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Ionic Regulation of the Unicellular Green Alga *Dunaliella tertiolecta*: Response to Hypertonic Shock

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Summary. The evolution of the volume, the Na^+ and K^+ contents and the glycerol and ATP contents were investigated after subjecting *Dunaliella tertiolecta* cells to hypertonic shocks. It was found that the variations in the glycerol and the ion contents superimpose as the cell regulates its volume. Hypertonic shock induces a rapid increase (some minutes) in the Na^+ influx and Na^+ content followed by a decrease until a new steady value is reached after 30 min of cell transfer. The regulatory mechanism extruding Na^+ out of the cells was dependent on the presence of K^+ or Rb^+ ions in the external medium. A transient pumping of K^+ ions was found after subjecting the cells to a hypertonic shock. This increase in K^+ content resulted from the transient increase in the K^+ influxes. The K^+ pumping mechanism was blocked by the absence of Ca^{++} and Mg^{++} ions in the external medium and was inhibited by DCCD, FCCP and DCMU, whereas ouabain, cyanide and PCMBS were ineffective. The increase in K^+ content was observed if the hypertonic shock was induced by the addition of NaCl, glycerol or choline chloride. These results are interpreted on the basis of two distinct mechanisms: a Na^+/K^+ exchange pump and a Na^+ independent K^+ pump. These ionic transfer mechanisms would participate in the osmoregulation of *Dunaliella* cells and would be of importance, particularly during the onset of the osmotic shock when glycerol synthesis is incomplete.

Key Words *Dunaliella* · unicellular algae · Na/K exchange · osmoregulation · glycerol · ATP · ion transport · cell volume

Introduction

The euryhaline green alga *Dunaliella* is known to respond to variations of external osmotic pressure by changing its cell volume (Ben Amotz, 1973); this change is very rapid and is followed by a slow return to a volume close to the original (Ben Amotz, 1973; Rabinowitch, Grover & Ginzburg, 1975; Gimmler, Schirling & Tobler, 1977; Riisgard, 1979). The return phase has been largely attributed to changes in endogenous concentrations of the organic compound glycerol (Ben Amotz & Avron, 1973; Borowitzka & Brown, 1974).

The role of glycerol in the osmoregulation of *Dunaliella* cells has received considerable attention but the little that is known of the role of inorganic substances such as salts is contradictory. Gimmler and Schirling (1978) and Ginzburg (1981*a*) found that in *D. parva* Na^+ ions are involved in osmoregulation whereas K^+ ions are not. In another species, *D. marina*, Riisgard, Norgard-Nielsen and Sogaard-Jensen (1980) suggested that both Na^+ and K^+ ions play a part in volume regulation.

Ehrenfeld and Cousin (1982) have recently shown that ions in *D. tertiolecta* are compartmentalized: they postulated that a large compartment regulates its ion concentration, maintaining low Na^+ and Cl^- and high K^+ concentrations, while a second compartment is in equilibrium with the external medium. They described a Na^+/K^+ exchange mechanism helping to regulate the ionic composition of the cells over a wide range of salinities.

This paper is concerned with the changes of the volume and of the ion (Na^+ and K^+) and glycerol contents of *Dunaliella* cells subjected to hypertonic shock. Simultaneously, the influxes of these ions were followed. The aim of these experiments was to elucidate the mechanisms by which the cell regulates its volume and ionic content and to follow changes of these parameters after osmotic shock. The relative contributions of the ion and glycerol contents in this regulation will be discussed.

Materials and Methods

Growth of Organisms

The experimental conditions for culturing *Dunaliella tertiolecta* Butcher and the composition of the culture medium are given in a previous paper (Ehrenfeld & Cousin, 1982). The sodium chloride concentration of the medium is indicated in each experiment.

Media A and E refer to the basic medium plus 15 and 410 mM of NaCl giving osmotic pressures of 160 and 923 mOsm, respectively. The cells were adapted for several months in medium A before experimentation. Those used in experiments were at the late logarithmic growing phase.

Determination of Cell Volume and Cell Water of *Dunaliella* Cells

The cells (1.2×10^8 cells) were suspended in the incubation medium with ^{14}C dextran 70,000 (15 μCi/ml medium) and centrifuged in cytocrit tubes of 1.14 mm internal diameter for 30 sec at $12,000 \times g$ in a rapid microcentrifuge (Hettich, Germany). The supernatant was drawn off and kept for counting. The length of the cell column was measured and the volume of the pellet calculated. The cells were then weighed with a Mettler balance (Type H6T). After drying at 85°C for 24 hr the pellet was reweighed. The difference between the two recorded weights gave the amount of water in the pellet. The extracellular space of the cell pellet was calculated from the amount of ^{14}C dextran sedimenting with the cells. The cell volume was calculated as the difference between the volume pellet and the extracellular space. Proteins were determined in each sample according to the method of Lowry, Rosebrough, Farr and Randall (1951). Cell volume and cell water are expressed in $\mu l \cdot mg^{-1}$ of protein.

Determination of Na^+ and K^+ Content of the Cells

Two techniques were used to measure the Na^+ and K^+ content of the cells. In the first, very rapid centrifugations and washings of the cells with an ice-cold isotonic glycine medium to avoid contamination with the highly saline outside medium were made before the subsequent procedure described in detail by Ehrenfeld and Cousin (1982). The second technique makes use of an extracellular marker (^{14}C dextran, mol wt 70,000) to measure the concentration of a given extracellular ion contaminating the pellet of centrifuged cells. By subtracting this value from the total concentration of this ion in the pellet, the ion content of the cell was determined. Criticisms of these techniques are given by Ehrenfeld and Cousin (1982). Ion contents are expressed in $neq \cdot mg^{-1}$ of protein.

