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Water Transport Parameters and Regulatory Processes in *Eremosphaera viridis*

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Summary. The water relations parameters and the osmoregulatory response of *Eremosphaera viridis* were investigated both by using the pressure probe technique and by analyzing the intracellular pool of osmotically active agents. In the presence of various concentrations of different salts a biphasic osmoregulatory response was recorded, consisting of a rapid decrease in turgor pressure due to water loss followed by an increase in turgor pressure to the original turgor pressure value (depending on the salt). The values of turgor pressure, volumetric elastic modulus and hydraulic conductivity depended on the composition of the media. Nonelectrolytes did not cause a turgor recovery after the initial water efflux. The second phase of turgor regulation in the presence of salts was characterised by the intracellular accumulation of ions and sugars and required at least 24 hr. Analysis of the cell sap showed that the increase in the internal osmotic pressure was mainly achieved by accumulation of sucrose. Additionally, accumulation of glucose was observed in illuminated cells in the presence of Rb and K. Electron micrographs suggested that the sucrose was produced by degradation of starch granules. Turgor pressure recovery after salt stress seemed to be dependent on temperature and is well correlated with the according photosynthetic activity. The data suggest that a temperature-dependent enzyme which is activated by potassium or rubidium is involved in the regulatory response.

Key Words *Eremosphaera viridis* · water transport parameters · osmoregulation · salt stress · sugar accumulation

Introduction

The ability to regulate and to adjust internal osmotic pressure and thus turgor pressure (and volume) in response to salt and water stress has been observed in many algae and higher plant cells. Some species are capable of maintaining constant turgor pressure (or volume) over a large range of salinity, e.g. *Chaetomorpha linum* (Steudle & Zimmermann, 1971), *Dunaliella parva* (Ben Amotz, 1974), *Valonia utricularis* (Zimmermann & Steudle, 1974) and *Poterioochromonas* (Kauss, 1967). Experimental evidence is available that the regulation of the internal osmotic pressure is under direct control of turgor pressure (or volume) and/or specific ions which are present in the external medium. Intracellular osmotic adjustment is achieved by regulation of the concentration of ions and/or of low molecular weight organic solutes. The latter appear to occur mainly in the cytoplasm because of the sensitivity of enzymes to salts. It is important to emphasize that both the maintenance of the correct ionic and solute environment for biochemical activity and the adjustment of water balance and cellular osmotic and turgor pressures are equally important for successful adaptation.

Despite the great advances made in the field of osmoregulation in recent years (Dainty, 1963, 1976; Levitt, 1972; Hellebust, 1976; Flowers, Troke & Yeo, 1977; Kauss, 1977; Gutknecht, Hastings & Bisson, 1978; Zimmermann, 1978; Wyn-Jones, 1984; Kirst, 1985), we are still far from understanding the complicated signal-reaction sequence which enables cells to maintain normal values for turgor or volume in conditions of water and salt stress. Various mechanisms have been discussed, e.g. activation of enzymes that convert high and low molecular weight substances (Kauss, 1973) or change the transport of osmotically active substances (Bisson & Kirst, 1980). A more recent idea is that turgor regulation occurs by pressure detection by electromechanical compression of membranes in conjunction with changes in the translocation rate of mobile charges which are linked with ion-specific carrier systems (Zimmermann, Büchner & Benz, 1981), as well as the activation of enzymes that convert high molecular substances into osmotically active ones of low molecular weight and vice versa (Kauss, 1973).

One of the reasons for the lack of understanding of this signal chain is almost certainly the fact that ionic and osmotic regulation of the vacuolar contents has been examined using predominantly biophysical techniques. Therefore, giant algal cells were examined because they have a vacuole which is directly accessible to measurements with micro-

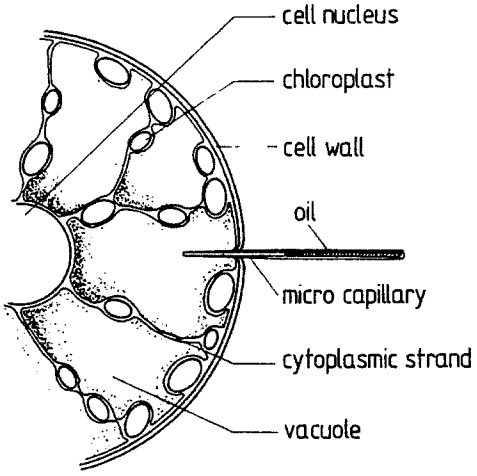

Fig. 1. Schematic cross section of a cell of *E. viridis* with inserted microcapillary

electrodes and pressure probes. In microscopic cells the biochemical aspects have been of more interest to many investigators because of the limitations imposed on the physical methods of measurement by cell size. The apparent differences between osmoregulation by inorganic ions in giant algal cells and the regulation by organic compounds in isolated microscopic and tissue cells may therefore be superficial. These days many authors tend to believe that both types of regulation are present in every osmoregulating plant system, but that their respective contribution to the osmotic relations of the whole cell depends on the size of the vacuole with respect to the cytoplasmic compartment.

One important step towards elucidating the mechanism of osmoregulation would be to carry out combined biophysical and biochemical investigations of the same species under various conditions of osmotic stress. Cells of the fresh-water alga *Eremosphaera viridis* have the right geometric prerequisites for this purpose. Having a mean diameter of 140 μm, the cells of this alga are large enough for the introduction of microelectrodes (for membrane potential and membrane resistance measurements with the aid of the single-electrode method and the charge-pulse technique) (Schefczik, Simonis & Schiebe, 1983) and pressure probes (for measurements of water relations parameters). At the same time, the cells are sufficiently small for measurements of the internal conductivity and electrical properties of the membrane (resistance and capacitance) by means of the electrorotation method (Arnold et al., 1985), and for biochemical analysis of the cell sap under osmotic stress so that conclusions can be drawn about the various regulatory pathways. Simultaneously, investigations by means of an electron microscope would provide further information about structure and structural changes under osmotic stress. This information aids the interpretation of measurements obtained with the pressure probe and microelectrode technique.

Table 1. Osmotic pressure (Π) and ionic composition of the culture media

Medium	Na^+ (μM)	K^+ (μM)	Cl^- (μM)	Ca^{2+} (μM)	Π (mOsm)
I	0.2	2095	—	—	5
I + Ca	0.2	2095	—	1000	8
II	3755	115	13.2	118.7	11

In this communication we report on the osmotic response of cells of *E. viridis* due to external osmotic stress, using both the biophysical and biochemical approaches. The results show that—like marine, litoral and fresh-water algae—*E. viridis* exhibits a biphasic regulation which consists of a rapid water transport phase and a subsequent, substantially slower turgor pressure recovery phase. This regulation exhibits many similarities with the processes of osmotic adjustment observed in other species; but there are also some remarkable differences.

Materials and Methods

The chlorococcal peat-bog alga *Eremosphaera viridis* (strain 71.80 from the algae collection of the Institute of Plant Physiology, University of Göttingen) grows to a diameter of up to 170 μm during its 2 to 4 day cell cycle. The cell nucleus is centrally suspended in the spherical cells. From the nucleus, thin, sometimes branched cytoplasmic strands radiate out through the large vacuole and link up with the thin layer of cytoplasm adhering to the cell wall (*see* Fig. 1). Only spherical cells that maintained constant pressure after impalement were used for measurements. The algae were grown in synthetic culture media. These were either that described by Schlösser (1982) with the addition of vitamins B_1 (0.1 mg/liter) and B_{12} (1 mg/liter) instead of soil-extract and peptone (medium I) or that of Smith and Bold (1966), with the addition of 0.12 mM $Ca(NO_3)_2$ at pH 5.5 (medium II). In some cases $Ca(NO_3)_2$ (1 mM) was added to medium I. The osmolarity of the media were 5 mOsm (medium I) and 11 mOsm (medium II), respectively. The main differences between the media concerning the contents of Na^+, K^+, Ca^{2+} and Cl^- as well as the osmotic pressure are given in Table 1. Cells were cultured in an illuminated thermostat (Fa. Kniese) at 30°C and 140 μE sec^{-1} m^{-2} in a 16 : 8 hr light/dark cycle and agitated with air containing 1% CO_2. Unless otherwise stated, experiments on osmoregulation were carried out under these conditions.

