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The response of the green halotolerant alga *Dunaliella* to osmotic stress: effects on adenine nucleotide contents

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Dunaliella tertiolecta **was subjected to osmotic stress by upward (salt stress) and downward (dilution stress) shifts in NaCl concentration, and the effects on adenine nucleotide and glycerol contents determined. Dilution of the algal suspensions with growth medium at constant NaCl concentration (as control experiments) caused oscillations and, over a 30 min time-course, small changes in the ATP/ADP quotient. Higher values of this quotient were observed in cells kept in the dark than in the light. The effect of salt stress on the adenine nucleotides depended on the size of the stress: salt stress from 0.17 M to 0.7 M caused damped oscillations in ATP and ADP contents about a mean close to the initial value, while a larger salt stress to 1.0 M NaCl caused the ATP content and ATP/ADP quotient initially to drop, followed by a slow and partial recovery over the 30 min time-course of the experiment. The cell shrinkage that immediately follows salt stress could nevertheless be sufficient to cause a transient increase in ATP and ADP concentrations during both stresses. In both cases, the glycerol content increased linearly after salt stress. The application of dilution stress decreased ADP and increased the ATP/ADP quotient in the dark but not the light, although the glycerol content decreased more rapidly in the light. These results do not support a direct role for the content or concentration of ATP or ADP, or of the energetic status of the cell as indicated by the ATP/ADP quotient, in regulating the synthesis and dissimilation of glycerol involved in osmoregulation.**

Introduction

Dunaliella is a green unicellular alga that is able to grow in wide range of salt concentrations, and to withstand changes in salt concentration. The ability of this alga to set the internal glycerol concentration at the level required to maintain a constant cell volume at different external salt concentrations appears to be the basis for its osmoregulatory capability. The metabolism of glycerol is relatively simple, involving a two-step synthesis and dissimilation to and from dihydroxyacetone phosphate [1]. The mechanism by which these reactions respond to osmotic stress may be central to osmoregulation in *Dunaliella*.

The effect of salt stress on the adenine nucleotide contents of Dunaliella has been investigated by Gimmler and Möller [2], who reported a decrease in ATP/ADP quotient and phosphorylation potential when *D. parva* was subjected to an increase in salt concentration. More recently, Belmans and Van Laere [3] reported a significant decrease in ATP content of *D. tertiolecta* following the application of a salt stress. Here, we present the results of experiments designed to clarify the effect of osmotic stress on the adenine nucleotide contents and to examine whether there is any relation between the changes in glycerol level and the adenine nucleotide content of *D. tertiolecta*.

The adenine nucleotide content of cells can be expressed in a number of ways in relation to metabolic status, including ATP concentration, ATP/ADP quotient, phosphorylation potential ($[ATP]/[ADP] \cdot [P_i]$) and adenylate energy charge [4]. We have elected to determine the ATP/ADP quotient because of its fundamental importance in describing the energy status of cells [5], its sensitivity to metabolic state for values above unity [6], and because it does not involve any assumptions about adenylate kinase activity which is absent from the mitochondrial matrix in plants [7]. An important practical advantage of the ATP/ADP quotient is that it is independent of the cell volume, which

Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; P_i, inorganic orthophosphate.

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changes rapidly following osmotic stress. The measurement of the intracellular volume of *Dunaliella* has been controversial, and the best method available [8] is not suited to following rapid changes in volume. Additionally, the use of the ATP/ADP quotient avoids the need to measure AMP which can be difficult to determine accurately [7].

Materials and Methods

Growth and treatment of cultures. Dunaliella tertiolecta was maintained and cultured in the defined medium of Johnson et al. [9], but with Tris-Cl^- replaced by 20 mM K^+-Hepes and with NaCl concentrations between 0.17 and 0.7 M as required [10]. The following modifications were made: the experimental cultures were grown at $27 \pm 2°C$ with continuous aeration with 5% CO_2 in air and shaking. The light intensity was 200 $\mu E \cdot m^{-2} \cdot s^{-1}$, with a light/dark regime of 16/8 h. The culture was harvested by centrifugation at $2000 \times g$ for 60 s in the late exponential growth phase, 7 days after inoculation. The algae were resuspended in iso-osmotic growth medium (pH 7.5).

