THE IONIC RELATIONS OF *CHAETOMORPHA DARWINII* (HOOKER) KUETZING

by

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge no material has been included which has been written or published by another person except where due reference is made in the text.

WILLIAM A. DODD.

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SUMMARY

Chaetomorpha darwinii is a marine green alga with large coenocytic cells and is suitable for ion permeability experiments.

The movement of sodium and potassium ions across the cell boundaries was studied. An analysis of the rate at which isotope diffuses out of the tissue shows three distinct phases in series and this is consistent with the model suggested for the *Characean* cells. An analysis of each compartment of the cell was made and the metabolic inhibitor DNP was used to elucidate the active and passive fluxes.

Using a microelectrode technique it was found that the vacuole was -35 mV and the cytoplasm was +10 mV relative to the external solution. The coenocyte of *Chaetomorpha* thus has a significant potential difference across the tonoplast.

A range of metabolic inhibitors was used to trace the source of energy used in the ion transport. In *Chaetomorpha* energy for ion transport appears to be derived from ATP or ATP mediated reactions.

SYMBOLS USED

a	Ion activity coefficient
ATP	Adenosine tri phosphate
ADP	Adenosine di phosphate
C	Concentration of ions (m.equiv./l)
DNP	2-4 di nitro Phenol
E_m or E_j	Transmembrane potential (mV) for any ion (j) from Nernst equation
F	Faraday's constant
FS	Free space
k_1	Rate constant for exchange of ions from Cytoplasm
P_1	Permeability constant[1] of plasmalemma
R	Universal gas constant
S_0	Specific activity of the external solution (counts/min/10ml)
S_2	Specific activity of the cytoplasmic phase
S_3 or S_v	Specific activity of the vacuole
Q_e or Q_2	Amount of ion in the vacuole (m.equiv./kg)
Q_c^*	Amount of isotope in cytoplasm (counts/min/10ml)

[1] P_1 defined as $\beta_1 = P_1 \dfrac{\ln \exp E_1 F/RT}{1 - \exp EF/RT} \cdot C_2$

where C_2 = 600 m.equiv./l

T	Absolute temperature ($^{\circ}K$)
t	Period in solution
$t^{\frac{1}{2}}$	Time for half exchange of isotope from a compartment
z	Charge on the ion
ϕ_1	Fluxes between free space and cytoplasmic phase (net flux = zero)
ϕ_2	Fluxes between cytoplasmic phase and vacuole (net flux = zero)

CHAPTER I

REVIEW OF LITERATURE

Much of the early work on ion transport was carried out on animal tissues such as muscle fibres, erythrocytes and nerve fibres. The principles developed in these studies are applicable to plants but it is only in relatively recent years that work has been carried out to place plant studies on a comparable basis.

The earliest workers in the field of ion transport in plants were Blinks (1930a) (1930b) (1940) and Osterhout (1922) (1925) who investigated the potentials, resistances and ionic content of several large algal cells. This work was important because it developed a basis for electrical measurements which was later used by the animal physiologists. While the earlier work of Blinks, Osterhout and others raised many interesting possibilities, it did not help to explain the distribution of ions in the cytoplasm and vacuole or the mechanisms involved.

The less advanced state of the work on plant cells is due partly to the more complicated structure of the plant cell.

Instead of a simple plasma membrane dividing the protoplasm from the external solution, as in the animal cell, there are in the plant cell two membranes separating the thin protoplast from the external solution on one side, and the vacuole on the other. In addition the cell wall adjacent to the plasmalemma is a complicating feature which is absent from the animal cell.

Ion uptake by plant cells and tissues appears to consist of a two stage process; a passive non-metabolic entry of ions into the free space, followed by an active metabolic transport of ions across a diffusion barrier. Free space is defined as that part of the cell or tissue into which solutes move relatively freely by passive diffusion. It may be stated that this passive uptake is only a necessary part of diffusion of ions to the site of uptake.

The limits of the free space were in doubt for some time. Briggs (1957) and Briggs and Robertson (1957) defined free space as comprising "the intercellular spaces, wet cell walls and much of the cytoplasm". The main barrier to ion diffusion was thought to be at the tonoplast. Opinions have long varied as to the function of the plasmalemma. Since the beginning of this century biologists have recognized that a lipid-protein membrane constituted the external barrier to the protoplast, (Blackman 1912) but there has been some doubt about the efficiency of the plasmalemma as a permeability barrier. Electron micrographs by Mercer et al (1955) failed to show the presence of a plasmalemma while showing membranes at the tonoplast and surrounding the mitichondria. However since MacRobbie and Dainty (1958) and Diamond and Solomon

(1959) published papers on the ionic fluxes and distribution of monovalent ions in large algal cells, there has been a mounting body of evidence in favour of the existence of a plasmalemma as a diffusion barrier, limiting the free space to the cell wall and intercellular spaces. Haapala (1960) using colour reactions to determine the rate of uptake of Na_2CO_3 and NaOH into the internodal cells of several genera of large celled plants, found that the plasmalemma constituted a barrier to diffusion. Whaley et al (1960) published electron micrographs of meristemmatic cells showing a well defined membrane in the plasmalemma region. A plasmalemma was shown in plasmolysed beet cells. (Briggs, Hope and Robertson 1962). Low potentials have been found at the tonoplast, (Walker 1957) (Hope 1961) (MacRobbie 1962) and hence most of the trans-cellular potential is at the plasmalemma. This means that much of the transport must be here too.

Ion uptake experiments with intact plants are difficult to interpret at the cellular level because the different types of cells present constitute a complex diffusion path. One way of reducing complexity is to use homogeneous tissue instead of whole plants as the experimental material, hence the wide use of parenchyma discs of beet and carrot. But even with homogeneous tissues slices such as these, complications arise due to the diffusion path, so that accurate flux determinations can be made only at low temperatures when there is sufficient difference between the diffusion path from the tissue and the cell membrane. (Pitman 1963) Consequently it is not surprising that the first significant

quantitative measurements on ion movement at the cellular level have been made on the ecorticate, large celled algae of the family Characeae. These plants have large internodal cells, and thus a simple and uniform diffusion path to their internodal solution. They have large vacuoles which can be sampled for chemical analysis and electrodes can be inserted for electrical measurement.

Ion	Sap Concentration m Molar	Concentration of External Solution m Molar	Ion Equilibrium Potential (mV)
Na	54	30	-15
K	113	0.65	-30
Cl	206	35	+45

Table 1:1 The ion concentrations in the sap and external solutions of Nitellopsis obtusa and the equilibrium potential calculated using the Nernst equation. from MacRobbie and Dainty (1958b)

MacRobbie and Dainty (1958b) using Nitellopsis obtusa and Diamond and Solomon (1959) using Nitella axillaris published results of experiments in which they were able to estimate the cation fluxes across both the tonoplast and the plasmalemma. It is convenient to cite the work of MacRobbie and Dainty (1958b) to illustrate the principles involved in a three phase model of the cell.

Nitellopsis obtusa is an ecorticate Characean from brackish water. It has large internodal cells with large vacuoles and a relatively thin layer of cytoplasm bounded by a cell wall. The authors showed that under the experimental conditions used, the cells were permeable to K, Na and Cl ions and that each ion was in approximate flux equilibrium between the vacuole and external solution. However an analysis of vacuolar sap (Table 1:1) showed that for at least two of the ions there must be an electrochemical potential difference between sap and external medium.

A direct determination of the transmembrane potential gave -150mV between the sap and external solution, showing that for Na $\bar{\mu}_i < \bar{\mu}_o$, for Cl $\bar{\mu}_i > \bar{\mu}_o$ and for K $\bar{\mu}_i \simeq \bar{\mu}_o$. These figures and the fact that influx was approximately equal to efflux for each ion, suggested that sodium efflux against the electrochemical gradient was an active transport, i.e. a sodium pump was operating and the chloride influx must have been due to an outwardly directed chloride pump. From a kinetic analysis of the exchange of sodium, potassium and chloride, between the cell and the external medium, MacRobbie and Dainty suggested that the chloride pump was at the tonoplast. They were able to conclude that the plasmalemma was similar to a typical membrane, was the site of the sodium extrusion pump and was five times more permeable to potassium than sodium. The fluxes in Nitellopsis were of the same order as those in the animal cells. The tonoplast was suggested to be the site of the chloride pump, which was a necessity

for maintaining turgor pressure. The low fluxes across the tonoplast suggested that the electrical resistance between the cytoplasm and the external medium would be high, and that most of the potential difference would be across the plasmalemma and not more than a few millivolts across the tonoplast.

Walker (1955) (1957) using microelectrodes showed that in Chara australis practically all the potential difference and resistance between the vacuole and external solution was across the plasmalemma, with only a few millivolts across the tonoplast. Blount and Levedahl (1960) with Halicystis ovalis and Etherton and Higinbotham (1960) using Avena root hairs have agreed with this. However Spanswick and Williams (1964) using Nitella translucens showed that there was negative potential of 18mV with respect to the vacuole, across the tonoplast, and Findlay and Hope (1964) using Chara australis also showed a potential difference across the tonoplast.

These data presented by MacRobbie and Dainty (1958b) and Diamond and Solomon (1959) fit very well with the model for a three phase system of a cell, three phases being in series. Diamond and Solomon (1959) showed that the free space potassium in Nitella axillaris had a half time for exchange of 23 seconds. The cytoplasmic potassium had a half time for exchange of 300 minutes and the vacuolar compartment a half time of forty days. The data of MacRobbie and Dainty (1958b) differed in that the compartments had a faster rate of exchange. However these data

the flux data of Hope and Walker (1960) on Chara australis and MacRobbie (1962) on Nitella translucens, fit the three phase model very well.

In the members of the Characeae which have been studied, the potassium fluxes are of the order 0.2 to 0.7 p moles/cm^2/sec and sodium fluxes generally a little less. Diamond and Solomon (1959) showed that the ratio of $\phi_1:\phi_2$ was about 0.6 in Nitella axillaris. The fluxes in marine plants may be very much higher since Blount and Levedahl (1960) measured a sodium efflux of up to 226 p moles/cm^2/sec in Haliclystis, using the short circuit method of Ussing.

Thus the algal cells studied, (and also beet and barley) appear to be best treated as a system of three phases in series, namely free space, cytoplasm and vacuole. The membrane between the cytoplasm and environment resembles the animal cell membrane and can be twenty to a hundred times more permeable than the tonoplast. Cation discrimination has been shown so far at the plasmalemma, involving sodium extrusion and perhaps potassium accumulation, while the chloride accumulating mechanism has been assigned to the tonoplast. Spanswick and Williams (1964) using Nitella translucens have suggested from potential measurements and cation analysis that sodium is pumped from the cytoplasm into the vacuole as well as into the external medium.

The ability of multicellular plants to survive in the sea has attracted much attention and such studies may assist in solving some of the more general problems of salt absorption. Two

main types of marine algae have been used in ion permeability studies. The first type, of which Porphyra perforata and Ulva lactuca are examples, has flattened fronds consisting of two layers of uniform cells. Diffusion effects which confuse the situation in bulkier algae are minimized. Porphyra appears to have no vacuoles and in Ulva they are certainly very small. In the second group, of which Valonia, Halicystis and Chaetomorpha are examples, the thallus consists of large coenocytes having a large vacuole and a thin layer of cytoplasm. This group provide likely material for the study of osmotic effects, permeability and electrical phenomena because of their size and the ease with which the sap can be sampled for analysis and electrical measurements made. Though the coenocytic marine algae are convenient subjects for permeability studies, care should be taken when drawing analogies between vacuolated cells and coenocytes. Coenocytes have probably evolved from multicellular tissue after reduction of the intervening walls.

Though much earlier work had been done on marine algae from 1920 to 1935 by Osterhout, Blinks, Jaques and others, these studies did not suggest mechanisms which could explain the ionic distributions observed. The recent study by MacRobbie and Dainty (1958a) on the red alga Rhodymenia palmata is as yet the only one in which the kinetics of ion movement and electrical measurements were carried out at the same time. Unfortunately the thallus of Rhodymenia had two different types of cells, each with a different permeability; consequently while this work allowed some con-

clusions to be drawn about sodium and potassium distribution and its maintainance in Rhodymenia, accurate flux figures could not be quoted nor could any really reliable assessment be made of the contribution of the different cellular phases to the ion distribution and exchange.

Blount and Levedahl (1960) studied the active transport of sodium and chloride ions in Halicystis ovalis but determined only the nett fluxes and not the individual fluxes using Ussing's short circuit technique.

Scott and Hayward (1953) (1954) using Ulva lactuca and Appley (1957) (1958) using Porphyra perforata studied the changes which light and metabolic inhibitors produced in these respective marine algae both of which exclude sodium and accumulate potassium. Scott and Hayward concluded that Ulva lactuca maintained its ionic composition by two alternate processes, either a photosynthetic reaction or a glycolytic reaction. Some agents such as ATP and phosphoglycerate dissociate potassium and sodium movement; potassium content became steady and sodium was gained. This fact, plus that the time course for potassium and sodium exchange differed, led the authors to conclude that the potassium and sodium movements were mediated by different processes. MacRobbie (1962) also suggested that potassium and sodium were acting independently in Nitella translucens. In 1957 Scott et al observed that 90% of sodium in the fronds of Ulva exchanged with sodium ions from sea water in five seconds. Potassium was exchanged less rapidly than sodium, (Scott and Hayward 1953b) but much more

readily than the bulk of potassium in the higher plant cells and coenocytes. Potassium ions were rapidly lost from Ulva fronds in the dark and reabsorbed in the light. It is possible that a large fraction of the sodium and potassium content of Ulva exists in metabolically bound forms in the cytoplasm and in the thick mucilaginous cell wall, while relatively little is accumulated in the vacuoles which are very small in this plant. The situation may be similar to that found in the bacteria and microorganisms. Conway and Downey (1950) found that 26 - 33% of the volume of yeast cells was free space.

Eppley (1957) (1958) working with Porphyra perforata studied the effect of a number of metabolic inhibitors on the cell's ability to exclude sodium and retain potassium. A variety of metabolic inhibitors, including 2.4 dinitrophenol and lack of oxygen, reduced the ability of the cells to retain potassium and exclude sodium. Lack of light had the same effect. While no final picture of sodium extrusion and potassium accumulation could be drawn, Eppley suggested that there was a coupled sodium and potassium cyclic mechanism similar to that proposed by Hodgkin and Keynes (1955) for squid giant axon. In contrast to the marine algae, salts accumulated in the vacuoles of higher plant cells are retained by the cytoplasm and vacuole, even under conditions of reduced metabolism.

The purpose of this study was to obtain quantitative information of the sodium, potassium and chloride content, transmembrane potentials and cation fluxes between the various compart-

ments of a marine algal cell.

Chaetomorpha darwinii was selected as a suitable alga on which to work. This plant is a marine alga consisting of chains of coenocytes (multinucleate cells) up to six millimetres in diameter. It is readily available locally and can be maintained in culture under physiological conditions for up to six weeks. While having large cells it is not complicated by layers of different types of cells as was found by MacRobbie and Dainty in Rhodymenia.

Preliminary experiments showed that Chaetomorpha had a K:Na ratio of 18.3 in the vacuole compared with 0.026 in sea water. Chaetomorpha thus excludes sodium and accumulates potassium against large concentration gradients.

The simplicity of the plant, its ease of handling and its ability to exclude sodium and accumulate potassium made Chaetomorpha darwinii an interesting and suitable subject for an ion permeability study.

CHAPTER II

METHODS AND MATERIALS

Chaetomorpha darwinii is a green alga and is a marine member of the order Cladophorales. The alga grows at the sublittoral zone, down to a depth of at least ten metres. It is frequently an epiphyte on various marine algae and in the area from which material was collected, Chaetomorpha was epiphytic on the marine monocotyledon Cymodocea. The alga was plentiful at Port Elliot (S.A.) and could be collected conveniently at low tide.

Chaetomorpha darwinii comprises strands of multinucleate cells or coenocytes (figure 2:1). Each cell is nearly spherical (figure 2:2) and is usually about four millimetres in diameter. This means that the volume of an average cell is about 0.03 cc. The cell consists of a cellulose wall, at least 3 microns thick surrounding a layer of cytoplasm 15 microns thick. (Dawes 1954). Thus the cytoplasm occupies about 2 - 5% of the total cell volume, the remainder being vacuole. Table 2:1 shows

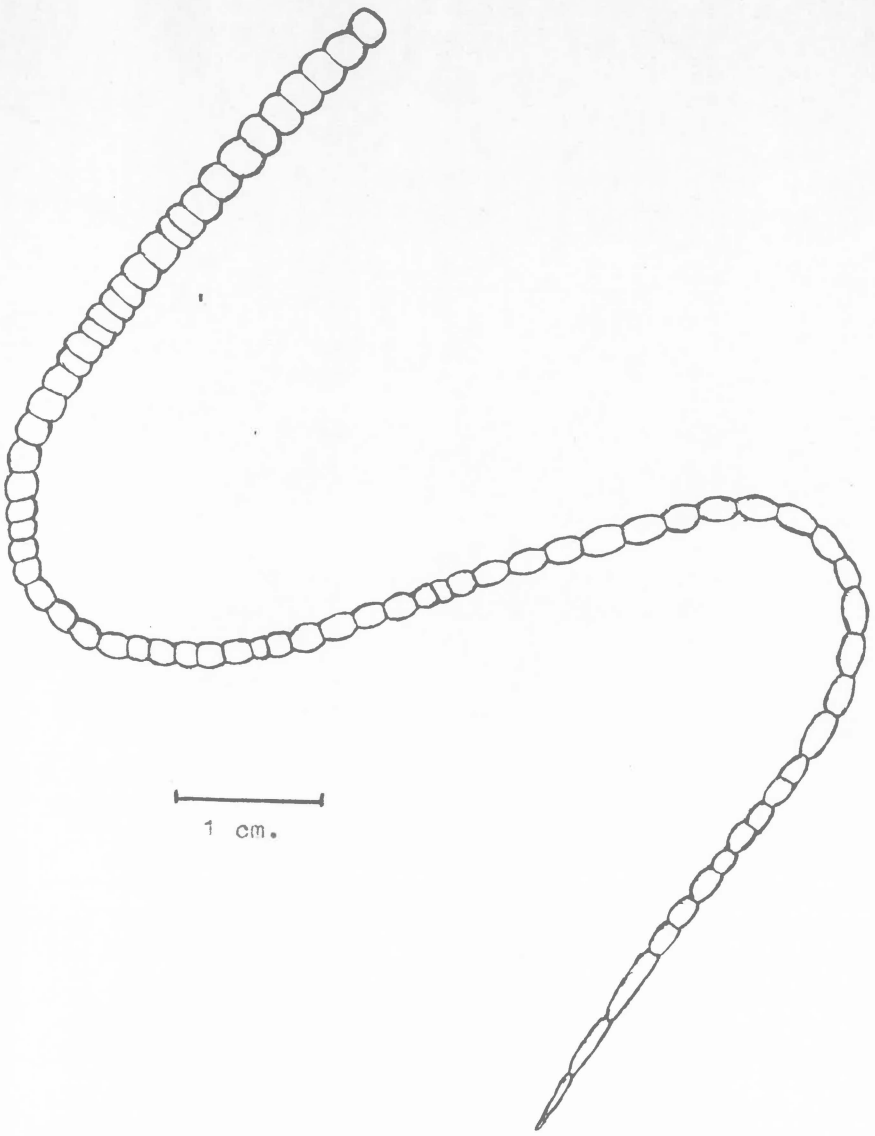

Figure 2.1 Strand of Chaetomorpha darwinii.

