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Osmotic Regulation in the Marine Alga, *Codium decorticatum*

II. Active Chloride Influx Exerts Negative Feedback Control on the Turgor Pressure

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Summary. *Codium decorticatum* maintains its internal osmotic pressure 2.3 bar above the osmotic pressure of the external medium, which results in a hydrostatic pressure difference (turgor) of 2.3 bar. During hypoosmotic stress the primary mechanism for decreasing the internal osmotic pressure is a decrease in active Cl^- influx. The decreased Cl^- influx is accompanied by a 6.9 mV depolarization of the negative vacuole potential, while there is no consistent change in the protoplast resistance. The net Cl^- efflux which results is balanced primarily by a passive net loss of K^+. During hyperosmotic stress the Cl^- influx increases, accompanied by a slight (2.6 mV) hyperpolarization and a decrease of 90 Ω cm^2 in the membrane resistance. A passive net uptake of Na^+ accompanies the net active influx of Cl^-. Following the restoration of normal turgor, which requires about 2 hr, concentrations of Na^+ and K^+ are slowly readjusted over a period of about 30 hr until the normal K^+/Na^+ ratio of 1.0 is restored. *Codium* regulates its turgor as rapidly in the dark as in the light, which suggests that the Cl^- pump is not dependent directly on photosynthesis for its energy supply.

Turgor pressure, i.e., the hydrostatic pressure gradient across the plant cell wall and plasma membrane, is generated by water flowing down an osmotic gradient into a cell which maintains its internal osmotic pressure (Π^i) above that of the surrounding solution. When the external osmotic pressure (Π^o) varies, cells must respond by altering Π^i or suffer a change in turgor, volume, or both.

Codium decorticatum is an estuarine alga which is regularly subject to changes in salinity. In the laboratory it maintains its turgor at 2.3 bar over a range of Π^o from 16.7–27.7 bars (23–37 parts per thousand (ppt) salinity). The principle solutes in the large (400 μm diameter) vacuolated

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cells are inorganic ions, especially Na^+, K^+ and Cl^-. These are also the solutes which change most in concentration as salinity changes (Bisson & Gutknecht, 1975).

Our earlier study did not determine which of these three ions is primarily responsible for turgor regulation, since at steady state all had increased or decreased by the same proportion. The changes in ion concentrations during the regulatory phase, as discussed here, indicate that Cl^- fluxes are involved most directly in the turgor regulatory system. Isotopic flux measurements show that the activity of the inwardly directed Cl^- pump is increased during hypertonic regulation and decreased during hypotonic regulation.

Materials and Methods

Methods for culturing the alga and for measuring ion concentrations, osmotic pressure, and electrical properties were presented earlier (Bisson & Gutknecht, 1975) and will be described only briefly here. The seaweed was collected from shallow waters near Beaufort, North Carolina. Cylinders 1 cm long were cut near the young, growing tips and incubated in aerated, buffered, enriched seawater under continuous light at room temperature for 2–3 days before the experiments. For dark experiments, tissue segments were incubated in the light for 1 day, then in opaque flasks for 1–2 days before the experiments. Segments were incubated in opaque flasks during the experiments.

Ion concentrations were measured in hot-water extracts of tissue. Na^+ and K^+ concentrations were measured with an atomic absorption spectrophotometer and Cl^- concentrations with a chloridometer. Osmolality was measured by freezing point depression of intracellular fluid which was expressed from the tissue by crushing and centrifuging. Osmolality was converted to osmotic pressure by multiplying by the gas constant and absolute temperature. Intracellular ion concentrations and osmotic pressure were estimated from whole tissue extracts by correcting for extracellular space (about 13% of the tissue volume). Under steady-state conditions turgor pressure (ΔP) was assumed to be equal to $\Delta\Pi$, since J_v (volume flux) is small and L_p (hydraulic conductivity) is large.

Two Ag – AgCl, KCl-filled microelectrodes were inserted into the vacuole to measure the electrical potential. Current was injected from one and the change in potential in the other was used to calculate the membrane (protoplast) resistance.