Application of osmotic stress. Algal suspensions were placed in a glass cylinder (28 mm diameter) and stirred continually with a magnetic stirrer. The glass cylinder was held in a constant temperature water bath ($27 \pm 2°C$) in darkness or illuminated from opposite sides, when appropriate, by two slide projectors with quartz-iodine lamps (photosynthetic photon flux; 800 $\mu E \cdot m^{-2} \cdot s^{-1}$). The suspension was preincubated for 15 min under these conditions before the start of the experiment.

Salt stress was imposed by adding, to a stirred 30 ml sample of algal suspension (10–25 μg chlorophyll $\cdot ml^{-1}$), 10 ml culture medium containing 0.17 M NaCl (control), 2.29 M NaCl (stress to 0.7 M) or 3.49 M NaCl (stress to 1.0 M). Dilution stress was imposed by adding, to a stirred 5 ml sample of algal suspension (130 μg chlorophyll per ml), 15.59 ml culture medium containing 0.7 M NaCl (control) or no NaCl (stress to 0.17 M). The osmolality of each of the three media, determined with a Wescor 5100C vapour pressure osmometer, was as follows: medium containing 0.17 M, 0.7 M and 1.0 M NaCl; osmolality was 384, 1385 and 1938 $mmol \cdot kg^{-1}$, respectively. Samples were taken before each stress was imposed and at each subsequent sampling time. The sampling and metabolic quenching of the cell suspension was achieved by withdrawing a 1 ml sample and ejecting it rapidly into a small glass test-tube containing the quenching agent, held adjacent to the glass cylinder. The transfer of the sample was done in a way that maintained the illumination or dark conditions until mixing of the quenching agent with the suspension.

Extraction of nucleotides. The quenching agent used was 70% perchloric acid (125 μl, room temperature). Perchloric acid extracts were centrifuged and the supernatants neutralised with KOH (5 M in 1 M triethanolamine). Some initial experiments were also done using a procedure in which algal suspensions were quenched by addition of a 10-fold larger volume of 80% ethanol containing 1 mM EDTA. This was immediately boiled until the volume reached 1/5 the initial volume, after which the extract was cooled and adjusted with water to a volume which was twice that of the original algal suspension. Extracts were stored at $-85°C$ for up to 10 days.

Determination of ATP and ADP. Adenine nucleotides were determined by the luciferin/luciferase procedure using a luminometer (LKB-Wallac 1251) operated in the automatic mode with dispensers for the luciferin/luciferase reagent. The luminometer was interfaced with an Apple Macintosh Plus computer which received and stored the data for subsequent analysis (software: Apple Macterminal). Each reaction mixture (25 °C) contained buffer (100 mM tricine, 50 mM magnesium acetate, 2.5 mM EDTA, 0.1 mM tetra-sodium pyrophosphate (pH 7.75 with KOH); 100 μl), luciferin/luciferase reagent (100 mM Tricine-KOH (pH 7.75), 0.5 mM dithiothreitol, 5 $mg \cdot ml^{-1}$ defatted bovine serum albumin, 375 μM D-luciferin and 2.25 $\mu g \cdot ml^{-1}$ luciferase; 50 μl), extract (containing 5–50 pmol ATP) and water to give a final volume of 500 μl. Each assay was started (zero time) by the addition of the luciferin/luciferase and the maximum peak height of light emission determined. At 2 s intervals, five 10 μl aliquots of standard ATP (50 pmol) were added and the incremental peak height signal from each determined. From a regression analysis of the five incremental signals, the theoretical internal standard signal at zero time was computed. This internal standard signal was used to calculate the quench factor applying to that assay. For the determination of ADP + ATP, the reaction mixture contained, additionally, 80 nmol P-enol pyruvate and 0.4 units pyruvate kinase, and was preincubated for 30 min before the ATP was determined as before. The ADP content of samples was then calculated by difference. Data analysis was performed by the Macintosh computer (software: Microsoft Excel). D-luciferin, firefly luciferase and other substrates and enzymes were purchased from Boehringer Mannheim.

