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Ionic Regulation of the Unicellular Green Alga *Dunaliella tertiolecta*

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Summary. Different techniques were investigated in order to determine the Na, K and Cl concentrations of *Dunaliella tertiolecta* cells adapted to a large range of salinity (20 to 1640 mM NaCl). The K cell concentrations were 6 to 13 times higher than the K concentration of the external medium (11 mM). The The Na and Cl cell concentrations, on the other hand, were lower than in the external medium at all salinities tested. Considerable differences in the absolute values of Na and Cl were, however, found according to the technique employed. These results are interpreted in terms of compartmentalization of the cells (at least two compartments). It is postulated that the larger compartment regulates its ion concentrations, maintaining low Na and Cl and high K concentrations, whereas the second compartment equilibrates with the external medium. The cation permeability of the membrane limiting the regulating compartment is altered by the antibiotics nystatin and monensin. Incubation of cells in K-free medium leads to a decrease of K and to an increase of the cell Na, this effect being reversed by addition of KCl to the medium. A good correlation is found between gain of K and loss of Na, suggesting a stoichiometric exchange of these two ions. The magnitude of this apparent Na/K exchange increases as the salinity increases. The external K concentration necessary to mediate half-saturation of the Na/K exchange is a function of the NaCl concentration of the adaptation medium. This Na/K exchange is partially light-dependant and inhibited by cold, cyanide and DCCD. It is suggested that this mechanism helps in the regulation of the ionic composition of *Dunaliella* cells.

Key words *Dunaliella*·unicellular algae·Na/K exchange·osmoregulation·ion transport·cell volume

Introduction

The euryphaline alga *Dunaliella tertiolecta* Butcher has the ability to survive in salinities ranging from 3 to 120% (McLachlan, 1960). This remarkable capacity for osmoregulation has been linked to the ability of these cells to synthesize glycerol under hypertonic conditions and to release this organic compound into the medium under hypotonic conditions (Craigie & McLachlan, 1964; Wegmann, 1971; Ben Amotz & Avron, 1972; Borowitzka & Brown, 1974; Ben Amotz, 1975; Jones & Galloway, 1979). Although the role of glycerol in *Dunaliella* cell osmoregulation is largely accepted, the nature and contribution of inorganic substances, i.e. salts, remains a subject of discussion. Marrè and Servettaz (1959), Trezzi, Galli and Bellini (1965), Ginzburg (1969), and Frank and Wegmann (1974) have claimed that the intracellular salt concentration is high and constitutes the main osmotic substance. The studies of Gimmler and Schirling (1978) on *Dunaliella parva* also showed high values for Na concentrations (Na_{int}/Na_{ext} varying between 0.4 and 0.5). These authors suggested that Na and even K ions contributed to the osmotic balance. More recently, Ginzburg (1981a, b) confirmed, in the same species, that Na^+ concentrations were of high magnitude. On the other hand, Johnson et al. (1968), Ben Amotz and Avron (1972), Borowitzka and Brown (1974), and Balnokin, et al. (1979) argued that the cell maintains considerably lower salt concentrations than those present in the surrounding medium.

The large differences in cell ionic concentration reported in the literature have at least two explanations: firstly, the different species of *Dunaliella* studied have different degrees of salt resistance and the regulation of the cell sap is only effective in a given range of salinity (*see* Balnokin et al., 1979); secondly, the measurement of the cell ionic concentrations (especially Na and Cl) is particularly difficult when the external medium contains very high concentrations of these ions and the results may differ considerably according to the technique used.

In this paper we have attempted, by the use and comparison of different techniques, to measure the Na, K and Cl concentrations of *Dunaliella* cells adapted to a large salinity range. High K and low Na and Cl cellular concentrations were found. Antibiotics, drugs and depletion of cellular K were used to ascertain the importance of these ions in cell ionic regulation.

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Materials and Methods

Growth of Organisms

Dunaliella tertiolecta Butcher was grown in sterile plastic flasks (Corning, France) lit with a combination of day-light (General Electric, U.S.A.), grolux (Sylvania, Germany) and coolwhite lamps (General Electric, U.S.A.) for 12 hr a day. The light intensity was about 35,000 lux (490 μmol m^{-2} sec^{-1}) on the surface of the culture flask. The temperature was maintained at 20 °C in the dark period and 24 °C in the light period.

The basic inorganic medium consisted of (mM): 8 Tris; 45 $MgSO_4$; 10 $CaCl_2$; 10 KCl; 1 $NaNO_3$; 1.2 $NaHCO_3$; 2.5 Na_2HPO_4 and 12 mg Na_4 EDTA; 2 mg $ZnSO_4$ 7 H_2O; 1 mg Na_2Mo_4; 0.5 mg $FeCl_3$ 6 H_2O; 0.2 mg $MnCl_2$; 2 μg $CoCl_2$; 2 μg $CuSO_4$; 620 μg H_3BO_4 per liter. The level of sodium chloride in the medium is indicated in each experiment. Media A, E, G refer to media containing 20, 410 and 1,640 mM of NaCl, respectively. The final pH was adjusted to 7.8 by a concentrated HCl solution. Cells were used at the late logarithmic growing phase. *Dunaliella* cells were adapted for several months at a given salinity before experimentation.

Cell Counting

Culture densities were determined with an electronic particle counter (Coultronics, France, Model D Ind). To avoid coincidence, culture samples were diluted to contain no more than 10,000 cells /ml.

