers in sample A due to the contribution of the higher NaCl concentration. The density of cells grown at 1 M NaCl is between 1.18 and 1.22 g/mL (Fig. 1A); it increases to 1.25 to 1.27 g/mL immediately after the shock (Fig. 1B), and it returns to 1.19 to 1.23 g/mL 2h following the shock (Fig. 1C). It can be concluded, therefore, that the change in density of the cells during the osmotic response, can be followed using sucrose gradients.

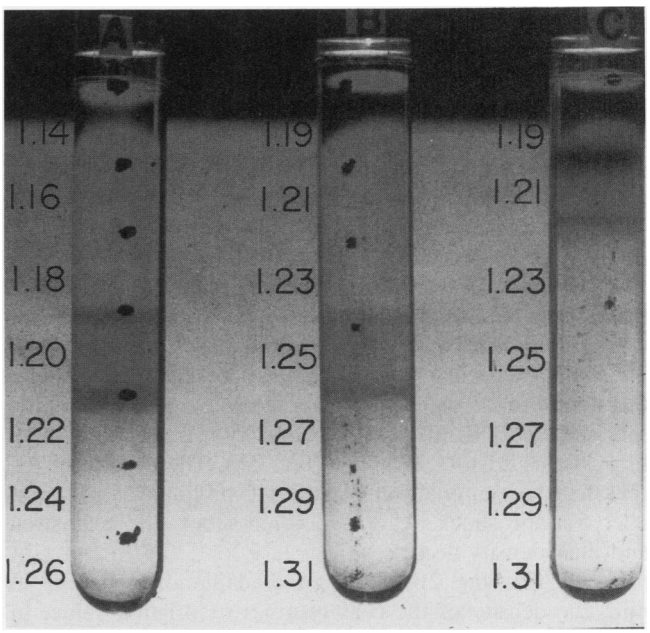

Figure 1. Density changes in *D. salina* cells following hypertonic shocks. A (left), Cells grown at 1 M NaCl were layered on a sucrose gradient containing 1 M NaCl. The densities of the layers (from the top) are: 1.14, 1.16, 1.18, 1.20, 1.22, 1.24, and 1.26 g/mL. B (center), Immediately after osmotic shock, cells were layered on a gradient containing 2.5 M NaCl. The densities of the layers (from the top) are: 1.19, 1.21, 1.23, 1.25, 1.27, 1.29, 1.31 g/mL. C (right), Two h after osmotic shock, cells were layered on a gradient with the same densities and NaCl content as in B.

The observation that cells resume almost exactly their original density following recovery from hyperosmotic shocks is somewhat surprising in view of the high specific weight of glycerol which is accumulated inside the cells. We have compared densities of *D. salina* cells adapted to 0.2 to 4 M NaCl and observed that at higher salinities cells indeed have higher densities than low salt adapted cells, consistent with the higher glycerol contents of the former. However, it appears that additional factors, and in particular the starch content, contribute to the overall cell density. It is possible that the decrease in starch content compensates the effect of accumulated glycerol on cell density following hyperosmotic shocks to preserve the original density.

Since the sucrose concentrations needed to obtain the densities required for cell separation are high, the osmotic contribution of sucrose, on top of NaCl becomes significant and should cause further cell shrinkage and an increase in density. Therefore, a comparison was made between different chemicals commonly used for density gradient separation of cells, including polysaccharides which have negligible osmotic contribution.

As is demonstrated in Table I the apparent densities obtained before and after hypertonic shocks with ficoll, dextran,

sucrose, and metrizamide density gradients are quite similar, indicating only a slight increase in density in sucrose at 1 M NaCl, compared to ficoll and dextran. It may be noted that separation on ficoll and dextran density gradients requires longer and faster centrifugation times due to their higher viscosity and often results in cell aggregation, while separation on sucrose density gradients is fast, and the viability of the separated cells is excellent. Therefore, for most practical purposes we have used sucrose density gradients.