The concentrations of luciferin, luciferase and of the minor components of the assay mixture (for further information, see Refs. 11 and 12) were selected for optimum sensitivity and stability of the emitted light signal for the conditions described. The luciferin/luciferase reagent was prepared in bulk in darkness using nitrogen-saturated solutions, dispensed into 1 ml aliquots in sealed tubes and stored for up to 1 year at $-85°C$ without significant loss of activity.

Glycerol and chlorophyll determination and cell-volume estimation. The glycerol content of algal extracts was determined by the procedure of Eggstein and Kuhlmann [13]. Chlorophyll was determined by extracting cell suspensions in 80% acetone and determining the extinction at 652 nm [14]. The cell volume was estimated from the packed cell volume [15] obtained by centrifuging samples of the alga at $2000 \times g$ for 3 min.

Results and Discussion

The extraction of *D. tertiolecta* suspensions with ethanol yielded ATP levels that were more variable and about half those obtained with perchloric acid extraction. The latter procedure, which is used widely for metabolic quenching [7], was therefore adopted.

The ATP and ADP contents of *D. tertiolecta*, expressed on a chlorophyll basis, varied substantially between different cultures. When the alga was grown in 0.17 M NaCl, contents (nmol per mg chlorophyll) of between 80 and 270 for ATP and between 40 and 150 for ADP were observed in the light with a range of cultures grown under similar conditions. The ATP/ADP quotients were less variable, ranging between 1.6 and 2.5. However, when measurements were made on an individual cell culture over a limited time period, the adenine nucleotide contents and ATP/ADP quotient gave almost constant values. When, in a different experiment, 24 separate determinations were made over a 30 min period on an illuminated culture grown in 0.17 M NaCl, the mean ($\pm$standard deviation) values obtained were as follows: ATP; 130 ± 4, ADP; 63 ± 5, ATP/ADP quotient; 2.08 ± 0.15. The ATP content of the supernatant from centrifuged suspensions was generally less than 2% of the total ATP present in the cell culture.

When *D. tertiolecta* suspensions grown in 0.17 M NaCl were diluted with culture medium without changing the NaCl concentration (Fig. 1), the ATP/ADP quotient oscillated about a median that increased in darkness during the 30 min time-course of these control experiments. In an identical experiment with a different algal culture, oscillations in the ATP/ADP quotient were also observed in the light (data not shown). There

