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Osmotic Regulation in the Marine Alga, *Codium decorticatum*

I. Regulation of Turgor Pressure by Control of Ionic Composition

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Summary. *Codium decorticatum* regulates its internal ionic composition and osmotic pressure in response to changes in external salinity. Over a salinity range of 23 to 37‰ (675 to 1120 mosmol/kg) *Codium* maintains a constant turgor pressure of 95 mosmol/kg (2.3 atm), observed as a constant difference between internal and external osmotic pressures. The changes in internal osmotic pressure are due to changes in intracellular inorganic ions. At 30‰ salinity the major intracellular ions are present in the following concentrations (mmol/kg cell H_2O): K^+, 295; Na^+, 255; Cl^-, 450. At different salinities intracellular ion concentrations remain in constant proportion to the external ion concentrations, and thus the equilibrium potentials are approximately constant. The potential difference between the vacuole and seawater (−76 mV), which is predominantly a K^+ diffusion potential, is also constant with changing salinity. Comparison of the equilibrium potentials with the vacuole potential suggests that Cl^- is actively absorbed and Na^+ actively extruded, whereas K^+ may be passively distributed between the vacuole and seawater. Turgor pressure does not change with environmental hydrostatic pressure, and increasing the external osmotic pressure with raffinose elicits a response similar to that obtained by increasing the salinity. These two results suggest that the stimulus for turgor regulation is a change in turgor pressure rather than a change in internal hydrostatic pressure or ion concentrations.

Codium decorticatum is a macroscopic green marine alga, which is common in southeastern North America and in southern Europe. It grows attached to rocks and shells in shallow water, where it is subject to sudden dilutions due to rainfall and is occasionally exposed to air at low tide. In the estuarine area around Beaufort, North Carolina, where our plants were collected, salinity regularly ranges from 23–37‰ (gm solids/kg seawater), i.e., 675 to 1120 mosmol/kg. Occasional values outside this range occur, and on a single day salinity may vary by as much as 13‰, although a variation of 2–5‰ is more usual.

It would seem likely that algae growing in this environment would have a mechanism for adjusting to such large changes in external osmo-

lality. An aquatic plant might conceivably maintain its internal osmotic pressure (Π_i) large and constant, and protect itself from osmotic volume changes by having a rigid inextensible cell wall. However, this would result in large changes in the internal hydrostatic pressure (turgor pressure), which is determined by the difference between internal and external osmotic pressures ($\Delta\Pi$). Since the turgor pressure itself performs the important functions of maintaining cell shape and plant structure, as well as providing the force for cell expansion and plant growth (Ray, Green & Cleland, 1972), it would seem more likely that aquatic plants would evolve mechanisms for regulating the turgor pressure in the face of changes in external salinity. Some algae apparently maintain a constant turgor in the face of external salinity changes (Kesseler, 1964*a*, 1965; Hastings & Gutknecht, 1974; *also, unpublished observations*), and in some species the rates of active ion uptake are controlled by the turgor pressure (Gutknecht, 1968; Graves, 1974; Hastings & Gutknecht, 1974; Zimmermann & Steudle, 1974) or by Π_i (Nakagawa, Kataoka & Tazawa, 1974). For a recent review of regulation and control mechanisms in plants *see* Cram (1975).

The purpose of this study is to determine whether *Codium* can regulate its turgor in response to changes in salinity. If *Codium* is capable of turgor regulation, then other questions arise. What solutes are responsible for $\Delta\Pi$ and how do they change when Π_o changes? Which solutes are actively transported? Is the stimulus for turgor regulation a change in concentration of one or more specific external ions, or is it the change in osmotic or hydrostatic pressure *per se*? The experiments reported here show that *Codium* is capable of regulating its turgor, as measured by $\Delta\Pi$, and that Π_i is due almost entirely to inorganic ions, primarily Cl^-, K^+, and Na^+. Turgor regulation is initiated by a change in external osmotic pressure, rather than a change in external ion concentrations, which implies that the stimulus for regulation is a change in turgor pressure.

Materials and Methods

Culture Conditions

Codium was collected near Beaufort, North Carolina. Cylindrical segments of tissue were cut from sections of thallus 2–5 mm in diameter. The segments were 1–1.5 cm long and weighed 0.2–0.4 g each. 10–20 g of tissue segments were grown in 800 ml of aerated medium in Erlenmeyer flasks. Offshore seawater was used, and salinity was adjusted either with distilled water or with seawater which was concentrated approximately twofold by freezing out water. Final salinity was checked by refractive index and osmotic pressure, and the measured ionic concentrations agreed with those tubulated by Barnes (1954) for

each salinity. The culture solutions were enriched with 120 μM $NaNO_3$ and 60 μM NaH_2PO_4 and were buffered with 5 mM Tris chloride at pH 8.0. The culture medium was changed every 12 hours. The flasks were aerated and light was provided by a bank of fluorescent lights, giving an intensity of 15 watts/m^2 at the surface of the flasks. Temperature was 24–25 °C. Two days at a given salinity was sufficient to acclimate the tissue, that is, to establish steady-state values of Π_i and internal ion concentrations. All experiments were completed within five days.

When *Codium* segments were to be grown under increased hydrostatic pressure, they were placed in a 500-ml Erlenmeyer flask filled with enriched, buffered seawater. A flexible, watertight seal was placed over the flask, and it was submerged in a NaCl solution isosmotic with the seawater in a Plexiglas pressure chamber. Pressure was exerted from the water tap and monitored on an Ametek U.S. Gauge.