Determination of the Cell Volume, the Cell Water and the Dry Weight 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 0.58 mm internal diameter for 45 sec at 6,000 *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. The dry weight of the cells and the cell water were calculated taking into account the density of the medium (1.015 for medium A, 1.025 for medium E and 1.075 for medium G) and its dry weight content (0.015, 0.038 and 0.110 g/ml for medium A, E and G, respectively). For each sample the number of cells, the proteins and the chlorophylls were determined. Chlorophyll *a* and *b* were assayed according to Wegmann and Metzner (1971) and proteins according to the method of Lowry, Rosebrough, Farr and Randall (1951). The cell volume and cell water are expressed as μl/10^6 cells and the dry weight as μg/10^6 cells.

The cell volume was also estimated by measuring the length and width of *Dunaliella* with the aid of a microscope connected to a video tape recorder. The volume of the cells was taken to be a revolution elipsoid and calculated according the formula ($V = 4/3\pi\ a\ b^2$ (*a*, taken as the half-length and *b* the half-width of the cell).

Determination of Na, K and Cl Contents of the Cells.

Two difficulties had to be overcome in order to measure the ion contents of *Dunaliella* cells: contamination of the cells with NaCl from the highly saline outside medium and possible loss of ions from the cells. The chosen method also had to avoid cell damage. Our preliminary experiments eliminated the technique of centrifugation of cells through silicone oil (Rhodorsil 190 and G40 V, France) since the cells were partly damaged and not sufficiently "washed" free of extracellular medium. The first technique tested was a washing technique using very rapid centrifugation. About 2.5×10^8 cells from each salinity were washed 3 times with an ice-cold isotonic glycine medium containing 4 mM magnesium and 1 mM calcium. Each centrifugation lasted 10 sec at 6,000 *g* with a rapid microcentrifuge (Mikrorapid, Hettich, Germany). The total washing procedure did not exceed 2 min. Microscopic observations confirmed that no cell damage occurred in these conditions. No Na was detectable in the last washing medium. After addition of 4 or 5 ml of distilled water, the cell pellets were disrupted by means of an Ultraturax (Janke & Kundel, Germany) and the samples were frozen at −30 °C. The Na and K contents were determined with an Eppendorf flame photometer and the Cl content with an electrometric chloride titrator (Buchler Instr. Div., USA).

The second technique tested involved determining the concentration of a given extracellular ion contaminating the pellet of centrifuged cells by using an extracellular Marker (^{14}C sorbitol, ^{14}C dextran 20,000 or ^{14}C dextran 70,000). By subtracting this amount from the total concentration of this ion in the pellet the cell ion content is obtained. The experimental procedure was identical to the one already described in the paragraph "Determination of cell volume..." and the measurements of the ion concentrations have already been described.

For a given adaptation salinity, the variability of the cell proteins of different batches of cells was smaller than that of the chlorophyll; for this reason the ion contents of *Dunaliella* cells will be referred either to cell volume (meq/liter cell) or to protein (neq/mg protein).

Results

Cell Volume of Dunaliella Cells

The chlorophylls *a* plus *b*, proteins, dry weights, cell volumes and cell water weights of *Dunaliella* cells as referred to 10^6 cells have been measured and are summarized in Table 1. *Dunaliella* cells were adapted in media of three different salinities: medium A, E and G containing 20, 410 and 1,640 mM of NaCl, respectively. The total chlorophyll content does not change significantly whereas the dry weight and proteins increase considerably as a function of the salinity of the adaptation medium (a twofold change between media A and G). The cell volumes and cell water weight of *Dunaliella* cells adapted to media A and G were slightly larger than for cells adapted to medium E.

The volume of one cell was also estimated by microscopic determinations of the length and the width of *Dunaliella* cells. The lengths (μm) were

Table 1. Proteins, chlorophyls, dry weight, cell water weight and cell volume of *Dunaliella* cells adapted to three different salinities[a]

Medium (mM)	Proteins ($\mu g/10^6$ cells)	Chlorophyls ($\mu g/10^6$ cells)	Dry weight ($\mu g/10^6$ cells)	Cell water weight ($\mu g/10^6$ cells)	Cell volume ($\mu l/10^6$ cells)
A (20)	25.1 ± 0.7 ($n=8$)	1.88 ± 0.28 ($n=19$)	58 ± 2 ($n=19$)	200 ± 8 ($n=19$)	0.227 ± 0.005 ($n=19$)
E (410)	30.8 ± 1.1 ($n=8$)	1.88 ± 0.23 ($n=20$)	65 ± 2 ($n=20$)	156 ± 8 ($n=20$)	0.211 ± 0.010 ($n=20$)
G (1,640)	49.3 ± 2.5 ($n=8$)	1.95 ± 0.15 ($n=19$)	109 ± 4 ($n=19$)	185 ± 10 ($n=19$)	0.259 ± 0.015 ($n=19$)

Media A, E and G contain 20, 410 and 1,640 mM of NaCl, respectively. Dextran 70,000 used as extracellular marker. Number of experiments in brackets.

11.5 ± 0.1, 10.2 ± 0.1 and 11.7 ± 0.1 and the widths (μm) 6.7 ± 0.1, 5.8 ± 0.7 and 6.8 ± 0.1 for *Dunaliella* cells adapted to media A, E and G, respectively. The calculated volumes (μm^3) were 274 ($n=124$), 179 ($n=164$) and 283 ($n=81$) for cells adapted to media A, E and G, respectively. These values are similar to the ones found with dextran 70,000.