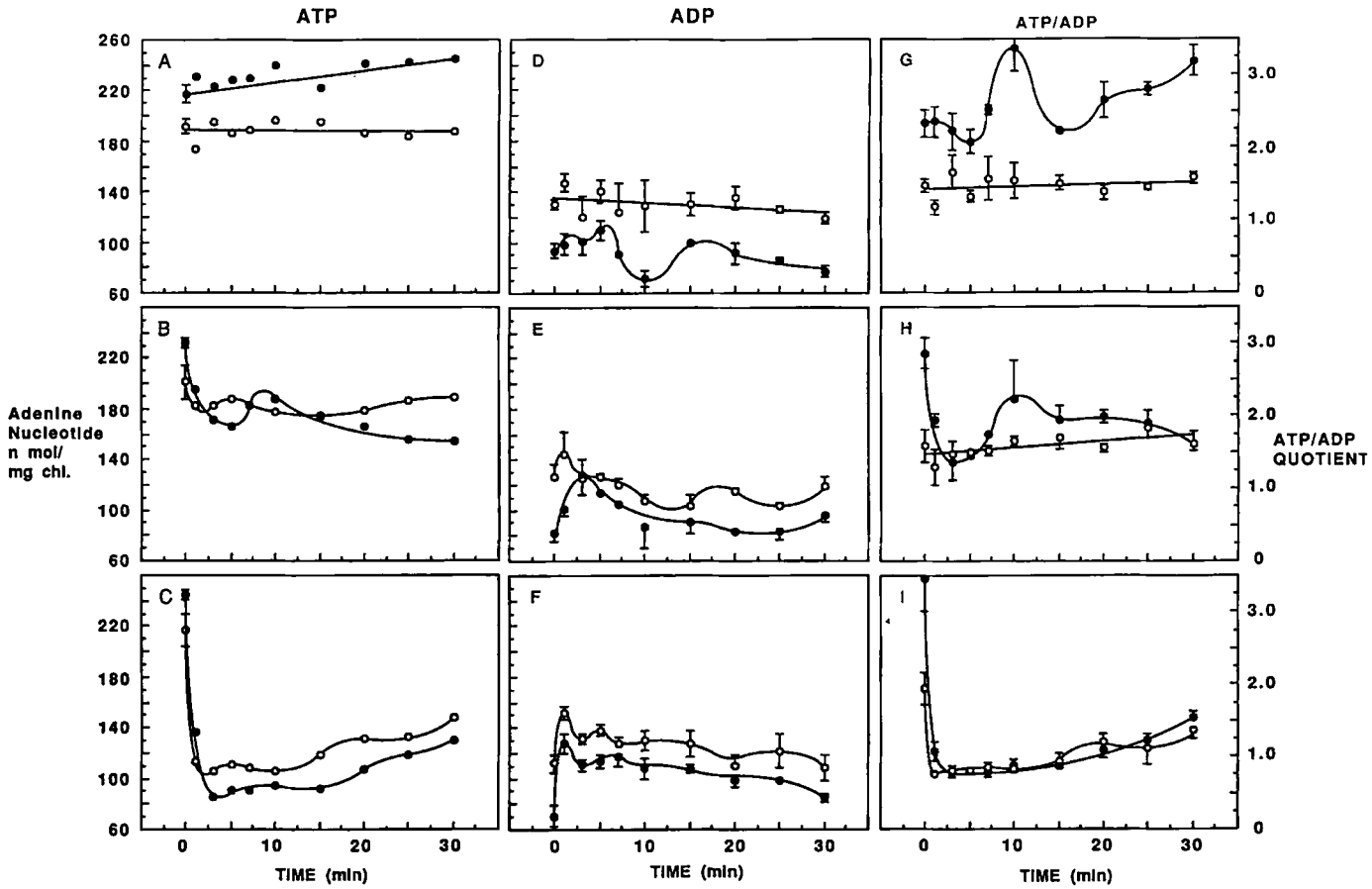

Fig. 1. The response to salt stress of the ATP content (A–C), the ADP content (D–F) and the ATP/ADP quotient (G–I) of *D. tertiolecta* (cultured for 6 days in 0.17 M NaCl). At zero time, growth medium containing NaCl (see Methods) was added to the culture in the light (○) or dark (●) to give final concentrations of 0.17 M (A, D, G), 0.7 M (B, E, H) or 1.0 M (C, F, I). Each value is the mean of at least four determinations where the standard deviation is represented by a bar, or otherwise of duplicate determinations. A linear regression line is drawn for each set of values when the standard deviations of the zero time point and at least seven other points intersect the line. If otherwise, curves were fitted to each set of values by hand.

was no change in the glycerol content of the alga under these conditions (Fig. 3a). While the data are insufficient to allow the amplitude and frequency of the oscillations in *Dunaliella* to be accurately determined, it can be seen that, in general, the oscillations damped out over the 30 min time-course of measurement.

Oscillatory behavior is well documented with soluble enzyme systems, isolated organelles, single cells and cell cultures [16]. The oscillations in ATP and ADP contents, observed here on dilution of the algal suspension at constant salinity, cannot be explained by our own results. They probably have their origin in changes in concentration of a metabolite such as CO_2 or O_2, or of a molecule that affects metabolism [17,18]. The demonstration here that diluting the algal culture (at constant osmolality) can cause changes in adenine nucleotides has implications for the design of experiments intended to test the effect of osmotic stress or any other modification involving dilution of algal cultures.

When the alga was subjected to salt stress by increasing the NaCl concentration from 0.17 M to 0.7 M with the addition of the same volume of medium as used in the control experiment (Fig. 1), the ATP and ADP contents again exhibited damped oscillations. In darkness, the ATP/ADP quotient oscillated about a median that decreased, although in an identical experiment with a different culture, no such decrease was seen (data not shown). In the light there was little change in ATP/ADP quotient. The glycerol content of the cells began to increase linearly after a lag of about 2 min over the 30 min course of the experiment (Fig. 3a and b). The estimated volume of the cells in 0.17 M NaCl was 125 ± 18 μl per mg chlorophyll (mean $\pm$ S.D.) of measurements on five different *Dunaliella* suspensions. This had decreased by about 40% to 77 ± 10 μl per mg chlorophyll at approx. 7 min after salt stress to 0.7 M NaCl.