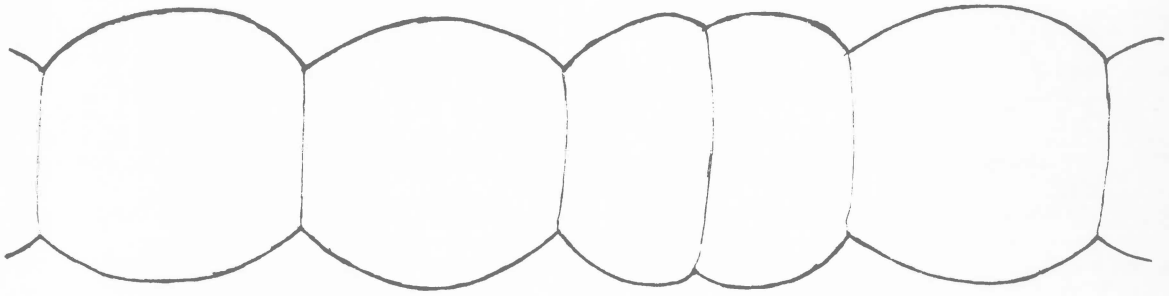

Figure 2.2 Details of multinucleate cells of *Chaetomorpha darwinii*.

the effect that diameter has on surface area of the cell and volume of the cytoplasm.

TABLE 2:1

THE EFFECT OF CELL SIZE ON THE VOLUME OF CYTOPLASM AND SURFACE AREA OF CELL

Diameter of cell	Cytoplasm as a % of total cell volume	Surface area of cell per gram of tissue
2 mm	4.2	175 cm^2/gm
3 mm	3.3	140 cm^2/gm
4 mm	2.5	100 cm^2/gm

It has been observed with other species of Chaetomorpha, (Hartmann 1929 and Christensen 1956) that there is in summer an abundance of material corresponding to the sporophyte generation, and in winter a scarcity of material corresponding to the gametophyte generation. The availability of Chaetomorpha darwinii follows the same pattern, and while no reproductive stages were observed, the life cycle of this alga probably resembles those of other species of Chaetomorpha and indeed most Cladophoraceae (Fritsch 1935) in having an alternation of isomorphic generations, a fact which must be remembered when comparing results from different seasons of the year.

Culturing Chaetomorpha

In preliminary experiments artificial seawater was prepared using the method of Provasoli (1956), but because the salts used were present in concentrations approaching saturation, precipitation often resulted. Thus the ionic content of these artificial media varied and cells soon perished. Efforts were made to grow Chaetomorpha in culture. Trials using varying intensities of light and additives such as nitrate, phosphate, trace elements and soil extract, were set up in tanks at 17°C, but natural sea water proved superior in every case and tissue could be maintained for at least six weeks in healthy condition. The cells were illuminated by natural daylight. Light reaching the tissue was one twentieth of the intensity of bright sunlight. This would correspond to the amount of light reaching a depth of approximately five metres in agitated sea water (Svedrup et al. 1961). Although tissue could be maintained in healthy condition for six weeks, experiments were usually completed within five days of collection.

Radioactive Solutions

Because preliminary experiments with artificial sea water were unsuccessful, isotope solutions were prepared by adding a small amount of the isotope as the chloride to natural sea water. Any concentration changes were taken into account by addition of salts of the major cations. The isotopes Na^{24} and K^{42} were obtained

from the AAEC as the carbonate (spec. pure) and converted to the appropriate chloride by titration with N/10 HCl. To minimise long-lived impurities, the K_2CO_3 and Na_2CO_3 were irradiated at medium neutron flux for a short period only. The activity of uptake solutions was high. For Na^{24} experiments it was necessary to use activities as high as 2.4 micro curies per milli-litre of solution to attain a high specific activity. The activity though high, caused no noticeable effects on the tissue and levels were lower than those of MacRobbie and Dainty (1958) using Nitellopsis.

Isotope Measurements

Uptake of K^{42} - Uptake of isotope by intact cells was measured by directly counting the cells on a perspex slide under an end-window G.M. tube. These cells were of equal diameter to eliminate variation due to geometry. The counting rate was related to potassium uptake by digesting the cells in normal nitric acid at the end of the experiment and estimating the activity of the resulting solution by liquid counting in an M6H liquid counting tube.

Uptake of Na^{24} - End window counting was not satisfactory with sodium isotopes. It was found that there was poor agreement between liquid counting and end window counting for sodium isotopes because sodium distributions in the cell cause over-estimation of surface content (Free space) in proportion to the vacuole.

The error in liquid counting was not greater than two percent. Since many measurements were necessary to calculate a single flux value, this error would be negligible compared with the variability in tissue behaviour. Activity remaining in the cells at the end of an experiment was estimated by liquid counting of a nitric acid digest of the cells.

Chemical Analysis

Analysis of Chaetomorpha was carried out in two ways. The first method was used with strands of intact cells, which were removed from sea water and blotted twice. To remove extracellular potassium and sodium, the tissue was placed in two rinses of isotonic sucrose for a total of 45 seconds and blotted again. The tissue was then digested in hot normal nitric acid.

The second method involved direct analysis on the cell sap. Samples were extracted by inserting a finely drawn glass micropipette into the vacuole and withdrawing a sample of the vacuolar sap. Direct analysis could be carried out on suitable dilutions of this sap.

Sodium and potassium analyses were carried out on an EEL flame photometer, standard reference being a curve constructed from dilutions of a culture solution. The error in cation measurement by this method was about 2%. For chloride analysis the potentiometric titration method of Ramsay et al. (1955) was used, error in the final analysis being about 1%. The calcium and

magnesium determinations were made using the E.D.T.A. titration method of Johnston et al. (1960).

Experimental Errors

The data from all experiments were analysed and are quoted in the form mean $\pm$ standard error of mean, with the number of determinations in brackets.

Fluxes

The fluxes are expressed in terms of pequiv. $gm^{-1}.hr^{-1}$. These may be converted to p moles.$cm.^{-2}sec.^{-1}$ by using the ratio 1 μe.$gm.^{-1}.hr.^{-1}$ is equal to 13.5 p moles $cm.^{-2}sec.^{-1}$.

Potential Measurements

Cells of Chaetomorpha were secured in a perspex perfusion cell, (figure 2:3) through which it was possible to maintain a constantly flowing solution and to change the perfusion solution without disturbing the preparation. The potential difference was measured between a micro electrode inserted into the cell vacuole and a calomel reference electrode connected through an agar salt bridge to the perfusion solution. The potential was measured on a Vibron electrometer model 33B and a Varian pen recorder was included in the circuit.

The microelectrodes were made of capillary tubing drawn to a tip diameter of 3 to 8 microns. The reference electrode had a tip diameter of up to 30 microns. Both electrodes were

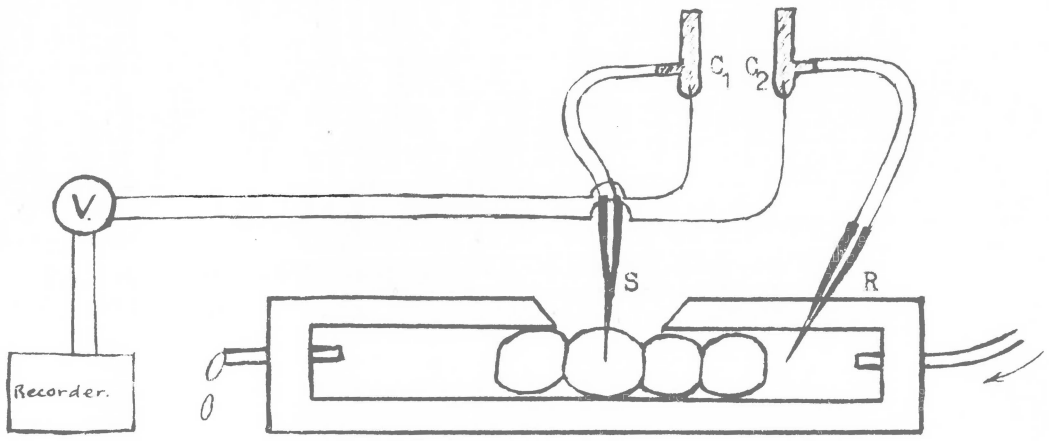

Figure 2.3 The perfusion cell used in potential measurements on Chaetomorpha. C_1 and C_2 are calomel electrodes. S and R are the cellular and reference electrodes.

filled with 2M KCl solution. Placing the electrode tip inside the vacuole without destroying the organisation of the cell required a certain amount of precision. A Leitz micromanipulator was used in conjunction with a mechanical stage and each operation was performed under a microscope at a magnification of 100 times. By this method, damage to the cell would be detected and there was little doubt that the electrode was in the correct position in the cell when the measurement was made.

CHAPTER III

CELLULAR ION BALANCE

From early experiments it was hoped that Chaetomorpha could be grown under physiological conditions in the laboratory over long periods. A preliminary experiment was designed to measure the content of major ions in the cell vacuoles over an extended period. It was thought that a knowledge of the plant's ability to maintain a balance of major ions would give an indication of any physiological change during this period. Samples of Chaetomorpha were taken at intervals over eleven days. The first sample was taken on collection from the sea and subsequent samples (during the next 11 days) from aerated sea water in a glass storage tank. Approximately one gram of tissue was used in each replicate and each sample had from six to twelve replicates. The results of this experiment are shown in figures 3:1 and 3:2. The analyses in this experiment include free space ions which are, 46.5 $\pm$ 3.0 µe/gm. of fresh tissue for sodium, 1.1 $\pm$ 0.3 µe/gm. for potassium and 60 $\pm$ 7.2 for chloride.

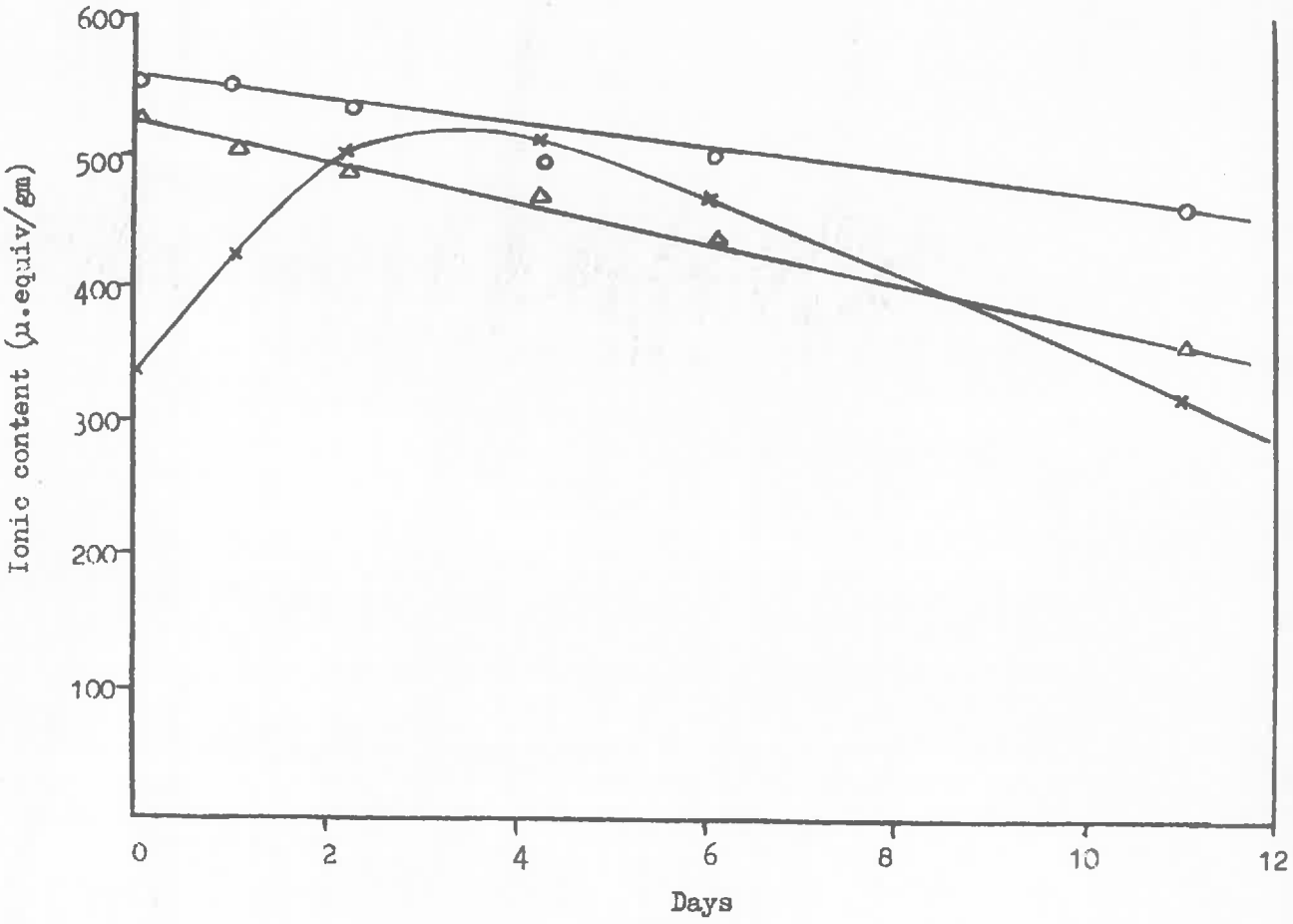

Figure 3.1 The ion content in Chaetomorpha tissue sampled over an eleven day period.

(o) = chloride; (x) = sodium; (Δ) = potassium.

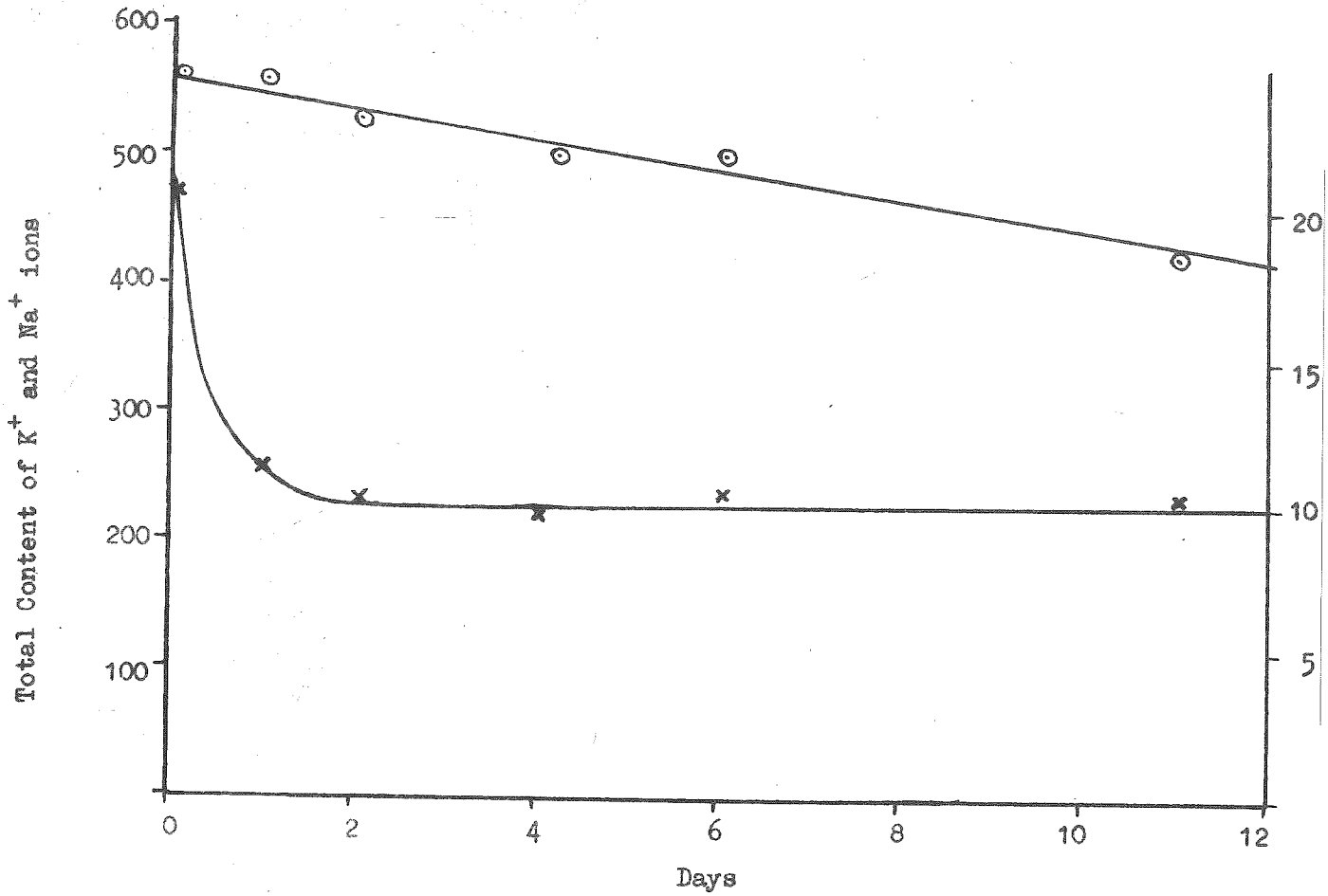

Figure 3.2 Change in cation content in Chaetomorpha tissue over eleven days.