Ion Flux Measurements

Unidirectional K^+ influxes were measured by the use of ^{42}K (20 μCi/ml); the cells were incubated 1.5 min with the isotope and an extracellular marker, ^{14}C dextran 70,000 (10 μCi/ml), then centrifuged for 10 sec in a rapid microcentrifuge at $12,000 \times g$ and the supernatant drawn off and kept for counting. The total procedure took 2 min. The pellet was then resuspended in 1 ml of distilled water. After additional of 5 ml of the scintillation medium, A.C.S. (Amersham, USA), the samples were counted for ^{14}C and ^{42}K in a liquid scintillation counter. All samples were counted again 10 days later after complete decay of ^{42}K. After correction for the contaminating extracellular K^+, the cell K^+ uptake was calculated and expressed in $neq \cdot mg^{-1}$ proteins $\cdot min^{-1}$. Preliminary experiments showed that equilibration of the cells with the isotope ^{42}K is very slow. The K^+ uptake time of 2 min was therefore chosen since the K^+ uptake of cells adapted to either medium A or E is linear over this time interval, which is also suitable for studying rapid phenomena. Unidirectional Na^+ influxes were measured with the use of ^{24}Na (20 μCi/ml), the technique being identical to that for estimating unidirectional K^+ fluxes. In a second technique, similar to that used for Na^+ content determination, the cells were centrifuged and washed with an ice-cold isotonic glycine solution after an incubation period of 2 min with the isotope ^{24}Na.

Glycerol Determination

The glycerol content of cell extracts was determined enzymatically with the Biochemica test kit "glycerol uv-method" (Boehringer, Manheim). They are expressed in $mg \cdot mg^{-1}$ of protein.

ATP Content

The ATP content of the cells was determined by the firefly bioluminiscent procedure described by Holm-Hansen and Booth (1966) with a Chem. Glow photometer (Aminco, USA). Results are expressed in $moles \cdot mg^{-1}$ of protein.

Results

Variations in Cell Volume and Glycerol Content of *Dunaliella* Cells after a Hypertonic Shock

The changes in the cell volume and cell water of *Dunaliella tertiolecta* following a hypertonic shock are represented in Fig. 1 (the cells were transferred from media A to E). Both determinations were performed on the same batch of cells. Comparison of the two curves shows that, as expected, cell volume variations are due to cell water movements which have two phases: an immediate shrinkage followed by a slower swelling of the cells to levels inferior to the original. The initial water loss is considerable, reducing the cell water content to a third of the original value: the partial recovery restores the value to two-thirds the initial. Microscopic observations indicate that immediately after the osmotic shocks the motility of the cells is severely affected for approximately 20 min, then the cells return progressively to their initial motility. Although the changes in the glycerol content of *Dunaliella* cells after hypertonic shock is well documented (Wegman, 1971; Ben Amotz & Avron, 1973), we repeated this study with *Dunaliella tertiolecta* under our experimental conditions. Figure 2 illustrates the changes with time of the glycerol content of cells transferred from medium A to medium E. This experiment shows that the synthesis of glycerol is

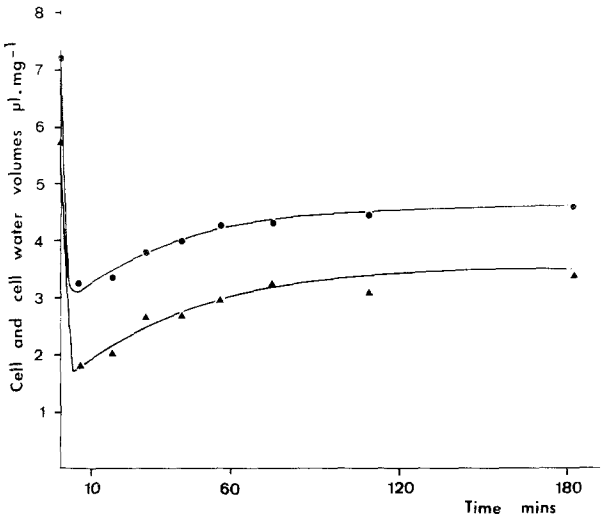

Fig. 1. Change with time of the cell volume and cell water of *Dunaliella* cells subjected to a hypertonic shock. The cells were transferred from medium A to E. The cell volume and cell water are expressed in $\mu l \cdot mg^{-1}$ protein and the time in minutes

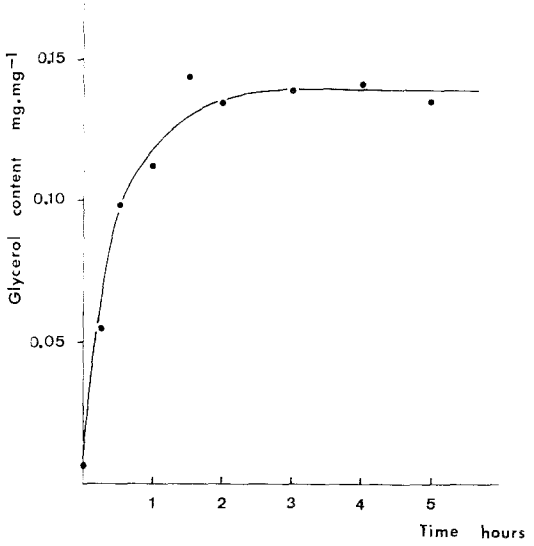

Fig. 2. Evolution with time of the glycerol contents of *Dunaliella* cells subjected to a hypertonic shock. The cells were transferred from medium A to E. Glycerol contents are expressed in mg · mg^{-1} protein and time in hours

immediate after the transfer of the cell to a hypertonic medium. After 1 hr, 94% of the total glycerol concentration (maximum: 460 mM of cell water) is reached. The cell water volumes given in Fig. 1 were taken into account in calculating the glycerol concentration.