Intracellular Na, K and Cl Concentrations

The changes in the ion contents (Na, K, Cl) of *Dunaliella* cells were followed as a function of the salinity of the preadaptation medium (20 to 1,640 mM of NaCl). Different extracellular markers were tested. Figure 1 and Table 2 summarize the results obtained using ^{14}C dextran 70,000 as extracellular marker (without any washing procedure). The concentrations of sodium and chloride increase linearly as the salinity of the incubation medium increases; however, the cells maintain low cellular Na and Cl concentrations, about 5 times smaller than these of the external medium. On the other hand, the K concentrations of *Dunaliella* cells are six, 10 and 13 times higher than the K concentrations (11.5 meq/liter) of media A, E and G, respectively.

Dextran 20,000 gives results similar to those obtained with dextran 70,000 (*see* Table 2). Another extracellular marker, sorbitol, has been used with *Dunaliella parva* cells by Gimmler, Schirling and Tobler (1977). We found that the space of distribution of this sugar is larger than that occupied by dextran 70,000. For instance, with medium E preadapted cells, the space in percent of the pellet was 49.7 ± 1.0 ($n=20$) for ^{14}C sorbitol and 37.0 ± 1.3 ($n=32$) for ^{14}C dextran 70,000. The measured cell volume (in μl/mg protein) using sorbitol was 4.21 ± 0.07 ($n=7$), 2.89 ± 0.10 ($n=8$) and 2.28 ± 0.06 (for cells adapted to media A, E and G, respectively. Taking into acount the correlation between proteins and cell number (Table 1), the calculated cell volume in μm^3 was found to be 106, 89 and 111 for the three different batches of cells tested. These volumes are less than half those found with the use of dextran 70,000 or from microscope measurements. Owing to the larger extracellular space found with sorbitol, the calculated K concentrations were higher than those obtained using dextran 70,000 (*see* Table 2); the Na content of cells preadapted in medium A was similar to that calculated with dextran 70,000, but for cells adapted to media E or G negative values were found. This last result suggests that either the Na pool is very small and not accessible with this technique or that the extracellular space is overestimated by an absorption of sorbitol or a filling of an intracellular compartment with the sugar. In the latter case, the penetration of sorbitol would have to be extremely rapid since in sorbitol uptake experiments as a function of time (from 3 min to 1 hr), no accumulation of the sugar in the cells was observed after the first 3 min.

It appears from these experiments that the choice of the extracellular marker is essential when estimating cell ion concentrations; even small variations in the extracellular space will lead to considerable differences in the estimation of the cell ion concentrations, particularly with media of high salinity for which the corrections due to Na contamination of the extracellular medium represent more than 80 and 100% of the toal amount of Na measured using dextran 70,000 and sorbitol, respectively.

For this reason, a second technique involving rapid washing of the cells with an ice-cold glycine solution was tested. Figure 2 and Table 3 illustrate and summarize the results obtained for a range of adaptation salinities varying from 20 to 1,640 mM NaCl. The cell K contents are close to those found with the technique involving extracel-

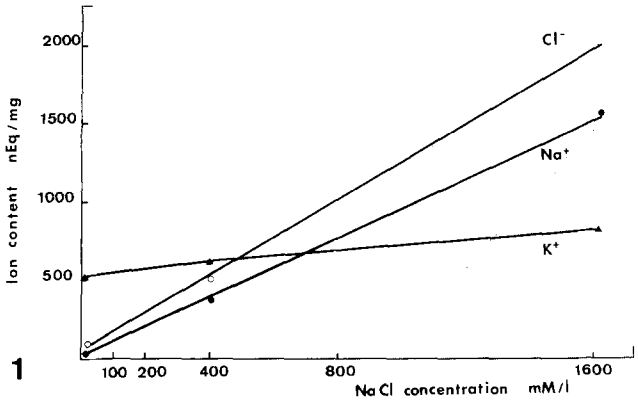

Fig. 1. Evolution of the Na, K and Cl contents of *Dunaliella* cells as a function of the NaCl concentration of the preadaptation medium using ^{14}C-dextran as extracellular marker. Ion contents are expressed in neq/mg protein and the NaCl concentration of the medium in meq/liter. Number of experiments: Na and K = 16, Cl = 8

Table 2. Ion contents of *Dunaliella* cells adapted to different salinities[a]

Extracellular marker	Medium	Na		K		Cl	
		Ion content	Ion conc.	Ion content	Ion conc.	Ion content	Ion conc.
Dextran 70,000	A	34± 4	4.6± 0.5	520± 4	70.7±0.5	95± 21	12.9± 2.9
		$n=16$		$n=16$		$n=8$	
Dextran 70,000	E	369± 30	71.7± 5.8	620±15	120.4±2.9	494± 25	96.7± 4.9
		$n=16$		$n=16$		$n=8$	
Dextran 70,000	G	1571±198	291.5±36.7	810±36	150.3±6.7	2040±215	330±34.7
		$n=16$			$n=19$	$n=8$	
Dextran 20,000	E	258± 31	53.5± 6.4	626±13	129.8±2.7	–	
		$n=11$		$n=11$			
Sorbitol	A	32± 8	7.6± 0.5	455± 2	108.1±0.5	–	
		$n=7$		$n=7$			
Sorbitol	E	−56± 18	−19.4± 6.2	516± 7	178.5±2.4	–	
		$n=8$		$n=8$			
Sorbitol	G	−88± 80	−38.9±35.4	600± 9	265.5±4.0	–	
		$n=8$		$n=8$			

Media A, E and G contain 20, 410 and 1640 mM of NaCl, respectively. Ion content is given in neq/mg protein and ion concentration calculated in meq/liter cell. n = number of experiments.