Ionic Concentrations

Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+} and PO_4^{3-} concentrations were measured in hot-water extracts of fresh or frozen tissue (approx. 2 g/10 ml H_2O). SO_4^{2-} and NO_3^- were measured in more concentrated extracts obtained as described below under *Osmotic Pressure.* Cation concentrations were measured with a Varian Techtron atomic absorption spectrophotometer. Cl^- concentrations were measured on a Buchler-Cotlove chloridometer. Phosphate was determined by a high-temperature modification of the Fiske-Subbarow method (Griswold, Humoller & McIntyre, 1951). Sulfate was determined indirectly by precipitation with Ba^{2+}, after which the amount of Ba^{2+} remaining in solution was measured by atomic absorption. Nitrate was reduced to nitrite by *E. coli* nitrate reductase (Worthington Biochemicals) and then measured colorimetrically (McNamara, Meeker, Shaw & Hageman, 1971).

Osmotic Pressure and Turgor Pressure

Intracellular fluid was obtained by freezing and thawing the tissue several times to fracture the cells, grinding with a homogenizer pestle, and centrifuging at about $13{,}000 \times g$ for about 10 min at room temperature. The clear supernatant was decanted immediately and used at once or frozen. Osmolality was determined cryostatically with an Advanced Osmometer Model 65-31, and osmotic pressure was estimated from the van't Hoff relation, i.e.,

$$\Pi = RT(\text{osmolality}) \tag{1}$$

where R is the gas constant and T is the absolute temperature. For convenience, we will use Π as a symbol for both osmolality (mosmol/kg) and osmotic pressure (atm), since the two parameters can be easily distinguished by their units. Turgor pressure (ΔP) is estimated by $\Delta\Pi$.

Extracellular Space and Percent Tissue Water

The extracts obtained by either of the two methods given above will contain some extracellular fluid. Thus, corrections must be made to convert the measured concentrations to intracellular concentrations. The following equations were used for computing intracellular ionic concentrations and osmotic pressures:

$$C_i = \frac{C_{\text{tiss}}/\rho - (\text{ECS})\,C_{\text{ECS}}}{\text{ICW}} \tag{2}$$

$$\Pi_i = \frac{\Pi_{\text{ex}} - (\text{ECS/TW})\,\Pi_{\text{ECS}}}{\text{ICW}'}. \tag{3}$$

C_i is the intracellular molal concentration of a given ion (mmol/kg cell H_2O), C_{tiss} is the concentration in the tissue (mmoles/liter tissue), and C_{ECS} is the concentration (mM) in the intracellular space. Π_i, Π_{ex} and Π_{ECS} are the osmolalities (mosmol/kg) of the intracellular fluid, extract, and extracellular space, respectively. ρ is tissue density, and is assumed to be approximately equal to that of seawater, i.e., 1.03 kg/liter. TW is the fraction of tissue water, and the ECS is the fraction of extracellular space, both in liters/kg. ICW is the fraction of intracellular water. In computing ionic concentrations of hot-water extracts of whole tissue samples (Eq. 2), ICW is calculated as TW-ECS, in liters/kg tissue. This is liters of cell water, not intracellular solution, and is thus equivalent to kg cell H_2O/kg tissue. To calculate ionic concentrations or osmolality of the supernatant of centrifuged tissue (Eq. 3), ICW′ is calculated as the fraction, (TW-ECS)/TW, in kg cell H_2O/kg tissue H_2O.

Tissue water was measured by weighing blotted segments of thallus, drying at 100 °C for 24 hr, and weighing again. Extracellular space was estimated by allowing the tissue to equilibrate for 3–4 hr with a radioactively labeled nonelectrolyte, i.e., inulin, sucrose, or mannitol, which was assumed to be impermeant. The tissue was transferred to a label-free solution, and the amount of label that washed out was measured.

Electrical Measurements

Microelectrodes were made of micropipettes pulled on a vertical pipiette puller (David Kopf Instruments). The micropipettes were filled with 2.5 M KCl under reduced pressure. Silver/silver chloride wires were inserted into the pipettes and connected to an amplifier and recorder. Microelectrode resistances were $5\text{–}10 \times 10^6$ ohms. A reference electrode was filled with 2.5 M KCl in 2% agar and placed in contact with the bathing solution. To measure the intracellular potential a microelectrode was inserted into the vacuole of a utricle (large outer cell), and the potential difference was recorded when it became steady. If the potential continued to fluctuate for more than 20 min, the highest potential attained was recorded.

Protoplast resistance was measured by inserting two microelectrodes into the same utricle. The second microelectrode was connected to a constant current device (Gage & Eisenberg, 1969) from which 0.4 A of current was injected. We measured the change in potential (usually 5–15 mV) after 1 sec of applied current, and the resistance was calculated from Ohm's law. To estimate utricle surface area, the most common shape was assumed, i.e., a tapering cylinder with lower diameter equal to 2/3 of the upper diameter. During an electrical experiment only the upper diameter could be measured; the length was assumed to be 1 mm. In separate experiments with dissected utricles the average length of 25 utricles was found to be 1.1 mm $\pm$0.04. (Throughout the text, data are presented as $\pm$SE, and the number of measurements is given in parentheses or stated in the text.)

For ion substitution experiments, *Codium* segments were acclimated to 37‰ seawater. Potential difference was first measured with the segment bathed in artificial seawater (NaCl, 520 mM; KCl, 10 mM; $MgSO_4$, 35 mM; $MgCl_2$, 10 mM). The bathing solution was then changed to one with the same concentrations of Cl^-, SO_4^{2-}, Mg^{2+} and Ca^{2+}, but with K^+ and Na^+ concentrations altered to give the desired K^+ concentrations and with $[K^+]+[Na^+]=530$ mM. The bathing solution was changed back to the artificial seawater between each solution change to determine that the potential difference returned to normal.