Evolution of Na Content and Na Influxes of *Dunaliella* Cells Subjected to a Hypertonic Shock

In a previous paper, Ehrenfeld and Cousin (1982) reported that the cells are compartmentalized into at least two compartments: a large one regulating its Na^+ concentration to low levels and a second one in equilibrium with the external medium; they showed that the total Na^+ content of both compartments can be measured by the use of ^{14}C dextran 70,000 as extracellular marker, whereas the "washing technique" measured only the Na^+ content of the first compartment, the second being removed by the successive washings of the cells.

Figures 3 and 4 represent the variations in Na^+ content of cells transferred from medium A to medium E as measured by the above two techniques. In both cases the curves are biphasic. Hypertonic shock induces a rapid increase in Na^+ content followed by a decrease to a new steady value 30 min after cell transfer. Although the two techniques give different absolute values it should be noted that the decreases are of the same order of magnitude (100 neq · mg^{-1} against 150 neq · mg^{-1}, with the washing and the extracellular marker techniques, respectively). These experiments confirm the existence of a well-regulated Na^+ compartment; the washing technique permits its measurement more accurately although an underestimation is still possible because of Na^+ loss during the washing procedure.

In order to see if the transient Na^+ content is the result of changes in Na^+ transport (influx) after the shock, the following experiments were performed. The ^{24}Na influxes were followed as a function of time after subjecting the cells to a hypertonic shock. In this purpose, "uptake pulses" of ^{24}Na (2 min) were measured at various times after the osmotic shock. Figures 5 and 6 show the results obtained with the two procedures.

Transfer to hypertonic media induces a considerable and immediate increase in the Na^+ uptake followed by a rapid, equal decrease all in less than 5 min after transfer. The influx is then maintained at a steady level 8 to 10 times higher than the initial level. The second phase of the effect of hypertonic shock on the cell Na^+ content (*see* Figs. 3 and 4) shows characteristics of a regulatory mechanism. Since an Na/K exchange mechanism has been postulated in *Dunaliella* cells (Gimmler & Schirling, 1978; Ehrenfeld & Cousin, 1982) we studied the effects of K^+ ions in the medium on the Na^+ content of such cells to test whether such a mechanism is involved. Table 1 gives the values of the Na^+ con-

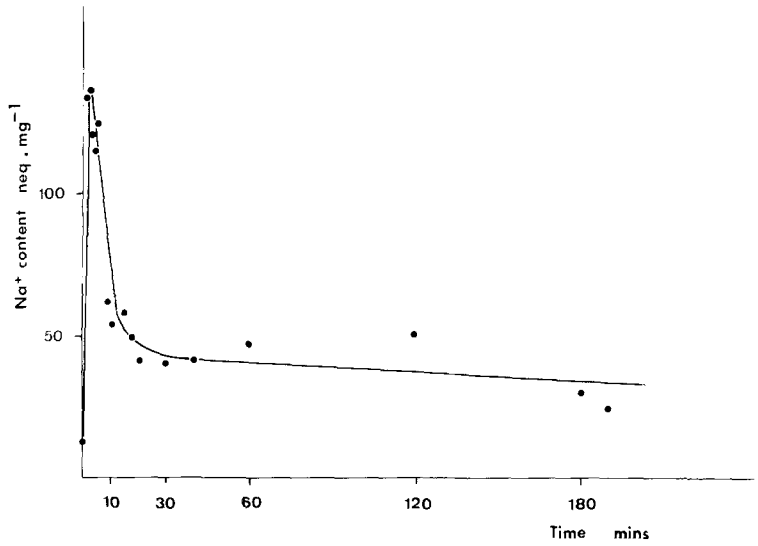

Fig. 3. Evolution with time of the Na^+ content of *Dunaliella* cells subjected to a hypertonic shock due to transfer from medium A to E. "The washing technique" was used to measure the Na^+ contents which are expressed in neq · mg^{-1} protein, and time in minutes

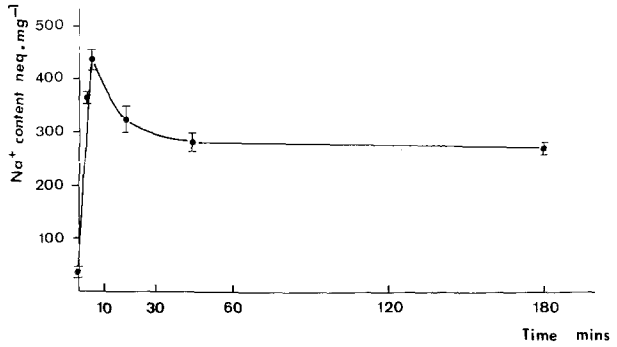

Fig. 4. Evolution with time of the Na^+ content of *Dunaliella* cells subjected to a hypertonic shock. The cells were transferred from medium A to E. In this technique dextran 70,000 was used as extracellular marker to determine the cell Na^+ contents. Na^+ cell contents are expressed in neq · mg^{-1} protein, and time in minutes

