

PHYSIOLOGICAL EFFECTS OF SALINITY ON
CHARA CORALLINA

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requirement for the degree of Doctor of Philosophy

by

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DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, it contains no material previously published or written by any other person, except where due reference is made in the text.

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SUMMARY

The effect of salinity on the transport of ions across the plasma membrane of *Chara corallina* was investigated. Detached internodes of *Chara corallina* tolerated solutions containing 100 mol m^{-3} NaCl if the concentration of Ca^{2+} in the external solution was greater than 1 mol m^{-3} . Elevated Na^+ concentrations in the external solution resulted in an increase in vacuolar Na^+ and to a lesser degree, a decrease in vacuolar K^+ . Charge balance was maintained by accumulation of Cl^- . Growth rate of *Chara* was inhibited by low turgor, the yield threshold for cell expansion being 0.29 MPa . However over the long term, low turgor or ion concentration did not affect HCO_3^- fixation or cytoplasmic streaming rate.

The presence of Ca^{2+} in the external solution was essential for the protection of the cell from salt damage from a variety of group 1A cations including Na^+ , K^+ , Li^+ and Rb^+ . The cell responded to elevated levels of these cations in the external medium by one of two methods. In the case of Na^+ and Li^+ the cell maintained a relatively constant internal osmotic potential, whereas in solutions containing K^+ or Rb^+ the cell accumulated K^+ or Rb^+ and thus maintained a relatively constant turgor. Charge balance was maintained by accumulation of Cl^- and/or the production and accumulation of the carboxylic acid, malate.

Malate was accumulated in the vacuole when the cell was bathed in the K^+ salts of SO_4^{2-} , NO_3^- , MES^- or Cl^- at a rate of up to $0.45 \mu\text{mol g}^{-1} \text{ h}^{-1}$. Malate synthesis occurred by the carboxylation of PEP and was sensitive to the external HCO_3^- concentration. During malate accumulation H^+ was pumped from the cell at a rate of approximately $40 \text{ nmol m}^{-2} \text{ s}^{-1}$; therefore the H^+ -pump was operating despite the cell

being depolarized ($PD \cong -42 \text{ mV}$).

Both the influx of Na^+ and K^+ increased with increasing external concentrations of these ions. Influx of both ions appeared to be biphasic. Influxes of Na^+ and K^+ were sensitive to the concentration of Ca^{2+} in the external medium, with both fluxes having a Ca^{2+} sensitive and Ca^{2+} insensitive component. The similarities between the influx of Na^+ and K^+ such as the inhibition of influx by external Ca^{2+} and Ba^{2+} suggest a common transport mechanism across the plasma membrane.

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SYMBOLS AND ABBREVIATIONS

APW	- artificial pond water
ATP	- adenosine 5'-triphosphate
ATPase	- adenosine triphosphatase
C_4	- photosynthetic dicarboxylic acid pathway
C_N	- total DMO per unit volume in phase N
CAPS	- 3-[cyclohexylamino]-1-propane-sulphonic acid
CAM	- crassulacean acid metabolism
CHES	- 2[N-cyclohexylamino]ethane sulphonic acid
CPW	- chara pond water
CTC	- chlorotetracycline
DCCD	- N,N'-dicyclohexylcarbodiimide
DMO	- 5,5-dimethyl-2,4-oxazolidinedione
ϵ	- elastic modulus
E_N	- Nernst equilibrium potential for an ion N
EDTA	- ethylenediaminetetraacetic acid
EGTA	- ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N' tetraacetic acid
EPSP	- N-[2-hydroxyethyl]piperazine-N'-3-propanesulphonic acid
I_d	- incident radiation at depth d
I_o	- incident radiation at the waters surface
IAA	- indole-3-acetic acid
IBA	- indole-butyric acid
F	- Faraday's constant (= 9.65×10^4 C mol ⁻¹)
HEPES	- N-[2 hydroxyethyl]piperazine-N'-[2 ethane sulphonic acid]
k	- extinction coefficient

J_N	- net influx of ion N
Li-Ca-CPW	- Li^+ and Ca^{2+} supplimented CPW
m	- yielding tendancy of the cell wall
MES	- 2-(N-morpholino)ethanosulphonic acid
NAA	- naphthaleneacetic acid
Na-CPW	- Na^+ supplimented CPW
Na-Ca-CPW	- Na^+ and Ca^{2+} supplimented CPW
NAD^+	- nictotinamide adenine dinucleotide (oxidized form)
NADH	- nictotinamide adenine dinucleotide (reduced form)
OAA	- oxaloacetate
P	- turgor potential
PEP	- phosphoenolpyruvate
pH_o	- pH of the external solution
pH_c	- pH of the cytoplasm
pH_v	- pH of the vacuole
Π	- osmotic potential
r	- growth rate
R	- gas constant
Rb-Ca-CPW	- Rb^+ and Ca^{2+} supplimented CPW
T	- absolute temperature
TAPS	- N-tris[hydroxymethyl]-3-aminopropanesulphonic acid
TEA	- tetraethylammonium
TES	- N-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid
U_N	- unionized DMO per unit volume in phase N
V	- cell volume
Y	- threshold turgor
Z	- valency of ion

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CHAPTER 1.

Introduction and Review

1.1 INTRODUCTION

The mechanisms of salt tolerance are some of the most studied subjects in plant physiology. Many of these studies have focussed on the interaction of external Ca^{2+} and salt tolerance. However, most of these studies have been undertaken using vascular plants where the complexity of plant structure complicates the interpretation of results. In contrast, there are few studies on the mechanisms of salt tolerance at the cellular level; yet understanding the cellular mechanisms of salt stress and salt tolerance is essential for elucidating mechanisms at the level of tissues or whole plants (Katsuhara and Tazawa 1986).

The giant internodal cells of the charophyte algae have been used to study ion transport and recently studies have been devoted to the topics of salt stress and salt tolerance (eg. Katsuhara and Tazawa 1986, 1987, 1988; Bisson and Bartholomew 1984; Bisson and Kirst 1983). In this thesis I attempt to further the understanding of salt tolerance in plants by investigating the physiological effects of "higher than normal" concentrations of K^+ and Na^+ on internodal cells of *Chara corallina*. Included in this thesis are studies concerning the survival, growth and ionic relations of the cell. The transport of K^+ and Na^+ across the plasma membrane has also been studied. The role that Ca^{2+} plays in the above processes has also been investigated.

1.2 Problems associated with salinity.

Salinity imposes problems for the plant cell because the concentrations of ions in the environment are higher than those to

which the species has become adapted for optimum growth (Greenway and Munns 1980). The degree to which growth and development can be maintained during non optimal ionic or osmotic conditions is referred to as salinity resistance (Yeo 1983). When challenged with a saline habitat a plant cell can either adapt osmotically by accumulation of solute or otherwise contend with a reduction in turgor. Herein lies the Scylla-Charybdis dilemma alluded to by Greenway and Munns (1980). Accumulation of solutes may lead to ion excess; however, a reduction in cell turgor may inhibit both the structural integrity of the plant as well as growth and development (Gutknecht et al. 1978).

Salt concentrations in the environment cause the cell a number of problems. Many enzymes and certain metabolic processes are sensitive to ionic concentration (Greenway and Munns 1980, Yeo 1983). Higher than "normal" concentrations of ions in the external environment are likely to influence membrane potential. For example millimolar concentrations of K^+ can result in the depolarization of the cell membrane potential (Hope and Walker 1961). On the other hand nutrient deficiencies can occur where the total tissue ion content is not damaging *per se* but where the quantity of a particular ion is inadequate.

It need not be the total concentration but the ratio of one ion to another that causes problems for the cell. For example Jeschke (1984) has discussed the specific effects of a high Na^+ to K^+ ratio in the cytoplasm. An ionic imbalance where there is a high ratio of one ion to another may be due to the failure of the selectivity of a transport process which has not evolved to cope with such ranges in salinity. Also the contribution of non-specific or relatively minor transporters may become dominant or membrane leakage of ions may occur as the concentration of ions in the environment increases.

Plants in saline environments have to contend with a lowered water potential of the medium. This results in a reduction in turgor unless the plant cell regulates internal osmotic potential. Turgor is the primary driving force for expansion growth as well as maintaining the form and rigidity of plant cells and tissues (Gutknecht *et al.* 1978).

1.3 The use of giant algal cells to study salinity tolerance.

Much has been written about salinity resistance in the whole plant (see reviews of Greenway and Munns 1980, Yeo 1983). However, understanding salinity tolerance in the whole plant is complicated by the specialization, compartmentation and transport of ions between cells and between tissues. To understand salt resistance at the whole plant level an understanding of the cellular mechanisms of salt tolerance is essential. It is here that the isolated internodes of the charophyte algae are potentially useful. The giant cells of these algae alleviate many of the problems associated with studies on the whole plant therefore allowing a study concentrated on the cellular mechanisms of salinity tolerance.

Giant celled algae belong to a variety of taxonomic groups but most are green algae in the broadest sense (Raven 1976) and include members of the Chlorophyceae and the Charophyceae. Members of the Charophyceae live in a wide variety of habitats from fresh to hypersaline waters and not surprisingly, different charophytes have evolved different responses to salinity. To date, salinity responses for the following charophytes have been described; from brackish waters, *Chara buckellii* (Hoffmann and Bisson 1986), *C. vulgaris* (Kirst *et al.* 1989), *Lamprothamnium papulosum* (Bisson and Kirst 1980) and *L. succinctum* (Ozaki *et al.* 1984) and from fresh waters, *Chara corallina* (Bisson and Bartholomew 1984), *Nitella translucens* (Tazawa and Nagai

1966) and *Nitellopsis obtusa* (Katsuhara and Tazawa 1986). This study concentrates on the effects of salinity on *Chara corallina*.

1.3.1 Chara corallina.

The giant internodal cells of *C. corallina* have a large central vacuole surrounded by a layer of cytoplasm. Internodes of *C. corallina* used in this study were typically 3-5 cm long and 1mm wide having a volume of about 20 μ l. The vacuole occupies between 90% (Okihara and Kiyosawa 1988) and 95% (Sakano and Tazawa 1984) of the cell volume.

The main cations in the vacuole of *C. corallina* are K^+ and Na^+ (Table 1) with the ratio of K^+ to Na^+ usually exceeding one. However, this ratio can change. For example young cells have a higher ratio of K^+ to Na^+ than older cells (MacRobbie 1962). Ion concentration of the vacuole will also vary with culture conditions. Other cations found in the vacuole include Ca^{2+} and Mg^{2+} (Okihara and Kiyosawa 1988). Cl^- is the main anion in the vacuole with smaller amounts of SO_4^{2-} and PO_4^{2-} .

Okihara and Kiyosawa (1988) measured the concentrations of various ions in the cytoplasm (Table 1). As in the vacuole there is a higher concentration of K^+ than Na^+ . The cytoplasmic ratio of K^+ to Na^+ does vary. Katsuhara and Tazawa (1988) report that in *Nitellopsis obtusa* high concentrations of NaCl cause an increase in cytoplasmic Na^+ and a corresponding decrease in cytoplasmic K^+ .

1.3.2 The effect of salinity on Chara corallina.

Prior to the commencement of this project Bisson and Bartholomew (1984) had reported that 25 mol m^{-3} NaCl was lethal to *C. corallina* and Lucas and Alexander (1981) had shown 20 mol m^{-3} NaCl to be lethal under turgor reduced conditions. In both of these studies the external Ca^{2+} concentration was 0.1 mol m^{-3} . At that time however, *C. corallina*

TABLE 1.1 Ion composition of vacuole and cytoplasm of *C. corallina*

	reference	ion concentration (mol m ⁻³)								II
		K ⁺	Na ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	H ₂ PO ₄ ²⁻	NO ₃ ⁻	SO ₄ ²⁻	
vacuole	1	108	56	7.6	5.4	104	6.9	3.3	-	
	2	80	54	4.5	3.2	106	9.5	0.5	6.4	284
	3	112	28			161				
	4	65	51			89				
	misc.	70 ^a	50 ^c		2.6 ^d	110 ^b			17 ^e	
cytoplasm	1	80	21	4.7	13.2	40	21	22		
	3	112	3			21				
	4	108	4.2			29				
	5	115				10				

1. Okihara and Kiyosawa (1988)
2. Bisson and Bartholomew (1984)
3. Tazawa *et al.* (1974)
4. Sakano and Tazawa (1984)
5. Vorobiev (1967)
- a. Hope and Walker (1961)
- b. Vorobiev (1967)
- c. Coster (1965)
- d. Coster and Hope (1968)
- e. Robinson (1969)

was being cultivated in the laboratories of Dr F. A. Smith (University of Adelaide), in solutions containing up to 25 mol m^{-3} NaCl. The essential differences between Smiths cultures and the cells in the experiments described above were a) the studies described by Bisson and Bartholomew (1984) and Lucas and Alexander (1981) used detached internodes whereas the cultures of Smith were growing *in situ* and b) the culture medium used by Dr F A Smith contained approximately $2 \text{ mol m}^{-3} \text{ Ca}^{2+}$. One of the initial aims of this project was to investigate the role of Ca^{2+} in protecting the cell from salinity damage particularly to detached internodes.

Since then Tufariello *et al.* (1988) and Hoffmann *et al.* (1989) have shown that detached internodal cells can survive for at least a few weeks in 70 mol m^{-3} NaCl whilst the ratio of Na^+ to Ca^{2+} in the external medium is maintained at 10 to 1. They found that increasing the concentration of NaCl whilst maintaining a constant Ca^{2+} concentration (at 0.1 mol m^{-3}) resulted in a depolarization of cell membrane potential difference, an influx of Na^+ and an efflux of K^+ from the cell (Tufariello *et al.* 1988). When Ca^{2+} was increased simultaneously (so the ratio of Ca^{2+} to Na^+ remained at 10:1) Na^+ influx remained at the lower pre-salt treatment level (Hoffmann *et al.* 1989). These results led Tufariello *et al.* (1988) to suggest that *C. corallina* fails to occupy saline habitats because of a failure to regulate turgor not because of sensitivity to Na^+ .

Salt tolerance of the fresh water charophyte *Nitellopsis obtusa* has been extensively studied by Katsuhara and Tazawa (1986, 1987, 1988, 1990). It is worthwhile reviewing their research because *N. obtusa* appears in many respects to behave as *C. corallina* when exposed to high NaCl concentrations.

1.3.3 The effect of salinity on *Nitellopsis obtusa*.

Katsuhara and Tazawa (1986) reported that detached internodes of *N. obtusa* did not survive 100 mol m^{-3} NaCl when the external Ca^{2+} concentration was 0.1 mol m^{-3} . The deleterious effects of 100 mol m^{-3} NaCl were not due to osmotic stress since isotonic sorbitol solutions had no effect on cell survival. Cell death in high NaCl was preceded by a simultaneous increase in cytoplasmic Na^+ and decrease in cytoplasmic K^+ . Cytoplasmic Cl^- remained constant. Addition of 100 mol m^{-3} also caused a depolarization of the cell membrane. These results led Katsuhara and Tazawa (1986) to conclude that Na^+ enters the cell in exchange for K^+ , and both processes were assumed to be passive.

Addition of 10 mol m^{-3} Ca^{2+} to the medium ameliorated the effects of adding 100 mol m^{-3} NaCl. Cytoplasmic K^+ and Na^+ remained constant and the cells remained alive for many days. Two mechanisms by which external Ca^{2+} could prevent salinity damage were postulated: Ca^{2+} might reduce plasma membrane permeability to Na^+ or external Ca^{2+} might stimulate active Na^+ extrusion. Ca^{2+} was effective in protecting cells against salt injury at 0°C when active processes would normally be assumed to be inhibited. Therefore, Katsuhara and Tazawa (1986) concluded that addition of millimolar levels of Ca^{2+} makes the plasma membrane impermeable to Na^+ .

The protective role of Ca^{2+} was also observed in perfused cells. However, when ATP was removed from the perfusion medium, addition of Ca^{2+} was ineffective. Katsuhara and Tazawa (1987, 1990) concluded that cytoplasmic ATP as well as external Ca^{2+} are necessary for reducing plasma membrane permeability to Na^+ . ATP was not being used as an energy source nor as a substrate for protein phosphorylation. Therefore, Katsuhara and Tazawa (1987, 1990) concluded that ATP was acting as a co-effector with Ca^{2+} in regulating the Na^+ permeability of

the membrane.

1.4 Terminology.

1.4.1 Osmo-terminology.

The terminology currently used for reporting studies of the osmotic responses of plant cells to salinity can be misleading. In an attempt to standardize the use of "osmo-terminology" Reed (1984) suggested the term osmoregulation be abandoned because of its inconsistent usage in the past. In its place are recommended the terms osmotic regulation and turgor regulation. Osmotic regulation is used to describe the process where osmotic potential of the cell is maintained constant by the cell in the face of external perturbation. The term turgor regulation should be used when turgor potential is maintained constant in response to changes in external osmotic potential. This convention has been adopted throughout this thesis.

1.4.2 Water Potential.

Water potential is generally accepted as the most suitable term for specifying the state of water in biological systems. It is derived from the chemical potential of the water and is frequently expressed as the sum of two components; P , the hydrostatic pressure or turgor potential and Π , the osmotic potential. Like all expressions for energy, water potential is relative, only differences are meaningful. For this reason osmotic potential is measured against a convenient reference point; free pure water at the same pressure and temperature as the system under investigation (see Dainty 1976). Therefore, in most biological systems, osmotic potential is negative and turgor potential is positive.

For small changes in cell volume, δV , we can write

$$\delta P = \epsilon \delta V/V$$

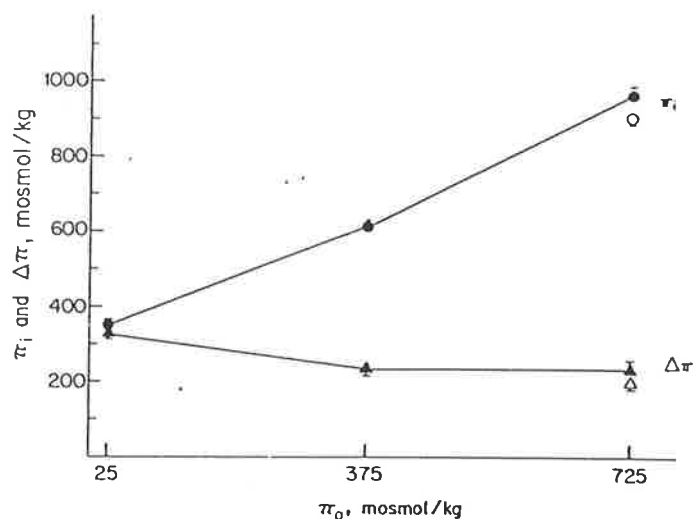
where ϵ is the elastic modulus and V is the cell volume (Dainty 1976). ϵ is an important parameter in cell water relations because it is often much greater than osmotic potential and so will control the way in which osmotic potential changes with cell volume. Measurements of elastic modulus for charophyte cells show that it can be quite high (eg. 760 bars for *C. corallina*; Dainty et al. 1974). A consequence of a large elastic modulus is that the pressure component handles changes in water potential (until plasmolysis) leaving osmotic potential relatively constant.

1.5 Osmotic regulation or turgor regulation in Chara.

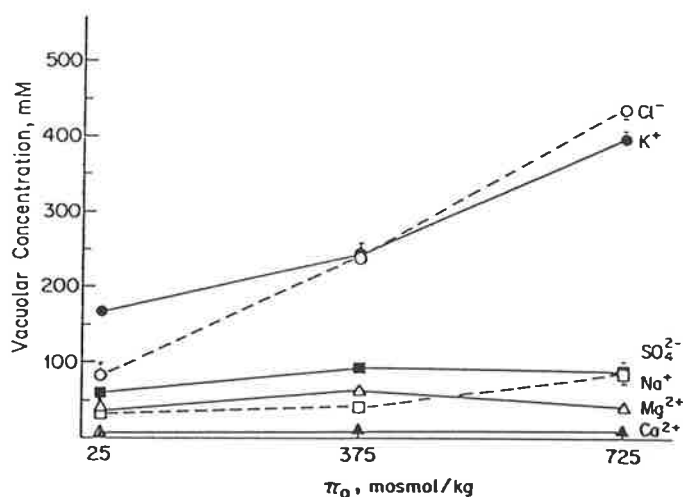
Many algae that colonize brackish water alter internal osmotic potential to maintain a constant osmotic gradient between the cell and the environment, thus resulting in turgor regulation. This strategy is clearly an advantage in environments where the osmotic gradient across the plasma membrane is greater than the osmotic potential of the cell, and variations in the external osmotic potential occur. Turgor regulation has been reported to occur in a number of marine algae (Gutknecht et al. 1978) and for the halotolerant charophytes *Lamprothamnium papulosum* (Bisson and Kirst 1983), *L. succinctum* (Ozaki et al. 1984), *Chara buckellii* (Hoffmann and Bisson 1986), *C. vulgaris* (Kirst et al. 1989) and *C. inflata* (Smith 1989). Turgor regulation is usually achieved by the accumulation of K^+ and Cl^- in the vacuole (eg. *C. buckellii* (Hoffmann and Bisson 1986) and *L. papulosum* (Bisson and Kirst 1983), see Figure 1.1). However, some algae achieve turgor regulation by accumulating Na^+ and Cl^- in their vacuole, for example *C.*

Figure 1.1 Turgor regulation in *C. buckellii*.

Figure reprinted from Hoffmann and Bisson (1986).



Internal osmotic pressure (π_i) and turgor pressure ($\Delta\pi$) of AWW-cultured *Chara buckellii* cells as a function of the external osmotic pressure (π_o) on day 10 of acclimation experiments for dilute ($\pi_o = 25$ mosmol/kg), control ($\pi_o = 375$ mosmol/kg), and evaporative ($\pi_o = 725$ mosmol/kg) treatments. Results from sorbitol treated cells ($\pi_o = 725$ mosmol/kg) are indicated by the open symbols for both π_i and $\Delta\pi$. Mean \pm SE, $n = 11-16$.



Vacuolar ionic concentrations (millimolar) of AWW-cultured *Chara buckellii* cells as a function of external osmotic pressure (π_o) on day 10 of acclimation experiments for dilute ($\pi_o = 25$ mosmol/kg), control ($\pi_o = 375$ mosmol/kg), and evaporative ($\pi_o = 725$ mosmol/kg) treatments. Mean \pm SE, $n = 5-17$.

inflata (Smith 1987).

Freshwater algae live in an environment where the external osmotic potential is much lower than the osmotic pressure of the cell. Changes in the osmotic potential of the external solution are generally small relative to the difference between internal and external osmotic potential so cell turgor remains relatively constant (Bisson and Kirst 1983). Consequently many freshwater algae maintain a constant internal osmotic potential to maintain a constant cell turgor (Kamiya and Kuroda 1956, Sanders 1981a, Bisson and Bartholomew 1984, Reed 1984).

Bisson and Bartholomew (1984) reported that *C. corallina* maintains a reasonably constant internal osmotic potential when external osmotic potential is increased with a non-permeating osmoticum (such as raffinose). However, Sanders (1981a) argued that it is unlikely that osmotic potential is an efficiently controlled parameter in *C. corallina* because a 30% increase in osmotic potential after isolation of internodal cells was observed. Bisson and Bartholomew (1984) also observed an increase in osmotic potential after addition of 25 mol m^{-3} NaCl to the external solution. They concluded that this increase in osmotic potential, whilst significant was not enough to compensate for the difference in external water potential, therefore cell turgor declined. Bisson and Bartholomew (1984) considered this partial turgor regulation as a failure of the osmotic regulatory system of *C. corallina* at high external NaCl concentrations. The results of Sanders (1981a) and Bisson and Bartholomew (1984) support the hypothesis that *C. corallina* does not effectively control turgor and they have also shown that under certain conditions *C. corallina* does not regulate osmotic potential.

1.6 Effect of salinity on ion transport.

1.6.1 HCO_3^- .

Sanders (1981a) and Lucas and Alexander (1981) reported that turgor reduction has a negligible affect on HCO_3^- and OH^- transport (until the cell is plasmolyzed). However, there was a 10 fold stimulation of the apparent carbon fixation rate following turgor reduction when carbon availability was not limiting. Enhanced carbon fixation was not reflected in an increase in net synthesis of vacuolar osmoticum. Sanders (1981a) concluded that fixation of carbon was probably not a good indicator of net synthesis and may simply reflect a faster turnover of organic compounds.

1.6.2 Na^+ .

Thermodynamic calculations indicate the influx of ions across the plasma membrane of *C. corallina* is a reaction favored by an electrochemical potential difference. Therefore it follows that the removal of Na^+ from the cell must be an active process (Smith 1967, Findlay et al 1969).

Little has been reported on Na^+ influx in *C. corallina*. It has been generally assumed that Na^+ moves into the cells by diffusion and independently of other ions (Hope and Walker 1975). If this is the case then net influx should be represented by the Goldman Flux equation:

$$J_{\text{Na}_{\text{co}}} = -P \cdot \frac{zFE_{\text{co}}}{RT} \left[\frac{\text{Na}_o - \text{Na}_i \exp(zFE_{\text{co}}/RT)}{1 - \exp(zFE_{\text{co}}/RT)} \right] \quad \text{equation 1}$$

where J = net Na^+ influx, P = passive permeability of the membrane to Na^+ , z = valency of Na^+ (=1), F = the Faraday, R = Gas constant, T = absolute temperature, Na_i^+ and Na_o^+ internal and external concentrations

of Na^+ and E_{co} = electrical potential difference of the cell cytoplasm relative to the bathing medium (see Smith 1967).

The net Na^+ influx is the difference between Na^+ influx and Na^+ efflux. Applying equation 1, Na^+ influx is expressed by the following (Smith 1967):

$$J_{\text{Na}_{\text{co}}} = -P \cdot \frac{FE_{\text{co}}}{RT} \left[\frac{\text{Na}_o}{1 - \exp(FE_{\text{co}}/RT)} \right] \quad \text{equation 2}$$

This equation assumes passive, independent movement of Na^+ across the plasma membrane and a linear potential gradient within the membrane (Hope and Walker 1975).

Hope and Walker (1960) reported Na^+ influx into *C. corallina* to be reduced in the dark and suggested that uptake may be mediated by transport systems that are controlled by chemical reactions. Findlay et al. (1969) reported that Na^+ influx was generally reduced in the dark and in the absence of external K^+ influx increased marginally. Smith (1967) proposed that Na^+ influx into *Nitella translucens* is linked to an inward Cl^- pump. Links between Cl^- and Na^+ could be maintained by effects of Cl^- on membrane potential difference or alternatively by chemical coupling of the two processes.

Hoffmann et al. (1989) found Na^+ influx into *C. corallina* to increase as a function of external Na^+ concentration. During the increase in Na^+ concentration the permeability of the plasma membrane to Na^+ remained constant so the increase in Na^+ influx was due to the increase in external concentration alone.

Clint and MacRobbie (1987) suggested that sodium efflux from *C. corallina* proceeds via an antiporter coupled to proton influx. Using

perfused cells they were able to show that sodium efflux to be ATP dependent, stimulated by low pH, high internal Na^+ concentrations and to be sensitive to DCCD and amiloride.

1.6.3 K^+ .

There have been many studies of K^+ influx into characean cells (eg. Hope 1963, Findlay et al. 1969, Beilby 1985, 1986a and b, Smith Smith and Walker 1987, Smith J R 1987, Smith and Walker 1989). These studies have shown that K^+ is generally close to thermodynamic equilibrium across the plasma membrane, especially when the H^+ -pump is inactivated. Often the membrane potential difference is more negative than the K^+ diffusion potential, E_k , (such as when the H^+ -pump is operational) and K^+ influx is then an electrogenically "downhill" process.

Voltage clamp studies using *C. corallina* have shown that under certain conditions membrane potential difference is dominated by K^+ transport across the plasma membrane via a K^+ -uniport; this is referred to as the K^+ -state (Beilby 1985, 1986). A number of experimental procedures including increasing external K^+ concentration or removing external Ca^{2+} result in the membrane potential difference becoming sensitive to external K^+ concentration. Voltage clamp studies using *C. corallina* have shown that in the K^+ -state, membrane potential difference is dominated by K^+ transport via channel proteins (Beilby 1985, 1986). The application of the patch clamp technique to giant algal cells has led to the description of a large conductance K^+ -channel from cytoplasmic droplets of *C. corallina* (Laver and Walker 1987). The K^+ -channel type described has voltage dependent properties very similar to the K^+ -uniport system described by Beilby (1985).

A comprehensive study using both electrical and tracer techniques

suggested that the transport of K^+ ions across the plasma membrane is usually passive, and independent of other ions during passage across the membrane (Smith JR. 1987, Smith JR. et al. 1987a,b, Smith and Kerr 1987). However, Tester (1988) has shown the selectivity of K^+ -channels to various monovalent ions to change depending upon the method of measurement. This led to the suggestion that ion movement through K^+ -selective channels may not be independent. The results from papers of Smith JR. (1987), Smith JR. et al. (1987 a,b) and Smith and Kerr (1987) suggest that there are two different modes of K^+ transport across the plasma membrane. The relative contributions to total K^+ flux depend upon the composition of the external medium and membrane potential difference.

The application of various animal K^+ -channel inhibitors showed that K^+ influx could be reduced to what was termed a "leak current" (Smith and Kerr 1987, Tester 1988a). Tester (1988a) concluded that there were two mechanisms of K^+ influx, one of which could be inhibited by K^+ -channel blocking agents and the other could not. Smith and Kerr (1987) also suggested that $^{42}K^+$ influx could be split into two components, one that could be inhibited by externally applied Ca^{2+} and TEA⁺ and one that which was uninhibitable.

K^+ fluxes across the plasma membrane are usually discussed in terms of passive diffusion. Net passive movement of K^+ , whether through channels or by simple diffusion can only occur when K^+ influx is an energetically downhill process. In many situations the cell membrane potential is hyperpolarized with respect to E_k . Under steady state conditions there would have to be an energetically uphill efflux mechanism. To account for this Walker and Smith (1977) postulated a K^+ - H^+ antiport where K^+ efflux is coupled to the energetically favored H^+ influx. This scheme was modeled on the K^+ - H^+ antiport described

from *E. coli* (Brey, Rosen and Sorenson 1980).

Smith and Walker (1989) have recently described Na^+ linked K^+ influx in *C. corallina*. The most likely mechanism is symport of K^+ with Na^+ with a stoichiometric ratio of 1:1. This porter is electrogenic and its functional significance seems to be for the active short term uptake of K^+ using the electrochemical potential difference for Na^+ .

1.7 The carrier-kinetic approach: dual mechanisms of ion transport.

Studies on the influx of K^+ and other ions into roots of higher plants led Epstein and Hagen (1952) to suggest that transport for a number of ions was mediated by two transport mechanisms involving carriers. Since then dual transport mechanisms have been described for a wide variety of both ions and tissue types (see review of Epstein 1976). Epstein proposed two mechanisms to describe the situation where there is a low and a high range of concentration at which the rate of absorption of the ion increases markedly with increasing concentration. These ranges are separated by an intermediate range where there is little or no change in the range of absorption. To explain this dual pattern of ion transport Epstein, Rains and Elzam (1963) proposed that two carrier systems were located at the plasma membrane and operated in parallel. The carrier systems could be characterized by having different K_m values. The high affinity carrier operated at low concentrations (mechanism 1) and the lower affinity carrier operated at higher concentrations (mechanism 2).

Laties (1969) also described dual mechanisms of ion uptake but offered a different explanation as to their cause. Laties (1969) suggested that dual mechanisms were the result of two carriers where the high affinity carrier was located at the plasma membrane and the

low affinity carrier was located at the tonoplast, and the two carriers operated in series. For various reasons this explanation has been discarded (MacRobbie 1971, Epstein 1976).

It has been argued that dual isotherms are an artifact of diffusion into roots at low external K^+ concentrations (Göring 1976). At low concentrations the uptake saturation observed is due to transport into the root epidermal cells and uptake at higher concentrations is the result of carriers on the root cortical cells. However, complex kinetics have been observed in a wide range of tissues (see MacRobbie 1971 and references therein) and in unicellular algae (see Nissen 1974 and references therein, Raven 1976). For unicellular plants, at least, this explanation is invalid.

Cheeseman (1982) proposed that at low external K^+ concentrations K^+ influx was an active, electrogenic process linked to a H^+ -ATPase. At higher concentrations ($>1\text{mol}\cdot\text{m}^{-3}$) K^+ influx occurs via a passive K^+ uniport. This is analogous to the K^+ channels proposed by Kochian et al. (1985) to describe the uptake of K^+ into corn roots at high external K^+ .

The concept of dual mechanisms for ion uptake has been criticised. Nissen (1974) argued that two or more uptake mechanisms operating simultaneously should give a continuously curved double reciprocal plot of concentration versus rate of uptake. However, Nissen's (1989) analysis of uptake rate versus external concentration suggests a single multiphasic uptake mechanism operating at the plasma membrane. Recently Nissen re-analyzed the data of Kochian et al. (1982, 1985) and dismissed their "uptake + linear" model claiming that a multiphasic model would better describe their results. The hypothesis championed by Nissen (1989) suggests that solutes are transported across the plasma membrane by multistate entities having carrier-like properties

at low external solute concentrations and channel-like properties at high concentrations. There is still a need for the carrier-kinetic approach, which depends on flux measurements to be integrated with a more rigorous biophysical approach. Why for example, changes in membrane potential difference as a driving force might produce changes in influx. Opening and closing of different families of channels could now be a way of involving known mechanisms.

1.8 Excess cation uptake.

1.8.1 Causes of excess ion uptake.

In higher plant tissue it is well documented that uptake of inorganic cations can exceed uptake of anions and that excess cations are balanced by the synthesis of organic acids in the cytoplasm (Ulrich 1941). Excess ion uptake has been associated with the production of a number of organic acids including malate, which is considered to be the most prevalent (see Cram 1985). Measurements of cation uptake from solutions containing KCl showed that during the early stages of uptake, K^+ uptake exceeded Cl^- uptake and the anion deficit was balanced by the synthesis of malate and efflux of the associated H^+ . As a general rule excess ion uptake and malate synthesis are most pronounced when SO_4^{2-} or HCO_3^- are the balancing anions, although excess cation uptake from solutions containing Cl^- has also been demonstrated (Pitman 1971).

A key feature of the control of ion uptake is the signal which determines whether a cell will synthesize organic acids or accumulate inorganic anions. Hiatt and Hendricks (1967) suggest K^+ accumulation from K_2SO_4 can be broken down into two steps, (1) initially K^+ exchanges for H^+ associated with organic acids and (2) the resulting change in cytoplasmic pH controls the rate of malate synthesis (see also "Davis pH stat" in Smith and Walker (1975)). However, Jacoby and

Laties (1971) argued that cytoplasmic HCO_3^- concentration and not pH_c induced malate formation. Jacoby and Laties (1971) concluded that K^+-H^+ exchange is a causative event in salt induced carboxylation. K^+-H^+ exchange results in HCO_3^- formation in the cytoplasm, which in turn stimulates carboxylation by PEP-Carboxylase. This scheme has been questioned by Smith and Raven (1979) and Osmond (1976) on the grounds that PEP-Carboxylase has a very high affinity for HCO_3^- . Therefore it would be unlikely that HCO_3^- could be an effective regulator of PEP-Carboxylase. Experimental evidence to reduce these cause-and-effect issues is lacking.

1.8.2 Regulation of malate formation.

Regardless of the precise signal that controls organic acid synthesis there is general agreement that K^+-H^+ exchange is associated with organic acid synthesis. However, little attention has focussed on the nature of plasma membrane K^+-H^+ exchange and in the literature there appears to be a some confusion of its characteristics. Some reports have implied that a K^+-H^+ antiport operates at the plasma membrane with some metabolic input of energy (eg. see Figure 2 in Smith (1973) and Figure 6 in Jacoby and Laties (1971)). However, there is no direct evidence for a K^+-H^+ antiport at the plasma membrane, therefore the two processes should be considered separately. Such a system where the H^+ pump operates simultaneously with a K^+ uniport has been proposed by Smith and Walker (1975).

1.8.3 Excess cation uptake in Chara.

The results discussed above were obtained from experiments using root tissue of higher plants. Although concentrations of organic acids in the vacuoles of charophytes are thought to be low under natural conditions, there have been a few indirect reports of *C. corallina* producing malate in response to excess cation uptake. Sanders (1981b) grew *C. corallina* in Cl^- -free solutions. An excess of inorganic cation relative to inorganic anion was reported. It was proposed that the plants were synthesizing malate and this was balancing vacuolar cations. During the uptake of the weak base, imidazole, from Cl^- -free solutions Smith and Whittington (1988) observed an excess of cation relative to inorganic anion in the vacuole. It was considered that an organic anion (such as malate) was being stored in the vacuole to balance the cations. Recently, Ryan (1988) described malate accumulation in *C. corallina* during uptake of NH_4^+ from Cl^- -free solutions. After 6 days in $150 \text{ mmol m}^{-3} \text{ NH}_4^+$ the vacuole of the cell contained 8.5 mol m^{-3} malate. Increases in pH_c or reduced cytoplasmic Cl^- concentration were implicated in the stimulation of malate accumulation in the vacuole.

1.9 Effects of Ca^{2+} on the plasma membrane.

1.9.1 Introduction.

Calcium is required for the maintenance of normal cell transport processes. There have been many studies reporting the effects of Ca^{2+} on the transport of molecules across the plasma membrane of plant and animal cells. It is important to separate the effects of Ca^{2+} at the external surface of the membrane from those occurring at the internal surface of the plasma membrane. Free cytoplasmic Ca^{2+} is tightly regulated by the cell and is in the micromolar range. It seems likely that in a normal resting cell cytoplasmic Ca^{2+} concentration is not sensitive to changes in external Ca^{2+} concentration (Tester 1988a), therefore the internal surface of the plasma membrane is probably subjected to a relatively well controlled Ca^{2+} concentration (Tester 1990). This is in contrast to the external surface of the plasma membrane where there can be widely fluctuating concentrations of Ca^{2+} . This thesis confines itself to the effects of Ca^{2+} on the external surface of the plasma membrane.

1.9.2 Effect of removing external Ca^{2+} .

When Ca^{2+} is removed from solution, the plasma membrane of *Chara* cells usually depolarizes and membrane conductance increases (Bisson 1984). Keifer and Lucas (1982) found that exposure to Ca^{2+} -free solutions did not, in itself cause a depolarization of membrane potential difference, but a stimulus such as high external K^+

concentration or an action potential was required. The addition of EGTA (Bisson 1984) in Ca^{2+} -free solution resulted a rapid depolarization of membrane potential difference and increase in membrane conductance, suggesting that removal of Ca^{2+} from the external surface of the plasma membrane resulted in changes to plasma membrane conductance and to potential difference. The removal of external Ca^{2+} has also been shown to increase membrane conductance of squid neurons (Armstrong and Lopez-Barneo 1987).

Removal of external Ca^{2+} reduces the ability of *Chara* cells to assimilate bicarbonate (Lucas 1977) by restricting bicarbonate transport activity. Smith (1984) showed that the removal of Ca^{2+} from solutions of pH_o 6 or below caused a slight drop in cytoplasmic pH (0.2-0.5 units) but had no effect above pH_o 6. This was used as evidence to support the hypothesis that the proton pump was operational in the absence of Ca^{2+} . This is in contrast to Bisson (1984) who suggested that removal of Ca^{2+} slowly deactivates the proton pump. Keifer and Lucas (1982) also present evidence that removal of Ca^{2+} does not inhibit the proton pump. During experiments designed to measure cytoplasmic pH Smith (1984) noted that the amount of 5,5-Dimethyloxazolidine-2,4-dione (DMO), in the vacuole relative to the amount of unionized DMO outside the cell was lower in the absence of Ca^{2+} . A simple explanation for this is that the plasma membrane, in the absence of Ca^{2+} , is more permeable to the ionized form of DMO.

1.9.3 Effect of increasing external Ca^{2+} .

Increasing Ca^{2+} concentration in the external media decreased K^+ conductance without affecting membrane potential difference in *Chara* (Beilby 1986, Tester 1988, Kourie and Findlay 1990b). Addition of Ca^{2+} reduced Na^+ influx and increased Rb^+ influx in maize roots (Riedell

1987). Ca^{2+} supplementation at high salinity (to keep the ratio of Na^+ to Ca^{2+} at approximately 10) increased survivorship of both *Chara buckellii* and *C. corallina* (Hoffmann and Bisson 1988, Tufariello et al 1988).

1.9.4 Effect of external Ca^{2+} on membrane rigidity.

There are a number of hypotheses relating to the action of Ca^{2+} at the external surface of the plasma membrane. However, there seems to be agreement between most workers that Ca^{2+} has at least two main roles at the exterior surface of the plasma membrane.

A major role of external Ca^{2+} is the maintaining of structural integrity of the plasma membrane. Roberts et al. (1986) showed that Ca^{2+} rigidifies microsomal membranes from beans. By using paramagnetic and fluorescent lipid-soluble probes Roberts et al. (1986) showed that the fluidity of microsomal membranes was greatly reduced upon the addition of Ca^{2+} . They also showed that Ca^{2+} had its effect at the membrane surface rather than within the membrane core. Using artificial phospholipid monolayers Landau and Lesham (1988) showed a clear interaction between Ca^{2+} concentration and monolayer surface tension. This condensation of the bilayer was attributed to Ca^{2+} cross linking with the negatively charged phospholipid molecules.

Ca^{2+} that is bound to the plasma membrane can be competitively displaced. Cramer et al. (1987) used a Ca^{2+} -sensitive fluorescing probe, (CTC) to show that as the concentration of Na^+ in the external media increased the amount of Ca^{2+} bound to cotton root plasma membranes decreased. Other cations did not reduce the Ca^{2+} -CTC fluorescence, indicating that the reduction was Na^+ -specific. Their evidence suggests that Ca^{2+} being displaced from the membrane was associated to ligands other than the phospholipids. EGTA treatments

also reduced the Ca^{2+} -CTC fluorescence to the same extent as Na^+ . The displacement of Ca^{2+} by Na^+ from the plasma membrane resulted in a large efflux of K^+ . It is interesting to note that auxins IBA, IAA and NAA also competitively displace Ca^{2+} (Landau and Lesham 1988).

Riedell (1987) claims that Ca^{2+} cross links the phospholipid head groups of the plasma membrane. This not only rigidifies the membrane but also neutralizes the surface negative charge of the membrane. A reduction in surface negative charge reduces the concentration of cations (such as Na^+) at the membrane surface, thus reducing their passive influx. Low pH also has similar effect, the negatively charged phospholipids becoming protonated due to the high concentrations of protons therefore decreasing surface negative charge of the membrane. Landau and Lesham (1988) observed a significant increase in membrane surface tension as pH (thus surface negative charge) was increased at constant Ca^{2+} concentration. It has also been suggested that in maize roots polyamines can substitute for Ca^{2+} in this role as a membrane rigidifier (Riedell 1987).

1.9.5 Effect of external Ca^{2+} on the operation of transport proteins.

A second important role of Ca^{2+} is the requirement of it by various transport proteins for their operation. The activity of the K^+ channel in squid neurons is dependant upon Ca^{2+} (Armstrong and Lopez-Barneo 1987). Armstrong and Matteson (1986) showed that the opening kinetics of the channel are slowed by binding of Ca^{2+} to the negative charged parts of the gating apparatus that are on the external surface of the channel protein. External K^+ competes with Ca^{2+} for channel occupancy. In effect Ca^{2+} has a latching or gating effect on K^+ channels. Evidence for a similar effect in *Chara* comes from Tester (1988) and Keifer and Lucas (1982) who showed that channels remain open

in solutions that have a high ratio of K^+ to Ca^{2+} . Armstrong and Lopez-Barneo (1987) showed that when the squid neuron K^+ channel was open for a long time it slowly assumed an unusual conformation with abnormal selectivity. When Ca^{2+} was present, the open time of the channel was so short the channel did not have time to deform. Lucas (1977) suggested that Ca^{2+} must be bound to the HCO_3^- transport complex before HCO_3^- transport can occur.

The results of Smith (1984) indicate that external Ca^{2+} is not required for the transport of protons from the cell. However, there may be a requirement for Ca^{2+} at the cytoplasmic side of the transport complex for proton transport to occur since the cytoplasm is the source of the protons.

1.10 Conclusions

In this thesis the effect that salinity has on the physiology of *C. corallina* is investigated. Included are measurements of the effects of high ionic concentration upon dominant vacuolar ions and membrane potential difference. From this information hypotheses for the movement of ions to and from the cell in response to elevated salinity can be formulated. Radiolabelled compounds are used to elucidate the fluxes of these ions.

Given the importance of Ca^{2+} effects in relation to salt sensitivity the role of Ca^{2+} has been a major part of this thesis. The effect of elevated Na^+ concentration has been compared to elevated concentrations of other ions, particularly K^+ . It is shown that *C. corallina* behaves not unlike glycophytes in accumulating significant amounts of organic acid in the vacuole in response to high external K^+ concentration.

CHAPTER 2

METHODS

2.1 Plant material.

Isolated internodal cells of giant celled alga *Chara corallina* = *C. australis* (see Figure 2.1 for morphology) were used in this study. *C. corallina* was cultured in containers which contained a 1 : 1 mixture of sterilized sand and garden loam (approximately 13 kilograms) and seventy litres of water (see Table 4.2, for chemical composition). Illumination was provided on a 16 h/8 h light/dark cycle by a combination of 40W cool white (Philips) and Gro-Lux (Sylvania) fluorescent tubes. This gave a light intensity of between 30 and 60 $\mu\text{E m}^{-2} \text{s}^{-1}$ at the surface of the water. Temperature was maintained between 22-26°C. Internodal cells that were to be used in an experiment were cut from a plant and the whorl cells removed. Only the second, third or fourth internode down from the apical cell

were used in experiments. Detached internodes were left overnight in artificial (chara) pond water, (CPW) (see section 2.2 for details) at pH8 to recover.

2.2 Solutions.

Chara pond water formed the basis of the solutions used in the experiments described in this study. CPW consists of the following: 1 mol m^{-3} NaCl, 0.1 mol m^{-3} K_2SO_4 , 0.5 mol m^{-3} CaSO_4 . Solutions were buffered with the appropriate zwitterionic buffer¹: \leq pH6 MES, pH7 HEPES, pH8 EPPS or TAPS, pH9 TAPS, \geq pH10 CAPS. Buffer concentration was 2.5 mol m^{-3} unless otherwise stated. Solutions were bought to the

¹MES = 2-[N-Morpholino]ethanesulphonic acid

HEPES = N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]

EPPS = N-[2-hydroxyethyl]piperazine-N'3-propanosulphonic acid

TAPS = N-tris[Hydroxymethyl]-3-aminopropanesulphonic acid

CAPS = 3-[Cyclohexylamino]-1-propane-sulphonic acid

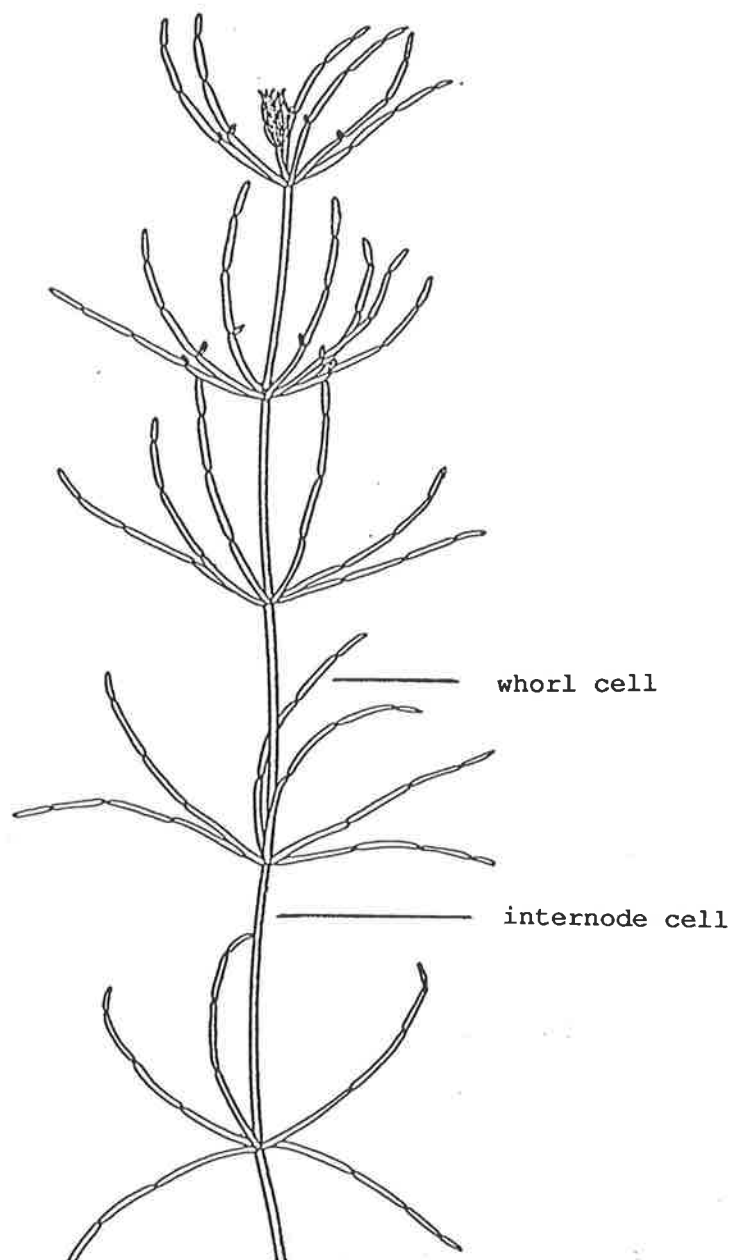


FIGURE 2.1 Habit of *C. corallina* (Ryan 1988), actual size.

required pH using freshly prepared NaOH (unless otherwise specified). The ionic concentration of solutions which were modified from CPW will be specified at the relevant point in the text. Most experiments were undertaken at pH 8. pH 8 was chosen because this pH is similar to the slightly alkaline waters in which *C. corallina* usually grows.

Solutions were made using rain water that was passed through a reverse osmosis unit (Permutit Australia) and a mixed bed exchange deionizer (Abtech). Deionized water prepared in this way had a resistivity of greater than 10 M Ω . Carbon-free solutions were prepared using the method of Lucas (1975). Chemicals used throughout were AR grade.

Ca²⁺-free solutions contained no added Ca²⁺. Ca²⁺ concentration was not buffered with EGTA and some Ca²⁺ would be carried over to the experimental solutions with the cells therefore the solutions would have a small but variable Ca²⁺ concentration.

2.3 Cell sizes and streaming rate.

Cell length was measured to the nearest 0.5 mm using mm ruled graph paper. Cell diameter was measured to the nearest 0.05 mm using an Olympus CO-11 binocular microscope. Cytoplasmic streaming rate was calculated by recording the time taken for particles in the cytoplasm to travel 0.5 mm and converting this to the units $\mu\text{m s}^{-1}$. This was done two or more times for each cell and then the rates averaged.

2.4 Vacuole: collection of sap, osmolality, ionic composition and pH.

The large size of the internodal cell of *C. corallina* enables direct sampling of the vacuole sap. To measure osmolality and ionic composition of the vacuole, cells were removed from solution, gently blotted and allowed to dry for a few minutes until cell turgor was slightly reduced. The cell was then placed on a wax block and the

nodes removed with a razor blade. The vacuole was allowed to run out of the cut end and collected using a 10 μl micro pipette (either Drummond Microcap or Blaubrand Intramark). 10 μl of sap could be collected from a typical cell in this way. This method is believed to give uncontaminated vacuole sap samples (eg. Smith and Whittington 1988).

Osmolality of the cell sap was determined using a WESCOR 5100B vapor pressure osmometer. Measurements were made on 7 μl of sap injected onto a filter paper planchette. The osmometer was calibrated using fresh WESCOR osmolality standards.

K^+ and Na^+ concentrations of cell sap were determined by diluting the sap 1:300 with deionized water. K^+ and Na^+ were then measured using a Corning 400 Flame Photometer.