Measurement of Cellular Dimensions

Size of utricles was measured with a dissecting microscope. Size of filaments and cytoplasmic thickness were measured on a Zeiss Photomicroscope II, using Nomarski differential interference optics.

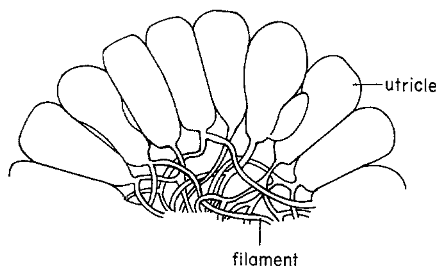

Fig. 1. Diagrammatic representation of cross-section of a *Codium* thallus. After Morris (1967)

Results

Morphology

To evaluate the cytoplasmic and vacuolar contribution to internal ionic concentrations, *Codium*'s unique and complicated anatomy must be taken into account (*see* Fig. 1). The central part of the thallus is comprised of branching, intertwined coenocytic filaments, 41.3 ± 3.5 µm (12 measurements) in diameter. These filaments are vacuolated, with the cytoplasm 3.2 ± 0.2 µm (11) thick. The filaments are thus 85% vacuole. The tips of the filaments swell to form large utricles, which comprise the outer surface of the thallus. The utricles are quite variable in size and shape, but are usually elongate cylinders, tapering slightly towards the inside. The utricles are 1.1 ± 0.04 mm (25) in length, and thus they comprise about 75% of the tissue volume in the cylindrical segments used in these experiments. The upper diameter is 380 ± 18 µm (36). The cytoplasm is 5.3 ± 0.6 µm (6) thick. Thus, in the utricles 97% of the cell is vacuole. In the tissue as a whole, then, 94% of the intracellular space is vacuole, and our intracellular concentrations are good estimates of the vacuolar concentrations.

Tissue Water and Extracellular Space

The fraction of tissue water in *Codium* is 0.95 ± 0.009 (15) over a salinity range of 23 to 37‰. In 30‰ seawater +210 mM raffinose, the fraction of tissue water is 0.93 ± 0.006 (7). This decrease is attributable to the volume which raffinose occupies in the extracellular space (ECS).

The fraction of ECS is 0.13 ± 0.01 liter/kg tissue (9), which agrees with the value of 0.145 obtained by Kesseler (1964*b*) using Li^+ as an ionic marker for ECS. Mannitol, sucrose and inulin gave similar values for ECS.

Growth Rates and Osmotic Volume Changes

The growth rate of *Codium* segments in culture is 6–15% per day over a salinity range of 20 to 45‰. The growth rate is about 4% per day at 50‰, and 0 at 15‰. When cultured tissue is subjected suddenly to an increase in salinity, it loses weight rapidly, then regains the lost weight within about 5 hr, and continues to grow at the previous rate (Fig. 2). When subjected to a sudden decrease in salinity, the tissue gains weight, loses some of the gained weight, then resumes its previous growth rate. The slight initial loss of weight of the control tissue is due to dehydration during handling. This suggests that the osmotic gain of water is slightly underestimated (upper curve), and the loss slightly overestimated (lower curve). These data indicate that *Codium* recovers from moderate hypotonic or hypertonic stress and, furthermore, that the response to a change in salinity involves substantial shrinking or swelling of the cells. The observed maximum volume changes of about −7% for shrinking and +10% for swelling are smaller than the −18 and +28% expected if the cells were behaving as perfect osmometers. Thus, unless salt movements are extremely rapid, changes in cell turgor must occur during the osmotic regulation process. The time course of the changes in turgor

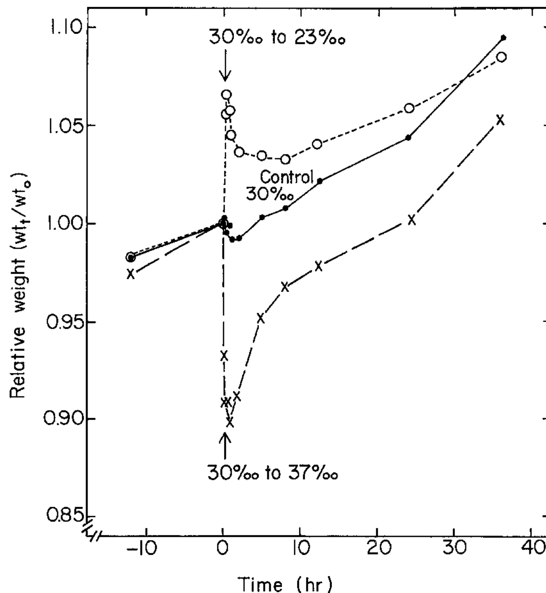

Fig. 2. Changes in tissue weight following transfer from 30 to 23‰ seawater, and from 30 to 37‰ seawater. Tissues were grown in 30‰ seawater for 2 days before transfer. Tissue weight is expressed as fraction of the weight at $t=0$ (time of transfer)

pressure and ionic composition will be dealt with in a future publication. In the present paper we will describe only the steady-state changes in ionic and osmotic concentrations which result from changes in external salinity or osmolality.