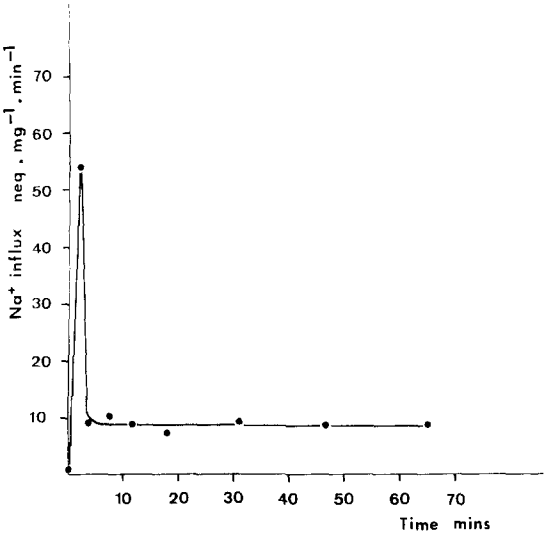

Fig. 5. Evolution with time of the Na^+ influxes of *Dunaliella* cells subjected to hypertonic shocks by transfer from medium A to E. The Na uptake time was 2 min and the technique used the "washing technique." Na^+ influxes are expressed in neq · mg^{-1} · min^{-1} and time in minutes

tent of cells maintained in medium A and of cells transferred from medium A to medium E in the absence or presence of 10 meq of K^+ (KCl salt). In the cells bathed in medium A the Na^+ cell content followed by a considerable decrease after 30 min but this decrease in Na^+ content is not observed in cells bathed with a K^+-free medium. Thus the elimination of Na^+ clearly depends on the presence of K^+ in the external medium, which suggests that a Na^+/K^+ exchange mechanism is involved in this regulatory process.

The range of K^+ concentrations necessary for this Na^+ excretion was investigated. Figure 7 shows the Na^+ contents of *Dunaliella* cells 30 min after hypertonic shock (medium A to E) in the presence of various KCl concentrations. Before transfer the cells were washed twice in a K^+-free medium A. As it is not possible to eliminate the K^+ in the medium completely, the minimum K^+ concentration tested was 50 μeq. The apparent affinity of K^+ for the regulatory mechanism could not therefore be determined precisely. This affinity must, however, be high since the maximal effect is already observed for 0.5 to 1 meq of K^+.

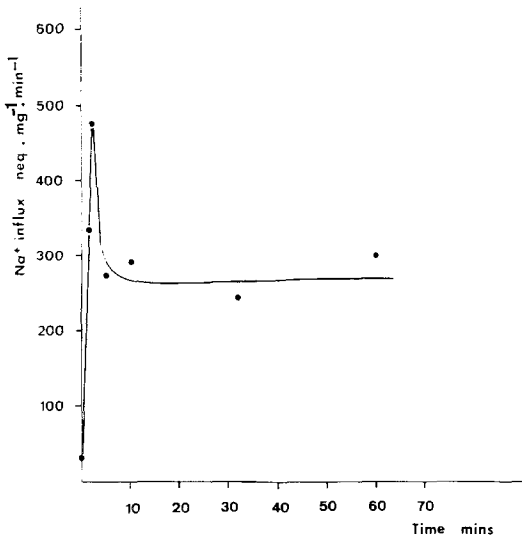

Fig. 6. Evolution with time of the Na^+ influxes of *Dunaliella* cells subjected to a hypertonic shock by transfer from medium A to E. The Na^+ uptake time was 2 min and the technique made use of dextran 70,000 as an extracellular marker to calculate the Na^+ influxes. Na^+ influxes are expressed in neq · mg^{-1} · min^{-1} and time in minutes

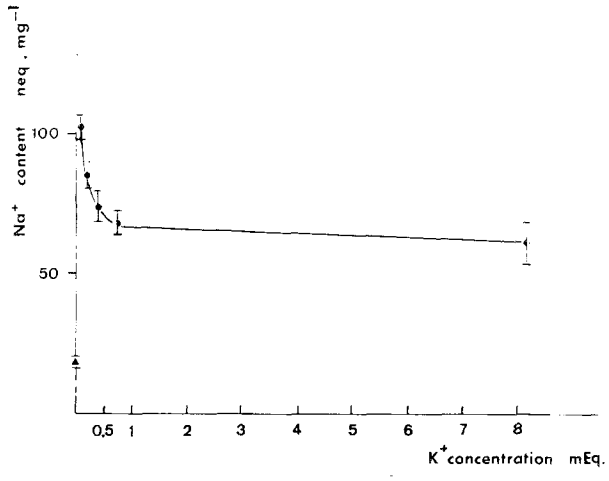

Fig. 7. Evolution of the Na^+ content of *Dunaliella* cells in relation to the KCl concentrations of the external medium 30 min after hypertonic shock. The cells were transferred from medium A to a medium E of varying KCl concentration. The "washing technique" was used to determine the Na^+ cell content expressed in neq · mg^{-1} protein. KCl concentration in meq

Figure 8 illustrates a similar experiment but in which rubidium ions were substituted for K^+. It can be seen that the Na^+ content decrease is also strongly dependent on Rb^+ ions and the apparent affinity of Rb^+ is close to that of K^+.

Table 1. Effect of the potassium in the external medium on the sodium content of *Dunaliella* cells subjected to hypertonic shock

Media	A	A → E (2 min after transfer)	A → E (30 min after transfer)
K^+-free medium	17 ± 1		102 ± 5
A	(n = 11)		(n = 12)
10 meq of K^+ medium	15 ± 2	105 ± 20	47 ± 7
B	(n = 13)	(n = 5)	(n = 7)

The "washing technique" was used to measure the Na^+ content. Cells were transferred from medium A to E in the presence (B) or absence (A) of K^+ ions. Number of experiments n, in brackets. The Na^+ content is expressed in neq · mg^{-1} of protein ± standard error of the mean.

Evolution of K^+ Content and K^+ Influxes of *Dunaliella* Cells Subjected to Hypertonic Shock

Figures 9 and 10 represent the change as a function of time of the K^+ content of *Dunaliella* cells transferred from medium A to medium E. In these experiments the cell pellet was not washed with a cold isotonic solution since the extracellular K^+ contaminations can be neglected (since they represent less than 1/100 of the total pellet K^+ when there is 1 meq of K^+ in the extracellular medium). Figure 9 shows that the K^+ cell variations can be followed either by measuring the K^+ content of the cells or that of the K^+ concentration of the external medium when relatively low K^+ concentrations (less than 1 meq) are present.