Determination of Cell Volume in Relation to Age

A 63% synchronization of the algae was achieved by diluting a culture from 9000 cells/ml to 1500 cells/ml. The size distribution

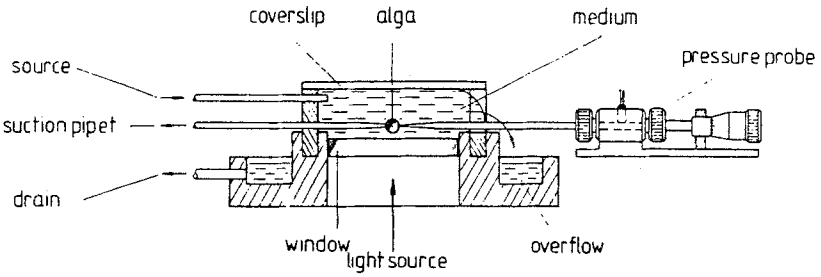

Fig. 2. Pressure probe and measurement chamber with suction pipette for the determination of water transport parameters of *E. viridis*

in the 3 days following the first cell division was determined by measuring the cell dimensions under the microscope and calculating the cell volumes of 120 to 150 cells. The maximum error for the volume determination was only 13%, i.e., fairly small compared to geometrically irregular cells.

Pressure Probe Technique

Turgor pressure and water relation parameters were measured by means of the pressure probe (Zimmermann, Räde & Steudle, 1969). The cell was fixed at the tip of a suction pipette (Fig. 2). In spite of the very small cell volume in relation to the volume of the pressure probe (1 : 10,000), it was possible to take measurements in these small cells because the position of the meniscus in the capillary tip was chosen as the reference point. In this way, the compressible volume of the apparatus was limited to that of the capillary tip. Turgor pressure changes were hydrostatically induced by displacement of the oil/cell sap boundary using the metal rod mounted in the pressure probe. A more detailed account of the technique is provided by Hüsken, Steudle and Zimmermann (1978). The ratio of the insertion area to the total surface was well below the critical value above which correct measurements of the water relation parameters are not possible because of leakage (Zimmermann & Hüsken, 1979). We calculated the internal osmotic pressure (Π_i) from the measured turgor pressure (P) and the cryoscopically determined osmotic pressure of the external medium (Π_o) by assuming that the reflection coefficients of all solutes are equal to unity:

$$\Pi_i = P + \Pi_o. \tag{1}$$

In the experiments on osmoregulation, at least 10 cells were measured and averaged for each turgor pressure determination. The osmotic pressure of the media was determined cryoscopically with an Osmomat 030 (Gonotec, Berlin, FRG). The volumetric elastic modulus ε, an inverse measure of the elasticity of the cell wall, is defined as follows (Philip, 1958):

$$\varepsilon = V \cdot dP/dV \tag{2}$$

where dV is a small volume change and dP the resulting small pressure change. dP is measured directly with the pressure probe, and dV is determined under the microscope from the advance of the meniscus. In order to determine ε at a different pressure, the osmolarity of the external medium was increased in stages by the addition of sorbitol. The pressure relaxations are described by exponential functions. The half-times for water exchange between cells and environment ($T_{1/2}$) were calculated from the gradient, k, of the semi-logarithmic plots of the pressure relaxations

$$T_{1/2} = \ln 2/k. \tag{3}$$

From $T_{1/2}$ it is possible to calculate the hydraulic conductivity (Lp) (Dainty, 1963, 1976; Zimmermann & Steudle, 1978)

$$Lp = V \cdot \ln 2/A \cdot T_{1/2}(\varepsilon + \Pi_i). \tag{4}$$

V = cell volume; A = cell surface area; ε = volumetric elastic modulus; Π_i = osmotic pressure of the cell sap. Lp values were calculated exclusively from hydrostatically induced water flows.

Electron Microscopy

The algae were fixed for 2 hr at room temperature in 4% glutaraldehyde and then for 2 hr in 2% osmium tetroxide at 4°C, in both cases in 0.1 M phosphate buffer. The samples were dehydrated with alcohol and embedded in Spurr's medium (1969). After staining with uranyl acetate and lead citrate (Reynolds, 1963), thin sections were cut and examined under a JEM 100 C electron microscope (Jeol, Japan).

Preparation of Cell Extracts and Analyses

In order to produce cell extracts for ion analysis the algae were washed five times in double distilled water, shock frozen with liquid N_2, and then thawed (Munns et al., 1983). The product was agitated and then centrifuged 10 min at 4000 × g. If the cell extract was destined to be analyzed for sugar content, each sample was first treated for a few minutes with a Potter homogenizer. It was found that the extraction of cellular contents could be improved by heating the cells to 100°C before homogenization. Potassium and sodium were determined with a flame photometer (M20, Zeiss, Oberkochem, FRG) and chloride with a chloride titrator (American Instrument Co., Silver Spring, Md.) For the determination of amino acids, an amino acid analyzer LC 2000 (Biotronik, Maintal-Dörnigheim, FRG) was used, and for organic acids a Tachophor (LKB, München, FRG). The sugars were analyzed by the sucrose and glucose test marketed by Boehringer (Mannheim, FRG). O_2 evolution during photosynthesis was measured with an oxygen electrode (Oxigraph EO 40, Gilson Medical Electronics, Kiddleton, Wisc.) in medium containing 0.5 mM $KHCO_3$ and under illumination of a slide projector (Leitz, Wetzlar, FRG). The nitrate and sulfate content were determined photometrically (Lange & Vejdelek, 1980). Chlorophyll content was determined by the method of Nusch and Palme (1975).

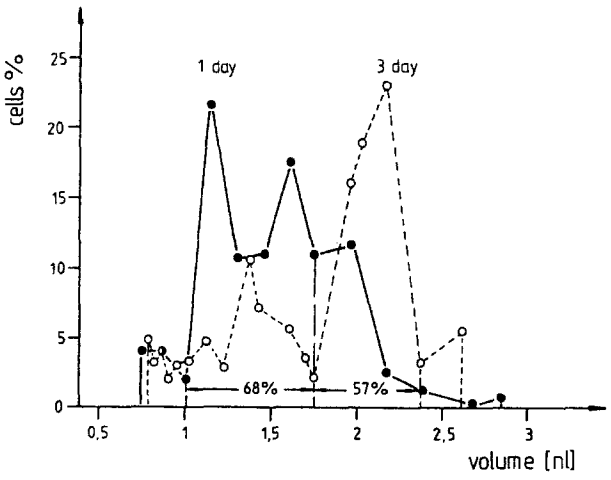

Fig. 3. Size distribution of a 63% synchronous culture of *E. viridis* on the first and third day of the cell cycle. The size distribution of the entire population in medium I on the first and third day of the development is shown. The curve for the first day exhibits two distinct maxima. The first maximum was attributable to the autospores. These arise by quaternary division of cells and are therefore smaller than those cells resulting from binary division which give rise to the second maximum. The lateral shoulder of the second maximum was caused by the nonsynchronized cells which were older than one day and therefore larger. The planimetrically determined area below the maxima represents 68 and 57% of the total

Results

Volume/Age Relationship

To arrive at a clear-cut interpretation of the data on a cell population of broad size and age distribution it is important to reveal the cell volume/age relationship. Such measurements are required because osmoregulatory parameters can depend on volume and/or age which need not necessarily be correlated. For this purpose the size distribution of a culture that was 63% synchronous was determined during a three-day cell cycle. In this experiment a total of 392 cells were measured. Figure 3 shows the size distribution of the entire population on the first and third day of the developmental cycle following partial synchronization. The curve for the first day has two distinct maxima, with the second maximum also exhibiting a shoulder. The first maximum was attributable to the autospores. These arise by quaternary division of a cell and are therefore necessarily smaller than those cells resulting from binary division; the latter gave rise to the second maximum. The shoulder of the second maximum is caused by the nonsynchronized cells which were older than one day and therefore larger. The planimetrically determined area below the two main maxima, not taking into account the shoulder of the second maximum, represents 68% of the total. This value is in the same range as the proportion of synchronized cells, which was determined by counting to be 63%. The volume distribution of the third day of the cell cycle exhibits a distinct shift in the main maximum, which accounted for 57% of the total area. The second, smaller maximum could be attributed to the asynchronous cells. It appears from the above, that to a good approach the volumes of the cells can be correlated with their ages. This had the important consequence that the following investigations could be carried out on asynchronous cultures because differences in volume could be interpreted as being due to differences in age.