Cl^- concentration of dilute sap samples was determined spectrophotometrically using a method developed in our laboratory. The vacuole sample was diluted with deionized water such that the final Cl^- concentration was between 10-100 mmol m^{-3} (approx. 1:2500). Then excess AgNO_3 was added and the resulting absorbance by AgCl at 260nm was measured using a Beckman DB Spectrophotometer (Beckman Australia). Absorbance at 260 nm was linear with concentration between 5 and 200 $\text{mmol m}^{-3} \text{Cl}^-$. The quartz cuvettes required acid rinsing (0.1N H_2SO_4) between readings to avoid contamination by AgCl precipitate adhering to the walls of the cuvette. This proved to be a reliable and sensitive method for determining Cl^- concentration.

The pH of vacuole sap was determined using a flat bulb isoelectric focusing pH electrode (Activon BJ332). 10 μl of sap was collected (as above) and placed in a shallow recess (8mm diameter, 5mm deep) cut into a perspex block. The pH electrode was then lowered into the recess and pH recorded.

2.5 Measurement of pH_c .

Cytoplasmic pH (pH_c) was determined using two methods: from the distribution of the weak acid, 5,5-dimethylloxazolidine-2,4-dione (DMO) between the cytoplasm and the bathing solution (described by Smith 1980, 1984 and references therein) and by the use of double-barrelled pH microelectrodes (described by Reid and Smith 1988). The DMO method was preferred because many samples could be measured simultaneously. However, microelectrodes have a very fast response time (approximately 30s) when compared to the DMO technique (approximately 300s at low pH (Smith 1986) but up to 2h when the bathing solution is alkaline). For this reason, microelectrodes were used when a fast response time was required.

2.5.1 Estimation of pH_c using [^{14}C]DMO.

pH_c was determined following the addition of radiolabelled [^{14}C]DMO (supplied by Amersham Australia) for 120 minutes. Cells were then given a two minute rinse in non-radioactive experimental solution to remove [^{14}C] from the cell wall. Cells were then blotted dry. After removal of the nodes, an aliquot of vacuole was taken (usually 10 μ l) and then this and the remainder of the cell were assayed separately by liquid scintillation counting. The concentration of [^{14}C]DMO in the cytoplasm was determined by subtraction of vacuolar [^{14}C]DMO. It was assumed that the cytoplasm occupied 95% of the cell volume. pH_c was then calculated from the following equation:

$$C_c = U_o [1 + 10^{(pH_c - pK_a)}]$$

where C and U are respectively the amounts of total and unionized DMO per unit volume and the subscripts c, v and o refer to the cytoplasm, vacuole and bathing solution respectively. The pK_a of DMO is taken as

6.38 in all phases since under the experimental conditions employed the small variations in pK_a with temperature and ionic strength would not significantly alter the results (Boron and Roos 1976). U_o is related to C_o as follows:

$$C_o = U_o [1 + 10^{(pH_o - pK_a)}]$$

Combining these two equations pH_c can be determined from the equation:

$$pH_c = pK_a + \log[(C_c/C_o (1 + 10^{(pH_o - pK_a)}) - 1]$$

2.5.2 Estimation of pH_c using pH microelectrodes.

Double-barrelled pH electrodes were manufactured by R. J. Reid following the procedure outlined in Reid and Smith (1988). pH_c was measured from small (≤ 15 mm long) internodal cells and whorl cells. It was found that with internodal cells the electrode tip was excluded from the cell within a few minutes. However, it took much longer for the electrode tip to be excluded from whorl cells. Therefore whorl cells were used when continuous recording was required.

2.6 Growth.

Growth of *C. corallina* internodal cells has been measured *in vitro* using a method similar to that of Franceschi and Lucas (1982). The terminal three internodal cells from vegetative apices were cut from the laboratory culture and rinsed thoroughly in experimental solution for three hours. Shoots were trimmed to remove some of the whorl cells (Fig 2.2). Shoots were then placed in petri dishes (diameter 150mm by 30mm deep) with 300 ml of experimental solution. The length of each of

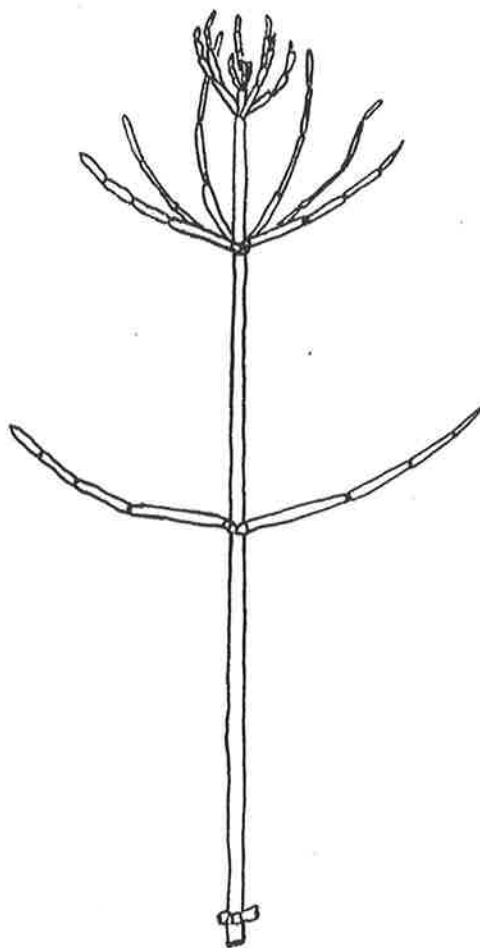
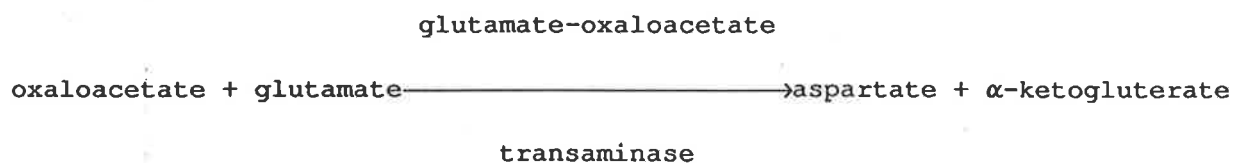
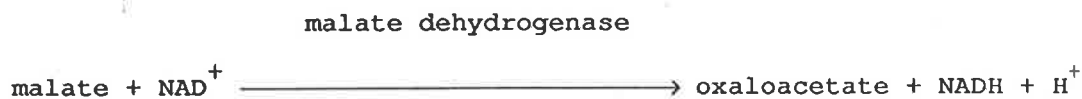


FIGURE 2.2 Diagram of the shoot region used for measuring the growth of *C. corallina*.

the terminal three internodes was measured to the nearest 0.1mm with a pair of calipers. Experimental solutions were changed daily throughout the experiment.

2.7 Malate.

Malate concentrations in the vacuole and whole cell were determined using an enzyme assay described by Lowry and Passonneau (1972). By coupling the oxidation of malate to the reduction of NAD the appearance of NADH is measured by its absorbance at 340nm. The reactions are shown below:



Reagents for the assay were made as stock solutions at 5 or 10 fold the required concentration and kept frozen until required. Vacuole sap or whole cell extract was assayed for malate by adding a 10 μ l vacuole sample to 2.5 ml of assay medium. The final concentrations of the assay medium were:

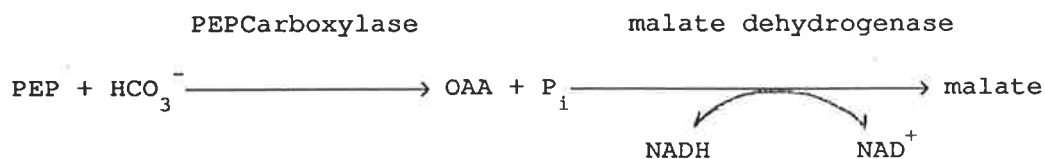
2 mol m⁻³ NAD⁺ (grade 2, 98% pure),
100 mol m⁻³ 2-amino-2-methylpropanol buffer, pH 9.9 (50% free
base, 50% as the hydrochloride),
40 mol m⁻³ glutamate (adjusted to pH 9.9 with fresh NaOH),
malate dehydrogenase (from pig heart, EC 1.1.1.37) 2.4 U ml⁻¹,
glutamate-oxaloacetate transaminase (from pig heart,
EC 2.6.1.1) 4 U ml⁻¹.

The reagents were obtained from Boehringer Mannheim Australia. The assays were carried out in quartz cuvettes at 25°C and the absorbance recorded after 15 minutes with a Philips PU8800 UV/VIS Spectrophotometer. L-malate standards were measured concurrently.

2.8 Phosphoenolpyruvate Carboxylase activity.

Phosphoenolpyruvate carboxylase (PEPCarboxylase) (EC 4.1.1.31) activity from *C corallina* extract was determined. For comparison the activity of this enzyme from a C₄ plant *Pennisetum clandestinum* (Kikoyu grass) was measured (Edwards and Walker 1983). *P. clandestinum* was collected from the Botany Lawn, University of Adelaide. By coupling the synthesis of oxaloacetate from phosphoenolpyruvate

(catalyzed by PEP Carboxylase) to NADH oxidation the the activity of PEP Carboxylase can be determined. The reactions are shown below:



An enzyme extract was prepared by grinding approximately 0.5 gm of plant tissue with a mortar and pestle in 3 ml of ice-cold extraction medium. This was then filtered through a single layer of miracloth and centrifuged at 20000g (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments) at 2°C for 10 minutes. The supernatant (which shall be referred to as the enzyme extract) was decanted and stored on ice until the commencement of the experiment.

50 μl of enzyme extract was added to 2.5 ml assay medium. The reaction was initiated by the addition of 50 μl of 80 mol m^{-3} PEP. The oxidation of NADH was then recorded at 340nm on a Philips PU8800 UV/VIS Spectrophotometer at 25°C. The reactions were carried out in quartz cuvettes.

The composition of the extraction medium is:

- 50 mol m^{-3} HEPES, adjusted to pH 7.2 with fresh KOH,
- 5 mol m^{-3} MgCl_2 ,
- 1 mol m^{-3} EGTA,
- 5 mol m^{-3} Dithiothreitol.

The final composition of the assay medium was:

- 30 mol m^{-3} HEPES adjusted to pH 8 with fresh KOH,
- 6 mol m^{-3} MgCl_2 ,
- 1 mol m^{-3} Glucose-6-phosphate,
- 4 mol m^{-3} Dithiothreitol,

0.2 mol m⁻³ NADH,
8 mol m⁻³ NaHCO₃,
16 U ml⁻¹ malate dehydrogenase (from pig heart, EC 1.1.1.37),
0.16 mol m⁻³ PEP.

The reagents were obtained from Boehringer Mannheim Australia.

2.9 Electrical measurements.

The vacuolar resting membrane potential difference was measured by inserting a glass microelectrode into the cell. The glass microelectrode was pulled from borosilicate glass capillary tube (o.d. 1.0 mm) with an internal filament, to fine taper (tip diameter 1-5 μm) in a microelectrode puller (model PW-6, Narishige, Tokyo) and then backfilled with 3M KCl saturated with AgCl. The electrode and reference were connected to a custom made electrometer using a salt bridge via calomel half cells. The salt bridge consisted of silicon tubing containing 3M KCl saturated with AgCl, set in 2% agar. The electrometer was connected to a Rikadenki Chart Recorder (model B-281HS).

The internodal cell to be measured was mounted in a perspex chamber and connected to a solution flow through system.

2.10 Radiolabelled influx experiments.

2.10.1 General.

The isotopes ¹⁴C, ²²Na, ²⁴Na, ⁴²K, ⁴⁵Ca, and ³⁶Cl were used to measure ion influx into *C. corallina*. Essentially the same protocol was used for all experiments using radiolabelled chemicals. The generalized protocol will be described, then each ion will be discussed in more detail.

Internodal cells were cut from the culture tanks and left overnight (usually in CPW pH8) to recover. Experiments were usually

performed in disposable plastic petri dishes (diameter 90mm by 15mm deep) with 25-30 ml of solution. Generally cells were given a 30 minute pretreatment in non-radioactive experimental solution. This was then replaced with radioactive experimental solution for 60-120 minutes. Experiments were conducted between 22-25°C at a light intensity of $40 \mu\text{E m}^{-2} \text{s}^{-1}$. After this cells were rinsed in non-radioactive solution for three minutes unless otherwise specified. During the rinse period the length and diameter of the cells were recorded.

The amount of radioactive label in each cell was determined by liquid scintillation counting. Each cell was placed in a 10 ml plastic vial with 5 ml of Ready Value Scintillation Cocktail (Beckman Australia). The samples were then counted for 10 minutes on a Beckman Liquid Scintillation Counter (model LS 3801, Beckman Instruments (Australia)). Influx was calculated on a per unit area basis with the units $\text{nmol m}^{-2} \text{s}^{-1}$.

2.10.2 $\text{H}^{14}\text{CO}_3^-$

Freshly prepared solutions were adjusted to the correct pH using freshly prepared 1N NaOH and were then placed in air tight 30 ml specimen jars. Cells were then added and allowed to equilibrate for 20 min before $12 \text{ kBq ml}^{-1} \text{H}^{14}\text{CO}_3^-$ (supplied as NaHCO_3^- by Amersham Australia) was injected through the cap and mixed by gentle shaking. After 210 minutes cells were rinsed in ice cold solution (pH5) for 5 minutes. 0.5 ml 1N NaOH was added to the scintillation fluid in each vial to trap any unfixed ^{14}C .

2.10.3 [^{14}C]Imidazole, [^{14}C]Methylamine and [^{14}C]DMO.

[^{14}C]Imidazole was added at 4.5 kBq ml^{-1} and was obtained from NEN Research Products. [^{14}C] Methylamine Hydrochloride was added at 10

kBq ml⁻¹ and was obtained from Amersham Australia. [¹⁴C]DMO was added at 10 kBq ml⁻¹ and was obtained from Amersham Australia.

2.10.4 ²²Na, ²⁴Na.

²²Na was added at 4-8 kBq ml⁻¹ and was obtained from Amersham Australia. ²⁴Na was added at 10-150 kBq ml⁻¹ and was obtained from the Australian Atomic Energy Commission, Lucas Heights, NSW. Both isotopes were obtained as chloride solutions.

2.10.5 ⁴⁵Ca.

The method used for measuring Ca²⁺ flux was similar to that described by MacRobbie and Banfield (1988). Cells used in these experiments were less than three weeks old and had no noticeable CaCO₃ encrustations. However, upon cutting, cells were rinsed in Ca²⁺-free CPW (pH5) for 180 minutes to remove Ca²⁺ from the cell wall. Decalcified cells were then placed in CPW (pH8) overnight before the commencement of the experiment. Cells were given the usual pretreatment and then were placed in radioactive solution (⁴⁵Ca obtained from Amersham Australia and added at 5 kBq ml⁻¹) for 15 minutes. After loading the cells were briefly rinsed twice then given a 20 minute rinse in ice-cold CPW containing 2 mol m⁻³ LaCl₃.

2.10.6 ³⁶Cl⁻.

³⁶Cl was obtained as the Na⁺ salt from Amersham Australia and was added at 7.2 kBq ml⁻¹.

2.10.7 ⁴²K⁻.

⁴²K was added obtained as ⁴²K₂SO₄ and ⁴²KCl. ⁴²K₂SO₄ was only used for the Cl⁻-free experiments. Both salts of ⁴²K were added at 2-300 kBq ml⁻¹ and were obtained from Australian Atomic Energy Commission, Lucas Heights, NSW.

CHAPTER 3

What salinity can *Chara corallina* tolerate?

3.1 INTRODUCTION.

C. corallina inhabits freshwater lakes, pools in rivers and roadside ditches (Wood 1972). To my knowledge, there are no reports of *C. corallina* occurring naturally in brackish water. In the laboratory Bisson and Bartholomew (1984) have shown that 50 mol m^{-3} NaCl in artificial pond water, APW¹, to be lethal to isolated internodal cells of *C. corallina*. Similarly, Lucas and Alexander (1981) found 20 mol m^{-3} NaCl to be lethal to *C. corallina* under turgor reduced conditions (ie. when added with 205 mosmol sorbitol). However, Smith and Gibson (1985) grew *C. corallina* in the laboratory in solutions containing 25 mol m^{-3} NaCl. This external solution also contained approximately 2 mol m^{-3} Ca^{2+} , whereas the external solution of Bisson and Bartholomew (1984) and Lucas and Alexander (1981) contained 0.1 mol m^{-3} Ca^{2+} and 0.2 mol m^{-3} Ca^{2+} respectively. It is widely accepted that Ca^{2+} is a major factor in the regulation of ion transport and the maintenance of membrane integrity (see Chapter 1) and Ca^{2+} concentration is an important factor in the survival of various higher plants in saline conditions (see review by Hanson 1984).

In the preliminary experiments described in this chapter the relationships between cell survival and the concentrations of Na^+ and Ca^{2+} in the external solution have been investigated. Similar experiments have been undertaken in M Bissons laboratory, which they have subsequently published. Their results will be discussed and compared to mine later in this chapter.

¹APW = 1 mol m^{-3} NaCl; 0.05 mol m^{-3} K_2SO_4 ; 0.1 mol m^{-3} CaSO_4 .

Cytoplasmic streaming rate is a commonly measured parameter in *C. corallina*. A correlation has been established between ATP concentration in the cytoplasm and the rate of cytoplasmic streaming (Reid and Walker 1983). This correlation is thought to be due to a direct dependence of streaming rate on ATP concentration. In this study cytoplasmic streaming rate has been used as an indication of the general health of the cell during the experiment. The rate of cytoplasmic streaming is also influenced by factors other than ATP concentration. Williamson and Ashley (1982) report cytoplasmic streaming rate to be sensitive to cytoplasmic Ca^{2+} concentration. It must be recognized that treatments that increase cytoplasmic Ca^{2+} concentration will also decrease cytoplasmic streaming rate.

3.2 RESULTS.

The results presented in Figure 3.1 show that isolated internodal cells of *C. corallina* can survive in 100 mol m^{-3} NaCl. Cell survival was dependent upon the concentration of Ca^{2+} in the external medium; 1, 5 or 10 mol m^{-3} Ca^{2+} ameliorated the effect of 100 mol m^{-3} NaCl. Cells died in solutions containing 100 mol m^{-3} NaCl when the Ca^{2+} concentration was below 1 mol m^{-3} . A cell was considered dead when cytoplasmic streaming ceased, the chloroplasts had dislodged from their matrix and the cell had lost turgor.

The rate at which injury occurs in 100 mol m^{-3} NaCl is also dependent upon the Ca^{2+} concentration. 90% of cells in Ca^{2+} -free treatments were dead within 24 hours, whereas with 0.5 mol m^{-3} Ca^{2+} all cells survived for 24 hours, however, 70% were dead after 96 hours. This toxicity is probably due to Na^+ not Cl^- since Na_2SO_4 is as toxic as NaCl (results not shown); (see also Tufariello et al. (1988).

Figure 3.2 shows cytoplasmic streaming rate of the surviving cells as a function of time that the cells were bathed in 100 mol m^{-3} NaCl.

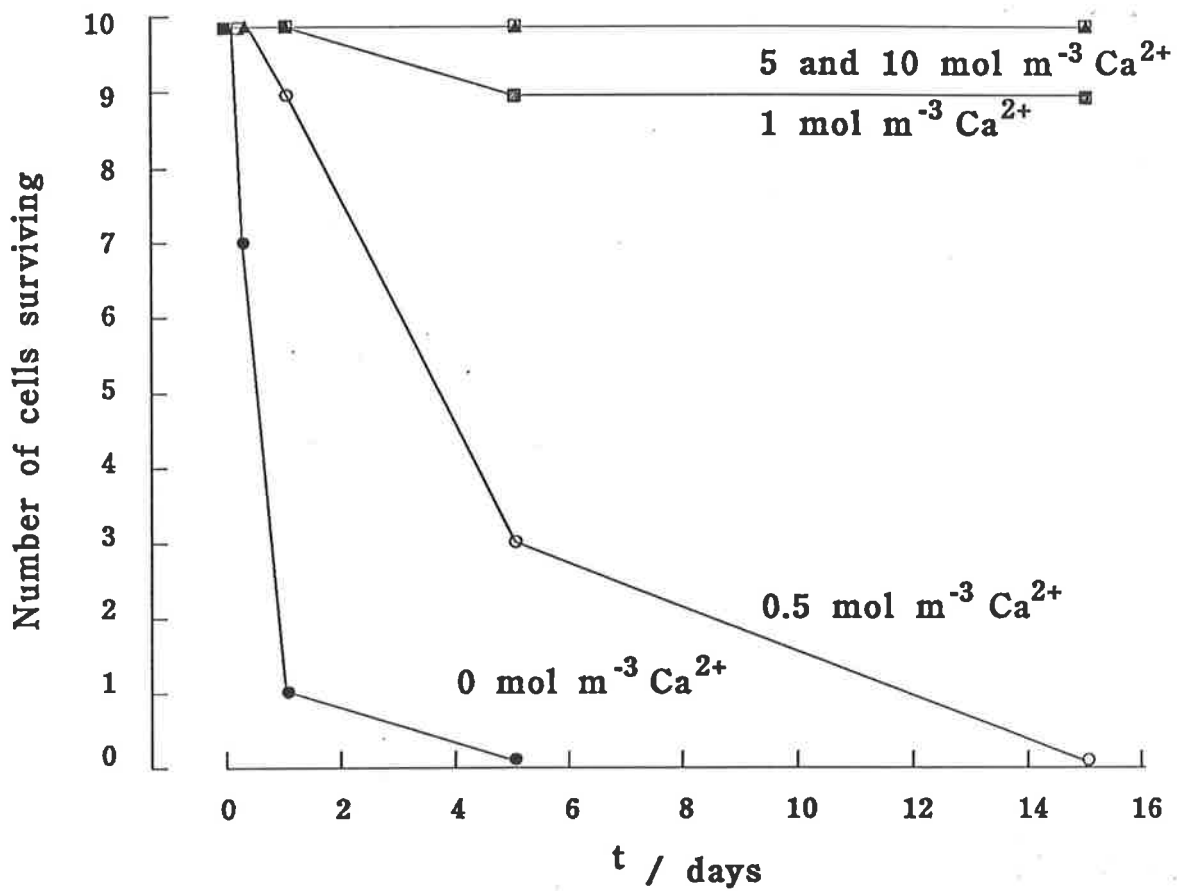


Figure 3.1 Effect of Ca^{2+} concentration on survivorship at 100 mol m^{-3} NaCl.

● Ca^{2+} -free, ○ $0.5 \text{ mol m}^{-3} \text{ CaSO}_4$, ■ $1.0 \text{ mol m}^{-3} \text{ CaSO}_4$,
 □ $5.0 \text{ mol m}^{-3} \text{ CaSO}_4$, ▲ $10.0 \text{ mol m}^{-3} \text{ CaSO}_4$.

Culture: LES

Solution: $0-10 \text{ mol m}^{-3} \text{ CaSO}_4$, Na-CPW, EPPS pH 8

Solutions replaced each day during experiment.

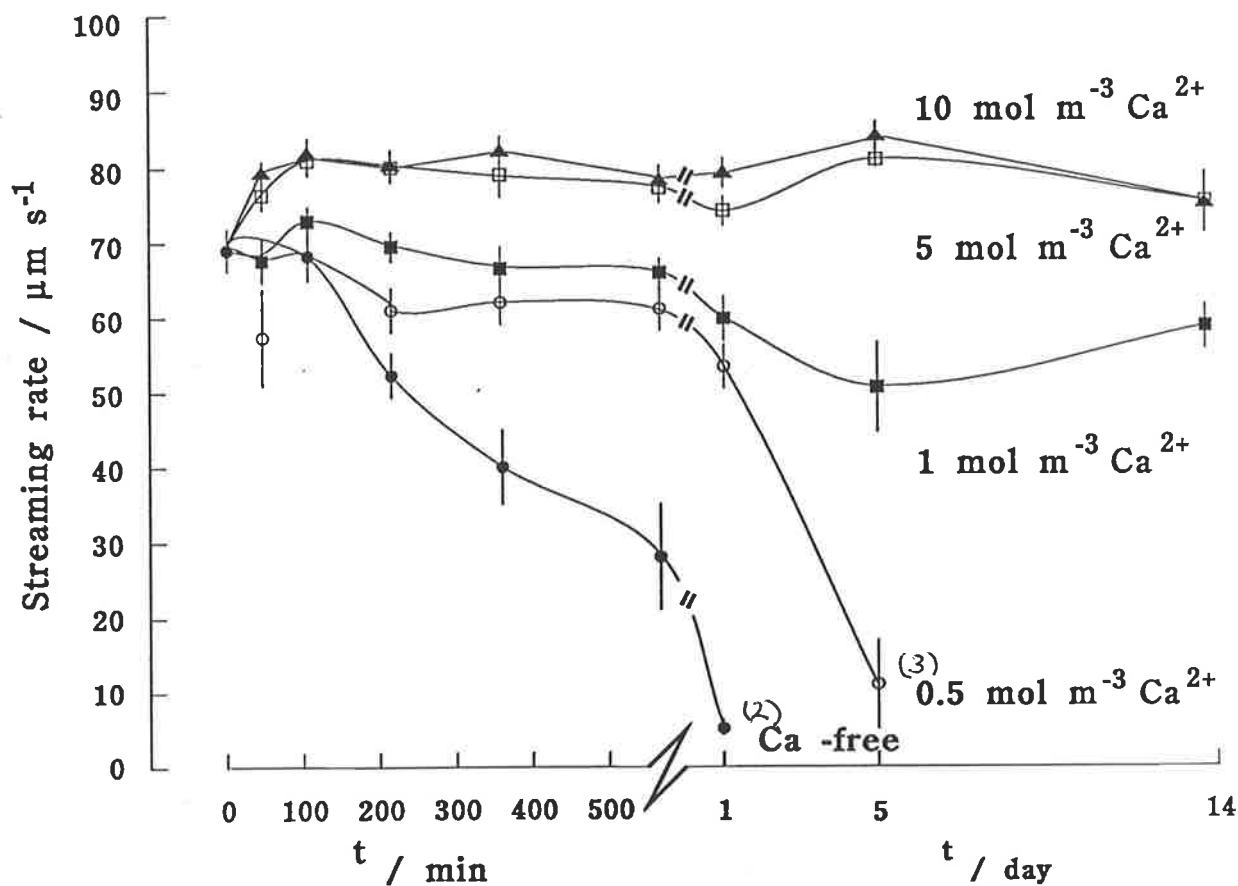


FIGURE 3.2 Effect of Ca²⁺ concentration on cytoplasmic streaming rate at 100 mol m⁻³ NaCl.

● Ca²⁺-free, ○ 0.5 mol m⁻³ CaSO₄, ■ 1.0 mol m⁻³ CaSO₄,
□ 5.0 mol m⁻³ CaSO₄, ▲ 10.0 mol m⁻³ CaSO₄.

Each point represents the mean and standard error of the number of surviving cells, ($n=10$ unless otherwise indicated).

Culture: LES

Solution: 0-10 mol m⁻³ CaSO₄, Na-CPW, EPPS pH 8

Solutions replaced each day during experiment.

Plotting cytoplasmic streaming rate rather than survival gives a better indication of the condition of the cell. Figure 3.2 shows that cytoplasmic streaming rate is reduced within a few hours after addition of NaCl. Another point to note is that although all the cells in $1 \text{ mol m}^{-3} \text{ Ca}^{2+}$ survived for 14 days cytoplasmic streaming rate of these cells was reduced by 30% relative to the higher Ca^{2+} treatments.

3.3 DISCUSSION.

C. corallina can survive in solution containing 100 mol m^{-3} NaCl if the Ca^{2+} concentration is higher than 1 mol m^{-3} . At 100 mol m^{-3} NaCl, an external Ca^{2+} concentration below 1 mol m^{-3} results in cell death. Since the addition of Ca^{2+} -free CPW did not reduce cytoplasmic streaming rate, I propose that it is a combination of high external Na^+ and low external Ca^{2+} that damages the cell. This observation agrees with the results of Tufariello et al. (1988) who show that raising external Ca^{2+} to 7 mol m^{-3} in the presence of 70 mol m^{-3} NaCl enhanced survival of *C. corallina*. Tufariello et al. (1988) concluded that a Na^+ to Ca^{2+} ratio of 10 to 1 is required for *C. corallina* to survive in saline waters. The results presented in this chapter indicate the Na^+ to Ca^{2+} ratio required for cell survival is between 100:1 and 20:1. This will be discussed further in later chapters.

A similar study was undertaken with the freshwater charophyte *Nitellopsis obtusa* (Katsuhara and Tazawa 1986). *N. obtusa* was able to survive $100 \text{ mol m}^{-3} \text{ Na}^+$ if the external solution was supplemented with $10 \text{ mol m}^{-3} \text{ Ca}^{2+}$.

The experiments described in this chapter show *C. corallina* is able to tolerate NaCl concentrations that approach a concentration that would plasmolyze the cell if no osmotic adjustment were to take place. However, *C. corallina* does not colonize brackish water that has a high Ca^{2+} concentration. To understand this puzzle, the effect that NaCl has upon growth and the ionic relations of *Chara* will be the subject of the next chapter.

CHAPTER 4

Effects of elevated Na^+ and Ca^{2+} concentration on growth and physiology of *Chara corallina*.

4.1 INTRODUCTION.

As was shown in the previous chapter isolated internodal cells of *C. corallina* can tolerate solutions containing up to $100 \text{ mol m}^{-3} \text{ Na}^+$ whilst the ratio of Na^+ to Ca^{2+} is maintained at 20:1 or less. However, *C. corallina* is only found growing in freshwater. In this chapter the physiology of *C. corallina* in saline solutions with adequate Ca^{2+} for survival is investigated with the aim of determining why *C. corallina* does not colonize brackish water.

4.1.1 Isolated internodal cells versus intact cells.

The studies of Bisson and Bartholomew (1984) and of Lucas and Alexander (1981) discussed in the previous chapter used detached internodes of *C. corallina* in their experiments. Whilst the use of isolated internodes is convenient it also poses limitations on any conclusions drawn from the experiments. Some of the problems associated with using isolated internodes are discussed below.

Charasomes are compact membranous structures at the plasma membrane that result to a tremendous increase in membrane surface area (Franceschi 1981) and it has been suggested that they are involved with Cl^- uptake. Franceschi and Lucas (1982) showed that most of the charasome material in the shoot was contained in the branch cells. Franceschi and Lucas (1982) suggested that the branch cells of *Chara* play a major role in Cl^- accumulation in the shoot. This is particularly important when Cl^- is being accumulated, such as when the cell is growing or if Cl^- is accumulated to increase osmotic potential.

Box et al. (1984) reported transport of ions between rhizoids and

shoots of *Chara hispida* and showed a close correlation between cytoplasmic streaming rate and intercellular transport. The use of isolated internodal cells nullifies any advantage to the cell associated with intercellular transport.

In the present experiments, growth and ionic relations of isolated internodes transferred to solutions containing elevated Na^+ is compared to growth of internodes from plants cultured in similar solutions.

4.1.2 Ionic composition of the vacuole.

The ionic composition of the vacuole from the charophyte algae is variable (Raven 1976, 1984) and can change with salinity (Bisson and Kirst 1980, Winter and Kirst 1990), age (MacRobbie 1962, Winter et al 1987), season (Winter et al. 1987) and developmental state (Kirst et al. 1987)

Na^+ , K^+ and Cl^- account for approximately 84% of the osmotic potential of the vacuole of *C. corallina* (Bisson and Bartholomew 1984) (see also Table 1.1). Bisson and Bartholomew (1984) found that increasing NaCl in the bathing medium from 1 to 25 mol m^{-3} resulted in an increase in vacuolar Na^+ and Cl^- as well as osmotic potential. The concentration of K^+ in the vacuole did not change. The combined effect of increasing external Na^+ was to decrease the ratio of K^+ to Na^+ in the vacuole.

4.1.3 Osmotic regulation at elevated Na^+ .

Bisson and Bartholomew (1984) described a significant increase in osmotic potential of the cell upon addition of 25 mol m^{-3} Na^+ but this was not sufficient to compensate for the increase in osmotic potential of the external solution, therefore cell turgor decreased. Bisson and Bartholomew (1984) concluded that *C. corallina* normally regulates osmotic potential but the regulation of osmotic potential fails at high

concentrations of external NaCl. However, their experiments were conducted without Ca^{2+} supplementation.

Sanders (1981b) argued that osmotic potential is not an effectively controlled parameter in *C. corallina*. The osmotic potential of isolated internodes kept in APW¹ for two weeks increased by up to 30%. Whether or not osmotic potential is an effectively regulated parameter in saline media is considered in this chapter.

4.2 RESULTS.

4.2.1 Effect of NaCl on the growth of *C. corallina* in culture.

C. corallina was cultured in solutions of varying Na^+ concentration where the ratio of Na^+ to Ca^{2+} was maintained at 10 to 1 (Table 4.1). Concentrations of Na^+ up to 50 mol m^{-3} did not greatly affect the appearance of the culture. However, cultures in solutions containing greater than $50 \text{ mol m}^{-3} \text{ Na}^+$ were less dense and exhibited poor whorl cell development relative to those in fresh water. Cultures in solutions containing $\geq 50 \text{ mol m}^{-3}$ were also generally slower growing than those below $50 \text{ mol m}^{-3} \text{ Na}^+$. I was unable to establish cultures in solutions containing greater than $75 \text{ mol m}^{-3} \text{ Na}^+$. It is assumed that approximately 75 mol m^{-3} is the maximum Na^+ concentration at which *C. corallina* can grow. No attempt to quantify the growth rate of *C. corallina* growing *in situ* was attempted.

4.2.2 Effect of NaCl on the growth of detached branches of *C. corallina*.

Growth was measured using the technique described by Franceschi and Lucas (1982). This involved measuring increases in the length of

¹APW = $1 \text{ mol m}^{-3} \text{ NaCl}$; $0.02 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$; $1 \text{ mol m}^{-3} \text{ CaSO}_4$

TABLE 4.1 Chemical composition of *C. corallina* culture solutions.

culture tank	ion content (mol m ⁻³)		
	NaCl	CaSO ₄	K ₂ SO ₄
1	1.0	0.2	0.2
2	25	2.5	0.2
3	50	5.0	0.2
4	58	5.8	0.2
5	67	6.7	0.2
6	75	7.5	0.2
LES	1.5	0.8	1.0
REG	3.3	1.1	1.4
JLG	1.4	0.3	1.4

All tanks contained 1/2000 strength Hoagland nutrient solution.

Tanks 1 to 6 were maintained at 16°C with 16/8 hour, day-night cycle with a light intensity at the waters surface shaded to 5-6 μmol m⁻² s⁻¹.

the terminal three internodal cells of a *Chara* branch that had been detached from the plant and placed in a petri dish. Most whorl cells were not trimmed from the branch (see Figure 2.2) because Franceschi and Lucas (1982) suggested that whorl cells play an important role in ion transport, particularly Cl^- uptake. Franceschi and Lucas (1982) observed increases in the length of the apical internode of up to 220% and 66% in the sub apical internode over an 11 day period.

As a preliminary experiment the increase in length of the terminal 3 internodal cells of isolated branches was measured for 21 days. Cells were kept in CPW supplemented with 1:2000 strength Hoaglands solution or in LES Tank culture solution (see Table 4.1 for chemical composition). There was no change in length of any cells during the first 5 days (Figure 4.1a). However, by day 15 the terminal apical cell had doubled in length. No increase in length of the second and third internodes was observed throughout the experiment (Figure 4.1b). Cells in LES tank water grew at a faster rate than cells in CPW supplemented with Hoagland's solution.

Having established that elongation of the apical internodal cell could be reliably measured, an experiment was conducted where growth was estimated in a range of NaCl and sorbitol concentrations (Figure 4.2). The results indicated that concentrations of Na^+ up to 50 mol m^{-3} were not inhibitory to growth, whereas no growth was observed at Na^+ concentrations of 100 mol m^{-3} . Cell elongation was reduced at all concentrations of sorbitol, with the greatest inhibition occurring at 180 mol m^{-3} sorbitol. The inhibition of growth at all sorbitol concentrations that indicates sorbitol may inhibit cell elongation independantly of an inhibition of growth due to a reduction in turgor.

Cell turgor potential was estimated by measuring the osmotic potential of each cell and subtracting this from the theoretical

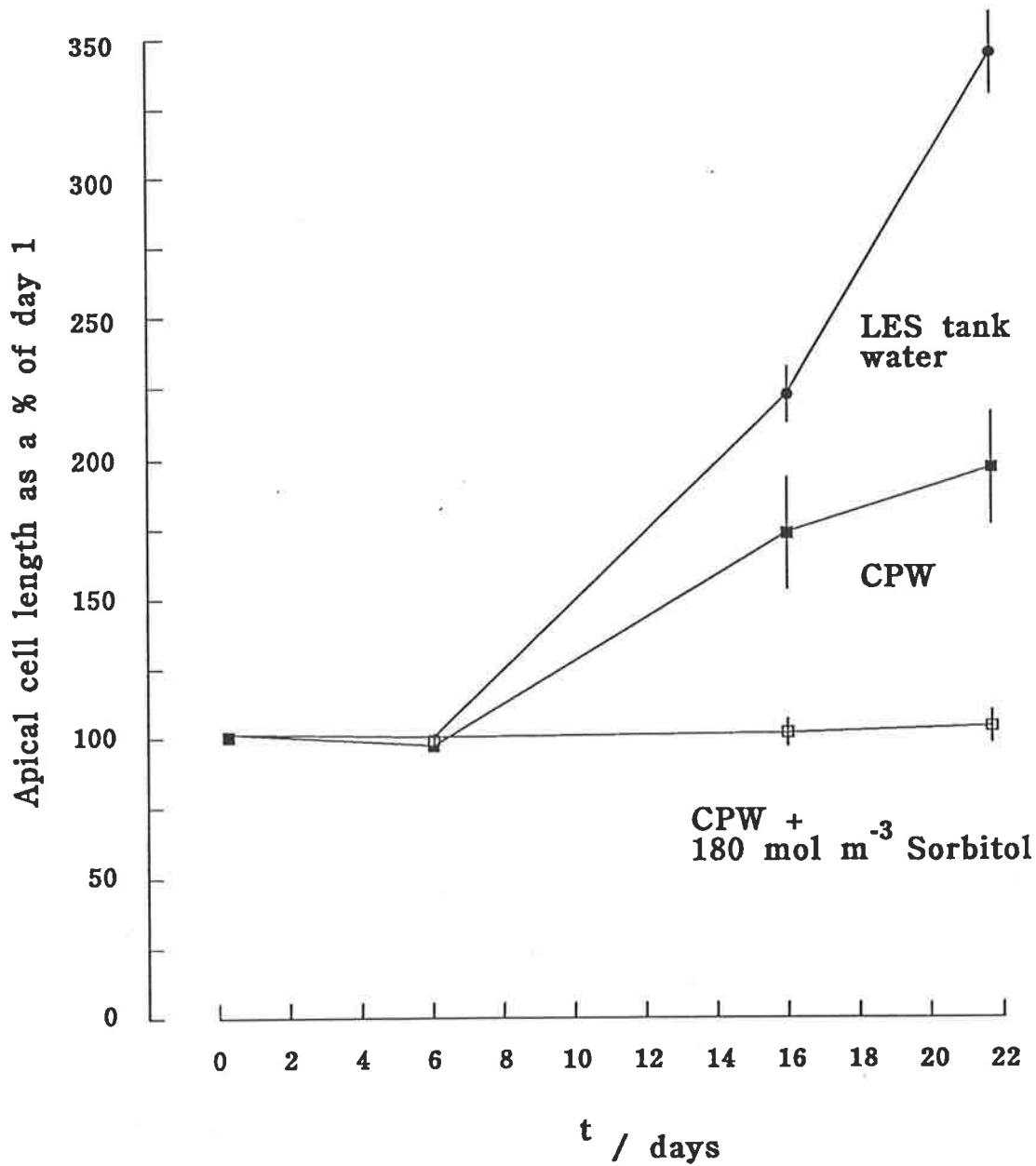


FIGURE 4.1a Growth of apical internodal cell in CPW, tank water or 180 mol m⁻³ sorbitol.

■ CPW, ○ LES tank water, □ 180 mol m⁻³ sorbitol.

Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: CPW, EPPS pH 8 or LES tank water (see table 4.1) or 180 mol m⁻³ sorbitol, CPW, EPPS pH 8. Solutions replaced each day during experiment.

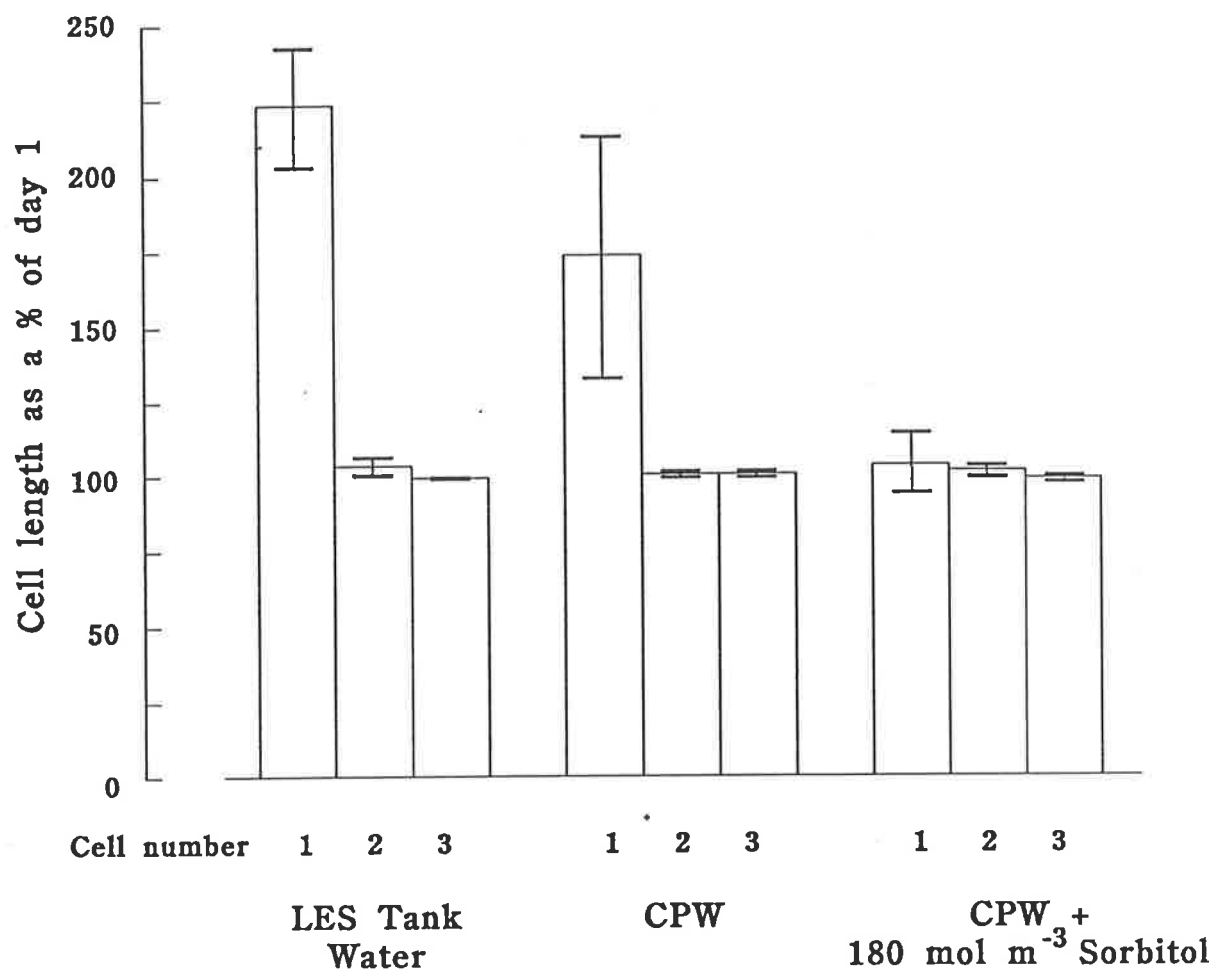


FIGURE 4.1b Growth of terminal 3 internodal cells in CPW, tank water or 180 mol m⁻³ sorbitol after 15 days.

Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: CPW, EPPS pH 8 or LES tank water (see table 4.1) or 180 mol m⁻³ sorbitol, CPW, EPPS pH 8. Solutions replaced each day during experiment.

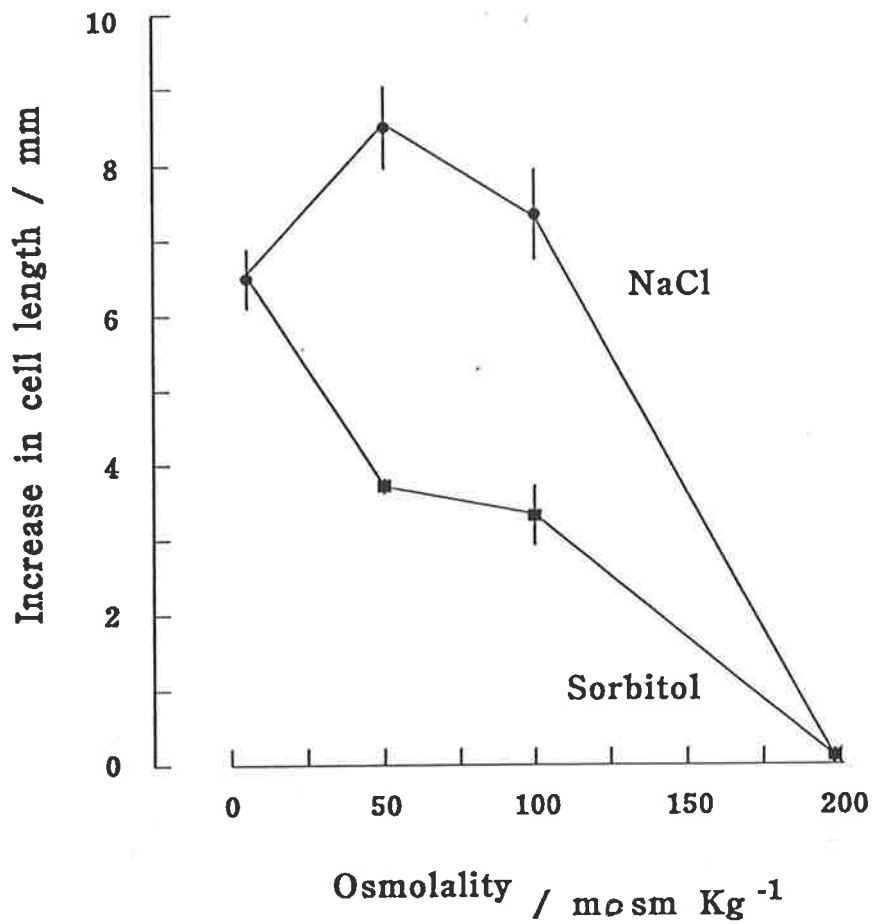


FIGURE 4.2 Growth of apical internodal cell.

■ sorbitol, ● NaCl.

Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: Na-Ca-CPW, EPPS pH 8

180 mol m⁻³ sorbitol, 10 mol m⁻³ CaSO₄, EPPS pH 8. Solutions replaced each day during experiment.

osmotic potential of the bathing solutions. There was a relationship between turgor potential and elongation rate of the apical internodal cell (Figure 4.3). These results indicate that cell elongation was dependant upon a high enough turgor being maintained within the cell. However, there remains the possibility sorbitol and high concentrations of Na^+ inhibit cell elongation due to a toxicity effect rather than simply to an osmotic effect.

4.2.3 Vacuolar ion content of high salt cultures.

The ion content of the vacuole from internodal cells of plants cultured in saline solutions are shown in Figures 4.4 and 4.5. The Na^+ concentration of the culture solution had only a small effect on the osmotic potential of the vacuole sap. The sum of Na^+ and K^+ concentration in the vacuole was relatively constant. However there was an "exchange" of K^+ for Na^+ such that the ratio of K^+ to Na^+ in the vacuole reversed between 1 and 25 mol m^{-3} Na^+ (Figures 4.4, 4.5).

Osmotic potential of the cell was between 2% and 10% higher at 50 mol m^{-3} Na^+ compared to 1 mol m^{-3} Na^+ . This translates to an increase in vacuolar osmotic potential of 0.072 MPa. However, turgor potential (calculated from the difference in osmotic potential of the cell and the bathing solution) decreased from 0.69 MPa to 0.5 MPa. Therefore the decrease in turgor potential was only slightly modified by the accumulation of Na^+ and Cl^- and to a lesser extent loss of K^+ from the vacuole.

The increase in cation content of the vacuole is balanced by the accumulation of Cl^- (Figure 4.5). The difference between Cl^- concentration and the sum of Na^+ and K^+ is probably balanced by Ca^{2+} and to a lesser extent Mg^{2+} (cf. Table 1.1).

The cytoplasmic streaming rates of internodal cells cultured in solutions of different salinities were similar (approximately 85

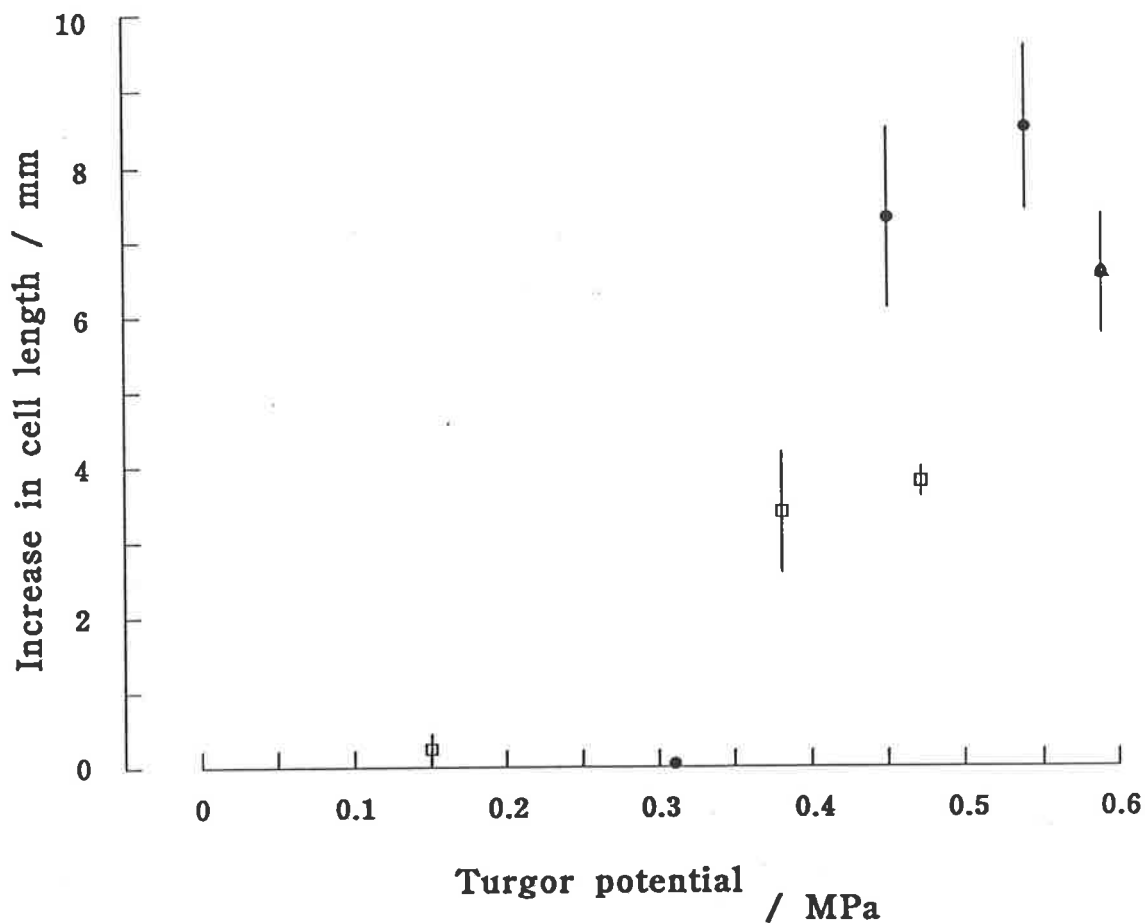


FIGURE 4.3 Relationship between growth and turgor potential.

Turgor was computed by subtracting the osmotic potential of the cell sap from the osmotic potential of the solution.

□ sorbitol, ● NaCl.

Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: Na-Ca-CPW, EPPS pH 8

180 mol m⁻³ sorbitol, 10 mol m⁻³ CaSO₄, EPPS pH 8. Solutions replaced each day during experiment.