Steady-State Flux Measurements

Unidirectional fluxes were measured with ^{36}Cl. The basic tracer flux equation for a two-compartment system is

$$\frac{dQ^{*i}}{dt} = J^{in}\frac{C^{*o}}{C^o} - J^{out}\frac{C^{*i}}{C^i} \tag{1}$$

where Q^{*i} is the amount of radioactivity inside (cpm), J^{in} and J^{out} are the flows of material into and out of the cell (moles sec^{-1}) and C^{*o}/C^o and C^{*i}/C^i are the specific activities outside and inside the cell. Since at steady state J^{in}, J^{out}, C^i and C^o (the internal and external concentrations) are constant, the equation can be integrated with respect to

time. For washout into a large extracellular bath, $C^{*o}=0$, and the efflux can be obtained by plotting ln C^{*i} *vs.* time. The efflux, normalized to membrane area, can be calculated as $J^{out}=-\text{slope } C^i \text{ (area/vol)}^{-1}$. For uptake experiments, $\ln[1-(C^{*i}\ C^o/C^{*o}\ C^i)]$ was plotted against time, and $J^{in}=-\text{slope } C^i \text{ (area/vol)}^{-1}$.

Nonsteady-State Fluxes

After a change in salinity, $J^{in}\neq J^{out}$, and the fluxes and C^i change with time. Hence Eq. (1) cannot be integrated over time. Instead, the following method was used. J^{out} was measured during isotope washout experiments, and J^{in} was estimated from values for $J^{net}=J^{in}-J^{out}$. During the efflux experiments $C^{*o}=0$ and

$$\frac{dC^{*i}}{dt}=-\frac{J^{out}}{V^i}\frac{C^{*i}}{C^i}. \tag{2}$$

dC^{*i}/dt can be estimated at any time by averaging the rate of change ($\Delta C^{*i}/\Delta t$) from the time intervals preceeding and following the given time. Thus, at every point the value of J^{out} can be calculated from Eq. (2) and normalized to membrane area. J^{net} at each time was obtained from the change in C^i by a similar method of averaging slopes. Finally, J^{in} was calculated from the relation, $J^{net}=J^{in}-J^{out}$.

Results

For the purposes of discussion, the *Codium* cells will be considered to be in one of three states. At steady state, turgor, Π^i, and ion concentrations are constant. However, since the tissue is growing, the cells are not in true steady state; although turgor and Π^i are constant at a constant salinity, there is a small net influx of water and ions. In addition, the cation concentrations shift slightly during a 5-day incubation at constant salinity, increasing the K^+/Na^+ ratio inside the cell. However, these changes are small, and the tissue is considered to be in steady state after 2 days incubation at a given salinity. After a salinity change, Π^i and C^i_j change rapidly. This "regulatory phase" of the turgor regulation cycle ends when turgor is restored to its normal value. As seen below, however, the tissue is not now at steady state, since cation concentrations and fluxes are not at their steady-state values for the new salinity. There is then a "post-regulatory phase", during which Π^i remains constant while the cation ratio is gradually restored to its steady-state value.

Changes in Π^i and C^i_j during Turgor Regulation

Fig. 1 shows the changes in internal osmotic pressure and Na^+, K^+ and Cl^- concentrations as a function of time. The top line shows the