Water Relation Parameters

The turgor pressure of *Eremosphaera viridis* was 6–12 bar immediately after introduction of the probe and remained constant over several hours. Measurements from cells of different sizes (i.e., different ages) showed that there was obviously no correlation between cell volume and cell turgor pressure. However, turgor pressure was influenced by the composition of the media in which the cells were cultured. Cells cultured in medium I containing neither Na^+ nor Ca^{2+} ions exhibited the lowest steady-state turgor pressure values with a mean of 7.6 bar. Cells cultured in Ca^{2+}-enriched medium I or in medium II (containing both Na^+ and Ca^{2+} ions) exhibited turgor pressures that were about 2 bar higher (9.3 and 9.6 bar, respectively).

The increase of turgor pressures due to these culture media corresponded to an increase in the values of the volumetric elastic moduli. The ε values for cells from medium I were 203 ± 93 bar (22 measurements); from the same medium with calcium, 244 ± 75 bar (21 measurements); and from the Na-containing medium II, 355 ± 107 bar (33 measurements). The values show that the presence of sodium in the culture medium has a pronounced influence on the elasticity of the cell wall. Following the progressive reduction in turgor pressure after the addition of sorbitol to the external medium and the establishment of the new quasi-stationary turgor pressure, it was found that ε is pressure dependent, as is the case in giant algae and in some higher plant cells (*cf.* Zimmermann, 1978). In the pressure range of about 0.5 to 5 bar there is a direct proportionality to turgor pressure, whereas above about 6 bar ε seems to be independent of pressure. Two typical curves are shown in Fig. 4. By replacing the initial external medium it was possible to bring the cells back to their original pressure within seconds, so

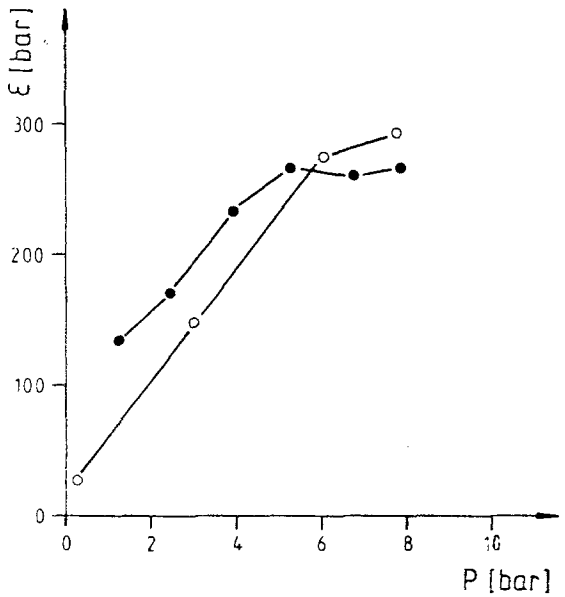

Fig. 4. Dependence of the volumetric elastic modulus (ε) on turgor pressure (P) in a single cell of *E. viridis*; (cell volume: 1.15 nl; cell surface area: 53×10^{-3} mm²). The turgor pressure of the cells was lowered by adding appropriate amounts of sorbitol to the medium

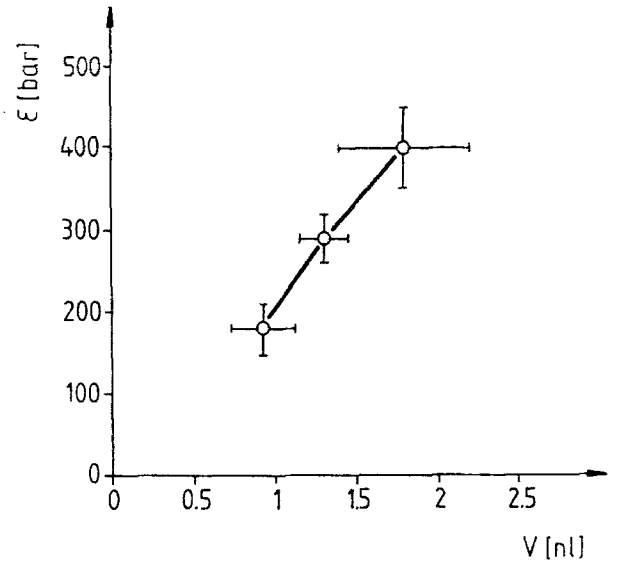

Fig. 5. Dependence of the volumetric elastic modulus (ε) on the volume (V) of cells of *E. viridis*. For the evaluation of the data a total of 18 cells have been investigated

Table 2. Half-time of water transport ($T_{1/2}$) and hydraulic conductivity (Lp) of *E. viridis* in different media

Medium	$T_{1/2}$ (sec)	Lp ($\times 10^{-6}$ cm · sec^{-1} · bar^{-1})
I	0.84 ± 0.17 (16)	7.34 ± 0.23 (15)
I + Ca	0.50 ± 0.11 (9)	6.16 ± 2.30 (9)
II	1.23 ± 0.36 (17)	3.72 ± 1.84 (17)

that irreversible changes in the cells due to a reduction in turgor pressure could be excluded. It is interesting that ε is also dependent on cell volume or age (Fig. 5). When the volume of cells cultured in the same medium doubled in size, the value for ε increased from 200 to 400 bar measured at constant turgor pressure. Investigations with the electron microscope showed (Fig. 6) that the cell wall of old cells had more layers than those of younger cells (e.g. a mother cell wall had 13 layers with a mean thickness of 0.5 μm whereas the cell wall of one of its autospores had only 5 layers and was 0.2 μm thick).

As can be seen from Table 2, $T_{1/2}$ and Lp are strongly correlated with the composition of the culture media. In the presence of sodium, which is contained in medium II, Lp decreases significantly. Experiments carried out at the initial turgor pressure, showed no significant differences between endosmotic and exosmotic $T_{1/2}$ or Lp (t test: confidence interval of >0.95 for 16 measurements). This means there is no polarity of water transport such as has been observed in other plants [e.g. *Chara australis* (Kiyosawa & Tazawa, 1977)]. No correlation of Lp with turgor pressure or with the osmotic concentration of the external solution could be found (Fig. 7) when the turgor pressure was reduced by addition of sorbitol.

Osmoregulatory Response

In osmoregulatory systems the rapid water transport phase is followed—after a quasi-stationary turgor pressure has been established—by a second phase. In this phase the original turgor pressure is nearly restored by changes of the internal osmotic pressure, independent of the nature of the osmoticum used in the external medium. Adaptation of internal osmotic pressure and turgor pressure during this very slow phase is normally achieved by transport of ions and/or by intracellular build-up or breakdown of osmotically active organic compounds.