Intracellular Ionic Composition

The steady-state concentrations of six major ions in *C. decorticatum* acclimated to three salinities are shown in Table 1 and Fig. 3. Intracellular Na^+ and K^+ concentrations are high and approximately equal, and Cl^- is the principal intracellular anion. The last two lines of Table 1 show that, within the limits of error in the measurements, the positive and negative charges are balanced. The *t*-test shows the differences between the internal concentrations of K^+, Na^+ and Cl^- to be significant ($p < 0.01$) at all three salinities. There is no significant difference between the internal Mg^{2+} concentrations ($[Mg^{2+}]_i$) at any salinity ($p > 0.2$). $[Ca^{2+}]_i$ does not change in a regular manner with salinity and is too low to contribute significantly to the total Π_i. Because of the large scatter in the sulfate data, principally due to imprecision of the assay, the values for $[SO_4^{2-}]_i$ do not differ significantly ($p > 0.2$) between 23 and 30‰, or between

Table 1. Concentrations of major inorganic ions in seawater and intracellular fluid in *Codium decorticatum* at 23, 30 and 37‰ salinity

	23‰		30‰		37‰	
	Seawater	Intracellular fluid	Seawater	Intracellular fluid	Seawater	Intracellular fluid
$[Na^+]$	327 ±5	212 ±7	419 ±10	257 ±9	520 ±9	302 ±2
$[K^+]$	6.8 ±0.2	210 ±8	8.4 ±0.1	295 ±15	10.6±0.2	368 ±11
$[Mg^{2+}]$	34.6±3	22.7±3	49.3±2.1	19.5±2	59.6±2	21.0±3
$[Ca^{2+}]$	7.3 ±0.2	11.1±0.9	9.6 ±0.5	10.0±0.4	12.0±0.3	11.1±0.5
$[Cl^-]$	375 ±5	387 ±9	492 ±10	454 ±17	605 ±9	568 ±20
$SO_4^{2-}]$	20.2±6	59.0±6	24.5±2	69.4±6	36.9±3	74.3±8
Σz_i [cation]	418 ±8	490 ±12	545 ±11	611 ±18	674 ±13	734 ±13
Σz_i [anion]	415 ±10	505 ±16	541 ±10	593 ±21	679 ±11	717 ±26

Results are quoted as mean ±SE ($n=4$). These are molal concentrations, i.e., mmoles/kg H_2O for seawater and mmoles/kg cell H_2O for the intracellular fluid. Molal concentrations for seawater are derived from the molar concentrations by dividing by kg H_2O/liter seawater (Barnes, 1954). The sums of the cation and anion concentrations shown in the last two lines are in mequiv/kg H_2O.

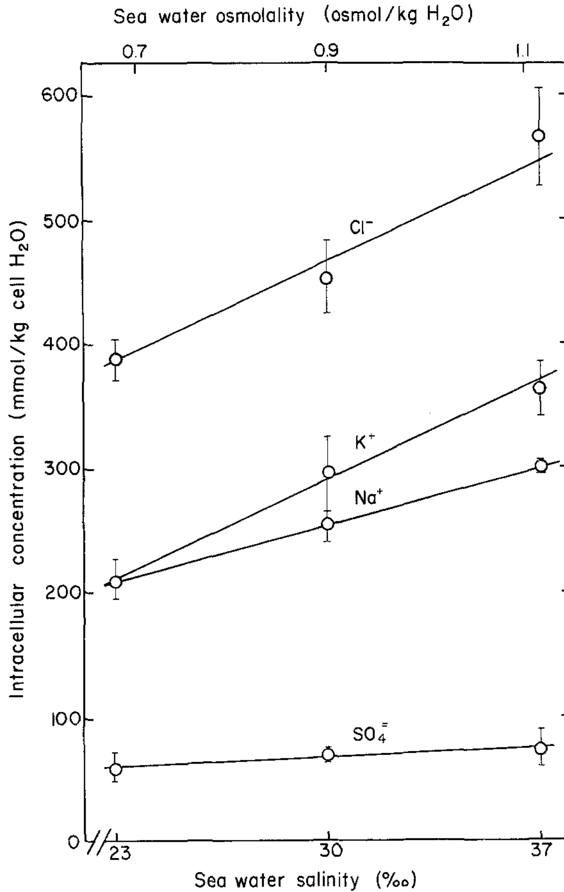

Fig. 3. Intracellular ion concentrations at three salinities (mean ±SD)

30 and 37‰. The values for 23 and 37‰ are significantly different ($p<0.1$) and this implies that sulfate may be important in altering Π_i in response to salinity changes. Both nitrate and phosphate are present intracellularly, but their concentrations are too low (<2 mM) to contribute significantly to Π_i.

Osmotic Pressure and Turgor Pressure

The osmolalities of intracellular and extracellular solutions at three salinities are shown in Table 2. The values for Π_i are significantly different ($p<0.01$) at the three salinities, whereas values for $\Delta\Pi$ are not significantly different ($p>0.5$). This implies that *Codium* is regulating $\Delta\Pi$ to about 95 mosmol/kg by altering Π_i in response to changes in salinity. This $\Delta\Pi$ corresponds to a turgor pressure of 2.3 atm.

Table 2. Osmolalities of intracellular fluid, artificial sap, and seawater at three salinities

	$23^0/_{00}$ (mosmol/kg)	$30^0/_{00}$ (mosmol/kg)	$37^0/_{00}$ (mosmol/kg)
Seawater	693 ± 6	899 ± 7	1115 ± 8
Intracellular fluid (Π_i)	791 ± 10	992 ± 6	1208 ± 9
Artificial sap	772	965	1142
$\Delta\Pi (= \Pi_i - \Pi_o)$	98 ± 7	93 ± 4	94 ± 9

All values are in mosmol/kg H_2O. The artificial saps are salt solutions made up to contain the same concentrations of the six major ions as contained in the intracellular fluid at each salinity (*see* Table 1) ($n=4$).