To exemplify the usefulness of this technique we followed the kinetics of density changes in *Dunaliella* cells after hypotonic and hypertonic shocks by sucrose density gradients (Fig. 2). *Dunaliella* cells grown either at 1 or 2.5 M NaCl were transferred to 2.5 or 1 M NaCl, respectively. After hypertonic shock, the cells shrink and their density increases from 1.19 to 1.20 to 1.23 to 1.25 g/mL (Fig. 2A). The density of the cells decreases gradually and reaches its original value 90 min after osmotic shock. After hypotonic shock, the cells swell and their density decreases from 1.19 to 1.20 g/mL to 1.14 to 1.18 g/mL (Fig. 2B). During the adaptation period of 90 min, the density of the cells resumes its original value. In both, hypertonic or hypotonic shocks, the recovery of the density under dark conditions is slower. It may be concluded that density changes as well as volume or glycerol content may be used to follow the recovery of *D. salina* cells from osmotic shocks.

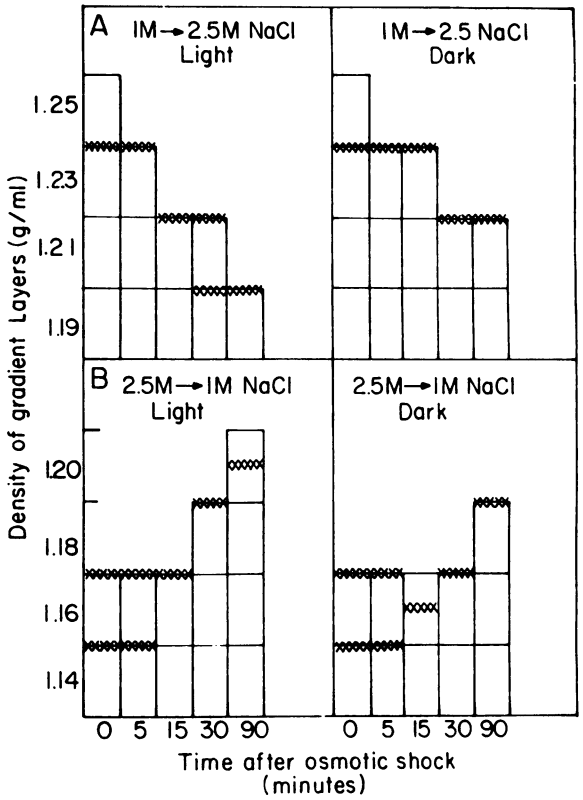

Figure 2. Kinetics of density changes in *D. salina* following hypertonic or hypotonic shocks in light or dark conditions. Cells grown at 1 or 2.5 M NaCl were transferred to 2.5 or 1 M NaCl growth media, respectively. The cells were allowed to recover from both hypertonic or hypotonic shocks either in light or dark. Samples of cells were centrifuged 0, 5, 15, 30, or 90 min after the osmotic shocks on sucrose stepwise gradients composed of seven layers of sucrose (1 mL each) containing either 2.5 or 1 M NaCl (hypertonic or hypotonic shocks, respectively). The density of cells grown at either 1 or 2.5 M NaCl was 1.19 to 1.20 g/mL.

Isolation of Mutants Defective in the Osmotic Response

The observation that recovery from hyperosmotic shocks is associated with changes in cell density may be used for selection of mutants defective in their osmotic response, the rationale being that mutants should not resume their low density during the recovery period and therefore can be physically separated from normal cells. The isolation procedure was as follows: *D. salina* cells were exposed either to UV light or to a chemical mutagen. After mutagenesis, cells were subjected to a hypertonic shock by a 2.5-fold increase in NaCl concentration, followed by incubation for 4 h in the light to allow recovery from the osmotic shock and applied to discontinuous sucrose gradients containing 2.5 M NaCl. After centrifugation, 1 mL of the 35 to 40% interphase, corresponding to shrunken cells (most of the cells recovered from the shock and were in the 20–25% interphase), was collected from the gradients with a syringe. The cells were washed from the sucrose and transferred to 1 M NaCl growth media.

Table I. *Density of D. salina Cells during Osmotic Shock and Recovery Measured on Different Density Gradients*

Cells grown in 1 M NaCl were subjected to hypertonic shock by raising the salt concentration to 2.5 M. Numbers indicate the densities at the interphase where the cells migrate in step-wise gradients. The gradients were made with isoosmotic NaCl solutions. After application of cell samples, the gradients were centrifuged for 30 min at 3000 rpm (sucrose or metrizamide) or at 5000 rpm (ficoll or dextran). Densities are expressed in g/mL.