To this end, cells of *E. viridis* were subjected to solutions of increasing concentrations of NaCl which had all been brought to the same total osmotic value with sorbitol (Table 3). After a rapid decline in turgor pressure due to the water transport phase a subsequent increase in cell turgor pressure was observed, depending on the external NaCl concentration. This increase in turgor pressure had a

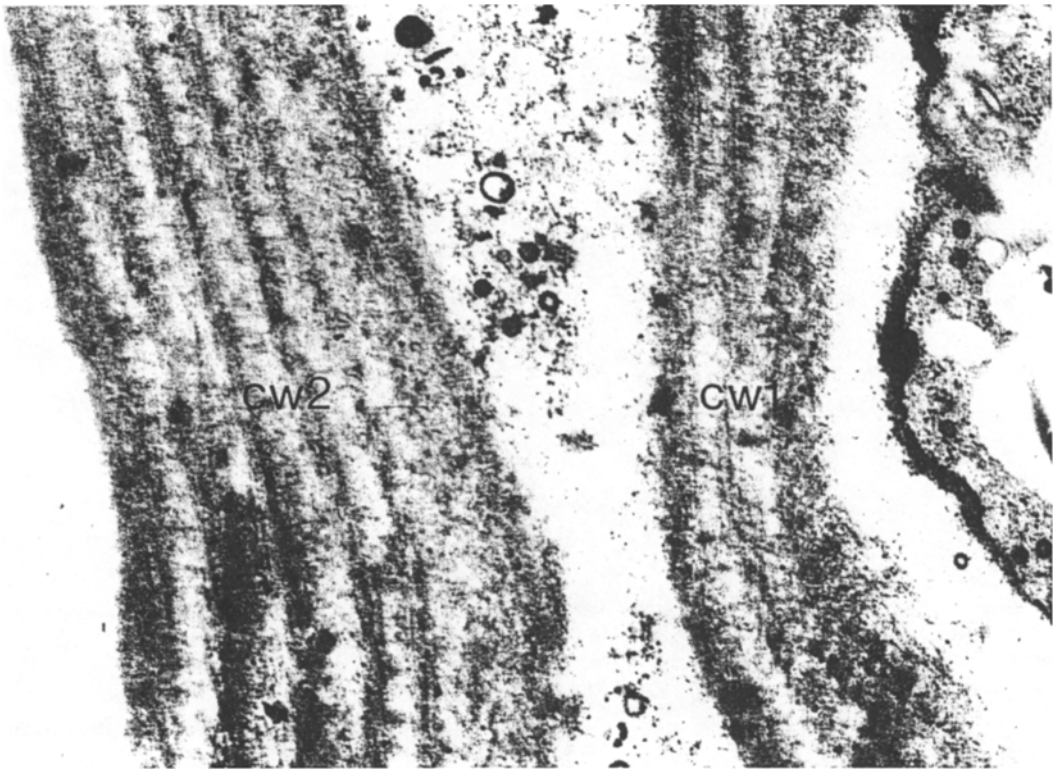

Fig. 6. Electron micrograph of a cell wall of an autospore (*cw*1) and of a mother cell (*cw*2) of *E. viridis*. Note that the mother cell wall has 13 layers with a mean thickness of 0.5 μm whereas the cell wall of one of its autospores has only 5 layers. Magnification: 60,000×

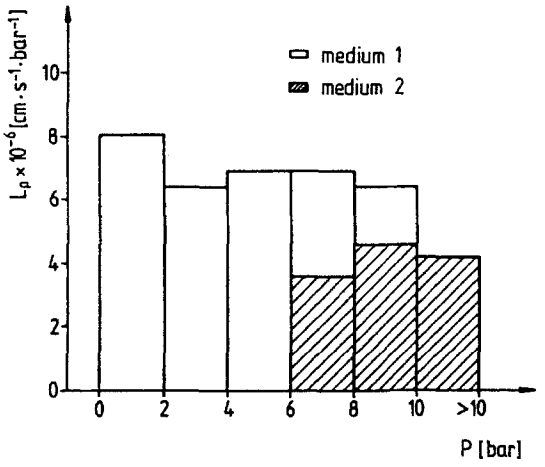

Fig. 7. Dependence of the hydraulic conductivity (*Lp*) of the cell membrane of *E. viridis* on turgor pressure (*P*). As in Fig. 5 the turgor pressure had been decreased by addition of sorbitol to the medium. The shaded area shows values measured on algae that had grown in medium II; the unshaded area shows values of algae from medium I

time constant of some hours, but did not reach the initial turgor pressure value within 25 hr. However, when isosmotic concentrations of sorbitol or sucrose were added in the absence of ions, no such rise in turgor pressure was detected (Table 3). The quasi-stationary turgor pressure (P_q), which was established within a few seconds at the end of the water transport phase, remained nearly constant over the entire measuring period of 48 hr. A concentration-dependent increase in turgor pressure was also observed when various concentrations of salts were added to the external medium without adjusting the osmotic pressure of the solutions to the same value with sorbitol.

Experiments in addition to those with NaCl

Table 3. Turgor pressure of *E. viridis* before (P), at the beginning (P_q) and after 25 hr incubation (P_{25}) in media of the same osmotic pressure but different NaCl concentrations[a]

Medium (mM)	P (bar)	P_q (bar)	P_{25} (bar)
125 NaCl	8.6 ± 0.8	3.5 ± 1.4	5.8 ± 1.0
75 NaCl +93 Sorbit	8.6 ± 0.8	3.5 ± 1.4	5.2 ± 1.1
25 NaCl +188 Sorbit	8.6 ± 0.8	3.5 ± 1.4	3.1 ± 1.4
230 Sorbit	8.6 ± 0.8	3.5 ± 1.4	1.2 ± 1.0

[a] Each value represents 10 measurements.

Table 4. Initial turgor pressure (P_0), turgor pressure at the end of the water transport phase (P_q), turgor pressure after 24 hr salt-incubation (P_{24}) and sugar content of the algae at 30°[a]

Salt	Conc. (mM)	P_0 (bar)	P_q (bar)	P_{24} (bar)	P increase (bar)	Sucr. (mM)	Sucr. (mM)	Gluc (mM)	Gluc (mM)
NaCl	0	9.2	9.2	9.2	—	5	—	7	—
	25	9.2	8.1	9.2	1.1	9	4	8	1
	75	9.2	6.0	7.9	1.9	15	10	8	1
	125	9.2	3.5	6.7	3.2	23	18	13	6
	175	9.2	1.3	5.4	4.1	19	14	14	7
D:	125	9.2	3.5	5.5	2.0	14	9	8	1
LiCl	0	8.6	8.6	8.6	—	7	—	1	—
	25	8.6	7.5	7.9	0.4	13	6	1	0
	75	8.6	5.4	7.9	2.5	22	15	5	4
	125	8.6	2.9	6.3	3.4	27	20	3	2
	175	8.6	0.7	4.7	4.0	29	22	1	0
D:	125	8.6	2.9	5.7	2.8	22	15	0	0
KCl	0	8.7	8.7	8.7	—	3	—	3	—
	25	8.7	7.6	8.5	0.9	9	6	4	1
	75	8.7	5.5	7.4	1.9	14	11	9	6
	125	8.7	3.0	5.6	2.6	23	20	13	10
	175	8.7	0.8	7.3	8.1	35	32	21	18
D:	125	8.7	3.0	3.6	0.6	23	20	4	1
RbCl	0	8.6	8.6	8.6	—	7	—	4	—
	25	8.6	7.5	7.2	0	10	3	4	0
	75	8.6	5.4	8.0	2.6	15	8	5	1
	125	8.6	2.9	10.3	7.4	29	22	8	4
	175	8.6	0.7	5.9	5.2	36	29	17	13
	225	8.6	−1.6	4.4	6.0	31	24	25	21
D:	125	8.6	2.9	4.9	2.0	13	6	2	0
CsCl	0	9.0	9.0	9.0	0	16	—	6	—
	25	9.0	7.9	8.1	0.2	18	2	5	0
	75	9.0	5.6	6.7	1.1	28	12	3	0
	175	9.0	1.1	3.1	2.0	41	25	0	0
$CaCl_2$	0	10.6	10.6	10.6	0	5	—	5	—
	10	10.6	9.4	10.8	1.4	29	24	14	9
	50	10.6	7.2	9.4	2.2	31	26	21	16
	100	10.6	3.8	5.2	1.4	38	33	19	14
D:	50	10.6	7.2	8.2	1.0	—	—	—	—

[a] The lines preceded by *D:* give the values of the measurements in the dark.

were also performed with increasing concentrations of LiCl, KCl, RbCl, CsCl, and $CaCl_2$. These also brought about a concentration-dependent increase in turgor pressure after the establishment of the quasi-stationary turgor pressure, P_q (Table 4). The addition of KCl and RbCl resulted in final pressures, 25 hr after the end of the water transport phase, which were very close to the original pressure or even exceeded it. In the experiments with high NaCl and LiCl concentrations the original turgor pressure was not re-established within this period of time, although the internal osmotic pressure increased again by about 3 bar. The same applies to CsCl and $CaCl_2$, which both induced an increase in the internal osmotic pressure of about 2 bar. We can assume that the original turgor pressure may be completely re-established after more than 24 hr in the case of higher salt concentrations. However, it was not possible to demonstrate this experimentally because the algae became progressively more damaged with increasing salt concentration so that an insufficient number of cells were available for the experiments.