The validity of our indirect method of obtaining internal osmolality was confirmed by comparison with microcryostatic measurements on pure vacuolar sap obtained by micropuncture. For three samples of vacuolar sap from *Codium* acclimated to $23^0/_{00}$ seawater, $\Delta\Pi$ was 88 ± 8 mosmol/kg. Our indirect method gave 87 mosmol/kg for tissue samples taken from the same batch. The samples differed in that the micropuncture sample contained only vacuolar sap, whereas our extract presumably includes sap plus cytoplasmic solutes. The cytoplasm and sap must have the same osmotic pressures, however, and observations in this lab indicate that when two salt solutions of the same Π are mixed, the resulting solution will have the same Π. Moreover, as we have shown above, the contribution of cytoplasmic solutes to the osmolality of the intracellular fluid will be small.

To determine the nature of the intracellular solutes contributing to Π_i, artificial intracellular fluids were made up to have the same concentrations of ions as the intracellular fluid at each salinity. Table 2 shows that the osmolalities of these artificial solutions are very close to the osmolalities of the intracellular fluid. This implies that Π_i is due mainly to inorganic ions in solution. The small differences between the two solutions may be due to organic solutes in the vacuole, although organic solutes in the cytoplasm must also have an effect. If 500 mM is taken as a reasonable concentration of organic solutes in the cytoplasm, then the concentration of organic solutes in the extracted intracellular fluid (cytoplasm + sap) would be about 30 mM, which would account for a large part of the 19 to 66 mosmol/kg difference between the artificial and natural solutions.

Stimulus for Regulation

To distinguish between the effects of osmotic pressure and salinity on osmotic regulation in *Codium*, the osmotic pressure of $30^0/_{00}$ seawater

Table 3. The effect of raffinose on ionic concentrations and osmolality of intracellular fluid

	30‰		30‰+210 mM raffinose		37‰	
	Seawater	Intracellular fluid	Seawater	Intracellular fluid	Seawater	Intracellular fluid
$[K^+]$	8.0 ±0.1	275±9	8.4 ±0.1	265±11	9.4 ±0.2	303±15
$[Na^+]$	386 ±8	249±5	390 ±8	293±11	482 ±10	297±11
$[Cl^-]$	481 ±5	448±14	469 ±7	565±7	582 ±6	561±25
Π_i	906±7	998±6	1083±13	1175±9	1075±12	1177±5
$\Delta\Pi$		92±6		92±12		102±12

Ionic concentrations are in mmol/kg H_2O, osmotic concentrations are in mosmol/kg H_2O. Molar ion concentrations in raffinose seawater include a correction factor for the increase in volume (6.6%) of seawater when raffinose is added. For 30‰, $n=15$ for ionic measurements, 11 for osmotic measurements; for 30‰+raffinose, $n=13$ for ionic measurements, 9 for osmotic measurements; for 37‰, $n=14$ for ionic measurements, 10 for osmotic measurements.

was raised with 210 mM raffinose to obtain a solution that had Π_o equal to that of 37‰ but salt concentrations similar to 30‰. Tissue acclimated to this solution shows an increase in Π_i equivalent to tissue in 37‰ seawater ($p>0.8$) (Table 3). Table 3 also shows that $[Na^+]_i$ and $[Cl^-]_i$ are greater in tissue acclimated to the raffinose solution than in tissue acclimated to 30‰ ($p<0.01$), and are, in fact, more like the concentrations in tissue acclimated to 37‰. This suggests that regulation of $[Cl^-]_i$ or $[Na^+]_i$ is stimulated by osmotic or hydrostatic pressure, whereas $[K^+]_i$ is controlled by $[K^+]_o$.

In order to assess the effects of external hydrostatic pressure on osmotic regulation, *Codium* segments were incubated under 3 atm of environmental hydrostatic pressure for 12 hr, which is sufficient time for response to a salinity change. Their turgor pressure was 2.3±0.1 atm (4), not significantly different ($p>0.7$) from the 2.2±0.2 atm (4) turgor of the controls at 1 atm environmental hydrostatic pressure. This indicates that internal hydrostatic pressure is not regulated, since no reduction in turgor pressure followed the imposed increase in P_i.

Electrical Potential and Resistance

The potential difference between the vacuole and seawater (V_{vo}) and the resistance of the protoplast (R_p) are shown in Table 4. For comparison, the equilibrium potentials of the major intracellular ions are shown also.

Table 4. Vacuole potential (V_{vo}), ionic equilibrium potential (E_j) and resistance of the protoplast (R_p) at three salinities

		Salinity of culture medium		
		23‰	30‰	37‰
V_{vo}	(mV)	-75.2 ± 1.7 (11)	-76.5 ± 1.2 (24)	-75.8 ± 1.3 (16)
R_p	(Ω cm^2)	270 ± 21 (11)	186 ± 17 (16)	215 ± 33 (9)
E_K	(mV)	-87.6 ± 1.2 (4)	-90.5 ± 1.2 (4)	-90.2 ± 1.0 (4)
E_{Na}	(mV)	11.2 ± 1.3 (4)	12.4 ± 0.6 (4)	13.1 ± 0.5 (4)
E_{Cl}	(mV)	1.0 ± 0.7 (4)	-1.8 ± 1.0 (4)	-1.4 ± 1.0 (4)
E_{SO_4}	(mV)	15.1 ± 4.2 (4)	13.3 ± 1.7 (4)	8.9 ± 2.3 (4)

Equilibrium potential $= \frac{RT}{z_j F} \ln \frac{C_j^o}{C_j^i}$, where R is the gas constant, T the absolute temperature, F the faraday. z_j is the valence of the jth ion and C_j^o and C_j^i are the external and internal concentrations. Numbers in parentheses following SE represent number of measurements.