(⊙) = potassium + sodium content; (x) = potassium : sodium ratio.

It can be seen that although total sodium and potassium ions show a decline over the experimental period, the K:Na ratio remains constant after an initial period. The sodium-potassium pump perhaps requires a few days of adjustment to adapt to different conditions. The laboratory conditions were similar to sea conditions for light and temperature but depth of sea water was less and water circulation was certainly quite different. All experiments were subsequently carried out on material that had been in culture conditions for two days and were normally completed by the sixth day. Tissue remained healthy and turgid in culture for at least four weeks.

The life cycle of Chaetomorpha darwinii is not known but the life cycle of the Cladophorales generally and of several species of Chaetomorpha, consists of an alternation of isomorphic generations. The sporophyte and gametophyte plants are identical in the sterile state. If Chaetomorpha darwinii has a similar life cycle it is important to know whether the two generations differ significantly in cell ion content and ion movement. As a means of examining this possibility, assays of samples taken over an eighteen month period were collated. Figure 3:3 shows that there was no significant difference in ion levels due to seasonal conditions or time of the year, so that if both sporophyte and gametophyte tissues have been used, it would appear that they were physiologically homogeneous as regards balance of major ions.

Whole tissue analyses and sap analyses of major ion were carried out on Chaetomorpha using the methods previously outlined.

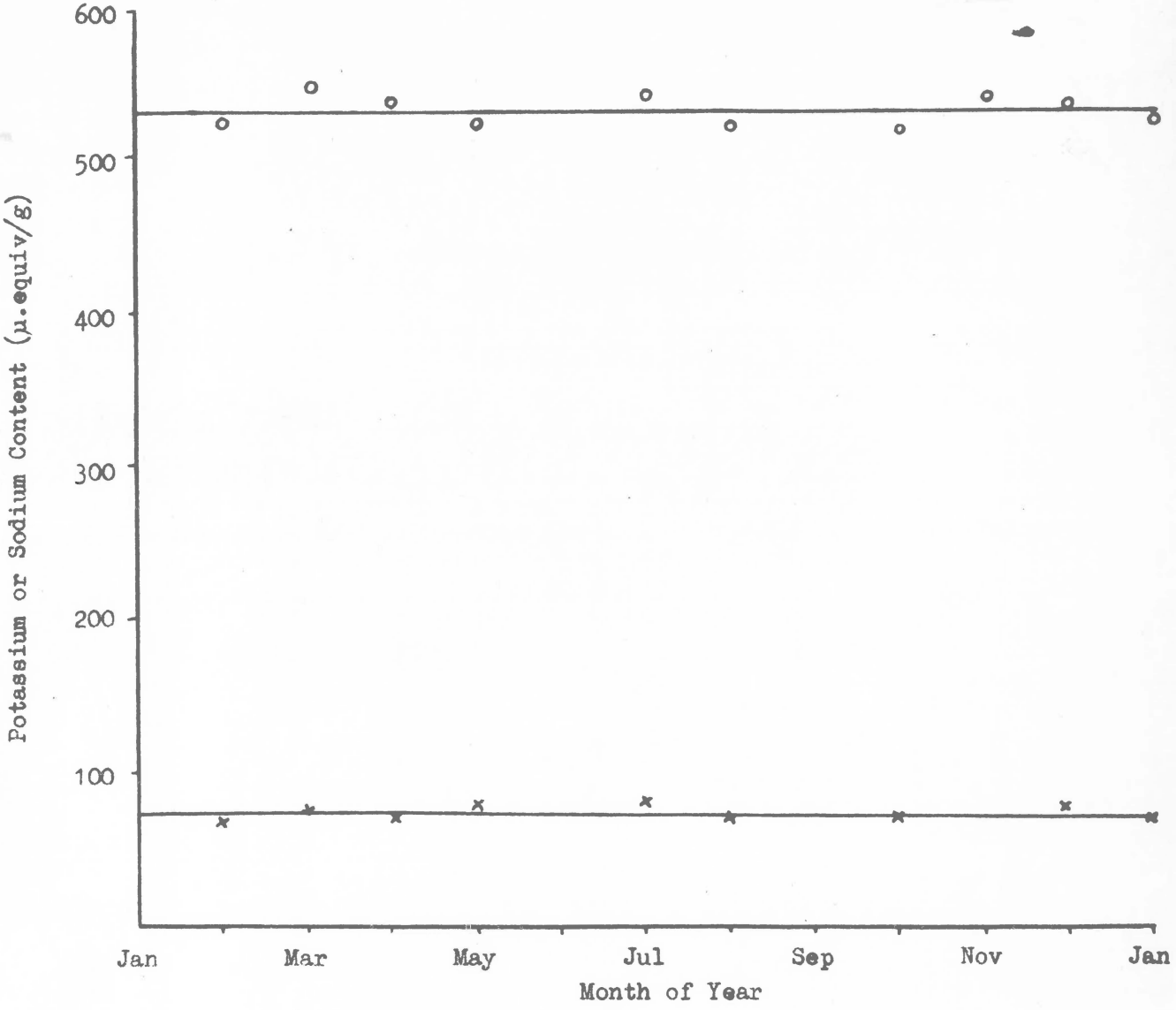

Figure 3.3 Sodium and Potassium Content in strands of Chaetomorpha at different times of the year.

(o) = potassium; (x) = sodium; (µ.equiv/gFWt)

In Table 3:1 the results of analyses using both methods are shown. Because the vacuole occupies such a large volume of the cell the concentrations of major ions shown in Table 3:1 do not differ very much in either method.

TABLE 3:1

ANALYSIS OF CHAETOMORPHA FOR MAJOR IONS, COMPARING THE VALUES FOR THE SAP AND TISSUE AS A WHOLE.

The figures in parenthesis show the number of separate estimations. Free space is not included in these figures.

Ion	Tissue Analysis (excluding free space) ue/gm. Fresh weight	Sap Analysis ue/ml. of sap
K	535 ± 4.7 (4)	541 ± 2.5 (4)
Na	25.8 ± 0.5 (4)	24.6 ± 2.7 (4)
Cl	575 ± 6.4 (4)	601 ± 18.0 (4)
Mg	22.1 ± 1.7 (4)	No Analysis
Ca	26.8 ± 2.5 (4)	No Analysis

The concentration of sodium and potassium in sea water is 500 me/l. and 13 me/l. respectively, which means that there is a high concentration gradient across the cell membranes for both ions.

Though the ion content of Chaetomorpha underwent some change when transferred from the sea to culture conditions, the constant K:Na ratio indicated that the fluxes became quite stable.

Cation analyses on samples taken throughout the year, showed that time of year or tissue heterogeneity were not sources of variation in ion balance.

CHAPTER IV

POTENTIAL MEASUREMENTS ON CELLS OF *CHAETOMORPHA*

Many plants and animals have higher concentrations of potassium than sodium in their tissues, even though the surrounding medium usually contains more sodium than potassium. Investigations have shown that low internal concentrations of sodium and high internal concentrations of potassium in plant, animal and bacterial cells, appear to be the result of an active efflux mechanism for sodium and perhaps an active influx pump for potassium.

The assymetric distribution of the ions resulting from ion pumps and their different mobilities across the membranes, results in a potential difference across the membranes. (the transmembrane potential). Because potassium has the highest mobility of the major ions in plant cells, its concentration has the most control over the resting potential, and in many cases the resting potential can be predicted with fair accuracy from potassium distribution alone, using the Nernst equation. (equation 4:1)

$$E_m = \frac{RT}{zF} \ln \frac{a^o_K}{a^i_K} \qquad 4:1$$

The assumption must be made, that the cell is in flux equilibrium for the situation under consideration and that potassium transport across the membrane is not by a pump. The approximation of replacing the activity constant of the Nernst equation by concentration, appears to be adequate in biological systems (Dainty 1961).

Once the concentration of ions, both inside and outside of the cell is known, as well as the transmembrane potential, the electrochemical potential gradient for each ion can be calculated. If conditions are such that the cell can be assumed to be in flux equilibrium, then for passively moving ions there should be no electrochemical potential difference as calculated by the Nernst equation. The Nernst equation is only valid where the mobility of all ions other than potassium is low. In this situation the Goldman equation approximates to the Nernst equation. The derivation and validity of the Goldman equation (equation 4:2) was discussed by Johnson et al. (1954) and Dainty (1962).

$$E_m = \frac{RT}{zF} \ln \frac{P_K [K_o] + P_{Na} [Na_o] + P_{Cl} [Cl_i]}{P_K [K_i] + P_{Na} [Na_i] + P_{Cl} [Cl_o]} \qquad 4:2$$

On this basis prediction can be made as to which ion is being actively transported. Thus in any study of ion transport, a knowledge of the transmembrane potential is essential.

Transmembrane potentials of plant cells have been measured for many years, beginning with the early work of Osterhout and Blinks

with large celled or coenocytic marine algae and extended to higher plant cells by Etherton and Higinbotham working with Avena coleoptiles. In recent years it has come to be believed that the total membrane potential was developed across the plasmalemma, because previous potential measurements on various types of cells had failed to detect a significant difference in potential across the tonoplast. [Walker (1955), Blount and Levedahl (1960) and Etherton and Higinbotham (1960)]

MacRobbie (1962) in a study of the ionic relations of Nitella translucens suggested that some potential drop should be expected across the tonoplast. Spanswick and Williams (1964), using the same species of Nitella from the same source and under the same conditions as MacRobbie, were able to show that there was a potential difference of +18 mV across the tonoplast. Findlay and Hope (1964) have shown that Chara australis has a +10 mV potential from cytoplasm to vacuole. One result of these small potential differences between cytoplasm and vacuole is that there should be little difference in concentration between cytoplasm and vacuole for ions in flux equilibrium.

Experimental Results

In preliminary experiments to determine the transmembrane potential of Chaetomorpha, a negative potential was recorded as soon as the electrode penetrated the cell. Over a period of about an hour there was a gradual depolarisation until a stable value of about +10 mV. was reached. Figure 4:1 shows the form of this pot-

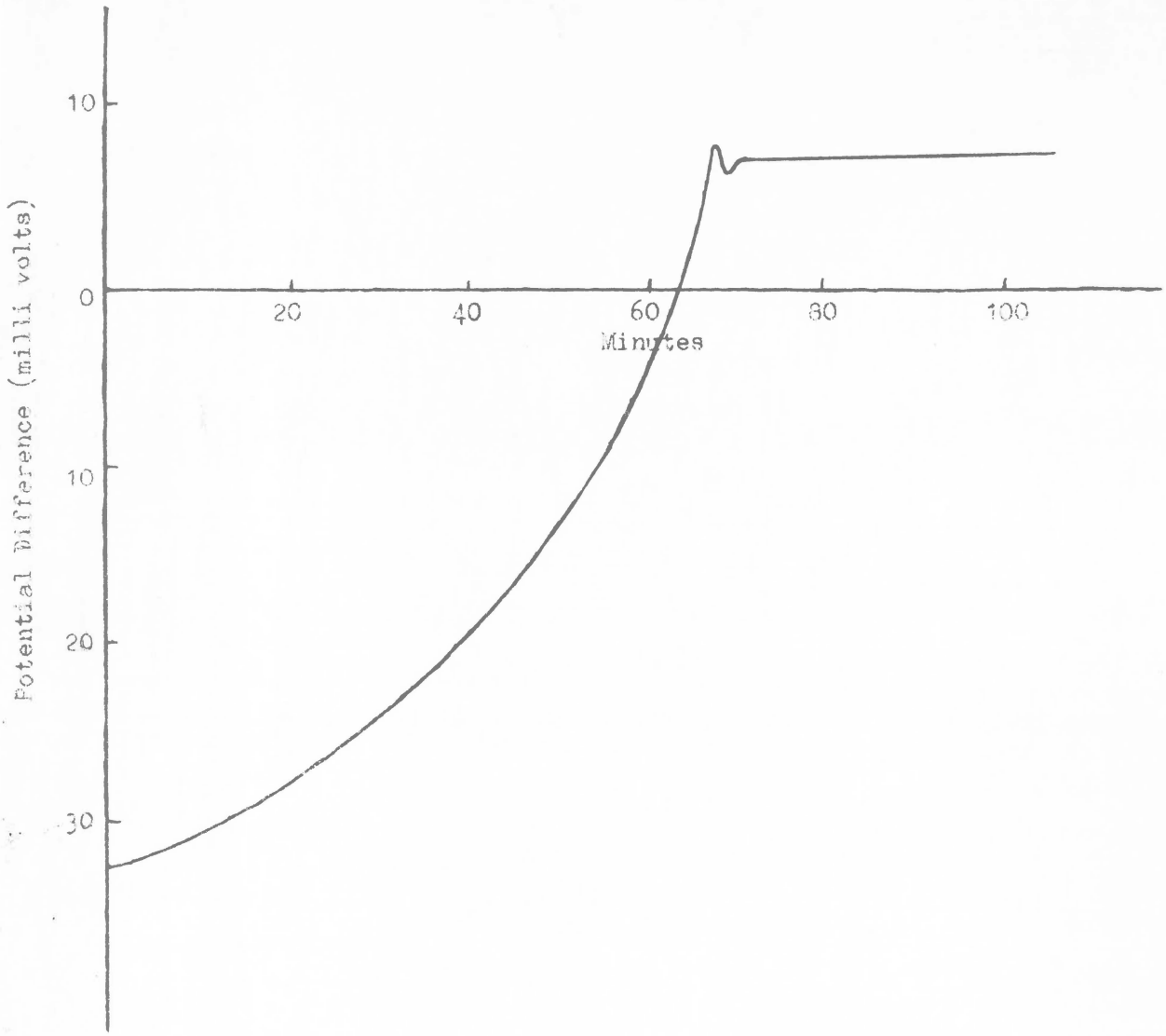

Figure 4.1 The potential change recorded when a microelectrode is pushed into the vacuole of a Chaetomorpha cell at t = o.

ential change. The negative potentials obtained were usually in the range -30 to -35 mV. and the results shown in the figure could be readily reproduced.

Walker, (private communication) using electrodes of different tip taper, measured potentials across the plasmalemma and tonoplast in Chaetomorpha. He found that when an electrode with a very obtuse taper and a tip diameter of 2 μ. was pushed into the cytoplasm, but not through the tonoplast, a potential of -75 mV. could be obtained. When an electrode with an oblique taper was pushed into the vacuole of Chaetomorpha, an immediate negative spike resulted which in this case built up more rapidly to +10 mV.

It is considered that the negative potential critically measured was the potential across the plasmalemma, while the +10 mV. eventually reached was the potential between vacuole and the solution. This means that the potential difference between cytoplasm and vacuole was +45 mV.

Because of difficulties in preparing the microelectrodes it was not possible to make one of a suitable taper that would penetrate only the plasmalemma. When the electrode is stuck into a cell, the cell wall is depressed by the tip and as more pressure is applied, the electrode passes through the cell wall and both membranes, into the vacuole. This meant that it was not possible to measure the potentials across the plasmalemma alone by this method. However it was found that if an electrode was pushed into the vacuole and the potential allowed to stabilise, then the electrode

could be slowly withdrawn so that its tip was in the cytoplasm. After a period of ten to twenty minutes the cytoplasm was able to "seal" the rupture in the tonoplast and the potential became negative. The phenomenon of cytoplasmic sealing was demonstrated by Walker (1955). Figure 4:2 shows the time course of the potential obtained by this method. The cytoplasmic potential usually did not fall to quite the same level as the initial reading, but it does confirm that the potential is a real effect probably due to the plasmalemma. The following table summarizes the data on the transmembrane potentials of Chaetomorpha.

TABLE 4:1

SUMMARY OF DATA OBTAINED FROM POTENTIAL MEASUREMENTS ON CHAETOMORPHA

The potential quoted is relative to the external solution.