The application of a larger salt stress to 1.0 M NaCl (Fig. 1) caused a rapid initial drop in ATP and rise in ADP content. The ATP/ADP quotient slowly and partially recovered over the 30 min time-course of the experiment. Prominent oscillations in these values were not then evident. The glycerol content also increased, but at a lower rate than after the smaller salt stress (Fig. 3). It is concluded that this salt stress caused either interference with the regulatory mechanisms that set the ATP and ADP levels and their quotient, or a perturbation of cell metabolism sufficiently large to exceed their range, as demonstrated by the rapid decrease in ATP content. Restoration of the adenine nucleotides to the initial level was then achieved without marked oscillations by a process with time-course exceeding 30 min. The application of an even larger stress to 1.3 M NaCl (results not shown) caused the ATP/ADP quotient to fall to below 0.5, and a proportion of the cells then never regained motility.

When *D. tertiolecta* was grown in 0.7 M NaCl, the ATP content was substantially lower on a chlorophyll basis than for cells grown in 0.17 M NaCl (Fig. 2). When the algal suspension was diluted by a factor of 4.1 while maintaining salinity at 0.7 M NaCl, oscillations in ADP content and ATP/ADP quotient similar to those of the control experiments at 0.17 M NaCl (Fig. 1) occurred. A small decrease in glycerol content was evident during the 30 min period after this control dilution (Fig. 3c).