The K^+ content of the cells remained stable for 10 min and then increased until a plateau was reached. Figure 10 reports identical experiments with a batch of cells bathed either in a medium containing an extremely low K^+(50 μeq) concentration (on left in Fig. 10) or in one containing 1 meq of KCl (on right).

It can be seen that hypertonic shock induces an uptake of K^+ even with very low K^+ concentrations in the medium suggesting an extremely high affinity for K^+. With a medium containing a K^+ concentration of 1 meq, a maximum K^+ content increase of 170 neq · mg^{-1} is reached 40 min after osmotic shock. In Fig. 11 the K^+ influxes were followed as a function of time after transfer of the cells from medium A to medium E. The K^+ concentration of the medium was 1.1 meq, the "uptake pulses" of $^{42}K^+$ were 2 min. After an initial decay during the first 6 min a sudden increase in K^+ influx occurs and then a decrease which levels out. The analysis of

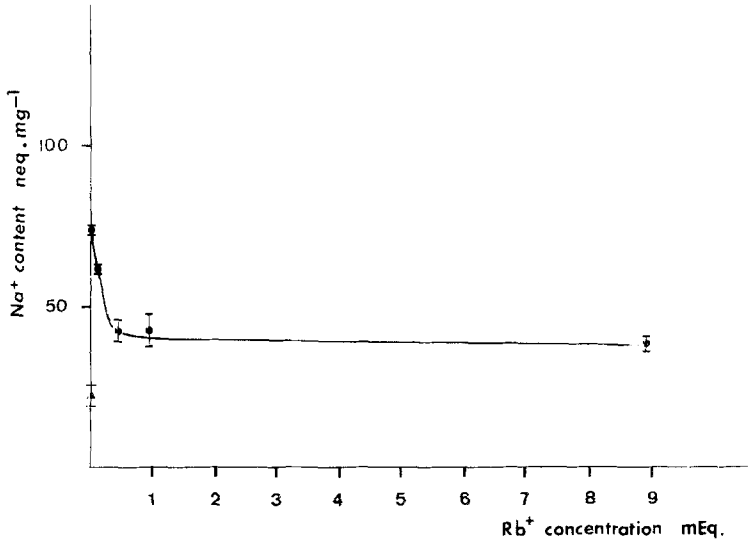

Fig. 8. Same legend as Fig. 7 except rubidium ions are used instead of K^+ ions

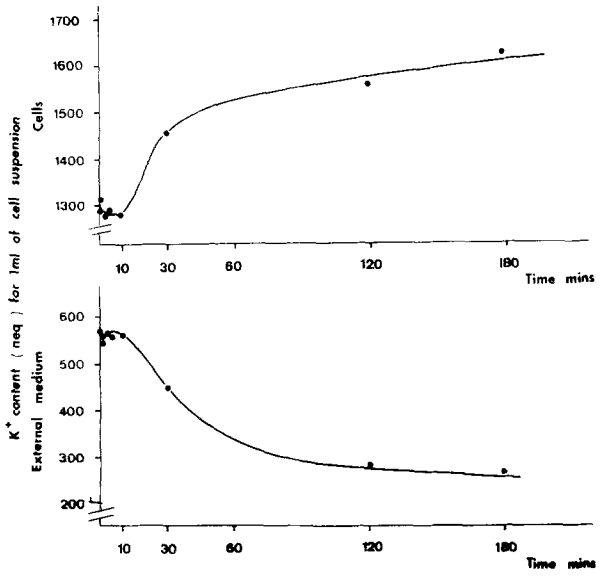

Fig. 9. Evolution with time of the K^+ content of the cells and of the external bath after subjection to hypertonic shocks. The cells were transferred from medium A to E containing an initial KCl concentration of 600 μeq. For comparison, results are expressed in the same units (neq per 1 ml of cell suspension). Time is expressed in minutes

Fig. 10, right side, and Fig. 11 indicates that a correlation exists between the time course of K^+ content and the K^+ influx, suggesting that the increase in K^+ content induced by the osmotic shock results from the transient increase in K^+ influx.

From the variations in cell water volume shown in Fig. 1, the cell K^+ concentrations have been calculated. The results are reported in Fig. 12. The K^+ concentration increases threefold immediately after the osmotic shock (86 to 280 meq of cell water); this increase in K concentration is due to the considerable water loss. In spite of the K^+ uptake subsequently observed during the first hour of transfer (*see* Figs. 9 and 10), the cell K^+ concentration in that period of time decreases as a consequence of the partial volume recovery. After 3 hr of transfer, a K^+ concentration of 200 meq of cell water can be calculated.

The Effect of Divalent Ions and of Some Inhibitors on the K^+ Content Increase Induced by Hypertonic Shocks

The effect of Ca^{++} and Mg^{++} was tested on the K^+ content increase induced by a hypertonic shock. A batch of cells was divided into 4 parts: the cells were transferred from medium A to E either in the presence of both divalent ions or in the absence of Ca^{++} or in the absence of both ions. The Ca^{++}-free media contain also 1 mM of EGTA. Each batch of cells was washed twice in the corresponding medium before the osmotic shock. Experiments consisted of measuring the variations of K^+ content 2 and 30 min after the osmotic shock. The results are given in Table 2. The data show that 2 min after transfer from medium A to medium E, the cell K^+ content was slightly lower in the Ca^{++} and Mg^{++}-free media and considerably lower in the medium containing neither Ca^{++} nor Mg^{++} suggesting that divalent ions are necessary for the integrity of the cell membrane.