Site of Electrode Tip	Potential measured	Potential	Number of Determinations
in vacuole	algebraic sum of tonoplast + plasmalemma	+10 ± 0.5	35
in cytoplasm	potential across plasmalemma	-32 ± 3.1	16

The concentration of potassium in the vacuole in equilibrium with seawater at a potential of +10 mV. would be 10

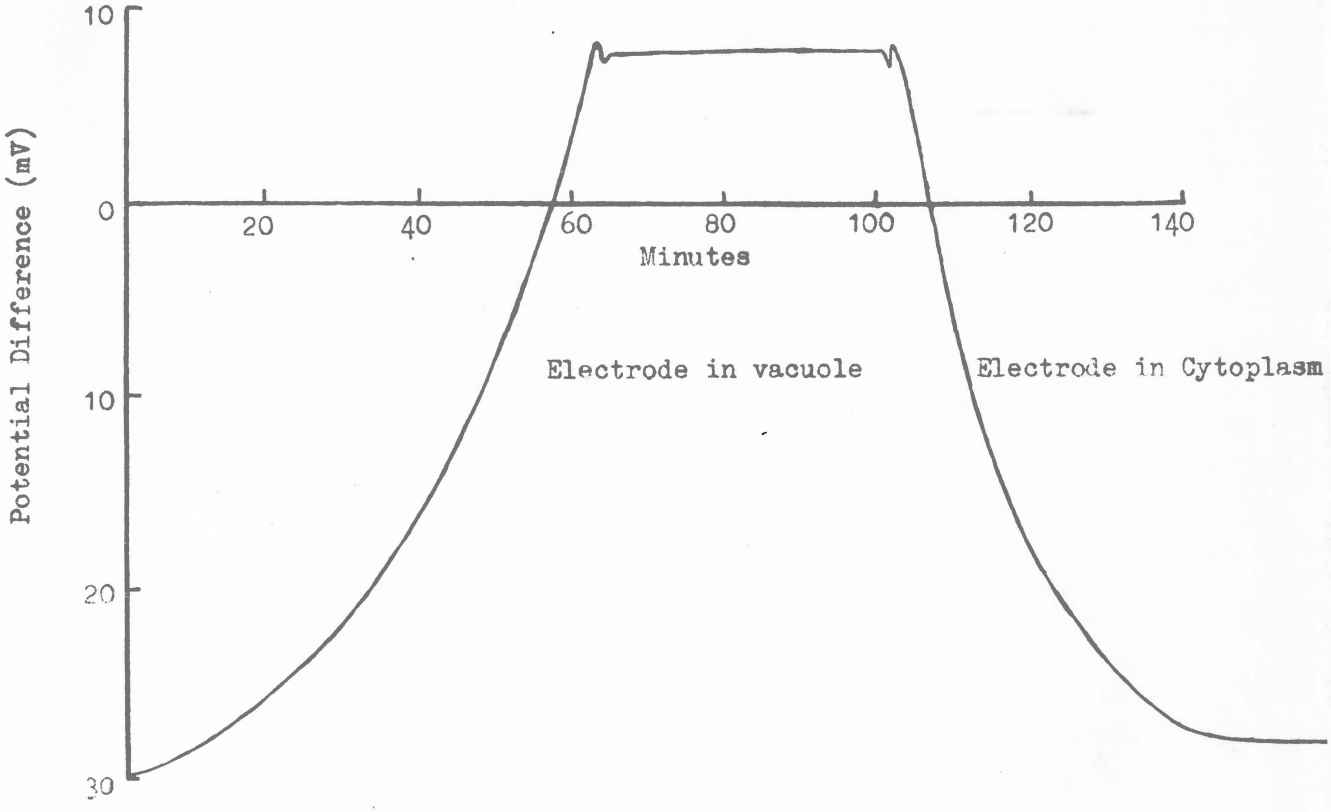

Figure 4.2 The potential changes recorded when an electrode tip is transferred from vacuole to cytoplasm accompanied by sealing of the tonoplast.

m.equiv./l; as the observed value was 541 m.equiv./l there must be a net inward flux of potassium. Similarly the sodium concentration expected in the vacuole if it is at +10 mV is 335 m.equiv./l and the low observed value shows that there is an active sodium efflux. The chloride concentrations do not provide conclusive evidence for a chloride pump as the observed concentration was 601 m.equiv./l and predicted concentration 845 m.equiv./l. This difference is equivalent to only a few mv. potential difference

CHAPTER V

SODIUM AND POTASSIUM FLUXES

The results of the experiments to measure isotope diffusing out of labelled tissue (using the method described above) were analysed using the method of MacRobbie and Dainty (1958). The logarithm of the rate of loss of radioactivity from the cell was plotted against time, a plot in which uniform exchange from a single compartment gives a straight line. Isotope efflux experiments with Chaetomorpha, as with Nitellopsis, were analysed and three compartments each having a distinct exchange constant could be distinguished. The bulk of the exchangeable ions in the cell were associated with a linear plot over a long time scale. (35 hours). But for several hours at the start of an experiment the rate of loss of activity was much greater than could be accounted for from the slow fraction. Subtraction of the slowest compartment from the total activity lost, gave a second straight line when plotted logarithmically, suggesting the

presence of a second compartment with a faster rate of exchange. In addition there was a rapid loss of isotope in the first few minutes of each efflux. Figure 5:1 shows a normal plot for the loss of isotope from Chaetomorpha tissue over forty hours. Figure 5:2 shows these results plotted logarithmically. It can be seen that the data of figure 5:1 has been resolved into at least two distinct phases, but the boundary between the free space and cytoplasmic phase is not clear. This plot can be made more sensitive and the boundary resolved if the contribution of the slowest (vacuolar) phase is subtracted from the total activity lost from the tissue (figure 5:3). Using this method, the data of figure 5:1 can be resolved into three reasonably distinct components. This three phase system has been substantiated for a number of plant cells, e.g. Nitellopsis obtusa (MacRobbie and Dainty 1958), Nitella translucens (MacRobbie 1962), Nitella axillaris (Diamond and Solomon 1958) and Beet parenchyma cells (Pitman 1963).

The rapid phase of exchange could be identified as the free space because there was a very high rate of exchange which is characteristic of the free space; the rate of exchange was not affected by very low temperatures; and in particular the ratio of $^{K}/_{Na}$ cations in this phase was reasonably close to the ratio present in seawater. Table 5:1 shows an analysis of this compartment based on isotope measurements.

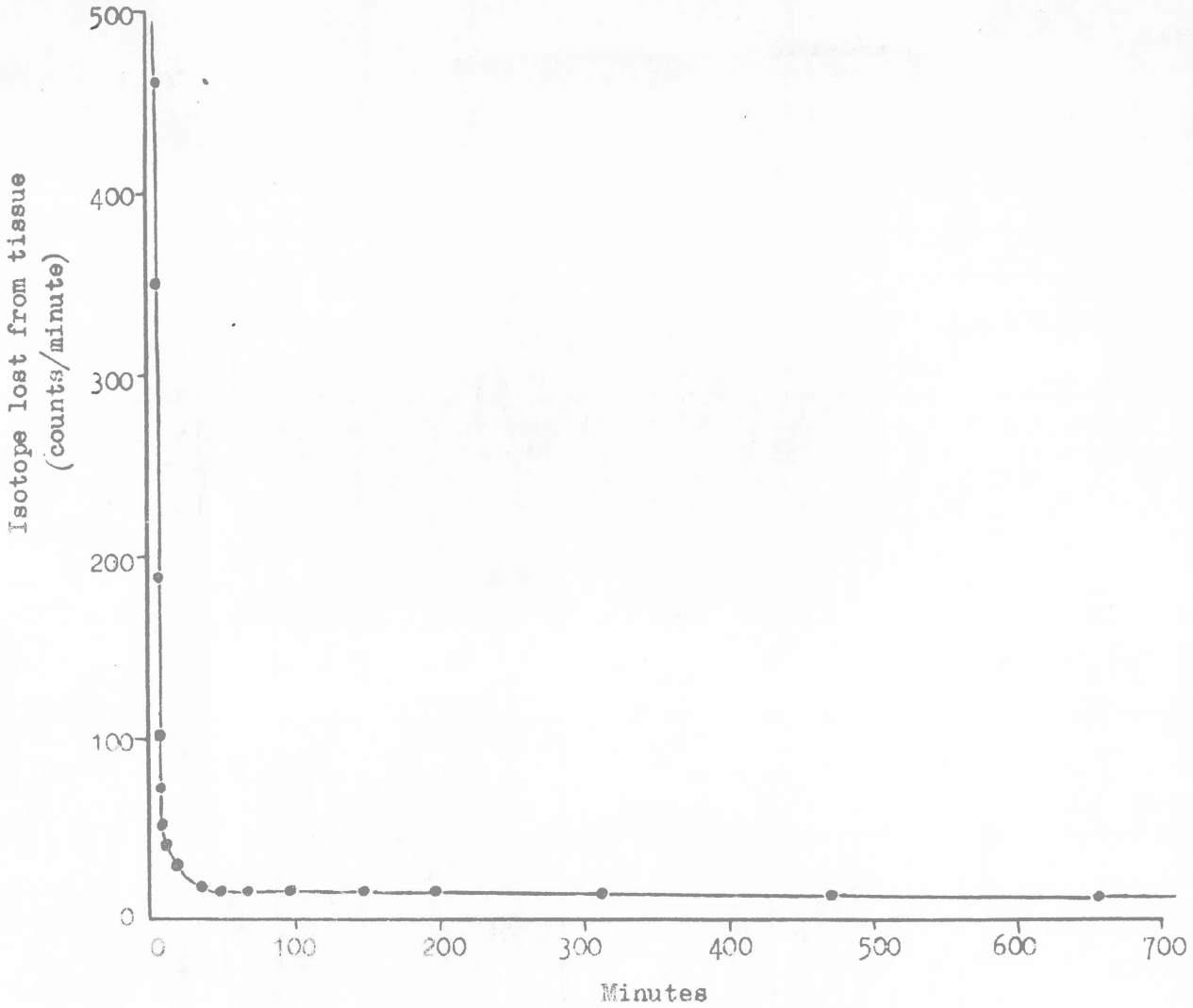

Figure 5.1 Standard Plot of the Relationship of Isotope loss vs. time in Chaetomorpha.

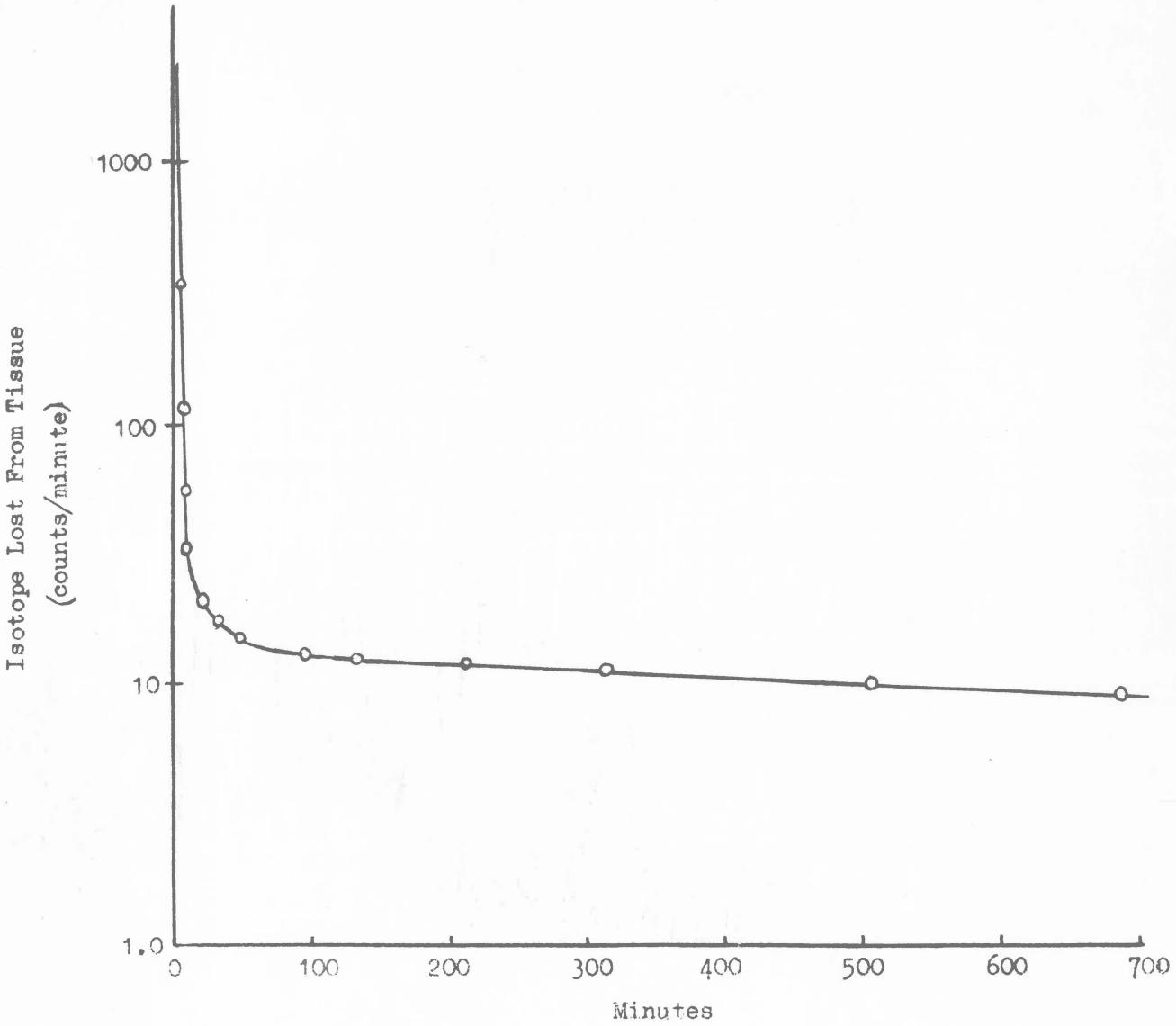

Figure 5.2 Logarithmic plot of isotope loss from tissue vs. time in Chaetomorpha.

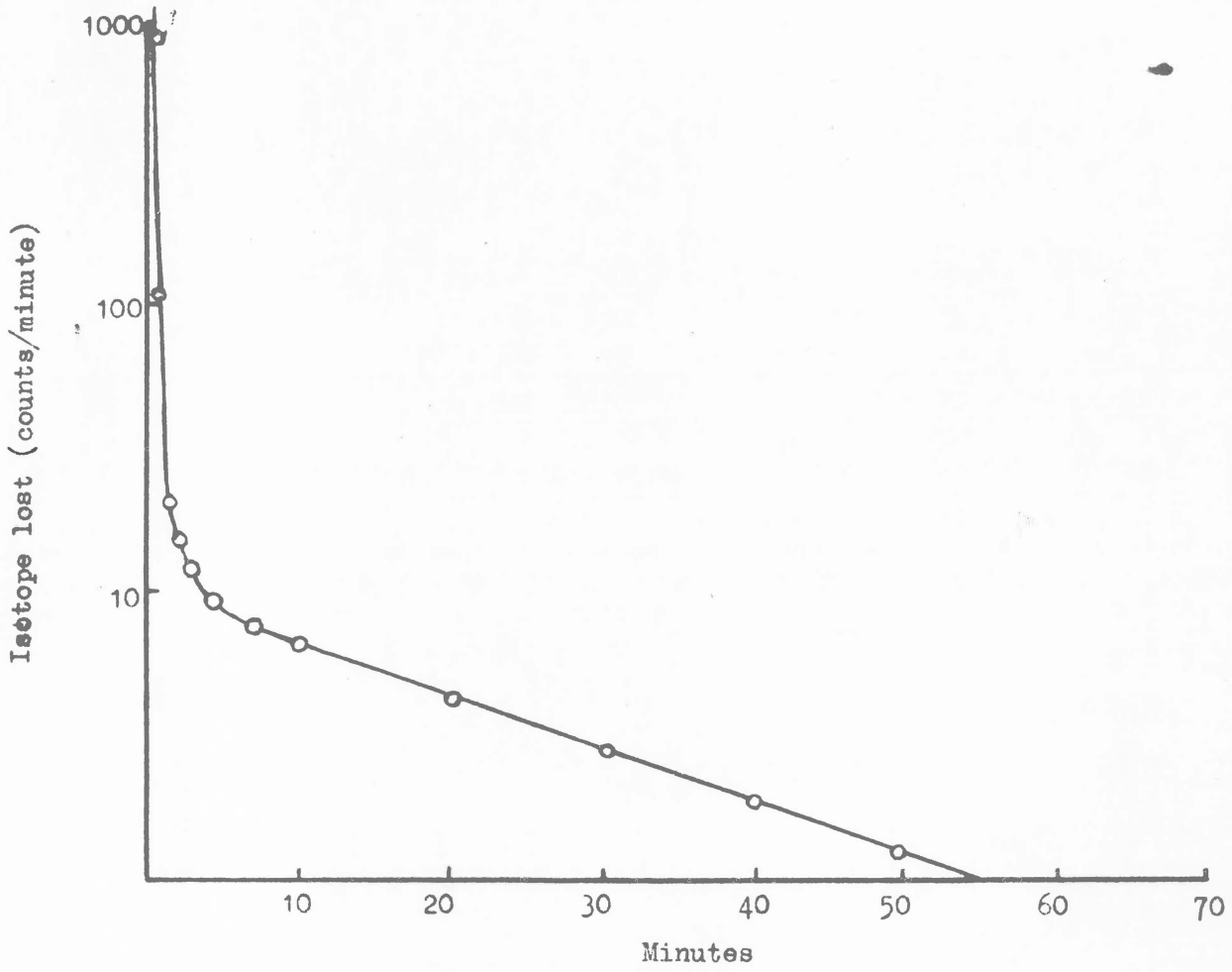

Figure 5.3 Logarithmic plot of isotope loss from the free space and cytoplasmic phase in Chaetomorpha.

TABLE 5:1

ANALYSIS OF THE APPARENT FREE SPACE BASED ON ISOTOPE MEASUREMENTS, SHOWING RATE OF EXCHANGE, THE CONCENTRATION AND VOLUME OF THE A.F.S.

Ion	½ time for exchange (secs)	Conc. of ion in A.F.S. (ue/gm F.W.)	Volume of A.F.S. (cc/gm F.W.)	Ratio K/Na of A.F.S.	Ratio K/Na in Seawater	Number of Analyses
Potassium	14.7±1.1	1.1±0.3	0.101±0.01	1/42	1/36	7
Sodium	16.8±1.0	46.5±3.0	0.098±0.017			9

The free space in Chaetomorpha probably comprises the cell walls and not the cytoplasm, as potential measurements showed a large potential difference across the plasmalemma.

The two other components were temperature sensitive and together comprise the non free space. One component was estimated from graphs such as figure 5:3, and the other slower phase from figure 5:1.

The rapid non free space component had a half time of exchange that was increased by reduction in temperature and contained much more potassium than sodium. Table 5:2 summarises observations of time for half exchange and apparent content of this component.

There is too little cytoplasm in cells of Chaetomorpha for it to be demonstrated that the faster component of the isotope exchange is due to the cytoplasm. Hence what is called "cytoplasmic component" is located in this region mainly by analogy with Characean cells. But as the other parts of the cells (vacuole and cell wall) are accounted for this assumption is not unreasonable.

The slowest component was identified with the vacuole because the rate of fall of the slowest efflux phase corresponded with the rate of fall of specific activity of the tissue. Separate measurements on the vacuolar sap showed that the specific activity of the whole tissue corresponded closely with that of the vacuole during the later stages of elution. The rate of ex-

TABLE 5:2

ANALYSIS OF THE CYTOPLASMIC PHASE BASED ON THE EFFLUX OF ISOTOPE FROM *CHAETOMORPHA* TISSUE

Ion	$t^{\frac{1}{2}}$ (mins)	Range	Number of experiments	u.equiv$^{\#}$/gm apparent content	Ratio of $t^{\frac{1}{2}}$ at 17° and 2°C
Potassium	35 $\pm$1.7	18-86	25	22 $\pm$1.2	2
Sodium	6.0$\pm$0.13	4-9	14	0.3$\pm$0.01	-

change from the vacuole was quite slow; a half time of about 50 hours for potassium exchange and 30 hours for sodium exchange were normal values.