V_{vo} is constant (-76 mV) at the three salinities, and the equilibrium potentials are also constant ($p > 0.1$). Fig. 4 shows that V_{vo} is close to E_K and is very sensitive to $[K^+]_o$, which suggests that V_{vo} is primarily a K^+ diffusion potential. However, K^+ diffusion alone cannot account for the curve shown in Fig. 4, because V_{vo} bends away from E_K at low $[K^+]_o$ and because V_{vo} is slightly but consistently more negative than E_K at high values of $[K^+]_o$. The implications of these data will be discussed later.

Subjecting the tissue to darkness causes a rapid (1–6 min), small (3–6 mV), positive shift in V_{vo}. This slight depolarization lasts throughout the dark period and is reversed by light. Cyanide at 1 mM has no effect on V_{vo}, but 5 mM cyanide causes a small (3–6 mV) positive shift in V_{vo} within 35 min.

The protoplast resistances are not significantly different ($p > 0.1$) at 30‰ (186 Ω cm^2) and 37‰ (215 Ω cm^2), but R_p at 23‰ (270 Ω cm^2) is significantly different ($p < 0.01$). This may be due to differences in salinity, or to the fact that these values were measured one month later than the others. The resistance values are highly variable, possibly due to the variability in the shape of the utricles, which causes errors in the surface area calculations. The measured values probably underestimate the true resistance, since they do not take into account the protoplasmic connection between the utricle and the filament. This connection almost certainly acts as a low resistance pathway for current.

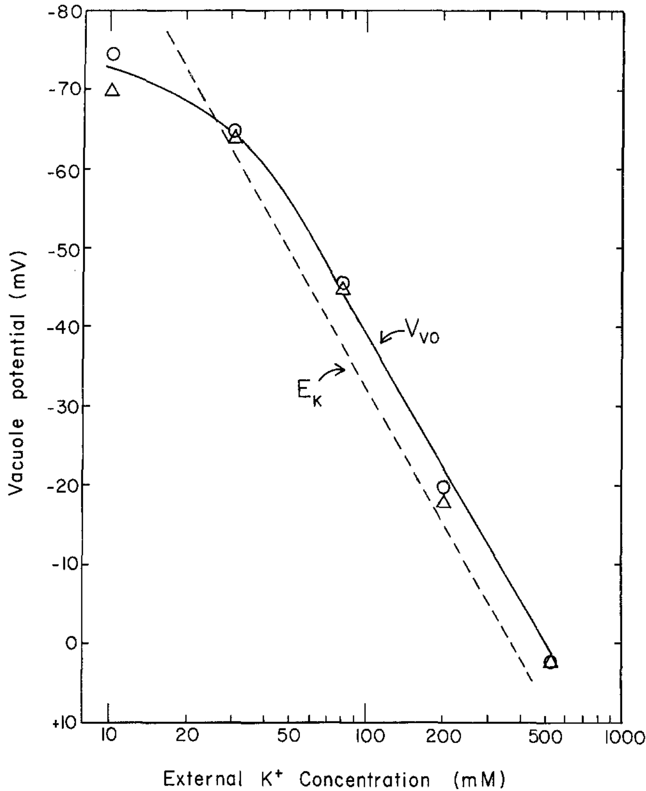

Fig. 4. The relation between vacuolar potential (V_{vo}) and external K^+ concentration. The circles and triangles represent results from two different utricles. The dashed line represents the equilibrium potential for K^+ (E_K)

Discussion

Before the data on intracellular ion concentrations and osmotic pressures can be discussed, it must be established that our treatment of the data is adequate. Errors in intracellular ionic concentrations and osmolalities can arise from at least three sources: (1) errors in our estimate of percent tissue water, (2) errors in either the volume or assumed composition of the extracellular fluid, and (3) existence of substantial amounts of "nonsolvent" water or "bound" ions in the tissue. The values for percent tissue water are easily obtained and do not vary significantly with season or salinity. Our estimate of the fraction of extracellular space is derived from three nonelectrolytes of quite different sizes, and agrees with that of Kesseler (1964*b*), who used Li^+ as the extracellular marker. Moreover, the calculation of intracellular concentrations and osmotic pressures is not very sensitive to the value assumed for extracellular space,

which appears in both the numerator and the denominator of Eqs. (2) and (3). For example, a 20% change in the estimate of ECS causes less than 0.2% change in the estimate of Π_i and 0.5–3% change in the ion concentrations. There is no simple way of testing our assumption that the ECS has the same composition as seawater. In plant cells Donnan forces arising from fixed negative charges in the cell wall can affect the ionic composition of the ECS. However, in a solution of high ionic strength, such as seawater, these effects will be small, especially for univalent ions. Finally, the similarity between the osmolalities of the intracellular fluids and our artificial sap solutions (Table 2) indicates either that the amounts of both nonsolvent water and bound ions are small or that these two sources of error fortuitously cancel.