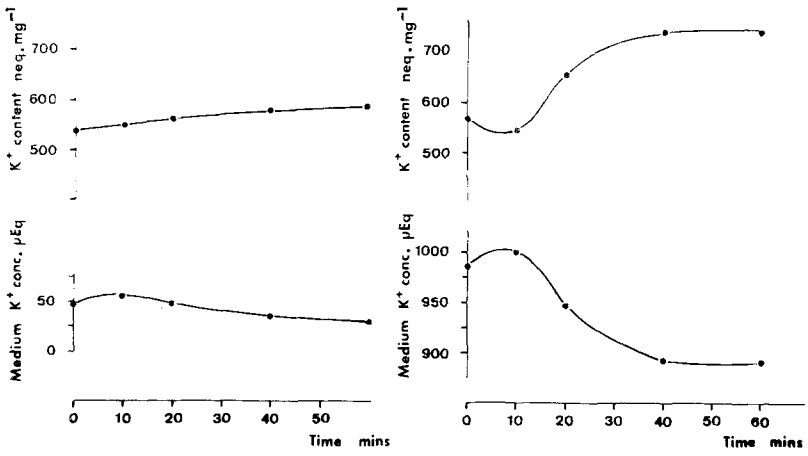

Fig. 10. Evolution with time of the K^+ content of the cells and of the external bath after subjection to hypertonic shocks. The cells were transferred from medium A to E containing either 50 μeq (left panel) or 1 meq (right panel). Cell K^+ content is expressed in neq mg^{-1} protein, K^+ concentration of the external bath in μeq and time in minutes

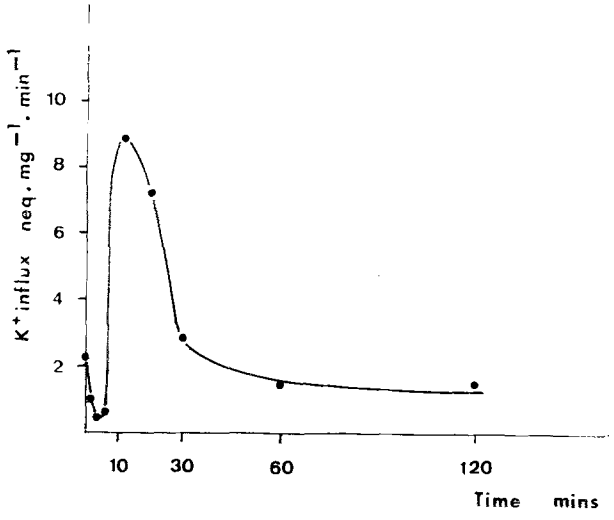

Fig. 11. Evolution with time of the K^+ influxes of *Dunliella* cells subjected to hypertonic shocks. The cells were transferred from medium A to E containing 1 meq of K^+ (KCl salt). The K^+ uptake time was 2 min. K^+ influxes are expressed in neq · mg^{-1} · min^{-1} and time in minutes

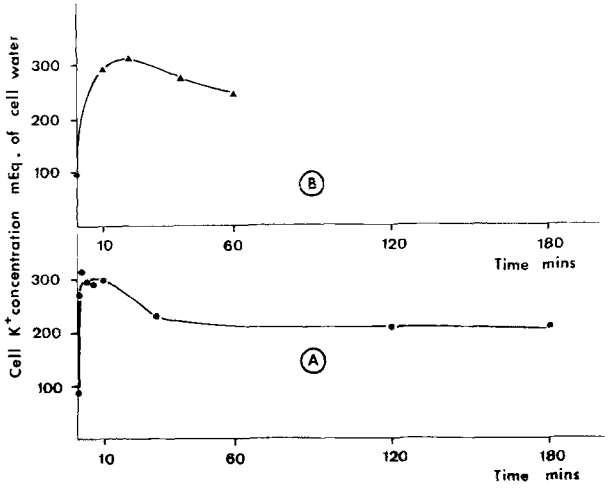

Fig. 12. Evolution with time of the cell K^+ concentrations of *Dunaliella* cells subjected to hypertonic shocks. The cells were transferred from medium A to E containing 600 μeq (A) or 1000 μeq (B) of K^+. K^+ concentrations in meq of cell water and time in minutes

However, the increase of K^+ after 30 min is completely normal if the medium contains either Ca^{++} or Mg^{++} ions, where as in a Ca^{++}- and Mg^{++}-free medium the cells continue to lose K^+ ions. These experiments show that Ca^{++} or Mg^{++} is essential for the maintenance of the transient K^+ uptake mechanism which follows a hypertonic shock.

In Table 3 the effects of some well-known inhibitors of ion transport in biological systems are shown. The K^+ increase of cells transferred from medium A to E was followed during the first 30 min. DCCD, FCCP and DCMU exert inhibitory effects whereas ouabain, cyanide and PCMBS were ineffective.

Table 2. Effect of Ca^{2+} and Mg^{2+} ions on the *Dunaliella* cell K^+ content increase induced by hyperosmotic shock

Time after transfer (min)	Control	Ca- and Mg-free	Ca-free	Mg-free
2	451 ± 7	270 ± 16	404 ± 1	367 ± 19
30	540 ± 15	224 ± 28	517 ± 10	481 ± 9
Difference	+ 89	− 46	+ 113	+ 114

Cells were transferred from medium A to E in the presence or absence of the divalent ions. Number of experiments = 4. The K^+ content is expressed in neq · mg^{-1} of protein ± standard error of the mean.

Table 3. Effect of some agents on the *Dunaliella* cell K content increase induced by hypertonic shock

Initial K^+ concentration of the external medium	Agents tested	ΔK^+
508 μeq	Control	79 ± 7
(n = 3)	DCCD 5×10^{-5} M	26 ± 6
	Control	68 ± 4
	Ouabain 10^{-3} M	81 ± 3
522 μeq	DCCD 10^{-4} M	3 ± 2
(n = 3)	FCCP 10^{-5} M	34 ± 4
	Acetazolamide 10^{-3} M	51 ± 4
	Control	114 ± 3
	DCMU 5×10^{-5} M	78 × 8
522 μeq	CN^- 10^{-3} M	129 ± 6
(n = 3)	DCMU (5×10^{-5} M) + CN^- (10^{-3} M)	66 ± 1
	PCMBS 2×10^{-4} M	118 ± 3

Cells were transferred from medium A to E. The K^+ content increase (ΔK^+) during the first 30 min after transfer were calculated from the variations of the K^+ concentrations of the external bath. They are expressed in neq · mg^{-1} of protein ± standard error of the mean. Number of experiments in brackets.