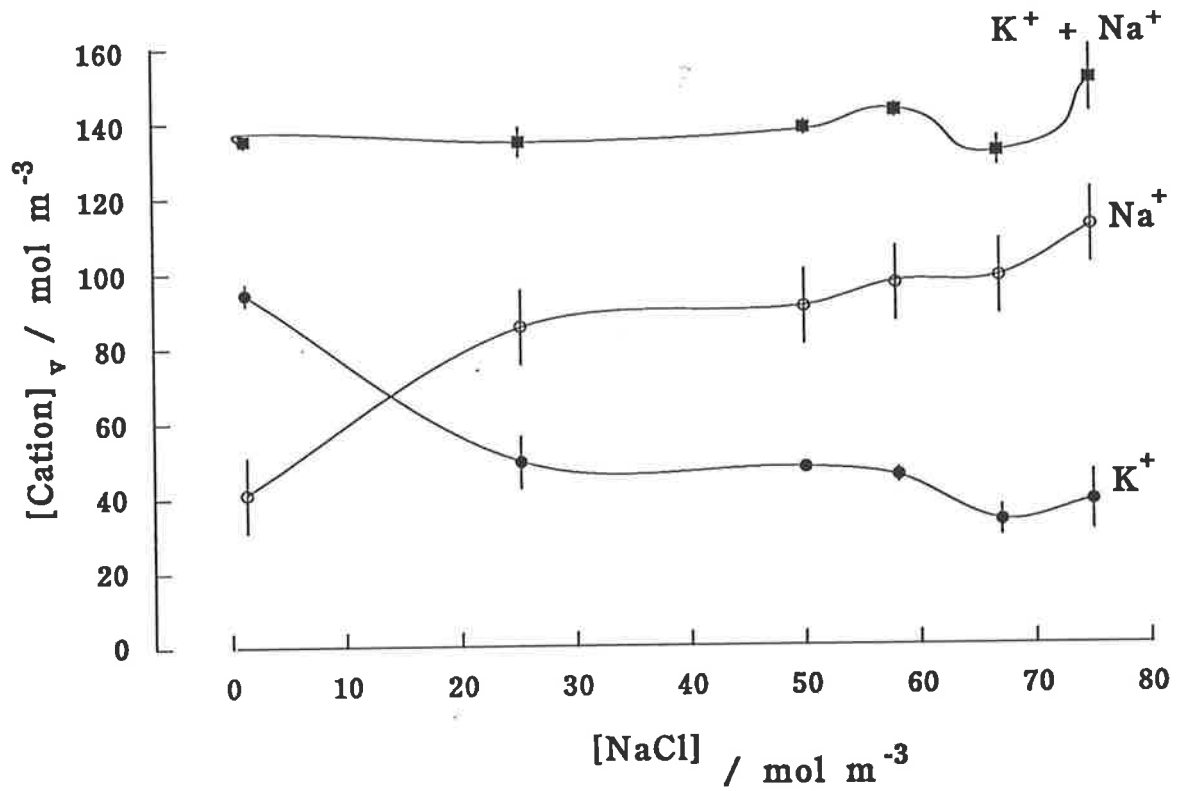


FIGURE 4.4 Concentration of cation in the vacuole cultured at various concentrations of NaCl.

● [K⁺], ○ [Na⁺] and ■ [K⁺ + Na⁺].

Each point represents the mean and standard error of 10 cells.

Culture: Tanks 1 to 6

Solution: see table 4.1

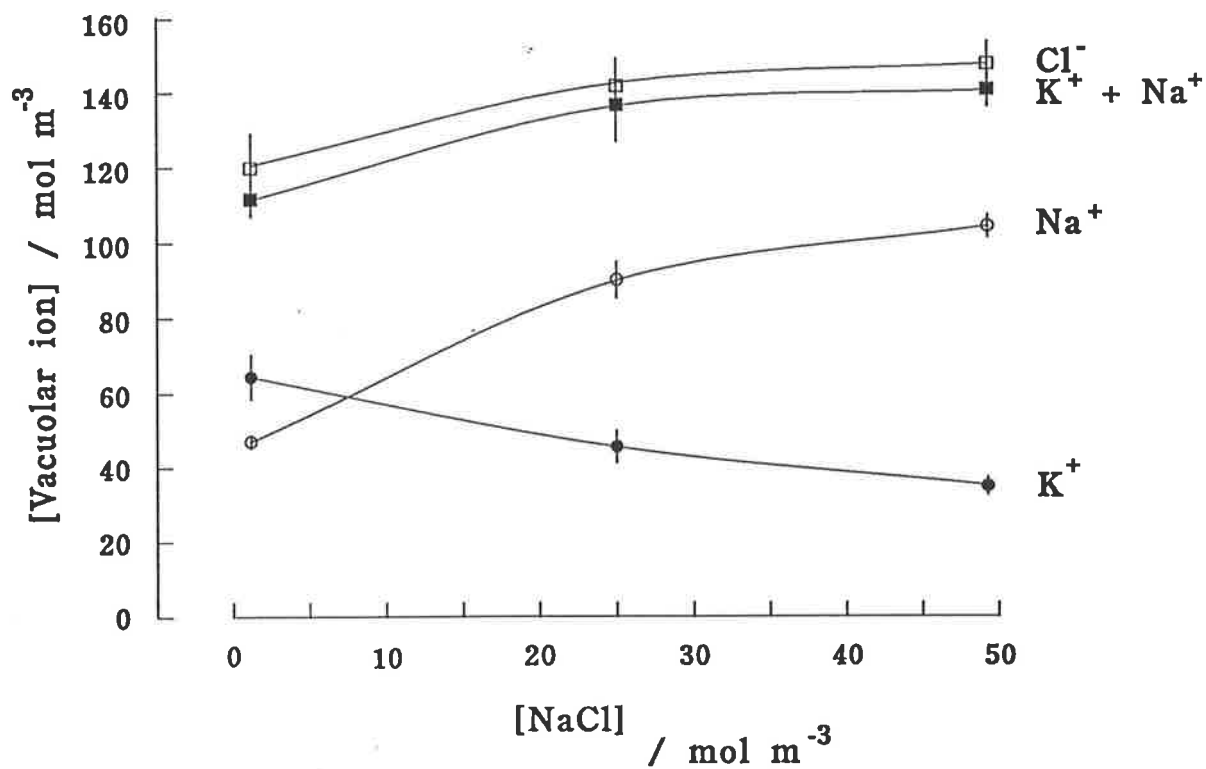


FIGURE 4.5 Concentration of ions in the vacuole cultured at various concentrations of NaCl.

● [K⁺], ○ [Na⁺], □ [Cl⁻] and ■ [K⁺ + Na⁺].

Each point represents the mean and standard error of 10 cells.

Culture: Tanks 1 to 3

Solution: see table 4.1

$\mu\text{m s}^{-1}$) indicating normal cytoplasmic streaming (see Chapter 3).

4.2.4 Ion content of isolated internodes.

The vacuolar cation concentration of cells transferred from culture tanks (see Table 2.1 for chemical composition) to 100 mol m^{-3} Na-Ca-CPW² is shown in Figure 4.6. During the first 10 days after transfer vacuolar Na^+ concentration doubled. For the remainder of the experiment Na^+ concentration was relatively stable. The K^+ concentration of the vacuole decreased as the experiment progressed. Initially the increase in Na^+ was greater than the loss of K^+ such that the sum of K^+ and Na^+ increased.

Generally, an elevation of external Na^+ concentration resulted in an increase in vacuolar Na^+ , a decrease in vacuolar K^+ and a small gain in total cation. The increase in vacuolar cation concentration was balanced by an increase in vacuolar Cl^- . The role played by Cl^- as the anion used by the cell to balance cation uptake is discussed in chapter 6.

4.2.5 Are the changes in vacuolar ion content an artifact of detaching internodal cells from the plant?

The results presented in Figure 4.7 show that cell osmotic potential increased by approximately 25% upon transfer to 100 mol m^{-3} Na^+ , assuming the changes in K^+ and Na^+ concentration were balanced by increases in Cl^- , and disregarding the contributions of any other ions to vacuolar osmotic potential. Sanders (1981b) has shown that when isolated *Chara* internodal cells are stored in APW the osmotic potential of the cell increased by up to 30%. However, Sanders (1981b) did not

²Na-Ca-CPW = 100 mol m^{-3} NaCl; 10 mol m^{-3} CaSO₄; 0.2 mol m^{-3} K₂SO₄

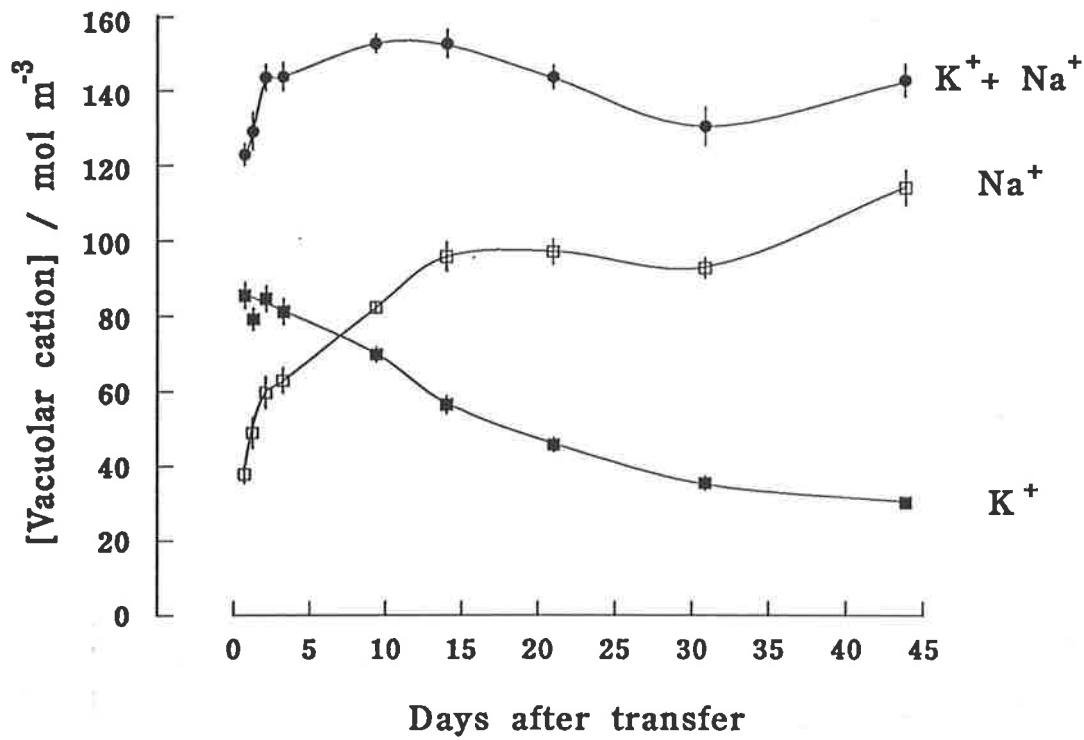


FIGURE 4.6 Concentration of cation in the vacuole after transfer to Na-Ca-CPW.

■ [K⁺], □ [Na⁺] and ● [K⁺ + Na⁺].

Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: Na-Ca-CPW EPPS pH 8

Solutions replaced each day during experiment.

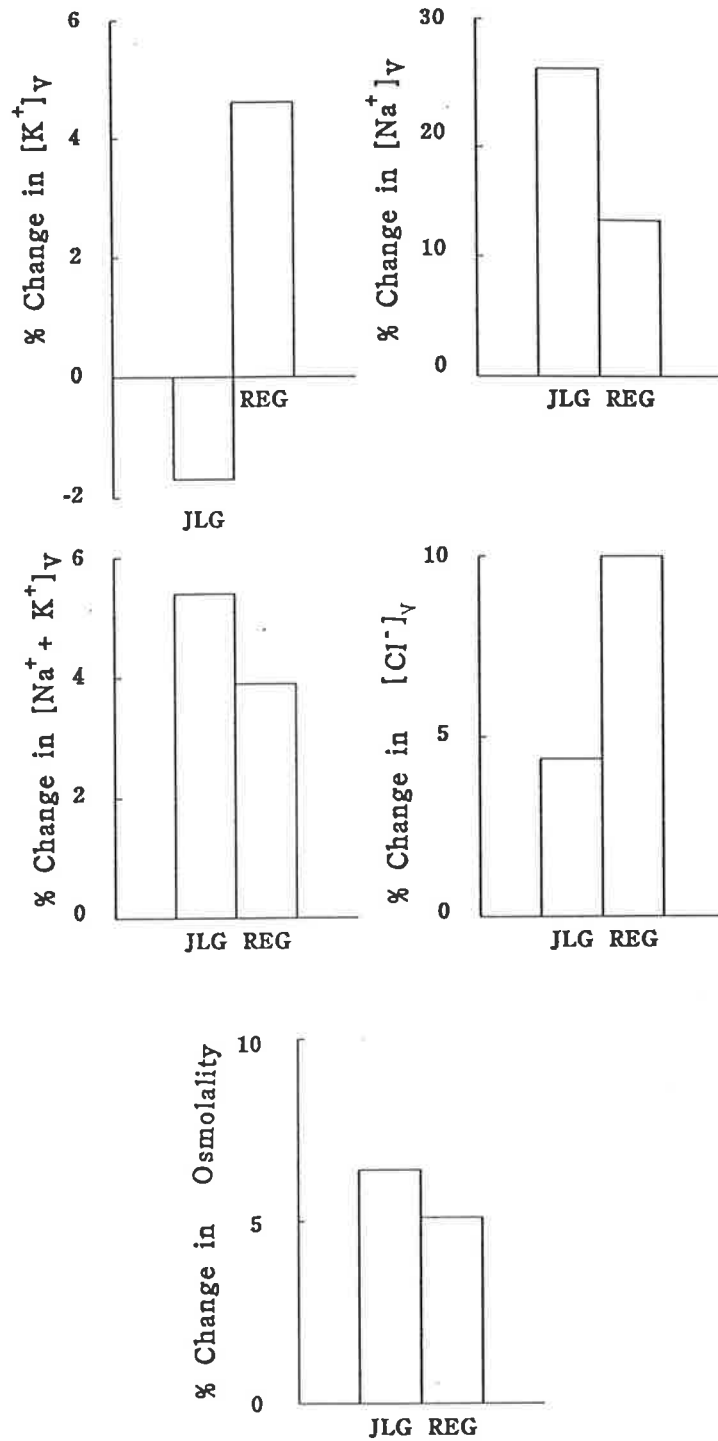


FIGURE 4.7 Changes in vacuolar ion content after detaching internodal cells from the plant.

Each value represents the mean of 10 cells.

Culture: REG and LES

Solution: CPW EPPS pH 8

Solutions replaced each day during experiment which lasted 7 days.

mention the ions responsible for this. The increase in cell osmotic potential reported by Sanders (1981b) is similar in size to that reported when internodes are transferred to $100 \text{ mol m}^{-3} \text{ Na}^+$ (see Figure 4.7). An experiment was designed to see if this phenomenon described by Sanders (1981b) could be responsible for the increase in the osmotic potential of the plant observed when cells were transferred to Na-Ca-CPW.

Cells were cut from JLG and REG cultures (see Table 4.2 for water chemistry). After 7 days the osmotic potential of cells stored in CPW increased by 5 to 6% (Figure 4.7). This was much smaller than the increases in cell osmotic potential described for cells transferred to solutions containing elevated concentrations of Na^+ and was also much smaller than the 30% increase described by Sanders (1981).

The increase in vacuolar osmotic potential in CPW was due to an increase in vacuolar Na^+ and to a lesser extent a decrease in vacuolar K^+ concentration. The ratio of K^+ to Na^+ in the vacuole decreased with time in CPW. The increase in cation concentration was balanced by an increase in Cl^- concentration in one experiment but not in the other.

The results presented show that there are changes in the ionic composition of the vacuole after isolating and placing in CPW. The trend is similar whether the cells are stored in CPW or Na-Ca-CPW however, the increases in vacuolar Na^+ and osmotic potential and the decrease in vacuolar K^+ were much greater in Na-Ca-CPW than in CPW.

4.2.6 What determines the ratio of K^+ to Na^+ in the vacuole?

An experiment was designed to determine whether changes in the vacuolar ion concentrations at elevated Na^+ were a result of the increased ion content of the external medium or of a decreased osmotic potential of the medium. The ionic composition of the vacuole from cells stored in CPW was similar to cells stored in 180 mol m^{-3}

sorbitol³ for 7 days (Figure 4.8). This suggests that the increase in osmotic potential of the cell when stored in elevated Na⁺ was not a reponse to low cell turgor but was a result of either the high external Na⁺ concentration or the ratio of K⁺ to Na⁺ in the external solution.

Whether the ratio of K⁺ to Na⁺ in the vacuole is a function of the external ratio of K⁺ to Na⁺ or elevated Na⁺ in the external media was tested by changing external K⁺ from 0 to 20 mol m⁻³ at 100 mol m⁻³ Na⁺ (Figure 4.9). When the external K⁺ ranged from 0 to 20 mol m⁻³ the increase in vacuolar K⁺ concentration was approximately 37 mol m⁻³. A decrease in vacuolar K⁺ occurred in all treatments except when external K⁺ was 20 mol m⁻³. The ratio of K⁺ to Na⁺ increased with increasing external K⁺ concentration. To summarize it appears that addition of 100 mol m⁻³ Na⁺ caused an increase in vacuolar Na⁺ concentration independent of the external K⁺ concentration. The degree to which K⁺ is lost from the vacuole was dependant upon the concentration of K⁺ in the external medium.

4.2.7 Effects of low turgor and external Na⁺ on ¹⁴C influx.

An experiment was conducted to measure the effect of elevated Na⁺ and turgor reduction (using sorbitol) on the transport of ¹⁴C into *C. corallina*. This experiment was conducted at pH 8 so that at least 90% of ¹⁴C influx would be via transport of ¹⁴HCO₃ and the remainder by diffusion of ¹⁴CO₂ (Lucas 1975a,b). The vacuolar concentration of K⁺ and Na⁺ and cytoplasmic streaming rate of the cells used in this experiment was measured.

The results presented in Table 4.2 show that changes in the ionic

³ 180 mol m⁻³ Sorbitol included 10 mol m⁻³ CaSO₄, 0.2 mol m⁻³ K₂SO₄ and was chosen because it has the same osmolarity as 100 mol m⁻³ NaCl, 10 mol m⁻³ CaSO₄, 0.2 mol m⁻³ K₂SO₄ treatment (Weast 1971).

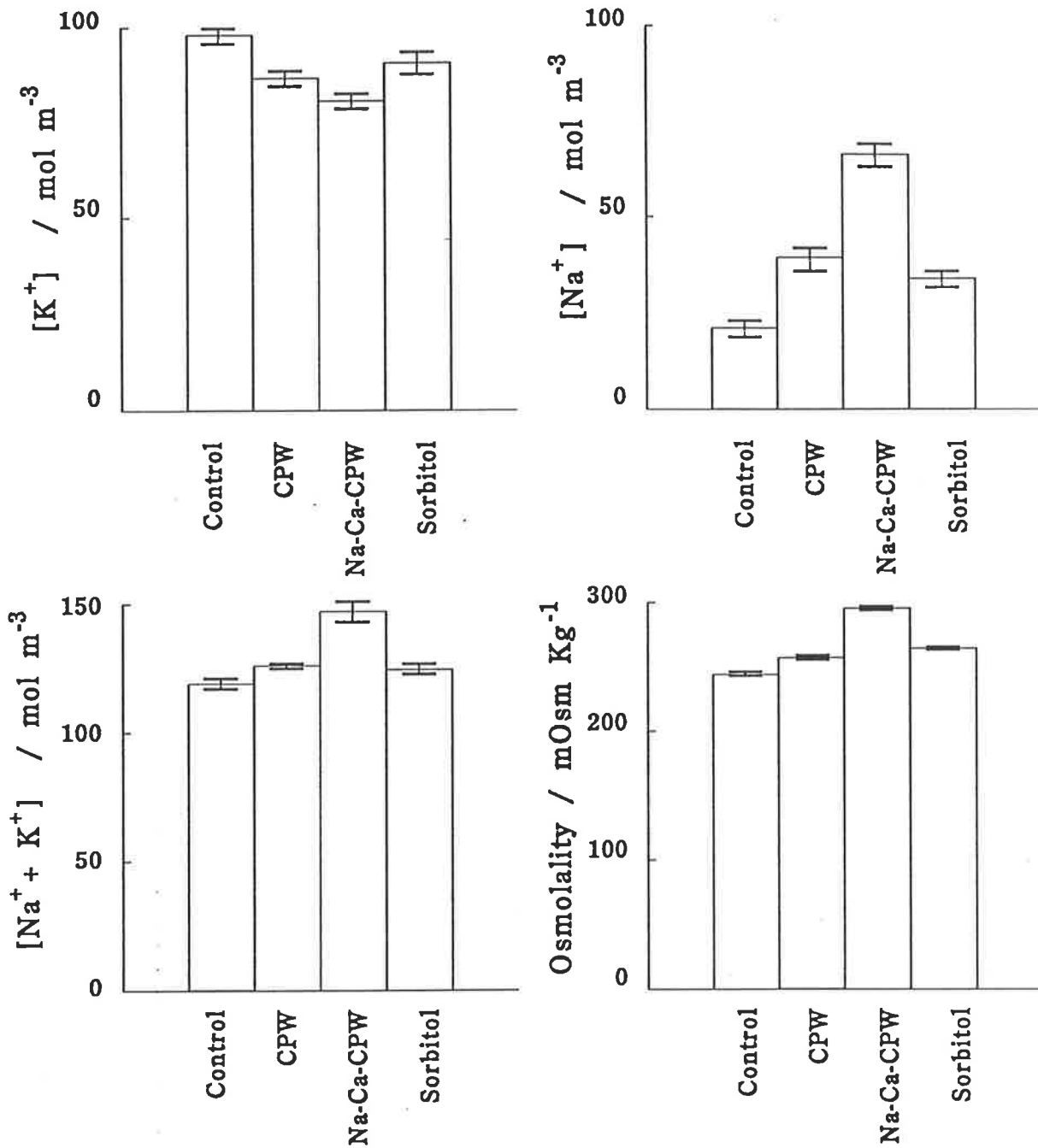


FIGURE 4.8 Vacuolar cation concentration after transferring internodal cells to CPW, Na-Ca-CPW or 180 mol m^{-3} sorbitol.

Control represents the pre-treatment ion content.

Each value represents the mean and standard error of 10 cells.

Culture: JLG

Solution: EPPS pH 8 + either CPW, Na-Ca-CPW or 180 mol m^{-3} sorbitol + CPW

Solutions replaced each day during experiment.

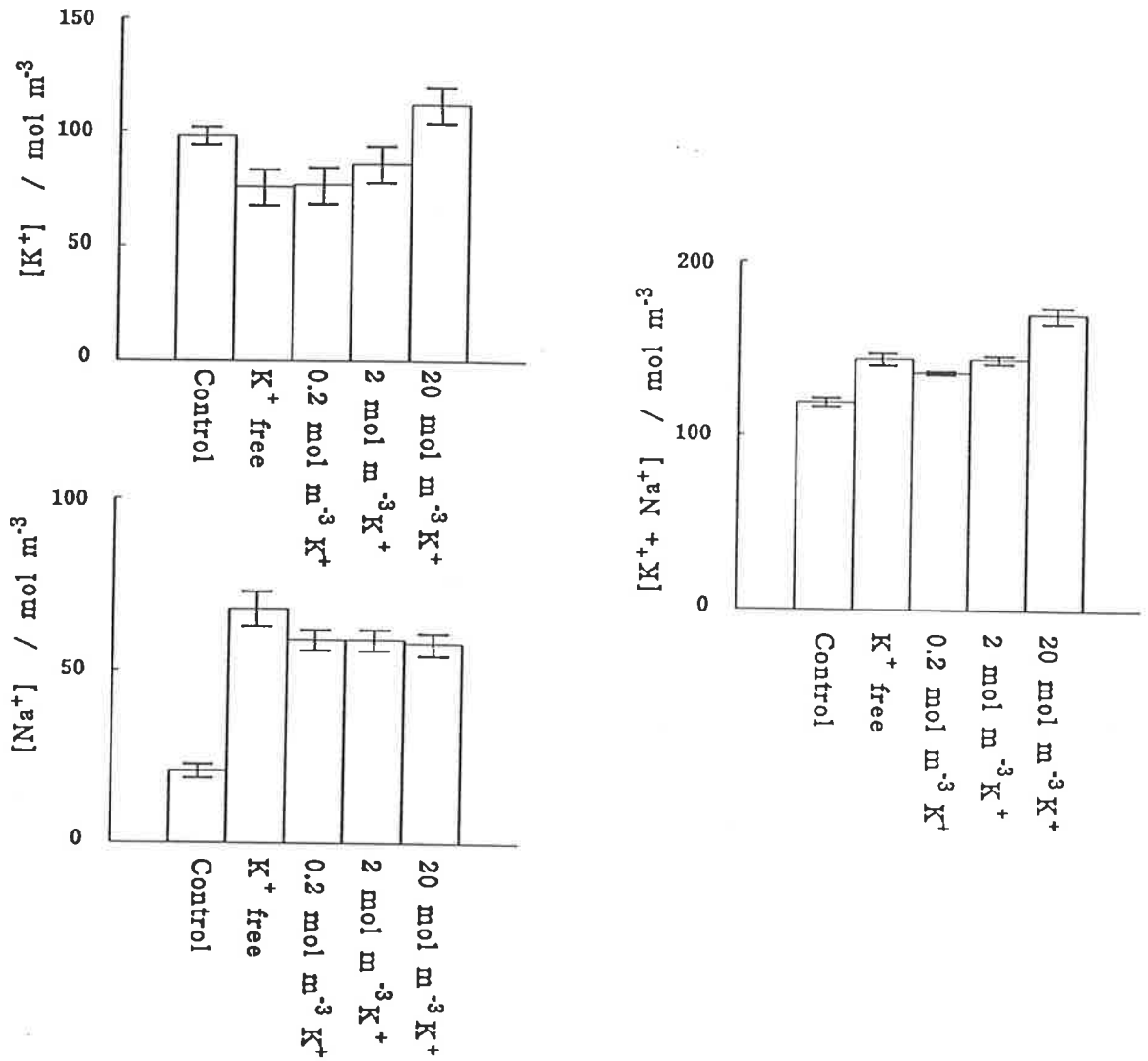


FIGURE 4.9 Vacuolar cation concentration after transferring internodal cells to various concentrations of K^+ in Na-Ca-CPW.

Control represents the pre-treatment ion content.

Each value represents the mean and standard error of 10 cells.

Culture: JLG

Solution: $0-10 \text{ mol m}^{-3} \text{K}_2\text{SO}_4$, Na-Ca-CPW EPPS pH 8

Solutions replaced each day during experiment.

TABLE 4.2 Ionic composition of the vacuole of cells used for HCO_3^- influx experiments (see Section 4.2.7).

Treatment	Vacuole ion composition (mol m^{-3})		
	CPW	sorbitol	100 mol m^{-3} NaCl
control			
	$[\text{K}^+]$	78 ± 2	-
	$[\text{Na}^+]$	36 ± 1	-
Day 6			
	$[\text{K}^+]$	64 ± 2	64 ± 2
	$[\text{Na}^+]$	49 ± 2	80 ± 2
Day 20			
	$[\text{K}^+]$	65 ± 1	58 ± 2
	$[\text{Na}^+]$	52 ± 1	70 ± 1

Vacuolar ion content of the start and after 6 and 20 days in CPW or 180 mol m^{-3} sorbitol, 0.1 mol m^{-3} K_2SO_4 , 10 mol m^{-3} CaSO_4 or 100 mol m^{-3} NaCl, 0.1 mol m^{-3} K_2SO_4 , 10 mol m^{-3} CaSO_4 . All solutions contained 2.5 mol m^{-3} EPPS pH 8. Each value represents the mean and S.E. of 10 cells.

content of the vacuole followed the typical pattern described earlier (Section 4.2.4). Isolation of internodal cells resulted in a reduction in K^+ and an increase in Na^+ . The increase in vacuolar Na^+ was much greater in Na-Ca-CPW than in either the sorbitol or CPW. The vacuolar ionic composition of turgor reduced cells (using sorbitol) was the same as that from the CPW treatment. There was a slight (though less than 10%) reduction in streaming rate in both the sorbitol and Na-Ca-CPW treatments (Table 4.2).

The influence of turgor reduction and elevated Na^+ on $H^{14}CO_3^-$ assimilation is shown in Figure 4.10. Fixation of $H^{14}CO_3^-$ in CPW varied between $264 \text{ nmol m}^{-2} \text{ s}^{-1}$ on day 1 and $454 \text{ nmol m}^{-2} \text{ s}^{-1}$ on day 6. HCO_3^- fixation was much higher on day 6 than any other day. The reason for this is unknown. HCO_3^- fixation of cells bathed in Na-Ca-CPW was initially similar to cells bathed in CPW (ignoring day 6) but increased to $378 \text{ nmol m}^{-2} \text{ s}^{-1}$ by day 20. HCO_3^- fixation during the first 6 hours after turgor reduction using sorbitol increased from $296 \text{ nmol m}^{-2} \text{ s}^{-1}$ to $589 \text{ nmol m}^{-2} \text{ s}^{-1}$. The rate of HCO_3^- fixation in sorbitol gradually reduced during the remainder of the experiment but always remained higher than HCO_3^- fixation in CPW.

There was no correlation between vacuolar concentrations of K^+ and Na^+ on the fixation of $H^{14}CO_3^-$. Similarly there was no correlation between cytoplasmic streaming rate and $^{14}HCO_3^-$ assimilation.

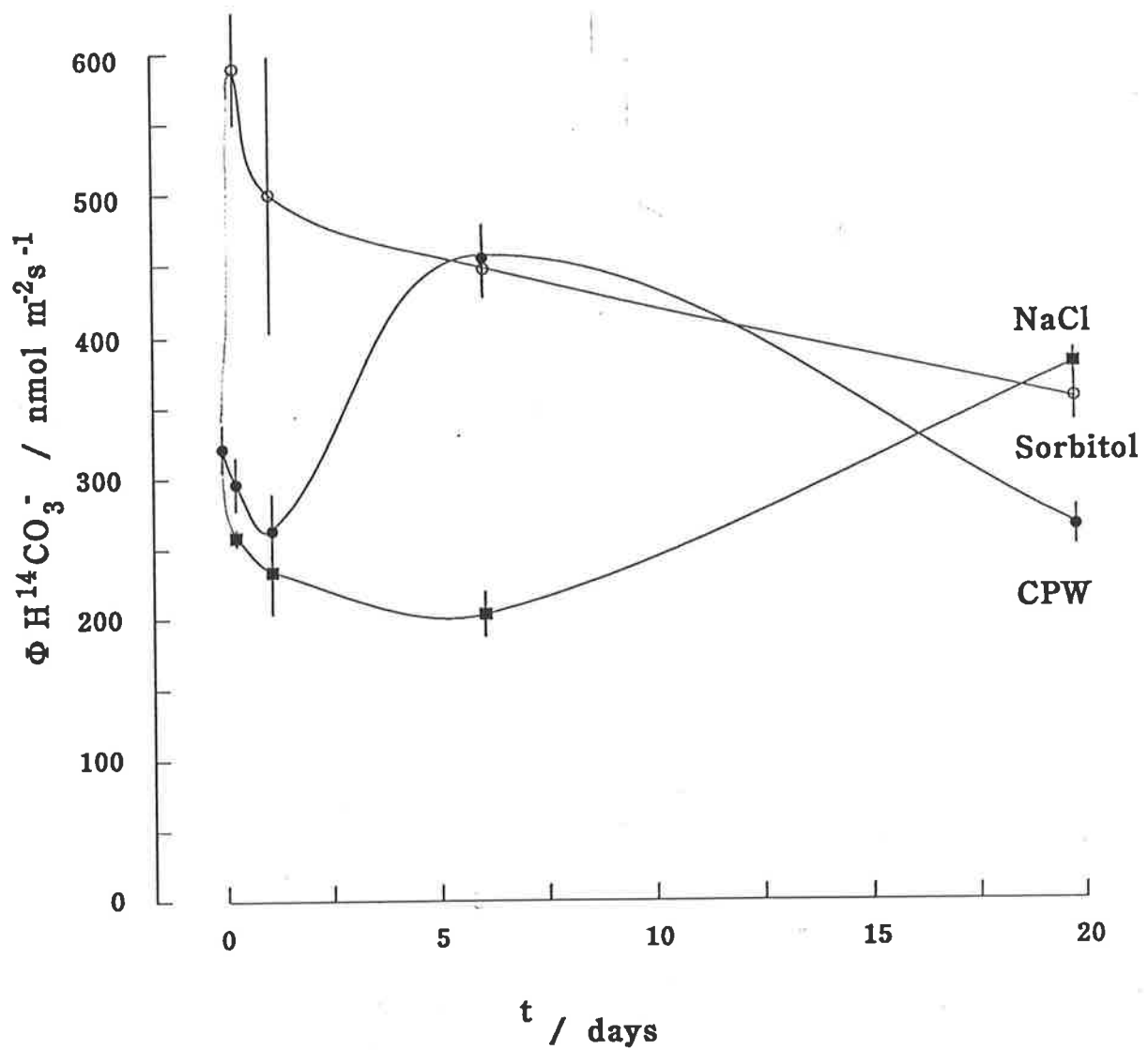


Figure 4.10 Effect of Na-Ca-CPW and 180 mol m^{-3} sorbitol on $\text{H}^{14}\text{CO}_3^-$ influx.

Each value represents the mean and standard error of 10 cells.

Culture: LES

Solution: CPW EPPS pH 8

Na-Ca-CPW EPPS pH 8

180 mol m^{-3} sorbitol, 10 mol m^{-3} CaSO_4 , EPPS pH 8

Solutions replaced each day during experiment.

4.3 DISCUSSION.

4.3.1 Growth of cultures.

There are few references to the growth rate of *Chara*, or the conditions under which Characean cells can be grown in culture. Hope and Walker (1975) claimed that it is difficult to grow *Chara* in a chemically defined medium. They recommend the most successful way to culture *Chara* was to plant cuttings in soil or mud collected from locations where *Chara* flourishes and then fill the tank with tap water or a dilute nutrient solution. The bathing medium should then contain approximately $2 \text{ mol m}^{-3} \text{ Na}^+$ and $0.15 \text{ mol m}^{-3} \text{ K}^+$ and whatever nutrients that leached from the soil. To my knowledge culturing *C. corallina* in conditions other than this has not been reported. Other charophytes have been cultured in much the same way (eg. Hoffmann and Bisson (1986) cultured *C. buckellii* collected from a brackish lake in a brackish medium).

The results presented in this chapter show that *C. corallina* can be cultured in quite brackish water. However, at higher salinities ($>50 \text{ mol m}^{-3} \text{ Na}^+$) growth was slower and the plants appeared to be less healthy. Cells grown at external Na^+ concentrations greater than 50 mol m^{-3} had very few whorl cells and the internodal cells were a pale or brownish green. Cells in this condition look similar to those from senescing plants. Winter, Meyer and Kirst (1987) and Winter, Jansen and Kirst (1988) describe a similar morphological progression in ageing *C. vulgaris*.

I initially tried to grow these salt series cultures in a constant temperature room (20°C) with a 16/8 hour, day-night cycle at a light intensity at the waters surface of between $45\text{-}55 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Under these conditions the plants grew very slowly and had short, bleached internodes. Once shade cloth was placed over the tanks so that the light intensity at the waters surface was reduced to $5\text{-}6 \mu\text{mol m}^{-2} \text{ s}^{-1}$

the plants grew much more quickly and produced longer, greener internodal cells. This low light stimulation of growth was observed at all of the salinities at which plants were grown.

Raven, Smith and Glidewell (1979) consider *C. corallina* to be a shade plant, since it is able to carry out photosynthetic carbon fixation and grow phototrophically at low irradiances. *C. corallina* achieves this by having low dark respiration and consequently the irradiance required for photosynthesis to compensate respiration (the compensation point) is also low. Raven and Smith (1978) report the irradiance for saturation of photosynthesis for *C. corallina* to be between 15 and 20 W m^{-2} and the compensation point to be 0.5-1 W m^{-2} for mature isolated internodes and 3 W m^{-2} for the apical cells. Raven and Smith (1978) measured irradiance between 400nm and 700nm. Their values for light measurement can be converted to a photon flux density according to the conversion described by Richardson, Beardall and Raven (1983). The values for saturation of photosynthesis and compensation point of Raven and Smith (1978) are then, 75-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 2.5-5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ respectively. These results show that in the experiment described above *C. corallina* grew at photon flux densities only slightly above the reported compensation point for this species and appeared to look healthier than *C. corallina* grown at a photon flux density closer to the saturating level for photosynthesis. Two possible explanations for this are as follows: it is possible that photon flux densities that are below saturating level for photosynthesis can inhibit growth or it may be that the relatively long fat, cells produced at low irradiances are a response to low irradiance and what appears to be a culture of pale and stunted cells is a healthy culture.

The photon flux density measured at the waters surface of the *Chara* tanks from which most of the material for the experiments

undertaken in our laboratory was between 20-40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, yet these cells resembled the low irradiance cells described from the previous experiment. This may be explained by the size and shape of the two types of tank used. In the salt series of experiments *Chara* was cultured in 7l aquaria whereas laboratory cultures were grown in 70l tanks. The smaller glass tanks used in the salt series of experiments let light in from all sides as well as the top surface such that no cell was further than 100 mm from a light penetrating surface. In contrast, very little light passed through the sides of the large plastic containers and the size of the tub was such that a cell could be up to 500 mm from the waters surface.

Light is attenuated exponentially with depth according to the Lambert-Beer Law:

$$I_d = I_o \cdot e^{-kd}$$

where d = depth, I_o = is the incident radiation at the surface, I_d = is the photon flux density at depth, d , and k is an extinction coefficient. The extinction coefficient of the water in the *Chara* tanks would be such that the photon flux density would be significantly reduced at the bottom of a 70l tank relative to the surface. This may account for the differences in growth patterns found between cultures in the two types of tank.

These results raise the question, how much light is needed by a *Chara* culture for optimum growth and indeed what is optimum growth? For the experiments reported in this thesis the relatively long, fat, dark green cells like those from the "low light" cultures were used. Whatever the "preferred" or optimal internodal cell morphology for *C. corallina* is, this morphology is likely to vary depending upon the environment.

4.3.2 Growth of detached shoots.

The salt series tanks showed a salinity range in which *C. corallina* could be cultured. However, this method did not allow for easy comparisons of growth rate between cultures, nor did it give much flexibility in regulating the elemental composition of the bathing solution. For these reasons I measured growth of detached branchlets of *C. corallina*.

Franceschi and Lucas (1982) measured growth rate of *C. corallina* by measuring the rate of elongation of the apical internodal cells of isolated branchlets. Their results indicate that the apical internode of *C. corallina* can increase in length by 218% during an 11 day period. In their experiments part of the branchlet was exposed to Cl^- -free solution, a treatment which they claimed to reduce growth rate. Franceschi and Lucas (1982) observed similar growth rates when 20 mol m^{-3} NaCl was added to the solution. Green, Erickson and Buggy (1971) measured growth rate of isolated internodal cells of *Nitella sp.* They reported growth rate as a percentage change in length of the cell and measured growth rates of approximately $1\% \text{ h}^{-1}$. The growth rates measured by Green et al. (1971) are of the same order of magnitude as those measured by Lucas and Alexander (1982).

The method of Franceschi and Lucas (1982) proved to be useful way of measuring growth of *Chara*. The results obtained using this technique compared favorably with the observations made on the salt series cultures described in Section 4.3.1.

When most plants are exposed to moderate concentrations of Na^+ , their growth rates fall (Greenway and Munns 1980). The processes that induce this slower growth are not fully understood. Growth rate of the *Chara* internode was suppressed by concentrations of Na^+ in excess of 50 mol m^{-3} . A similar pattern of suppression of growth is seen in

salt-sensitive nonhalophytes such as beans, soybeans, citrus, avocado and stone fruits (Greenway and Munns 1980). Whether the suppression of growth of *C. corallina* is a result of adverse water relations or ion excess will be discussed.

The rate of apical cell elongation in *C. corallina* is loosely correlated with cell turgor (Figure 4.3). Plant cell growth is generally assumed to be the result of turgor potential acting on a yielding cell wall (eg. Green et al. 1971, Greenway and Munns 1980). However, recent experiments in saline soils show that turgor might not control the rate of leaf expansion of plants (Termaat et al. 1985, Munns 1988). Green et al. (1971) showed the growth of *Nitella* sp., in the short term (<15 minutes), to be extremely sensitive to turgor potential. Growth of *Nitella* was described by the following equation (Green et al. 1971):

$$r = (P - Y)m$$

where r = rate of growth, P = turgor potential, Y = threshold turgor and m = the yielding tendency of the cell wall. However initial responses of growth rate to changes in P were compensated for by changes in Y such that growth rate reverted back to its original value. Changes in Y are thought to be the result of metabolic-dependant reactions that loosen the cell wall (Green et al. 1971). The larger the decreases in turgor potential the less well compensated growth rate became. This led to lower growth rates of *Nitella* until turgor fell to about 0.2 MPa (from about 0.5 MPa). Growth did not resume once turgor dropped to 0.2 MPa. Therefore, 0.2 MPa represented the lowest yield threshold, Y_1 , of the *Nitella* cell wall. The results of Green et al. (1971) have been used (eg. Termaat et al. 1985) to suggest that turgor potential does not control the rate of cell growth.

The results I obtained show that turgor reduction using NaCl did not affect growth when turgor was reduced from 0.60 MPa (in CPW) to 0.45 MPa (in 50 mol m⁻³ NaCl). The results presented for *C. corallina* in Figure 4.3 suggest the minimum value of the yield threshold of the cell wall was approximately 0.29 MPa. This is a similar value to the 0.2 MPa obtained by Green et al. (1971). The growth rate of *C. vulgaris* dropped to zero when cell turgor dropped below 0.57 MPa, which was still 70% of the initial turgor (Winter and Kirst 1990).

Sorbitol is frequently used as a non permeating osmoticum in various types of experiment (eg. Bisson 1989, Lucas and Alexander 1981) to lower the water potential of the medium. These experiments assume sorbitol itself is not injurious to the cell and that any effect upon the cell is solely due to a reduction in osmotic potential of the medium. In my experiments lowering the water potential of the medium using sorbitol resulted in an inhibition of growth that was greater than that produced by NaCl at the same osmolality. This suggests that sorbitol is inhibitory to growth, not because it lowers the water potential of the medium, but because it affects growth more directly.

Sorbitol has been shown to be deleterious to cell survival when it is added with NaCl. Bisson (1989) reported that sorbitol enhanced Na⁺ toxicity in *C. corallina*, though sorbitol itself was not toxic. Bisson (1989) suggested that sorbitol increased Na⁺ toxicity by decreasing the ability of Ca²⁺ to protect against salt injury. A similar result was described by Lucas and Alexander (1981) who suggested that using sorbitol to lower turgor potential increased membrane permeability to Na⁺. The interaction between Ca²⁺ and membrane permeability to Na⁺ will be discussed in Chapter 7.

Whilst sorbitol appears to inhibit growth rate it does not affect cytoplasmic streaming, membrane potential difference or short-term carbon fixation in *C. corallina*.

4.3.3 Does *C. corallina* regulate osmotic potential or turgor potential?

It is generally accepted that *C. corallina* like most freshwater algae does not regulate cell turgor (Bisson and Bartholomew 1984). Turgor regulation seems to be a requirement for plants which come from environments which have a high and variable osmotic potential, such as in estuarine situations (Bisson and Kirst 1983). Freshwater plants live in an environment in which the external osmotic potential is much lower than the osmotic potential of the plant. Because the osmotic potential of the solution is small relative to the internal osmotic potential, significant changes in external osmotic potential will generally cause only minor changes in plant turgor. Therefore, osmotic regulation is an effective form of turgor regulation in freshwater environments (Bisson and Kirst 1983, MacRobbie and Burman 1989).

Sanders (1981a) argued that osmotic potential is not an effectively regulated parameter in *C. corallina* because the osmotic potential of internodal cells increases after isolation. The increases in osmotic potential observed by Sanders (1981a) were of the order of 30%. In the experiments described in this chapter osmotic potential of isolated internodes in CPW increased by only 5-6%. The results I obtained are similar to those of Bisson and Bartholomew (1984) who found no consistent trend in changes in osmotic potential of cells stored in APW. The increases in osmotic potential described by Sanders (1981a) may have been due to any of several environmental changes eg. from low to high (laboratory) temperature, light, or changes in water chemistry.

Bisson and Bartholomew (1984) did find a slight increase in osmotic potential when the cells were incubated in 25 mol m^{-3} NaCl. They argued that osmotic regulation failed at high concentrations of

external Na^+ . The solutions used in their experiments did not contain supplementary Ca^{2+} . Experiments described in this chapter show cellular osmotic potential increased in Na-Ca-CPW. Whilst Ca^{2+} is vital to survivorship of *C. corallina* at high salinity (see Chapter 3) it does not protect the cell from what Bisson and Bartholomew (1984) describe as a failure of osmotic regulation at high external Na^+ concentrations. The failure to fully regulate osmotic potential when incubated in high concentrations of Na^+ results in a slight amelioration of turgor reduction. The results described in this chapter reinforce those of Bisson and Bartholomew (1984) that *C. corallina* generally regulates osmotic potential but this regulation is not fully effective at high external Na^+ concentrations. Using the strategy of maintaining osmotic potential to regulate turgor relies on changes in external osmotic potential being much smaller than the internal osmotic potential of the cell. In the experiments described in this chapter the concentrations of NaCl in which the cells were bathed were high enough to make osmotic regulation an ineffective method of turgor regulation.

4.3.4 Net Na^+ and K^+ fluxes in cells transferred to Na-Ca-CPW.

Generally Na^+ and K^+ are equally available to the plant yet most species tend to accumulate far more K^+ than Na^+ (Flowers and Läuchli 1983). An exception to this generalization is the accumulation of Na^+ by some plant species growing on saline soils. The cytoplasm of the charophyte algae generally contains far more K^+ than Na^+ (see Table 1.1); however, the cytoplasm is only between 5% and 10% of the cell volume (Okihara and Kiyosawa 1988, Sakano and Tazawa 1984). The vacuole, which comprises the remainder of the cell has a variable ratio of K^+ to Na^+ .

The experiments described in this chapter show that elevated Na^+

in the external solution results in an increased Na^+ concentration in the vacuole. Coincident with the increase in Na^+ is a decrease in the vacuolar K^+ concentration. The decrease in vacuolar K^+ is a result of the decreased ratio of K^+ to Na^+ in the external solution, rather than a high Na^+ concentration *per se*.

These results are in partial agreement with those presented for *C. corallina* by Tufariello et al. (1988). Tufariello et al. (1988) showed that cells in Na-Ca-APW had significant increases in vacuolar Na^+ over a 6 day period. However, they also observed a significant increase in vacuolar K^+ during the first 3 days of this treatment, after which vacuolar K^+ remained constant. Vacuolar K^+ also increased upon addition of external Na^+ in *C. buckellii* (Hoffmann and Bisson 1988). In contrast repeated additions of Na-Ca-APW to *Nitellopsis obtusa* resulted in an increase in vacuolar Na^+ but a decrease in vacuolar K^+ (Katsuhara and Tazawa 1988). The response to Na-Ca-APW by *N. obtusa* described by Katsuhara and Tazawa (1988) is similar to that described for *C. corallina* in this chapter.

The results describing vacuolar K^+ concentration of *C. corallina* after addition of external Na^+ by Tufariello et al. (1988) do not agree with the results presented in this study. In the experiments described by Tufariello et al. (1988) the bathing medium contained $0.1 \text{ mol m}^{-3} \text{ K}^+$ and $70 \text{ mol m}^{-3} \text{ Na}^+$; this is compared to $0.2 \text{ mol m}^{-3} \text{ K}^+$ and $100 \text{ mol m}^{-3} \text{ mol m}^{-3} \text{ Na}^+$ in my experiments. The ratio of K^+ to Na^+ in my experiments was higher than in the experiments of Tuffariello et al. (1988). However, the results presented in Figure 4.9 predict a higher retention of K^+ in our experiments than in theirs. In the experiments described by Tufariello et al. (1988) vacuolar K^+ at the start of the experiment was approximately 40 mol m^{-3} whereas in the experiments described in this study vacuolar K^+ was initially 84 mol m^{-3} . MacRobbie (1962) has shown the vacuole of young cells to

have about twice as much K^+ than Na^+ , but as the cells aged the proportion of Na^+ increased. The cells used by Tufariello et al. (1988) may be older than those used in this study or the differences in vacuolar K^+ may reflect the water chemistry of the respective culture solutions. It may be that cells of different ages or ion content have different responses to salinity.

In view of the conflicting results concerning changes in vacuolar K^+ upon transfer to Na-Ca-CPW it is interesting to compare the ionic content of the vacuole from isolated internodes transferred to Na-Ca-CPW with the vacuole of cells cultured in NaCl. *Chara* cultured in saline conditions had a lower ratio a K^+ to Na^+ in the vacuole than plants cultured in freshwater. The ratio of K^+ to Na^+ in the vacuole decreased with increased salinity. This finding supports the results observed in my experiments using isolated internodes; vacuolar K^+ decreased as the ratio of K^+ to Na^+ decreased in the external solution.

The role of K^+ and Na^+ in the vacuole of most plants appears to be largely osmotic and together with Cl^- comprise the major part of the osmotic potential of the plant vacuole (Cram 1976, Flowers and Läuchli 1983). If the role of both Na^+ and K^+ in the vacuole is purely osmotic then substitution of Na^+ for K^+ should not be a problem to the cell. To some extent this happens; as cells age Na^+ is substituted for K^+ and at higher salinity the concentration of K^+ in the vacuole decreases.

4.3.5 Comparison of cytoplasmic and vacuolar ion concentrations.

The concentration of ions in the vacuole is regulated by the transport of ions across the tonoplast. The cytoplasmic concentration of these ions is a function of the plasma membrane and tonoplast permeability. It is generally accepted that the tonoplast resistance is less than that of the plasma membrane and that the membrane potential difference across the tonoplast is small (Hope and Walker

1975, Smith JR 1983). Tracer fluxes of K^+ and Na^+ at the tonoplast of *C. corallina* and *Nitella translucens* are much greater (1-2 orders of magnitude) than the equivalent fluxes across the plasma membrane (Raven 1976 and references therein). This suggests that ionic conditions in the vacuole may reflect those in the cytoplasm and therefore the flux of ions across the plasma membrane. For example an increase in Na^+ influx across the plasma membrane (without a corresponding efflux of Na^+) would result in an increase in cytoplasmic Na^+ concentration. This may result in an increase in the vacuolar flux, and therefore vacuolar concentration of Na^+ . However, there is some evidence that cytoplasmic and vacuolar concentrations of inorganic ions are not tightly coupled. Sanders (1980) was able to starve the cytoplasm of Cl^- by placing the cell in Cl^- -free solution and recently Smith and Walker (1989) claimed to reduce cytoplasmic K^+ by storing cells in K^+ -free solutions. Starving the cytoplasm of K^+ induced a large but transient Na^+ -coupled influx of both K^+ and Na^+ . Smith and Walker (1989) suggest the reason for the transient nature of this current is that the cytoplasmic Na^+ concentration increased thus inhibiting the cotransport system. Therefore it appears that even though there can be large ion fluxes occurring across the tonoplast, the changes in the concentration of an ion in one compartment do not necessarily reflect changes in another.

The changes in concentration of vacuolar K^+ , Na^+ and Cl^- after addition of 100 mol m^{-3} NaCl must represent fluxes of these ions across both the plasma membrane and tonoplast because the cytoplasmic pool of these ions is too small to supply the required concentrations of these ions. The cytoplasmic volume of internodal cells of *C. corallina* occupies between 5% and 10% of the total cell volume (Okiyama and Kiyosawa 1988, Sakano and Tazawa 1984). Therefore, to change the vacuolar concentration of an ion by 1 mol m^{-3} the cytoplasmic

concentration has to change by 10-20 mol m⁻³. Considering that changes in vacuolar concentration observed were up to 50 mol m⁻³ it is clearly untenable that the cytoplasm could have acted as a pool or repository for these ions.