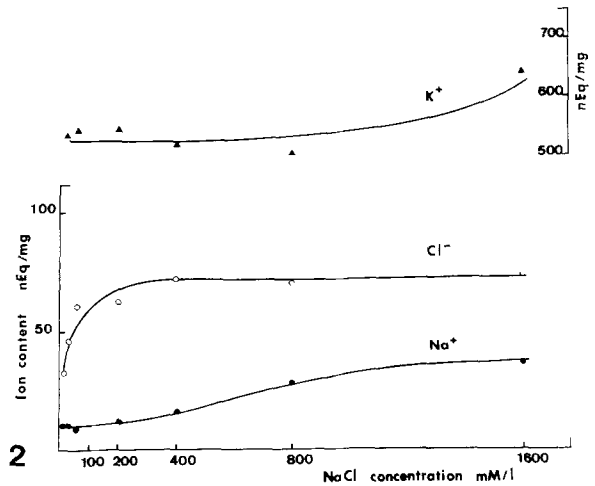

Fig. 2. Evolution of the Na, K and Cl contents of *Dunaliella* cells as a function of the NaCl concentration of the preadaptation medium. These experiments were performed with the "washing technique". Ion contents are expressed in neq/mg protein and NaCl concentrations of the medium in meq/liter. Number of experiments: $n=4$

lular markers. The main difference lies in the values of the Na and Cl contents of *Dunaliella* cells, which are much smaller than those found when using Dextran 70,000. Only small variations in the Na and Cl contents of *Dunaliella* cells are observed as the salinity of the predaptation media increases; the Na contents increase slightly with increasing salinity and the chloride contents follow a saturation curve, the maximum being reached at concentrations of the adaptation medium close to 400 mM of NaCl.

Nystatin and Monensin Modify Cellular Ion Contents

In order to test the validity of the above results we artificially modified the ion contents of the cells by means of two ionophores, nystatin and monensin. Nystatin is a polyene antibiotic that greatly and unspecifically increases the monovalent ion

Table 3. Ion contents of *Dunaliella* cells adapted to different salinities[a]

Medium	Na^+		K^+		Cl^-	
	Ion content	Ion conc.	Ion content	Ion conc.	Ion content	Ion conc.
A	16± 2	2.2±0.3	492± 9	66.9±1.2	32± 6	4.4±0.8
	$n=17$		$n=17$		$n=4$	
E	34±10	6.6±1.9	546±15	106.0±2.9	73± 8	14.2±1.6
	$n=8$		$n=8$		$n=4$	
G	39± 2	7.2±0.4	637±20	118.2±3.7	74±14	13.7±2.6
	$n=6$		$n=6$		$n=4$	

The technique used involved rapid washings of the cells with an ice-cold solution. Media A, E and G contain 20, 410 and 1640 mM of NaCl, respectively. Ion content is given in neq/mg protein and ion concentration calculated in meq/liter cell, using dextran 70,000 as extracellular marker. n = number of experiments.

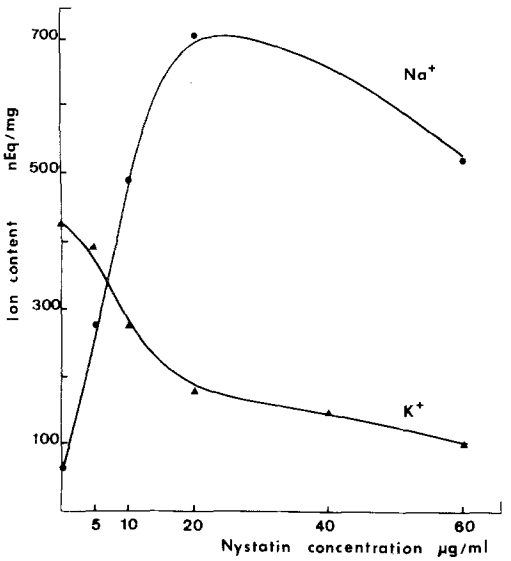

Fig. 3. Effect of different concentrations of nystatin on the Na and K contents of *Dunaliella* cells. This typical experiment was performed with the "washing technique". Ion contents are expressed in neq/mg protein. Cells were incubated 30 min in the presence of nystatin in a K-free medium containing 410 mM of NaCl. Constant illumination was provided during the experiment

permeability of natural or artificial membranes (Lampen, 1966; Finkelstein & Cass, 1968; Cass & Dalmark, 1973). Monensin is a polyether antibiotic which preferentially mediates a Na/H exchange through bacterial or animal culture cell membranes (MacDonald, Lanyi & Greene, 1977; Smith & Rozengurt, 1978).

A typical experiment showing the effect of nystatin on the Na and K contents is illustrated by Fig. 3. Determination of ion contents was made by the washing technique. *Dunaliella* cells were incubated 30 min with different concentrations of nystatin in a K-free medium containing 410 mM of NaCl. Nystatin induces drastic changes of Na and K contents; with the use of 20 µg/ml, a 12-fold increase of Na content is observed and the K content of *Dunaliella* cells is reduced to less than half the control value. At the highest concentrations tested or with longer times of incubation with the antibiotics, cells were damaged. No reversibility of the effect of nystatin by washing the cells several times was found. It should be noted that in the absence of nystatin incubation of the cells in a K-free medium also induces changes in the ion contents (i.e. 30 min); however, these modifications are considerably smaller than in the presence of nystatin (*compare* Fig. 3 and 5).