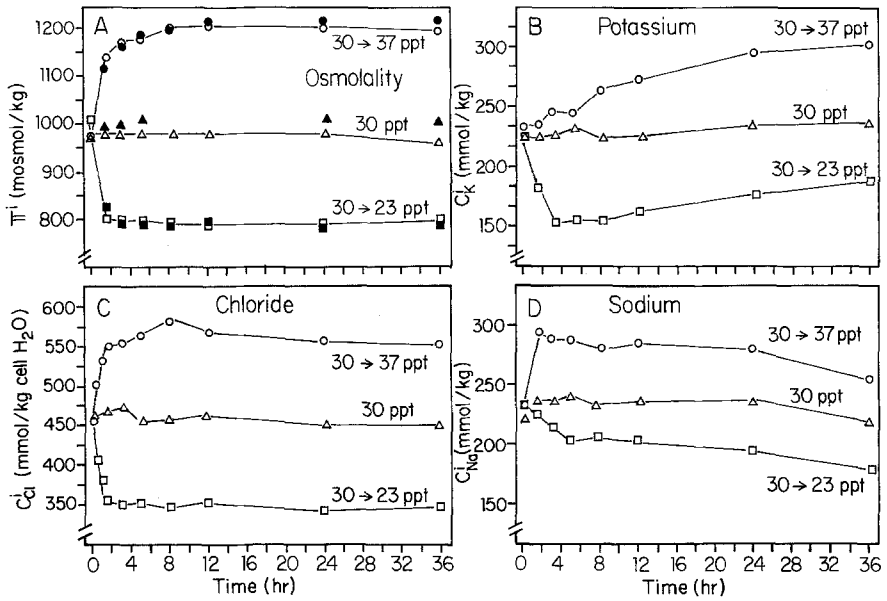

Fig. 1. Time course of changes in internal osmotic pressure (Π^i) and ion concentrations (C_j^i) following a change in salinity. At time 0, *Codium* segments acclimated to 30 ppt seawater were transferred to 37 ppt (circles), 23 ppt (squares) or maintained in 30 ppt (triangles). Open symbols represent the mean of four experiments in the light, and the dark symbols represent one experiment in the dark. Fig. 1*C* includes data from additional experiments (four experiments for 30→23 ppt, six for 30→37 ppt) during which chloride concentrations were measured at shorter intervals (0–1.5 hr after salinity change)

results from tissue transferred from 30 to 37 ppt (i.e., 22.3 to 27.6 bar), the bottom line shows the results from tissue transferred from 30 to 23 ppt (22.3 to 16.7 bar) and the middle line shows the results from control tissue kept at 30 ppt. The open symbols represent experiments in the light, and the closed symbols experiments in the dark. Fig. 1*A* shows the changes in Π^i. Note that by 2 hr osmotic regulation is 80–100% complete. Fig. 2 shows an experiment in which Π^i was measured frequently during this period. Here the data are plotted as $\Delta\Pi$ ($=\Pi^i - \Pi^o$) to illustrate the return to normal turgor. Fig. 1*C* shows that the intracellular Cl^- concentration (C^i_{Cl}) follows the same time course as the changes in Π^i. This graph includes data from both short-term and long-term experiments. Concentrations of intracellular cations, as shown in Fig. 1*B* and 1*D*, change with a time course different from that of Π^i. During hypertonic regulation (following transfer to higher Π^o) C^i_K increases slowly and is, in fact, still increasing at 36 hr. In contrast, C^i_{Na} increases rapidly, then decreases slowly for the duration of the experi-