If the cells were incubated in Na_2SO_4, the turgor pressure no longer changed after the end of the water transport phase, which suggests that Na_2SO_4 does not enter the cell.

The increase in turgor pressure observed in *E. viridis* may be due to a metabolically dependent uptake of ions and/or to a metabolically dependent synthesis of osmotically active substances, which is triggered by ions but not by nonelectrolytes (sug-

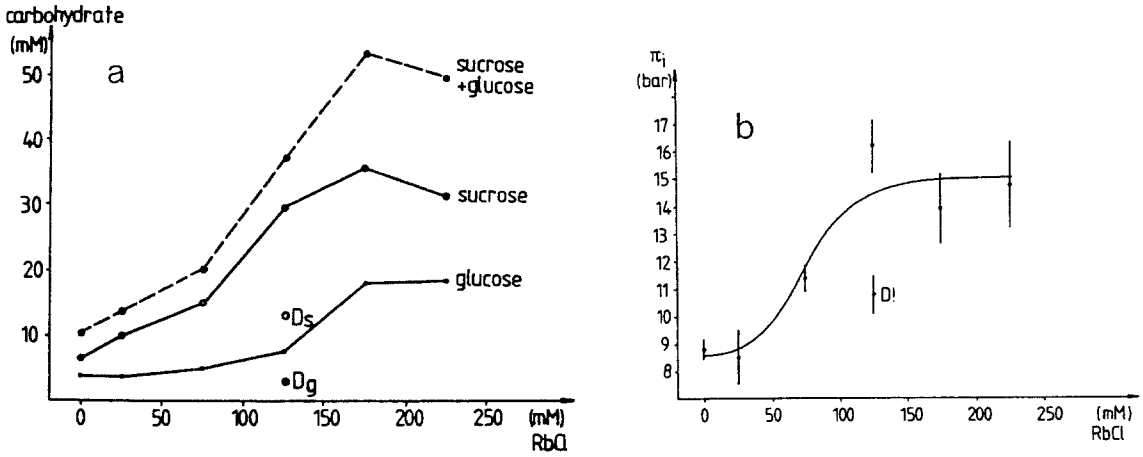

Fig. 8. (*a*) Dependence of the intracellular sugar concentration on the RbCl concentration in the medium. Cells had been incubated for 24 hr prior to determination of the different sugars. The dashed line represents the numeric addition of sucrose and glucose concentrations. The dots denoted by *D* give the concentrations of glucose (*Dg*) and sucrose (*Ds*) after incubation at 125 mM RbCl in the dark. (*b*) Dependence of the internal osmotic pressure (as calculated from the turgor pressure and the osmolarity of the external medium) on the RbCl concentration. At low concentrations there is a linear correlation between the internal osmotic pressure and external RbCl concentration. Above ca. 100 mM RbCl the internal osmotic pressure reaches a saturation value of about 15 bar. The value denoted by *D!* represents the internal osmotic pressure in the dark. Each value (mean ± SD) represents 10 measurements

ars). For this reason the Na^+-, K^+ and Cl^- contents of the algae were determined under various conditions of osmotic stress.

Under normal culture conditions the internal K^+ concentration was 90–110 mM. If the algae were incubated in 125 mM KCl, the internal K^+ concentration increased by a maximum of 18% at high external K^+ concentrations. This corresponds to an osmotic pressure increase of about 0.5 bar, i.e., the K^+ concentration remained relatively constant. The Na^+ content increased from 2 to a maximum of 40 mM with increasing external NaCl concentration up to 125 mM. This corresponds to an increase in the internal osmotic pressure of almost 1 bar, which does not explain the observed increase in the internal osmotic pressure.

The Cl^- content of the cells was normally 1–5 mM. With increasing external NaCl concentration, the chloride content of the cells increased to a maximum of 10 mM. No changes were observed in the nitrate, sulphate and phosphate contents of NaCl-treated algae and their controls.

These results show that the increase in turgor pressure after the water transport phase under conditions of salt stress can only partly be explained by the uptake of ions, and that the production of an osmotically active organic substance has to be considered as a possibility. An examination of cell extracts from algae incubated in the presence of various concentrations of NaCl and their controls revealed no differences in the contents of amino organic acids after 24 hr. The presence of SH-derivatives could also be excluded. Analysis (trimethyl derivation followed by gas chromatography) showed that the production of glycerol could be ruled out. The occurrence of isofloridosides would also have been detected by gas chromatography. (They would have split into glycerol and sugar after being heated in HCl.)

In the algae treated with chloride salts, on the other hand, there was a marked increase in the sugar content. It was found that cells treated with LiCl, NaCl and $CaCl_2$ formed sucrose almost exclusively and only very little, if any, glucose, while the KCl–, RbCl– and CsCl-incubated cells showed a marked accumulation of glucose in addition to sucrose, provided that light had been supplied (Table 4). The sugar content could be related qualitatively to the increase in the internal osmotic pressure (Fig. 8*a*,*b*). It was not possible to establish a quantitative relationship between sugar accumulation and the increase in turgor pressure measured with the pressure probe. If we take into account that the nonosmotic volume of the cells is of the order of 25% (as calculated from the geometrical dimensions of a median cross section of the alga) an extraction rate of about 78 ± 13% can be estimated.

In order to provide further evidence for the assumption that there is metabolic involvement in the observed reactions of the algae to osmotic stress, experiments were carried out at different temperatures and at the same external NaCl concentrations (Fig. 9). Surprisingly, it was found that the turgor recovery after 24 hr was significantly more pronounced at low temperature (10°C) than at 30°C. This reaction cannot be attributed to different inter-

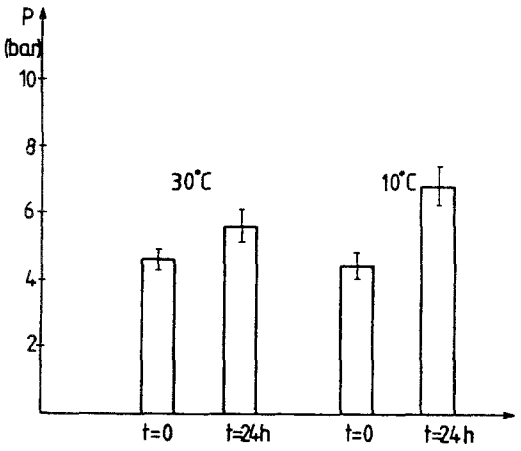

Fig. 9. Determination of the turgor pressure of *E. viridis* at the beginning ($t = 0$) and after 24 hr ($t = 24$) of NaCl incubation (125 mM) at temperatures of 10 and 30°C, respectively. Values (mean ± SD) were calculated from at least 10 different measurements