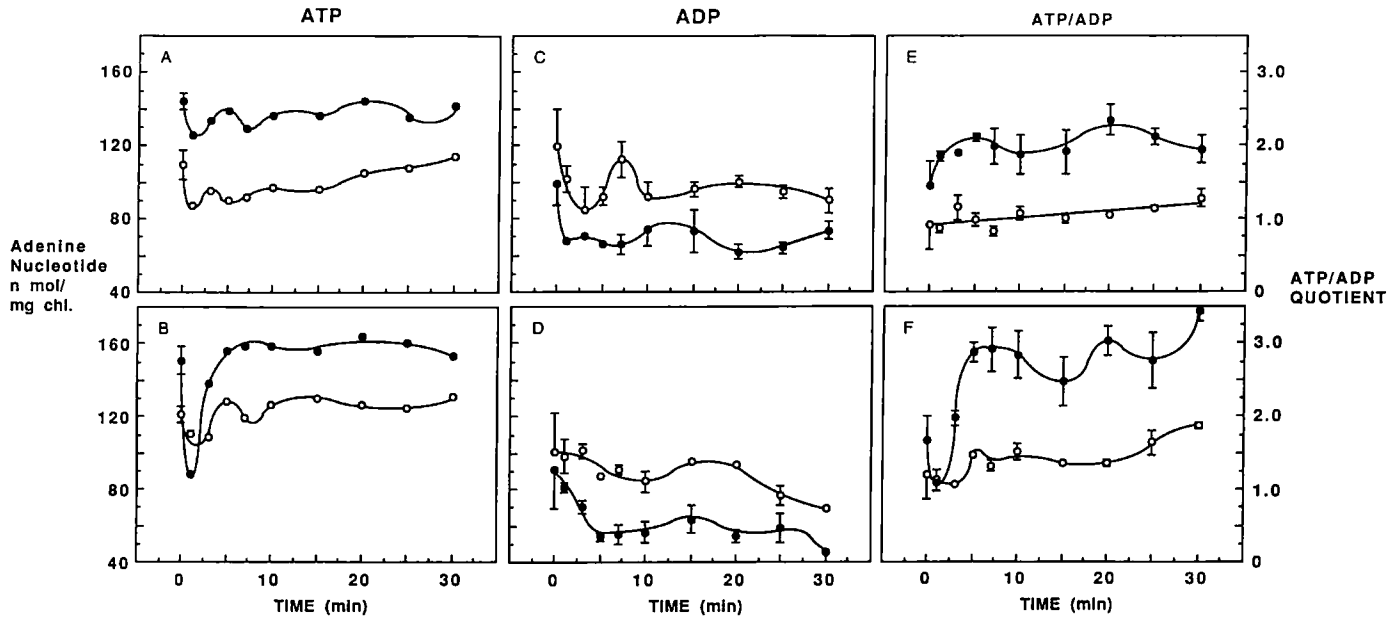

Fig. 2. The response to dilution stress of the ATP content (A, B,), the ADP content (C, D) and the ATP/ADP quotient (E, F) of *D. tertiolecta* (cultured for 7 days in 0.7 M NaCl). At zero time, growth medium containing NaCl (see Methods) was added to the culture in the light (○) or dark (●) to give final concentrations of 0.7 M (A, C, E) or 0.17 M (B, D, F). Data values and curve fitting as for Fig. 1.

Dilution stress from 0.7 to 0.17 M NaCl had little effect on ATP content in the light, but caused a rapid transient decrease in ATP content in darkness (Fig. 2), which then recovered within 5 min to a value close to the original. The ADP content exhibited a slower decrease, of greater magnitude in the dark, and did not recover over the 30 min time-course of the experiment. The ATP/ADP quotient exhibited oscillations and a substantial increase in darkness. The rate of decrease in the glycerol content was non-linear with time (Fig. 3c).

The decrease in ATP/ADP quotient seen here with stress from 0.17 M to 1.0 M NaCl is consistent with the effects of salt stress reported in *D. parva* [2], and on the ATP content in *D. tertiolecta* [3]. On the other hand, the small effect of salt stress from 0.17 M to 0.7 M NaCl observed here is comparable to the response of suspension-cultured carrot cells to the addition of 100 mM NaCl, which Reuveni et al. [19] showed to cause little effect on the adenine nucleotide contents. The changes in glycerol content after salt and dilution stress were generally similar to earlier reports for this alga [1,20] and were more rapid in the light than in the dark as observed previously [21]. Although it is facilitated by light, the increase in glycerol after salt stress is not fully dependent on photosynthesis, since it also occurs in the dark at the expense of starch [22,23].

These results demonstrate that there is no apparent relation between the increased rate of glycerol synthesis following salt stress and the ATP and ADP contents or their quotient. The highest observed rate of glycerol synthesis, following the salt stress from 0.17 to 0.7 M NaCl in the light, was not accompanied by any significant changes in ATP or ADP content or their quotient. Following the larger salt stress to 1.0 M NaCl in both light and dark, the rate of glycerol synthesis was constant from 5 to 30 min, although the ATP content and ATP/ADP quotient were changing rapidly during this period.

The synthesis of glycerol from dihydroxyacetone phosphate via the oxidative pentose phosphate pathway may occur without net ATP consumption if one triose is oxidised per five glycerol molecules synthesised and if two ADP molecules are phosphorylated per NADH oxidised [23]. This may confer the independence of glycerol synthesis from the energy state of the cell suggested by our data. ATP, however, is a strong inhibitor of the first enzyme of the glycerol synthesis pathway, glycerol phosphate dehydrogenase, which is also subject to general anion inhibition [24]. A full assessment of the potential for this effect to couple ATP concentration to glycerol synthesis requires additional information on the inhibitory effects of ADP and inorganic phosphate on the enzyme. These effects of salt stress on the adenine nucleotides are in marked contrast to the effects on the pyridine nucleotide contents [21], where the increase in glycerol after salt stress was always associated with increases in NADPH and $NADP^+$ contents, a decrease in NAD^+ and an initial increase in $NADH/NAD^+$ quotient.