The experimental results that can be obtained from this approach are

(1) Apparent uptake

(2) Apparent efflux

(3) Apparent content of the cytoplasmic phase

(4) Rate constant for exchange of the cytoplasmic phase

(5) Rate constant for exchange of the vacuolar phase

Estimation of Fluxes

Fluxes were calculated from these data by the method given by Pitman (1963), though in the present example the tissue was in flux equilibrium and the net flux was zero. Hence the following relationships can be used when the rate of change of isotope in the cytoplasmic phase is small

(1) Uptake of isotope is $(\phi_1 S_0 - \phi_1 S_2)$

which is equal to $(\phi_2 S_2 - \phi_2 S_3)$

hence $S_2 = \dfrac{\phi_1 S_0 + \phi_2 S_3}{\phi_1 + \phi_2}$

and net tracer uptake (apparent uptake) is $\dfrac{\phi_1 \cdot \phi_2 \cdot (S_0 - S_3)}{\phi_1 + \phi_2}$

(2) The apparent content estimated from graphs such as figure 5:4 is equal to

$$\frac{\phi_1}{\phi_1 + \phi_2} \cdot Q_2 \cdot \left\{ \frac{S_2 - S_3 \cdot \phi_2}{\phi_1 + \phi_2} \right\}$$

or $$\frac{\phi_1^2}{(\phi_1 + \phi_2)^2} \cdot Q_2 \cdot S_0$$

As the rate constant for the cytoplasmic phase, K_1, is equal to

$$\frac{\phi_1 + \phi_2}{Q_2}$$

the term $\frac{\phi_1^2}{\phi_1 + \phi_2} \cdot S_0$ can be estimated

(3) The apparent efflux is $\frac{\phi_1}{\phi_1 + \phi_2} \cdot (\phi_2 \cdot S_3)$

From these relationships ϕ_1, ϕ_2 and Q_2 can be calculated

This method assumes that (1) the fluxes do not change during the experiment and that net flux is zero, (2) the three phases are arranged in series. The first assumption is reasonable and is supported by measurements of isotope uptake and of potassium or sodium content. There is no unequivocal evidence for the second assumption, though it is appropriate to the cellular organisation.

Potassium fluxes

The apparent uptake of potassium was much larger than

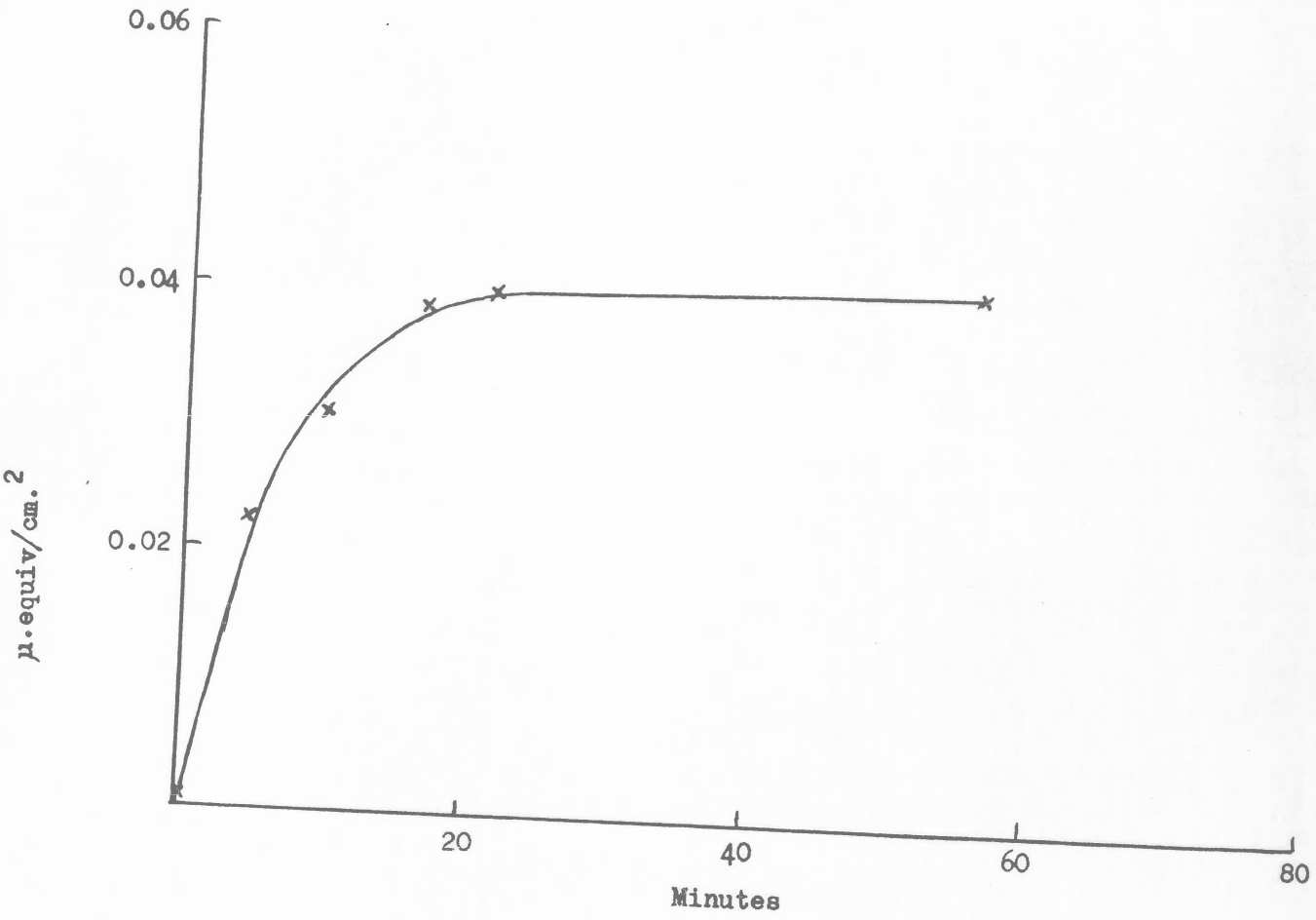

Figure 5.4 Uptake of potassium isotope into the cytoplasm of Chaetomorpha.

that of sodium, the usual value being about 80 - 120 p.moles. $cm^{-2}.sec^{-1}$ but values of up to 240 p.moles.$cm^{-2}sec^{-1}$ and as low as 40 p.moles.$cm^{-2}sec^{-1}$ were measured.

The determination of the fluxes of potassium was much less satisfactory than of sodium. The graphs of log (Y) vs. t and log ($^{dY}/_{dt}$) vs. t were treated to yield separable components, but the subsequent calculation of β_1, β_2 and Q_2 led to some ridiculously high values of Q_2. Some values are summarised in Table 5:3. For example, if Q_2 were 90 µ.equiv./gm then the concentration in the cytoplasm would be equivalent to 1500 µ.equiv./ml, a concentration three times larger than was found in the vacuole by direct analysis.

TABLE 5:3

SOME OF THE VALUES OBTAINED FOR Q_2 WITH THE EQUIVALENT CONCENTRATION IN THE CYTOPLASM

Q_2 µ.equiv./gm	Number of determinations	Concentration in Cytoplasm µ.equiv./ml
99 ± 7.5	5	1540
90 ± 9.0	2	1500
68 ± 5.3	4	1050
29 ± 12.1	4	450

The primary data from such an experiment are shown in figure 5:5 plotted as (a) log (Y) vs. t and (b) log ($^{dY}/_{dt}$) vs. t. The overestimation of Q_2 is mainly due to the persistent curve in (b), which is a more sensitive estimate than (a) of the rate constant k_1.

The equation for isotope loss from the tissue includes the assumption that,

isotope flux = flux x specific activity in bulk of phase

When fluxes are large compared with diffusion in the phase, the specific activity at the surface could be very different from that in the bulk of the phase and the simple equation is no longer applicable. This difference is suggested as an explanation for the very large values of Q_2 when apparent uptake was high. In these conditions observed isotope diffusion out of the tissue would be smaller than expected and the time for exchange of the cytoplasmic phase would be increased, making k_1 an underestimate. In this case the most reliable estimates are the lowest ones, i.e. about 17 to 20 µ.equiv./gm, but even these may be too large. Taking the cytoplasmic volume as 3% these estimates give reasonable potassium concentrations in the cytoplasm of 500 - 600 µ.equiv./ml. Fluxes in this particular experiment were at the low end of the range (ϕ_1= 60, ϕ_2= 100) and it must be recognised that higher values are found. As apparent uptake is equal to $\phi_1 \cdot \phi_2/\phi_1 + \phi_2$ both ϕ_1 and ϕ_2 must be larger than the observed

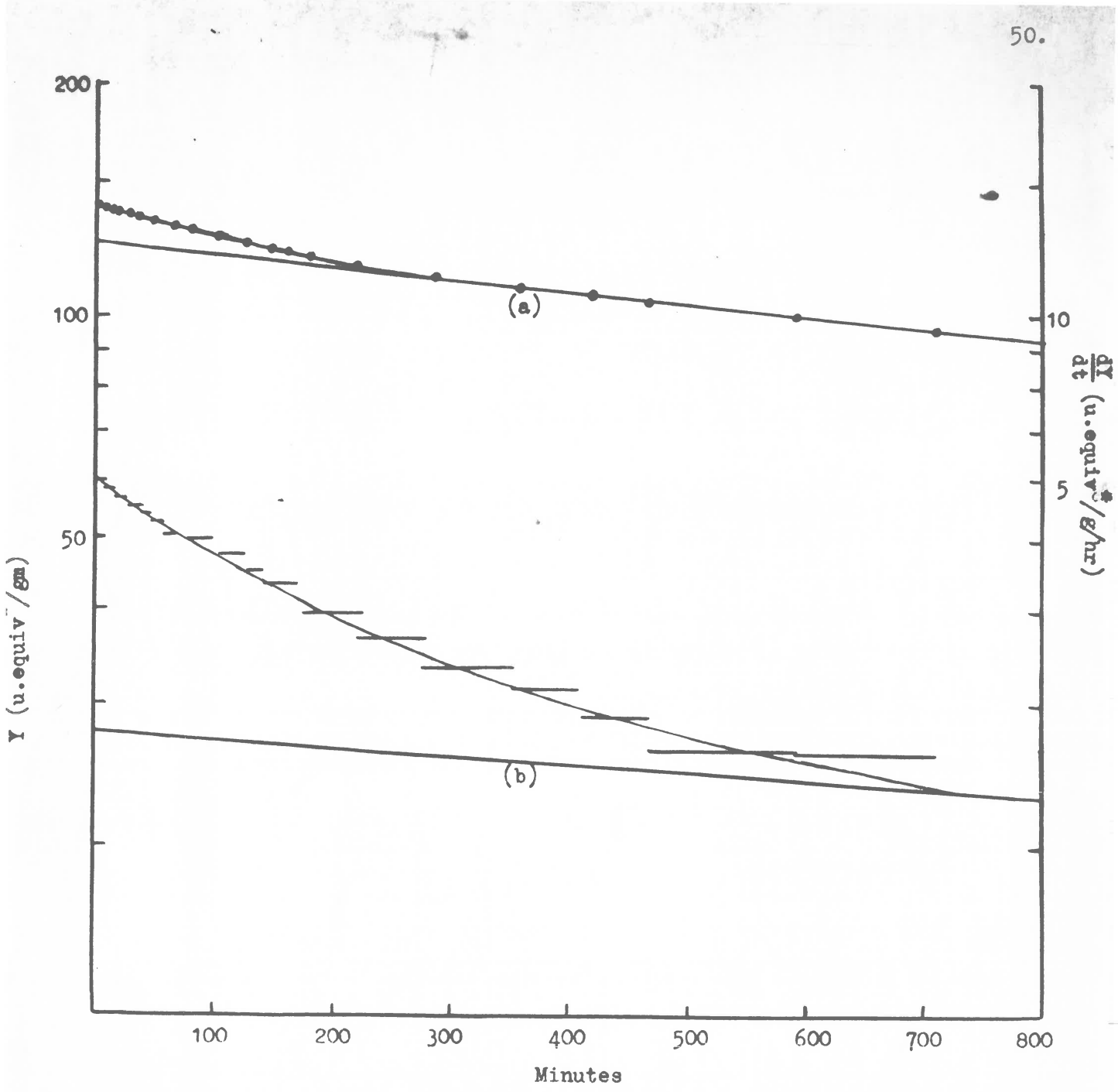

Figure 5.5 Efflux of isotope from Chaetomorpha.

(a) Log (Y) vs time and (b) Log $\frac{dY}{dt}$ vs time

$Y = \mu.equiv^*/gm$ $\frac{dY}{dt} = \mu.equiv/gm/hr$

apparent uptake and in some cases over 160 p.moles.cm.$^{-2}$ sec.$^{-1}$.

Spanswick and Williams (1964) in a direct analysis of cell components found that Nitella translucens had a higher concentration of potassium in the cytoplasm than in the vacuole. It was suggested by MacRobbie (1962) that the organelles of the cytoplasm may be able to concentrate ions by utilising pumps at their surface membranes. This could explain the higher values of potassium obtained in both Nitella and Chaetomorpha.

The chloroplasts, because of their size were thought to be the only organelles large enough to retain such a high concentration of potassium. To investigate this possibility, chloroplasts and cell fractions were isolated in isotonic sucrose at 4°C using a density gradient method of separation. (Pierpoint 1962). Subsequent cation analyses on these fractions suggested that there was not a significantly high $^{K}/_{Na}$ ratio in the chloroplasts of Chaetomorpha.

Sodium Fluxes

The uptake of tracer sodium (apparent sodium uptake) was only 3 - 4 p.moles*.cm.$^{-2}$sec.$^{-1}$, and after 13 hours in labelled sea water the amount of tracer in the vacuole and cytoplasmic phase was a small proportion of the total in the tissue, due to the large free space content (40 u.equiv./gm.). However it was possible to make a good separation between free space and cytoplasmic phase as the half time for free space exchange, (10 seconds)

was short compared with that of the cytoplasmic phase (6 mins.). The distinction between cytoplasmic phase and vacuole was also clear and the graphs of log (Y) vs. t and log ($^{dY}/_{dt}$) vs. t both rapidly become straight lines of the same slope (k_2). In all, determinations of k_1, k_2, A and B were most satisfactory. Estimates of fluxes and sodium content of the cytoplasmic phase are given in the following tabulation; the values are means of six separate determinations.

TABLE 5.4

SODIUM FLUXES AND CONTENT OF THE CYTOPLASMIC PHASE OF CHAETOMORPHA

ϕ_1 p.moles.cm^{-2}sec^{-1}	ϕ_2 p.moles.cm^{-2}sec^{-1}	Content μ.equiv/gm	Apparent Uptake p.moles.cm^{-2}sec^{-1}
110 ± 12	3.7 ± 0.4	1.15 ± 0.2	3.6

The low apparent uptake is clearly due to the low flux across the tonoplast, but this low flux is a result of the low sodium concentration in the cytoplasmic phase. There is a good reason to believe that the inward movement of sodium is passive, so the permeability of the outer membrane P_1, can be defined by

$$\phi = - P_1 \cdot \frac{zEF}{RT} \cdot \frac{C}{1 - \exp \frac{zEF}{RT}}$$

where C is the external concentration and E the potential of the inside relative to outside, say -35mV. The permeability of the inner membrane for sodium can be defined by a similar expression using vacuolar concentration and the potential between vacuole and cytoplasm, i.e. -45mV. In this case P_1 is 1.2×10^{-7} and P_2 is 0.7×10^{-7} cm.sec.$^{-1}$. The average concentration of sodium in the cytoplasmic phase would be between 60 and 120 u.equiv. per ml. if volume is 1 - 2%.

The ion exchange from Chaetomorpha tissue can be analysed as shown above. A large number of experiments have shown that the sodium fluxes and content can be estimated with good reproducibility, however the potassium fluxes and content were more variable and difficult to estimate because, (as suggested above), the diffusion out of the tissue is smaller than expected, resulting in an underestimate of k_1.

CHAPTER VI

METABOLISM AND THE UPTAKE OF SODIUM AND POTASSIUM

The measurements of concentrations and potentials in the cells described above, show that sodium is actively pumped out of the cell and the potassium is pumped in. The relationship of these processes to metabolism was studied using inhibitors such as DNP, oubain, arsenite, cyanide and oligomycin. Three kinds of measurements were made. Firstly, the apparent uptake is related to the fluxes by the relation

$$\text{apparent uptake} = \frac{\phi_1 \cdot \phi_2}{\phi_1 + \phi_2} \qquad 6:1$$

This quantity can be measured for potassium by direct counting of the cells under an end window G-M tube. Secondly, the fluxes can be estimated in some conditions when there is no change in

fluxes over a reasonable period of time. Thirdly, the effect of inhibition on potentials can be measured.

Apparent Uptake of Potassium

The apparent uptake of potassium by cells of Chaetomorpha was very nearly linear over periods of about six hours (figure 6:1) and the rate of tracer uptake was easily measured.

TABLE 6:1

EFFECT OF TEMPERATURE ON APPARENT INFLUX OF K^{42} INTO CELLS OF CHAETOMORPHA

Temperature degrees C	Apparent influx u.e/gm/hr	Range	Number of determinations
10	1.83 ± 0.04	1.75 - 1.95	4
15	3.25 ± 0.042	2.6 - 4.1	4
20	5.9 ± 0.07	5.4 - 6.2	4

This uptake was very sensitive to temperature as shown in Table 6:1, which gives the rate of uptake at different temperatures. The temperature coefficient (Q_{10}) in the range 10 - 20°C was about 3.2. The values given in this table are a little low and are equivalent at 20°C to about 100 p.moles/cm^2/sec.