Gradient Composition	Control Cells	Osmotically Shocked Cells
Sucrose	1.18–1.20	1.23–1.24
Ficoll	1.17	1.24
Dextran	1.15–1.17	
Metrizamide	1.16–1.19	1.23–1.24

Cloning of the Mutants

With the purpose of obtaining single cell cultures, the cells were plated in 96 well microplates in high dilution (1 cell/4 wells). To avoid evaporation, the plates were sealed with masking tape. After 10 culturing days in continuous illumination, 350 colonies were observed in 18 plates. Samples from each colony were transferred to microplates containing 2.5 M NaCl growth media. Growth was monitored by the increase in absorption at 450 nm. The colonies which failed to grow were selected and cultured in 1 M NaCl media.

Following chemical mutagenesis, 7 out of 350 colonies failed to respond normally to upshocks. They are denoted: D3, D4, D6, D7, D8, D9, and D10 (see Fig. 3). Following UV irradiation, 1 out of 100 colonies was unable to recover after upshock. It is denoted U22.

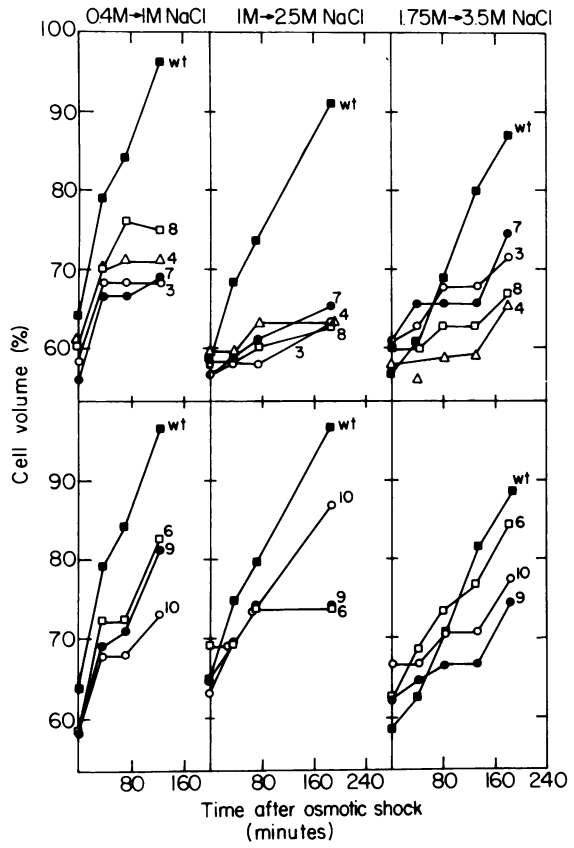

Figure 3. Volume changes in *D. salina* mutants following hypertonic shocks in different salt concentrations. The mutants D3, D4, D6, D7, D8, D9, D10, and wild type, adapted to 0.4, 1, or 1.75 M NaCl were transferred to 1, 2.5, or 3.5 M NaCl, respectively. Samples of cells were taken at the indicated times following hypertonic shock and the volume measured using a coulter counter. Note that the factor of shock for cells adapted to 0.4 and 1 M NaCl is higher (2.5-fold) than for cells adapted to 1.75 M NaCl (2-fold).