To evaluate any possible role of ATP fully, the time-course during salt stress of its concentration, rather than content (ideally in the chloroplast stroma, the site of glycerol phosphate synthesis), should be known. To determine accurately the intracellular volume at precise times during the rapid initial shrinkage that follows salt stress is technically difficult. Our estimations of intracellular volume at approx. 7 min after salt stress from 0.17 M to 0.7 M and to 1.0 M NaCl show that it was reduced to 62% and 58%, respectively, of the initial volume (125 μl per mg chlorophyll) (data not shown). Katz and Avron [8] measured the intracellular volume of *D. parva* at a range of salt concentrations at 0 °C when glycerol metabolism was inactive. Under these conditions the cell volume was linearly related to osmolality and the non-osmotic volume was 10% of the intracellular volume of unstressed cells. If these findings are applied to our conditions, then stress from 0.17 M to 0.7 M and to 1.0 M NaCl would be expected to reduce the volumes to 36% and 29% of the initial volume, respectively; representing the maximum possible change before volume recovery commenced as a result of glycerol synthesis. From these figures and representative ATP and ADP contents before and at 1 min after salt stress, the relevant concentrations have been calculated (Table I). The calculations show that the intracellular concentrations of ATP and particularly ADP may increase immediately after salt stress. In contrast, Belmans and Van Laere [3] suggested that a temporary decrease in ATP concentration occurs which then stimulates glycerol 3-phosphate formation and hence glycerol synthesis. In any case, these concentrations will change rapidly as the volume recovery proceeds, whereas the rate of glycerol accumulation remains constant for most of the 30 min after salt stress (Fig. 3a).

TABLE I

Calculated intracellular adenine nucleotide concentrations before (0.17 M NaCl) and 1 min after salt stresses to 0.7 and 1.0 M NaCl

Cell volumes were estimated as in Materials and Methods (0.17 M NaCl) and from Kanz and Avron [8] (0.7 and 1.0 M NaCl, see Discussion). Representative contents of ATP and ADP at 1 min after salt stress were taken from Fig. 1.

NaCl concentration (M)	Cell volume (μl per mg chlorophyll)	Representative content (nmol per mg chlorophyll)		Concentration (mM)	
		ATP	ADP	ATP	ADP
0.17	125	200	110	1.6	0.9
0.7	45	190	120	4.2	2.7
1.0	36	120	140	3.3	3.9