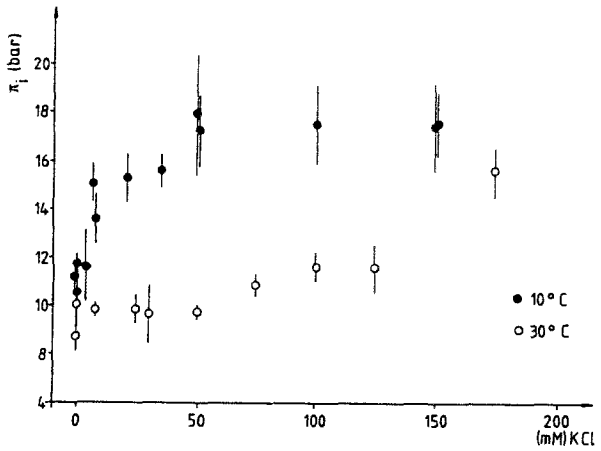

Fig. 10. Dependence of the turgor pressure of *E. viridis* on increasing KCl concentration of the incubation medium. Cells have been investigated at temperatures of 10°C (●) and 30°C (○), respectively. The mean value of 10 measurements on different cells has been determined at a given KCl concentration to evaluate the curve

nal ion concentrations since the ionic content remained constant at 10 and 30°C. After incubation with KCl at 10°C the algae exhibited in the second regulation phase a marked increase in internal osmotic pressure of about 7 bar as calculated from the turgor pressure (Fig. 10) even at low KCl concentrations (15–20 mM). At these concentrations, turgor pressure was found to have a linear relation with the external KCl concentration, which was not the case above 50 mM. At 30°C the turgor response was quite different: at low concentrations the increase in pressure was very small, increasing more when the external concentration exceeded 100 mM KCl. The proportion of internal sucrose and glucose concentrations behaved in a corresponding manner. When the cells were incubated with NaCl, on the other hand, the internal osmotic pressure increased gradually with the external concentration both at 10 and 30°C.

In order to elucidate the process involved in supplying energy for the production of sugars, the photosynthetic rate of oxygen evolution was measured during incubation with increasing concentrations of KCl at 10 and 30°C. It was found that photosynthetic production of oxygen correlated well with the pressure curves. At 10°C it increased rapidly even at low concentrations, whereas at 30°C an increase in oxygen production was only observed above an external KCl concentration of 100 mM (Fig. 11*a,b*). The respiration remained unchanged.

Discussion

The results presented here show that in comparison with other fresh-water algae, *Eremosphaera viridis* exhibits significant differences in its osmotic pressure regulation while at the same time sharing many of the fundamental regulatory processes. As could be further shown by electron microscopy, the rise in ε with increasing volume could be related to the progressive increase in the number of cell wall layers. However, we cannot exclude the possible influence of the so-called "stress-hardening effect" (Steudle, 1980). In spite of the very wide range of cell volumes, the absolute values for ε of about 200 to 400 bar at normal turgor pressure are in the same range as for most fresh-water algae. In the pressure range under investigation, reported values vary between 22 and 496 bar. Gerdenitsch (1979) found similar ε values of 82 to 499 bar for *E. viridis* on the basis of pressure volume curves. These were also measured at reduced turgor, but without taking into account the pressure dependence of ε. In the studies presented here, measurements of ε at different turgor pressure values show that it is strongly pressure dependent below 5 bar. It is also interesting to note that ε measured at a given volume and turgor was changed significantly when the growth medium was altered. The media differed in their Na^+ and K^+ content and slightly in their salinity (2 or 5 mM). The addition of Na^+ to the medium led to the formation of a thick layer of mucus around the cells, which indicates a change in metabolic activity. Investigations showed that, as in the case of *Halicystis parvula* and *Acetabularia mediterranea* (Büchner & Zimmermann, 1982), calcium ions have no causal effect on the elasticity of the cell wall. The increase in the absolute value of ε in *E. viridis* can therefore be attributed to the specific effect of low NaCl con-

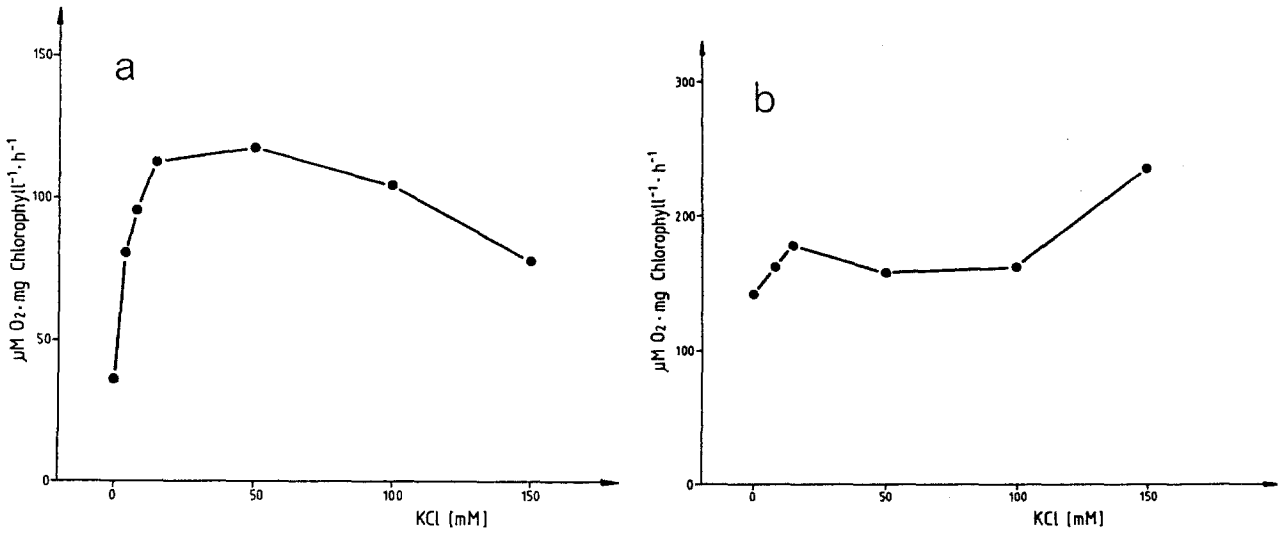

Fig. 11. Photosynthetic O_2 production of *E. viridis* after 24 hr incubation in media with increasing KCl concentration at different temperatures (*a*: 10°C; *b*: 30°C). It should be noted that the oxygen release occurs in parallel with the internal osmotic pressure of the cells (*see* Fig. 10)

centrations. Investigations have shown that Na^+ ions are to some extent taken up by the cell and that cells grown in media containing NaCl exhibit, on average, a 50% increase in volume, which is probably due to the prolonged generation time. With a value of 2 to 12 $\times$ 10^{-6} · cm · sec^{-1} · bar^{-1} the hydraulic conductivity is in the same range as that of *Lamprothamnium* [4 to 19 $\times$ 10^{-6} · cm · sec^{-1} · bar^{-1} (Wendler & Zimmermann, 1985)]. Although the half-time for water exchange is very short (0.5 to 1.5 sec), *Lp* is relatively low. The reason for this is the small surface area/volume ratio of this spherical alga compared to that of the cylindrical internodal cells of the *Characeans*. It could be further shown for the first time that the water permeability of the cell membrane may be changed by the culture conditions. NaCl reduces the value for *Lp* by a factor 2. These results demonstrate that the water transport parameters of these algae will change significantly even under minor salt stress. *E. viridis* is capable of re-establishing near-normal turgor pressure within 24 hr after water loss when the osmotic pressure of the medium is changed by salts (Table 4). The addition of equivalent amounts of nonelectrolytes did not lead to a renewed increase in the internal osmotic pressure after the initial rapid loss of water, at least not within the 24 to 48 hr period of investigation. The absence of a biphasic turgor response to changes in the external osmotic pressure in the presence of nonelectrolytes excludes a true osmoregulation process. Tazawa (1961) reports that in *N. flexilis* the regulation of the vacuolar osmotic pressure is induced only by the addition of ions to the external medium. However, in this alga the osmotic pressure is predominantly regulated by the transport of ions (mainly K ions) and not by the synthesis of low molecular weight substances as in *E. viridis*. Despite this these two algae show the same ion specificity in their osmotic response. As with *E. viridis* KCl is more effective for the re-establishment of the original turgor pressure than NaCl or $CaCl_2$, which has no effect at all in *N. flexilis*. In the presence of KCl both *N. flexilis* and *E. viridis* may under certain conditions exhibit an over-reaction, i.e., the original turgor pressure is exceeded. The dependence of the osmotic reaction on the concentration of the specific salt is also characteristic of the two algae. In both species salt-induced osmoregulation was also found to be light-dependent. It is interesting to note that a difference in the behavior of illuminated or nonilluminated *E. viridis* cells was only found in the case of KCl, RbCl or $CaCl_2$ addition. The salts NaCl, LiCl and CsCl, on the other hand, only caused a minor light effect on the internal osmotic pressure. In *Nitella* a light-induced increase in the internal osmotic pressure in the presence of KCl has been observed by Tazawa (1961). In Tazawa's work Li^+, Rb^+ and Cs^+ salts were not investigated.