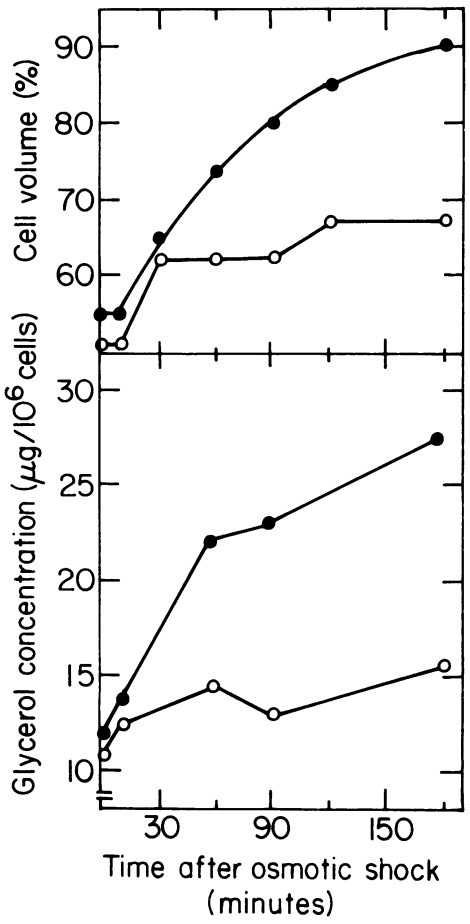

Figure 4. Volume and glycerol changes in U22 following hypertonic shocks. Wild-type (●) and U22 (○) *Dunaliella* grown at 1 M NaCl were transferred to 2.5 M NaCl. Samples of cells were taken before and after the shock for volume and glycerol measurements.

Response to Hyperosmotic Shocks of *Dunaliella* Osmotic Mutants, Adapted to Different NaCl Concentrations

To characterize the response of the *D. salina* osmotic mutants to hyperosmotic shocks, the kinetics of volume recovery and glycerol synthesis following upshocks was measured (Figs. 3 and 4). The UV-induced mutant (U22) completely fails to recover from hypertonic shocks (1–2.5 M NaCl) within 2 to 3 h as is demonstrated from the kinetics of glycerol production and of volume changes in comparison to the wild type (Fig. 4). Similarly, all seven nitrosoguanidine mutants either completely fail to recover within 3 h or recover slower than the wild type (Fig. 3, center). Glycerol production closely parallels the rate of volume recovery in all cases (not shown). It may be noted that the growth rate of all mutants in 1 M NaCl is similar to that of the wild type (doubling time 7–8 h except for D4 which was slightly slower).

To check whether the failure to recover from hypertonic shocks results from a defect in triggering glycerol synthesis or from inhibition by high NaCl concentration, we tried to expose the mutants to hyperosmotic shocks at different NaCl concentrations. All mutants could be adapted either to 0.4 M NaCl or to 1.75 M NaCl, but the adaptation to the latter required several days in comparison to several hours in the wild type. As is demonstrated in Figure 3, all the nitrosoguanidine mutants recover slower than the wild type both at the lower and at the higher NaCl concentration. These results suggest that the defects in these mutants are not due to inhibition by high salt.

In contrast to the severe inhibition in recovering from hypertonic shocks, the U-22 mutant recovers like the wild type from hypotonic shocks (Fig. 5, bottom). Similar results were obtained for all the nitrosoguanidine mutants, indicating that the defect in triggering glycerol production is specific and unrelated to the trigger of glycerol elimination. Na^+ or Cl^- ions do not seem to be specifically involved in the defected response of U-22 to upshocks since osmotic shocks in Tris-cloride or Na phosphate media give identical results to the NaCl media for both wild type and U-22.

Changes of Volume and ATP Content in U22 and Wild Type *D. salina* during Hyperosmotic Response

It has been demonstrated that the level of cellular ATP in *Dunaliella* decreases dramatically within minutes after hyper-

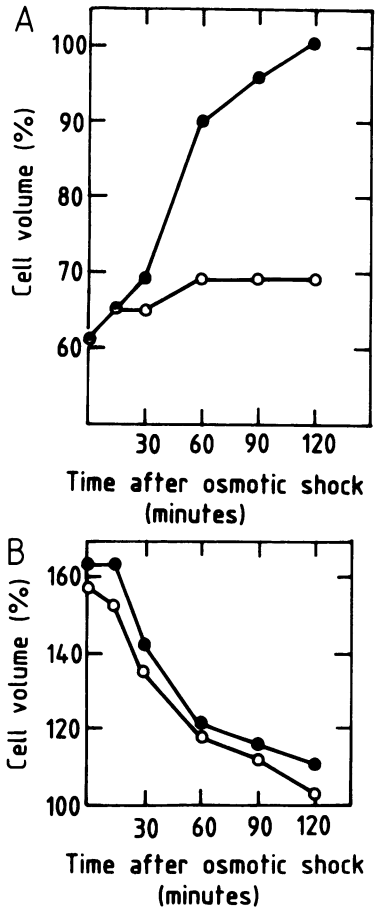

Figure 5. Volume changes in *D. salina* wild type (●) and U22 (○) following hypertonic or hypotonic shocks. A, Cells were tested for their response to hypertonic shock as in Figure 4. B, Wild-type *Dunaliella* and U22 adapted to 1 M NaCl were transferred to 0.5 M NaCl. Samples of cells were taken following the hypotonic shock for volume measurements.