Table 4. Effect of nystatin (20 µg/ml) on ion contents of *Dunaliella* cells[a]

	Cell volume	Na	K
control	7.22±0.14	535±28	877±10
nystatin	7.10±0.30	1025±58	658±21
difference	0.12±0.10	490±60	219±31

Cell volumes and ion determinations are performed with the use of ^{14}C dextran 70,000 as extracellular marker. The incubation time with nystatin was 30 min. The cells were illuminated during the experiment. The cells were preadapted to medium E (410 mM of NaCl). Ion content is given in neq/mg protein. Cell volume is expressed in µl/mg protein. Number of experiments = 5

Table 4 shows the effect of nystatin (20 µg/ml) on ion contents of *Dunaliella* cells using ^{14}C dextran 70,000 as an extracellular marker (without washing the cells). During this experiment the cell volume does not change significantly; the cell Na contents increase by 490 neq/mg protein and the K contents decrease by 219 neq/mg protein. These changes in ion contents are similar to those found previously with the "washing technique" (*compare* with Fig. 3). It can be concluded that nystatin induces considerable modification of the Na and

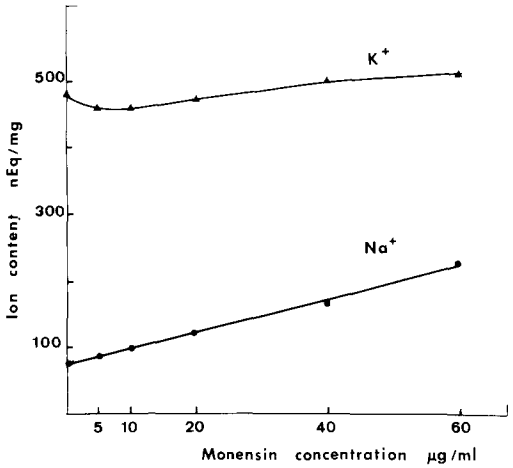

Fig. 4. Effect of different concentrations of monensin on the Na and K contents of *Dunaliella* cells. This typical experiment was performed with the "washing technique". Ion contents are expressed in neq/mg protein. Cells were incubated 30 min in the presence of monensin in a K-free medium containing 410 mM of NaCl. Constant illumination was provided during the experiment

Table 5. Modification of the ion contents of *Dunaliella* cells incubated in K-free media[a]

Medium	Period	Na		K	
		Ion content	Ion conc.	Ion content	Ion conc.
E	control	336± 98	70±20	488±11	102±2
$n=4$	K-free	427± 44	90± 9	382± 9	80±2
	reversal	431± 42	86± 8	561± 7	112±1
G	control	899±136	269±41	520± 7	156±2
$n=4$	K-free	901± 6	263± 2	341± 8	100±2
	reversal	876± 46	238±13	532±17	145±5

Cells were incubated 24 hr in a K-free medium, followed by a reversal period of 2 hr (addition to the medium of 10 mM of KCl). Media E and G contain 410 and 1640 mM of NaCl, respectively. Ion content is given in neq/mg protein and ion concentration calculated in meq/liter cell using dextran 70,000 as extracellular marker. n = number of experiments.

K contents which can be followed either with the washing technique or with the use of dextran; although the magnitude of the ion content variations are identical with the two techniques, the absolute values of the Na contents are notably different (*see above*).

The effect of monensin is illustrated by Fig. 4. Monensin was found to modify specifically the Na content of *Dunaliella* cells, the K content remaining unchanged in the range of concentrations of monensin tested in this study. The increase in Na content was directly proportional to the concentration of monensin used and the effect was not so drastic as with nystatin. Reversibility was partially achieved after several cell washings and the cells were not injured.

Table 6. Modifications of the ion contents of *Dunaliella* cells incubated in K-free media[a]

Medium	Period	Na	K
A	control	16± 2	492± 9
$n=5$	K-free	43±14	373±12
	reversal	16± 7	412±19
E	control	34±10	546±15
$n=8$	K-free	134±13	385±21
	reversal	33± 5	465±20
G	control	39± 2	637±20
$n=7$	K-free	193±15	394±32
	reversal	76± 7	524±23

The technique used involved rapid washings of the cells with an ice-cold solution. Media A, E and G contain 20, 410 and 1640 nM of NaCl, respectively. Cells were incubated 24 hr in K-free medium, followed by a reversal period of 2 hr (after addition to the medium of 10 mM of KCl). Ion content in neq/mg protein. n = number of experiments.

Evidence for a Na/K Exchange Mechanism

Changes in the ion contents of *Dunaliella* cells, after incubation in K-free media, were investigated. A first series of experiments using dextran 70,000 as extracellular marker was made. Table 5 summarizes the Na and K contents of two batches of cells incubated in medium E or G. The experiment consisted of three successive periods, the first being a control period, the second entailing incubation of the cells for 24 hr without potassium and the third being a reversal period of 2 hr (in presence of 10 mM of KCl) following 24 hr in the absence of potassium.

Incubation of the cells in K-free media led to a decrease in the K content of the cells. The loss of K was greater in medium G-adapted cells. Reversal was achieved when incubating the cells with a medium containing 10 mM KCl. On the other hand, no significant modifications in Na content were observed in cells incubated in either of the K-free media tested. This could have been due to the lack of precision in the determination of the Na content inherent in the technique when the cells are bathed in high salinity media.