Incubation in salt solution induced the accumulation of sucrose in *E. viridis* irrespective of any illumination. Müller and Wegmann (1978) reported the same behavior from *Dunaliella tertiolecta*. The sucrose is a secondary product of the starch breakdown. Under salt stress the starch grains in *E. viridis* became smaller (Fig. 12*a* and *b*). Breakdown

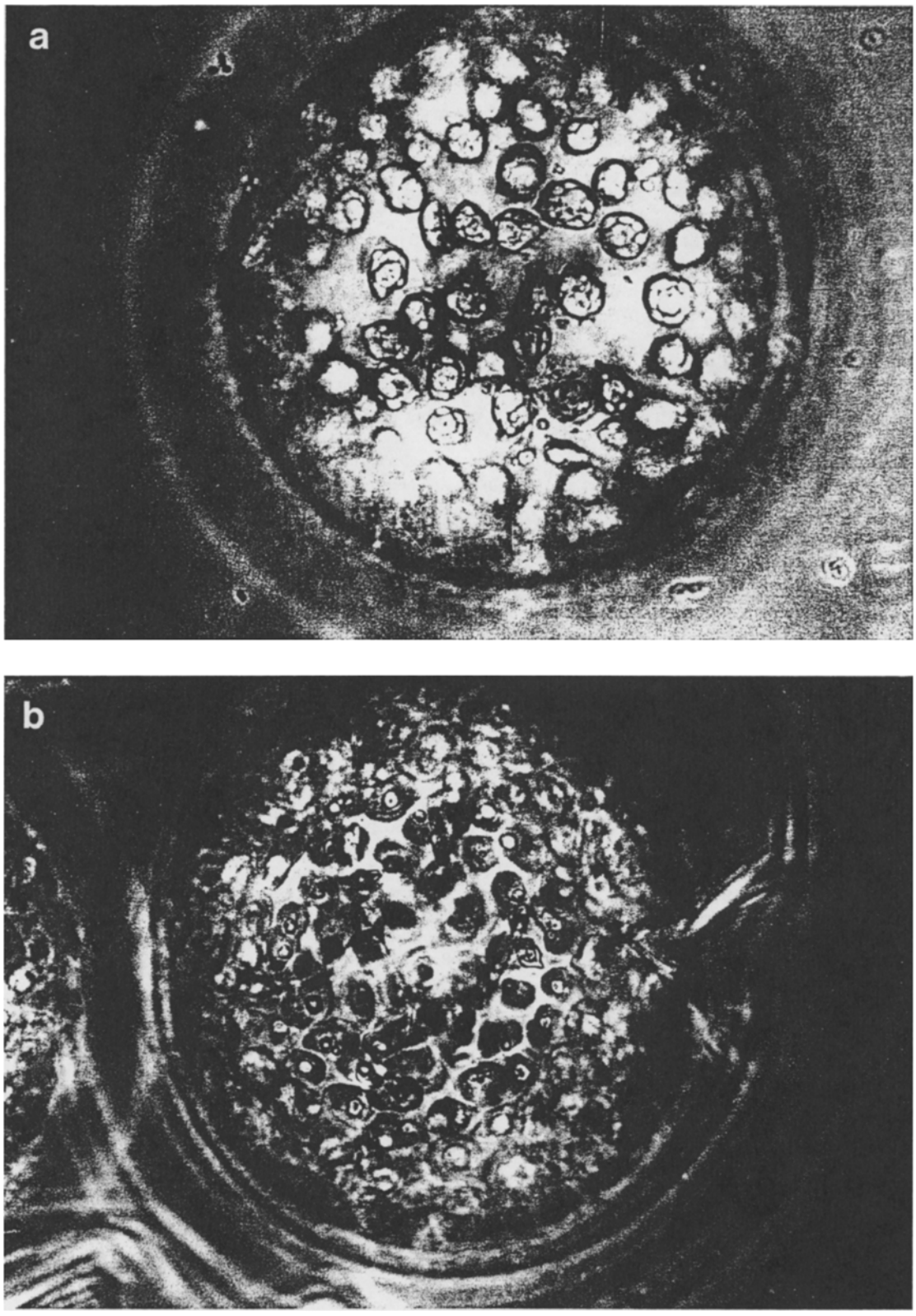

Fig. 12. Phase contrast light micrograph of chloroplasts of *E. viridis*. As can be seen, the chloroplasts are extended by large starch grains when the cells had been grown in the light under normal culture conditions (*a*). However, the starch grains in the chloroplasts are markedly smaller after incubation in NaCl under the same light conditions (*b*)

of starch could occur via the maltose catalyzed by β-amylases. This could explain why the level of glucose in the dark was always very low under conditions of stress. The glucose measured in *E. viridis* was accumulated only in conditions of light and under normal culture conditions stored as starch or used as an energy source. An enzyme in the starch breakdown pathway is α-glucan-phosphorylase (Steup & Melkonian, 1981). This enzyme cannot use maltose as a substrate. Therefore synthesis of starch from glucose and breakdown of starch which finally leads to sucrose formation are completely separated. Our investigations show that glucose accumulation is only triggered by Rb^+, K^+ and Ca^{2+}, whereas Na^+ has little effect and Li^+ and Cs^+ none at all. Usually Rb^+ can substitute for K^+ as an enzyme activator, whereas Na^+ shows only reduced activation and Li^+ none at all. This pattern of ionic effects seems to be valid for the results presented here, in which K^+ and Rb^+ show responses that are not elicited by Na^+ and Li^+. If it is assumed that the binding of ions to enzymes depends on their hydratation enthalpy, one should expect that the binding properties of Cs^+ are similar to those of K^+ or Rb^+. However, our results suggest a mechanism similar to that described by Benz (1978) for the binding of ions to ion carriers. According to this mechanism, the selectivity of the binding is determined by the selectivity of the binding port. In the case reported here Rb^+ seems to have the highest selectivity. Lowering the temperature to 10°C seems to increase the affinity of the ions to the enzyme, as also reported by Benz and Läuger (1976) for the binding of alkali ions to valinomycine.

At 30°C the osmoregulatory activity of the cells increases with increasing salt concentration. However, Tables 3 and 4 show that the cells do not react to changes in the osmolarity but rather to changes in the concentration of the specific ions in the media. Each of the four solutions used in the experiment had the same osmolarity but the reaction of the cells was different and clearly only dependent on the ionic content. In other words, the formation of osmotically active substances in *E. viridis* is not due to common sense osmoregulatory activity but results from changes in the ionic content of the external medium. Such ionic effects are also observed in the photosynthetic processes of the alga. Measurements of photosynthetic O_2 production as a function of the external K^+ concentration revealed a weak dependence at 30°C, whereas at 10°C a marked increase was observed even at low K^+ concentrations. We summarize by stating that, for a fresh-water algae, *E. viridis* exhibits a remarkable tolerance to salt in experiments, although the natural biotope of this alga has only a very low salinity. The capacity for osmotic adaptation (maintenance of turgor pressure or volume) must therefore be an inherent property of cells. This ability must be closely linked with specific membrane transport systems and intracellular enzymes required *per se* for the maintenance of cellular and membrane functions. *E. viridis* is a most suitable cell for investigations of this effect (as pointed out in the introduction). The elucidation of the signal-response sequence in this model system should provide information applicable to the mechanisms of turgor pressure (or volume) regulation in other algae and plant cells. An interesting starting point for further investigations could be the long-term behavior of the osmoregulatory processes as well as ion-specific interactions during the activation of regulatory processes and their kinetics.