There are additional systematic errors in our estimates of osmotic pressure and turgor. In concentrated solutions such as seawater the van't Hoff equation (Eq. 1) overestimates the true $\Delta\Pi$ by about 2% (Hastings & Gutknecht, *unpublished*). Since this error is about the same at all salinities, it will not hinder our comparisons of $\Delta\Pi$ at different salinities. In addition, turgor (ΔP) is slightly overestimated by $\Delta\Pi$. The relationship between ΔP and $\Delta\Pi$ is described by the equation

$$J_v = L_p(\Delta P - \sigma \Delta\Pi) \tag{4}$$

where J_v is the volume flow, L_p is the hydraulic conductivity, and σ is the reflection coefficient for the osmotically important solutes (Kedem & Katchalsky, 1958). In growing cells, such as those of *Codium*, ΔP will be slightly overestimated by $\Delta\Pi$ due to the fact that $J_v < 0$. (By convention, an inward volume flow is designated as negative.) The true turgor pressure (ΔP) can be estimated from J_v and Eq. (4). From morphological estimates of the surface area of the tissue (about 200 $cm^2\ g^{-1}$) and the growth rate, J_v is roughly minus $7 \times 10^{-9}\ cm^3\ cm^{-2}\ sec^{-1}$. Form the initial rate of shrinking when *Codium* is placed in hypertonic seawater we estimate L_p to be about 7×10^{-7} cm $sec^{-1}\ atm^{-1}$. From Eq. (4) we can compute a minimum error of about 0.5% in estimating ΔP from $\Delta\Pi$, using the above figures for J_v and L_p, $\Delta\Pi = 2.3$ atm, and $\sigma = 1$, since from Eq. (4),

$$\Delta P = \sigma\Delta\Pi + J_v/L_p = 2.3 + (-7 \times 10^{-9}) = 2.29.$$

If $\sigma < 1.0$, this error will increase. However, water permeability is several orders of magnitude higher than the inorganic ion permeabilities in all the large algal cells which have been studied, and our preliminary measurements suggest that this is also true for *Codium*. Thus, our assumption that $\sigma \cong 1.0$ is a reasonable one. Moreover, as in the case of estimates

of $\Delta\Pi$, these errors will not change with salinity, and hence will not invalidate our comparisons of ΔP.

From the results shown in Tables 1 and 2 we conclude that *Codium* regulates its ionic and osmotic composition in response to changes in external salinity. Since $\Delta\Pi$ is maintained constant at 95 mosmol/kg (about 2.3 atm) over the entire range of salinities normally encountered by *Codium,* we conclude that *Codium* is regulating its turgor pressure. The slope of the linear regression of $\Delta\Pi$ (mosmol/kg) on salinity ($^0/_{00}$) is -0.29 ± 0.62, which is not significantly different from zero. Thus, we conclude that turgor regulation is 100% efficient over this range. Turgor regulation has been established in two other green algae, *Valonia* (Hastings & Gutknecht, 1974; Zimmermann & Steudle, 1974) and *Chaetomorpha* (Kesseler, 1946*a*; *see also* Steudle & Zimmermann, 1971; Zimmermann & Steudle, 1971).

Having established that osmotic regulation of turgor does occur, we wanted to determine whether the response of *Codium* to hypertonic stress is triggered by the increase in external ionic concentrations or by the increase in external Π. Substituting raffinose for salts enabled us to separate the two variables. Table 3 shows that the increase in Π_i in response to 210 mM raffinose in $30^0/_{00}$ seawater is the same as the increase which would have been produced if the increased Π_o were due to salt. Thus, the response to increased salinity can be attributed to the increase in Π_o.

We have eliminated P_i as the regulated parameter, since an increase in external hydrostatic pressure did not cause a change in turgor pressure. If the cells were regulating to a constant $P_i = \Delta P + P_o$, a 2 atm increase in P_o should have caused a decrease in ΔP to almost zero. We have not clearly distinguished between $\Delta\Pi$ and ΔP as possible stimuli for turgor regulation, because we are unable to vary these two parameters independently in *Codium.* In *Valonia* both $\Delta\Pi$ and P_i have been eliminated as possible stimuli, and turgor regulation is triggered by changes in ΔP (Gutknecht, 1968; Hastings & Gutknecht, 1974; *also, unpublished observations*). In *Nitella,* however, Π_i rather than ΔP appears to be regulated (Nakagawa *et al.,* 1974), but in fresh water $\Pi_o = 0$ and is constant, so that $\Delta\Pi = \Pi_i$ (*see* Cram, 1975). If *Codium* were regulating Π_i rather than $\Delta P,$ it would have to measure both Π_i and Π_o, and compute $\Pi_i - \Pi_o$, which is a small difference between two large numbers (*cf.* Table 2) and thus subject to considerable error of measurement. Although at this point we cannot rule out a change in $\Delta\Pi$ as the stimulus for turgor regulation in *Codium,* we infer from this study and by analogy with *Valonia* that a change in turgor pressure (ΔP) is the signal for turgor regulation.

In *Codium,* Cl^- appears to be regulated perfectly in response to Π_o; the intracellular concentrations of Cl^- are indistinguishable in tissues acclimated to 37⁰/₀₀ seawater and to 30⁰/₀₀ seawater + raffinose ($p > 0.2$) (Table 3). However, $[K^+]_i$ appears not to be affected by Π_o, but solely by $[K^+]_o$. $[K^+]_i$ is actually less in 30⁰/₀₀ + raffinose than in 30⁰/₀₀, apparently due to a decrease in molarity of K^+ due to the large partial molar volume of raffinose. Under these conditions, $[Na^+]_i$ increases to a concentration higher than that found in tissue acclimated to 37⁰/₀₀, apparently to compensate in charge and osmolality for the "missing" K^+.