These experiments were therefore repeated using the washing technique. Table 6 summarizes the values of the Na and K contents of cells incubated 24 hr in K-free media and after 2 hr of reversal by addition of 10 mM KCl. Control values are also given in the first line of the Table. Three batches of cells adapted to media A, E and G were tested. As already seen, incubation of the cells with

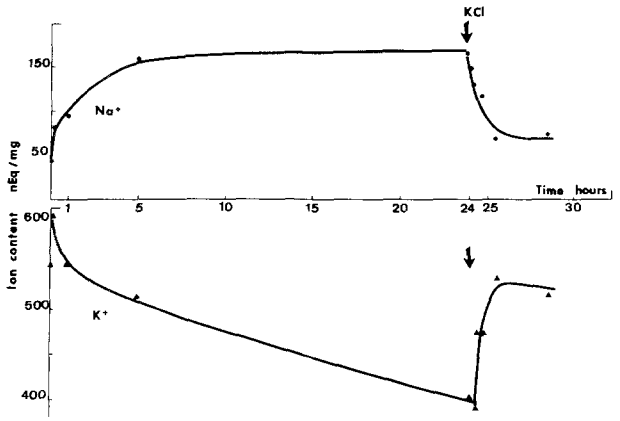

Fig. 5. Evolution, as a function of time, of the Na and K contents of *Dunaliella* cells in the presence or absence of K in the incubation medium. The addition of 10 mM of KCl is illustrated by an arrow. The cells were preadapted to medium G (1640 mM of NaCl). Ion content in neq/mg protein. Time in hours

K-free media led to a K content decrease but a parallel Na increase was observed. In all three cases, the K decrease was greater than the Na increase, and the higher the salinity of the medium the greater was the modification of the cellular Na, K content. Addition of 10 mM KCl reversed the phenomenon, i.e. the cell K increased and the Na content decreased. Figure 5 illustrates the time course of the phenomenon. The Na content increases to a constant level in about 5 hr whereas the K content continues to decrease after 24 hr of incubation in a K-free medium. Addition of KCl (10 mM) to the external medium induces a reversion of the phenomenon. The kinetics of the reversion are similar for Na and K; after 90 min, steady-state values are reached and the magnitude of the ion variations are similar. However, it can be seen that the cell K content is lower than in the control period. These observations suggest that a fraction of the K lost during incubation of the cells in a K-free medium is not reabsorbed by the cells during the reversal period and is not associated with Na movements.

The next Figure (Fig. 6*A* and *B*) correlates the variations in the Na and K contents (ΔNa, ΔK) of *Dunaliella* cells induced by the addition to the medium of 10 mM KCl (reversion time 2 hr); these cells were previously incubated for 24 hr in K-free media. Analysis of the data leads to the following equations for media E and G, respectively:

	n	r
$\Delta K = (0.97 \pm 0.15)\ \Delta Na + 15.37 \pm 10.60$	12	0.89
$\Delta K = (1.06 \pm 0.20)\ \Delta Na + 21.65 \pm 14.75$	15	0.83

The correlations are highly significant. $p < 0.001$. It can be concluded from these analyses that the

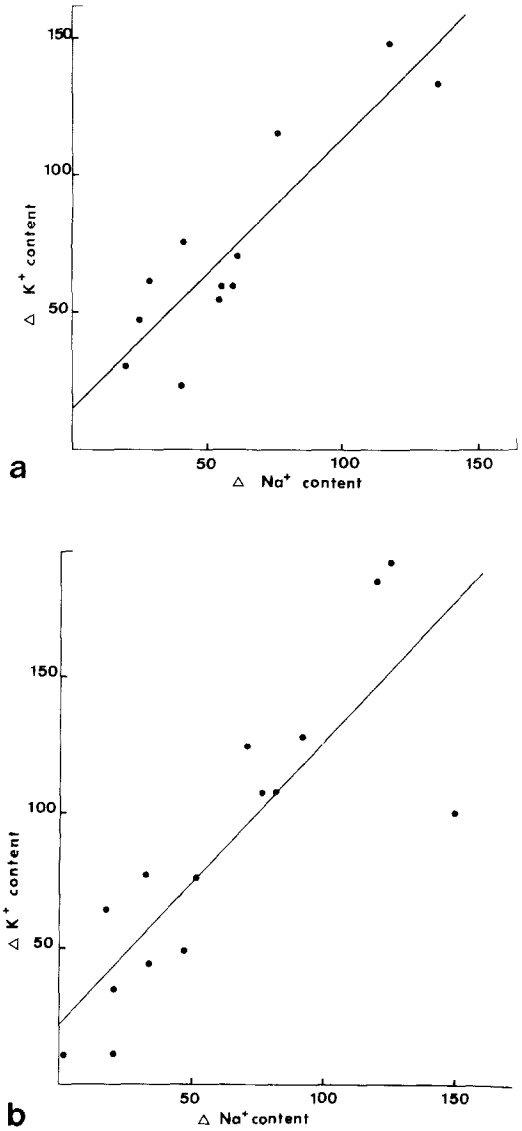

Fig. 6. *A* and *B*: Correlation between the Na and K variations of the *Dunaliella* cell contents. The cells were successively incubated 24 hr in a K-free medium and 2 hr in this medium after the addition of 10 mM of KCl. The Na and K cell contents of *Dunaliella* cells were measured at the end of each period and the variations were plotted. Part *A* and *B* represent experiments on cells adapted to media containing 410 and 1,640 mM of NaCl, respectively. Ion content variations in neq/mg protein. $n = 12$ and $n = 15$ for parts *A* and *B*, respectively

increase in K content of *Dunaliella* cells is associated with a Na decrease in a 1 to 1 ratio, suggesting an exchange of K ions against Na ions.