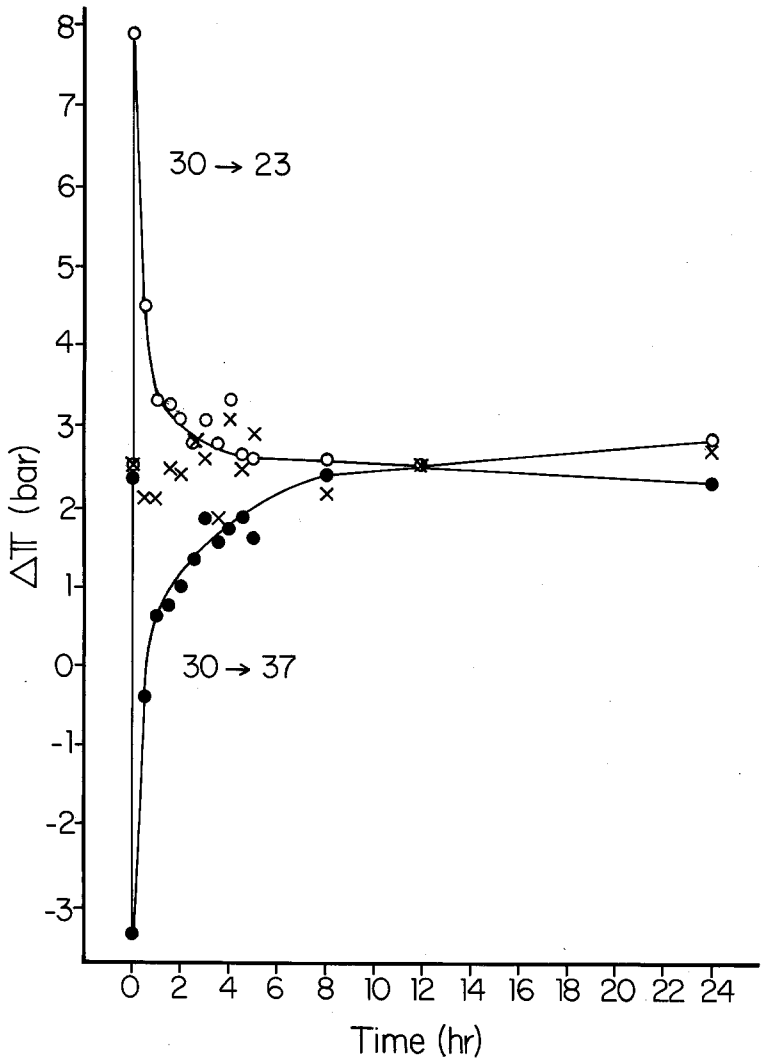

Fig. 2. Changes in $\Delta\Pi$ following changes in external salinity. Open circles show hypotonic regulation (30→23 ppt), closed circles represent hypertonic (30→37 ppt) and crosses indicate control material maintained at 30 ppt. Data are from a single experiment

ment. During hypotonic regulation, C^i_K falls rapidly and then increases, while C^i_{Na} decreases slowly throughout the experiment.

Vacuole Potential and Protoplast Resistance during Turgor Regulation

When a *Codium* cell impaled with two microelectrodes is subjected to a salinity change, the voltage trace often becomes quite unstable, show-

ing periods of depolarization which may be due to a number of causes. The electrode may be excluded from the cell. With two microelectrodes recording potential, this is easily identifiable, as only one trace will show the depolarization. Reinsertion of the microelectrode will restore the high potential, although there is often, as in the case of any insertion, a period during which the potential rises as the cytoplasm seals around the electrode. Frequently, both electrodes show parallel depolarization. This may be accompanied by a lowered resistance, in which case the depolarization is presumed to be due to breaking of the cytoplasmic seals around the electrode as the cells shrink or swell. Sometimes the depolarization is accompanied by an increased resistance, the cause of which is unknown. In order to assess changes in electrical parameters associated with turgor-regulated ion fluxes, only the experiments with steady potentials throughout were included in the analysis given in Figs. 3 and 4. Six experiments of hypotonic regulation (30 → 23 ppt) and four experiments of hypertonic regulation (30 → 37 ppt) were included. Other experiments which gave qualitatively similar results were not included in the quantitative analysis because they showed more of the above-described changes in vacuole potential (V_{vo}) and protoplast resistance (R_p) which were not directly associated with turgor regulation.

Figs. 3 and 4 show the results of these ten experiments. In all these experiments, one utricle in a segment of *Codium* thallus acclimated to 30 ppt seawater was impaled by microelectrodes and steady-state readings were made of V_{vo} (normally -70 to -80 mV) and R_p (normally 230–460 Ω cm^2). The seawater was then changed for 37 ppt (dark rectangles) or 23 ppt seawater (light rectangles). Fig. 3 shows that during hypertonic regulation the utricles hyperpolarize slightly (2.6 mV), whereas during hypotonic regulation they depolarize an average of 6.9 mV. There is no large or consistent change in resistance during hypotonic regulation ($+5.7\ \Omega$ cm^2), but during hypertonic regulation resistance falls about 90 Ω cm^2.

Steady-State Cl^- Fluxes

Since chloride ions are most important in altering Π^i during both hypertonic and hypotonic regulation, we decided to look more closely at the unidirectional fluxes of chloride. It was first necessary to determine these fluxes during steady state. Efflux was determined from washout experiments, and influx from uptake experiments. When plotted semi-logarithmically, as described in Materials and Methods, the data points