Katsuhara and Tazawa (1986, 1987) using *Nitellopsis obtusa* measured changes in cytoplasmic concentration of K⁺, Na⁺ and Cl⁻ after the addition of 100 mol m⁻³ NaCl. In the absence of supplementary Ca²⁺ they observed a rapid increase in cytoplasmic Na⁺ and a corresponding decline in cytoplasmic K⁺. Addition of supplementary Ca²⁺ stopped and then reversed this trend. Katsuhara and Tazawa (1987) suggested a model whereby passive Na⁺ influx and K⁺ efflux from the vacuole regulate the cytoplasmic Na⁺ and K⁺ concentration.

4.3.6 Cl⁻ accumulation.

As has been described previously, increases in vacuolar Na⁺ that occur as a result of treatment in Na-Ca-CPW are partially balanced by the accumulation of Cl⁻. The studies of Tuffariello *et al.* (1988) and Katsuhara and Tazawa (1986) also show vacuolar Cl⁻ to increase when the cells are subjected to elevated Na⁺. Given the electrochemical gradient for Cl⁻ at the plasma membrane and tonoplast, Cl⁻ accumulation has to be an active flux. MacRobbie and Burman (1989) used the technique of transcellular osmosis to show that Cl⁻ influx is stimulated by lowering the osmotic potential of the cell. However, Cl⁻ influx was insensitive to decreased cell turgor obtained by lowering the solution osmotic potential with sucrose.

Observations of net Cl⁻ flux described in this chapter agree with the results of MacRobbie and Burman (1989). Vacuolar Cl⁻ did not increase in cells in which turgor had been reduced by addition of sorbitol. However, Cl⁻ influx increased in cells where turgor had been reduced by addition of NaCl.

As MacRobbie and Burman (1989) show, Cl^- influx is not regulated by cell turgor. Cl^- influx is generally believed to be controlled by cytoplasmic pH (Smith and Walker 1976) and/or cytoplasmic Cl^- concentration (Sanders 1980). There are no reasons to suspect that Cl^- influx is responding to changes in cytoplasmic pH since cytoplasmic pH was unaffected by Na-Ca-CPW (results not shown). The calculations of MacRobbie and Burman (1989) suggest that the Cl^- flux changes they observed were too big to be simply due to changes in cytoplasmic Cl^- concentration. They suggested that changes in either cytoplasmic Ca^{2+} or cytoplasmic osmotic potential (and hence volume of cytoplasmic organelles) stimulated Cl^- influx.

The net Cl^- influx described in cells placed in Na-Ca-CPW could be due to a number of factors including those outlined by MacRobbie and Burman (1989). Cytoplasmic Cl^- concentration could be significantly reduced if Cl^- was being transported to the vacuole as a counter ion to Na^+ . The resultant decrease in cytoplasmic Cl^- could stimulate Cl^- influx.

4.3.7 Effect of NaCl and turgor reduction on HCO_3^- influx.

The transport of HCO_3^- into cells of *C. corallina* is able to proceed at very high rates. Lucas (1975a,b) reports the maximum rate at which HCO_3^- is transported across the plasma membrane at pH_o 8 to be $600 \text{ nmol m}^{-3} \text{ s}^{-1}$. This value was obtained under conditions of saturating irradiance (25 W m^{-2}) and $^{14}\text{HCO}_3^-$ concentrations (3 mol m^{-3}).

$^{14}\text{HCO}_3^-$ transport rates obtained for cells in CPW in this study were similar to those reported for *C. corallina* by Lucas (1975a,b). At the light intensity used this study ($40 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \cong 8 \text{ W m}^{-2}$) Lucas (1975a,b) reported HCO_3^- transport rates of about $350 \text{ nmol m}^{-2} \text{ s}^{-1}$. The values of HCO_3^- fixation reported by Sanders (1981b) for *C. corallina* in similar conditions (10 W m^{-2} , $1 \text{ mol m}^{-3} \text{ HCO}_3^-$) are about an order of

magnitude lower than the rates described in this thesis or those of Lucas (1975) or Lucas and Alexander (1981). Lucas and Alexander (1981) observed only slight changes in HCO_3^- assimilation when turgor was reduced using either sorbitol, mannitol or sucrose. In a similar series of experiments Sanders (1981a) also found no effect of turgor reduction on HCO_3^- transport. However, Sanders (1981a) reported a stimulation of the apparent ^{14}C fixation rate by reduced turgor (this experiment was conducted at pH 6 when entry of C would be by diffusion of CO_2). Sanders (1981a) did not give a reason for this although it was pointed out that fixation of ^{14}C was not a good indicator of net synthesis since it may simply be reflecting a faster turnover of organic compounds. The experiments described in this chapter show that lowering turgor with NaCl did not stimulate HCO_3^- fixation. In contrast to the results of Sanders (1981a) and Lucas and Alexander (1981) lowering turgor using sorbitol stimulated $^{14}\text{HCO}_3^-$ fixation. This stimulation may be reflecting a faster turnover of organic compounds since it did not result in the production of organic osmoticum.

4.4 CONCLUSIONS.

C. corallina can be cultured in solutions containing up to 75 mol m^{-3} NaCl. Above 50 mol m^{-3} NaCl *C. corallina* was slow growing and had few whorl cells. Growth of isolated branchlets was not inhibited by concentrations of NaCl up to 50 mol m^{-3} .

Transfer of cells to Na-Ca-CPW resulted in an increase in cell osmotic potential. The increase in osmotic potential was a result of elevated Na^+ concentrations in the external solution since osmotic potential did not increase in isotonic sorbitol solutions. The increase in osmotic potential was brought about by an increase in vacuolar Na^+ and a smaller decrease in vacuolar K^+ . Charge balance was maintained by Cl^- influx. The ratio of K^+ to Na^+ in the vacuole is a

function of the ratio of K^+ to Na^+ in the bathing solution. Cytoplasmic streaming rate and HCO_3^- fixation were unaffected by Na-Ca-CPW.

The results summarized above suggest that *C. corallina* remained in a healthy state in Na-Ca-CPW. Reduction in growth rate of internodal cells occurred when turgor potential dropped below the yield threshold of the cell wall, approximately 2 MPa. These experiments support the recent conclusions of Tufariello *et al.* (1988) that *C. corallina* fails to occupy saline habits because of its failure to regulate turgor, not because of sensitivity to Na^+ .

CHAPTER 5

Effect of salinity on membrane potential difference.

5.1 INTRODUCTION

All living plant cells exhibit transmembrane potential differences in which the cytoplasm is usually negative with respect to the external medium (Findlay and Hope 1976). A membrane potential difference is the result of ionic concentration differences across the membrane which can be caused by two factors: differences in diffusivity of different ions across the membrane and, the presence of active transport of ions across the membrane.

A knowledge of membrane potential difference enables the electrochemical gradient for ions to be calculated. This information indicates which ions are not in electrochemical equilibrium across the membrane and therefore what, if any, ion pumps are operating.

Hoffmann and Bisson (1986) report that the properties of the plasma membrane of *C. buckellii* cultured in saline water are different to those of cells cultured in freshwater. The effect of NaCl on the behaviour of the membrane of *C. corallina* is described.

5.2 RESULTS

5.2.1 Effect of Na-Ca-CPW on membrane potential difference.

Figure 5.1 shows the membrane potential difference of *C. corallina* in CPW and in Na-Ca-CPW. The results presented were obtained from a series of experiments conducted over a 6 month period. Upon transferring cells to Na-Ca-CPW the plasma membrane usually underwent a transient depolarization but then repolarized within 1 minute (Figure 5.1). After the initial depolarization the membrane potential remained relatively constant between -190 and -160 mV for up to 4 weeks. Membrane potential difference was not measured after this time.

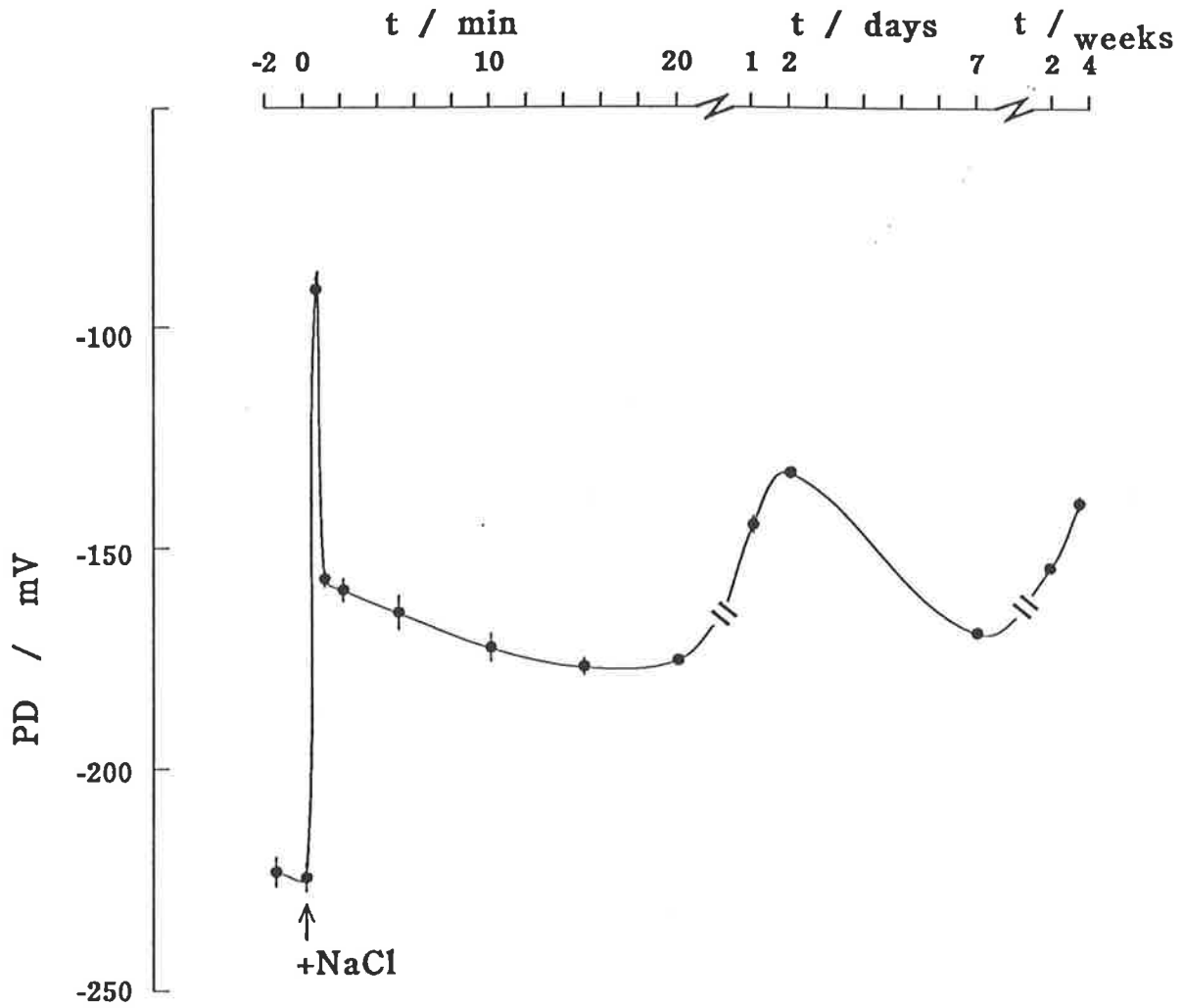


FIGURE 5.1 Membrane potential difference after transfer to Na-Ca-CPW.

Each value represents the mean and standard error of 6-10 cells.

Culture: LES

Solution: Cells initially in CPW. Solution replaced with Na-Ca-CPW EPPS pH 8 at $t=0$. Solution replaced each day during experiment.

Figure 5.2 shows membrane potential of cells as a function of external K^+ concentration. The cells used in this experiment had been stored in Na-Ca-CPW for 3 weeks prior to the experiment. Cells were exposed to different concentrations of K^+ for the minimum time required for the membrane potential difference to adopt a new "steady state", usually 2 minutes. Membrane potential difference was hyperpolarised relative to E_K^1 at all except the lowest K^+ concentration used. Membrane potential difference was insensitive to K^+ concentration between 0.05 and 5 mol m^{-3} . The results obtained by Hoffmann and Bisson (1987) with salt water grown *C. buckellii* are given for comparison.

Figure 5.3 shows the effect of changes in external pH (pH_o) on membrane potential difference. Cells hyperpolarized by 55 mV as pH_o was increased from 5 to 8. Membrane potential difference then depolarised by 40 mV as pH_o was increased from 8 to 9.5. As pH_o was increased further (to pH 11) membrane potential difference hyperpolarized back to -180 mV. Published results of the dependence of membrane potential difference on pH_o for *C. corallina* in fresh water (Bisson and Walker 1982) and *C. buckellii* in saline water (Hoffmann and Bisson 1987) are shown for comparison.

The membrane potential difference of cells in which the turgor was

E_K^1 was calculated using the Nernst Equation, and assuming cellular K^+ to be 50 mol m^{-3} ;

$$E_K = - \frac{RT}{zF} \ln \frac{K_{in}^+}{K_{out}^+}$$

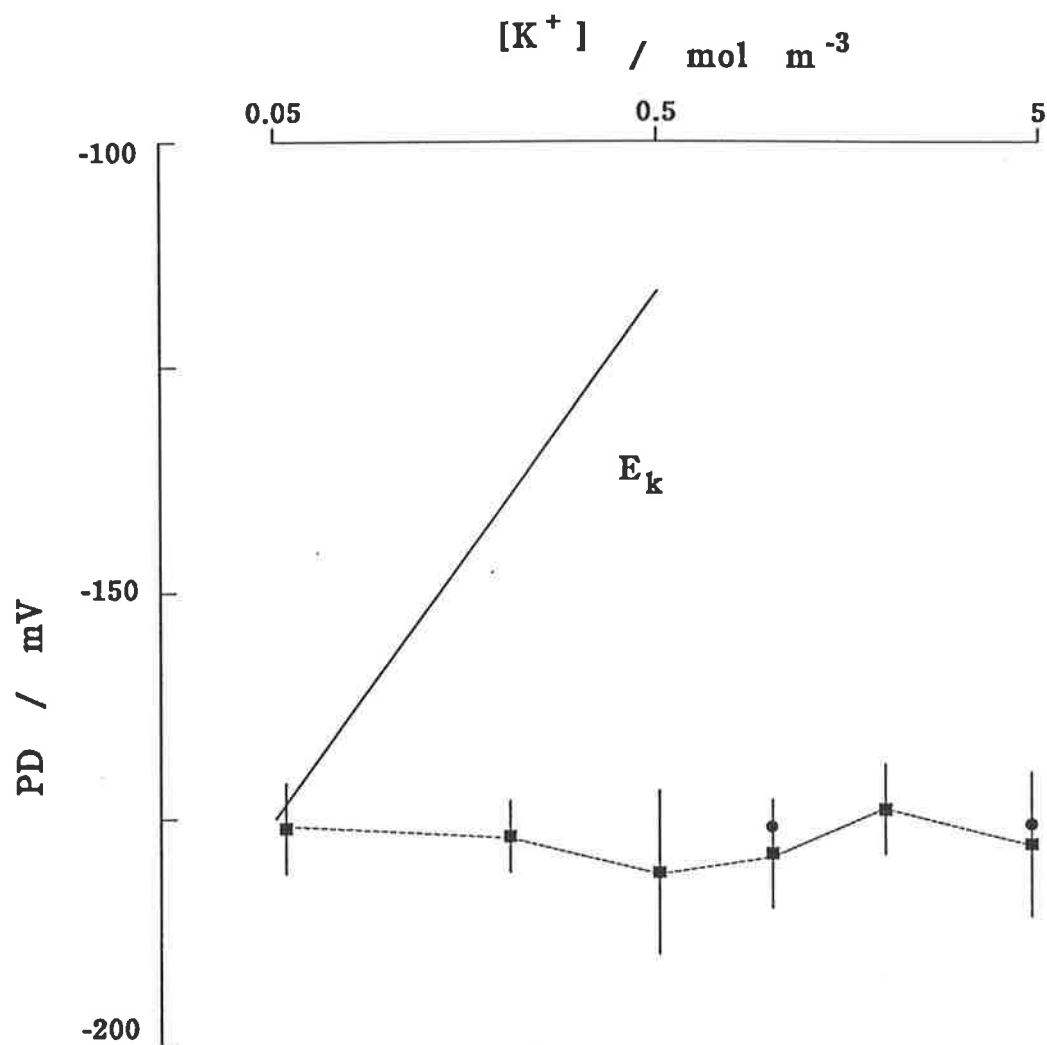


FIGURE 5.2 Membrane potential of *C. corallina* as a function of external K^+ concentration in Na-Ca-CPW.

■ results from this study, ● redrawn from Hoffman and Bisson (1987). E_k is shown and was computed assuming cellular K^+ to be 50 mol m^{-3} (see Figure 4.6). Each value represents the mean and standard error of 5-30 cells.

Culture: LES

Solution: $0.05 - 5 \text{ mol m}^{-3}$ KCl, Na-Ca-CPW EPPS pH 8

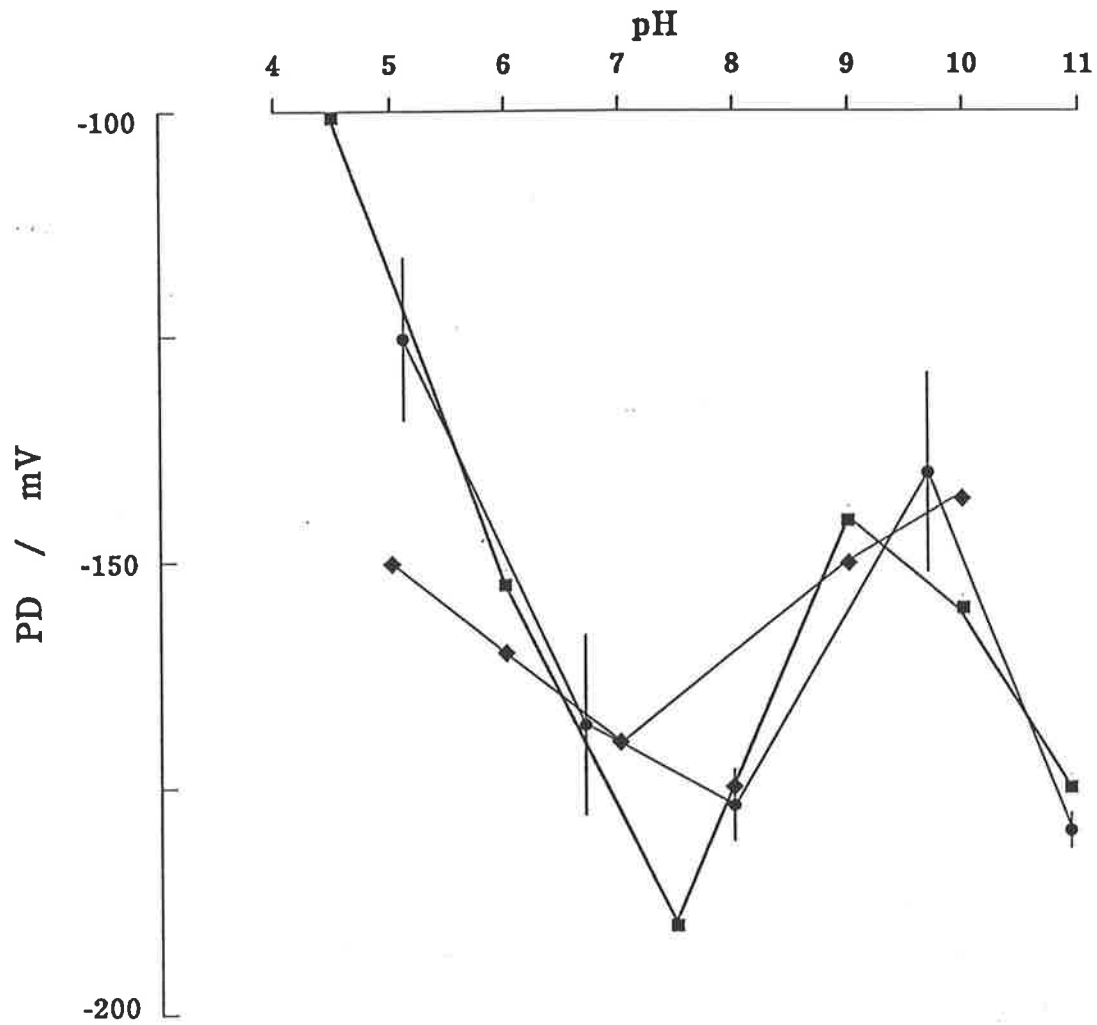


FIGURE 5.3 Membrane potential of *C. corallina* and *C. buckellii* as a function of external pH in Na-Ca-CPW.

● results from this study, ◆ redrawn from Hoffman and Bisson (1987),
 ■ redrawn from Bisson and Walker (1982). Cells pretreated by
 storing in
 Na-Ca-CPW EPPS pH 7.5 for 20 hours prior to the experiment. Each value
 represents the mean and standard error of 5-21 cells.

Culture: LES

Solution: $100 \text{ mol m}^{-3} \text{ NaCl} + 10 \text{ mol m}^{-3} + 0.1 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4 + \text{MES pH } 5.1 \text{ or}$
 $\text{TES pH } 6.7 \text{ or EPPS pH } 8 \text{ or CHES pH } 9.7 \text{ or CAPS pH } 11.$

reduced using 180 mol m^{-3} sorbitol are shown in Figure 5.4. Addition of up to 180 mol m^{-3} sorbitol resulted in a membrane potential being approximately 30 mV less negative than cells that were stored in CPW for the same length of time. Kourie and Findlay (1990a) showed the membrane potential difference of *Chara inflata* decreased by 75 mV from -215 to -140 mV upon addition of a similar concentration of sorbitol. This depolarization was accompanied by an increase in membrane conductance.

In this experiment the addition of 100 mol m^{-3} NaCl resulted in a change in membrane potential to a less negative potential than normally measured (Figure 5.1).

5.2.2 Effect of Na-CPW on membrane potential difference.

Cells were pretreated in CPW or Na-Ca-CPW for 48 hours and then mounted in an electrode chamber and impaled with a microelectrode. Cells were then subjected to 100 mol m^{-3} NaCl with or without addition of 10 mol m^{-3} CaSO_4 .

Addition of Na-CPW induced a series of action potentials from which the membrane potential difference did not recover. The membrane potential difference fell to approximately -35 mV over a period of 20 minutes (Figure 5.5). Removal of Ca^{2+} from the external solution of cells which had been pretreated in Na-Ca-CPW did not induce an action potential. However, membrane potential difference did decrease in a similar way to those cells pretreated in CPW. This result indicates that pretreatment of cells in high concentrations of Ca^{2+} and Na^+ did not protect the cell when exposed to Na-CPW.

5.3 DISCUSSION.

5.3.1 General electrophysiology.

The plasma membrane of *C. corallina* exists in one of three states

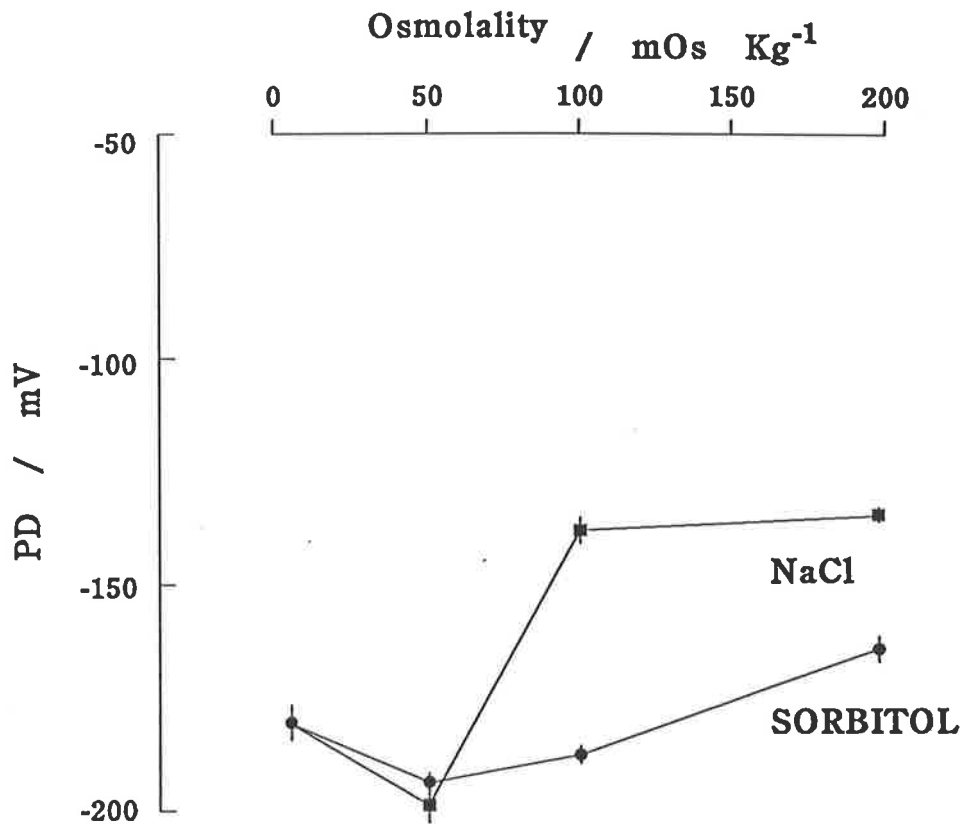


FIGURE 5.4 Effect of turgor reduction using sorbitol or NaCl on the membrane potential difference of isolated internodal cells.

■ Na-Ca-CPW, ● 180 mol m⁻³ sorbitol.

Each point represents the mean and standard error of 6 cells.

Culture: LES

Solution: Na-Ca-CPW EPPS pH 8

180 mol m⁻³ sorbitol, 10 mol m⁻³ CaSO₄, 0.1 mol m⁻³

K₂SO₄, EPPS pH 8.

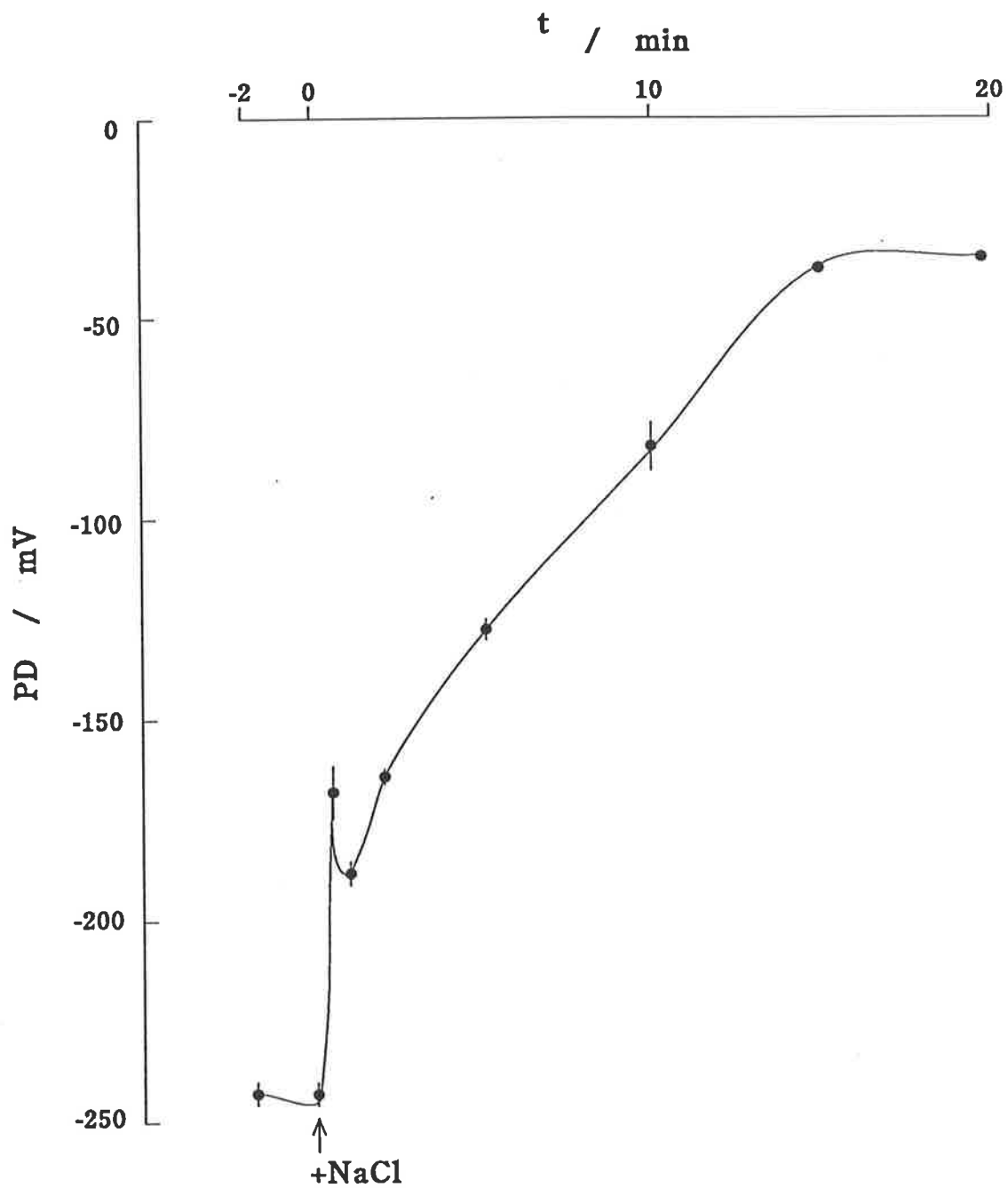


FIGURE 5.5 Membrane potential difference after transfer to Na-CPW.

Each value represents the mean and standard error of 8 cells.

Culture: LES

Solution: Cells initially in CPW. Solution replaced at $t=0$ with Na-CPW EPPS pH 8.

which can be described by the dominance of membrane potential difference by a transport system running at, or near to thermodynamic equilibrium (Bisson and Walker 1982, Bisson and Kirst 1980). These states have been referred to as the P-state, H-state and the K-state. Below pH_o 9 the membrane potential may be dominated by passive diffusion of K^+ (K-state). In this mode the membrane potential difference will be moderately negative, insensitive to pH_o and strongly dependant on K^+ concentration. In the K-state the membrane potential difference is well approximated by E_K and can be induced by inhibiting the H^+ -pump (or presumably by greatly increasing permeability to K^+ (Bisson 1984).

When the membrane is in the P-state membrane potential difference is dominated by the H^+ -pump. The membrane potential difference tends to be more negative than in the K-state, is independent of K^+ concentration and is characterized by a biphasic response to pH_o with the most negative value at about pH_o 7 (Bisson 1982). Above pH of about 10.5 the membrane potential difference is characterized by H^+ diffusion. During the H-state membrane potential difference is approximated by E_H with membrane potential difference becoming more negative with increasing pH and is independent of K_o^+ concentration (Bisson and Walker 1980,1981).

5.3.2 Effect of salinity on membrane potential difference.

Hoffmann and Bisson (1987) performed a series of electrophysiological experiments on *C. buckellii* cultured in saline water and found the membrane not to be dominated by any one of the states described above for *C. corallina*. However, *C. buckellii* cultured in fresh water had membrane properties in common with *C. corallina*. Freshwater cells appeared to be in the P-state between pH_o 5 and 7 but the K-state and H-state were never observed. The

results of Hoffmann and Bisson (1987) show that the plasma membrane of *C. buckellii* behaves differently in freshwater than in saline water.

Electrophysiological experiments were performed on *C. corallina* incubated in Na-Ca-CPW for up to 4 weeks to see if high external concentrations of Na^+ resulted in a change in behavior of the plasma membrane. The results presented in this chapter indicate that elevated concentrations of NaCl (with supplementary Ca^{2+}) resulted in a small depolarization of membrane potential difference. However, membrane potential difference appeared to be under the same control as cells in CPW. It is suggested that between pH_o of 4.5 to 9 that efflux of H^+ by the proposed H^+ -ATPase (Smith and Walker 1976) is being maintained in elevated Na^+ . The continued functioning of this pump is particularly important to the cell at elevated NaCl because the electrochemical potential for H^+ ($\Delta\mu\text{H}^+$) generated by this pump can be chemiosmotically coupled to other ion fluxes. Examples include Cl^- influx (Smith and Walker 1976) and Na^+ efflux (Clint and MacRobbie 1987).

The plasma membrane of *C. corallina* in Na-Ca-CPW was hyperpolarized relative to E_K and yet under the same conditions K^+ was gradually lost from the cell. With external K^+ concentration at 0.2 mol m^{-3} and a membrane potential difference of approximately -177mV (Figure 5.1) the theoretical equilibrium K^+ concentration for the cytoplasm would be 217 mol m^{-3} . This value for cytoplasmic K^+ concentration is approximately 2 times higher than those previously measured for *C. corallina* (see Table 1.1) and would be an unrealistically high concentration of K^+ to expect on osmotic grounds alone. This calculation predicts that efflux of K^+ from cells observed during these experiments would be energetically unfavorable and so require active efflux of K^+ . The need to postulate uphill, outward transport of K^+ is not unique to experiments described in this chapter. In many situations the membrane potential difference is

hyperpolarized relative to E_K (Walker and Smith 1977). The realization that the internal concentration of K^+ is often below its electrochemical equilibrium led to the suggestion that a steady state for K^+ would involve uphill outward transport of K^+ (Walker and Smith 1977, Smith and Walker 1989) and the proposal for an electrically silent K^+ antiport (Smith and Walker 1989). They suggested that this antiporter would operate in a similar way to the K^+/H^+ antiporter described from *Escherichia coli* by Brey et al. (1980). At present there is no direct experimental evidence to support such a transport process in *C. corallina*. However, it is a problem that requires further investigation in the general context of regulation of intracellular K^+ . For example Smith (1987) suggested *C. corallina* does not grow in brackish water because a high external concentration of Na^+ causes a progressive loss of K^+ from the cell. Eventually the K^+ concentration in the cytoplasm becomes low enough to interfere with normal enzyme functioning, particularly those enzymes associated with protein synthesis. The hypothesis presented by Smith (1987) implied that the loss of K^+ from the cell is an unavoidable cost of being in an environment which has a high external concentration of Na^+ . However, the results presented above show K^+ efflux to be an active process. An active process is subject to some form of control. It would seem unlikely that a cell suffering from a K^+ deficiency would continue to actively pump K^+ from the cell. On the other hand, *C. buckellii* and *L. papulosum* regulate turgor by increasing internal K^+ (see Figure 1.1). Here we have an intriguing conundrum, as yet unresolved.

5.3.3 Effect of removal of external Ca^{2+} from salt treated cells.

Bisson (1984) showed that when *C. corallina* was bathed in Ca^{2+} -free APW the membrane depolarized toward E_K over 1 to 2 hours. This depolarization was accompanied by an increase in plasma membrane

conductance which was caused by an increase in K^+ conductance (Bisson 1984). Tufariello et al. (1988) found when cells were placed in Na-APW² membrane potential difference decreased to -108 mV over 2 days but increased slightly to -137 mV by the third day. The Day 2 value was significantly below E_K but on Day 3 membrane potential difference was close to E_K . However, removal of Ca^{2+} from the external medium of salt-stressed *Nitellopsis obtusa* resulted in membrane potential difference decreasing to -30 mV within 30 minutes (Katsuhara and Tazawa 1986). This depolarization was due to an increase in plasma membrane permeability to Na^+ .

In the experiments described in this chapter removal of Ca^{2+} from cells previously bathed in Na-Ca-CPW resulted in a rapid depolarization of the membrane to approximately -35 mV at which membrane potential difference remained until the cell died (usually 24 hours). Under these conditions (external NaCl = 100 mol m^{-3} , external K_2SO_4 = 0.1 mol m^{-3} and a membrane potential difference of -35 mV) the hypothetical equilibrium concentration for K^+ in the cell would be 0.8 mol m^{-3} , for Na^+ 401 mol m^{-3} and for Cl^- 25 mol m^{-3} . These results indicate that the membrane potential difference stabilized close to the equilibrium membrane potential difference for Cl^- (E_{Cl}), and that membrane potential difference was well below E_K . This is a similar result to that described for *Nitellopsis obtusa* by Katsuhara and Tazawa (1986, 1988). Katsuhara and Tazawa (1988) also reported that 30 minute treatment in 100 mol m^{-3} NaCl resulted in an increase in cytoplasmic Na^+ from 11 to 90 mol m^{-3} . During the same treatment cytoplasmic K^+ decreased from 90 to 33 mol m^{-3} . Tufariello et al. (1988) suggested a similar sequence of events were responsible for the toxicity of NaCl to *C. corallina* in the absence of Ca^{2+} . The electrophysiological data

²Na-APW = 70 mol m^{-3} NaCl, 0.1 mol m^{-3} $CaSO_4$

presented for *C. corallina* supports this assumption. The effect of Ca^{2+} on Na^+ influx is the subject of Chapter 6.

In summary, the results presented in this chapter show that addition of 100 mol m^{-3} NaCl depolarizes the membrane potential difference of *C. corallina*. However, the concentration of Ca^{2+} in the external solution determines the degree to which the membrane is depolarized.

CHAPTER 6

How does Ca^{2+} protect the cell from salt damage?

6.1 INTRODUCTION.

Ca^{2+} is essential for plasma membrane integrity. Ca^{2+} cross-links the negatively charged phospholipid head groups of the plasma membrane and therefore rigidifies the plasma membrane and also neutralizes the surface negative charge of the plasma membrane (Reidell 1987). Ca^{2+} is also required by various transport proteins for their operation. Ca^{2+} has a gating effect on squid neuron K^+ channels (Armstrong and Lopez-Barneo 1987) and similarly Ca^{2+} is implicated in the closing of K^+ channels in *C. corallina* (Tester 1988, Keifer and Lucas 1982). The removal of membrane-bound Ca^{2+} by elevated Na^+ concentration could be the cause of salt damage in *C. corallina*.

The results presented in this chapter are from a range of experiments aimed at determining whether the the primary cause of salt damage is the failure of Ca^{2+} in one of the above mentioned roles.

6.1.1 Is displacement of Ca^{2+} from the plasma membrane Na^+ -specific?

It has been suggested that displacement of membrane-associated Ca^{2+} by Na^+ is a primary response to salinity and that this ultimately leads to a disruption of membrane integrity and selectivity (LaHaye and Epstein 1969; Cramer et al. 1985). The displacement of membrane-associated Ca^{2+} by Na^+ is mitigated by high external Ca^{2+} . Using cotton roots, Cramer et al. (1985) showed Ca^{2+} displacement to be Na^+ -specific and acting at the external surface of the plasma membrane. To see if a Na^+ -specific displacement of Ca^{2+} occurred in *C. corallina*, cytoplasmic streaming rate and vacuolar ion content were measured after addition of the Cl^- salts of 100 mol m^{-3} Na^+ , K^+ , Rb^+ and Li^+ at various Ca^{2+} concentrations.

6.1.2 Polyamines and sterols: possible substitutes for Ca^{2+} ?

6.1.2.1 Polyamines.

Polyamines are thought to elicit a number of responses in plant tissue including: regulation of senescence, promotion of growth, stabilization of protoplasts against lysis and a reduction in membrane permeability (Agazio et al. 1988).

It is not clear how polyamines act in plants. Roberts et al. (1986) reported that application of exogenous polyamine to the plasma membrane resulted in a decrease in membrane fluidity. Roberts et al. (1986) argued that at physiological pH, polyamines are polycationic and therefore could associate non-specifically with negatively charged phospholipids. The reduction in membrane permeability seen on addition of polyamines was considered by Roberts et al. (1986) to be a non-specific cross-linking of phospholipids similar to that reported for Ca^{2+} . However, Agazio et al. (1988) provide evidence that polyamines behave as competitive inhibitors of proton extrusion. They argue against a non-specific effect on the plasma membrane such as rigidification, as this would indiscriminately alter the biochemical functions of the membrane.

6.1.2.2 Sterols.

The passive permeability of the plasma membrane of plant cells is decreased by the addition of sterols (Grunwald 1968, 1971). The addition of planar sterols such as cholesterol and campesterol to the external solution reduced a methanol-induced leakage of electrolytes from barley roots (Grunwald 1968). Douglas and Walker (1983) reported the salt exclusion abilities of three lines of citrus to be correlated with the ratio of the more planar to less planar free sterols in the roots. The less substituted sterols conferred the greater salt

resistance. Erdei et al. (1980) suggested that the salinity tolerance of various *Plantago sp.* could be correlated to the sterol lipid content of the plants. They reported a decrease in sterol content of plants exposed to NaCl but a higher lipid content in the salt tolerant plants. The work of Grunwald (1968, 1971) suggests that the sterol content of the plasma membrane can be altered by incubating the plant tissue in a medium containing the appropriate sterol.

The following experiments were performed to see whether sterols or polyamines could substitute for Ca^{2+} in protecting *C. corallina* from salt injury.

6.2 RESULTS.

6.2.1 Effect of various group 1A cations *C. corallina*.

6.2.1.1 Streaming rate.

Isolated internodes of *C. corallina* were placed in a solution containing 100 mol m^{-3} NaCl, KCl, LiCl or RbCl with either 0, 0.5 or 10 mol m^{-3} CaSO_4 . Removal of Ca^{2+} from CPW did not reduce cytoplasmic streaming rate, but Ca^{2+} removal combined with the addition of 100 mol m^{-3} LiCl, RbCl, KCl or NaCl resulted in a cessation of cytoplasmic streaming (Figure 6.1). Supplementing the external solution with 0.5 mol m^{-3} Ca^{2+} partially protected the cells (Figure 6.2) whilst supplementing with 10 mol m^{-3} Ca^{2+} protected cells in 100 mol m^{-3} Li^+ , K^+ , Na^+ or Rb^+ from dying (Figure 6.3). However, the cytoplasmic streaming rate of cells from the KCl or RbCl treatments was lower than for the NaCl or LiCl treatments. This could reflect a decrease in cytoplasmic ATP concentration (Reid and Walker 1983) or changes in cytoplasmic Ca^{2+} concentration (Williamson and Ashley 1982) of cells treated with Rb^+ or K^+ .

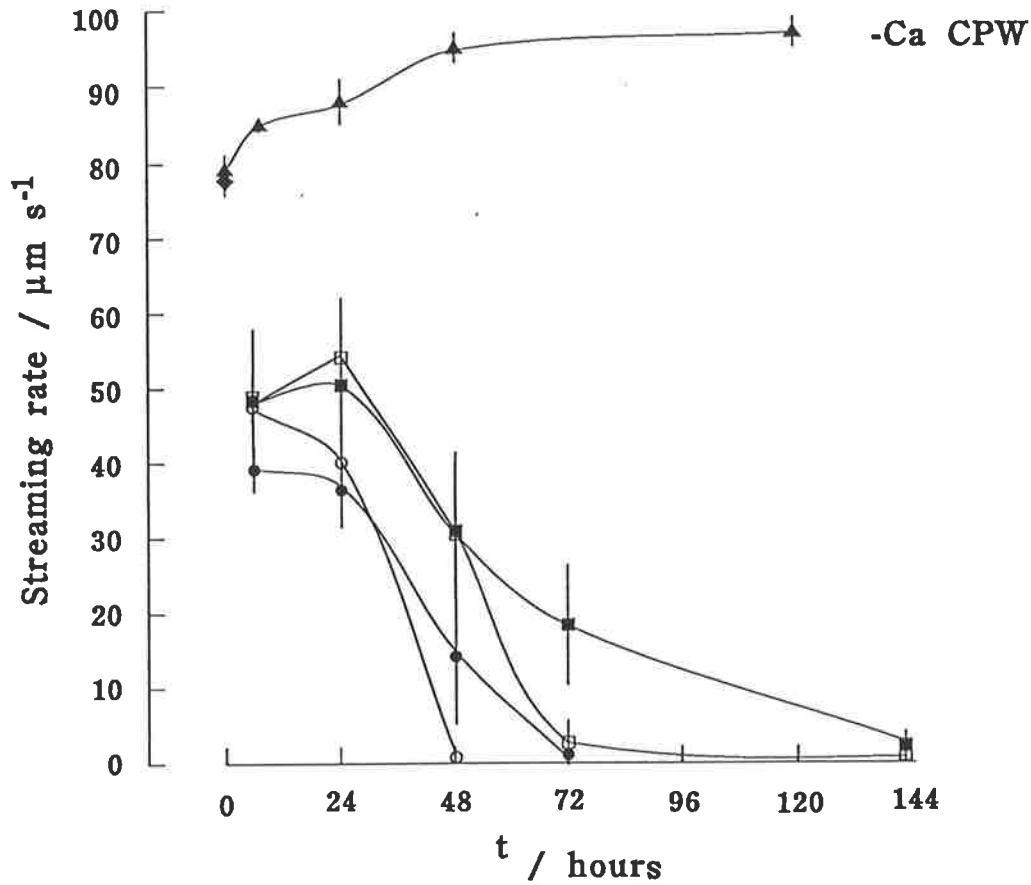


FIGURE 6.1 Effect of Ca^{2+} -free 100 mol m^{-3} alkali cations on cytoplasmic streaming.

▲ Ca^{2+} -free CPW ● RbCl, ■ KCl, ○ LiCl and □ NaCl.

Each value represents the mean and standard error of 10 cells.

Culture: LES

Solution: 100 mol m^{-3} RbCl or 100 mol m^{-3} KCl or 100 mol m^{-3} LiCl or
 100 mol m^{-3} NaCl, EPPS pH 8.

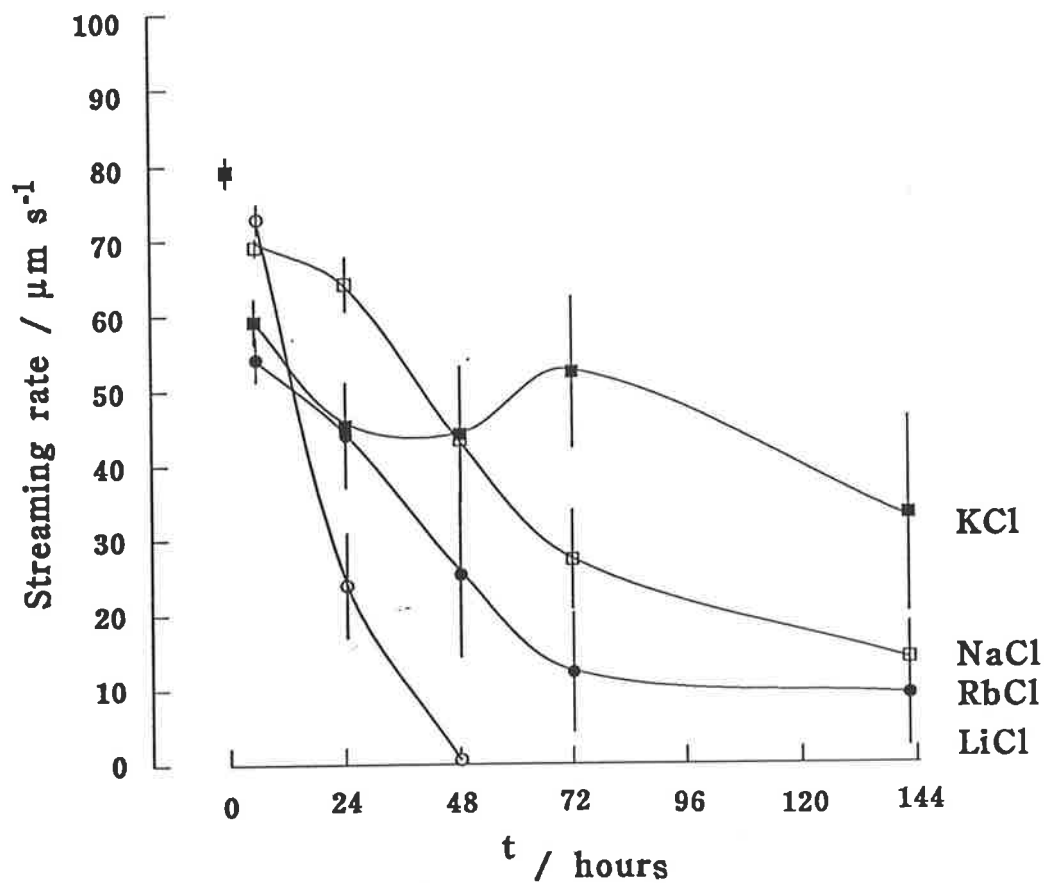


FIGURE 6.2 Effect of 100 mol m^{-3} alkali cations with $0.5 \text{ mol m}^{-3} \text{ Ca}^{2+}$ on cytoplasmic streaming.

● RbCl, ■ KCl, ○ LiCl and □ NaCl.

Each value represents the mean and standard error of 10 cells.

Culture: LES

Solution: 100 mol m^{-3} RbCl or 100 mol m^{-3} KCl or 100 mol m^{-3} LiCl or 100 mol m^{-3} NaCl, $0.5 \text{ mol m}^{-3} \text{ CaSO}_4$, EPPS pH 8.

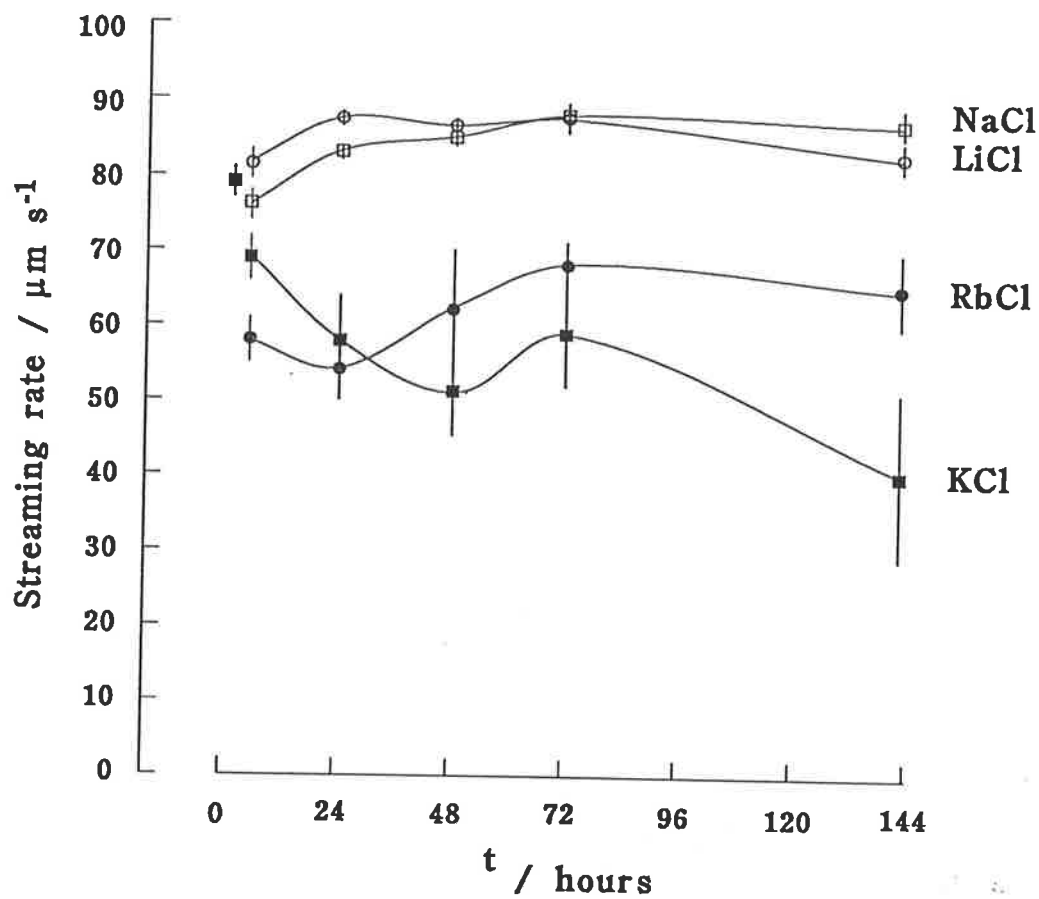


FIGURE 6.3 Effect of 100 mol m^{-3} alkali cations with $10 \text{ mol m}^{-3} \text{ Ca}^{2+}$ on cytoplasmic streaming rate.

● RbCl, ■ KCl, ○ LiCl and □ NaCl.

Each value represents the mean and standard error of 10 cells.