K Dependence of the Na/K Exchange. Effect of Certain Inhibitors

In order to characterize this Na and K exchange, we followed the variations of the Na and K cell contents induced by the addition of KCl to a medi-

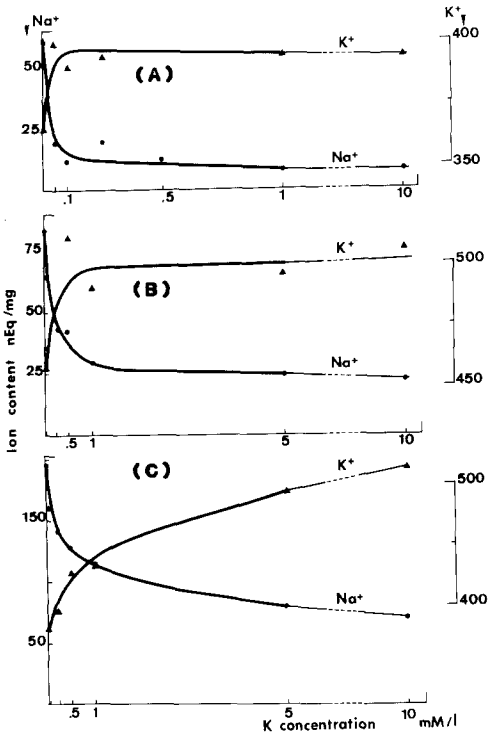

Fig. 7. *A–C:* Ion variations in *Dunaliella* cells as a function of the KCl concentration of the incubation medium. (Same protocol as in Fig. 6). *A–C* represent experiments with cells adapted to media containing 20, 410 and 1,640 mM of NaCl, respectively. Ion content variations in neq/mg protein and KCl concentration in mM. $n=4$

Table 7. Effect of different agents on the apparent Na/K exchange of *Dunaliella* cells[a]

Period	ΔNa	Inhibition (%)	ΔK	Inhibition (%)
control $n=6$	131± 5		152± 2	
darkness $n=4$	69±11	47	101± 7	34
cyanide $n=4$	99±16	24	120± 8	21
darkness +cyanide $n=4$	63±10	52	75±11	51
control $n=5$	82± 4		114± 9	
cold $n=5$	29± 3	64	47±17	59
control $n=6$	127± 6		140± 9	
DCCD $n=4$	31± 7	76	32±23	77

Washing technique used for Na and K determination. After incubating the cells 24 hr in a K-free medium (medium E), KCl (10 mM) was added to the medium. The variations in Na and K contents (neq/mg protein) between these two periods, in presence or absence of an inhibitor are reported in the Table. Cyanide concentration 10^{-4} M; DCCD concentration 5×10^{-5} M. Control experiments were performed at 22 °C and in the cold period at 2 °C. n=number of experiments.

um containing K-depleted cells under different experimental conditions.

Figure 7*A*, *B* and *C* show the variations of the ion contents of *Dunaliella* cells as a function of the concentration of the KCl present in the medium in three batches of cells (cells incubated in A, E and G media, respectively). In all cases saturation processes are observed. For a given batch of cells the KCl concentration corresponding to half-saturation of the ion variations (K_{app}) was of the same order of magnitude for Na and for K.

The "K_{app}" were identical ($\simeq$0.50 mM) for K and Na for cells adapted in medium G; about 0.20 mM for cells adapted in medium E and less than the smallest KCl concentration tested, i.e. 50 µM, for cells adapted in medium A. Thus the K_{app} decreases as the NaCl concentration of the adaptation medium decreases.

Table 7 shows the effects of some well-known inhibitors of ion transport in biological systems. The variations of Na and K cell contents, induced by the addition of 10 mM KCl to a medium containing cells previously incubated in a K-free medium, were followed in the absence or presence of a given inhibitor.

Cold, darkness and cyanide partially block the apparent Na/K exchange. The simultaneous action of obscurity and cyanide (10^{-4} M) seems to be additive. DCCD (5×10^{-5} M) inhibits the Na/K exchange considerably.

Discussion

The results reported in this study show that according to the technique used, very different values may be obtained when measuring cell ion concentration. If an extracellular marker is used to evaluate the contribution of external NaCl to the total NaCl value of the cell pellet, the nature of the extracellular marker is of great importance. Sorbitol, one of the extracellular markers tested, seems inadequate. Indeed, the cell volume estimated with this sugar is only half that calculated by the direct optical method. It appears that either sorbitol very quickly fills the cellular compartment (in less than 3 min) or that this sugar is adsorbed on to the

cells. "An unusual membrane permeability" of *Dunaliella parva* cells has been reported by Ginzburg (1969) who argues that the cells are permeable to inulin; similarly, Riisgaard et al. (1980) suggest absorption or entrance of inulin into the cells. In this respect sorbitol behaves like inulin.