With regard to the nature of the ionic transport processes involved in turgor regulation, we can tentatively conclude from the data at hand (Table 4) that Na^+, Cl^- and SO_4^{2-} are actively transported in *Codium.* This conclusion is based on the fact that in the steady state these ions are maintained far from equilibrium; $V_{vo} - E_{Cl} = -77$ mV, $V_{vo} - E_{Na} = -88$ mV, $V_{vo} - E_{SO_4} = -88$ mV. The direction of the driving forces indicates that Na^+ is pumped out of the vacuole and Cl^- and SO_4^{2-} are pumped in. Although E_K is somewhat more negative than V_{vo} ($V_{vo} - E_K = +14$ mV), the difference is too small to establish that K^+ is actively transported into the vacuole. If our recorded value of V_{vo} is slightly more negative than the true V_{vo}, and if cytoplasmic $[K^+]$ is higher than the vacuolar $[K^+]_i$, then K^+ could be in electrochemical equilibrium between vacuole and seawater.

V_{vo} is close to E_K and is very sensitive to $[K^+]_o$, which suggests that V_{vo} is predominantly a K^+ diffusion potential (Fig. 4). However, the relationship between V_{vo} and $[K^+]_o$ cannot be generated by the Goldman equation or by any other diffusion equation which takes into account only the diffusion of K^+, Na^+ and Cl^-, because the equilibrium potentials for all these ions are more positive than V_{vo} over most of the range shown in Fig. 4. The curve can be obtained, however, by making some reasonable assumptions about the cytoplasmic compartment, i.e., (1) $[K^+]^{cyt} > [K^+]^{vac}$, $[Na^+]^{cyt}$ and $[Cl^-]^{cyt} \leq 100$ mM, (2) the potential drop across the tonoplast is zero or slightly positive, and (3) the K^+ permeability of the plasmalemma is much higher than the Na^+ or Cl^- permeability. The curve can also be obtained using the ionic concentrations in Table 1 and by postulating a K^+-selective membrane and, in addition, an electrogenic pump which makes a small (-5 to -10 mV) contribution to V_{vo}. This latter hypothesis is consistent with the small depolarization caused by darkness and cyanide. However, without knowing the ionic concentrations or the electrical potential of the cytoplasm it is impossible to distinguish among the several plausible models.

The osmotic and ionic relations of two European *Codium* species were studied by Kesseler (1965), who showed that *Codium decorticatum* growing in 38‰ seawater near Naples has a $\Delta\Pi$ of 2.9 atm, similar to the $\Delta\Pi$ of 3.6 atm in *C. fragile* growing in 30‰ seawater near Helgoland (Table 5). This is indirect evidence for turgor regulation in *Codium,* now confirmed by the present study. There are distinct differences, however, among the different species and populations of *Codium.* The European population of *C. decorticatum* is "low K^+" ($[K^+]_i/[Na^+]_i=0.02$), whereas our populations is "high K^+" ($[K^+]_i/[Na^+]_i=1.1$). The difference is not due to any obvious environmental differences, since in our alga the high K^+/Na^+ ratio is maintained during all seasons and over a wide range of salinities. The ionic composition thus seems to be a genetic difference between the two geographically separated populations. Both the European and the North American *C. fragile* are low K^+ ($[K^+]_i/[Na^+]_i=0.02$ for the American alga and 0.03 for the European). An interesting question for future study is whether any turgor regulation mechanism which exists in the low K^+ populations is basically different from that in the high K^+ population.

Table 5. Na^+ and K^+ concentrations and estimated turgor pressures in two species of *Codium* from several locations

Species, collection site and salinity	Intracellular concentrations		Turgor	Reference
	Na^+ (mmol/kg H_2O)	K^+ (mmol/kg H_2O)	(atm)	
C. decorticatum				
Beaufort, N.C.				
23‰	212	210	2.4	
30‰	257	295	2.3	This study
37‰	302	368	2.3	
C. decorticatum (formerly *C. tomentosum*)				
Naples, 38‰	656	15	2.9	Kesseler, 1965
C. fragile				
Helgoland, 30‰	475	16	3.6	Kesseler, 1965
C. fragile				
Connecticut (salinity unknown)	660	16	—	Graves, 1974

The concentrations in the Kesseler (1965) study are in mM. The genus *Codium* was reorganized by Silva (1960) so that the species *C. tomentosum* studied by Kesseler is now classified as *C. decorticatum.*

In conclusion, we have shown that *Codium decorticatum* is capable of maintaining a constant turgor pressure over the range of salinities which it normally encounters. It regulates Π_i primarily by regulating the internal concentrations of K^+, Na^+, Cl^- and SO_4^{2-}. When Π_o is altered independently of salt concentrations, $[Na^+]_i$ and $[Cl^-]_i$ change with Π_o, but $[K^+]_i$ seems to be determined by $[K^+]_o$. Comparing the vacuole potential to the equilibrium potentials of the major ions suggests that Cl^- and SO_4^{2-} are actively absorbed, whereas Na^+ is actively extruded. Future papers in this series will describe the time course of changes in ionic and osmotic composition and the nature of the fluxes of Na^+, K^+ and Cl^-.

Dr. J.S. Graves did the experiments on the effect of light and metabolic inhibitors on the vacuole potential of *C. decorticatum*. Dr. H. Fyhn performed the microcryostatic determinations of osmotic pressure. Dr. W.F. Blankley provided the Nomarski microscope for our use. We thank D.F. Hastings for helpful discussions and advice throughout the course of this work. The proficient technical assistance of M.P. Bradley is gratefully acknowledged. This work was supported by USPHS Grant HL 12157.

Note Added in Proof: The species which Kesseler worked with in the Mediterranean, which he identified as *Codium tomentosum*, was misidentified by us as *C. decordicatum* (*see* legend of Table 5). The true identity of this species according to P.C. Silva is *Codium vermilara*. This is confirmed by the difference in ionic composition between *C. decorticatum* and *C. vermilara*.

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