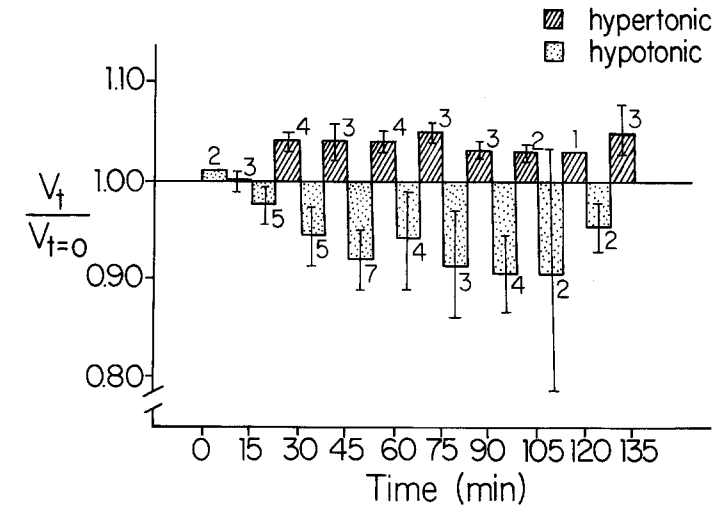

Fig. 3. Changes in vacuolar potential (V_{vo}) during turgor regulation. The heights of the rectangles show the relative changes, the vertical bars through them are the SEM's, and the numbers are the number of measurements during each time interval. Each rectangle represents readings taken during the entire 15-min interval, although for clarity the rectangles are split. Occasionally the same utricle was measured more than once during the interval; not every utricle was measured during every interval. The numbers are presented as a ratio ($V_t/V_{t=0}$) to normalize the values from utricles with different initial values of V_{vo}

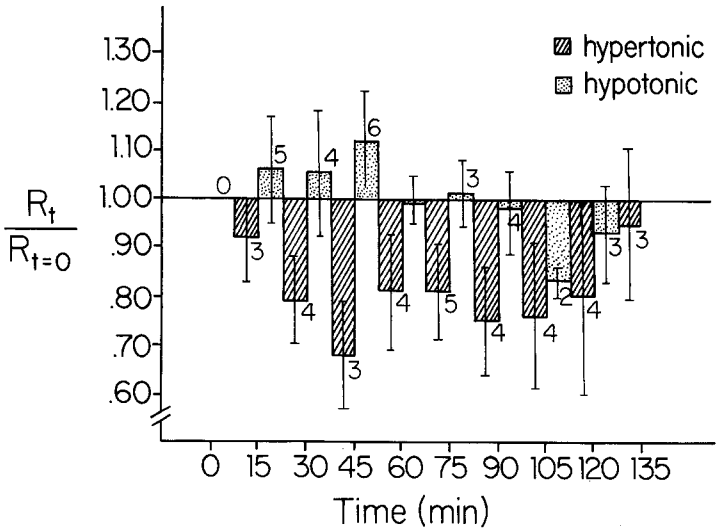

Fig. 4. Changes in protoplast resistance (R_p) during turgor regulation. For details, *see* the legend of Fig. 3

Table 1. One-way Cl^- fluxes and concentrations and vacuole potentials in *Codium* acclimated to three different salinities

Salinity (ppt)	C^o_{Cl}- (mmol/kg H_2O)	C^i_{Cl}- (mmol/kg cell H_2O)	Vacuole potential (mV)	Cl^- influx (pmol/cm^2 sec)	Cl^- efflux (pmol/cm^2 sec)
23	375	387	−75	46.9 ± 7.4	40.1 ± 1.4
30	492	454	−76	64.1 ± 0.7	53.4 ± 6.8
37	605	568	−76	63.2 ± 4.5	50.3 ± 2.1

Each flux value is the mean of three experiments (± standard error). Concentrations and potentials are from Bisson and Gutknecht (1975).

fall on straight lines (Bisson & Gutknecht, 1977) except for an early, steeper component, which is presumably due to fluxes into or out of small compartments, such as the extracellular space (13% of tissue volume) and cytoplasm (6% of tissue volume). The major linear portion represents fluxes into and out of the vacuole, which comprises 82% of the tissue volume (Bisson & Gutknecht, 1975). As shown in Table 1, flux values are similar for influx and efflux at all salinities. At 23 ppt both one-way fluxes are somewhat less than at higher salinities. Influx appears to be slightly higher than efflux in all cases. This would result in a small net influx, consistent with the growth rate (about 6% d^{-1}). The derivation of the formula for determining influx requires that $J^{in} = J^{out}$. The apparent inequality of J^{in} and J^{out} at each salinity does not significantly affect the determination, however. First, the differences between influx and efflux are not significantly different at the 0.5 level, except for 37 ppt, and here the difference is not significant at the 0.1 level. Second, if J^{out}/J^{in} is substantially different from unity, the graph of $\ln(1-(C^{*i}\,C^o/C^i\,C^{*o}))$ *vs.* time should curve away from the straight line, departing from a straight line more as time increases. This is not seen in the graphs (Bisson & Gutknecht, 1977), which indicates that the scatter in the data is greater than the error introduced by $J^{out}/J^{in} \neq 1.0$.