The Hypertonic Shock by Itself Induces the Transient K^+ Content Increase

Table 4 summarizes the increases of the K^+ content of cells subjected to hypertonic shock induced by the addition of NaCl, glycerol and choline chloride. The cells were transferred from medium A (osmolarity 160 mOsm) to a medium with a final osmolarity of 490 mOsm. This osmolarity, although only half of that of medium E was chosen because the concentrations of glycerol or choline chloride needed to raise the osmolarity further cause damage to the cells. A K^+ content increase is observed in all cases indicating that it is the osmotic shock itself and not the nature of the osmolalities which is responsible for the K^+ increase. No changes in cell Na^+ content were noted after the addition of glycerol or choline chloride to the medium. This result may be surprising since previous experiments (*see* Figs. 7 and 8) suggested a Na/K exchange mechanism. An explanation of this apparently controversial result will be given in the Discussion.

Changes in Cell ATP Content after Hypertonic Shock

Figure 13 illustrates the change in ATP content expressed in 10^{-8} mole · mg^{-1} of protein after subjecting the cells to a hypertonic shock (medium A to E).

Table 4. Variations in the K^+ and Na^+ content of *Dunaliella* cells after a hypertonic shock induced by the addition of NaCl, glycerol or choline chloride

	NaCl	Glycerol	Choline chloride
ΔK^+	82 ± 4	70 ± 27	72 ± 14
ΔNa^+	–	13 ± 11	1 ± 6

The cells were adapted to medium A and transferred from medium A to a medium containing NaCl, glycerol or choline chloride with a final osmotic pressure of 490 mOsm. ΔK^+ = increases of cell K^+ content calculated from the variations of K^+ in the external medium during the first 30 min after transfer. ΔNa^+ = cell Na^+ content variations in the same period (washing technique). ΔK^+ and ΔNa^+ are expressed in neq · mg^{-1} of protein ± standard error of the mean. Number of experiments = 6.

This curve is biphasic: an initial decrease is followed by a progressive increase in ATP content after approximately 10 min. If the volume of water in the cells (*see* Fig. 1) is taken into account when calculating the cell concentration of ATP it can be seen that in fact there was an initial increase in ATP concentration (8.9 mM in medium A to 13.1 mM after 5 min in medium E), after which the concentration remained constant at about 13.4 mM. There would appear to be a strict regulation of this nucleotide.

Discussion

When *Dunaliella* cells are subjected to hypertonic shock, the variations in the glycerol and the ionic content take place concurrently with the regulation of cell volume. Changes in cell volume, and cell constituents occur in less than 2 hr. At the same time there is considerable variation in the ATP content of the cell in response to the metabolic demands of osmoregulation.

The initial event after a hypertonic shock is immediate cell shrinkage until a new osmotic equilibrium is reached. This decrease of water potential has been observed in the marine algae *Dunaliella* and *Platymonas* (Ben Amotz, 1974; Gimmler et al., 1977; Krist, 1977) and in the freshwater alga *Ochromonas* (Kauss, Lüttge & Krichbaum, 1975). Gimmler et al., (1977) attribute this rapid phase (less than 1 min) entirely to rapid water movements. During this period cell ionic concentrations increase in proportion to the water loss.

Our data show that early in hypertonic shock, *Dunaliella* cells suddenly increase their Na^+ content as a consequence of a transient increase in Na^+ influxes through the cell membranes (*see* Figs. 3–

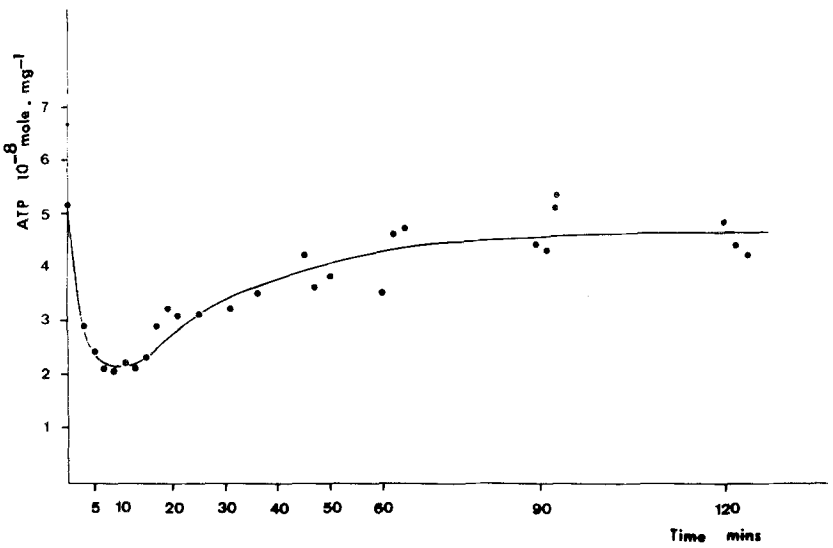

Fig. 13. Evolution with time of the ATP content of *Dunaliella* cells subjected to a hypertonic shock. The cells were transferred from medium A to E. ATP contents are expressed in 10^{-8} mole · mg^{-1} protein and time in minutes

6). Considering that the Na^+ permeability reported for marine algae is around 10^{-7} cm sec^{-1} (*see* Raven, 1976; Ginzburg, 1981*b*) and the water permeabilities of *Duinaliella bardawil* and *Dunaliella salina* are 1.8 and 1.5 × 10^{-3} cm sec^{-1}, respectively (Degani & Avron, 1982), the Na^+ entry into the cells, although very rapid (1 to 2 min), must be secondary to the water loss. This Na^+ uptake may have a role at the beginning of the cell shrinkage in limiting the cell water loss and thus acting as osmoregulatory mechanism until sufficient glycerol is synthetized by the alga.