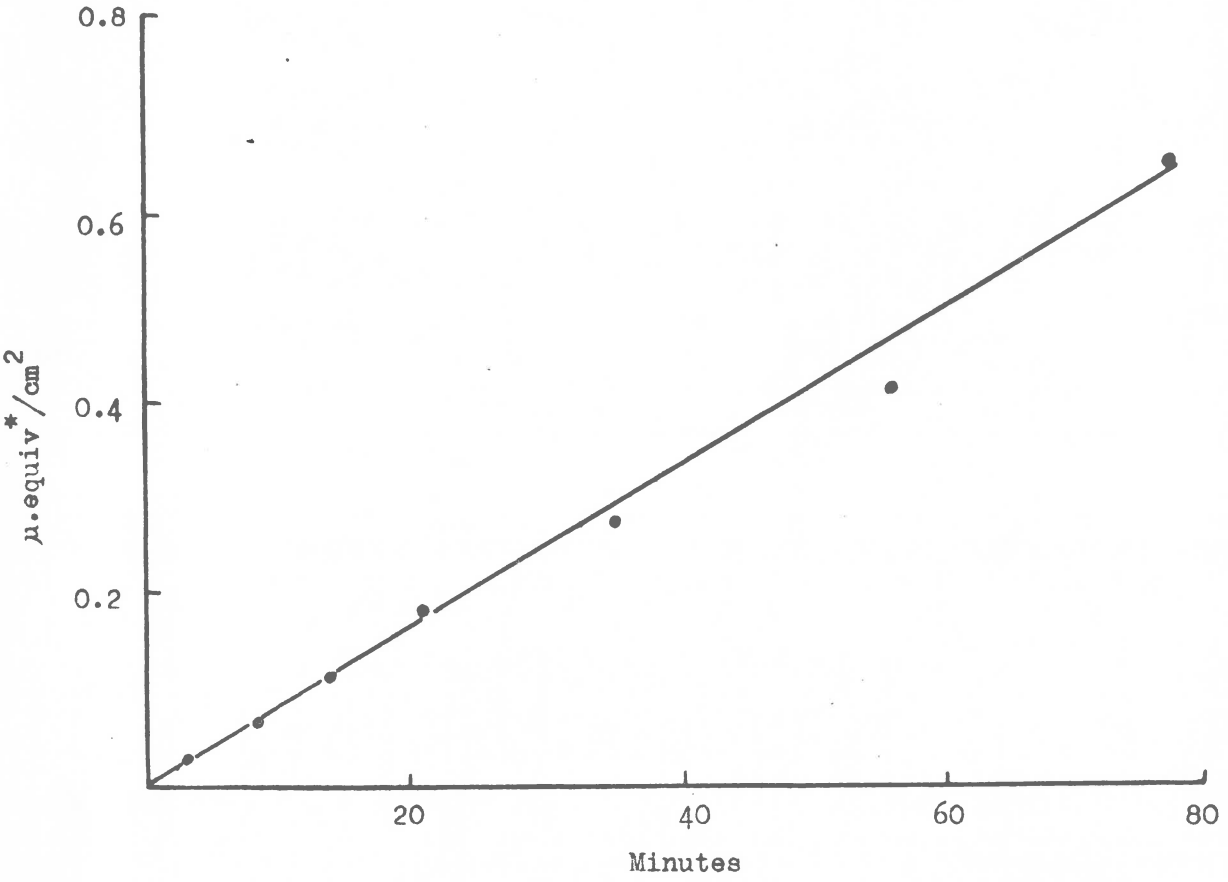

Figure 6.1 Uptake of potassium isotope into the cell vacuole of Chaetomorpha.

Earlier workers on ion balance in plant cells had been able to show that a change in the experimental conditions from light to dark in some cases resulted in a big change in the fluxes, particularly potassium. (Scott and Hayward 1953 and 1954), (Appley 1957) and (MacRobbie and Dainty 1958). Several experiments with Chaetomorpha on the uptake of K^{42} and efflux of K^{42} and Na^{24} in light and dark conditions failed to show any dependence on light.

Light had an effect on potassium and sodium transport when nitrogen was present instead of air. This behaviour is shown in figure 6:2 as the effect of nitrogen in light and dark on the maintainance of ion balance in Chaetomorpha.

In early experiments commercial nitrogen was bubbled through sea water containing tissue. When sap samples were analysed as in the DNP experiment, it was found in replicates in the dark and in the light, that the ion balance was unchanged. It was thought that commercial nitrogen may have contained sufficient oxygen to allow oxidation of the cytochrome system. Alkaline pyrogallol was used in an effort to trap the "oxygen" but experimental result was unchanged. However when medical nitrogen was used there was a definite change in ion balance in the dark. (figure 6:2)

Because the chloride in the vacuole is in approximate electrochemical equilibrium with the chloride in sea water, very

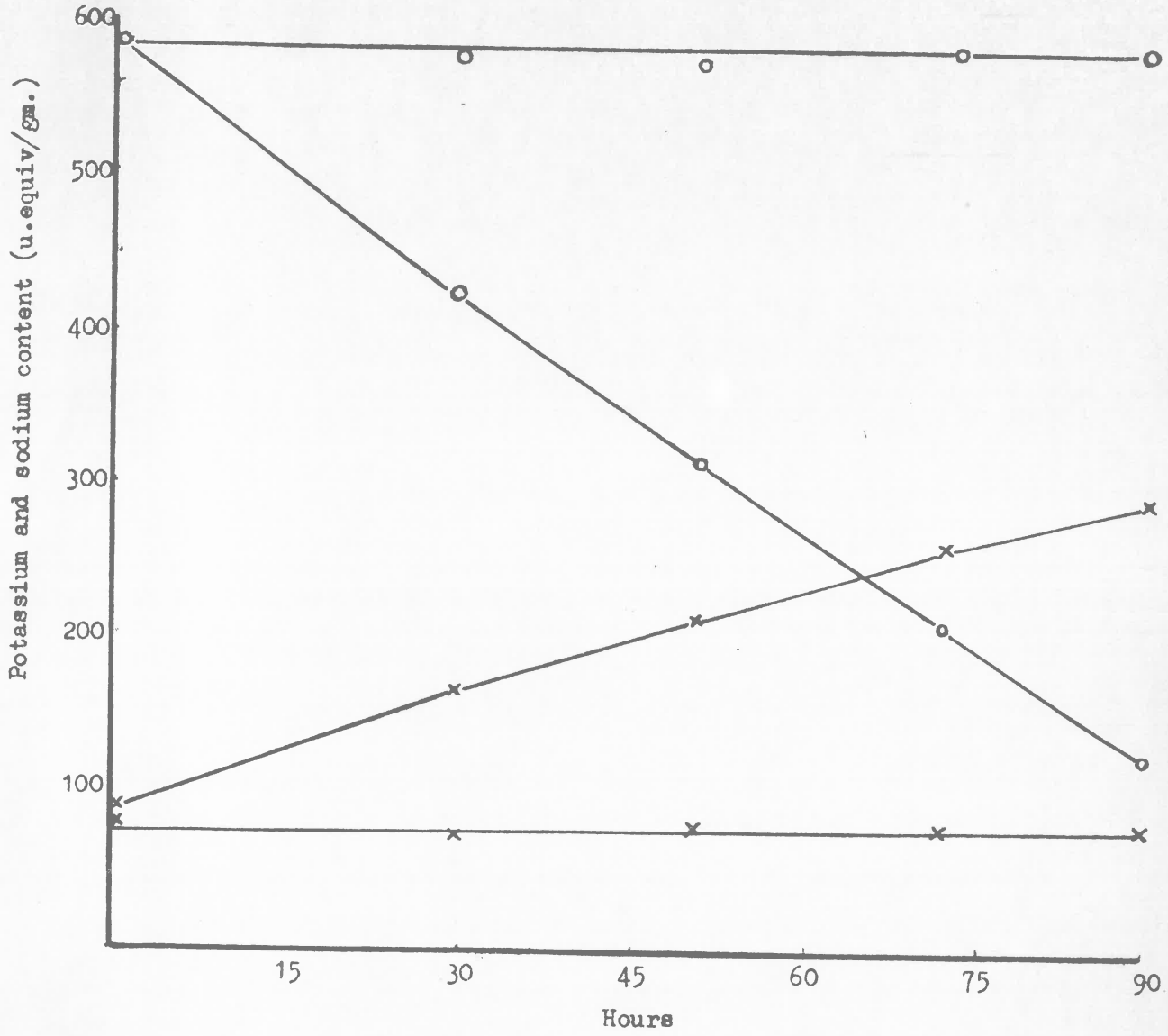

Figure 6.2 Effect of Nitrogen in the light and dark, on potassium and sodium content of cells of Chaetomorpha.

little change could be expected in this ion so analyses included only the sodium and potassium ions, both of which have very strong electrochemical potential gradients. This nitrogen inhibition effect in the dark can be released immediately by illumination or by bubbling oxygen instead of nitrogen.

Nitrogen is a specific block for any metabolic pathway having an oxidase, (usually the cytochrome system) where oxygen is required as the acceptor for hydrogen. It seems likely that the release of inhibition of uptake was due to oxygen formation in the light and its use as a hydrogen acceptor in respiration, and not to provision of alternative sources of ATP.

Inhibition of uptake was also produced by DNP. Early experiments using a range of DNP concentration in sea water were unsuccessful because the pH fell to 3.6 and tissue soon perished. An acetate buffer was subsequently added at a concentration of 2.85×10^{-3}M. This had the effect of maintaining pH at 5.7, in the physiological range for growth of Chaetomorpha and in the range of ionization of DNP. (Stenlid 1958).

In subsequent experiments the sodium acetate-acetic acid buffer was used at 2.85×10^{-3}M concentration and no detrimental effects were observed over long periods.

To measure the effect of DNP on the influx a steady rate of uptake was established in normal sea water, after which the DNP treatment was applied. The inhibition effect produced

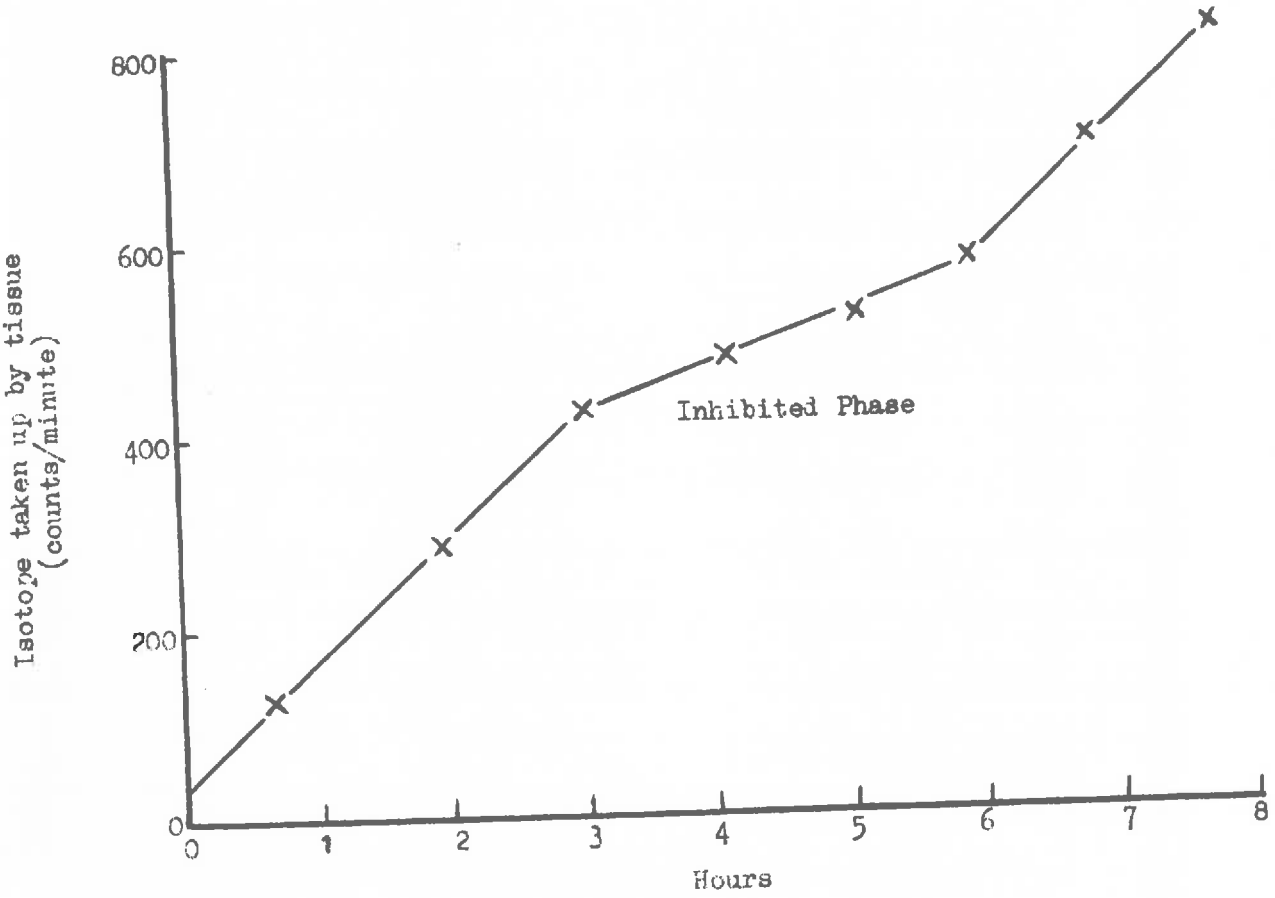

Figure 6.3 The effect of 10^{-4}M DNP on the influx of K^{42} in Chaetomorpha tissue. The inhibition produced over this time scale was completely reversible.

by the DNP could be reversed (figure 6:3).

DNP also inhibited the efflux of isotopic potassium from the tissue. Cells that had taken up tracer were put into unlabelled solution and the rate of loss of isotope measured.

TABLE 6:2

EFFECT OF BUFFER IN CONTROLLING pH OF MEDIUM FOR CHAETOMORPHA EXPERIMENTS USING 2.85×10^{-3}M SODIUM ACETATE BUFFER AND DNP AT 10^{-4}M

	pH	Range	No. of replicates
sea water	7.4	7.4	3
sea water + DNP	3.6 ± .08	3.4 - 3.7	3
sea water + buffer	5.7 ± .02	5.7 - 5.9	3
sea water + DNP + buffer	5.8 ± .03	5.6 - 5.9	3

When the rate of loss had fallen to a relatively steady value, the tissue was put into a DNP-seawater solution for 4.5 hours and then put back into the sea water. Figure 6:4 shows that the efflux was inhibited very quickly after addition of DNP but that the effect was reversible and after removal of DNP, the efflux rose again to a value close to that before inhibition.

The effect of 10^{-4}M DNP on general ion balance was measured by placing strands of cells of Chaetomorpha in a buf-

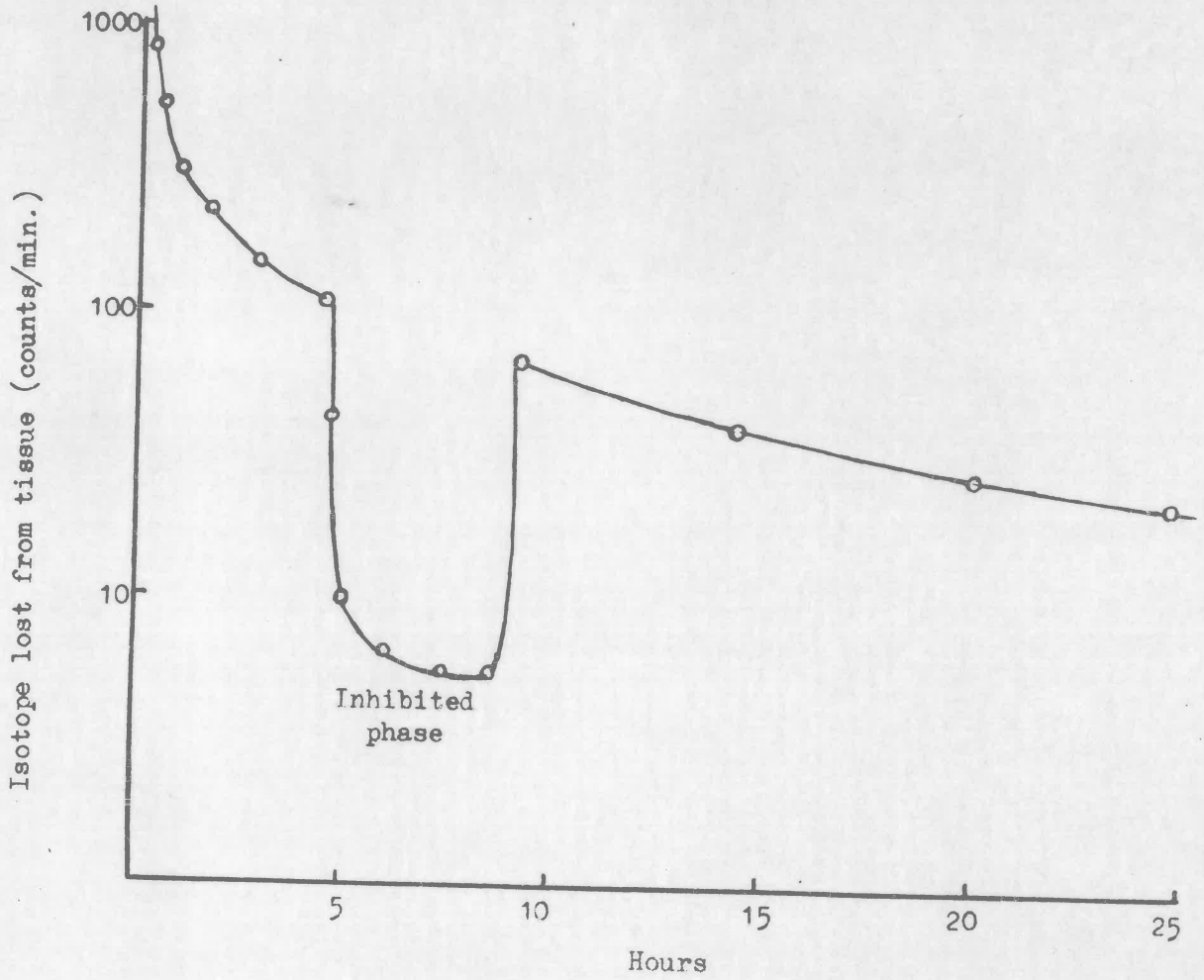

Figure 6.4 Effect of 10^{-4}M 2-4 Dinitrophenol on the efflux of K^{42} from Chaetomorpha tissue.

fered DNP seawater which was aerated. At suitable time intervals, strands of cells were removed and the sap sampled by means of a glass micropipette. Approximately 0.2 cc of cell sap was extracted and after suitable dilution, direct analysis of potassium, sodium and chloride was carried out. The results in figure 6:5 show data from one of many experiments on DNP effect on ion balance, which all gave similar results.

After 80 hours in the DNP-seawater, unsuccessful attempts were made to reverse the "effect". Until 35 hours after the start of this experiment, DNP-seawater could be replaced with natural seawater without any ill effects to the tissue. Thus effects encountered after forty hours in DNP-seawater appear to be due to tissue damage, perhaps membrane breakdown. The concentration of both cations changes markedly because there is a large electrochemical potential gradient for these ions across the membranes. The chloride concentration gradient is very small, hence drift is small.

Inhibition of apparent uptake and change in ion balance was also produced by 5 mM NaCN in seawater. The effect on uptake (figure 6:6) was readily reversible, but as found for DNP the ion content was unaffected for a period of about forty hours. The change in potassium and sodium levels that then occurred was irreversible (figure 6:7).