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References

Arnold, W.M., Wendt, B., Zimmermann, U., Korenstein, R. 1985. Rotation of a single swollen thylakoid vesicle in a rotating electric field. Electrical properties of the photosynthesic membrane and their modification by ionophores, lipophilic ions and pH. *Biochim. Biophys. Acta* **813**:117–131

Ben Amotz, A. 1974. Osmoregulation mechanism in the halophilic alga *Dunaliella parva*. *In:* Membrane transport in plants. U. Zimmermann and J. Dainty, editors. pp. 95–100. Springer-Verlag, Berlin

Benz, R. 1978. Alkali ion transport through lipid bilayer membranes mediated by enniatin A and B and beauvericin. *J. Membrane Biol.* **43**:367–394

Benz, R., Läuger, P. 1976. Kinetic analysis of carrier-mediated ion transport by the charge-pulse technique. *J. Membrane Biol.* **27**:171–191

Bisson, M.A., Kirst, G.O. 1980. *Lamprothamnium*, a euryhaline charophyte: I. Osmotic relations and membrane potential at steady state. *J. Exp. Bot.* **31**:1223–1235

Büchner, K.-H., Zimmermann, U. 1982. Water relations of immobilized giant algal cells. *Planta* **154**:318–325

Dainty, J. 1963. Water relations of plant cells. *Adv. Bot. Res.* **1**:279–328

Dainty, J. 1976. Water relations of plant cells. *In:* Encyclopedia of Plant Physiology, N.S. Vol. 2, Part A: Transport in Plants II. U. Lüttge and M.G. Pitman, editors. pp. 12–35. Springer-Verlag, Berlin

Flowers, T.J., Troke, P.F., Yeo, A.R. 1977. The mechanism of salt tolerance in halophytes. *Annu. Rev. Plant Physiol.* **28**:89–121

Gerdenitsch, W. 1979. Microscopic contributions to the pressure volume diagram of the cell water relations as demonstrated with single cells of *Eremosphaera viridis* and cell filaments of *Spirogyra spp. Protoplasma* **99**:79–98

Gutknecht, J., Hastings, D.F., Bisson, M.A. 1978. Ion transport and turgor pressure regulation in giant algal cells. *In:* Membrane Transport in Biology. Vol. 3, pp. 128–174. G. Giebisch, D.C. Tosteson, and H.H. Ussing, editors. Springer-Verlag, New York

Hellebust, J.A. 1976. Osmoregulation. *Annu. Rev. Plant Physiol.* **27**:485–505

Hüsken, D., Steudle, E., Zimmermann, U. 1978. Pressure probe technique for measuring water relations of higher plants. *Plant Physiol.* **61**:158–163

Kauss, H. 1967. Metabolism of isofloridoside (O-α-D-galactopyranosyl-(1 1)-glycerol) and osmotic balance in fresh water algae *Ochromonas*. *Nature (London)* **214**:1129–1130

Kauss, H. 1973. Turn over of galactosylglycerol and osmotic balance in *Ochromonas*. *Plant Physiol.* **52**:613–615

Kauss, H. 1977. Osmotic regulation in algae. *Prog. Phytochem.* **5**:1–27

Kirst, G.O. 1985. Osmotische Adaptation bei Algen. *Naturwissenschaften* **72**:125–132

Kiyosawa, K., Tazawa, M. 1977. Hydraulic conductivity of tonoplast-free *Chara* cells. *J. Membrane Biol.* **37**:157–166

Lange, B., Vejdelek, Z.J. 1980. Photometrische Analyse. Weinheim, Verlag Chemie, Deerfield Beach (Florida), Basel

Levitt, J. 1972. Responses of Plants to Environmental Stresses. Academic Press, New York

Müller, W., Wegmann, K. 1978. Sucrose biosynthesis in *Dunaliella:* I. Thermic and osmotic regulation. *Planta* **141**:155–158

Munns, R., Greenway, H., Setter, T.L., Kuo, J. 1983. Turgor pressure, volumetric elastic modulus, osmotic volume and ultrastructure of *Chlorella emersonii* grown at high and low external NaCl. *J. Exp. Bot.* **34**:144–166

Nusch, E.A., Palme, G. 1975. Biologische Methoden für die Praxis der Gewässeruntersuchung. *Gas- Wasserfach* **116**:562–565

Philip, J.R. 1958. Osmosis and diffusion in tissues: Half-times and internal gradients. *Plant Physiol.* **33**:275–278

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron opaque-stain in electron microscopy. *J. Cell Biol.* **17**:208–212

Schefczik, K., Simonis, W., Schiebe, M. 1983. Continuous registration of membrane input resistances of small plant cells using a double-pulse current clamp technique for single-electrode impalements. Comparison with the conventional two-electrode method. *Plant Physiol.* **72**:368–375

Schlösser, U.G. 1982. Sammlung von Algenkulturen. *Ber. Dtsch. Bot. Ges.* **95**:181–276

Smith, R.L., Bold, H.C. 1966. Physiological studies: VI. Investigations of the algal genera *Eremosphaera* and *Oocystis*. University of Texas Pub. 6612, Austin

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruc. Res.* **26**:31–43

Steudle, E. 1980. Effect of cell diameter and length on the overall elasticity of cylindrical plant cells. Significance for membrane transport and growth. *In:* Plant Membrane Transport. R.W. Spanswick, R.W. Lucas, and J. Dainty, editors. pp. 91–112. Elsevier/North-Holland, Amsterdam

Steudle, E., Zimmermann, U. 1971. Hydraulische Leitfähigkeit von *Valonia utricularis*. *Z. Naturforsch.* **26b**:1302–1311

Steup, M., Melkonian, M. 1981. α-1,4-glucan-phosphosphorylase EC-2.4.1.1. forms in the green alga *Eremosphaera viridis*. *Physiol. Plant.* **51**:343–348

Tazawa, M. 1961. Weitere Untersuchungen zur Osmoregulation der Nitella-Zelle. *Protoplasma* **53**:227–258

Wendler, S., Zimmermann, U. 1985. Determination of the hydraulic conductivity of *Lamprothamnium* by use of the pressure clamp. *Planta* **164**:241–245

Wyn-Jones, R.G. 1984. Phytochemical aspects of osmotic adaptation. *Rec. Adv. Phytochem.* **18**:55–78

Zimmermann, U. 1978. Physics of turgor- and osmoregulation. *Annu. Rev. Plant Physiol.* **29**:121–148

Zimmermann, U., Büchner, K.-H., Benz, R. 1981. Transport properties of mobile charges in algal membranes: Influence of pH on turgor pressure. *J. Membrane Biol.* **67**:183–197

Zimmermann, U., Hüsken, D. 1979. Theoretical and experimental exclusion of errors in the determination of the elasticity and water transport parameters of plant cells by the pressure probe technique. *Plant Physiol.* **64**:18–24

Zimmermann, U., Räde, H., Steudle, E. 1969. Kontinuierliche Druckmessung in Pflanzenzellen. *Naturwissenschaften* **56**:634

Zimmermann, U., Steudle, E. 1974. The pressure-dependence of the hydraulic conductivity the membrane resistance and membrane potential during turgor pressure regulation in *Valonia utricularis*. *J. Membrane Biol.* **16**:331–352

Zimmermann, U., Steudle, E. 1978. Physical aspects of water relations of plant cells. *Adv. Bot. Res.* **6**:45–117

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