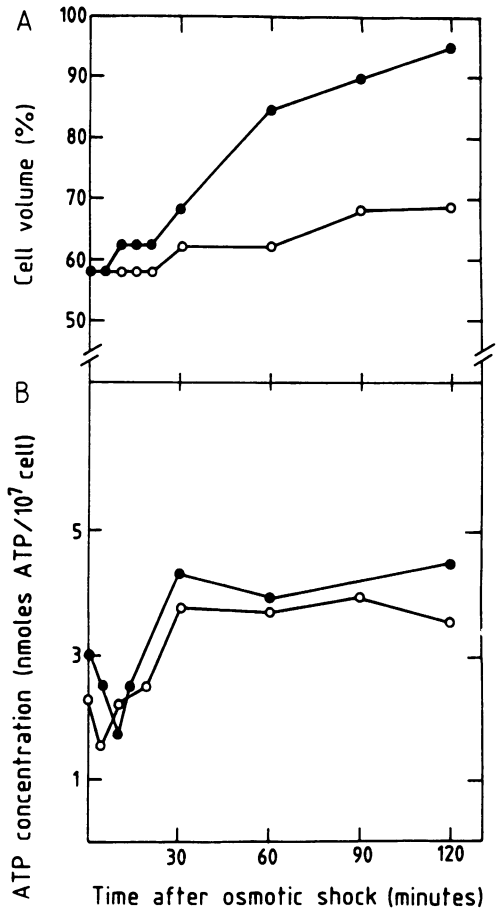

Figure 6. Changes in ATP content following hypertonic shock in *D. salina* wild type and U22. U22 (○) and wild-type (●) *D. salina* grown in 1 M NaCl are osmoticaly shocked in 2.5 M NaCl. Samples of cells were taken for volume (A) and ATP (B) measurements.

osmotic shocks. Furthermore, it appears that the capacity to recover from hyperosmotic shocks is closely correlated with ATP production and is perturbed by metabolic inhibitors (12). It seemed possible, therefore, that the defect in U-22 may be related to ATP supply and utilization in the response to upshocks. However, as is demonstrated in Figure 6, the transient decrease in cellular ATP level in wild type and U-22 *D. salina* following upshocks is similar, suggesting that the defect in U-22 is not related to ATP production or utilization.

Starch Utilization of U22 and Wild Type *Dunaliella* during Hyperosmotic Response in the Dark and in the Light

One of the possible defects in U-22, as well as the other mutants, is a metabolic deficiency in triggering starch mobilization to glycerol. To check this possibility, we searched for conditions under which recovery from hypertonic shocks will be solely dependent on starch reserves. Glycerol synthesis of *Dunaliella* can be driven either through starch breakdown or via photosynthetic CO_2 assimilation through the Calvin cycle. Cells recovering in the dark from hypertonic shock will utilize only starch for glycerol synthesis. Therefore, recovery from hypertonic shocks in the dark is more efficient in cells with high starch reserves. Recovery of wild type and U-22 from upshocks was compared in the dark in cells subjected to nitrate starvation, a treatment that causes an increase of starch concentration in the cells (1).

As is demonstrated in Table II, the mutant U22 cannot degrade starch needed for glycerol accumulation following osmotic upshocks either in the dark or in the light. However, the mutant appears to be capable of utilizing starch for other metabolic purposes, since the starch content of U22 cells decreases in the dark and the cells duplicate normally in synchronized culturing conditions, in the dark (not shown).

These results may indicate that the mutation in U22 may be in the triggering of starch utilization in response to hypertonic shock. An alternative metabolic defect that could block starch conversion to glycerol is the inactivation of one of the terminal enzymes leading to glycerol production from dihydroxyacetone phosphate. Such a defect could lead to accumulation of precursors of glycerol following upshocks, such as glycerol-3-phosphate or dihydroxyacetonephosphate. We have, therefore, compared the soluble carbon metabolites in wild type and U22 *D. salina* following osmotic upshocks, using ^{13}C NMR (3), but observed no difference in their

Table II. *Starch, Glycerol, and Volume Changes following Hypertonic Shock in D. salina Wild Type and U22 in the Light or in the Dark*

Cells cultured in low-nitrate for starch accumulation (A) or in regular growth medium (B) were osmoticaly shocked (1–2.5 M NaCl) in the dark (A) or in the light (B). Starch or glycerol contents were determined either before the shock or after a recovery period of 2 h (A) or 45 min (B) as described under "Materials and Methods."