Net Chloride Fluxes during Turgor Regulation

In order to determine the regulatory and post-regulatory periods, we considered the net fluxes during osmotic stress (Fig. 5). There is a distinct change in the net flux between 2.5 and 3 hr. Before 1 hr the cells are changing rapidly in size. Because these size changes complicate the method for calculating fluxes, values from these early times were not

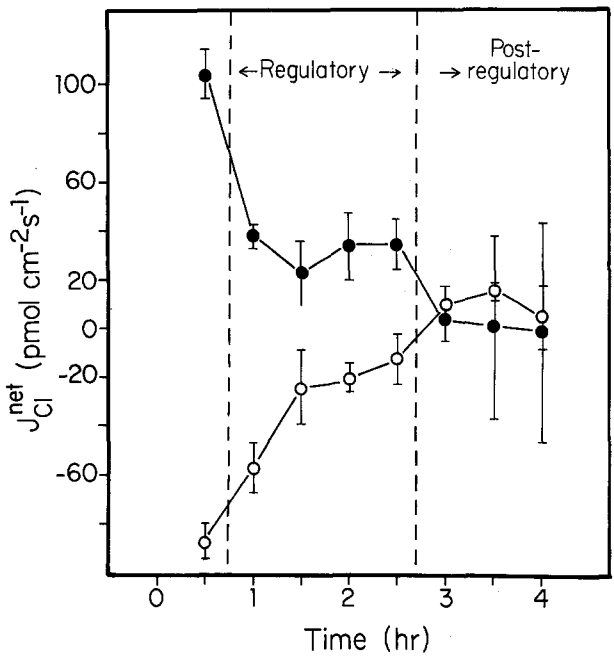

Fig. 5. Net fluxes of Cl^- during turgor regulation. Hypertonic regulation is shown by closed circles, hypotonic by open circles. Net influx is shown as positive and net efflux as negative

used, and the regulatory phase was considered to be from 1 to 2.5 hr. Since the net fluxes before one hour are quite high, we are actually underestimating the average change in flux during the regulatory period. The average net influxes during the regulatory period are -29.1 ± 6.9 pmol cm^{-2} sec^{-1} (mean $\pm$ SE) (16 measurements) for hypotonic regulation and 32.2 ± 5.2 (19) for hypertonic regulation.

One-Way Chloride Fluxes during Turgor Regulation

During the hypotonic regulatory period, $J^{out} = 40.9 \pm 10.4$ pmol cm^{-2} sec^{-1} (16), similar to the efflux at steady state. Influx, however, falls to 11.0 ± 9.7 pmol cm^{-2} sec^{-1} (16). After 2.5 hr, during the post-regulatory period, there is a small net influx of Cl^-, 10 pmol cm^{-2} sec^{-1}, consistent with the growth rate. During this period, one-way influx has increased to 30.2 ± 10.9 (12). Post-regulatory fluxes are less than the steady-state fluxes in 23 ppt seawater (*see* Table 1). This may be due to individual variation, or it may be that the post-regulatory fluxes increase slowly to reach the measured steady-state values.

The average net influx during the hypertonic regulatory period is 32 pmol cm^{-2} sec^{-1}. To achieve this net influx, *Codium* would have to increase its one-way influx to about 85 pmol cm^{-2} sec^{-1}, while maintaining its efflux at the steady-state value of 53 pmol cm^{-2} sec^{-1}. However, during this regulatory period Cl^- influx increases to 206 $\pm$25 pmol cm^{-2} sec^{-1} (19) while efflux increases to 174$\pm$25 (19). After 2.5 hr, when net influx drops to 3.8$\pm$13.5 pmol cm^{-2} sec^{-1} (9), the one-way fluxes still remain high, although more variable: influx = 187$\pm$101 (9) and efflux = 185$\pm$93 pmol cm^{-2} sec^{-1} (9). Since these fluxes are much higher than those measured in tissue acclimated to 37 ppt seawater for 2 days (*see* Table 1), this post-regulatory period appears to be different from the steady state. We must assume that the one-way fluxes eventually decrease to the levels measured at steady state.