Culture: LES

Solution: 100 mol m^{-3} RbCl or 100 mol m^{-3} KCl or 100 mol m^{-3} LiCl or 100 mol m^{-3} NaCl, $10 \text{ mol m}^{-3} \text{ CaSO}_4$, EPPS pH 8.

6.2.1.2 Ionic composition of the vacuole.

Vacuolar Na^+ , K^+ and Cl^- were measured from cells that had been in 100 mol m^{-3} NaCl , KCl , RbCl or LiCl (+ $10 \text{ CaSO}_4 \text{ mol m}^{-3}$ and 0.1 mol m^{-3} K_2SO_4) for five days (Table 6.1).

The ion content of the vacuole of cells in Na-Ca-CPW was similar to the experiments described previously (see Chapter 4). There was an increase in vacuolar Na^+ , a smaller decrease in vacuolar K^+ and charge balance was maintained by an increase in vacuolar Cl^- .

The Li-Ca-CPW treatment gave similar results to the Na-Ca-CPW treatment. Vacuolar Cl^- increased slightly while the concentration of K^+ in the vacuole decreased (Table 6.1). The sum of K^+ and Na^+ was 84 mol m^{-3} , 55 mol m^{-3} less than vacuolar Cl^- , suggesting that Li^+ was exchanged for K^+ and accumulated in the vacuole.

The K-Ca-CPW treatment resulted in vacuolar K^+ increasing to 214 mol m^{-3} over the 5 day period. The increase in vacuolar K^+ was partially balanced by an increase in vacuolar Cl^- . However, there remained an imbalance of approximately 75 mol m^{-3} between monovalent cation and anion concentration. Apart from Cl^- , the only anion in the external solution was $10.1 \text{ mol m}^{-3} \text{ SO}_4^{2-}$. Therefore the balancing anion in the vacuole could be either SO_4^{2-} or an internally produced organic anion. This will be the subject of further discussion in chapter 6.

The Rb-Ca-CPW treatment gave similar results to the K-Ca-CPW treatment. Vacuolar Cl^- of cells bathed in Rb-Ca-CPW increased to 202 mol m^{-3} while the sum of K^+ and Na^+ was 122 mol m^{-3} . This left a deficit of 80 mol m^{-3} of positive charge in the vacuole which probably represents the concentration of Rb^+ in the vacuole. This could be an under-estimate of the true Rb^+ concentration in the vacuole because in the KCl treatment the increase in vacuolar K^+ was about 75 mol m^{-3} greater than the increase in vacuolar Cl^- . Using this as a guide the vacuolar Rb^+ concentration could be up to 155 mol m^{-3} .

TABLE 6.1 Effect of 100 mol m^{-3} K^+ , Na^+ , Rb^+ and Li^+ on vacuolar ion content.

treatment	vacuolar ion content (mol m^{-3})			
	K^+	Na^+	$\text{K}^+ + \text{Na}^+$	Cl^-
control (CPW)	71 ± 3	47 ± 2	118 ± 4	123 ± 4
K-Ca-CPW	214 ± 9	53 ± 2	266 ± 9	191 ± 4
Na-Ca-CPW	42 ± 4	93 ± 3	135 ± 5	144 ± 6
Rb-Ca-CPW	62 ± 4	60 ± 2	122 ± 3	202 ± 7
Li-Ca-CPW	35 ± 2	49 ± 2	84 ± 2	139 ± 3

Ion content of the vacuole after 6 days in each treatment. Values are the mean \pm standard error ($n = 10$).

Culture: LES

Solution: 100 mol m^{-3} RbCl or 100 mol m^{-3} LiCl or 100 mol m^{-3} NaCl or 100 mol m^{-3} KCl , EPPS pH 8.

6.2.2 Effect of substituting polyamines and sterols for Ca^{2+} .

6.2.2.1. Spermidine.

Agazio et al. (1988) added up to 20 mol m^{-3} spermidine to maize and did not report any ill health to the plant. When 20 mol m^{-3} spermidine (in CPW, pH8) was added to isolated internodes of *C. corallina* at pH 8 cytoplasmic streaming rate was reduced to half the initial rate within 20 minutes and all the cells were dead within 90 minutes.

The experiments of Agazio et al. (1988) were conducted at pH_0 6 where spermidine is fully protonated, polycationic and is able to associate with negatively charged phospholipids (Roberts et al. 1986). The experiment was repeated at pH_0 6 to see if spermidine was as toxic to *C. corallina* under identical conditions to those used by Agazio et al. (1983). The results presented in Figure 6.4 show that 20 mol m^{-3} spermidine reduced cytoplasmic streaming at pH_0 6 as it did at pH_0 8. Addition of 1 mol m^{-3} spermidine did not alter cytoplasmic streaming rate in the first 8 hours but over a 24 hour period resulted in cell death.

It has been suggested that polyamines influence membrane permeability in a manner similar to Ca^{2+} (Reidell 1987), and so it was of interest to see if 1 mol m^{-3} spermidine would (at least in the short term) substitute for Ca^{2+} in protecting the cell from salt damage. The results of such an experiment are presented in Figure 6.5. Contrary to expectation, addition of 1 mol m^{-3} spermidine enhanced rather than diminished the toxicity of Na-CPW. Pretreatment of the cell in 1 mol m^{-3} spermidine for 1 hour before the addition of Na-CPW did not confer any greater tolerance.

6.2.2.2 Cholesterol.

Grunwald (1971) and Douglas and Walker (1983) report that the

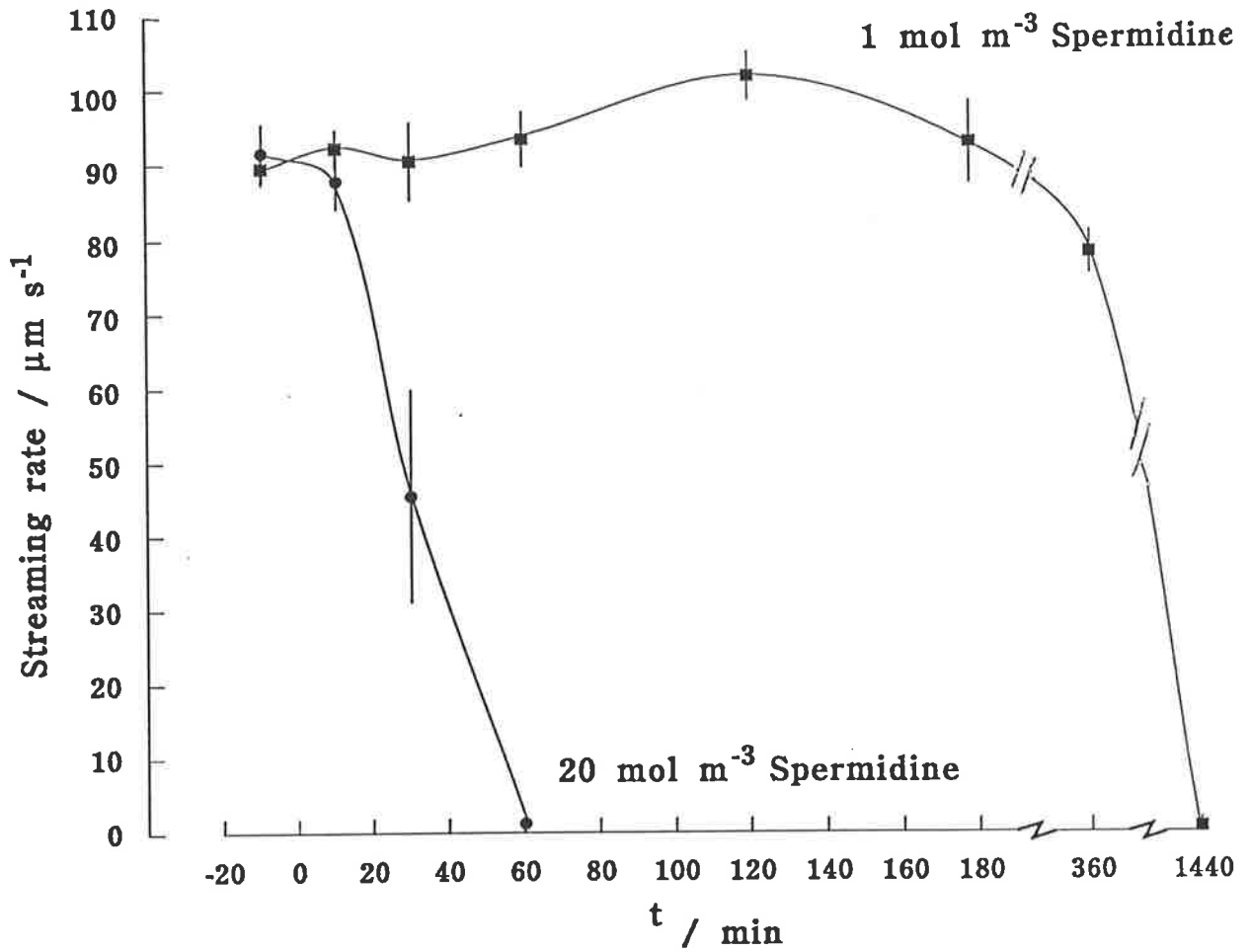


FIGURE 6.4 Effect of spermidine on streaming rate.

■ 1.0 mol m^{-3} spermidine and ● 20 mol m^{-3} spermidine.

Spermidine was added at $t = 0$. Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: 1 or 20 mol m^{-3} spermidine, CPW EPPS pH 8.

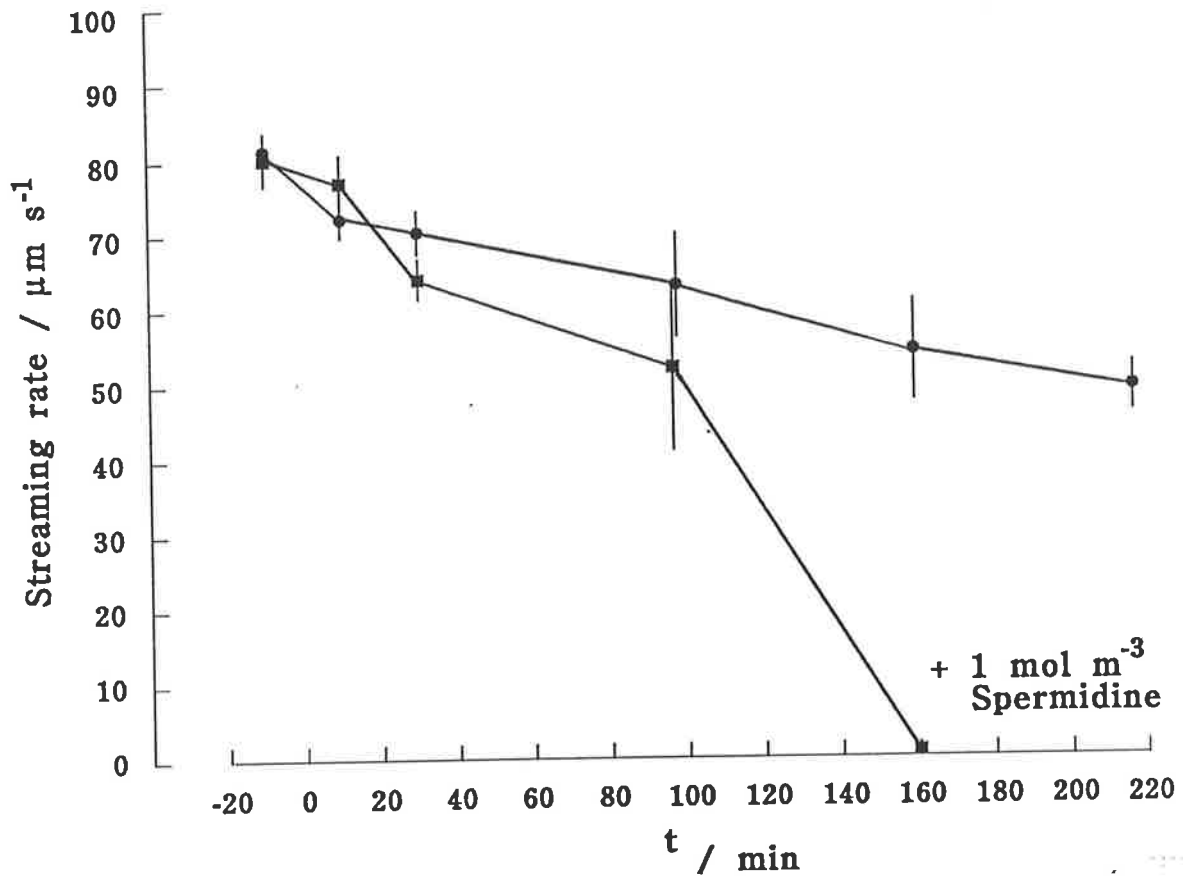


FIGURE 6.5 Effect of spermidine on streaming rate in the presence of Na-CPW.

● Na-CPW, ■ Na-CPW + 1 mol m⁻³ spermidine.

Na-CPW and spermidine were added at $t = 0$. Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: $\pm 1 \text{ mol m}^{-3}$ spermidine, 100 mol m^{-3} NaCl, 0.1 mol m^{-3} K₂SO₄, EPPS

pH 8.

"more planar" sterols (such as cholesterol) are more effective in increasing membrane permeability than the "less planar" sterols. How planar the molecule is refers to the degree of substitution of the side chain at 17C (Goodwin 1974). Cholesterol is one of the least substituted sterols and so is a relatively planar sterol. It is believed that only those sterols with a flat configuration can penetrate the phospholipids of the membrane (Grunwald 1971). For this reason cholesterol was chosen for testing in these experiments.

Cholesterol is practically insoluble in water (Weast 1971) so a stock solution containing 20 mol m^{-3} cholesterol dissolved in ethanol was used. Since ethanol was added with the cholesterol, the appropriate concentration of ethanol was added to the controls (final concentration of ethanol was $0.005\% / \text{mmol m}^{-3}$ cholesterol).

The effects of 10 mmol m^{-3} and 100 mmol m^{-3} cholesterol on cytoplasmic streaming rate in the presence of Na-CPW is shown in Figure 6.6. The streaming rate of cells in CPW (+ethanol) reduced dramatically on addition of Na-CPW. Addition of cholesterol did not alter this effect.

6.3 DISCUSSION.

6.3.1 Effect of Group 1A cations on *C. corallina*.

Cramer et al. (1985) showed that NaCl concentrations as low as 25 mol m^{-3} reduced the quantity of membrane-associated Ca^{2+} when the external Ca^{2+} concentration was low. Other cations, including Li^+ , K^+ and Rb^+ did not reduce membrane-associated Ca^{2+} , indicating that the displacement of Ca^{2+} was specific to Na^+ . In the experiments described in this chapter elevated Li^+ , K^+ , Rb^+ and Na^+ all caused cell death in the absence of external Ca^{2+} . This suggests that either cation displacement of Ca^{2+} was not Na^+ -specific, but occurred with any Group 1A cation or that a reduction in membrane-associated Ca^{2+} and the

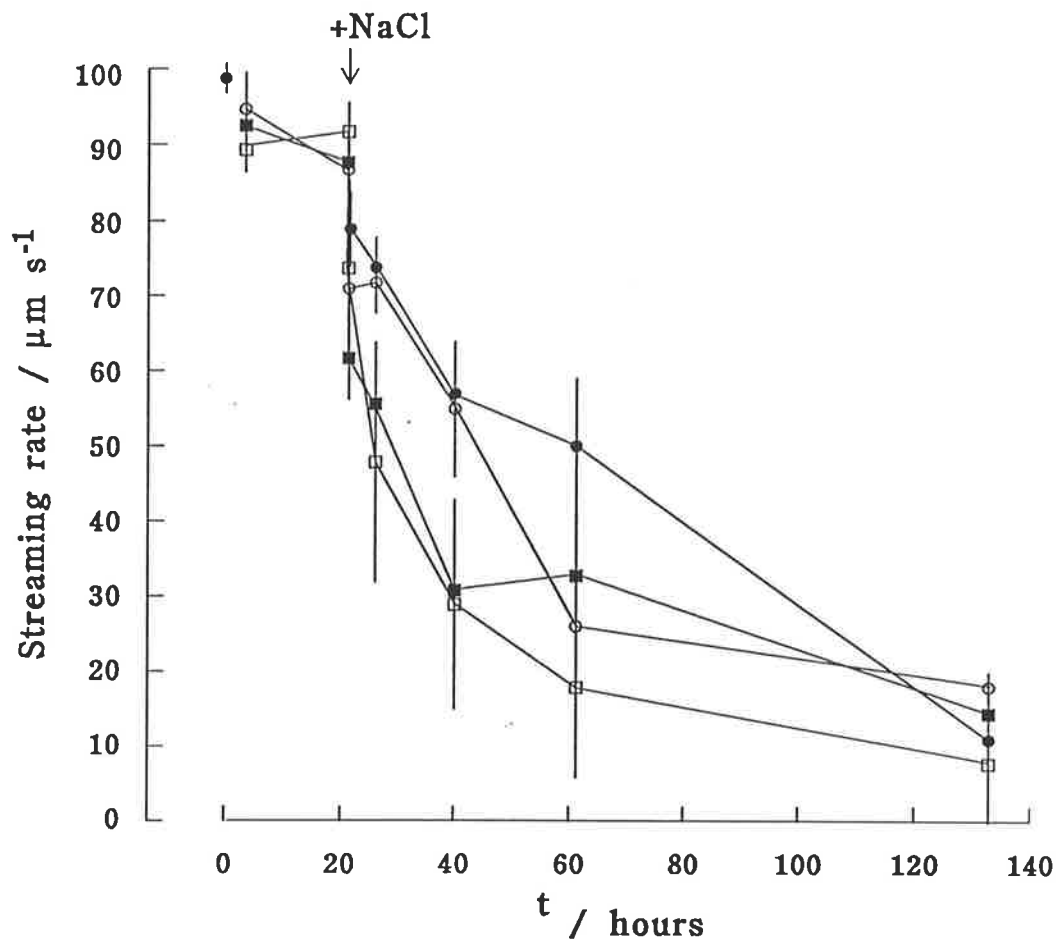


FIGURE 6.6 Effect of cholesterol on streaming rate in the presence of Na-CPW.

● 10 $\mu\text{mol m}^{-3}$ cholesterol + 0.05% ethanol, ○ 0.05% ethanol,
 ■ 100 $\mu\text{mol m}^{-3}$ cholesterol + 0.5% ethanol, □ 0.5% ethanol,
 Cholesterol was added at $t = 0.1$ hours. Na-CPW added a
 $t = 20.75$ hours. Each point represents the mean and standard
 error of 10 cells.

Culture: LES

Solution: Cells initially in CPW EPPS pH8

solution replaced at $t = 0.1$ with 0.05 or 5% ethanol \pm
 10 or 100 mmol m^{-3} cholesterol, CPW EPPS pH 8

solution replaced at $t = 20.75$ with Na-CPW EPPS pH 8
 + 0.05 or 5% ethanol \pm 10 or 100 mmol m^{-3} cholesterol.

subsequent loss of membrane integrity was not the primary cause of death in these treatments. No attempt was made to measure membrane-associated Ca^{2+} under each of these treatments so this question remains unresolved.

Cell injury associated with high concentration of salt was not specific to Na^+ . This agrees with the hypothesis of Hauser et al (1976) which suggested that the binding of cations to membrane phospholipids was determined by their charge density. Using the scheme of Hauser et al. (1976) Li^+ and K^+ should both displace Ca^{2+} at least as equally, if not more thoroughly than Na^+ .

High concentrations of Li^+ , K^+ , Rb^+ or Na^+ were not lethal to *C. corallina* when the external concentration of Ca^{2+} was 10 mol m^{-3} . *C. corallina* responded to Na-Ca-CPW and Li-Ca-CPW treatments in a similar manner. Essentially Li^+ or Na^+ replaced K^+ in the vacuole. The increase in external osmotic pressure caused by 100 mol m^{-3} LiCl or NaCl was much greater than the slight increase in osmotic pressure of the vacuole of *C. corallina*. Therefore cell turgor was reduced.

C. corallina responded to high external concentrations of Rb^+ and K^+ by accumulating large amounts of these ions in the vacuole. By accumulating Rb^+ , K^+ and Cl^- the cells effectively maintained turgor at the pre-treatment level. Before the cells were transferred to either elevated K^+ or Rb^+ cell turgor was about 0.6 MPa. After five days in K^+ or Rb^+ salts cell osmotic potential rose to about 1.16 MPa while the osmotic potential of the external media was about 0.53 MPa. Therefore turgor potential of cells bathed in 100 mol m^{-3} RbCl or KCl for five days was 0.63 MPa. These results indicate that *C. corallina* sustained cell turgor when bathed in 100 mol m^{-3} KCl and RbCl.

Cells bathed in KCl and RbCl had lower cytoplasmic streaming rates and possibly ATP concentrations than cells bathed in NaCl or LiCl. Presumably this is a result of the accumulation of K^+ or Rb^+ and Cl^- .

Accumulation of Cl^- against its electrochemical gradient is an energy consuming process. The lower cytoplasmic streaming rates of the cells that are regulating turgor may reflect the expenditure of ATP on Cl^- transport. For cells bathed in K-Ca-CPW and Rb-Ca-CPW there is a deficit of inorganic anion relative to inorganic cation in the vacuole. It is proposed that this deficit is balanced by the synthesis and accumulation of carboxylate (see chapter 7). This process may also consume ATP and thus lower cytoplasmic streaming rate. The lower ATP concentration in these cells may be a result of the high ionic strength of the cytoplasm. Alternatively, the changes in cytoplasmic streaming rate may reflect changes in the cytoplasmic Ca^{2+} concentration brought about by high external (or internal) cation concentration.

6.3.2 Sterols and polyamines.

Sterols and polyamines have both been shown to alter plasma membrane permeability to electrolytes in a similar manner to Ca^{2+} (eg. Grunwald 1968, 1971 and Roberts et al. 1986). The experiments in this chapter were designed to test the hypothesis that either or both spermidine and cholesterol could substitute for external Ca^{2+} in providing protection for the cell from salt damage. In the experiments described in this chapter concentrations of spermidine and cholesterol were used that have previously been shown to alter membrane permeability (eg Grunwald 1968, 1971; Roberts et al. 1986; Agazio et al.). However, these concentrations of spermidine were lethal whilst cholesterol had no effect on salt tolerance of *C. corallina*. Agazio et al. (1988) applied up to 20 mol m^{-3} spermidine to *Zea mays* root apical cells. This treatment reduced K^+ influx by 80%. However, there was no mention of the health of the cells during this treatment. Roberts et al. (1986) applied 50 mol m^{-3} spermidine to microsomal membranes isolated from *Phaseolus vulgaris*. At this concentration spermidine reduced membrane fluidity. In view of the effects of

spermidine on cytoplasmic streaming rate and membrane potential difference it would be interesting to see how healthy the cells used by Agazio et al. (1988) were at the end of the experiments they conducted.

Grunwald (1971) reported that cholesterol-induced changes in membrane permeability were dependant upon the sterol concentrations in the external medium. Cholesterol at 10 mmol m^{-3} greatly reduced leakage of electrolytes, while at 100 mmol m^{-3} greatly stimulated loss of electrolytes. The cholesterol-induced increase in plasma membrane permeability may be due to the formation of sterol clusters in the lipid matrix similar to those reported in senescing tissue by Lees and Thompson (1980).

The results presented in this chapter indicate that cholesterol at both 10 mmol m^{-3} and 100 mmol m^{-3} did not alter salt tolerance of *C. corallina* in the absence of Ca^{2+} . This suggests that either cholesterol was not being incorporated into the plasma membrane or that cholesterol once in the plasma membrane had no effect on salt tolerance. It would appear unlikely that cholesterol would not be incorporated into the plasma membrane since sterols have been shown to readily move into and out of membranes with a half time for movement across the bilayer in the order of seconds (Cooper and Strauss 1983) to about 1 hour (Demel and DeKruyff 1976).

In summary neither the sterol, cholesterol or the polyamine, spermidine were able to replace Ca^{2+} in protecting the cell from salt damage.

CHAPTER 7.

"Excess cation uptake" and the production of malate.

7.1 INTRODUCTION

It has long been recognized that in higher plants cation uptake can exceed anion uptake and that excess cations can be balanced by the synthesis of organic acids in the cytoplasm (Ulrich 1941). In a variety of tissues malate has been commonly associated with excess ion uptake (Osmond 1976, Cram 1985).

In most studies with giant algal cells ion balance is satisfactorily explained by movements of inorganic ions. However, there have been recent reports where tissue concentrations of inorganic cation exceed that of inorganic anion (Ryan 1988, Smith and Whittington 1988, Sanders 1981b). In these studies it was postulated that electroneutrality was maintained by production of carboxylate anion and extrusion of H^+ . However, Ryan (1988) was the only author to conclusively show accumulation of carboxylate: this was achieved by measuring accumulation of malate in the vacuole. In the above mentioned studies using *C. corallina*, electroneutrality was satisfied by movements of K^+ , Na^+ and Cl^- , when Cl^- was available. The need to propose carboxylate accumulation was only required during cation uptake in the absence of Cl^- .

The results presented in chapter 5 clearly showed an imbalance between inorganic cation and inorganic anion in the vacuole when the cell was bathed in K-Ca-CPW or Rb-Ca-CPW. In this chapter it is shown that malate is the carboxylate that is used to maintain electroneutrality. Two broad areas are discussed: the control and regulation of malate synthesis and the physiological consequences of malate production and accumulation.

7.2 RESULTS

7.2.1 Regulation of malate synthesis.

7.2.1.1 Is malate accumulated in the vacuole during excess cation uptake?

The results presented in Section 6.2.1.2 clearly showed that cells bathed in K-Ca-CPW or Rb-Ca-CPW accumulated large amounts of either K^+ or Rb^+ in their vacuole. This increase in vacuolar cation was partially matched by an increase in vacuolar Cl^- . However, despite the increases in vacuolar Cl^- there was a greater concentration of inorganic cation relative to inorganic anion of up to 75 mol m^{-3} in the vacuole. It was suggested that the deficit in inorganic ion concentration could be balanced by either accumulation of SO_4^{2-} or an organic carboxylate (such as malate).

A preliminary experiment was undertaken to see if excess accumulation of cation occurred in the absence of Cl^- . The results presented in Table 7.1 show that K^+ is accumulated in the vacuole of the cell in Cl^- -free solutions. Vacuolar Cl^- was lower in cells bathed in Cl^- -free solution than equivalent cells bathed in CPW. There was a deficit of inorganic anion relative to inorganic cation of 103 mol m^{-3} .

The results to this point suggested that when cells, particularly in the absence of Cl^- , accumulate Rb^+ or K^+ in the vacuole that an organic compound begins to appear in the vacuole. The work of Ryan (1988) suggested that electroneutrality could be maintained in the vacuole by synthesis of malate. Therefore, an enzyme assay (Lowry and Passonneau 1972) was used to quantify the concentration of malate in the cell. The experiment was conducted in the absence of Cl^- using concentrations of cation that were previously found to result in excess cation accumulation. The results presented in Figure 7.1 show the amount of malate accumulated in the vacuole of *C. corallina* in 100 mol

TABLE 7.1 Effect of $100 \text{ mol m}^{-3} \text{ K}^+ \pm \text{Cl}^-$ on the vacuole ion content of cells after 7 days.

	Ion content (mol m^{-3})			
	K^+	Na^+	$\text{K}^+ + \text{Na}^+$	Cl^-
7 Day CPW	71 ± 3	47 ± 2	118 ± 4	123 ± 4
7 Day 100 mol m^{-3} K-MES	177 ± 5	36 ± 2	213 ± 7	110 ± 2
7 Day 100 mol m^{-3} KCl	169 ± 5	30 ± 3	199 ± 7	170 ± 6

All solutions contained 2.5 mol m^{-3} EPPS pH_0 8. High K^+ solutions contained 10 mol m^{-3} CaSO_4 . 100 mol m^{-3} K-MES was made by neutralizing 100 mol m^{-3} KOH with MES until the solution was pH_0 8. Each value represents the mean and S.E. of 10 cells.

Culture: LES.

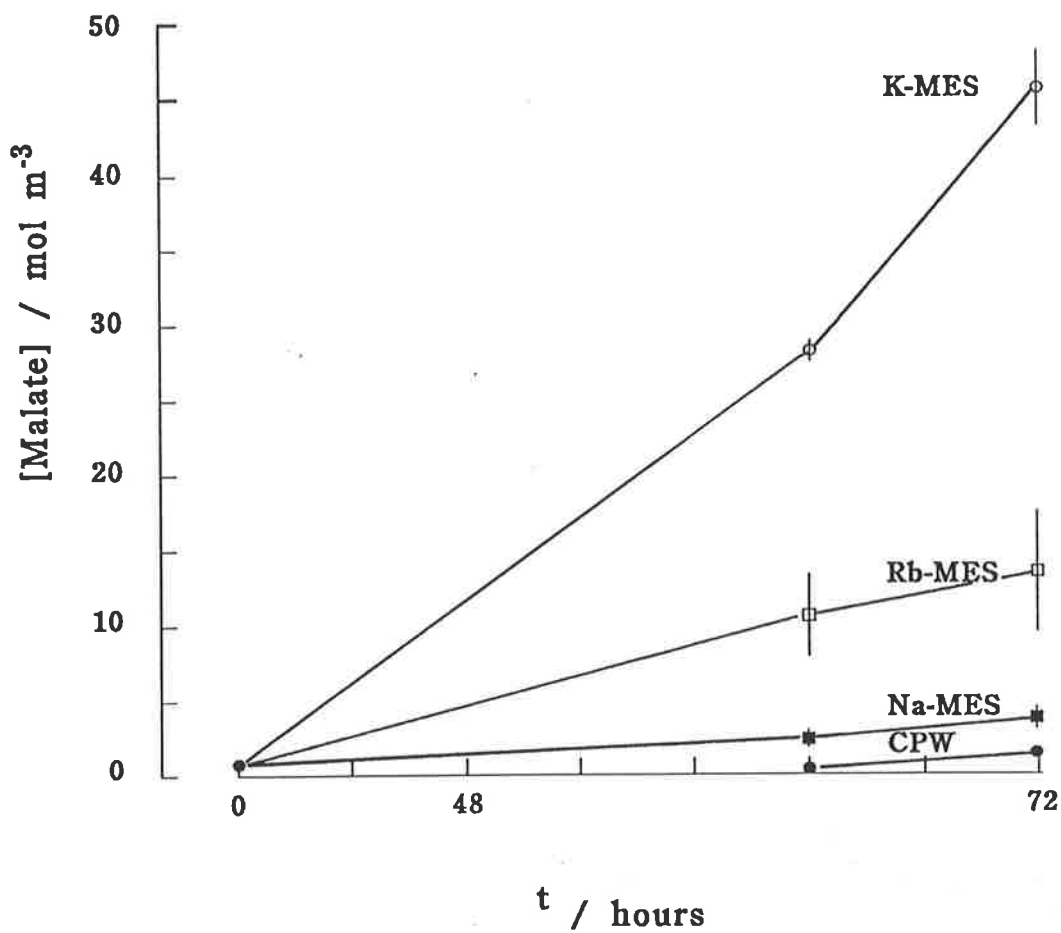


FIGURE 7.1 Effect of $100 \text{ mol m}^{-3} \text{ Na}^+$, K^+ and Rb^+ on malate accumulation in the vacuole.

○ K^+ -MES, □ Rb^+ -MES, ■ Na^+ -MES, ● CPW. Solutions made from the hydroxide by neutralizing with MES. Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: CPW pH8 EPPS or $100 \text{ mol m}^{-3} \text{ Na}^+$, K^+ or Rb^+ -MES + $10 \text{ mol m}^{-3} \text{ CaSO}_4$ pH8.

m^{-3} K^+ , Rb^+ and Na^+ in the absence of Cl^- . Malate concentration in cells bathed in CPW is also shown.

The main feature of this experiment is that *C. corallina* accumulated up to 48 mol m^{-3} malate in the vacuole. The largest concentration of malate previously measured from the vacuole of *C. corallina* was 16 mol m^{-3} (Ryan 1988). Also the concentration of malate balanced the anion deficit in the vacuole. There was minimal accumulation of malate in the Na^+ treatment and previous results show that *C. corallina* does not accumulate Na^+ in the vacuole. At the end of 6 days only two of the eight cells remained alive in the K^+ treatment. Cell death is a common feature of experiments conducted with 100 mol m^{-3} K^+ , even with Ca^{2+} supplementation.

7.2.1.2 Malate distribution between the cytoplasm and the vacuole.

Associated with the above experiment a crude measure of the distribution of malate within the cell was attempted. The malate concentration of isolated vacuole samples was compared to that of the remainder of the vacuole and the cytoplasm combined. There was no difference between the values obtained (results not shown). This indicates that the bulk of the malate in the cell was stored in the vacuole. This experiment did not allow the cytoplasmic malate concentration to be determined since the vacuolar malate concentration would swamp any contribution from cytoplasmic malate because of the relative volumes of the cytoplasm and vacuole. The concentrations of malate observed in the vacuole are large enough to suggest that malate accumulated in the vacuole was synthesized during the experiment rather than transported from the cytoplasm to the vacuole during the experiment. For example, to achieve a malate concentration in the vacuole of 20 mol m^{-3} cytoplasmic concentration of 400 mol m^{-3} would be required. A cytoplasmic malate concentration of this magnitude is

clearly untenable.

7.2.1.3 Time course and K^+ concentration dependence of malate accumulation.

The previous experiments have shown that malate is produced when K^+ or Rb^+ are accumulated in the vacuole to a greater concentration than vacuolar Cl^- . An experiment was designed to investigate both the time course of malate production and to determine how much K^+ is required in the external solution to induce malate production. The results of this experiment are presented in Figures 7.2 and 7.3 and Table 7.2.

Accumulation of malate in the vacuole was loosely correlated to K^+ concentration. There was a large increase in rate of malate accumulation between an external K^+ concentration of 2 and 10 mol m^{-3} . However, there was no difference in rate of accumulation between 10 and 20 $\text{mol m}^{-3} K^+$. The maximum rate of malate accumulation in the vacuole was 0.45 $\text{mol m}^{-3} \text{h}^{-1}$. Malate was formed at the same rate during the first six hours as it was for 48 hours, suggesting that the maximum rate of malate accumulation during excess cation uptake was 0.45 $\text{mol m}^{-3} \text{h}^{-1}$. Whether this represents a saturation of malate production or whether malate production is restricted by the rate of accumulation of K^+ in the vacuole remains unresolved.

7.2.1.4 Charge balance with malate.

The malate anion has two protonation states with pK_a 's of 3.40 and 5.11 (Weast 1971). The net charge on the malate ion at any given pH can be determined using the equations in Appendix 1. Assuming pH_c of *C. corallina* at pH_o to be approximately 8.0 (Smith and Walker 1976) then greater than 99.5% of malate in the cytoplasm would be dissociated into the free acid, and would therefore have two negative charges.

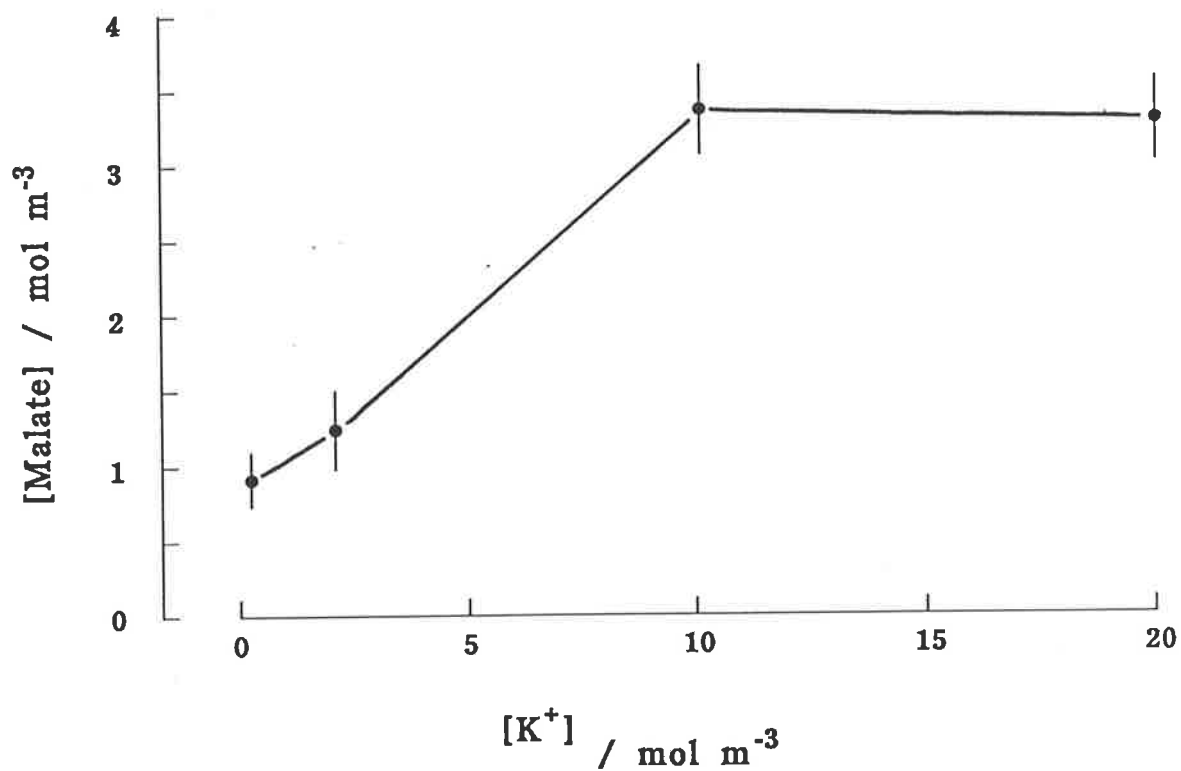


FIGURE 7.2 Effect of K⁺ concentration on malate accumulation in the vacuole over 6 hours.

Solutions were made up without Cl⁻. Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: 0.1 - 10 mol m⁻³ K₂SO₄, 0.5 mol m⁻³ CaSO₄ EPPS pH8.

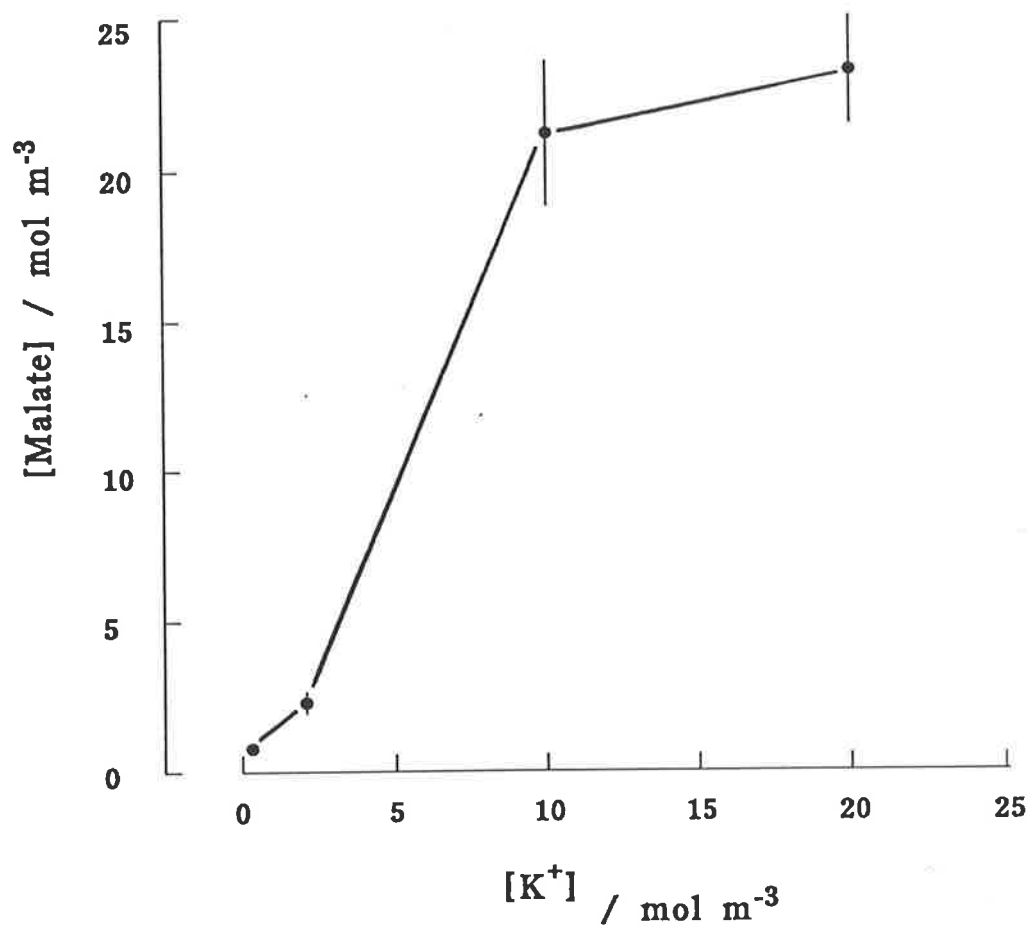


FIGURE 7.3 Effect of K^+ concentration on malate accumulation in the vacuole over 24 hours.

Solutions were made up without Cl^- . Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: $0.1 - 10 \text{ mol m}^{-3} K_2SO_4$, $0.5 \text{ mol m}^{-3} CaSO_4$ EPPS pH8.

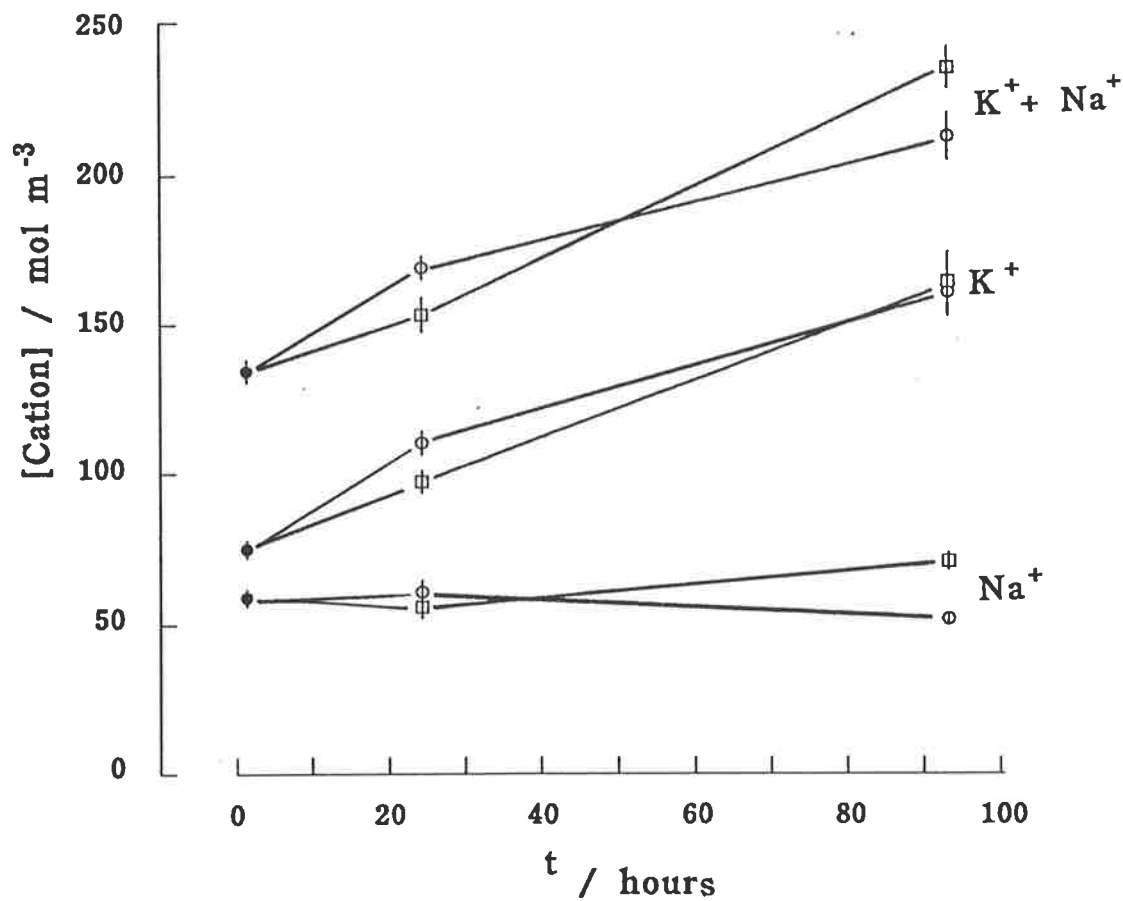


FIGURE 7.4 Concentration of cation in the vacuole after transfer to 10 mol m⁻³ K⁺ ± Cl⁻.

● CPW_{initial}, ○ 10 mol m⁻³ KCl, □ 5 mol m⁻³ K₂SO₄. Each point represents the mean and standard error of 10 cells.

Culture: LES

Solution: 10 mol m⁻³ KCl or 5 mol m⁻³ K₂SO₄ 0.5 mol m⁻³ CaSO₄ EPPS pH8.

Solutions replaced each day during experiment.

Vacuolar pH of *C. corallina* is acidic and is close to the pKa's of malate, therefore malate in the vacuole is less dissociated than in the cytoplasm. At pH 5.0 malate has an overall negative charge of 1.42 per molecule of malate and at pH 5.5 has a negative charge of 1.71 per molecule.

The results of previous experiments were analyzed to see if malate accounts for the charge imbalance measured during excess cation uptake. The maximum rate of malate accumulation was generally $0.45 \text{ mol m}^{-3} \text{ h}^{-1}$ (Table 7.2). This equates to an accumulation of negative charge of $0.77 \text{ mequiv m}^{-3} \text{ h}^{-1}$ in the vacuole. Under similar conditions the rate of change of $\text{K}^+ + \text{Na}^+ + (-\text{Cl}^-)$ varies between 0.55 and 0.96 $\text{mequiv m}^{-3} \text{ hour}^{-1}$. These results indicate that malate accumulation is similar to the rate of excess cation uptake. Ryan (1988) showed that malate accumulation maintains ionic balance during the uptake of amine from Cl^- -free solutions. It would be interesting to see if the observed inorganic ion imbalance in *C. corallina* during uptake of imidazole from Cl^- free solutions (Smith and Whittington 1988) is also balanced by malate.

TABLE 7.2 Vacuolar malate production (nmol h^{-1})

[K ⁺] mol m ⁻³	treatment	
	6 hours	48 hours
0.2	0	0
2	1.2	0.6
10	8.2	8.4
20	8.0	9.4

Rate of malate accumulation in the vacuole over 6 and 48 hours. Solutions contained 0.1, 1, 5, or 10 mol m⁻³ K₂SO₄ and 0.5 mol m⁻³ CaSO₄ at pH 8.

Values are computed from the data presented in Figures 7.2 and 7.3.

7.2.1.5 Effect of TEA⁺ on malate accumulation.

Tetraethylammonium (TEA⁺) is a classic blocker of K⁺ channels in many tissue types (Armstrong 1975) and clearly inhibits the conductance of both inward and outward K⁺ current in charophytes (Smith and Kerr 1987, Tester 1988b). The blockade of K⁺ channels by TEA⁺ is independent of membrane potential difference (Sokolik and Yurin 1986). Smith and Kerr (1987) showed TEA⁺ to substantially inhibit K⁺ transport in *C. corallina*: the concentration of TEA⁺ at which inhibition occurred increased as external KCl concentration was increased. However, even high concentrations of TEA⁺ (up to 10 mol m⁻³) did not reduce K⁺ flux by a factor of greater than 3. An experiment was conducted to see if inhibition of K⁺ influx with TEA⁺ resulted in a decrease in malate accumulation.

The results presented in Table 7.3 show malate accumulation to be insensitive to the concentrations of TEA⁺ used in this experiment. This suggests that K⁺ accumulation at external concentrations of 100 mol m⁻³ K⁺ was not inhibited by 10 mol m⁻³ TEA⁺. Smith and Kerr (1987) and Tester (1988b) found that TEA⁺ only inhibited a portion of K⁺ current. The uninhabitable fraction of current was termed the "leak current". Under conditions of high external K⁺ the "leak current" may be responsible for the accumulation of K⁺ in the cell. If this was the case then addition of TEA⁺ would not affect K⁺ accumulation and therefore malate accumulation. Both the inhabitable and uninhabitable modes of K⁺ transport will be discussed in chapter 8.

7.2.1.6 Effect of external Cl⁻, SO₄²⁻ and NO₃⁻ on malate accumulation.

Univalent cation absorption in excess of inorganic anions is most pronounced when the plant is bathed in a salt with a poorly absorbable anion such as SO₄²⁻ (Jacoby and Laties 1971, Osmond 1976) although some barley plants show excess cation uptake in KCl (Pitman 1971). In the

TABLE 7.3 Effect of TEA on malate accumulation in the vacuole.

[TEA]/mol m ⁻³	[malate]/mol m ⁻³
0.0	20.9 ± 1.0
3.0	22.8 ± 1.7
10.0	19.9 ± 1.5

Cells placed solution for 48 hours. Each value is the mean ± S.E.
of 10 cells

Culture: LES

Solution: 50 mol m⁻³ K₂SO₄, 10 mol m⁻³ CaSO₄ EPPS pH 8

experiments described so far, K_2SO_4 and K-MES have been used to induce malate accumulation. The results presented in Chapter 6 showed an excess of cation relative to inorganic anion in the vacuole when the cells were bathed in KCl. An experiment was designed to see if excess cation uptake was balanced by malate accumulation when cells were bathed in KCl.

C. corallina transports NO_3^- relatively slowly unless the cell is pretreated for a number of hours in NO_3^- (Deane-Drummond 1984b). Although, once induced, NO_3^- uptake can proceed at rates in excess of $60 \text{ nmol m}^{-2} \text{ s}^{-1}$ (Deane-Drummond 1984b). However, Ryan (*pers comm.*) was unable to measure any NO_3^- uptake into *C. corallina*. If equimolar quantities of NO_3^- and K^+ are absorbed by the cell and transported to the vacuole malate will not be required to maintain electroneutrality. However, if NO_3^- is not absorbed, then carboxylate will be accumulated in the vacuole to maintain electroneutrality with K^+ . This argument assumes that NO_3^- entering the cell is not reduced. If NO_3^- reduction did occur hydroxyl ions would be produced which would then increase cellular pH (Osmond 1976). This could stimulate production of malate as part of a biochemical pH regulatory system (Davies 1973a,b).

An experiment was designed in which cells of *C. corallina* were challenged with different K^+ salts. The quantity of malate in the vacuole was then measured after 16 hours. The results, presented in Figure 7.5a, show malate to be accumulated in all treatments which contained $10 \text{ mol m}^{-3} K^+$. There is no difference in accumulation of malate between SO_4^{2-} and NO_3^- salts. When the Cl^- salt of K^+ is used the vacuolar malate concentration was 59% of the Cl^- -free treatment. This experiment was repeated at a K^+ concentration of 20 mol m^{-3} (Figure 7.5b). In this experiment vacuolar malate concentration in the KCl treatment was 77% of the vacuolar malate concentration in the absence of Cl^- .