Cells	State of Cells	Cell Volume	Starch Content	Glycerol Content
		%	$\mu g/10^6$ cells	
(A)				
Wild type	Before shock	100	39.5	NT[a]
	After shock	61		
	Recovery period	78	21.0	NT
U22	Before shock	100	42.0	NT
	After shock	63		
	Recovery period	68	43.0	NT
(B)				
Wild type	Before shock	100	12.6	11.5
	After shock	52		
	Recovery period	76	8.0	18.8
U22	Before shock	100	23.0	14.0
	After shock	52		
	Recovery period	60	24.0	15.0

[a] Not tested.

metabolite contents except for the absence of glycerol accumulation in U22 (E. Chitlaru, M. Shamir, unpublished observations). From these results it may be concluded that the defect in U22 is most probably in triggering one of the osmoregulatory metabolic steps leading to glycerol production, such as starch degradation.

DISCUSSION

Little is known about the mechanism which triggers glycerol production in *Dunaliella* in response to hypertonic shocks. Mutants which are specifically defective in triggering glycerol production are in this context of special importance. However, so far there has been only one report about a *Dunaliella* mutant defective in responding to osmotic shocks, which is in fact a metabolic mutant, with an abnormal carbonic anhydrase (4).

Theoretically a minimum of four types of osmotic mutants may be expected: (a) mutants in which one of the enzymes involved in glycerol biosynthesis becomes rate limiting in recovery from hyperosmotic shocks; (b) mutants defective in the triggering mechanism of glycerol production, which is presumably located in the plasma membrane (7, 11); (c) metabolic mutants in which the production of ATP or NAD(P)H needed for glycerol production becomes rate limiting for the recovery from hyperosmotic shocks; (d) mutants which are particularly sensitive to high salt.

The selection method which is described here has several advantages over a general screening technique: (a) the physical separation of nonrecovering from recovering cells, following osmotic upshocks, avoids the need of screening a large number of mutants; (b) the fact that the permissive medium contains 1 M NaCl lowers the probability of selection of metabolic mutants or of mutants with major defects in glycerol production, since such mutants will not survive in 1 M NaCl. In some experiments, we have incubated cells following mutagenesis under light-dark cycles (8–16 h, respectively) to eliminate metabolic mutants defective in starch mobilization. The final elimination of metabolic mutants was made by avoiding mutants which failed to grow like wild type in the permissive medium. Nevertheless, metabolic mutants cannot be excluded altogether by this selection since a minor metabolic defect that may not be manifested in steady state growth may become a limiting factor under stringent osmotic stress conditions.

The possibility of salt-sensitive mutants seem unlikely in view of the observations that all the selected mutants can be adapted to high salt following prolonged incubation and respond poorly to osmotic upshocks in different salt concentration ranges (Fig. 3).

Although at present we have not identified the specific defect in any of the mutants, several conclusions can be drawn already from their preliminary characterization: (a) The observation that all the mutants respond like the wild type to hypotonic shocks suggests that the triggering mechanisms of glycerol production and elimination are different regulatory and metabolic elements. This conclusion is consistent with recent observations of different changes in plasma membrane lipids, notably inositol phospholipids, and ultrastructure following upshocks and downshocks in *Dunaliella* (7, 11) and with our recent observation that certain H^+-ATPase inhibitors specificaly inhibit recovery from upshocks but not from downshocks in *D. salina* (12). (b) We have noticed several differences in the response to osmotic upshocks among the mutants; for example, the rate of recovery differs in different salt concentrations (Fig. 3). We have preliminary indications suggesting that some of the osmotic mutants may fall into the category of metabolic mutants, other are limited in starch mobilization, and the rest are blocked at different stages. These differences suggest that the triggering of glycerol production is a sequence of steps involving several different enzymes. It is our hope that further characterization of these mutants will contribute to the understanding of the triggering mechanism of glycerol production in response to hypertonic shocks in *Dunaliella*.

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