It has been advanced in a previous paper (*see* Ehrenfeld & Cousin, 1982) that at least two cellular compartments coexist in *Dunaliella* cells. The larger, limited by a tight membrane, regulates its ion concentration, maintaining low Na^+ concentrations; and the second, limited by a leaky membrane, equilibrates with the external medium. Only the former can be investigated with the washing technique whereas the technique using Dextran 70,000 permits the study of both. From Figs. 3 and 4 it can be seen that after the initial phase of increasing cell Na^+ content, a phase of diminishing Na^+ content follows, the amplitude and duration of which are similar whichever technique is employed. These results are in complete agreement with the above assumption of Na^+ cell compartmentalization and of the existence of a well-regulated compartment maintaining low Na^+ concentrations. Similar transient Na^+ increases, after hypertonic shock, have been reported in *Dunaliella parva* (Ginzburg, 1981*a*), *Chlorella emersoni* (Greenway & Setter, 1979) and *Platymonas subcordiformis* (Kirst, 1977).

When *Dunaliella* cells are subjected to hypertonic shock, large variations in the K^+ content and K^+ influx are observed (*see* Figs. 10 & 11). After relatively constant values during the first 10 min, the K^+ content increases by approximately 170 neq · mg^{-1}, the maximum being reached in 40 min with 1 meq K^+ in the external medium. This K^+ increase may be interpreted as resulting from the transient increase in the K^+ influx during this period. Considering the calculated K^+ concentrations, Fig. 12 shows that after an initial increase in K^+ concentration due to the water loss induced by the osmotic shock, a period follows in which the concentration decreases. Three hours after transfer the value of 200 meq was calculated. Since this value is higher than that reported for cells adapted for several weeks in medium E (*see* Ehrenfeld & Cousin, 1982), it would appear that in the 3 hr after transfer *Dunaliella* cells have not completely performed their osmotic readjustment. In a recent work Ginzburg (1981*a*) reports a constancy of K^+ concentration in *Dunaliella parva* cells subjected to hypertonic shock. This discrepancy with our results may be due to the very simplified experimental medium used by these authors (K^+ and Ca^{++}-free medium) which may also explain the much lower kinetics of Na^+ extrusion reported in their experiments. We found that external K^+ plays a role in Na^+ extrusion (Table 1) and that the presence of Ca^{++} (20 meq) or Mg^{++} (90 meq) was essential for the increase of cell K^+ content after hypertonic shock (*see* Table 2). Similar increases in K^+ content have also been observed in the algae *Ochromonas malhamensis* (Kauss et al., 1975) and *Platymonas subcordiformis* (Kirst, 1977) after an increase of external osmotic pressure. The action of FCCP and DCMU as inhibitors of the K^+ content increase would indicate an energy-dependent pumping mechanism. The inhibi-

tory action of DCCD (energy transfer inhibitor) could also be interpreted as the effect of a change in the ATP available to the pump or in view of the relatively low concentrations (5×10^{-5} M) of this agent used as a direct action of this drug on the pump.

Over a wide range of salinities, *Dunaliella* maintains considerably lower Na^+ concentration and higher K^+ concentration than those of the surrounding medium, although the absolute values are controversial (Johnson et al., 1968; Ben Amotz & Avron, 1972; Borowitzka & Braun, 1974; Gimmler & Schirling, 1978; Balnokin, Strogonov, Kukaeva & Medvedev, 1979; Ginzburg, 1981*b*; Ehrenfeld & Cousin, 1982). An active process of Na^+ extrusion and K^+ uptake has been generally postulated on an electrochemical basis in euryhaline algae (Kirst, 1977; Gimmler & Schirling, 1978; Ginzburg, 1981*b*; Ehrenfeld & Cousin, 1982). By modifying the K^+/Na^+ ratio of *Dunaliella tertioelecta*, Ehrenfeld and Cousin (1982) demonstrated an apparent exchange mechanism of Na^+ ions against K^+ ions. The fact that the phase of Na^+ extrusion following hypertonic shock is dependent on the presence of K^+ or Rb^+ ions in the medium (*see* Figs. 7 & 8) would suggest that the Na^+ extrusion is linked to K^+ pumping. That the two phenomena are directly linked may, however, be questioned since a time delay was observed between Na^+ extrusion and K^+ content increase and a hypertonic shock produced by glycerol or choline chloride increased the K^+ content without changing the Na^+ content. A hypothesis which could explain these results is that two distinct mechanisms occur: a Na/K exchange pump and a Na^+-independent K^+ pump. The first would function in steady-state conditions maintaining a low Na^+ concentration and a high K^+ concentration and would be evoked by the initial passive entrance of Na^+ into the cell; its effect would be to decrease intracellular Na^+. The intracellular K^+ concentration would not necessarily change during this period (first 10 min) since the K^+ uptake could be balanced by a passive K^+ loss in view of the increased chemical gradient of this ion resulting from water loss. After this initial period a second Na^+-independent K^+ pump would temporarily increase the cellular K^+ content. The absence of known specific inhibitors and the complication of cell compartmentalization will make the experimental demonstration of these pumping mechanisms difficult.

In conclusion, although these is no doubt that glycerol is the main osmoticum in *Dunaliella*, Na^+ and K^+ ions contribute to the osmotic adaptation of the alga after osmotic shock and their effects are particularly important in the initial phases of shock.

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