Discussion

Any plant which regulates its turgor must be able to alter its internal osmotic pressure in response to changes in turgor pressure. The number of ways by which a plant can alter Π^i are numerous (*see*, for example, Cram, 1976; Hellebust, 1976; Gutknecht, Hastings & Bisson, 1977). *Codium* controls its osmotic pressure by controlling the concentrations of Na^+, K^+ and Cl^- in the vacuole. The concentrations of these ions are maintained by pumping Cl^- (and possibly K^+) into the vacuole and by pumping Na^+ out (Bisson & Gutknecht, 1975). Thus, changes in the concentrations of these ions may be achieved by various combinations of changes in pump rates and passive permeabilities. The simplest hypothesis is that a change in turgor has a direct effect on a single transport system, and that changes in the concentrations of other ions are secondary consequences of changes due to the regulatory process. We see from Fig. 1 that if this is so, Cl^- is the best candidate for the turgor-regulated ion. C^i_{Na} changes too slowly to cause the changes in osmotic pressure during hypotonic regulation; C^i_K changes too slowly during hypertonic regulation. Thus, *Codium* appears to alter Π^i by altering C^i_{Cl}, and the counterion is different during hypo- and hypertonic regulation.

A large electrochemical gradient favoring Cl^- efflux exists at all salinities ($V_{vo} - E_{Cl} \cong -76 - 1 = -77$ mV) (Bisson & Gutknecht, 1975). A decrease in C^i_{Cl} could thus be accomplished either by decreasing the active chloride influx or by increasing the permeability to Cl^-. The isotopic flux measurements indicate that during hypotonic regulation, active Cl^- influx decreases while the passive efflux remains the same.

Similarly, during hypertonic regulation, an increase in C^i_{Cl} is accomplished by increasing the rate of active Cl^- uptake.

Fig. 1*A* shows that turgor regulation in *Codium* occurs with equal speed and effectiveness in both the dark and the light. Preliminary experiments indicate that Cl^- is the principle turgor-regulated ion in the dark, since C^i_{Cl} closely follows changes in Π^i and the cation concentrations show the same trends in the dark as in the light. We have done no isotopic flux measurements in the dark, and therefore cannot say whether the one-way Cl^- fluxes are lower in the dark, as they are in many algae (MacRobbie, 1970; Hope & Walker, 1975). Nor do we know how the net fluxes during regulation in the dark arise from changes in the one-way fluxes. We do know, however, that *Codium* is capable of generating similar regulatory net fluxes of Cl^- in response to turgor changes in both the light and the dark.

Different cations serve as counterions to the net chloride fluxes during hyper- and hypotonic regulation. In each case, the cation with the higher net flux moves down its electrochemical gradient. There is a large electrochemical gradient for Na^+ influx ($V_{vo} - E_{Na} = -76 - 12 = -88$ mV) and a smaller gradient for K^+ efflux ($V_{vo} - E_K = -76 + 89 = 13$ mV) (Bisson & Gutknecht, 1975). During the regulatory period, the changes in V_{vo} and cation concentrations inside and outside the cell are small. Therefore the changes in electrochemical gradients alone are not sufficient to produce the observed regulatory changes in cation fluxes (*see* Fig. 6). Thus, there must be additional turgor-sensitive mechanisms which facilitate the regulatory net passive fluxes of cations, either by selectively increasing the permeability of the membrane or by reducing the rate of active transport.

Because the counterion to the net Cl^- flux is different for hypo- and hypertonic regulation, the internal K^+/Na^+ ratio is reduced under both conditions. At steady state, $K^+/Na^+ = 0.96 \pm 0.05$ (4). During hypotonic regulation the ratio falls to a minimum of 0.72 ± 0.01 (4) at 3 hr. During hypertonic regulation the ratio falls to a minimum value of 0.79 ± 0.03 (4) at 1.5 hr. In both cases the ratio is restored to about 1.0 over the next 12–24 hr by a slow net influx of K^+ and efflux of Na^+.