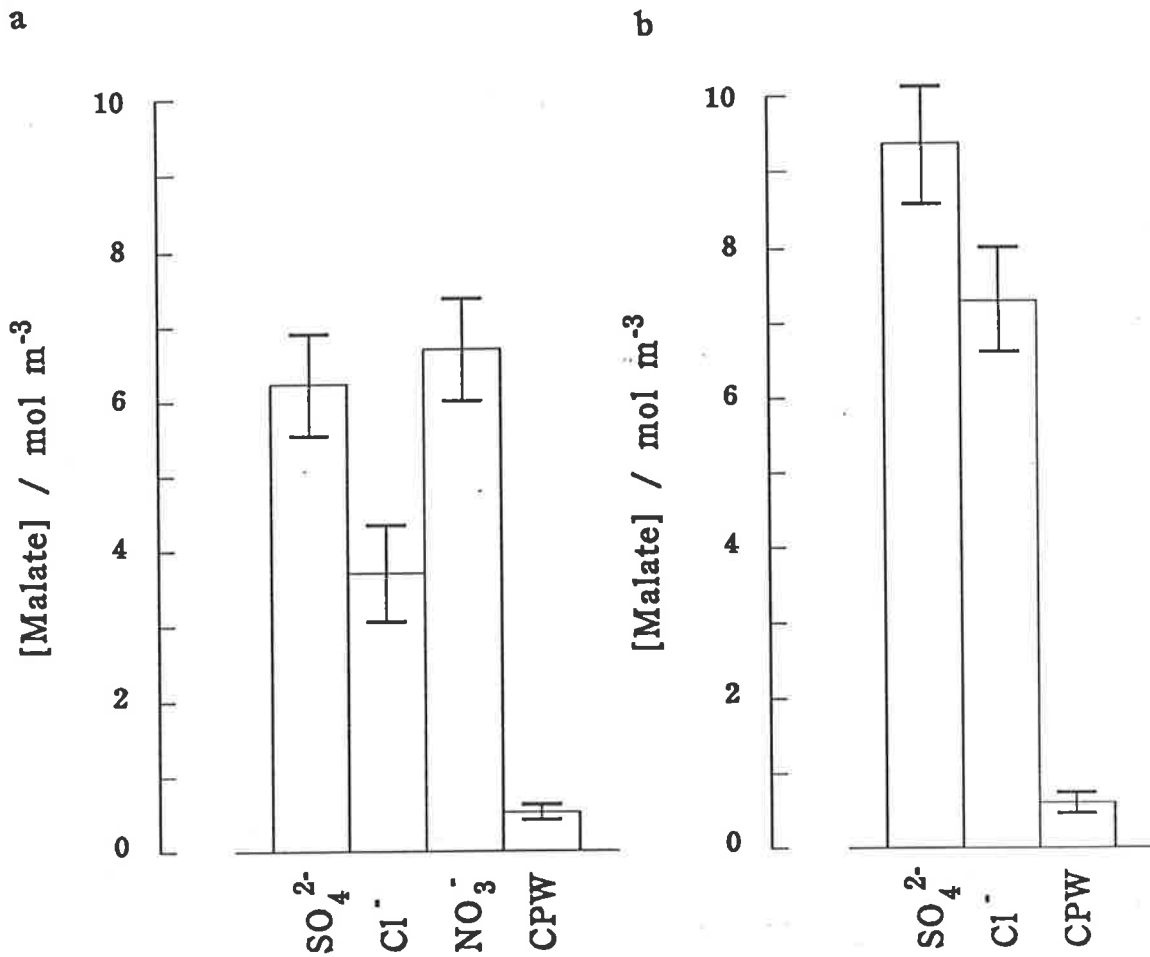


FIGURE 7.5 Effect of anion type on the accumulation of malate in the vacuole.

Cells were placed in solution for 20 hours prior to malate determination.

a) and b) represent separate experiments. Each value represents the mean and standard error of 10 cells.

Culture: REG

Solutions: a) either 10 mol m⁻³ KCl or 10 mol m⁻³ KNO₃ or 5 mol m⁻³ K₂SO₄ with 0.5 mol m⁻³ CaSO₄ EPPS pH8.

b) either 20 mol m⁻³ KCl or 10 mol m⁻³ K₂SO₄ with 0.5 mol m⁻³ CaSO₄ EPPS pH8.

The results presented in Figures 7.5 a and b show that bathing cells in solutions with a high external K^+ concentration caused *C. corallina* to accumulate K^+ -malate in the vacuole (stoichiometry of K^+ -malate will be discussed in section 7.2.2.2). The amount of inorganic anion relative to carboxylate in the vacuole is partially dependant upon the rate of transport of the relevant inorganic anion present.

7.2.1.7 Effect of pH_0 and HCO_3^- on malate accumulation.

The experiments describing the conditions under which malate is formed have been conducted at pH_0 8. Assuming atmospheric CO_2 concentration to be 0.033% (Weast 1971), CO_2 concentration in a solution in equilibrium with the atmosphere would be 13 mmol m^{-3} . Therefore at pH_0 8 the equilibrium HCO_3^- concentration in the experimental solution would be 622 mmol m^{-3} and at pH_0 5 the equilibrium HCO_3^- concentration would be 3 orders of magnitude smaller, 0.62 mmol m^{-3} .

The pH_c of *C. corallina* is sensitive to changes in pH_0 ; the relationship between pH_c and pH_0 has been described by Smith and Walker (1976);

$$pH_c = 6.28 + 0.22 pH_0 \quad (6.1)$$

This relationship has since been modified slightly (Smith 1980), but as a general rule, large changes in pH_0 result in only small changes in pH_c . Cells of *C. corallina* bathed in solutions at pH_0 8 would have pH_c of about 8.0-8.1 whereas cells in solution at pH_0 5 would have a pH_c of about 7.4-7.5 (Smith and Walker 1976, Smith 1980). An experiment was conducted to test if malate accumulation in the vacuole was sensitive to pH_0 .

The results presented in Figure 7.6 show malate accumulation to be strongly dependant upon pH_o . At pH_o 5 the vacuolar malate concentration was 1.13 mol m^{-3} whereas at pH_o 8 the vacuole concentration of malate was 5.3 mol m^{-3} . Two possible explanations for the lack of vacuolar malate at pH_o 5 are that accumulation of malate requires external HCO_3^- or that malate accumulation was sensitive to low pH_o . To test whether external HCO_3^- concentration is an important requirement for malate to be accumulated an experiment was conducted where carbon was removed from the solution before the commencement of the experiment. Removal of HCO_3^- from solution was achieved using the method described by Lucas (1975a). The results presented in Figure 7.6 show malate accumulation to be severely inhibited at pH_o 8 when HCO_3^- is removed from the external solution. This is evidence for the hypothesis that malate is formed by the carboxylation of PEP by PEP-carboxylase; a reaction for which HCO_3^- is a substrate (MacLennan et al. 1963). Assuming HCO_3^- is a substrate used for the synthesis of malate these results also suggest that HCO_3^- is transported into the cell as previously demonstrated by Lucas (1975a,b)

7.2.1.8 Effect of light on malate production.

Cells were harvested in the usual manner but were then pretreated in the dark for 20 hours before the commencement of the experiment. This experiment was conducted at two concentrations of external K^+ . In each case the accumulation of malate in the dark was 44% of the malate accumulation in the light (Table 7.4).

7.2.1.9 Pathway of malate synthesis.

Malate synthesis during excess cation uptake is thought to involve PEP-carboxylase (Figure 7.7, redrawn from Jacoby and Laties 1971). This pathway is similar to that responsible for malate synthesis in

TABLE 7.4 Effect of light on malate accumulation in the vacuole.

$[\text{K}_2\text{SO}_4]/\text{mol m}^{-3}$	light	dark
5	6.21 ± 0.68	2.72 ± 0.34
10	9.36 ± 0.78	4.13 ± 0.40

Cells placed solution for 20 hours. Each value is the mean \pm S.E.
of 10 cells

Culture: REG

Solution: K_2SO_4 as above, 0.5 mol m^{-3} CaSO_4 EPPS pH 8

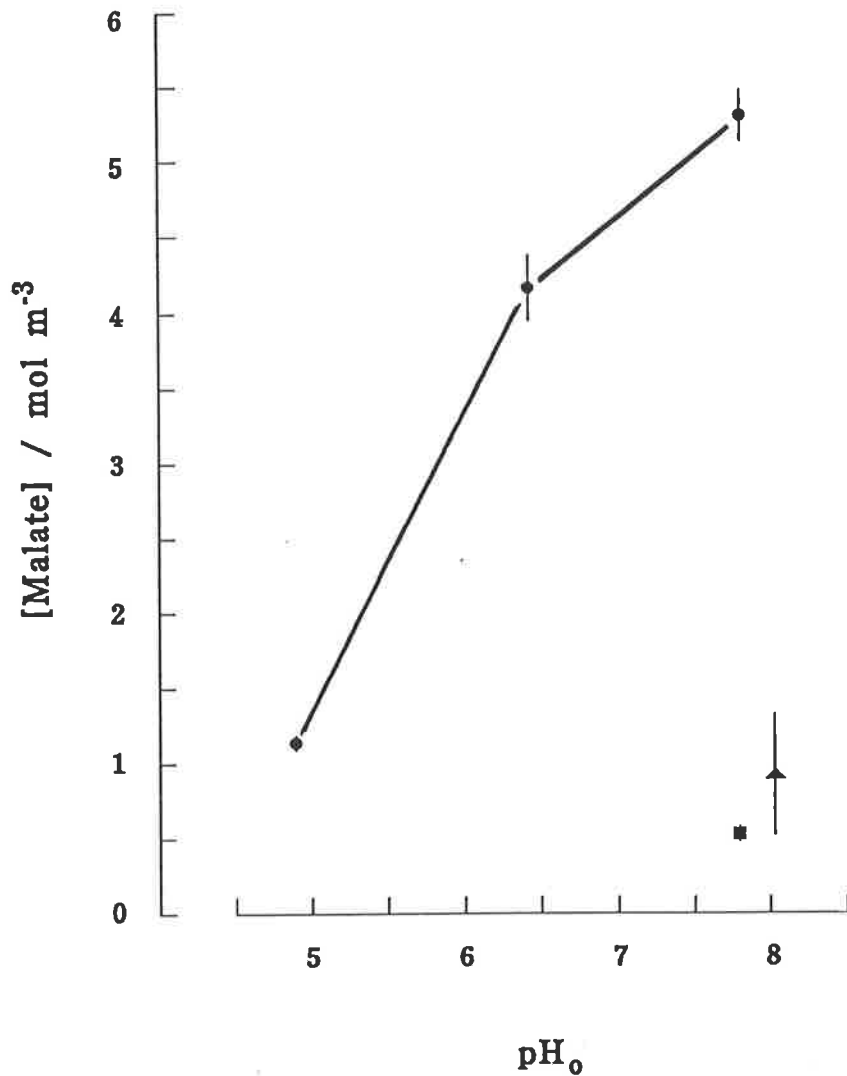


FIGURE 7.6 Effect of pH₀ on malate accumulation in the vacuole.

● 5 mol m⁻³ K₂SO₄, ■ CPW, ▲ Carbon free 5 mol m⁻³ K₂SO₄. Cells were placed in solution for 22 hours prior to malate determination. Each point represents the mean and standard error of 10 cells.

Culture: REG

Solution: CPW EPPS pH 8

5 mol m⁻³ K₂SO₄, 0.5 mol m⁻³ CaSO₄ with either MES pH 5, MES pH 6, EPPS pH 7 or EPPS pH 8.

Carbon free (see Chapter 2) 5 mol m⁻³ K₂SO₄, 0.5 mol m⁻³ CaSO₄ EPPS pH 8.

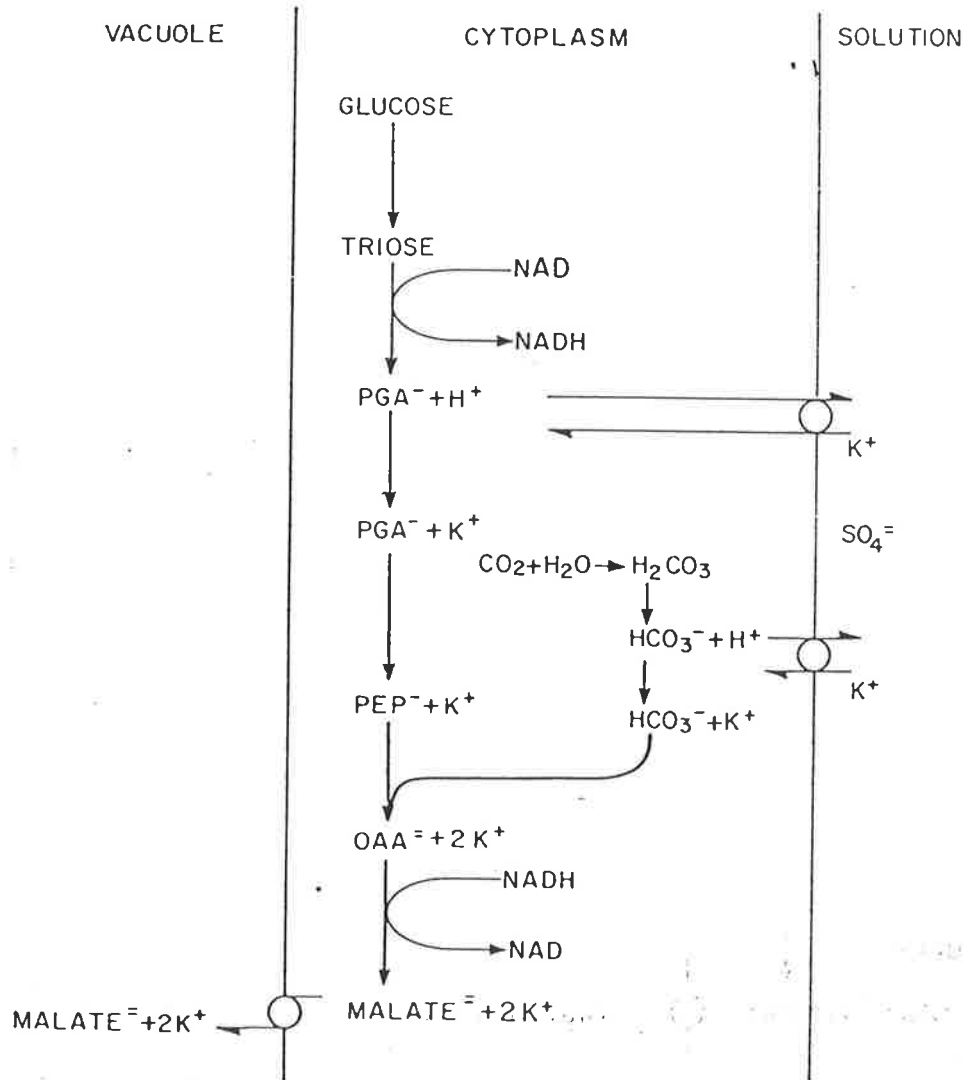


FIGURE 7.7 Schematic representation of organic acid synthesis in response to selective cation uptake. Abbreviations: PGA: phosphoglyceric acid; PEP: phosphoenolpyruvic acid; OAA: oxalacetic acid. The net negative charges depicted for PGA, PEP, OAA, and malate refer to dissociated carboxyl groups. CO_2 refers to respiratory CO_2 .

Redrawn from Jacoby and Laties (1971).

leaves of C_4 plants and also the pathway involved in malate accumulation by CAM plants (Osmond 1976). PEP-carboxylase activity in *C. corallina* was measured to see if this pathway could be responsible for malate accumulation measured. The activity of PEP-carboxylase from the C_4 grass *Pennisetum clandestinum* was also measured. *P. clandestinum* is a C_4 plant (Edwards and Walker 1983) and would therefore be expected to have a high PEP-Carboxylase activity. Therefore, *P. clandestinum* provides both a test of the extraction procedure and a source of PEP-carboxylase for later experiments.

The average rate of PEP-carboxylase activity measured for *P. clandestinum* was $1406 \mu\text{mol g}^{-1}(\text{fwt}) \text{h}^{-1}$ (Table 7.5). This rate is similar to the average rates of PEP-carboxylase activity from C_4 leaf extracts (Edwards and Walker 1983) which suggests that both the extraction and assay procedures were suitable. The average rate of PEP-carboxylase activity measured for *C. corallina* was $6.2 \mu\text{mol g}^{-1}(\text{fwt}) \text{h}^{-1}$. *C. corallina* is reported to contain between 0.5 mg chlorophyll $\text{g}^{-1}(\text{fwt})$ (Lucas 1975a) and 1 mg chlorophyll $\text{g}^{-1}(\text{fwt})$ (Reid pers. comm.). Therefore, the rate of PEP-carboxylase activity in *C. corallina* was between 6.2 and $12.4 \mu\text{mol mg}^{-1}(\text{chlorophyll}) \text{h}^{-1}$. This is lower than the usually reported rate for C_3 plants of $20-40 \mu\text{mol mg}^{-1}(\text{chlorophyll}) \text{h}^{-1}$ (Edwards and Walker 1983). However, the activity of PEP-carboxylase was more than sufficient to produce malate at the rates observed during cation uptake.

PEP-carboxylase activity was measured from cells of *C. corallina* that had been pretreated in for 4 hours in $10 \text{ mol m}^{-3} \text{K}_2\text{SO}_4$ and was found to be slightly less than in cells that had been pretreated in CPW (Table 7.5). This result indicates that PEP-carboxylase is normally in the cytoplasm but its activity is regulated.

The activities of samples of PEP-Carboxylase extract isolated from *P. clandestinum* and *C. corallina* were measured. Samples of the

TABLE 7.5 Rate of PEP-Carboxylase activity.

<i>Pennisetum clandestinum</i>	1501 $\mu\text{mol g(fwt)}^{-1} \text{h}^{-1}$
	1312 $\mu\text{mol g(fwt)}^{-1} \text{h}^{-1}$
<i>Chara corallina</i> (CPW)	6.10 $\mu\text{mol g(fwt)}^{-1} \text{h}^{-1}$
	6.22 $\mu\text{mol g(fwt)}^{-1} \text{h}^{-1}$
<i>C. corallina</i> (CPW + K_2SO_4)	4.3 $\mu\text{mol g(fwt)}^{-1} \text{h}^{-1}$
	5.6 $\mu\text{mol g(fwt)}^{-1} \text{h}^{-1}$

Culture: LES

extracts were mixed and PEP-carboxylase activity measured. The theoretical sum of the activities was 96% of the measured activity. This indicated that there were no inhibitors of PEP-carboxylase in the *Chara* extract.

7.2.2 Physiological consequences of malate production.

7.2.2.1 Production of H^+ in the cytoplasm.

Malate synthesis is generally accepted as occurring in the cytoplasm (Jacoby and Laties 1971, Osmond 1976). The production of malate from neutral sugars releases 2 H^+ malate⁻¹ molecule at cytoplasmic pH (eg. Jacoby and Laties 1971). This reaction forms the proton producing component of the "Davies pH-stat" (Davies 1973a,b). This calculation assumes that the carboxylation of PEP utilizes HCO_3^- that is produced from CO_2 within the cytoplasm. This production releases one H^+ per HCO_3^- produced and is therefore responsible for half of the proton load on the cytoplasm during malate synthesis. However, evidence presented in the previous section suggested that HCO_3^- required for carboxylation of PEP was supplied by HCO_3^- influx. $H^+-HCO_3^-$ symport is a favored mechanism for HCO_3^- influx in charophytes (Lucas 1982, Smith 1985a) though it is not proven (Smith 1985b). If $H^+-HCO_3^-$ symport provides the mechanism of HCO_3^- influx, and the stoichiometry is $1HCO_3^- : 1H^+$ (though the stoichiometry may be $2H^+ : 1HCO_3^-$ (Smith 1985b)), then the acid load on the cytoplasm would still be equivalent to $2H^+$ per malate synthesized.

Assuming that the above hypothesis to be correct, the rates of malate accumulation measured imposed an acid load on the cytoplasm of $1.8 \times 10^{-8} \text{ mol h}^{-1} H^+$. The buffering capacity of the cytoplasm of *C. corallina* has been calculated to be approximately $20 \text{ mol m}^{-3} \text{ pH unit}^{-1}$ (Ryan 1988, Reid et al. 1989). A hypothetical cell with a volume of 20 μl (assuming that the cytoplasm occupies 5% of the cell volume (Sakano

and Tazawa 1984)) would require 2.2×10^{-8} mol H^+ to reduce the pH_c by 1 unit. Therefore, malate produced in the cytoplasm at the rates observed, assuming no membrane transport of H^+ (ie. to the vacuole or the external solution) would reduce pH_c by 1 pH unit each 66 minutes. During a 6 hour experiment cytoplasmic pH would fall until a level where the malate produced would buffer pH_c (at approximately pH 3.4). Clearly this is untenable since cell processes such as cytoplasmic streaming would be totally inhibited at such a low pH_c yet they have been shown not to be under similar conditions (Figure 6.3). Therefore, it must be concluded that H^+ was transported from the cytoplasm to the vacuole or the external solution during malate synthesis.

7.2.2.2 Could regulation of pH_c be maintained by H^+ transport to the vacuole?

Malate is synthesized in the cytoplasm and transported to the vacuole where it accumulates as K-malate when K^+ is the cation balancing HCO_3^- uptake (Jacoby and Laties 1971, Ryan 1988). The exact charge on the malate molecule is determined by vacuolar pH; assuming this to be 5.3 (see section 7.2.2.3) malate would have an overall negative charge of 1.6 (Appendix 1). Therefore each divalent malate ion transported from the cytoplasm would consume 0.4 molecule of H^+ on entering the vacuole. Without H^+ transport this would result in an increased vacuolar pH. The vacuolar sap of *C. corallina* is weakly buffered relative to the cytoplasm, being approximately $0.63 \text{ mol m}^{-3} \text{ pH unit}^{-1}$ (Ryan 1988, Reid et al. 1989). Therefore (assuming malate accumulation of $0.45 \text{ } \mu\text{mol g}^{-1} \text{ h}^{-1}$ and no H^+ proton transport across the tonoplast) the pH of the vacuole would increase by 1 pH unit each 200 minutes.

It was shown in the previous section that for cytoplasmic pH_c to remain relatively constant during malate synthesis approximately

$1.8 \times 10^{-8} \text{ mol h}^{-1} \text{ H}^+$ would have to be transported from the cytoplasm. The accumulation of the malate in the the vacuole requires the supply of $3.6 \times 10^{-9} \text{ mol h}^{-1} \text{ H}^+$, otherwise vacuolar pH will rise. If the H^+ required for vacuolar pH maintenance were transported from the cytoplasm there would still remain an excess of $1.44 \times 10^{-8} \text{ mol h}^{-1} \text{ H}^+$ in the cell. This amount of H^+ would acidify the vacuole at the rate of 1 pH unit each 50 minutes.

These calculations show that the maintenance of pH_c during malate accumulation requires a net transport of H^+ from the cell to the external solution at a rate of approximately $40 \text{ nmol m}^{-2} \text{ s}^{-1}$.

7.2.2.3 pH_v , pH_c and membrane potential difference during malate accumulation.

A series of experiments were undertaken to measure pH_v , pH_c and membrane potential difference during malate synthesis. The results are discussed with reference to the calculations considered in the previous section.

Values of pH_v and pH_c were measured in cells bathed in $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ and in CPW for 6 hours. The results are presented in Table 7.6. The value of pH_v increased by 0.46 pH units whilst pH_c decreased by 0.2 pH units. The direction of the changes in pH are consistent with the calculations considered in section 7.2.2.2; the disassociation of malate produced in the cytoplasm reduced pH_c and the slight protonation of malate transported to the vacuole increased pH_v .

A time course of membrane potential difference after the addition of either $10 \text{ mol m}^{-3} \text{ KCl}$ or $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ was conducted (Figure 7.9 and representative trace 7.8). Membrane potential difference decreased by an average of 14 mV upon addition of $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ and remained at this level for between 2 and 21 minutes (mean = 14 minutes) before the cell underwent a further large depolarization to approximately -67 mV.

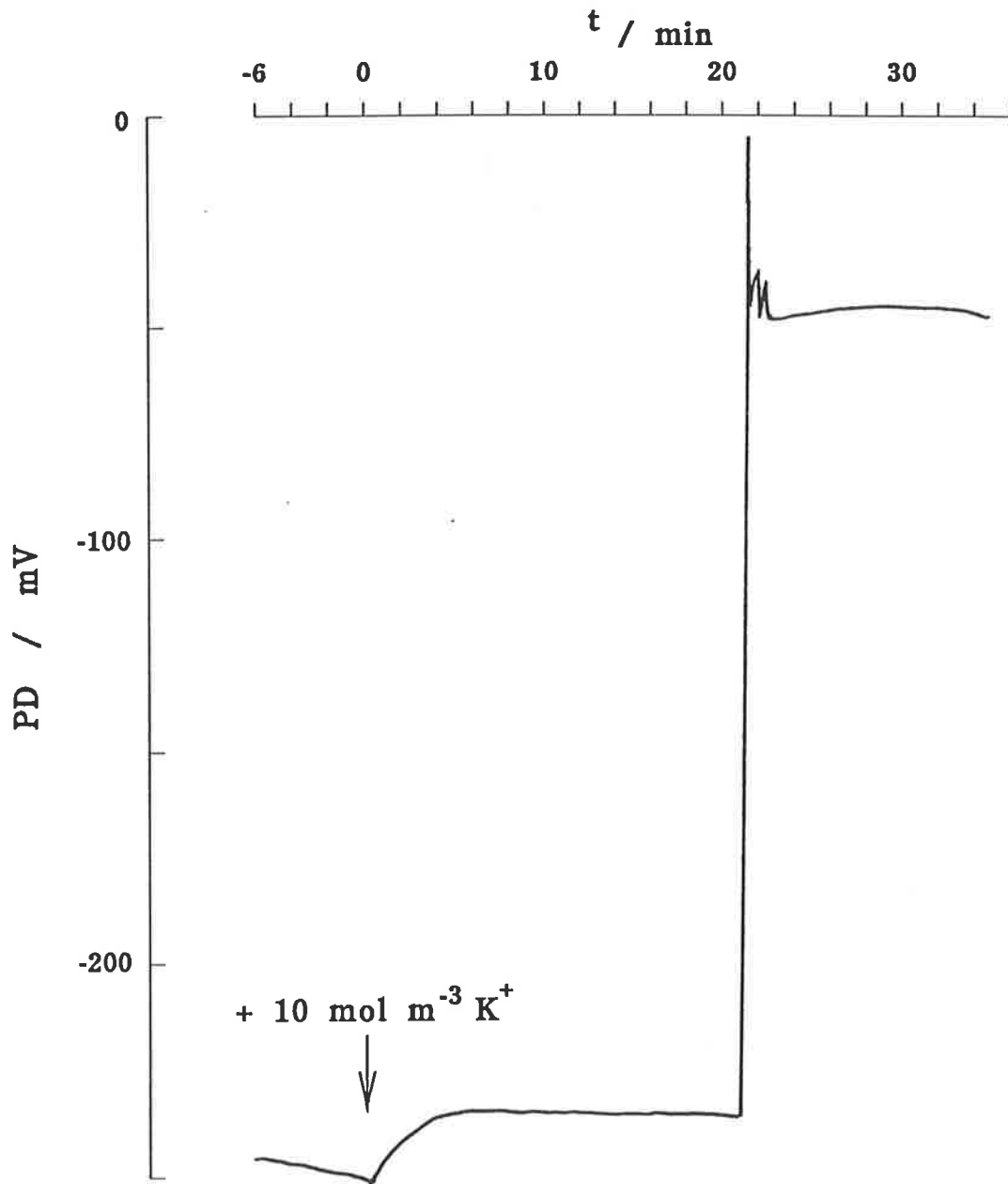


FIGURE 7.8 Effect of $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ on membrane potential difference.

$5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ added at $t = 0$. Data represents a single trace.

Culture: LES

Solution: Cells initially in CPW EPPS pH 8. Solution replaced with $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$, EPPS pH 8.

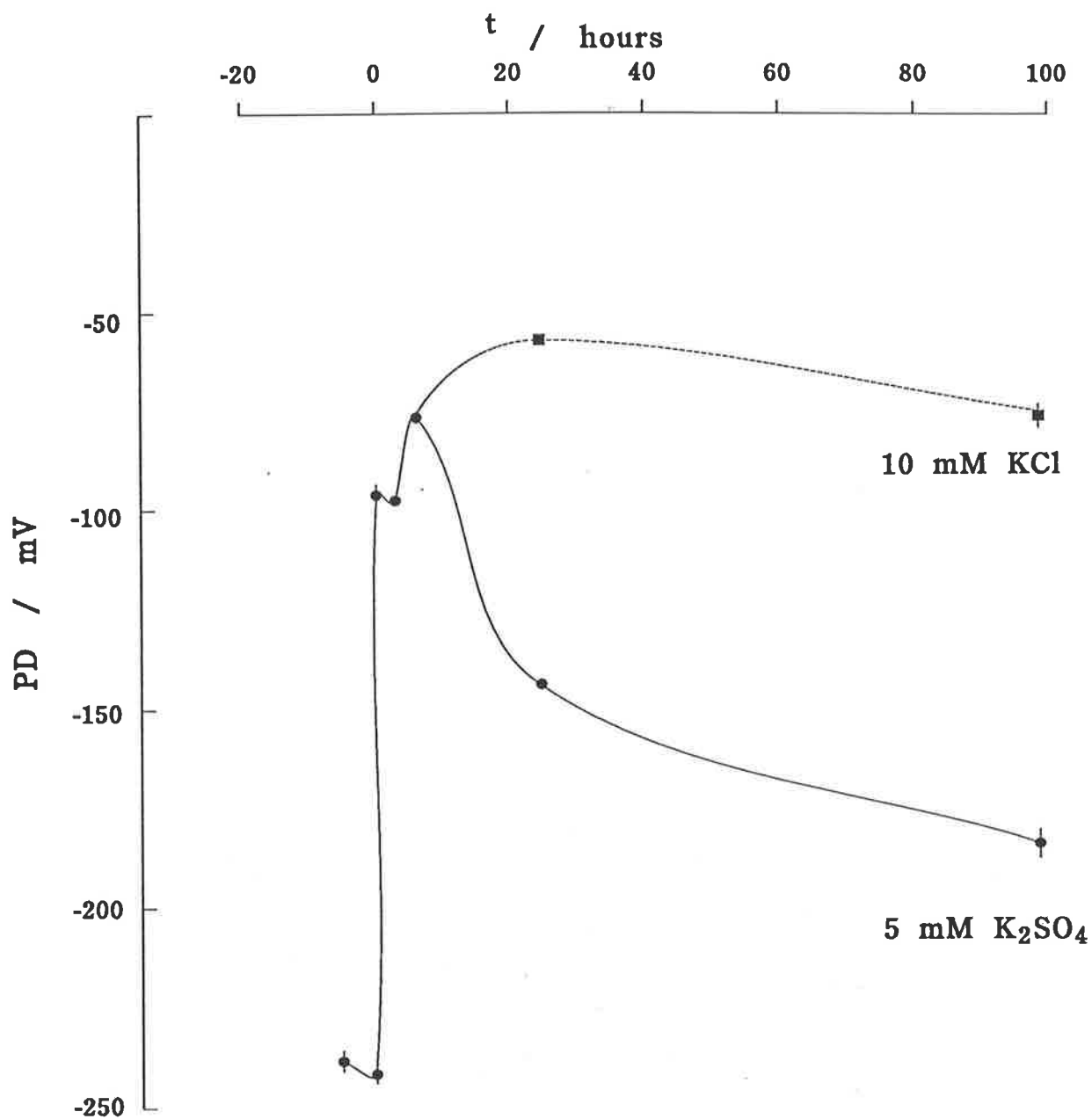


FIGURE 7.9 Effect of $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ and $10 \text{ mol m}^{-3} \text{ KCl}$ on membrane potential difference.

$10 \text{ mol m}^{-3} \text{ KCl}$ and $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$. $\text{K}^+ \pm \text{Cl}^-$ added at $t = 0$. Each point represents the mean \pm S.E. of 6 cells. Solutions changed daily throughout the experiment.

Culture: LES

Solution: Cells initially in CPW EPPS pH 8. Solution replaced with $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ or 10 mol m^{-3} , EPPS pH 8.

Associated with this depolarization were a series of action potentials. Membrane potential difference continued to depolarize to an average of -42 mV over the next 6 hours. It is interesting to note that throughout this 6 hour period that the H^+ -pump removed approximately $40 \text{ nmol m}^{-2} \text{ s}^{-1} H^+$ even though the cell membrane potential difference was -42 mV. This result shows that the H^+ -pump is not necessarily inoperative when the plasma membrane is depolarized. Cells bathed in $10 \text{ mol m}^{-3} \text{ KCl}$ remained depolarized throughout the experiment. However, cells bathed in $5 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ repolarized to approximately -165 mV during the same period.

During this experiment vacuolar concentrations of K^+ rose by approximately 22 mol m^{-3} per day throughout the experiment. Vacuolar Na^+ remained unchanged during the experiment (Figure 7.4). Whilst not measured in this experiment it would be expected that in the Cl^- free treatments malate would also be accumulated in these cells at a rate of up to 10 mol m^{-3} per day (see Section 7.2.1.3).

E_K for cells from each treatment has been calculated with the assumption that cytoplasmic K^+ is similar to vacuolar K^+ in each treatment. E_K at the beginning of the experiment was -51 mV. By the fourth day of the experiment E_K for the both the K_2SO_4 and KCl treatments was -70 mV. Whilst the membrane potential difference of cells in the KCl treatments were close to E_K cells in the K_2SO_4 treatment were hyperpolarized by 94 mV relative to E_K .

7.2.2.4 Do changes in pH trigger the formation of malate?

Three major models for the regulation of malate synthesis have been proposed. They include changes in the cytoplasmic pH (Davies 1973a,b) and associated changes in cytoplasmic HCO_3^- concentration (Jacoby and Laties 1971), low cytoplasmic Cl^- concentration (Ryan 1988) and cytoplasmic malate concentration (Ting 1968).

Davies (1973a,b) proposed a biochemical "pH-stat" to regulate pH_c . This hypothesis predicts that high pH_c will increase the rate of PEP-carboxylase activity resulting in malate synthesis and the production of H^+ . If this proposed pH-stat is responsible for the production of organic acids then it should be possible to measure an increase in pH_c during treatment with $10 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ since this treatment has been shown to stimulate malate production. Table 7.6 shows pH_c to be slightly lower in this treatment relative to CPW. However, pH_c was measured during the final 120 minutes of a 6 hour experiment. Once malate accumulation has been induced the associated H^+ would also have influenced cytoplasmic pH_c . Nevertheless, it is obvious that elevated pH_c is not required for the continued production of malate. The possibility still remained that elevated pH_c was not required to initiate malate accumulation.

In the above experiment pH_c was measured using a technique that relies on the equilibration of undissociated DMO between the bathing solution, cytoplasm and vacuole (described by Smith 1980, 1984 and references therein). The half time for DMO equilibration across the tonoplast is about 25 minutes so experiments are usually conducted over 2 hours (Walker and Smith 1975). Smith (1986) has shown that the DMO technique can be successfully used to estimate pH_c over a five minute period. However, this technique is unsuitable when the bathing solution is pH 8.

To overcome this problem changes in pH_c in the short term were measured using micro pH-electrodes (Reid and Smith 1988) as described in Chapter 2.5.2. Whorl cells were inserted in an electrode chamber and impaled with a double barrelled micro-electrode. One barrel records pH_c and the other membrane potential difference. Once a continuous recording of membrane potential difference and pH_c were obtained the bathing solution was replaced with $10 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$. Changes in

TABLE 7.6 pH of the vacuole and cytoplasm of *C. corallina* treated in
 0.1 mol m^{-3} or 5 mol m^{-3} K_2SO_4 .

$[\text{K}_2\text{SO}_4] / \text{mol m}^{-3}$	pH_v	pH_c
0.1	5.05 (+0.03, -0.02)	8.22 (+0.056, -0.050)
20.0	5.51 (+0.04, -0.04)	8.02 (+0.022, -0.021)

Cells placed solution for 6 hours. Each value is the mean \pm S.E.
of 10 cells.

Culture: LES

Solution: K_2SO_4 as above, 0.5 mol m^{-3} CaSO_4 EPPS pH 8

membrane potential difference were similar to those shown in Figure 7.9 and described in Section 7.2.2.3. In 4 of the 6 recordings obtained pH_c rose by 0.1 to 0.4 units on addition of $10 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ whilst in two cases pH_c did not change (Table 7.7, see Figure 7.10 for a representative trace). It is unlikely that such small, transient and inconsistent increases in pH_c would act as a trigger for malate synthesis.

TABLE 7.7 Summary of cytoplasmic pH and membrane potential difference measured with double barrelled microelectrodes.

cell	PD _{initial}	PD _{final}	pH _c initial	pH _c final	Δ pH _c
210989b	-220	-20	7.6	8.0	0.4
210989a	-155	-50	7.9	8.1	0.1
200989a	-160	-30	7.9	7.9	0
200989b	-205	-65	7.8	7.8	0
210989c	-190	-20	7.8	7.9	0.1
210989d	-110	-25	8.2	8.4	0.4

Initial refers to PD and pH before the addition of $25 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ and final refers to a stabilized value of PD and pH approximately 20 minutes after the addition of $25 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$.

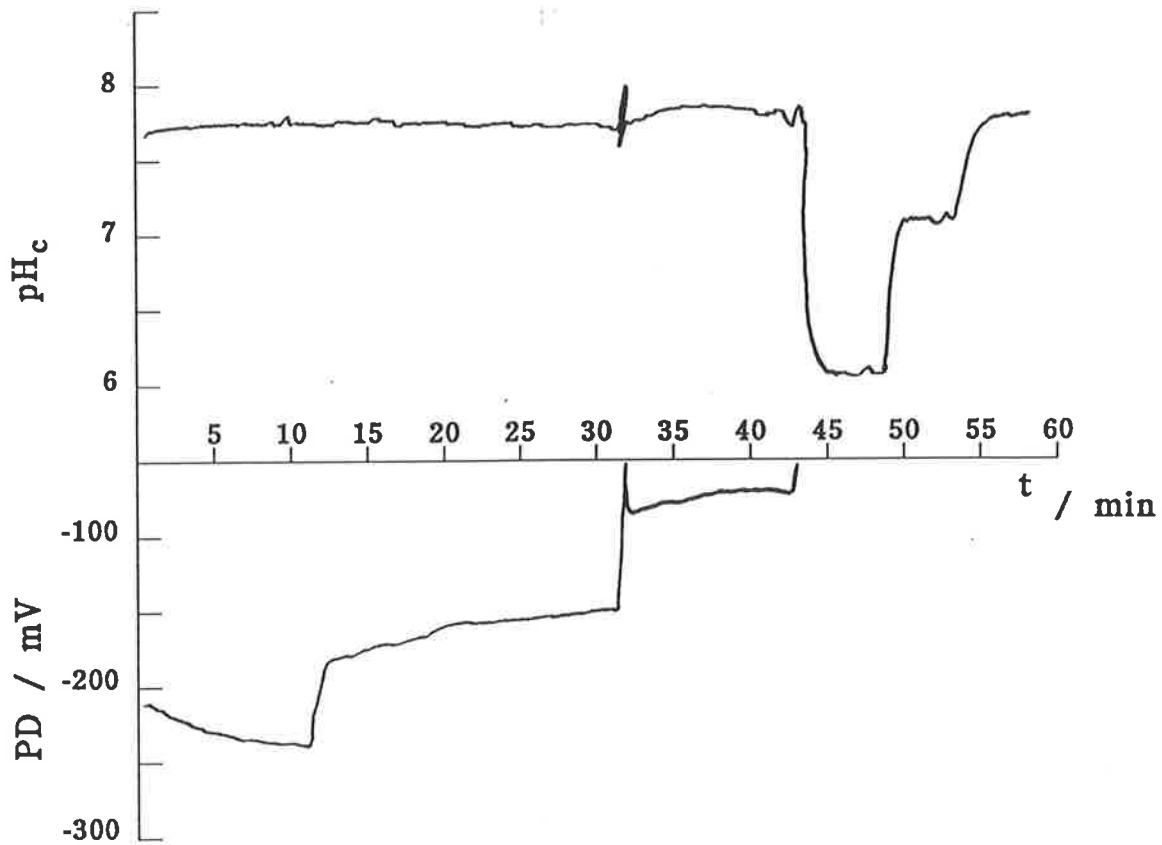


FIGURE 7.10 Effect of $25 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ on pH_c and membrane potential difference.

$25 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$ added at $t = 12$ minutes. Calibration curve commenced at $t = 42$ minutes. Data represents a single trace.

Culture: LES

Solution: Cells initially in CPW EPPS pH 8. Solution replaced with $25 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$, EPPS pH 8.

7.3 DISCUSSION

7.3.1 Malate production during "excess ion uptake".

Absorption of excess K^+ (or Rb^+) relative to inorganic anion, whether it be Cl^- , SO_4^{2-} or NO_3^- leads to synthesis and then accumulation of malate in the vacuole. The amount of malate produced is probably related to the rate of transport of ions present in the external medium across the plasma membrane, the lower the membrane rate of transport of the anion the more malate produced. The maximum rate of malate accumulation observed was $0.45 \mu\text{mol g}^{-1} \text{h}^{-1}$. This equates to an accumulation rate of $0.77 \mu\text{equiv g}^{-1} \text{h}^{-1}$ (assuming $\text{pH}_v = 5.3$). Malate accumulation saturated at an external K^+ concentration of between 10 and 20 mol m^{-3} and accumulation continued at this rate for at least 48 hours. The *in vitro* activity of PEP-carboxylase was sufficient to produce malate at a rate of $6.2 \mu\text{mol g}^{-1} \text{h}^{-1}$, an order of magnitude greater than the maximum *in vivo* rate observed. The rate of malate accumulation in *C. corallina* is generally lower than the maximum rates recorded for higher plants (eg. *Hordeum vulgare*, $2 - 10 \mu\text{mol g}^{-1} \text{h}^{-1}$ (Jacoby and Laties 1971, Hiatt 1967)).

In the vacuole of higher plants electroneutrality is usually maintained by inorganic cations, and a mixture of inorganic anions and carboxylate anions (Osmond 1976). As was mentioned in section 7.1 most studies with giant algal cells show ion balance to be satisfactorily explained by fluxes of K^+ , Na^+ and Cl^- although Sanders (1981b) and Smith and Whittington (1988) postulated that an organic anion contributed to electroneutrality in the vacuole of *C. corallina* under various Cl^- -free treatments. Ryan (1988) has since shown *C. corallina* to accumulate malate in the vacuole during amine uptake in the absence of Cl^- . The results presented in this chapter agree with those of Ryan (1988), showing that under certain conditions *C. corallina* maintains electroneutrality in the vacuole with malate.

It was presumed in this study that synthesis of malate occurred in the cytoplasm and that malate was then transported to the vacuole. Evidence for cytoplasmic synthesis of malate in higher plants is reviewed by Osmond (1976). In the experiments described in this thesis synthesis of malate in the cytoplasm resulted in an acid load on the cytoplasm of up to $1.8 \times 10^{-8} \text{ mol h}^{-1} \text{ H}^+$. Most of the malate produced was transported to the vacuole where it became slightly protonated; therefore malate transported to the vacuole consumed $3.6 \times 10^{-8} \text{ mol h}^{-1} \text{ H}^+$. Considering the buffering capacity of cell (Reid et al. 1989) and the lack of substantial cellular pH changes observed during the accumulation of malate it is concluded that transport of H^+ from the cell occurred during malate synthesis at a rate of approximately $40 \text{ nmol m}^{-2} \text{ s}^{-1}$. The results discussed above are presented as a diagrammatic model in Figure 7.11.

7.3.2 Regulation of pH_c during malate accumulation.

Regulation of pH_c in plants is achieved in part by biochemical and biophysical processes. External application of weak acids has been used as a means of imposing an acid load on the cytoplasm (Marrè et al. 1983, Reid et al. 1985, Sanders et al. 1981, Felle 1987, 1988, Reid et al. 1989). These studies have shown that different types of plants under different conditions rely to differing degrees on biophysical and biochemical pH_c regulation. However, pH regulation in *C. corallina* is thought to be primarily biophysical (Smith and Reid 1989). In this study, accumulation of malate imposed an acid load on the cytoplasm. The results summarized in Figure 7.11 indicate that the cell regulated pH_c by pumping H^+ from the cell; in effect biophysical pH regulation. But the question remains, what induces malate synthesis? Is malate synthesis during excess cation uptake part of a biochemical pH regulatory system such as that proposed by Davies (1973a,b)?

7.3 3 Regulation of malate accumulation.

It has been proposed that K^+H^+ exchange is a causative event in salt induced carboxylation (Jacoby and Laties 1971). Since there is no experimental evidence for a K^+H^+ antiport operating at the plasma membrane of *C. corallina* the transport of K^+ is considered as separate from the transport of H^+ . Such a system where a H^+ -pump operates simultaneously with a K^+ uniport system has been postulated by Smith and Walker (1975). This scheme suggested that H^+ were pumped from the cell in response to a decrease in membrane potential difference brought about by K^+ influx. This led to an increase in pH_c . Smith and Walker (1976) argued that regulation of pH_c is of paramount importance for the control of the H^+ -pump. Thus the cell faces a dilemma: continued pumping of H^+ from the cell to maintain membrane potential difference or maintaining pH_c by stopping H^+ -pumping. In the presence of Cl^- the cell can maintain pH_c and membrane potential difference by Cl^-2H^+ symport (eg. Smith and Walker 1976). In the absence of external Cl^- both $K-H$ exchange and pH_c could be maintained by the production of malate via the biochemical pH-stat proposed by Davies (1973b). For a number of reasons the results presented in this chapter do not fully support this hypothesis.

First, malate is produced in the presence of external Cl^- . It could be argued that K^+H^+ exchange causes K^+ to be accumulated faster than Cl^- can be accumulated via Cl^-2H^+ antiport and therefore malate is required to make up the anion deficit. Low concentrations of cytoplasmic Cl^- have been implicated in malate accumulation (Ryan 1988). In the absence of external Cl^- , cytoplasmic Cl^- could become low if cytoplasmic Cl^- was transported into the vacuole to balance K^+ accumulation. In the presence of external Cl^- this may still happen if cytoplasmic Cl^- is transported to the vacuole faster than Cl^- is

transport into the cell. Sanders (1980) has shown that the cytoplasm of *C. corallina* can be starved of Cl^- which resulted in a stimulation of Cl^- influx. The Cl^- influx in *C. corallina* at pH_o 8 is normally about $25 \text{ nmol m}^{-2} \text{ s}^{-1}$ (Smith and Walker 1976) but in the presence of amines (Smith and Walker 1978) or after cytoplasmic Cl^- starvation (Sanders 1980) Cl^- influx can increase to $60 \text{ nmol m}^{-2} \text{ s}^{-1}$. If this rate could be maintained it should balance the accumulation of K^+ .

Secondly, no significant or reproducible changes in pH_c were measured on addition of high concentrations of external K^+ . If the enzymes of the Davies pH-stat are regulated by pH_c then measurable changes in pH_c would be expected. Therefore these results argue against rises in pH_c being the trigger for malate accumulation.

In light of the above findings it is worth reconsidering the form of regulation which involves the feedback control of PEP-carboxylase by malate. Winter (1989) has shown that the activity of PEP-carboxylase extracted from CAM plants to be very sensitive to inhibition by malate. Osmond (1976) reports the K_i value for malate to range between 1 and 10 mol m^{-3} . *C. corallina* can accumulate malate at rates of up to $0.45 \text{ } \mu\text{mol g h}^{-1}$. If malate produced was not transported from the cytoplasm then malate concentration in the cytoplasm would rise by about 9 mol m^{-3} each hour. It would be expected that such a concentration of malate in the cytoplasm would inhibit PEP-carboxylase: thus the rate of transport of malate from the cytoplasm to the vacuole could be a regulator of malate accumulation in *C. corallina*. The regulation of malate transport across the tonoplast is an area for future research.

7.4 CONCLUSIONS

The results presented in this chapter show that malate was accumulated in the vacuole of *C. corallina* when the cell was bathed in the K^+ salts of SO_4^{2-} , NO_3^- , MES^- or Cl^- . Malate accumulation was

sensitive to pH_o and external HCO_3^- concentration. Malate synthesis occurred by the carboxylation of PEP by the enzyme PEP-carboxylase with HCO_3^- the substrate. During malate accumulation H^+ was pumped from the cell at a rate of about $40 \text{ nmol m}^{-2} \text{ s}^{-1}$, therefore the H^+ -pump was operating even though the cell was depolarized (membrane potential difference of about -42mV). The results presented indicate that changes in pH_c did not invoke malate accumulation nor was it likely that low cytoplasmic Cl^- was responsible. What invokes malate accumulation in *C. corallina* remains unresolved.

C. corallina responds in a similar way to other higher plants during imbalanced ion uptake and so could provide a useful tool for studying biochemical pH regulation particularly during the decarboxylation of malate.

CHAPTER 8

Effect of Ca^{2+} on Na^+ influx.

8.1 INTRODUCTION

Na^+ is the predominant cation in most water bodies in which charophytes grow. Despite this there are few published reports describing Na^+ influx into *Chara*. Movement of Na^+ into *C. corallina* is facilitated by an electrochemical potential gradient (Hope and Walker 1975). Smith (1967) has shown Na^+ influx in the freshwater Charophyte, *Nitella translucens*, to be linked to processes controlled by metabolism and suggested that Na^+ influx was partially linked to the inward Cl^- -pump.

Using *Nitellopsis obtusa* Katsuhara and Tazawa (1988) showed that increasing Na^+ in the absence of external Ca^{2+} resulted in a large increase in cytoplasmic Na^+ and a corresponding decrease in cytoplasmic K^+ . Hoffmann et al. (1989) reported that Na^+ influx increased in both *C. corallina* and *C. buckellii* when external Na^+ was increased from 1 to 70 mol m^{-3} . Both Hoffmann et al. (1989) and Katsuhara and Tazawa (1988) show that when Ca^{2+} was added to the external medium (maintaining the $\text{Na}^+:\text{Ca}^{2+}$ ratio at 10:1) Na^+ influx remained at the same rate as in 1 mol m^{-3} Na^+ . Using *Nitella translucens* and much lower concentrations of external Na^+ , Smith (1967) found that in the presence of Ca^{2+} , influx of Na^+ increased only slightly when the external Na^+ concentration was raised above 1 mol m^{-3} . However, in the absence of Ca^{2+} , Na^+ influx did not saturate. The results presented above indicate that Ca^{2+} is implicated in the regulation of Na^+ influx and that this is an important part of the protective role played by Ca^{2+} during salt treatment of charophyte algae.

In the early part of this chapter the influence of Ca^{2+} on the

influx of Na^+ will be investigated. The results show that there are two components to Na^+ influx, one that is independent of Ca^{2+} concentration and the other that is sensitive to Ca^{2+} concentration. The second part of this chapter describes some of the characteristics of these two components of Na^+ influx.

In the charophyte algae, the transport of K^+ has received much more attention than the transport of Na^+ , consequently much more is known about the transport of K^+ compared to Na^+ . External Ca^{2+} has been implicated in the regulation of both K^+ and Na^+ influx into various plant and animal cells, including *C. corallina* (eg. Bisson 1984, Beilby 1986, Smith and Kerr 1987, Kourie and Findlay 1990b). The effect of Ca^{2+} on the influx of Na^+ is compared to the effect of Ca^{2+} on the influx of K^+ . The similarities between the Ca^{2+} -sensitive influx of both of these ions suggests that they enter the cell via a common transport mechanism.

8.2 RESULTS

8.2.1 Time course of Na^+ uptake.

Figure 8.1 shows the rate of Na^+ uptake to be constant with time. Influx was measured for 120 minutes and found to be 41 ± 5 (10) $\text{nmol m}^{-2} \text{ s}^{-1}$. Influx was also measured for a 5 minute period at various times throughout the experiment. Apart from the first measurement (at 2 minutes) influx varied between 25 and 44 $\text{nmol m}^{-2} \text{ s}^{-1}$ ($x = 36 \pm 3$). The higher rate of uptake at 2 minutes probably reflects Na^+ bound to the cell wall that was not washed from the free space during the rinse period.