On the other hand there is quite good agreement between the cell volume measured with the use of dextran 70,000 and that measured with light microscopy. Taking this molecule as an acceptable extracellular marker for the estimation of cell ion concentrations, a linear function is found between the concentrations of Na or Cl in the cells and in the preadaptation media (*see* Fig. 1 and Table 2). In all cases the intracellular Na and Cl concentrations were lower than in the external medium and a constant ratio close to 5 was observed for Na_{ext}/Na_{int} and Cl_{ext}/Cl_{int}. These results are comparable with those found by Gimmler and Schirling (1978) for *Dunaliella parva* although higher cellular concentrations of sodium are reported in their study. In all preadaptation media tested, we found higher K concentrations in the cells than in the outside medium; the K_{int}/K_{ext} ratio increases as the cells are adapted to media of higher NaCl concentration. This last result confirms those obtained with other species of *Dunaliella* (Gimmler & Schirling, 1978; Ginzburg, 1978; Riisgard et al. 1980) and on other marine algae (Raven, 1976; Kirst, 1977).

If we now compare these results with those obtained with the fast washing procedure we find that this procedure leads to considerably lower Na and Cl contents. Furthermore, the changes in these ion contents as a function of the salinity of the adaptation media are also completely different with the second technique. Indeed, the Na contents increase very slightly and the Cl contents follow a saturation curve as the salinity increases. K concentrations of *Dunaliella* cells on the other hand are similar in both techniques studied. The simplest hypothesis which could explain the discrepancy in the results from the two techniques would be that two cellular compartments exist. A tight membrane would maintain very low concentrations of Na and Cl and high concentrations of K in the first cellular compartment. These concentrations would be relatively insensitive to external salinity variations. A second cellular compartment with a membrane extremely permeable to anions and cations would be in equilibrium with the external medium and could thus have an ionic composition identical with that of the incubation medium. The washing technique would eliminate the ions of this compartment by diffusion and thus leave for analysis the ion contents of the first compartment. The results of the experiments using antibiotics such as nystatin support this hypothesis. Thus, nystatin (20 μg/ml) increases the Na content of *Dunaliella* cells by about 500 neq/mg protein irrespective of the technique employed (*see* Fig. 3 and Table 4), which suggests that the antibiotic only equilibrates the ionic contents of compartment 1. The composition of compartment 2 remains unchanged since it is already in equilibrium with the external medium. The idea of a compartimentalization of *Dunaliella* cells has already been advanced by Ginzburg (1978, 1981 b), who suggested that the chloroplast was one of the compartments limited by a tight membrane and that the other was limited by a porous plasmic membrane. We have no indication on the localization of the cellular compartments but the dimension of the second is certainly small. In fact, it can be calculated that an external layer of 0.3 μ in equilibrium with the external medium would explain the discrepancy between the two techniques tested in this study. In view of this, the question arises whether this compartment is really a cellular compartment rather than an extracellular compartment. The experiments reported in this study demonstrate the presence of a well-regulated cellular compartment containing high K and low Na concentrations. Furthermore, a transport mechanism exchanging K ions for Na ions is suggested by the following observations: (*i*) there is a good 1 to 1 correlation between increase in K and decrease in Na contents; (*ii*) similar kinetics are observed for the two ions; (*iii*) the K concentration of the medium corresponding to half-saturation of Na and K cell variations are identical for these two ions. Such a Na/K exchange has been already suggested in *Dunaliella parva* (Gimmler & Schirling, 1978) and *Platymonas* (Kirst, 1977) and has been demonstrated in another unicellular alga, *Chlorella pyrenoidosa* (Barber, 1968; Shieh & Barber, 1971). This Na/K exchange shows saturation with increasing external potassium concentrations (*see* Fig. 7). In this respect *Dunaliella tertiolecta* behaves like *Chlorella pyrenoidosa* (Barber & Shieh, 1973). As the NaCl concentration of the external medium decreased we found a diminution of the K concentration corresponding to half-saturation of the Na/K exchange. This fact could be explained by postulating that Na competes with K for an external K-transporting site; nevertheless, taking into account the large difference existing between Na and K concentrations in the medium, the affinity of this site for K ions must be considerably greater than for Na ions.

A Na/H exchange contributing to the mainte-

nance of a low cell sodium has been suggested by Latorella and Vadas (1973) for *Dunaliella teriolecta* and by Kaplan and Schreiber (1981) for *Dunaliella salina*. This mechanism was found to occur when the cells were transferred to a medium of higher salinity. Our present results were performed in steady-state conditions; i.e., cells preadapted in a given salinity were treated during the experimental period in the same medium. The results therefore are not contradictory and Na/H and Na/K mechanisms functioning together or independently according to the osmotic equilibrium of the cells may contribute to assure the ionic regulation of the cell contents.

The inhibition of the linked Na/K exchange by dark, the respiratory inhibitor cyanide and the energy transfer inhibitor DCCD suggest that this exchange mechanism is energy dependent. Furthermore, although electrical potentials have not been measured in *Dunaliella* cells, from electrochemical considerations it may be postulated that the K and Na movements are active. Similar assumptions have been advanced by Ginzburg (1981*a*, *b*). A Na/K ATPase has been localized in the plasmalemma of *Dunaliella* cells (Tang Jokela, 1970) and it would be tempting to suggest that this enzyme is involved in the Na/K exchange observed in this study. However, there is no direct evidence of a link between this ATPase and ion transport; furthermore, ouabain has no effect on the Na/K exchange in *Dunaliella* cells (*unpublished results*). Additional experiments need to be made to test whether the Na/K exchange is mediated by an enzymatic carrier.

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