The changes in V_{vo} and R_p occurring during regulation could be due to changes in concentration gradients, ionic permeabilities, or electrogenic pump rates. The changes in concentration ratios are small and cannot alone account for the changes in V_{vo}. Although the ionic permeabilities may change, the changes in both V_{vo} and R_p cannot easily be explained in this way. For instance, during hypertonic regulation, high

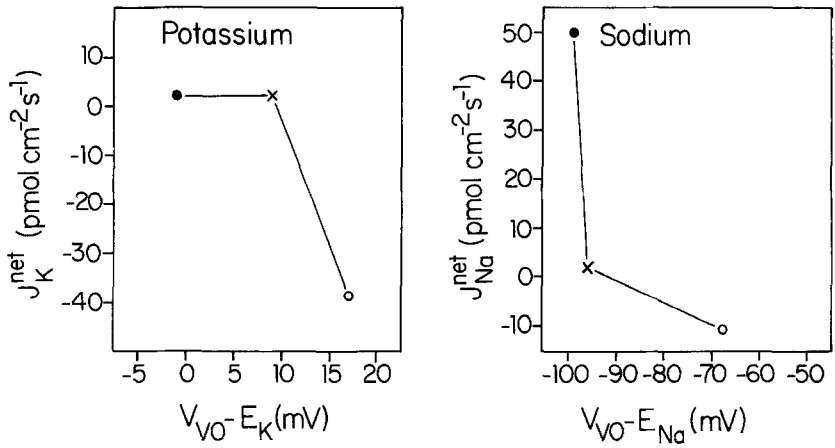

Fig. 6. Relationship between net Na^+ and K^+ fluxes and their electrochemical driving forces. Closed circles represent hypertonic regulation (1.5 hr after salinity change), open circles represent hypotonic regulation (1.5 hr after salinity change) and crosses represent steady state at 30 ppt. A positive value of $V_{vo}-E_j$ indicates an outward driving force on Na^+ or K^+

net Na^+ influx and the high one-way Cl^- efflux suggest that the permeabilities to these ions may increase. This is qualitatively consistent with the decrease in resistance. However, both increases would depolarize the membrane, which is, in fact, hyperpolarized during hypertonic regulation. Although V_{vo} is probably primarily a K^+ diffusion potential, there may be a small electrogenic component (Bisson & Gutknecht, 1975). If this is due to the Cl^- pump, then the decrease in pump rate during hypotonic regulation would depolarize V_{vo}, and the increase during hypertonic regulation would hyperpolarize V_{vo}. The changes in V_{vo} are small compared to the changes in chloride flux, but the cell resistance is so low that a large pump current would produce only a small potential. Of course, the changes may be complex in origin, affected by changes in both permeabilities and in the Cl^- pump rate.

Turgor regulation has been demonstrated in several other marine algae. For example, in *Valonia* a low turgor pressure is elevated by a turgor-sensitive inwardly directed K^+ pump (Gutknecht, 1968; Hastings & Gutknecht, 1976), and a high turgor pressure is reduced by an increase in K^+ permeability (Steudle, Lelkes & Zimmermann, 1977). Thus, the turgor-regulated ion in *Valonia* appears to be K^+. The same is probably true in *Chaetomorpha* (Kesseler, 1964; Zimmermann & Steudle, 1971). *Valonia*, however, is unusual in that it has a positive vacuole potential generated partly by an electrogenic cation pump, and Cl^- is distributed

at equilibrium (Hastings & Gutknecht, 1976). *Chaetomorpha* similarly has a positive vacuole potential, active K^+ uptake, and apparently passive Cl^- distribution (Dodd, Pitman & West, 1966). However, most algae (and higher plants) have a large negative vacuole potential and an inward Cl^- pump. Thus, as more algae are investigated, turgor-sensitive Cl^- transport may be shown to be prevalent. For example, the embryo of *Pelvetia* generates a turgor pressure during development by increasing active Cl^- influx (Allen, Jacobsen, Joaquin & Jaffe, 1972) and reduces excess turgor pressure by increasing Cl^- efflux (Nuccitelli & Jaffe, 1976*a*, *b*). In both cases the predominant counterion is K^+. *Halicystis (Derbesia)*, another turgor-regulating alga (Gutknecht *et al.*, 1977), has a Cl^- pump which may be turgor sensitive (Graves, 1974; Graves & Gutknecht, 1976). If Cl^- pumps are found to be the predominant effectors for turgor regulation, this will support a speculation by Dainty (1962) that anion pumps evolved in plant cells as a mechanism to increase Π^i and generate turgor pressure.

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