The influx of Na^+ remained relatively constant for two hours, therefore this time period was chosen as a standard for experiments describing Na^+ influx. Hoffmann et al. (1989) report a similar result. They showed uptake of Na^+ to be linear for 2 hours. Measuring influx

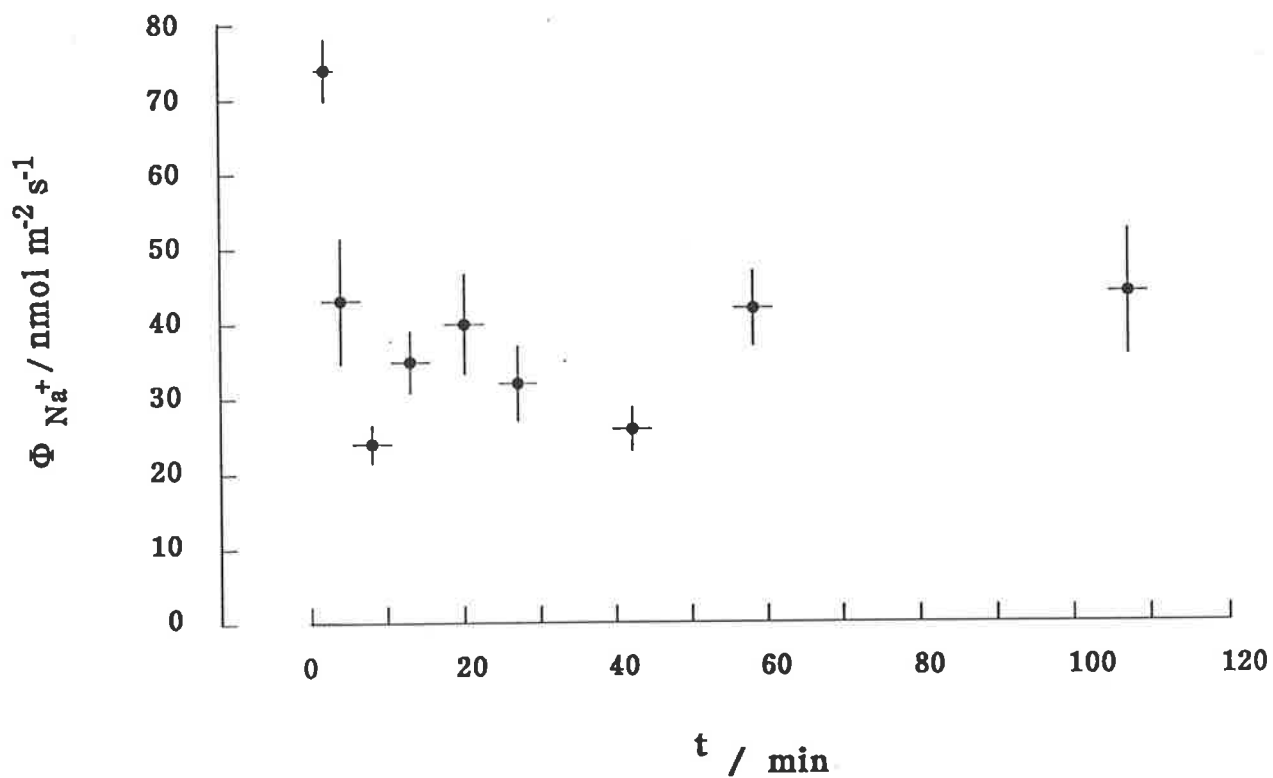


FIGURE 8.1 Time course of $^{24}Na^+$ influx in Ca^{2+} -free solutions.

Na^+ influx measured for the last 5 minutes of each period (represented by the horizontal bar). Each point represents the mean and S.E. of 10 cells.

Culture: LES

Solution: Ca^{2+} -free CPW 1 mol m^{-3} TAPS pH 8 ($[Na^+] = 1.36 \text{ mol m}^{-3}$).

for two hours rather than for a shorter time period has a number of advantages. The longer the uptake period, the lower the specific activity of Na^+ required in the external solution. This allows the use of $^{22}\text{Na}^+$ rather than $^{24}\text{Na}^+$, for most experiments. Na^+ influx was measured so as to describe the physiologically important mechanisms of Na^+ transport across the plasma membrane at high concentrations of external Na^+ . In this context influxes over the long term are probably more relevant to the survival of the cell than those over the short term.

8.2.2 Na^+ influx from solutions containing Ca^{2+} ions.

$^{22}\text{Na}^+$ influx was measured as a function of Na^+ concentration with an external Ca^{2+} concentration of 0.5 mol m^{-3} (Figure 8.2). Na^+ influx increased with Na^+ concentration: however, the curve describing Na^+ influx as a function of Na^+ concentration is steeper at concentrations of Na^+ below 5 mol m^{-3} and at Na^+ concentrations greater than $20\text{--}25 \text{ mol m}^{-3}$. At Na^+ concentrations below 20 mol m^{-3} influx of Na^+ into *C. corallina* resembles Na^+ influx into *Nitella translucens* as described by Smith (1967). The increase in the rate of Na^+ uptake at high concentrations of Na^+ resembles the biphasic influx K^+ described for various plant and algal cells (eg. Raven 1976, Kochian and Lucas 1982). Smith (unpublished) obtained similar results, showing Na^+ influx to be biphasic (Figure 8.3).

Experiments with $^{24}\text{Na}^+$ were undertaken to measure the relationship between Na^+ influx and higher external concentrations of Na^+ than could be achieved using $^{22}\text{Na}^+$. The results presented in Figure 8.4 show that the rate of Na^+ uptake is roughly proportional to external Na^+ concentration up to 100 mol m^{-3} . This pattern of Na^+ uptake is similar to K^+ transport into corn roots described by Kochian and Lucas (1982). The kinetics of Na^+ influx are probably resolvable into saturable and

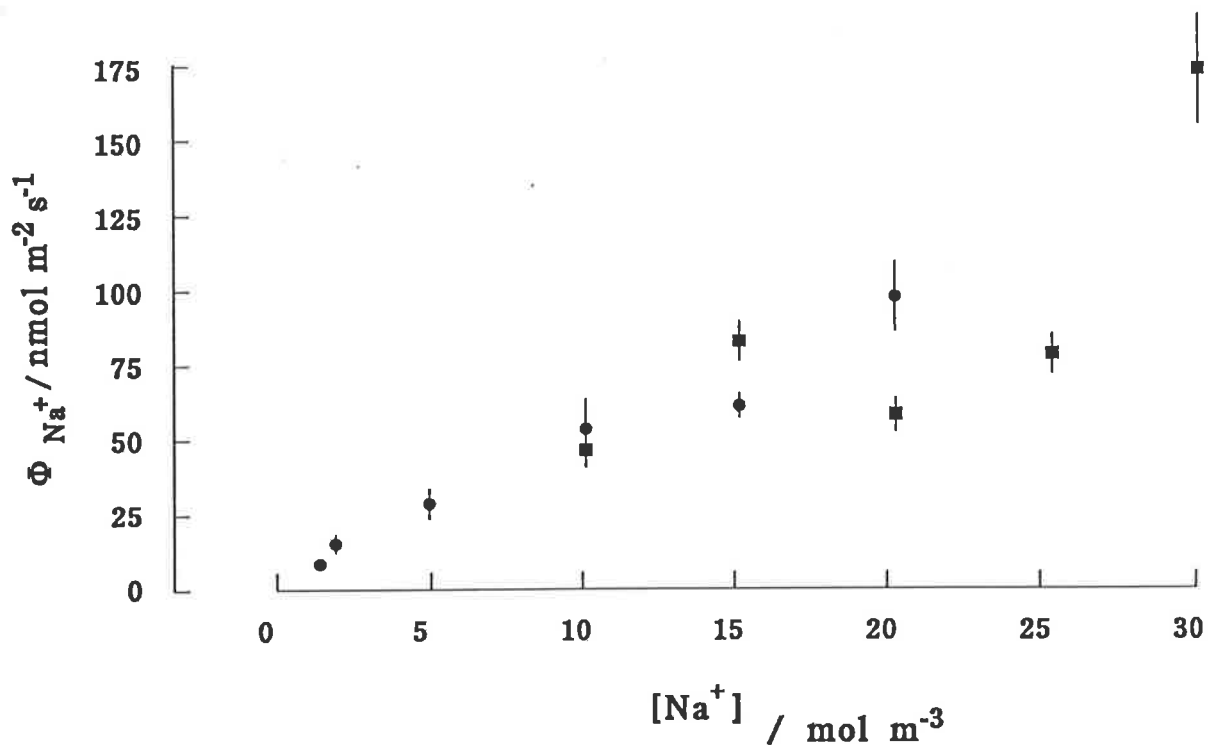


FIGURE 8.2 Effect of Na^+ concentration on Na^+ influx.

Results collected on two separate occasions, represented by the symbols ● and ■. Each point represents the mean and S.E. of 10 cells.

Culture: LES

Solution: CPW 1 mol m⁻³ TAPS pH 8 + the appropriate concentration of NaCl.

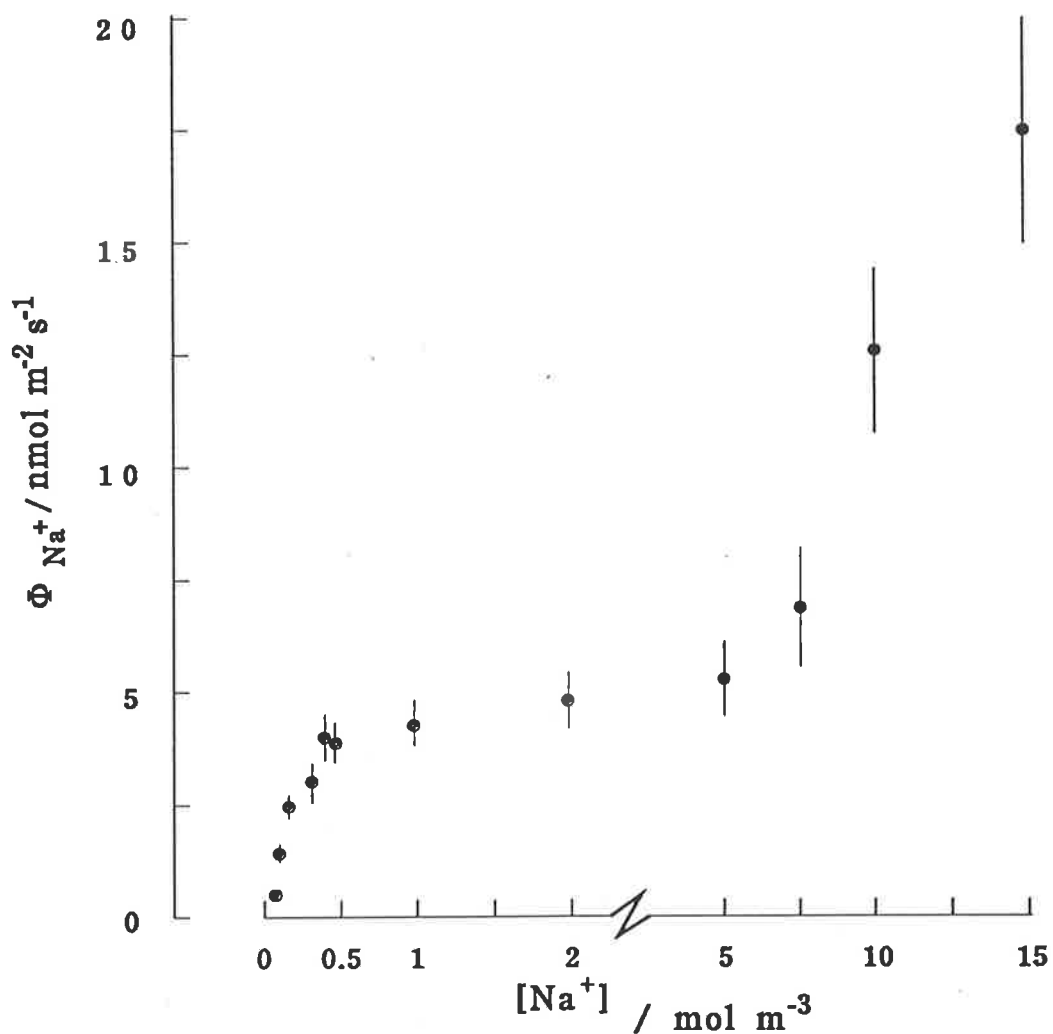


FIGURE 8.3 Effect of Na⁺ concentration on Na⁺ influx in *Nitella translucens* (Smith F. A. unpublished).

Each point represents the mean and S.E. of 10 cells. Experiments conducted in November 1967.

Solution: 0.5 mol m⁻³ CaSO₄, 0.1 mol m⁻³ K₂SO₄ + the appropriate concentration of NaCl.

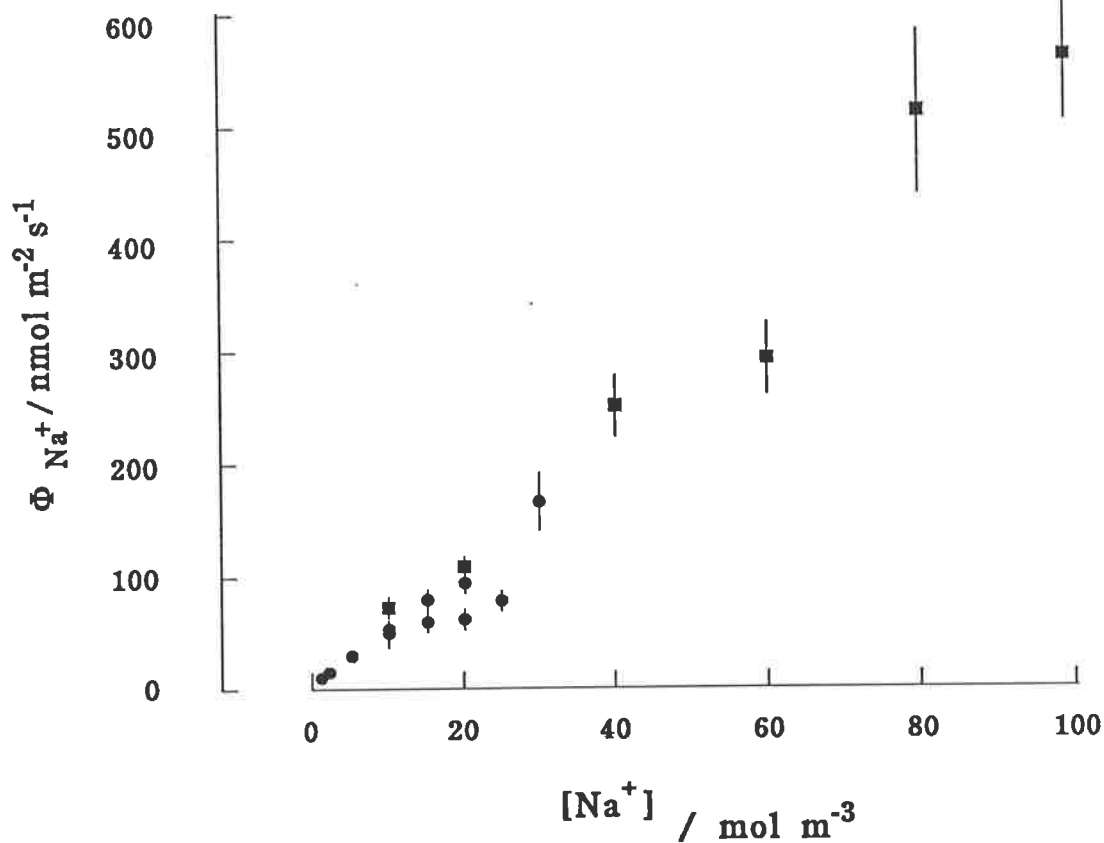


FIGURE 8.4 Effect of Na⁺ concentration on Na⁺ influx.

Results collected using ²⁴Na⁺ ■ and ²²Na⁺ ● . Each point represents the mean and S.E. of 10 cells.

Culture: LES

Solution: CPW 1 mol m⁻³ TAPS pH 8 + the appropriate concentration of NaCl.

linear components. Rains and Epstein (1967a,b) showed that Na^+ absorption into barley roots can be explained by 2 mechanisms. Over the concentration range 0.005 to 0.2 mol m^{-3} a single Michaelis-Menton term described the relationship between Na^+ concentration and influx. Over the higher concentration ranges a second low affinity mechanism was used to describe Na^+ transport.

8.2.3 The influence of Ca^{2+} on Na^+ influx.

Figures 8.5, 8.6 and 8.7 show the effect of external Ca^{2+} concentration on Na^+ influx at 3 concentrations of Na^+ . These results show that at lower concentrations of external Ca^{2+} , Na^+ influx was strongly dependent upon Ca^{2+} concentration. At higher concentrations of external Ca^{2+} , Na^+ influx was not sensitive to changes in external Ca^{2+} concentration. These results suggest that there are two mechanisms of Na^+ influx, one that is sensitive to external Ca^{2+} concentration and the other that is insensitive to external Ca^{2+} . For convenience, Na^+ influx that is insensitive to Ca^{2+} concentration shall be called mechanism 1 and Na^+ influx that is sensitive to Ca^{2+} concentration shall be referred to as mechanism 2. The Ca^{2+} insensitive Na^+ influx is similar to the high affinity, K^+ transport system that Epstein et al. (1963) termed mechanism 1. The Ca^{2+} sensitive Na^+ influx is similar to Epsteins' et al. (1963) low affinity mechanism 2.

The external Ca^{2+} concentration where Na^+ influx becomes insensitive to Ca^{2+} (that is the Ca^{2+} concentration where mechanism 2 is inhibited) is dependant upon the external Na^+ concentration. The higher the Na^+ concentration, the higher the Ca^{2+} concentration required for inhibition of mechanism 2. The point at which Na^+ influx via mechanism 2 is inhibited occurs at a ratio of Ca^{2+} to Na^+ of 8:1 when Na^+ concentration is 2.4 mol m^{-3} , 10:1 when Na^+ concentration is 10 mol m^{-3} and approximately 100:1 when Na^+ concentration is 100

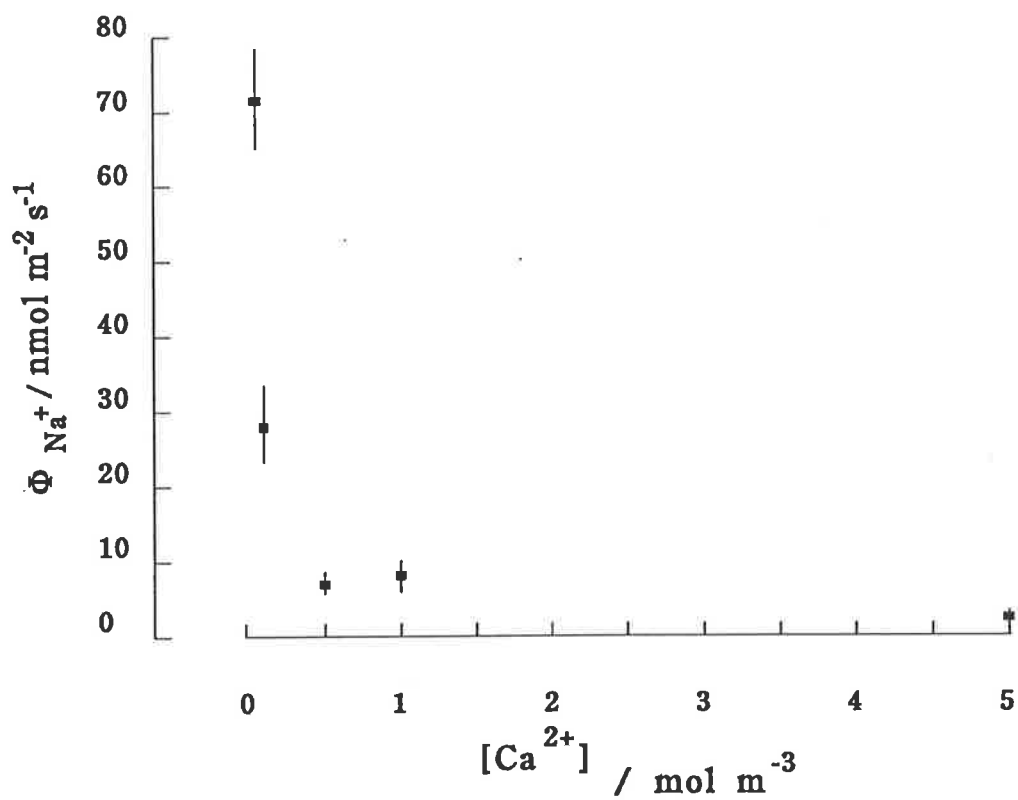


FIGURE 8.5 Effect of Ca²⁺ concentration on Na⁺ influx at 2.37 mol m⁻³ Na⁺.

Points represent the mean and S.E. of 10 cells.

Culture: LES

Solution: CPW 1 mol m⁻³ TAPS (actual [Na⁺] = 2.37 mol m⁻³) + appropriate concentration of Ca²⁺.

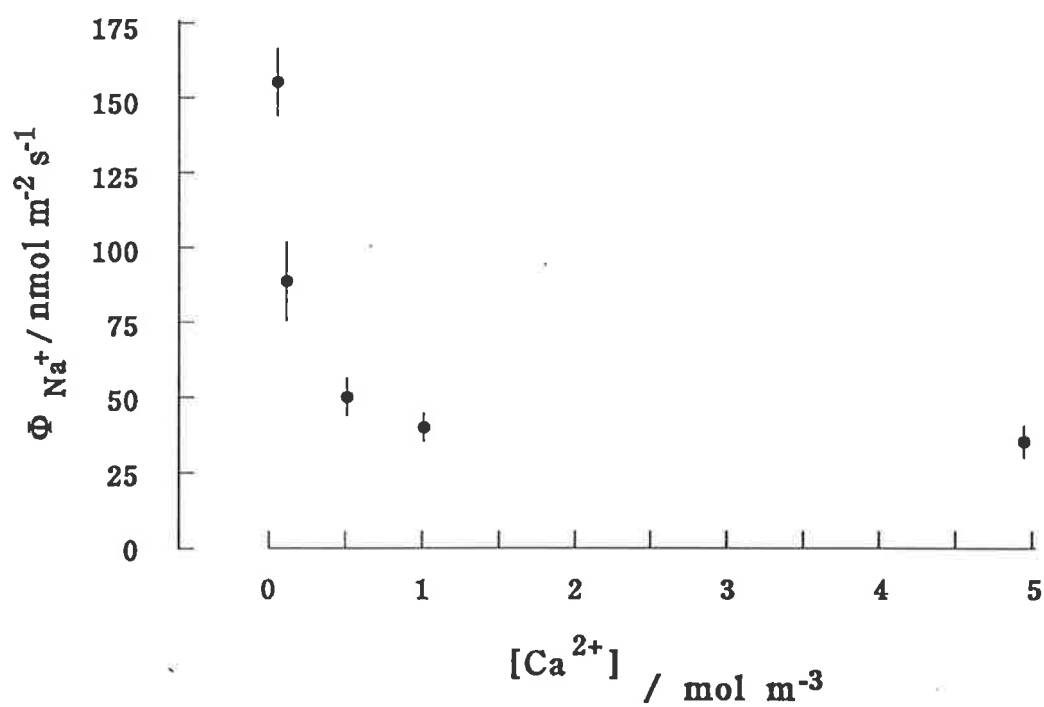


FIGURE 8.6 Effect of Ca^{2+} concentration on Na^+ influx at $10 \text{ mol m}^{-3} Na^+$.

Points represent the mean and S.E. of 10 cells.

Culture: LES

Solution: CPW 1 mol m^{-3} TAPS + 10 mol m^{-3} NaCl + appropriate concentration of Ca^{2+} .

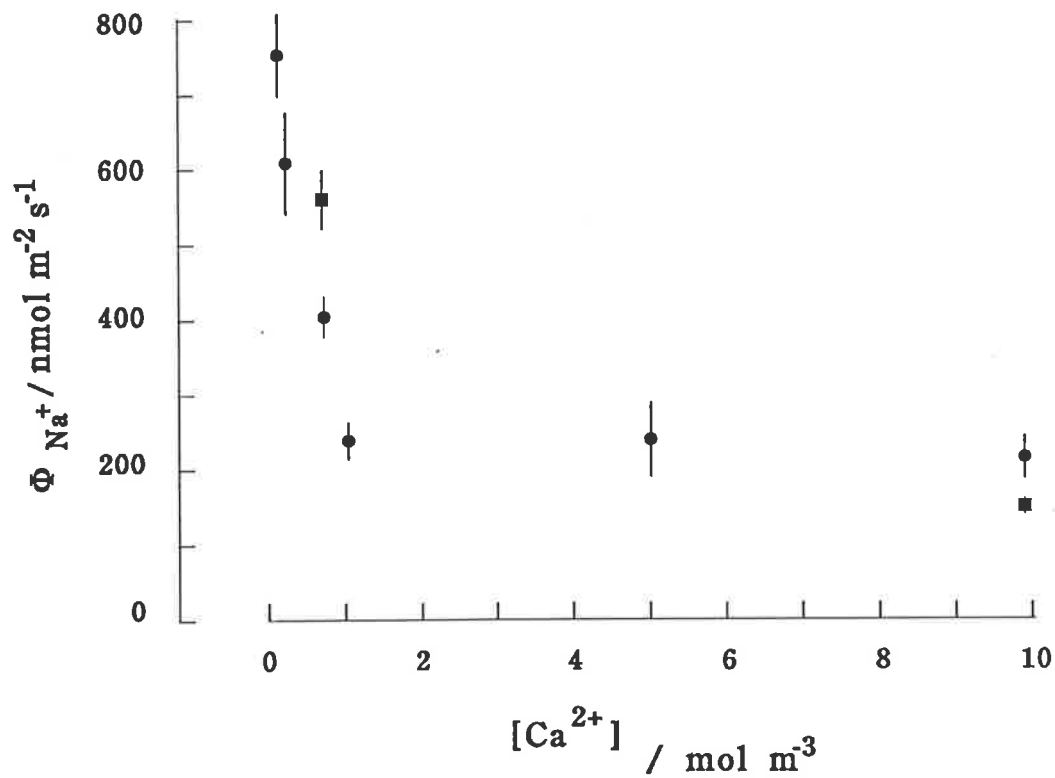


FIGURE 8.7 Effect of Ca^{2+} concentration on Na^+ influx at $100 \text{ mol m}^{-3} \text{ Na}^+$.

Results collected on two separate occasions, represented by the symbols ■ and ●. Points represent the mean and S.E. of 10 cells.

Culture: LES

Solution: CPW 1 mol m^{-3} TAPS + 100 mol m^{-3} NaCl + appropriate concentration of Ca^{2+} .

mol m^{-3} . Therefore, inhibition of mechanism 2 occurs below the minimum Ca^{2+} concentration required for survival of *C. corallina* in brackish water (see chapter 3).

8.2.4 The effect of external Na^+ on Na^+ influx.

In the previous experiment evidence was presented that suggested there were two mechanisms of Na^+ influx. These mechanisms were distinguished by their sensitivity to external Ca^{2+} concentration. In this series of experiments resolving that portion of Na^+ influx attributable to mechanism 1 from mechanism 2 was attempted. Initially Na^+ influx in the presence of $5 \text{ mol m}^{-3} \text{ CaSO}_4$ was measured. $5 \text{ mol m}^{-3} \text{ Ca}^{2+}$ should have inhibited Na^+ influx via mechanism 2. Na^+ influx was then be measured in the absence of Ca^{2+} where influx via mechanism 2 would be at a maximum. Subtracting Na^+ influx in the absence of Ca^{2+} from influx in the presence of $5 \text{ mol m}^{-3} \text{ Ca}^{2+}$ provides details of Na^+ influx via mechanism 2.

Na^+ influx in both the presence and absence of Ca^{2+} was proportional to external Na^+ concentration (figures 8.8, 8.9). Both in the presence and absence of Ca^{2+} , Na^+ influx plotted against external Na^+ concentration formed nonsaturating curves that approached linearity between 5 and $10 \text{ mol m}^{-3} \text{ Na}^+$.

The result obtained by subtracting the data generated in the presence of Ca^{2+} from the data generated in the absence of Ca^{2+} is presented in Figure 8.10. This relationship describes Na^+ influx via mechanism 2, the Ca^{2+} -inhibited Na^+ uptake mechanism. However, an assumption that Na^+ uptake via mechanism 1 is not affected by the rate of Na^+ uptake via mechanism 2 is made. This may not be valid since uptake via mechanism 2 is rapid and could quickly cause increases in cytoplasmic Na^+ concentration. Increased cytoplasmic Na^+ may feedback on mechanism 1 Na^+ transport and may also affect other cellular

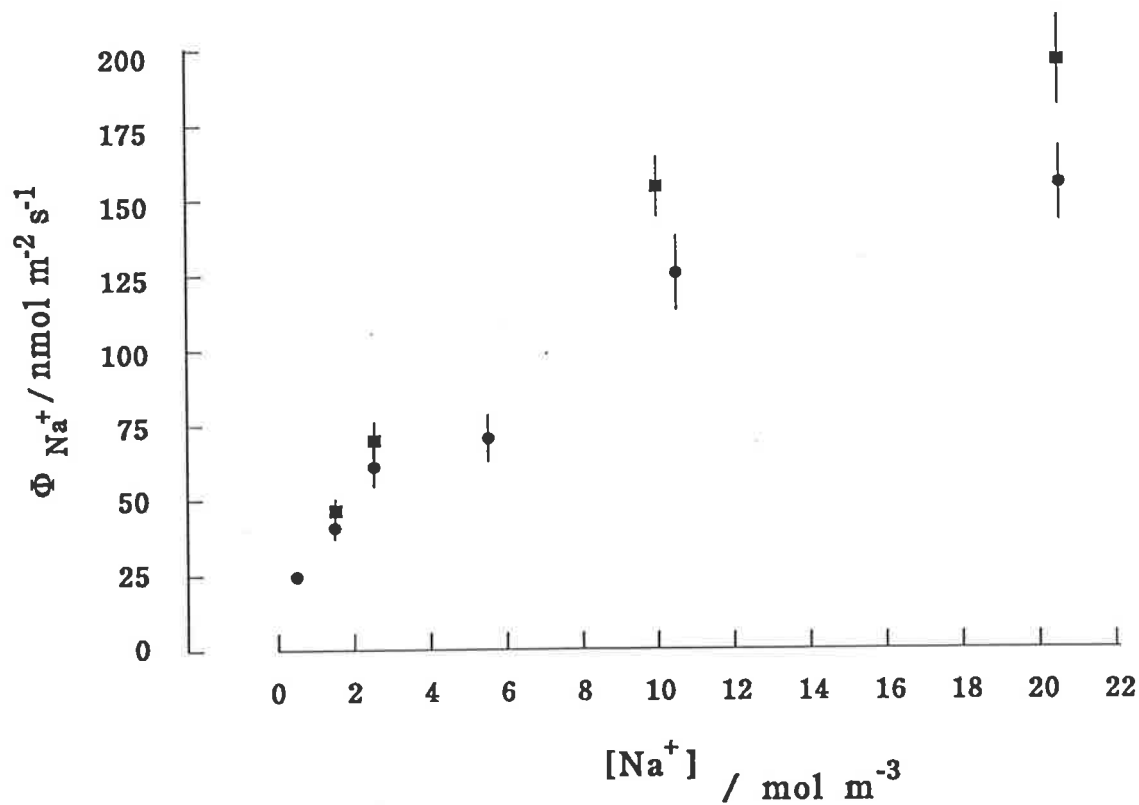


FIGURE 8.8 Effect of Na⁺ concentration on Na⁺ influx in Ca²⁺-free solutions.

Results collected on two separate occasions, represented by the symbols ■ and ●. Each point represents the mean and S.E. of 10 cells.

Culture: LES

Solution: 1 mol m⁻³ TAPS pH 8 + the appropriate concentration of NaCl.

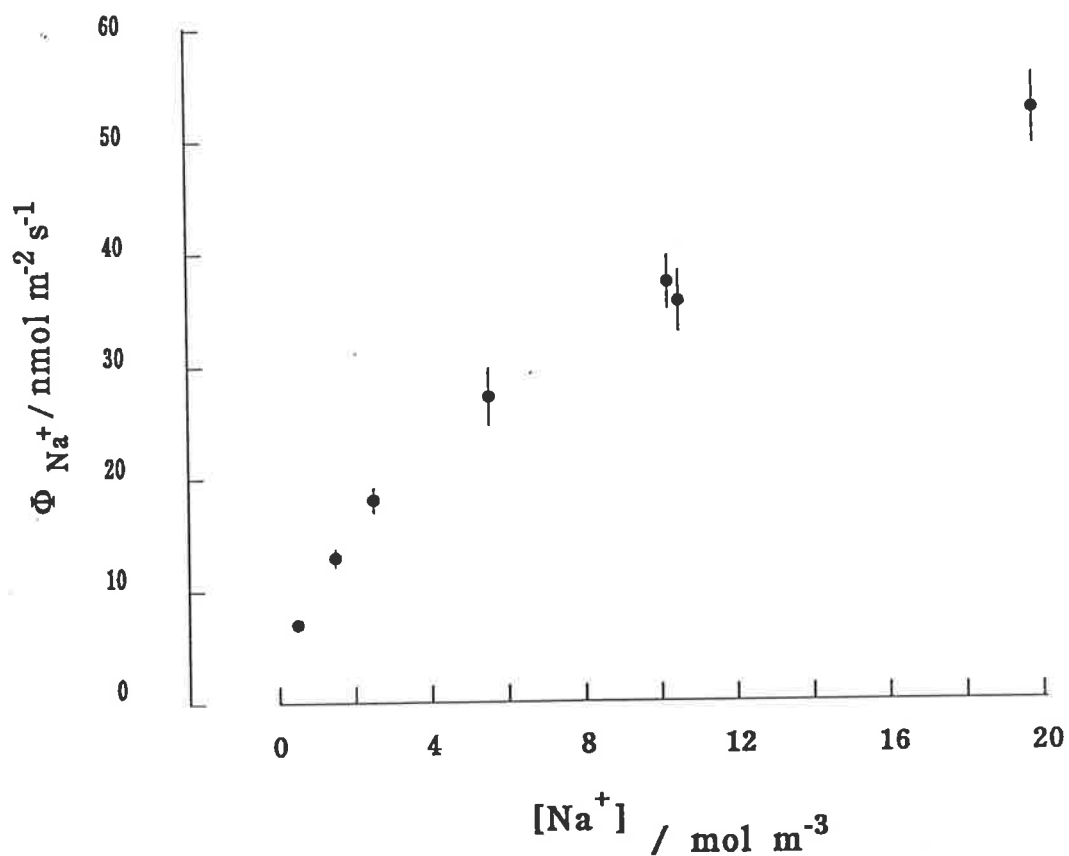


FIGURE 8.9 Effect of Na^+ concentration on Na^+ influx from solutions containing $5 \text{ mol m}^{-3} \text{ CaSO}_4$ solutions.

Each point represents the mean and S.E. of 10 cells.

Culture: LES

Solution: $5 \text{ mol m}^{-3} \text{ CaSO}_4$, $1 \text{ mol m}^{-3} \text{ TAPS}$ pH 8 + the appropriate concentration of NaCl.

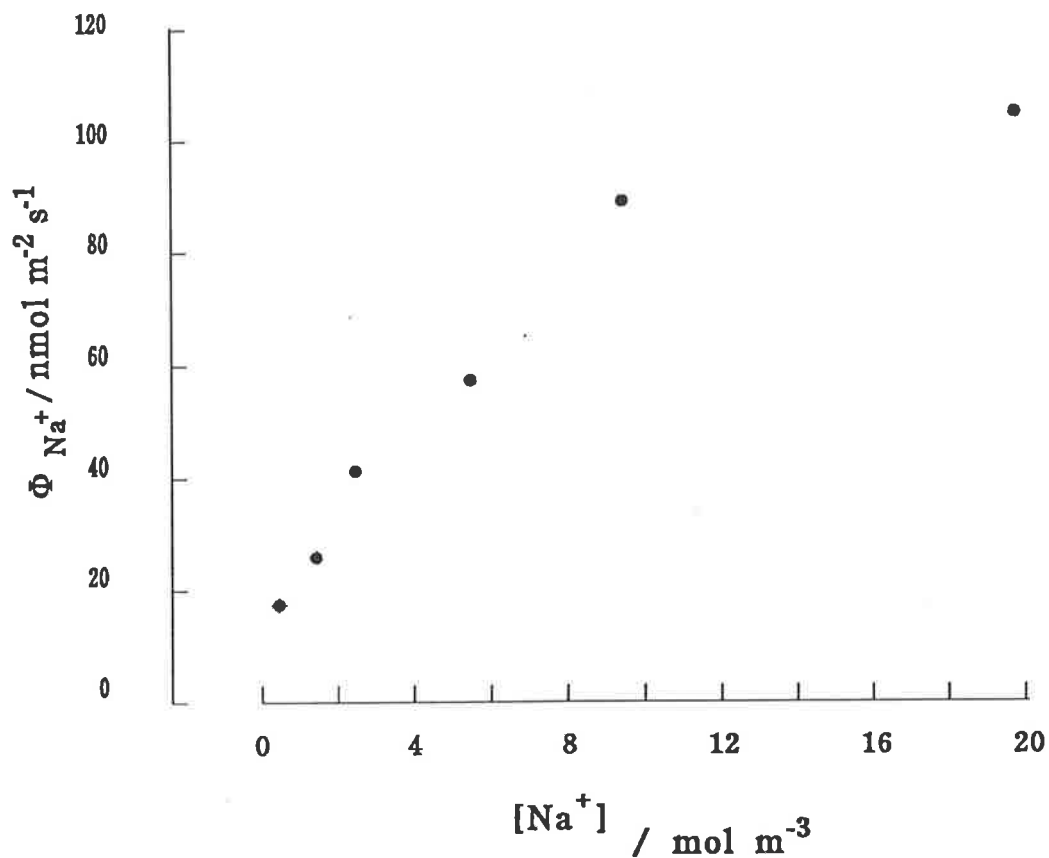


FIGURE 8.10 Effect of Na⁺ concentration on Na⁺ influx via the Ca²⁺ insensitive Na⁺ influx mechanism.

The data presented in this figure were generated by subtracting Na⁺ influx in the presence of 5 mol m⁻³ Ca²⁺ from Na⁺ influx in the absence of Ca²⁺.

Culture: LES

Solution: ± 5 mol m⁻³ CaSO₄, 1 mol m⁻³ TAPS pH 8 + the appropriate concentration of NaCl.

processes for example, Clint and MacRobbie (1987) have shown that Na^+ efflux from perfused internodal cells of *C. corallina* is increased by increasing internal Na^+ concentration.

8.2.5 Influx of K^+ .

In section 7.2.2 it was suggested that Na^+ influx into *C. corallina* had features in common with K^+ influx. In this section the results of experiments describing K^+ influx into *C. corallina* will be compared to measurements of Na^+ influx described in previous sections of this chapter.

The transport of K^+ ions into *C. corallina* is generally passive and independent of any other ions during passage across the plasma membrane (Smith JR 1987, Smith JR et al. 1987a,b, Smith and Kerr 1987). Measurement of K^+ fluxes by Smith and Kerr (1987) led to the proposal of two modes of K^+ transport (two populations of K^+ channels?) across the plasma membrane. It was suggested that only one of these modes of transport was inhibitable by cations such as Ca^{2+} and TEA^+ . Tester (1988b) reported that only part of the inward K^+ current in voltage clamped *C. corallina* cells could be inhibited by addition of K^+ channel blockers such as Ba^{2+} , Cs^+ and TEA^+ . Application of these inhibitors of K^+ transport reduced K^+ flux to what was termed a "leak current" (Tester 1988b). Beilby (1986) also reported that elevation of external Ca^{2+} decreases K^+ conductance to a "leak" conductance.

Uptake of $^{42}\text{K}^+$ was measured as a function of time in 10 mol m^{-3} $^{42}\text{K}^+$. After approximately 40 minutes the rate of $^{42}\text{K}^+$ accumulation reached a steady state (Figure 8.11). The remainder of experiments describing K^+ influx were conducted over 120 minutes for the same reasons that Na^+ influx was measured over 120 minutes. As with Na^+ uptake experiments there was probably a large efflux of K^+ occurring during the uptake period. The effect this has on the estimation of K^+

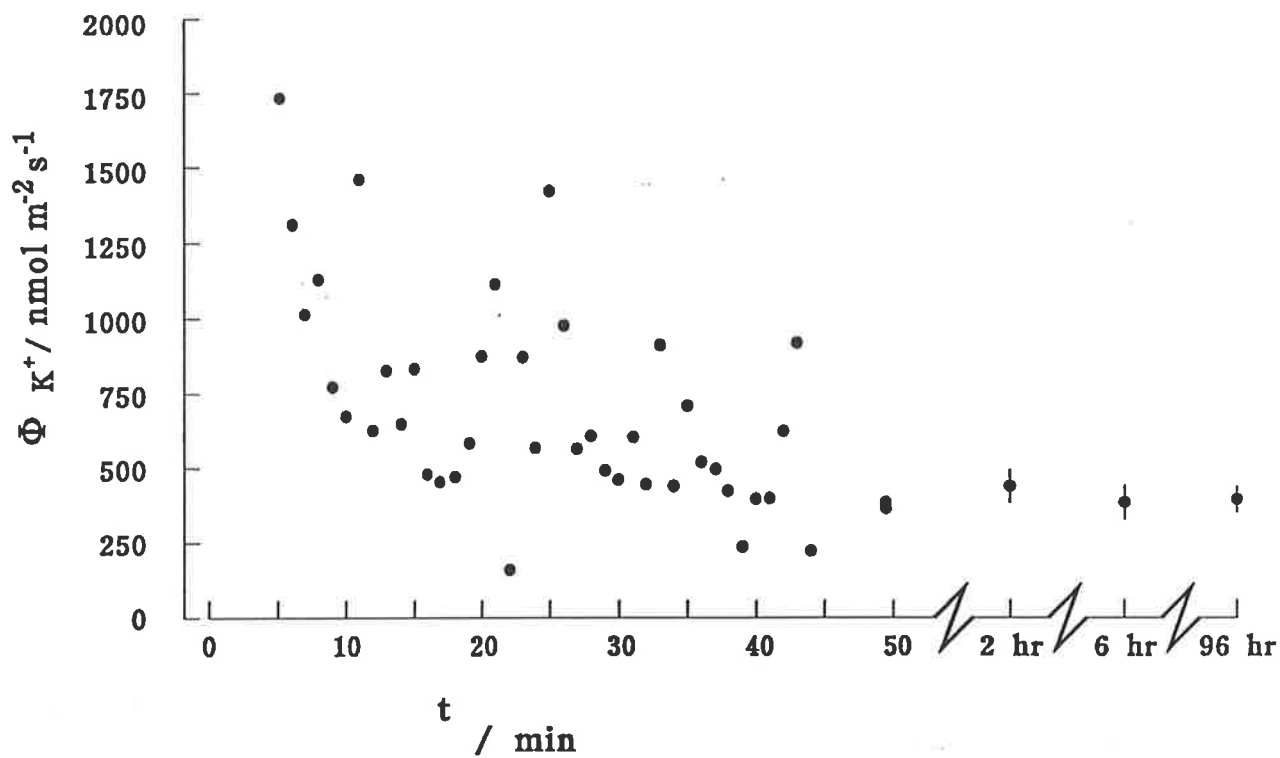


FIGURE 8.11 Time course of K^+ influx from solutions containing 5 mol m^{-3} K_2SO_4 .

Data up to 50 minutes represent values from single cells. Data for longer periods represents the mean and S.E. of 10 cells.

Culture: LES

Solution: 5 mol m^{-3} K_2SO_4 , 0.5 mol m^{-3} $CaSO_4$, 1 mol m^{-3} TAPS pH 8.

influx will be discussed in section 8.3.1.

Figure 8.12 shows K^+ influx plotted as a function of external K^+ concentration, with and without external Cl^- . Whilst this represents the results of only two experiments and so must be viewed with caution, K^+ influx appears to have two components. K^+ influx saturates at low concentrations of external K^+ but at higher concentrations influx increases rapidly. The limited data available make it difficult to speculate on whether K^+ influx saturates at high external K^+ concentrations.

The results of two experiments in which K^+ influx was measured as a function of external Ca^{2+} concentration are presented in Figure 8.13. The external K^+ concentration was 10 mol m^{-3} . These experiments show that K^+ influx was inversely proportional to Ca^{2+} concentration. This result supports the findings of Smith and Kerr (1987) who also showed $^{42}K^+$ influx to be partially inhibited by external Ca^{2+} .

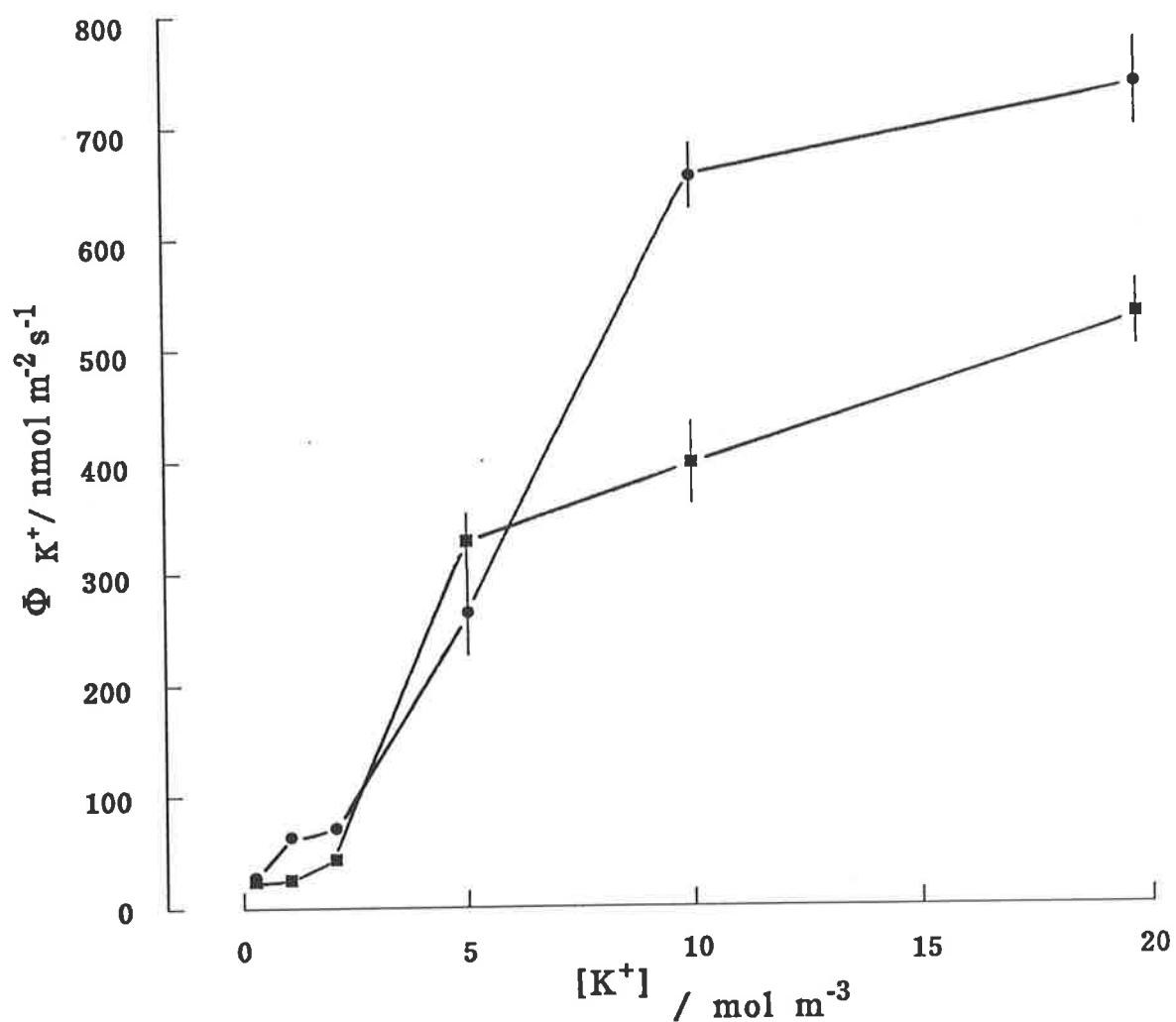


FIGURE 8.12 Effect of K^+ concentration on K^+ influx from solutions with and without Cl^- .

K^+ added as KCl ■ and K_2SO_4 ●. Each point represents the mean and S.E. of 10 cells.

Culture: LES

Solution: $0.5 \text{ mol m}^{-3} \text{ CaSO}_4$ $1 \text{ mol m}^{-3} \text{ NaCl}$ EPPS pH 8 + the appropriate concentration of either KCl or K_2SO_4 .

8.2.6 Comparison of Na⁺ influx with K⁺ influx.

When the influx of K⁺ is compared to the influx of Na⁺ a number of similarities become apparent. Both the influx of Na⁺ and K⁺ in the presence of 0.5 mol m⁻³ Ca²⁺ produced similar relationships when influx was measured against concentration of each ion (Figures 8.5, 8.6, 8.7, 8.13). Also, the influx of Na⁺ and K⁺ are affected by the concentration of Ca²⁺ in the external solution. Influx of both ions is greatest in Ca²⁺-free solutions and influx is inhibited by external Ca²⁺. In both cases Ca²⁺ inhibits influx by approximately 70-85% depending upon the external K⁺ or Na⁺ concentration.

I have suggested that there are two mechanisms for Na⁺ transport, one which is sensitive to external Ca²⁺ and one which is insensitive to external Ca²⁺ concentration. The results describing K⁺ influx presented in this chapter and those presented by Beilby (1986), Smith and Kerr (1987) and Tester (1989) suggest similar Ca²⁺ sensitive and insensitive mechanisms for K⁺ transport into *C. corallina*. The differences between K⁺ and Na⁺ transport relate to their relative rates of transport under the same conditions. Influx of K⁺ is generally faster than influx of Na⁺ under similar sets of conditions.

Because of the similarities between influx of Na⁺ and K⁺ a number of experiments were performed to see if the uptake of Na⁺ is via the same mechanism(s) as K⁺. The results of these experiments are presented in the following sections.

8.2.7 Effect of TEA⁺, Ba²⁺, Mg²⁺ and amiloride on Na⁺ influx.

TEA⁺ and Ba²⁺ have been shown to block K⁺ channels at the plasma membrane of *Chara* (Sokolik and Yurin 1986, Tester 1988b). The inhibition of inward current by Ba²⁺ was strongly voltage dependent, whereas inhibition of inward current by TEA⁺ was voltage independent. Both Ba²⁺ and TEA⁺ reduce inward current into *C. corallina* to a similar

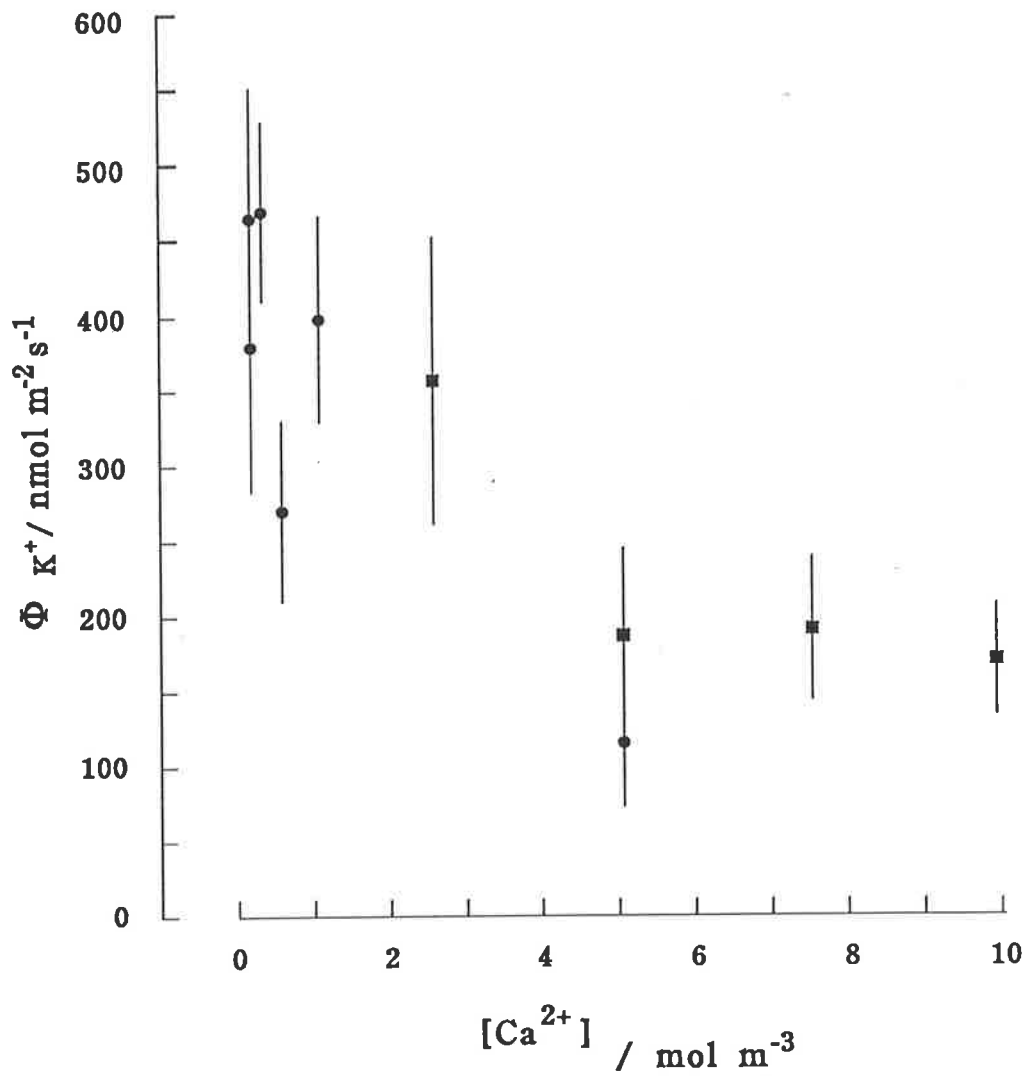


FIGURE 8.13 Effect of Ca^{2+} concentration on K^+ influx at $10 \text{ mol m}^{-3} \text{ K}^+$.