Arsenite effects on ion balance in Chaetomorpha were

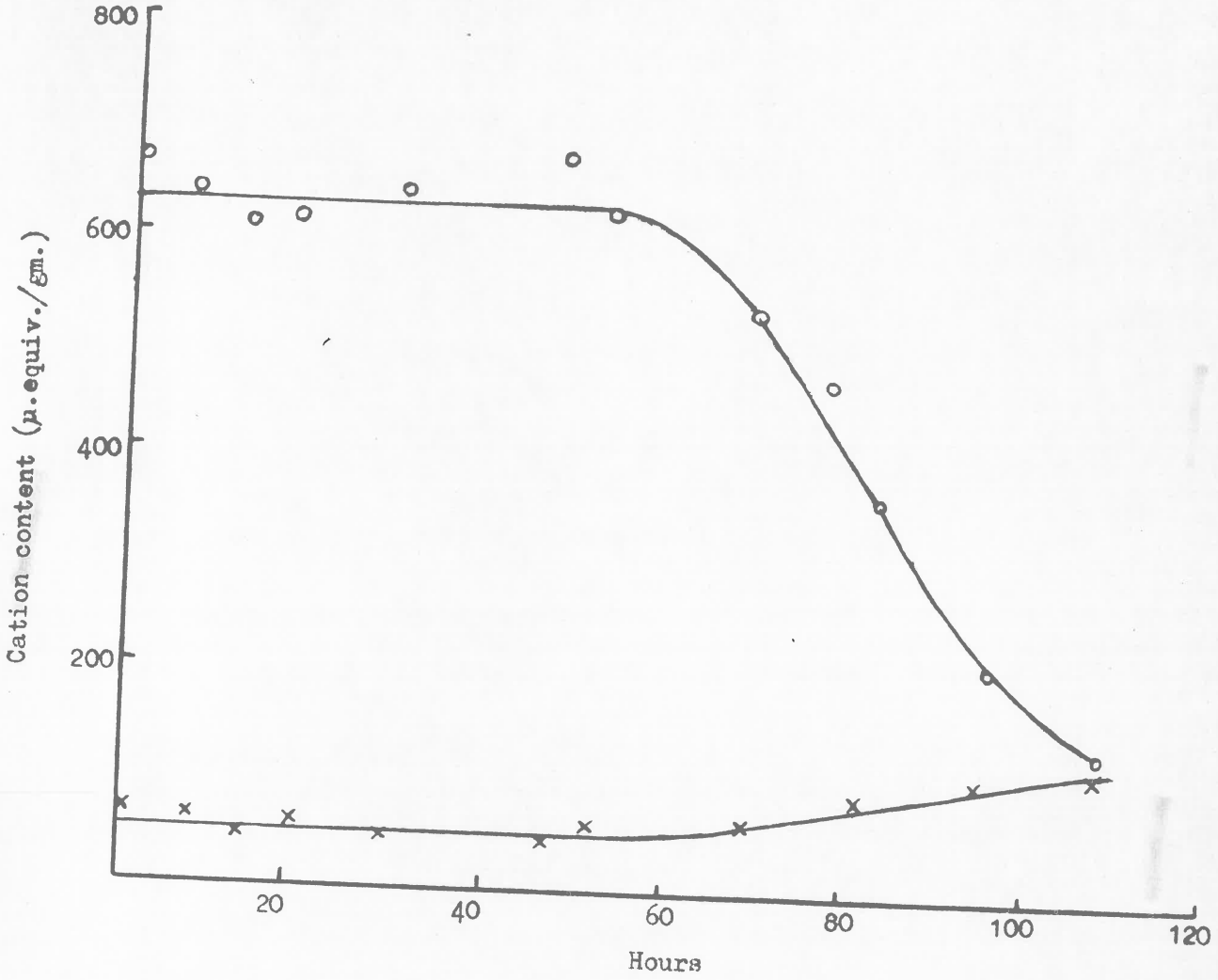

Figure 6.5 Effect of 2-4 Dinitrophenol on the ion content of Chaetomorpha darwinii.

(x) = sodium; (o) = potassium; (µ.equiv/gm Fresh Weight)

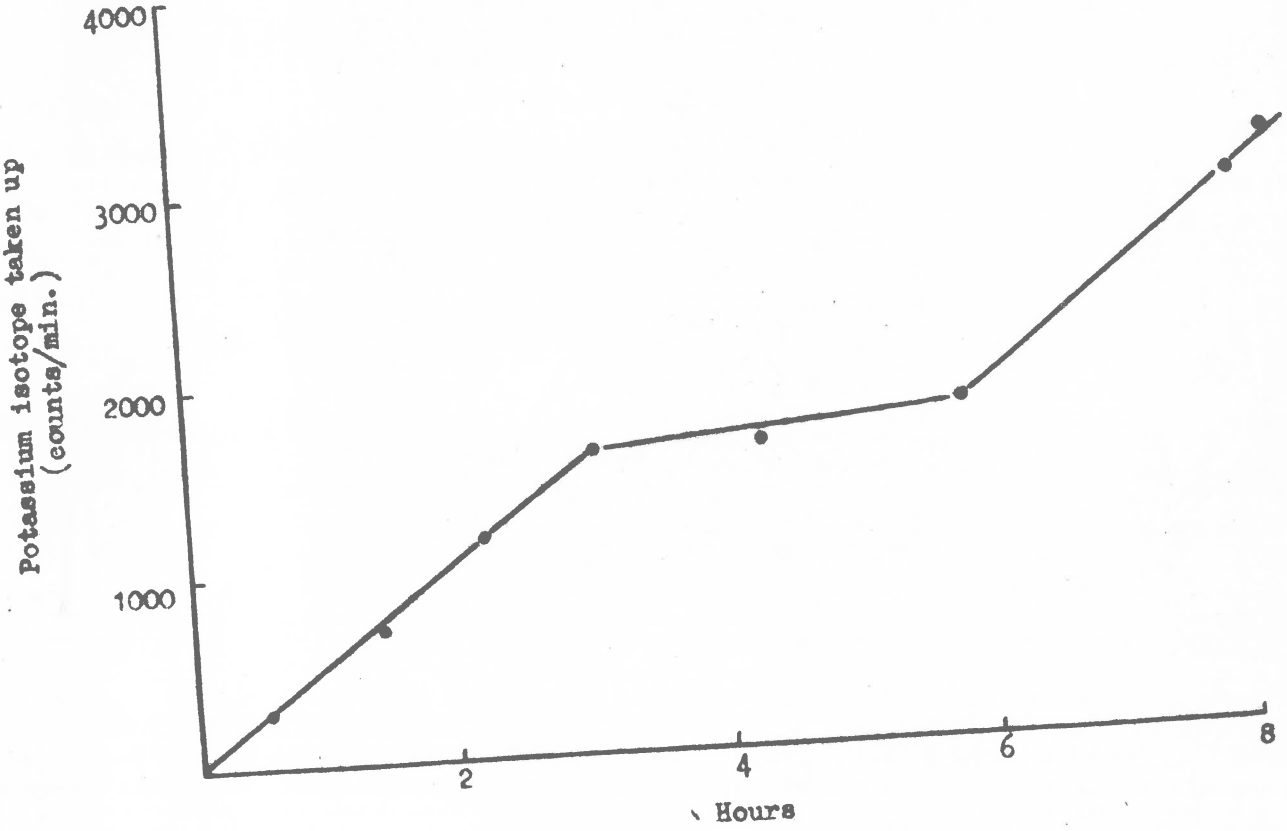

Figure 6.6 Effect of 5mM NaCN on the uptake of potassium isotope in Chaetomorpha.

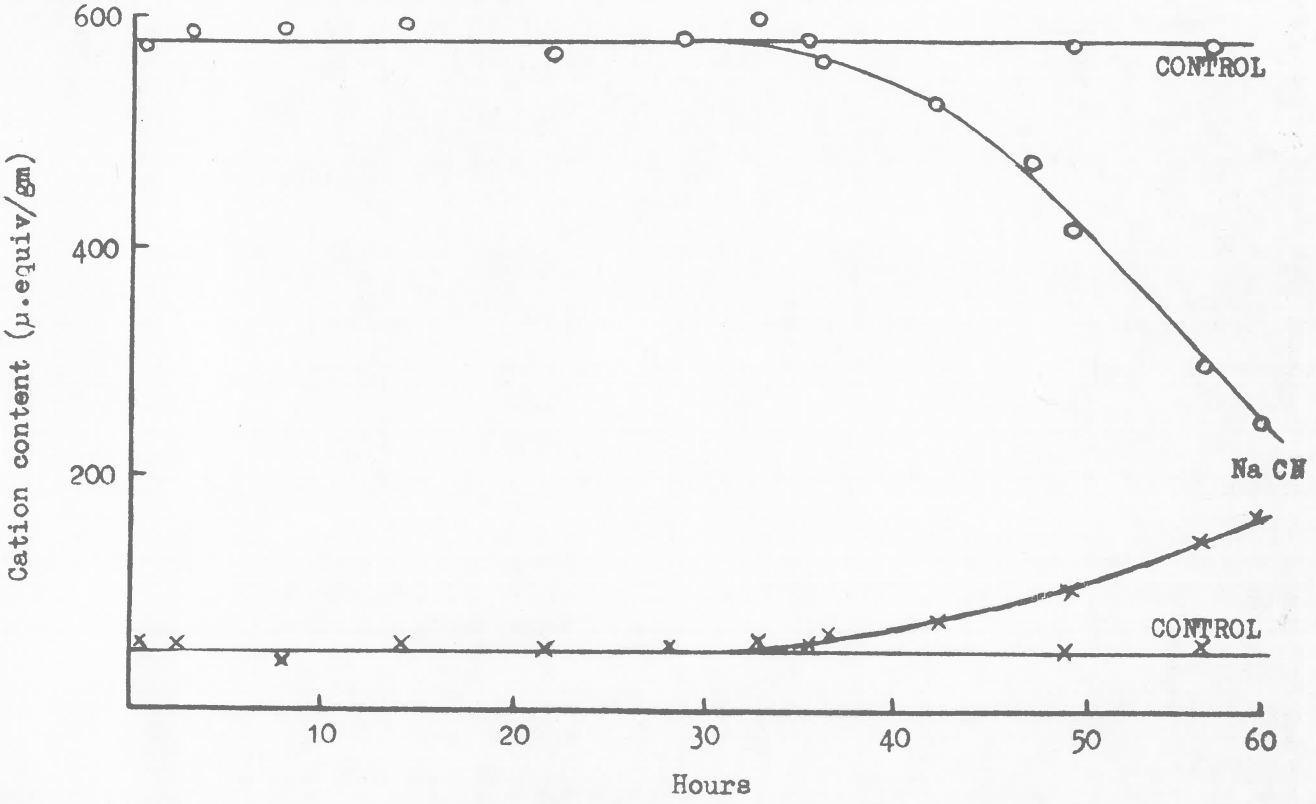

Figure 6.7 Effect of 5mM sodium cyanide on the ion content of Chaetomorpha.

(x) = sodium; (o) = potassium; (µ.equiv/gm Fresh Weight)

similar to those of DNP and NaCN, except that there was no rapid increase in the amount of sodium in the vacuole. The results of the inhibition effects by 3mM sodium arsenite on Chaetomorpha tissue are shown in figure 6:8.

Oligomycin is a specific uncoupler of the phosphorylation of ADP. Its effect on the ion balance of Chaetomorpha gave inconsistent results. Of five experiments, two gave results indicating that oligomycin did not significantly effect ion balance and the other three gave results as in figure 6:9. In these latter experiments the potassium distribution was changed but sodium balance was unchanged giving the same pattern as with arsenite.

The cardiac glycoside, oubain has been used in a wide range of tissues, both plant and animal, as a specific uncoupler of the sodium-potassium pump. However over a range of concentrations up to 20 milli molar oubain had no effect on the fluxes or ion content of Chaetomorpha over long periods. Experiments using the same oubain had shown that it was active in inhibition of the K-Na pump in human erythrocytes.

The effect of inhibitors on Cellular potentials

Chaetomorpha tissue was fixed in the perfusion cell, through which seawater was passing. An electrode was stuck into a cell and a constant vacuolar potential obtained. The following

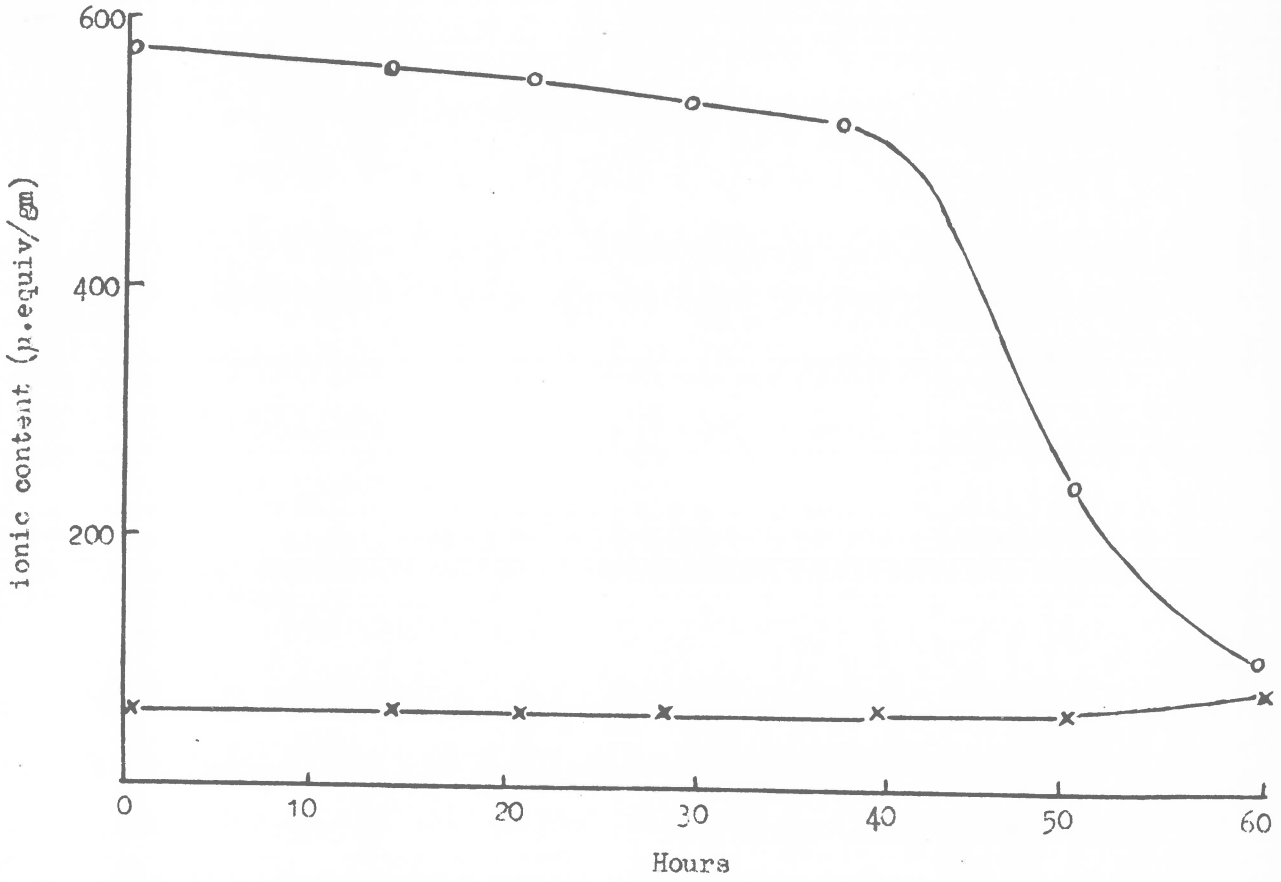

Figure 6.8 The effect of 3mM sodium arsenite on the ion balance of Chaetomorpha.
Mean of two experiments.
(x) = sodium; (o) = potassium.

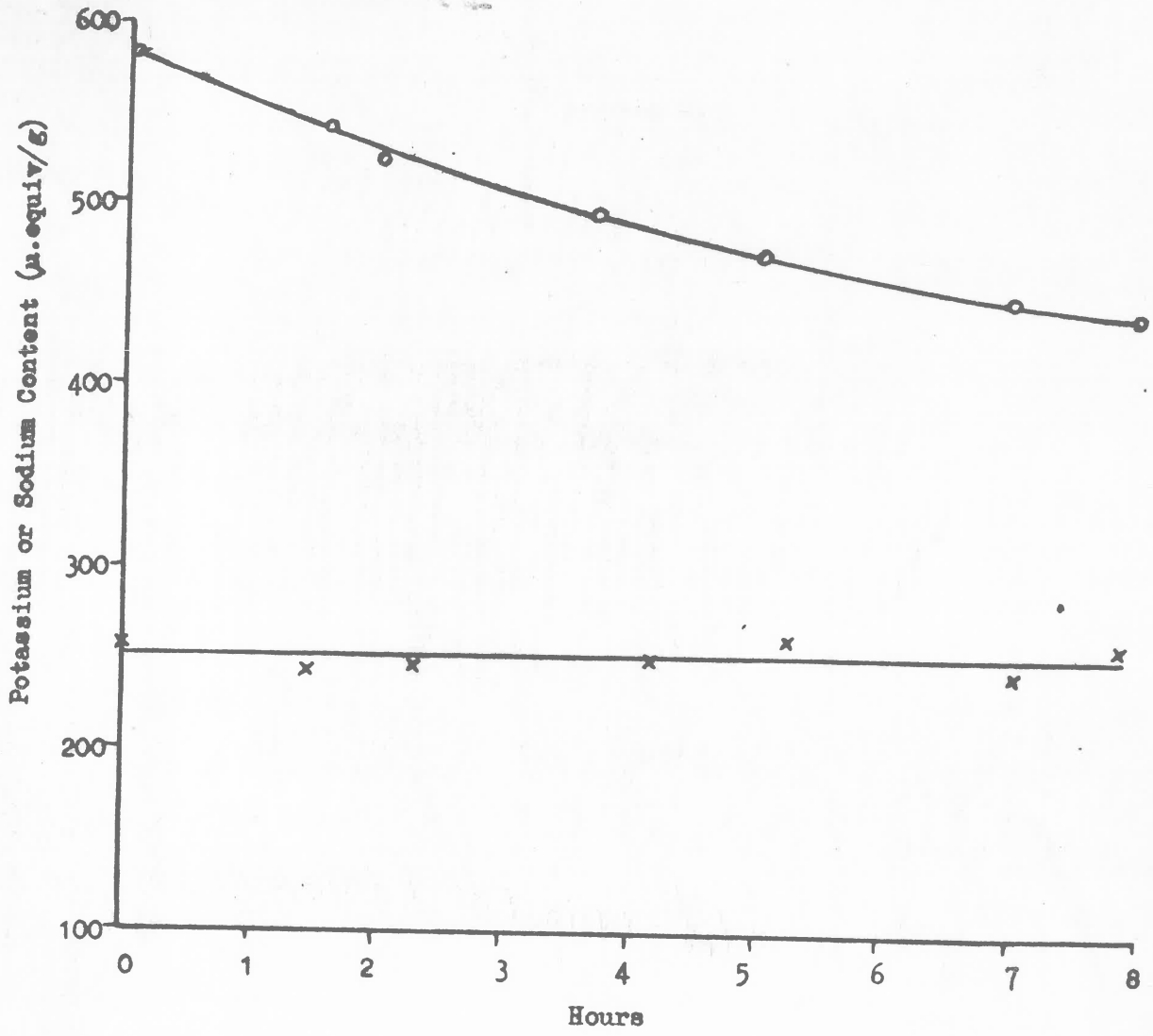

Figure 6.9 Effect of Oligomycin (2μ.gms/ml) on the ion balance of Chaetomorpha in the dark.