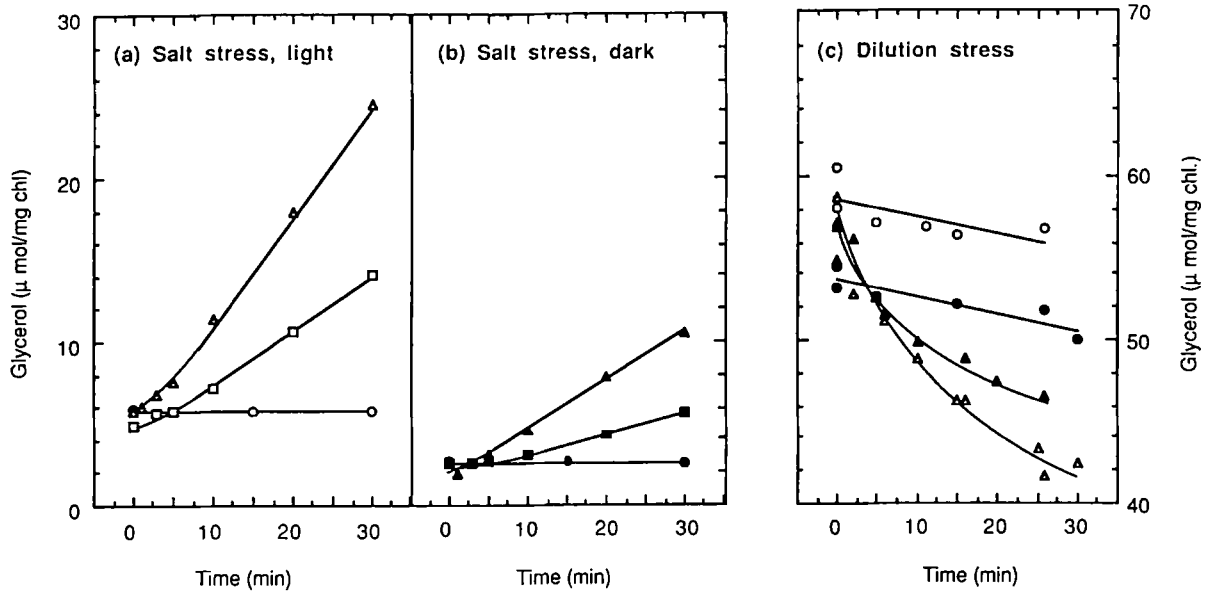

Fig. 3. (a, b) The response to salt stress of the glycerol content of *D. tertiolecta* (cultured for 9 days in 0.17 M NaCl). Final NaCl concentrations: A, 0.17 M (○, ●); B, 0.7 M (△, ▲); C, 1.0 M (□, ■). (c) The response to dilution stress of the glycerol content of *D. tertiolecta* (cultured for 7 days in 0.7 M NaCl). Procedures as in Fig. 2. Final NaCl concentrations: 0.7 M (○, ●); 0.17 M (△, ▲). Open symbols represent the response in light, closed in the dark.

The dissimilation of glycerol to dihydroxyacetone phosphate requires ATP for the dihydroxyacetone kinase reaction [25]. An increase in ATP or in the ATP/ADP quotient could thus conceivably increase the rate of glycerol dissimilation, although the overall conversion of glycerol to α-(1,4)-glucan can be without net change in ATP [23]. However, the application of dilution stress decreased the ADP content and increased the ATP/ADP quotient in the dark but not the light, although the glycerol content decreased more rapidly in the light. Because dilution stress will cause an initial swelling of the cell until the volume is restored after glycerol dissimilation, the intracellular concentration of ATP and ADP can be expected to show an additional temporary decrease. Hence these results do not support a direct role for the adenine nucleotides in regulating glycerol dissimilation. Dilution stress is associated with an increase in NADPH/$NADP^+$ quotient in the dark but not in the light [21].

The possible role of inorganic phosphate in the regulation of starch and glycerol metabolism has been discussed [2]. However, reported intracellular concentrations in *Dunaliella* of inorganic phosphate vary by more than one order of magnitude (see Ref. 26, compare Refs. 2 and 22). It has recently been shown that a very large proportion of inorganic phosphate (in excess of 97%) is bound [26]; acid extraction is unsuitable for determining the content of free inorganic phosphate in *Dunaliella* or any other organism. Likewise, intracellular binding of adenine nucleotides could alter the in vivo ATP/ADP quotient from that determined after acid extraction. The extent of compartmentation in *Dunaliella* of adenine nucleotides between bound and free pools is not known: recently, it has been shown that, in corn root tips, a proportion of the nucleoside diphosphate pool is metabolically inert [27]. On the other hand, Heber and his co-workers have presented evidence that in higher plant chloroplasts, there is no significant sequestration of either ATP or ADP [6,28].

The representative concentrations in *D. tertiolecta* of 1.6 mM for ATP and 0.9 mM for ADP (Table I) can be compared with measurements in other algae. The ATP concentration in the giant green alga *Chara corallina* has been found to be in the range 1.6–3.4 mM and ADP in the range 0.1–1.1 mM [29]. These are average cytoplasm values; the concentration of these compounds differs substantially between the chloroplast, mitochondrion and cytosol [7]. Darkening the alga caused the ATP/ADP quotient to increase (Figs. 1 and 2), despite the availability of photophosphorylating capacity in the light. This observation may be understood on the basis of the demonstration that the ATP/ADP quotient in the cytosol of wheat protoplasts decreases on illumination due to an apparent deenergisation of the mitochondria [7]. Thus, in *Dunaliella*, it would be predicted that the decrease in cytosolic ATP/ADP quotient outweighs the increase in the stroma on illumination. Decreases in ATP content on illumination of other green algae have also been reported previously [30,31].

The evidence from this work does not support an hypothesis that changes in the content or concentration of ATP or ADP, or in the energy status of the cells as indicated by the ATP/ADP quotient, directly control

the adjustment of glycerol content that occurs in response to variations in salinity. However, this conclusion is based on the average cell contents of the total ATP and ADP. A final exclusion of adenine nucleotides from this regulatory role must await a complete compartmental analysis of metabolically active intracellular adenine nucleotides in *Dunaliella*, a task which is not yet technically possible.

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