Results collected on two separate occasions, represented by the symbols ● and ■. Points represent the mean and S.E. of 10 cells.

Culture: LES

Solution: $10 \text{ mol m}^{-3} \text{ KCl}$ + EPPS pH 8 + appropriate concentration of CaSO_4 .

background "leak" (Tester 1988b, Smith and Kerr 1987). As far as I am aware there are no reports of the affect of either Ba^{2+} or TEA^+ on the influx of Na^+ in plant tissue.

The diuretic drug amiloride is a specific inhibitor of Na^+/H^+ antiport and of Na^+ -channel activity in some animal systems (Benos 1982). Clint and MacRobbie (1987) have shown amiloride to inhibit H^+ -coupled Na^+ efflux from perfused *Chara* cells. Smith and Walker (1989) report that inward current via K^+-Na^+ symport into *Chara* to be partially inhibited by external amiloride of $75 \mu\text{mol m}^{-3}$.

Tufariello et al. (1988) have shown Mg^{2+} to be a poor substitute for Ca^{2+} in protecting *C. corallina* from salt injury. Hoffmann et al. (1989) report that Mg^{2+} did not reduce Na^+ influx or Na^+ permeability at elevated Na^+ , however external Mg^{2+} could substitute for external Ca^{2+} in the brackish water charophyte, *C. buckellii*.

Na^+ influx was measured in the presence and absence of TEA^+ , Ba^{2+} and amiloride at concentrations that have previously been shown to inhibit either K^+ or Na^+ influx. The experiments were undertaken in both Ca^{2+} -free solutions and with $5 \text{ mol m}^{-3} \text{ Ca}^{2+}$. This was done so that any inhibition of Na^+ influx could be attributed to either mechanism 1 (Ca^{2+} insensitive) or mechanism 2 (Ca^{2+} sensitive) Na^+ influx.

Figures 8.14 and 8.15 show Na^+ influx in the absence of Ca^{2+} to be inhibited by Ba^{2+} and Mg^{2+} . However, Ba^{2+} and Mg^{2+} do not inhibit Na^+ influx in the presence of $5 \text{ mol m}^{-3} \text{ Ca}^{2+}$. Addition of $5 \text{ mol m}^{-3} \text{ TEA}^+$ resulted in a slight decrease in Na^+ influx (from 45 ± 5 to $35 \pm 5 \text{ nmol m}^{-2} \text{ s}^{-1}$) in the absence of external Ca^{2+} but had no effect in the presence of external Ca^{2+} . $75 \mu\text{mol m}^{-3}$ amiloride had no affect upon Na^+ influx in either 0 or $5 \text{ mol m}^{-3} \text{ Ca}^{2+}$.

These results provide further evidence for the existence of two mechanisms of Na^+ influx. Ca^{2+} insensitive Na^+ influx was not

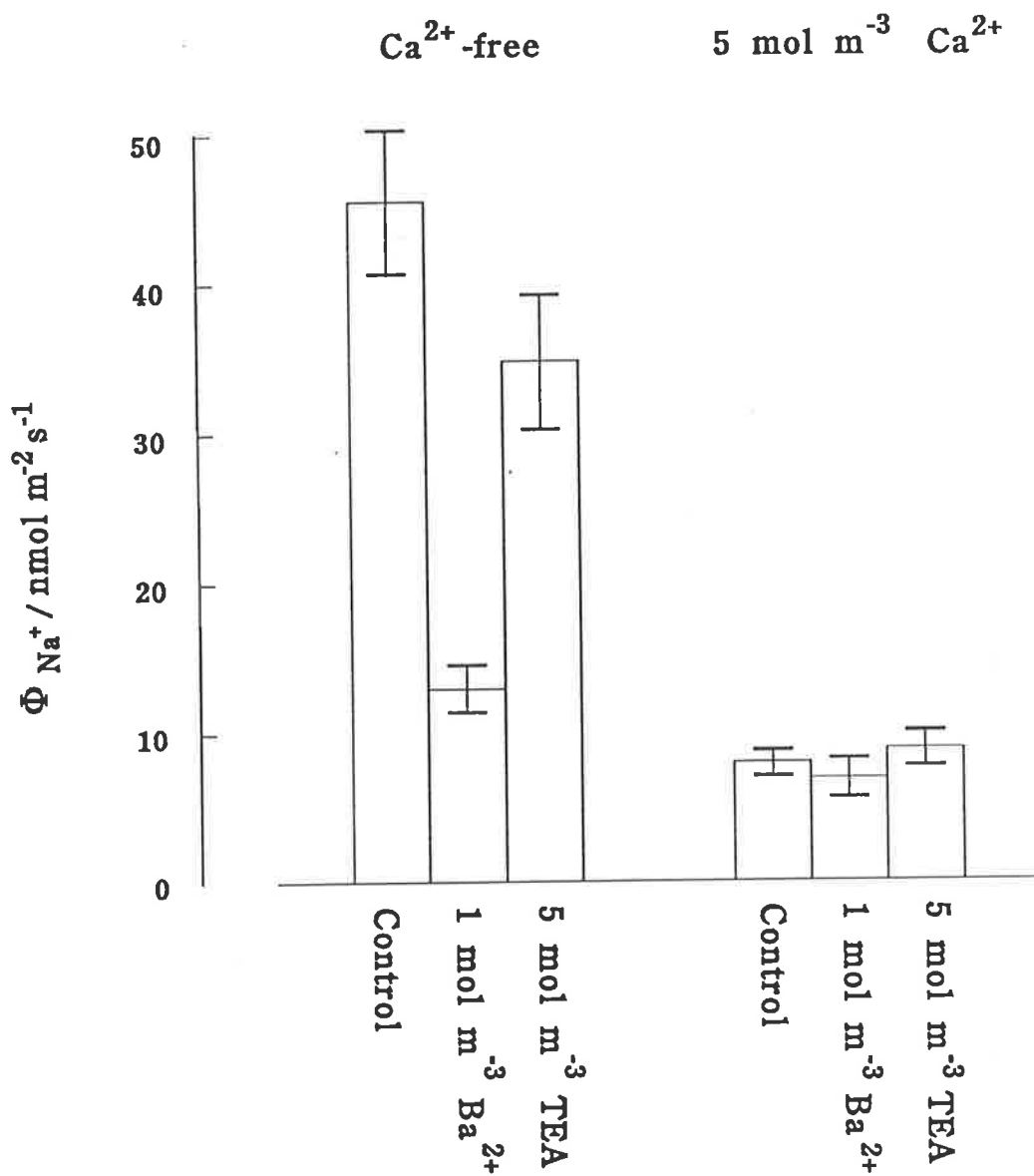


Figure 8.14 Effect of TEA and Ba^{2+} on Na^+ influx in the presence and absence of Ca^{2+} .

Values represent mean \pm S.E. for 10 cells.

Culture: LES

Solution: $1 \text{ mol m}^{-3} \text{ NaCl}$, $0.1 \text{ mol m}^{-3} \text{ K}_2\text{SO}_4$, TAPS pH 8 (actual $[\text{Na}^+] = 1.36 \text{ mol m}^{-3}$) = control + either $1 \text{ mol m}^{-3} \text{ Ba}^{2+}$ or $5 \text{ mol m}^{-3} \text{ TEA}$.

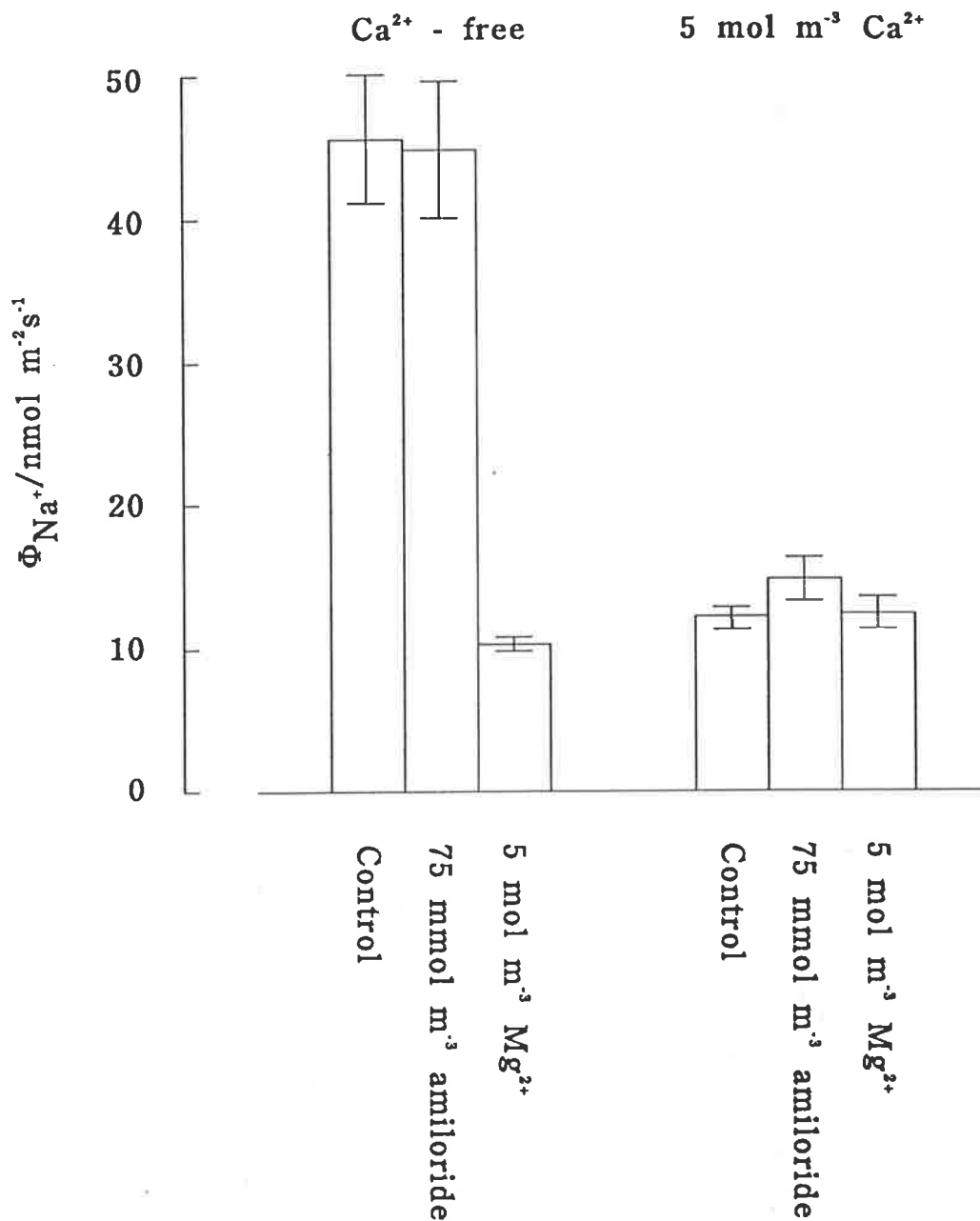


Figure 8.15 Effect of amiloride and Mg^{2+} on Na^+ influx in the presence and absence of Ca^{2+} .

Values represent mean \pm S.E. for 10 cells.

Culture: LES

Solution: 1 mol m⁻³ NaCl, 0.1 mol m⁻³ K₂SO₄, TAPS pH 8 (actual [Na⁺] = 1.36 mol m⁻³) = control + either 5 mol m⁻³ Ba²⁺ or 75 mmol m⁻³ amiloride.

inhibited by the divalent cations Ba^{2+} or Mg^{2+} . However, the component of Na^+ influx that was sensitive to external Ca^{2+} concentration was also inhibited by Mg^{2+} , Ba^{2+} and to a lesser extent TEA^+ . These results suggest that Ca^{2+} insensitive Na^+ influx has characteristics in common with the " K^+ -leak" current described from voltage clamped *Chara* cells (Beilby 1986, Tester 1988ab).

The results of Smith and Kerr (1987) show that a TEA^+ concentration of 5 mol m^{-3} inhibited K^+ influx by approximately two thirds. These experiments were performed in the presence of $0.1 \text{ mol m}^{-3} \text{ Ca}^{2+}$, thus making channels more receptive to closure than in Ca^{2+} -free solutions. Significantly more TEA^+ may be required for channel closure in the absence of Ca^{2+} . It would be interesting to measure $^{42}\text{K}^+$ influx in Ca^{2+} free solutions with TEA^+ to see whether inhibition of K^+ influx is similar under similar conditions.

The fact that amiloride did not inhibit Na^+ influx is surprising in view of its inhibition of Na^+/K^+ symport in *Chara* (Smith and Walker 1989) and Na^+ channel conductance and Na^+/H^+ antiport in animal cells (Benos 1982). This suggests Na^+ was entering the cell via an amiloride insensitive mechanism(s) independent unlike those described above.

8.2.8 The effect of Ca^{2+} on the influx of DMO, methylammonium and imidazole.

An experiment was undertaken to see whether the increase in Na^+ and K^+ influx observed upon the removal of external Ca^{2+} was specific to those ions or whether there was there a general breakdown of membrane integrity when Ca^{2+} was removed from the external solution. To this end influx of DMO, methylammonium and imidazole was measured in the presence and absence of Ca^{2+} .

At $\text{pH } 8$ the weak acid, DMO is a univalent anion ($\text{pK}_a = 6.38$ (Smith 1980)), methylamine is a univalent cation ($\text{pK}_a = 10.65$ (Smith and Walker

1978)) and imidazole is neutral ($pK_a = 6.96$ (Smith and Whittington 1988)). Under these conditions it would be expected that the influx of DMO would be by passive diffusion as would influx of imidazole (Smith and Whittington 1988). Influx of methylamine is expected to be facilitated by a selective porter (Smith and Walker 1978).

The results presented in Table 8.1 show that removal of Ca^{2+} results in an increase in the influx of methylammonium and imidazole and a slight decrease in the influx in DMO. However, the changes in influx are quite small when compared to the increases in Na^+ and K^+ influx when Ca^{2+} is removed from the external solution (eg. fig 8.5 and 8.13). This suggests that the increase in influx of K^+ and Na^+ upon removal of Ca^{2+} is not just the result of a general deterioration of the plasma membrane integrity, but that there is a more specific effect on the influx of (at least) Na^+ and K^+ .

8.2.9 Effect of external K^+ on Na^+ influx.

The experiments described in this chapter show the transport of Na^+ and K^+ into *Chara* to have many similarities. One explanation for this is that Na^+ and K^+ cross the plasma membrane via common transport system(s). If this hypothesis is correct then K^+ and Na^+ will compete for transport across the plasma membrane. Na^+ influx was measured in Ca^{2+} -free solutions in the presence of varying concentrations of K^+ .

Increasing the concentration of K^+ had only a small effect upon the influx of Na^+ (Figure 8.16). The decrease in influx beyond external Na^+ concentration of 0.2 mol m^{-3} was possibly due to a reduction of membrane potential difference decreasing the electrochemical potential difference for Na^+ . It would be predicted that in the absence of Ca^{2+} , membrane potential difference would depolarize as the concentration of K^+ was increased (Bisson 1984).

TABLE 8.1 Influx of DMO, imidazole and methylamine from CPW and Ca^{2+} -free solutions.

	Influx ($\text{nmol m}^{-2} \text{ s}^{-1}$)		
	$0.5 \text{ mol m}^{-3} \text{ Ca}^{2+}$	Ca^{2+} -free	% increase
0.2 mol.m^{-3} DMO	8.95 ± 0.18	7.04 ± 0.22	-21%
0.2 mol.m^{-3} Imidazole	52 ± 3	63 ± 2	+21%
0.2 mol.m^{-3} Methylamine	244 ± 15	173 ± 8	+41%

Uptake measured for 120 minutes. Each value is the mean \pm S.E. of 10 cells.

Culture: LES

Solution: as above \pm CaSO_4 CPW EPPS pH 8

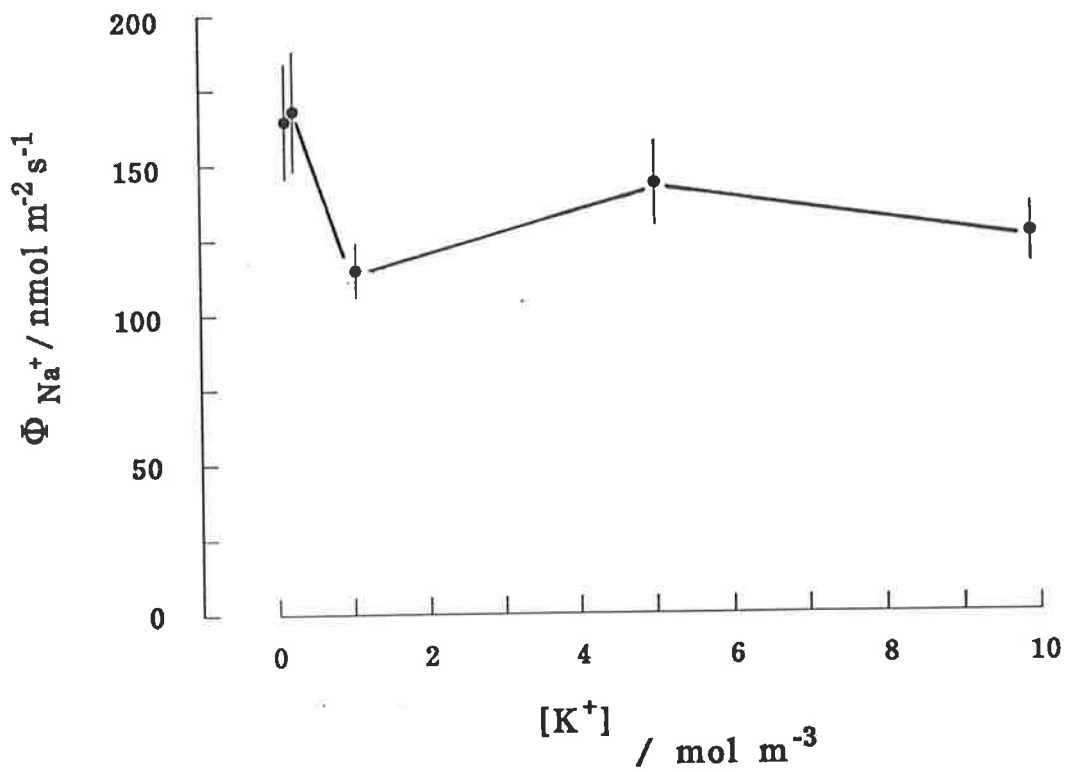


FIGURE 8.16 Effect of K⁺ concentration on Na⁺ influx in the absence of Ca²⁺.

Values represent mean ± S.E. for 10 cells.

Culture: LES

Solution: 10 mol m⁻³ NaCl, 1 mol m⁻³ TAPS pH 8 + the appropriate concentration of KCl.

Interestingly Na^+ influx did not decrease when the external K^+ concentration varied between 0.5 and 10 mol m^{-3} and yet K^+ influx increased from about 30 to 650 $\text{nmol m}^{-2} \text{s}^{-1}$ (Figure 8.12). The fact that Na^+ influx was not inhibited by such an increase in K^+ influx indicates that either Na^+ and K^+ do not compete for the same transport mechanism across the plasma membrane or that the transport mechanism for these ions is not saturated by an influx rate of approximately 800 $\text{nmol m}^{-2} \text{s}^{-1}$.

In this experiment I have made the assumption that the proposed transport system for these ions has a greater selectivity for K^+ than Na^+ . This was based on the assumption that Na^+ "leaked" through K^+ channels. Azimov *et al.* (1987) and Tester (1988b) have shown the permeability of K^+ to be greater than the permeability of Na^+ through this channel type. If however, this assumption is invalid then increasing K^+ concentration, as in the previous experiment, may not significantly decrease Na^+ influx. To overcome this problem, influx of K^+ at various concentrations of Na^+ should be attempted.

8.2.10 Effect of Na^+ and K^+ on the influx Ca^{2+} .

Ca^{2+} influx at the plasma membrane is thought to be via gated channels which can be modulated by membrane potential difference (MacRobbie and Banfield 1988). In this section the possibility that the Ca^{2+} sensitive influx of K^+ and Na^+ is due to "leakage" of these ions through Ca^{2+} channels is investigated. If K^+ and/or Na^+ were competing with Ca^{2+} for transport across the plasma membrane then increasing the concentration of external K^+ and/or Na^+ may result in a decrease in the rate of Ca^{2+} uptake as K^+ and Na^+ compete for channel occupancy. To this end, Ca^{2+} influx from a solution containing 0.1 mol m^{-3} Ca^{2+} in the presence of 0.2 and 20 mol m^{-3} K^+ and Na^+ was measured.

The results presented in Table 8.2 show that high concentrations of monovalent cation slightly increase Ca^{2+} influx. This experiment is a partial repeat of an experiment described by MacRobbie and Banfield (1988). They showed that bathing the cells in $20 \text{ mol m}^{-3} \text{ K}^+$ resulted in a stimulation of Ca^{2+} influx. This was attributed to the high concentration of external K^+ depolarizing the plasma membrane and thus causing Ca^{2+} channels to open. The results presented in this chapter agree with this model. It is interesting to note that $20 \text{ mol m}^{-3} \text{ Na}^+$ also results in a stimulation of Ca^{2+} influx. In a solution containing $0.2 \text{ mol m}^{-3} \text{ Ca}^{2+}$ increasing external Na^+ from 0.3 to 20 mol m^{-3} would be expected to depolarize membrane potential difference.

The above hypothesis assumes that increased flux of K^+ or Na^+ through Ca^{2+} channels will inhibit Ca^{2+} influx through these channels. This may not be a valid assumption if the selectivity of the channel strongly favours Ca^{2+} over K^+ or Na^+ .

The measured fluxes of Ca^{2+} shown in Table 8.2 are much less than the expected fluxes of K^+ and Na^+ under similar conditions (see for example Figures 8.4 and 8.12). It would seem unlikely that Ca^{2+} channels would pass Ca^{2+} at a rate not exceeding $25 \text{ nmol m}^{-2} \text{ s}^{-1}$ and yet pass K^+ at rates approaching $700 \text{ nmol m}^{-2} \text{ s}^{-1}$.

Nifedipine has been shown to inhibit Ca^{2+} influx into *Chara corallina* (MacRobbie and Banfield 1988). Nifedipine is thought to inhibit Ca^{2+} influx by blocking nifedipine sensitive Ca^{2+} channels. By measuring Na^+ influx in the presence of nifedipine an estimation of Na^+ crossing the plasma membrane through this channel type could be made. This was not attempted but could prove interesting.

8.2.11 Effect of external Cl^- on the influx of Na^+ , K^+ and Cl^- .

Smith (1967) has shown that the light promoted influx of Na^+ in *Nitella translucens* was inhibited in the absence of Cl^- . This result

TABLE 8.2 Effect of K^+ and Na^+ on Ca^{2+} influx.

	Ca^{2+} Influx ($nmol\ m^{-2}\ s^{-1}$)
0.3 mol m^{-3} NaCl	16.4 \pm 1.7
20 mol m^{-3} NaCl	21.7 \pm 2.7
0.1 mol m^{-3} K_2SO_4	17.1 \pm 1.5
10 mol m^{-3} K_2SO_4	23.3 \pm 1.1

Each value represents the mean and S.E. of 10 cells.

Culture: LES

Solution: 0.1 mol m^{-3} $CaSO_4$, 1 mol m^{-3} TAPS pH 8 + 1 mol m^{-3} NaCl or
0.1 mol m^{-3} K_2SO_4 unless otherwise specified.

led Smith to suggest that Na^+ influx in the light is partly linked to the inward Cl^- pump. This could be achieved either by the effects of Cl^- on the electrochemical driving forces controlling Na^+ entry or alternatively, chemical coupling between the two influxes could be involved. A series of experiments were undertaken to see if Na^+ influx is dependent upon external Cl^- and to see if Na^+ influx is coupled to Cl^- influx.

The rates of Na^+ influx from Ca^{2+} -free solutions containing 1.3 and 10 mol m^{-3} NaCl presented in Table 8.3 are similar to results presented previously (Figure 8.8). The removal of external Cl^- resulted in an increase in the rate of Na^+ uptake from 152 ± 14 to 341 ± 34 $\text{nmol m}^{-2} \text{ s}^{-1}$. Under identical conditions the influx of Cl^- was much lower than the influx of Na^+ . The large imbalance between Na^+ influx and Cl^- influx has to be balanced by the movement of another ion(s). It is likely that the imbalance between Na^+ and Cl^- influx is maintained by the efflux of K^+ . Katsuhara and Tazawa (1988) report that during treatment of *Nitellopsis obtusa* with high concentrations of Na^+ in the absence of Ca^{2+} that cytoplasmic K^+ concentration decreases rapidly. Similarly it was shown in chapter 4 that K^+ concentration in *C. corallina* decreased during treatment with high external Na^+ .

Influx of K^+ from solutions containing 10 mol m^{-3} KCl was greater than from solutions containing 5 mol m^{-3} K_2SO_4 (Figure 8.12) indicating that external Cl^- was required for the maximum uptake of K^+ . The role of Cl^- in this process may be directly linked to K^+ uptake as Smith (1967) suggested was the case with Na^+ uptake. However, this proposal does not fit views currently held concerning K^+ uptake (see chapter 1).

These results show that the influx of Na^+ into *C. corallina* is not inhibited by low external Cl^- concentration whereas K^+ influx is. This is in direct contrast to the results presented for *Nitella translucens* presented by Smith (1967). It should also be pointed out that the

TABLE 8.3 A comparison of Na^+ and Cl^- influx in Ca^{2+} -free solutions.

Treatment	influx ($\text{nmol m}^{-2} \text{ s}^{-1}$)	
	$\Phi \text{ Na}^+$	$\Phi \text{ Cl}^-$
$1 \text{ mol m}^{-3} \text{ NaCl}$	52 ± 5.4	19.3 ± 2.0
$10 \text{ mol m}^{-3} \text{ NaCl}$	152 ± 14	41 ± 5.0
$1 \text{ mol m}^{-3} \text{ NaCl} + 4.5 \text{ mol m}^{-3} \text{ Na}_2\text{SO}_4$	287 ± 35	28.9 ± 3.8
$5 \text{ mol m}^{-3} \text{ Na}_2\text{SO}_4$	341 ± 34	-

Uptake measured for 120 minutes. Each value is the mean \pm S.E. of 10 cells.

Culture: LES

Solution: as above + 1 mol m^{-3} TAPS (0.3 mol m^{-3} NaOH) pH 8

rates of uptake of Na^+ into *Nitella translucens* reported by Smith (1967) are lower than the rates measured for Na^+ uptake into *C. corallina*. For example Na^+ influx in Ca^{2+} -free solution with 5.5 mol m^{-3} NaCl was approximately $30 \text{ nmol m}^{-2} \text{ s}^{-1}$ in *N. translucens* (Smith 1967) whereas under similar conditions (however, the solutions were unbuffered) Na^+ influx for *C. corallina* in this study was $80 \text{ nmol m}^{-2} \text{ s}^{-1}$. Smith (*pers comm*) investigated the effect of external Cl^- on the influx of Na^+ into *C. corallina*. It was found difficult to obtain reproducible results but sometimes absence of external Cl^- gave lower Na^+ influx than in the presence of external Cl^- .

8.3 DISCUSSION

8.3.1 A comment on the measurement of Na⁺ and K⁺ influx.

It is likely that a significant efflux of K⁺ and Na⁺ would occur during an uptake period of 120 minutes. If the rate of uptake was sufficient for the amount of radiolabel absorbed to become a significant proportion of the internal pool, then efflux of the radiolabel would cause an underestimate of influx. For example, an uptake rate of 200 nmol m⁻² s⁻¹ would result in an increase in cytoplasmic concentration of approximately 36 mol m⁻³ h⁻¹ (this calculation assumes that all Na⁺ entering the cell remains in the cytoplasm). The cytoplasmic concentration of Na⁺ in *C. corallina* is reported to be between 3 and 21 mol m⁻³ (Tazawa et al. 1974, Okihara and Kiyosawa 1988). Therefore, the concentration of absorbed tracer would, over a two hour period result in the cytoplasm having a considerable specific activity. The most likely explanation of why efflux of K⁺ and Na⁺ ions does not have an impact on the apparent tracer influx is that radio-labelled Na⁺ and K⁺ ions are readily distributed between the cytoplasm and the vacuole. This would effectively dilute radiolabelled ion concentration in the cytoplasm by 20 fold, compared to accumulation in the cytoplasm alone. The distribution of tracer between the cytoplasm and the vacuole could be tested by sampling the radioactivity in each compartment in the same way as is done for estimating pH_c using the distribution of weak acids (see Section 2.5.1). This was not attempted because of the wish to minimize handling of ²²Na⁺, ²⁴Na⁺ and ⁴²K⁺.

8.3.2 Na⁺ influx.

The rate of Na⁺ uptake into *C. corallina* varies with Na⁺ concentration. The values of Na⁺ influx reported in this study are higher than those previously reported for *C. corallina* (Hoffmann et al.

1989) and *Nitella translucens* (Smith 1967). Na^+ influx in CPW (1 mol m^{-3} Na^+) was approximately $15 \text{ nmol m}^{-2} \text{ s}^{-1}$. If this is a steady state influx, then Na^+ must be transported from the cell at approximately $15 \text{ nmol m}^{-2} \text{ s}^{-1}$ for the cellular concentration to remain constant. Clint and MacRobbie (1987) report a maximum rate of Na^+ efflux from *C. corallina* of about $3 \text{ nmol m}^{-2} \text{ s}^{-1}$. However, this rate was obtained from perfused cells and so may not reflect steady state efflux from an intact *Chara* cell.

The work reported in this chapter shows that the absorption of Na^+ is via two mechanisms and the operation of one of these mechanisms is sensitive to Ca^{2+} concentration. In Ca^{2+} free conditions the Ca^{2+} sensitive mechanism transports Na^+ at a much greater rate than the Ca^{2+} insensitive mechanism. Under "normal" physiological conditions (eg. in CPW or APW) at which most *Chara* experiments are conducted, most Na^+ enters the cell via the Ca^{2+} insensitive mechanism. However, if the ratio of Na^+ to Ca^{2+} is increased beyond a critical level (the size of which is dependant upon the Na^+ concentration) influx via the Ca^{2+} sensitive mechanism will dominate. These results are similar to those described from barley roots by Rains and Epstein (1967a,b). They concluded that Na^+ absorption by barley proceeds via two mechanisms, one of which is severely reduced in effectiveness by the presence of external Ca^{2+} ions. However the existance of two mechanisms of Na^+ transport in plant cells is not a universally held hypothesis. Cheeseman (1982) argues that Na^+ influx into corn roots is due to a passive "leak" (based upon Goldman-type models) that increases linearly with external Na^+ concentration.

In Chapter 3, survival of *C. corallina* was shown to be dependent upon both external Na^+ and Ca^{2+} concentration. The ratio of Na^+ to Ca^{2+} at which salt damage occurred (measured as a reduction in cytoplasmic streaming rate) was approximately 20:1. The results

presented in this chapter show Na^+ influx to be dependent upon both external Na^+ and Ca^{2+} concentrations. Na^+ influx via the Ca^{2+} sensitive mechanism occurred when the ratio of Na^+ to Ca^{2+} exceeded a ratio of between 10 to 1 to 100 to 1, depending upon the external Ca^{2+} concentration. I believe that salt damage occurs in *C. corallina* as a result of uncontrolled Na^+ influx occurring via the Ca^{2+} sensitive mechanism. This occurs when Na^+ influx to the cell exceeds the rate at which Na^+ can be transported from the cell. This may be a simplified model because it takes no account of the effect of external Ca^{2+} on either cytoplasmic Ca^{2+} concentration or Na^+ efflux. Nor does this model allow for deleterious effects of high concentrations of external anion.

It is interesting to note that Na^+ influx from a solution with an external Na^+ concentration of 100 mol m^{-3} and Ca^{2+} concentration of 10 mol m^{-3} was about $200 \text{ nmol m}^{-2} \text{ s}^{-1}$. Under these conditions the cell survived for many weeks without a decrease in cytoplasmic streaming rate. Taking into account the increase in vacuolar Na^+ concentration of $7.5 \text{ mol m}^{-3} \text{ d}^{-1}$ (this equates to an influx of approximately $17 \text{ nmol m}^{-2} \text{ s}^{-1}$) the steady state efflux of Na^+ must be approximately $180 \text{ nmol m}^{-2} \text{ s}^{-1}$. This calculation assumes that Na^+ influx measured over 2 hours remains constant for the six days. Hoffmann et al. (1989) showed Na^+ influx in *C. corallina* to remain constant for 6 days (in 70 mol m^{-3} NaCl and $7 \text{ mol m}^{-3} \text{ Ca}^{2+}$). However, the Na^+ influx reported by Hoffmann et al. (1989) was an order of magnitude smaller than influx described in this paper.

8.3.3 K^+ influx.

As with Na^+ , the rate of K^+ influx is dependent upon both the external K^+ and Ca^{2+} concentration. The K^+ influx reported in this paper is similar to that reported by Smith and Kerr (1987). The

results also show that the influx of K^+ can be substantially inhibited by Ca^{2+} thus supporting the reports of Bisson (1984), Sokolik and Yurin (1986), Beilby (1986) and Smith and Kerr (1987). All but Smith and Kerr (1987) inferred this result from measurements of membrane conductance. Based on the above reports, and other experiments, Smith and Kerr (1987) concluded that K^+ transport into charophytes must occur via channels. Smith and Kerr (1987) assumed that there were two distinct types of channel, one type that was inhibitable by Ca^{2+} and the other type that was not. The suggestion that there are two types of K^+ channel is not new. Sokolik and Yurin (1986), Beilby (1986) and Tester (1988a,b) have all proposed the existence of two mechanisms of K^+ transport into charophytes.

8.3.4 Do Na^+ and K^+ share a common transport mechanism?

The influx of K^+ into *C. corallina* is greater than the influx of Na^+ under similar conditions. However, the influx of these two ions have a number of common features. Similarities such as the dependence of both fluxes on external Ca^{2+} and Ba^{2+} suggest that 1 transport mechanism might be responsible for Ca^{2+} insensitive K^+ and Na^+ influx.

The experiments presented in section 8.2.2.2 were undertaken to determine whether removal of external Ca^{2+} resulted in a large, non specific, influx of ions into the cell, or whether only the influx of K^+ and Na^+ was affected. The data presented in section 8.2.2.2 indicates that the removal of Ca^{2+} did not result in a complete loss of membrane integrity. Of the compounds tested large stimulations of uptake rate by removal of external Ca^{2+} was confined to K^+ and Na^+ (however, data in Table 6.1 suggest that the uptake of other Group 1A cations may also be stimulated). This result adds more circumstantial evidence to support the hypothesis that Ca^{2+} sensitive influx of K^+ and Na^+ is via the same mechanism.

However, the results presented in section 8.2.2.3 indicate that K^+ and Na^+ do not compete for transport across the membrane. It would be expected that if both ions were being transported via the same mechanism that addition of K^+ (when transport is saturated with Na^+) would cause a reduction in the rate of transport of Na^+ . This result leads to the proposal for a transport mechanism for Na^+ that is regulated by external Ca^{2+} concentration and independent of K^+ concentration. As far as I am aware nobody has postulated either the need for, or the existence of such a transport system at the plasma membrane of plants. Given that in most non-halophyte cells excess Na^+ accumulation rather than insufficient Na^+ is the problem and that the electrochemical gradient for Na^+ always favours Na^+ influx it is hard to envisage a role for a Na^+ channel at the plasma membrane. Rapid inward transport of Na^+ when external Na^+ is increased would be beneficial to cells that could accumulate Na^+ as an osmoticum, however *C. corallina* does not do this.

CHAPTER 9

Conclusions and Future Research

9.1 Effect of salinity on *C. corallina*.

The main objective of this study was to determine the effects of salinity on the physiology of *C. corallina*. This required a description of the concentrations of external ions in which *C. corallina* could survive. The results presented in Chapter 3 show that *C. corallina* survived in solutions containing up to 100 mol m^{-3} NaCl providing that the solution contained an adequate Ca^{2+} concentration. In the absence of external Ca^{2+} , 100 mol m^{-3} Na^+ resulted in the plasma membrane undergoing a number of action potentials from which the membrane did not repolarize. Cytoplasmic streaming ceased and the cells died. This is consistent with the results of Katsuhara and Tazawa (1986) and Tufariello et al. (1988) which both showed that increasing the ratio of Na^+ to Ca^{2+} in the bathing solution by addition of Na^+ resulted in the cells dying. In this thesis changes in salinity of the bathing medium were always made in one big jump. An area for further research is to see whether stepwise increments of external Na^+ , rather than a single jump, might improve survival of *C. corallina*.

If the ratio of Na^+ to Ca^{2+} in the bathing solution was maintained above approximately 20 to 1, isolated internodes survived for many weeks in solutions containing 100 mol m^{-3} NaCl without inhibiting cytoplasmic streaming rate or HCO_3^- fixation. This treatment resulted in a net influx of Na^+ and a net efflux of K^+ from the cell. During this process there was a slight increase in osmotic potential of the cell, however this was not enough to compensate for the increase in osmotic potential of the external solution, therefore turgor potential of the cell decreased. The results presented in this thesis confirmed those of Bisson and Bartholomew (1984) which showed *C. corallina* to

regulate osmotic pressure at the expense of cell turgor. This is in contrast to the brackish water characean plants such as *C. buckellii* (Hoffmann and Bisson 1986) which accumulate Na^+ and/or K^+ with Cl^- in the vacuole as a response to increasing salinity.

Cell elongation was severely inhibited when the turgor potential of the cell dropped below approximately 0.29 MPa. This represented the yield threshold of the cell wall and is similar to the value of 0.2 MPa obtained by Green et al. (1971) for *Nitella* sp..

9.2 How does Ca^{2+} protect the cell from salt damage?

At the external surface of the plasma membrane there are two major sites for the action of Ca^{2+} . Ca^{2+} bonds with the negatively charged phospholipid head groups that make up the matrix of the plasma membrane and Ca^{2+} is also required by various transport proteins for their operation. Cramer et al. (1985) suggested that the primary cause of salt damage to plant cells was the displacement of Ca^{2+} by Na^+ from the plasma membrane which ultimately led to a disruption of membrane integrity and selectivity. The results presented in Chapter 6 show that addition of sterols and polyamines did not alter the sensitivity of the cell to NaCl in the absence of Ca^{2+} . This suggested that if Ca^{2+} was displaced from the phospholipids by Na^+ , then it was not the major cause of Na^+ -induced damage to the cell. Whether or not Ca^{2+} displacement from transport proteins at the surface of the plasma membrane was the primary cause of salt damage in *C. corallina* was investigated by monitoring the effect of external Ca^{2+} on the transport of various ions, principally Na^+ and K^+ , across the plasma membrane of *C. corallina*.

This thesis focused on the effect of elevated levels of Na^+ and to a lesser extent K^+ , and their interaction with Ca^{2+} on the physiology of *C. corallina*. I have made no attempt to measure the cytoplasmic

concentrations of these ions. Katsuhara and Tazawa (1988) attempted to measure the cytoplasmic concentrations of K and Na in *Nitellopsis obtusa* during exposure to elevated Na^+ ; a similar technique could be applied to *C. corallina*. Tester and MacRobbie (1990) have recently shown that cytoplasmic Ca^{2+} affects the gating of K^+ channels in the plasma membrane of *C. corallina*. The effect of raising the external Na^+ concentration on the cytoplasmic Ca^{2+} concentration, and how this in turn affects the transport of K^+ and Na^+ and thus survival of *C. corallina*, is an area for future research.

9.3 Displacement of Ca^{2+} from the plasma membrane.

Cramer *et al.* (1985) suggested that displacement of membrane associated Ca^{2+} was Na^+ -specific and argued that the ratio of Na^+ to Ca^{2+} in the external solution determined the degree of displacement of Ca^{2+} , and therefore salt damage. The results presented in chapter 6 showed that elevated levels of Na^+ , K^+ , Li^+ and Rb^+ were equally toxic to *C. corallina* in the absence of external Ca^{2+} . Assuming that cell death was a result of the displacement of membrane-associated Ca^{2+} and the associated loss of membrane integrity, then my results indicate that Ca^{2+} displacement was not Na^+ -specific. This could be tested by measuring membrane bound Ca^{2+} using a Ca^{2+} -sensitive fluorescing probe (eg. CTC).

9.4 Excess cation uptake.

A large deficit of vacuolar Cl^- relative to vacuolar K^+ and Na^+ (and Rb^+) was discovered in treatments which contained elevated levels of K^+ (or Rb^+) in the external solution. Excess inorganic cation relative to inorganic anion was not observed in the treatments involving elevated Na^+ or Li^+ .

It was shown that when there was an inorganic anion deficit in the vacuole, electroneutrality was maintained by accumulation of malate. A similar phenomenon has been reported from a number of higher plants (see review by Osmond 1976), however it has only recently been reported for *C. corallina* (Ryan 1988). Malate accumulated in the vacuole during excess cation uptake was produced from the carboxylation of PEP with HCO_3^- . This reaction was catalyzed by the enzyme, PEP Carboxylase. Malate accumulation in the vacuole was favored when the balancing anion in solution was transported into the cell relatively slowly, however malate was produced in the presence of anions transported into the cell relatively quickly such as Cl^- . Malate accumulation was dependent upon the external concentration of HCO_3^- . Malate was not accumulated when external HCO_3^- was removed either by lowering external pH or using carbon free solutions.

The synthesis and accumulation of malate in the vacuole has a number of associated costs to the cell including the production of approximately 1.7 H^+ per molecule of malate accumulated. Given the rates of malate accumulation measured, the associated production of H^+ would impose a severe acid load on the cell. A calculated balance sheet of H^+ production and consumption (see Figure 7.11) showed that during malate accumulation there was a net transport of H^+ from the cell to the external solution at a rate of approximately $40 \text{ nmol m}^{-2} \text{ s}^{-1}$. Under conditions that promote malate accumulation the membrane potential difference was depolarized (eg. $10 \text{ mol m}^{-3} \text{ K}^+$ the

membrane potential difference was -42mV) . Therefore the H^+ -pump can be operational even when the cell is depolarized.

The results presented in Chapter 7 indicated it unlikely that the generally accepted signals for malate accumulation, namely changes in pH_c or low cytoplasmic Cl^- (Osmond 1976, Ryan 1988) induced malate accumulation in the experiments reported in this study. I suggested that the rate of PEP Carboxylase activity may be regulated by cytoplasmic malate concentration. Cytoplasmic malate concentration would be regulated by transport of malate across the tonoplast to the vacuole. Treatments that altered tonoplast transport and the distribution of malate within the cell would therefore control malate accumulation in the cell.

Treatments that have been shown to cause malate accumulation in *C. corallina* and other plants have involved the uptake of a cation in excess of anion and usually a depolarization of membrane potential difference. For example membrane potential difference of *C. corallina* was depolarized to approximately -60 mV (from -220mV) by the addition of $150\text{ mmol m}^{-3}\text{ NH}_4^+$; a treatment that was shown to induce malate accumulation (Ryan 1988). A sustained depolarization of membrane potential difference may affect the transport of various ions across the tonoplast. An area for further study would be to test whether malate accumulation can be induced by depolarizing the cell without inhibiting the proton pump. This line of research would also contribute to the understanding of the relative roles of biochemical and biophysical pH regulation in *C. corallina*.

Whilst the external solution contained a suitable ratio of Na^+ to Ca^{2+} the main impediment to the growth of *C. corallina* in brackish water was the reduction in cell turgor. However, in solutions containing high concentrations of K^+ or Rb^+ accumulation of K-malate (or Rb-malate) in the vacuole resulted in the maintenance of cell

turgor at the pre-salt treatment level. The question then arises: why does the cell not accumulate Na-malate in the vacuole, and therefore maintain turgor in brackish waters? Similarly, why does the cell not accumulate NaCl in the vacuole since cells are capable of accumulating KCl and RbCl in the vacuole? The answer to this may be associated with the concentrations of K^+ and Na^+ that the cytoplasm can tolerate coupled with the permeability of the tonoplast to those ions. If the tonoplast is relatively permeable relative to the rate at which these ions can be transported across the tonoplast, then cytoplasmic concentrations of these ions will rise. Cytoplasmic enzymes may be less tolerant of Na^+ ions than K^+ ions, therefore restricting the ability of the cell to accumulate Na^+ . This hypothesis could be investigated by perfusing the vacuole of *C. corallina* with solutions of increasing Na^+ concentration and comparing cell survival to cells in the vacuole is perfused with similar concentrations of K^+ . Attempts could also be made to measure the cytoplasmic concentrations of Na^+ and K^+ during these experiments.

The results presented in this thesis show that under the right conditions (such as high external K^+ concentration) *C. corallina* can accumulate significant concentrations of malate in the vacuole which can contribute to turgor regulation by the cell. This observation shows that cells of *C. corallina* could have a role as models for studying vacuolar organic acid accumulation in higher plants.

9.5 Effect of external Ca^{2+} on the transport of ions across the plasma membrane.

The concentration of Ca^{2+} in the external solution affected the rate of K^+ and Na^+ transport across the plasma membrane. I have argued that there are at least two mechanisms for the transport of these two ions across the plasma membrane; one that is insensitive to external Ca^{2+} and another that is dependent upon external Ca^{2+} . These mechanisms are similar to the mechanism 1 and mechanism 2 uptake described by Epstein et al. (1952). The rates of Na^+ uptake described in this thesis are higher than those described for *Nitella translucens* (Smith 1967) and for *C. corallina* (Hoffmann et al. 1989, Hope and Walker 1960). However, Smith and Walker (1989) report Na^+ influx up to $30.5 \text{ nmol m}^{-2} \text{ s}^{-1}$ in K^+ -starved *C. corallina* from solutions where the external Na^+ concentration was 1 mol m^{-3} ($\text{Ca}^{2+} = 0.5 \text{ mol m}^{-3}$). The steady state Na^+ influx under these conditions was $4.1 \text{ nmol m}^{-2} \text{ s}^{-1}$, a value similar to those reported in this thesis. In this study the measurement of Na^+ and K^+ influx was carried out in unstirred solutions over relatively long periods of time (usually 120 minutes), therefore rates of uptake measured would be under-estimates of the maximum flux rates across the membrane (MacFarlane 1985, Hone 1988). This was one reason that there has been little attempt to describe mechanistic models for Na^+ uptake in anything but a most general way.

The influx of Na^+ was shown to be dependent upon the concentration of Ca^{2+} in the external medium even at "low" concentrations of Na^+ (eg. see Figure 8.5). The dependence of Na^+ influx on the ratio of Na^+ to Ca^{2+} should be considered when undertaking experiments with *C. corallina*. For example, NaOH is used to neutralize various zwitterionic buffers which can result in relatively high concentrations of Na^+ (eg. Smith and Walker (1975) used NaOH to adjust the pH of experimental solutions containing up to 10 mol m^{-3} zwitterion buffer).

The results presented in this study show that the flux of Na^+ across the plasma membrane is significant relative to the flux of other ions and should not be discounted.

There were a number of similarities between the rate of uptake of Na^+ and K^+ across the plasma membrane, which prompted the question: are Na^+ and K^+ influx across the plasma membrane via a common transport system? Experiments described in section 8.2.2 were aimed at addressing this question; however, the results were inconclusive. There remains the scope for further work on elucidating the transport processes involved with the various mechanisms of Na^+ influx. There exist a number of possibilities to account for the influx of Na^+ . These include;

1. "leakage" of Na^+ through K^+ channels. The results of Tester (1988b) showed that the conductivity of the K^+ channel to Na^+ was 0.17 times that of K^+ . At high concentrations of external Na^+ the influx of Na^+ could be significant (ie. at an external K^+ concentration of 10 mol m^{-3} , K^+ influx in the presence of 0.5 mol m^{-3} would be approximately $400 \text{ nmol m}^{-2} \text{ s}^{-1}$ (figure 8.13). This would equate to a Na^+ influx of $0.17 \times 400 = 68 \text{ nmol m}^{-2} \text{ s}^{-1}$.)

2. the existence of a population of Ca^{2+} -sensitive Na^+ channels at the plasma membrane. As was mentioned in section 8.3.4, it is hard to envisage a role for such a population of channels at the plasma membrane of a freshwater cell given that the cell does not accumulate large quantities of Na^+ . Intuitively it would seem a disadvantage for the cell to possess such channels.

3. Na^+ "leaks" through a channel other than K^+ channels. The only likely candidate for this would be the Ca^{2+} channel. The results discussed in section 8.16 suggested that this did not occur.

The transport molecule responsible for the influx of Na^+ across the plasma membrane is regulated by Ca^{2+} and Ba^{2+} . Armstrong and

Matteson (1986) have shown that the opening kinetics of the K^+ channel in squid neurons are slowed by the binding of Ca^{2+} to the negative parts of the gating apparatus. These sites are located on the external surface of the protein. External K^+ competes with Ca^{2+} for channel occupancy, the channels remaining open when Ca^{2+} is excluded from the gating apparatus. Armstrong et al. (1987) have also shown that when a K^+ channel remains open it slowly assumes an unusual conformation which affects its selectivity of other ions. These results suggest that the biphasic influx of Na^+ and K^+ described in this thesis could be due to the ungating of K^+ channels when a critical ratio of monovalent cation to Ca^{2+} is reached. This scenario uses one transport protein to explain the dual mechanisms of uptake (eg. mechanisms 1 and 2 described by Epstein (1976)). Both Ba^{2+} and Mg^{2+} can substitute for Ca^{2+} in gating the transport complex. Tufariello et al. (1988) suggest that Mg^{2+} is not a good substitute for Ca^{2+} in saline water. It would be interesting to see if addition of either Mg^{2+} or Ba^{2+} could enhance survival of *C. corallina* in Ca^{2+} -free solutions containing 100 mol m^{-3} NaCl.

In this study the giant internodal cells of *C. corallina* were used to further understand the cellular mechanisms of salt stress and salt tolerance. There is much scope for further work in comparing the mechanisms of turgor regulation, organic acid accumulation and Na^+ transport with other characean algae, particularly salt tolerant species. Hopefully, this avenue of research combined with the results discussed in this study will lead to an understanding of the mechanisms of salt tolerance and salt stress at the level of tissues and whole plants.

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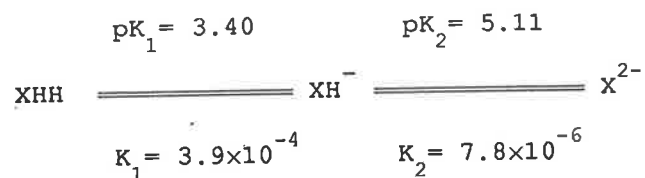
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APPENDIX 1 Equations to calculate net charge on a malate ion.

The net charge on a malate is pH dependant having two protonation states with pK_a^s at 3.40 and 5.11 (Weast 1971).



$$K_1 = \frac{[XH^-] \times [H^+]}{[XHH]} \quad \text{and} \quad K_2 = \frac{[X^{2-}] \times [H^+]}{[XH^-]} \quad \text{and} \quad 1 = [XHH] + [XH^-] + [X^{2-}]$$

These equations can be written in terms of each species as follows:

$$[XHH] + \frac{K_1 [XHH]}{[H^+]} + \frac{K_1 K_2 [XHH]}{[H^+]^2} = 1$$

$$[XH^-] + \frac{K_2 [XH^-]}{[H^+]} + \frac{[XH^-] [H^+]}{K_1} = 1$$

$$[X^{2-}] + \frac{[X^{2-}] [H^+]^2}{K_1 K_2} + \frac{[X^{2-}] [H^+]}{K_2} = 1$$

Therefore the proportion of each species can be determined and the mean charge on a malate molecule can be determined. These equations were adapted from those of Ryan (1988).