(o) = potassium; (x) = sodium; μ.equiv/ml cell sap.

treatments were applied by addition to the perfusing seawater, 2.85×10^{-3}M sodium acetate, 10^{-4}M DNP and 10^{-4}M DNP in sodium acetate. The sodium acetate was used as a buffer and was necessary to obtain the inhibitory action of DNP. It was used in this experiment to determine if it caused any effect on membrane potential. The results of this experiment are recorded in figure 6.10 where it can be seen that sodium acetate and sodium acetate-DNP mixture depressed the potential but DNP alone caused no change. The variables were introduced in different order and equilibration allowed for longer time periods but the same general result was obtained. The sodium acetate is responsible for lowering the potential by about six millivolts.

Several experiments with Oubain and Sodium Cyanide failed to show that these substances had any effect on the transmembrane potential of Chaetomorpha.

Nagai and Tazawa (1962) showed that upon illumination the transmembrane potential in Nitella flexilis was increased by up to 50mV. They were able to correlate this increase with uptake of cation upon illumination. An experiment designed to examine the effect of illumination on the stable transmembrane potential showed that this effect does not take place in Chaetomorpha, but no measurements of membrane resistance were made and this could have reacted to light conditions.

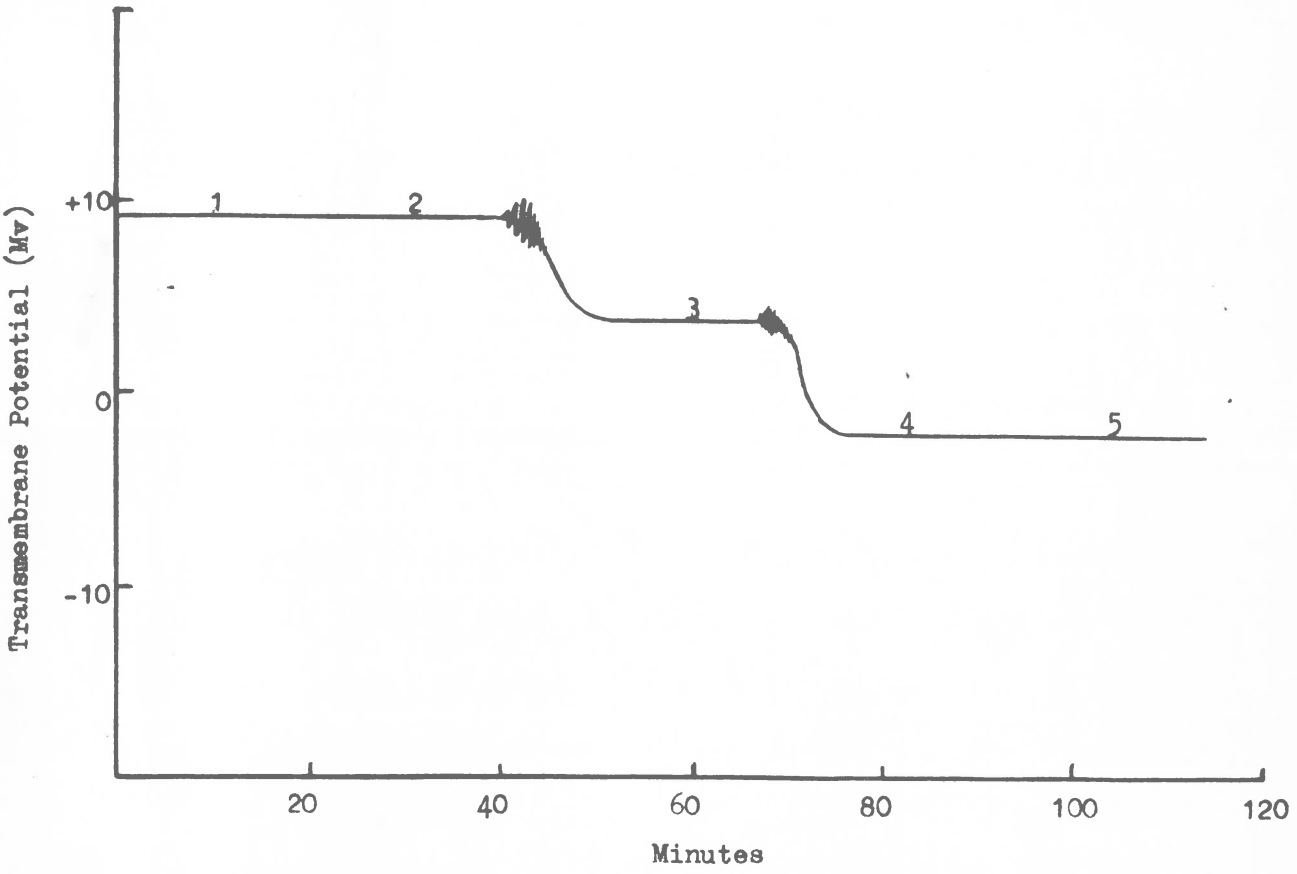

Figure 6.10 The effect of 10^{-4}M DNP and 2.85×10^{-3}M acetate buffer on the transmembrane potential in Chaetomorpha.

1. Control
2. 10^{-4}M DNP
3. Sodium acetate-DNP
4. Sodium acetate
5. DNP

Effect of DNP on the fluxes of sodium and potassium

From the experiments described above it is clear that DNP can inhibit potassium fluxes but that there is no immediate stimulation of a net potassium efflux and sodium uptake. This state of inhibition (which is reversible) can last for anything from 40 to 80 hours so tissue in this state can be used for estimation of fluxes by the method outlined above (P 45)

Experiments had shown that Chaetomorpha could be maintained indefinitely at pH 5.7 using an acetate buffer. This lower pH did not appear to have any adverse effect on the ion balance and was necessary to allow dissociation of DNP to the form that inhibits the active process. Measurements of this kind are given in table 6:3. Fluxes of tissue from the same collection of Chaetomorpha and used as controls are given for comparison with the inhibited state.

It can be seen from the results that DNP inhibits the potassium influx considerably, leading to a reduction in potassium in the cytoplasmic phase. Sodium content is correspondingly increased but there is no equivalent increase in sodium fluxes. The behaviour of the outer boundary is thus quite consistent with there being an inward active potassium transport and an outward sodium transport.

It would be expected from inhibition of these fluxes and consequent changes in q_2, that there would be a net efflux

TABLE 6:3

CONTENT OF THE CYTOPLASMIC PHASE, AND FLUXES OF POTASSIUM AND SODIUM IN DNP SOLUTIONS

Potassium	p.moles/cm^2/sec		Content (u.equiv/g)	Vacuolar Concentration (m.equiv/l)	
	ϕ_1	ϕ_2		K	Na
Control	65±0.3	100±4	17.7±0.3	520	45
DNP (1)	19	13	0.9	490	50
DNP (2)	11	30 3.0	0.2	524	28
Sodium					
Control (1)	110±12	3.8±0.4	1.15±0.20	500	28
DNP	90	5.0	6.7	560	20
Control (2)	40	2.6	0.40	385	95
DNP	58	62	15.0	320	150

of potassium from the vacuole due to reduction in the influx ϕ_2. Similarly there should be a net sodium uptake as the high value of ϕ_2 should lead to an increased influx. Throughout these studies with inhibitors this behaviour was found in only a few examples. For example, oligomycin and nitrogen led to immediate changes in vacuolar content. This behaviour is not explicable if potassium and sodium transport at the tonoplast are both passive. This problem will be considered again when discussing the general model for ion transport in this alga.

Experiments had shown that Chaetomorpha could be maintained indefinitely at pH 5.7 using an acetate buffer. This lower pH did not appear to have any adverse effect on the ion balance and this pH was necessary to allow dissociation of DNP and thus efficient inhibition. The results of these experiments are presented in table 6:4.

TABLE 6:4

SUMMARY OF DATA OBTAINED FROM EXPERIMENTS ON THE FLUXES OF INHIBITED CELLS OF CHAETOMORPHA VALUES FOR Q_2 ARE IN μe./gm TISSUE FRESH WEIGHT. VALUES FOR FLUXES ARE QUOTED μe./gm/hr.

	Number of separate determinations	Range	Mean ± S.E.M.
Q_2 K	3	1.5 - 2.3	1.73 ± 0.27
ϕ_1 K	3	1.76 - 4.3	3.17 ± 0.7
ϕ_2 K	3	0.18 - 0.54	0.33 ± 0.11
Q_2 Na	3	13.6 - 17.0	15.1 ± 1.0
ϕ_1 Na	3	3.6 - 4.8	4.2 ± 0.07
ϕ_2 Na	3	3.1 - 5.9	4.2 ± 0.8

CHAPTER 7

DISCUSSION

From the experimental data it would appear that the ions of the external solution are first exchanged with the free space ions and the cytoplasmic ions and finally the cytoplasmic ions exchange with the ions in the vacuole. The data presented fits closely the model for a three phase system postulated by MacRobbie and Dainty (1958b).

The concentrations of sodium and potassium in the vacuole are not in electrochemical equilibrium with sodium and potassium in the external solution, indicating that a mechanism of active transport may exist for these ions, although sites cannot be determined from this information alone.

During the period of isotope experiments there was no detectable net transport of sodium and potassium, thus any ion moving passively between external solution and the cell sap should

have its distribution governed by the Nernst equation (equation 7:1).

$$E_j = \frac{RT}{zF} \cdot \ln \frac{C_o}{C_i} \qquad 7:1$$

Table 7:1 gives the values of E_j if the ions concerned move passively across chemical and electrical potential gradients.

TABLE 7:1

ION CONCENTRATION POTENTIALS IN THE CELLS OF CHAETOMORPHA

E_o = +10 mV

ion	C_o/C_i	E_j	$E = E_o - E_j$
Na	560/25	+ 75.5	- 65
K	15/540	- 94	+ 104
Cl	560/601	+ 1.8	- 8

It would appear that the chloride ion in the vacuole is approximately in electrochemical equilibrium. Thus sodium and potassium must be undergoing active transport and it is clear from table 7:1 that sodium is actively transported outwards and potassium inwards. The probable sites of these active transports may be deduced from the relative concentrations of the three ions in each compartment (Table 7:2).

The reasons for associating the slow compartment with the vacuole have already been given. The volume of the second compartment is uncertain but some of its properties can be discussed without specifying its volume. The ratio $^{K}/_{Na}$ is 0.014 and does not differ significantly from the $^{K}/_{Na}$ ratio in the vacuole.

TABLE 7:2

ION CONTENT OF THE THREE COMPARTMENTS IN CHAETOMORPHA

Content in μ.equiv/gm

Ion	Free space	Cytoplasm	Vacuole
Na	500	22	24.6
K	15	0.3	541
$^{K}/_{Na}$	0.026	0.014	21.6

This suggests that K-Na selectivity is a property of the plasmalemma, due to an outwardly directed sodium pump and an inwardly directed potassium pump. as Hodgkin (1957) suggested to explain the selectivity in nerve and muscle.

Insufficient information is available concerning the chloride balance but from the electrochemical potential values it is unlikely that an active chloride pump exists in Chaetomorpha. Dawes (private communication) showed that the cytoplasm in Chaetomorpha darwinii was 15 μ wide. Based on this figure the cytoplasm would comprise about 2% of the total cell volume. On this basis the

concentration of potassium in the cytoplasm would be 1100 µ.equiv./gm. The suggested explanation for this high value is that, because fluxes are large compared to diffusion in any phase, then diffusion out of the tissue would be smaller than expected and the time for exchange of the cytoplasmic phase would be increased, making k_1 an underestimate. Thus the lowest values (those in the range 500 to 600 µ.equiv./gm.) are perhaps most accurate.

MacRobbie and Dainty (1958) have suggested a provisional scheme where the free space includes some of the cytoplasm. The boundary of the cytoplasm may be equally hard to define, particularly in Chaetomorpha where exact volume of the cytoplasm is unknown.

The suggestion that mitichondria may be sites of ion accumulation was discussed by Robertson (1956). Accumulation of potassium in the mitichondria could explain the high values present in the cytoplasm. In the present work the possibility that chloroplasts were sites of ion accumulation was investigated but there was insufficient evidence to support this theory.

In 1955 Hodgkin and Keynes showed by inhibitor studies that giant axons of Sepia and Loligo had secretory mechanisms driven by metabolism, which ejected sodium and absorbed potassium against electrochemical gradients. The principles elucidated by them were subsequently applied to plant cells. Eppley (1958)

concluded that the mechanism for ion balance in Porphyra perforata was a cyclic mechanism and similar to the one postulated by Hodgkin and Keynes. MacRobbie and Dainty (1958b) and MacRobbie (1962) using brackish water algae showed that the rate of exchange of radioactive cations was drastically reduced by a range of metabolic inhibitors and that the energy for the active transport process was closely geared to light dependent metabolism rather than respiration.

A wide range of metabolic inhibitors was used on Chaetomorpha in an attempt to establish the importance of metabolic energy in the maintainance of ion balance. The data presented indicates the necessity of cellular metabolism in maintainance of cation balance in Chaetomorpha, but failed to show that a light dependent mechanism was involved. Scott and Hayward (1954) were able to show that there was a loss of potassium and gain of sodium in Ulva tissue in the dark. While this result could be repeated with Ulva, light and dark conditions had no effect on the cation content of Chaetomorpha. The difference may be basically structural since Ulva has only small vacuoles and light may strongly effect permeability if there is only one permeability barrier. Nagai and Tazawa (1962) showed that light increased the resting potential in Nitella and also caused an increased permeability of the plasmalemma to ions. If Ulva has a plasmalemma as the important permeability barrier this plant

could be susceptible to ionic change with changing light conditions.

DNP, an uncoupling agent, was used in many experiments. The precise mechanism of DNP action is not known, and while it is thought that the main site of action is the prevention of ATP formation during aerobic respiration, DNP may also be involved in inhibiting other energy transfer reactions. The pH of seawater (7.4) was much higher than the pH for effective DNP action, so buffered solutions were used. Chaetomorpha had little tolerance to pH change and collapsed at pH 5.5. This left only a narrow range in which DNP was active and the cells were unaffected by low pH. That DNP ionization took place was confirmed by the immediate suppression of the potassium fluxes when DNP was introduced. Despite this inhibition of fluxes the amount of potassium and sodium in the tissue was unchanged for up to 40 hours. Subsequently there was a rapid loss of potassium and uptake of sodium which was irreversible. The inability of the cell to maintain its ion balance after a period of about 40 hours in DNP is probably due to deterioration of cell membranes.

As a result of DNP action, influx of potassium at the outer boundary was almost inhibited and must be predominantly due to an active pump. The influx of sodium at the outer boundary was little affected by DNP and yet the sodium content of the cytoplasmic phase increased, indicating that the efflux of Na

(ϕ_1) was at least partly active. The result of the active flux inhibition at the outer boundary was to change the cytoplasmic content from high potassium/low sodium to low potassium/high sodium. The effect of this change over of the K:Na ratio, on high fluxes into and out of the vacuole was unexpected. Not only was the potassium influx reduced but so was the potassium efflux and uptake of sodium did not increase proportionately with the change in sodium content.

Sodium cyanide and nitrogen in the dark are similar in that they are specific inhibitors for parts of the cytochrome chain. From the action of sodium cyanide, DNP and nitrogen in the dark on the ion balance of Chaetomorpha, it would appear that sodium extrusion and potassium accumulation is a coupled mechanism which derives its energy from ATP or an ATP linked reaction.

On the other hand oligomycin and arsenite both caused leakage of potassium but had little effect on sodium uptake. Oligomycin is a specific inhibitor for the transfer of high energy phosphate to ADP, preventing formation of ATP, while arsenite is a nonspecific inhibitor of the TCA cycle and EMP pathway. The inability of oubain to inhibit sodium-potassium transport is perhaps not unexpected since oubain inhibition of the sodium-potassium ATPase has only been shown once in plant tissue. The oubain used was known from other experiments to be active as an inhibitor. The results of arsenite and oligomycin inhibition point to there being two separate mechanisms for sodium and potassium transfer, but this could be due to high sodium permeability inwards.

It would appear that the active potassium influx alone is strongly inhibited by these two chemicals, resulting in a decrease in potassium content and presumably a change in potential seeing that sodium content is unchanged. Scott and Hayward (1954) suggested a similar system for Ulva. They postulated that potassium transfer depended on ATP, while sodium transfer was dependent upon energy from metabolism and light. Figure 7:1 is a diagrammatic interpretation of the data presented above.

TABLE 7:3

CONCENTRATION OF CATIONS AND POTENTIALS IN THE COMPARTMENTS OF CHAETOMORPHA (CONCENTRATION IN m.equiv./litre)

	External solution	Cytoplasm	Vacuole
K	13	600	541
Na	500	15	25
Potential to outside		+ 10	- 35

Agrees ≡ Summary but not ≡ p 74 Table 4:1

Table 7:3 is a summary of results of experiments on potential measurements in Chaetomorpha. The potential difference across the plasmalemma and tonoplast have been measured by many workers in different tissues. The conclusion from most of these experiments was that nearly all of the potential difference in the plant cell was developed across the plasmalemma.

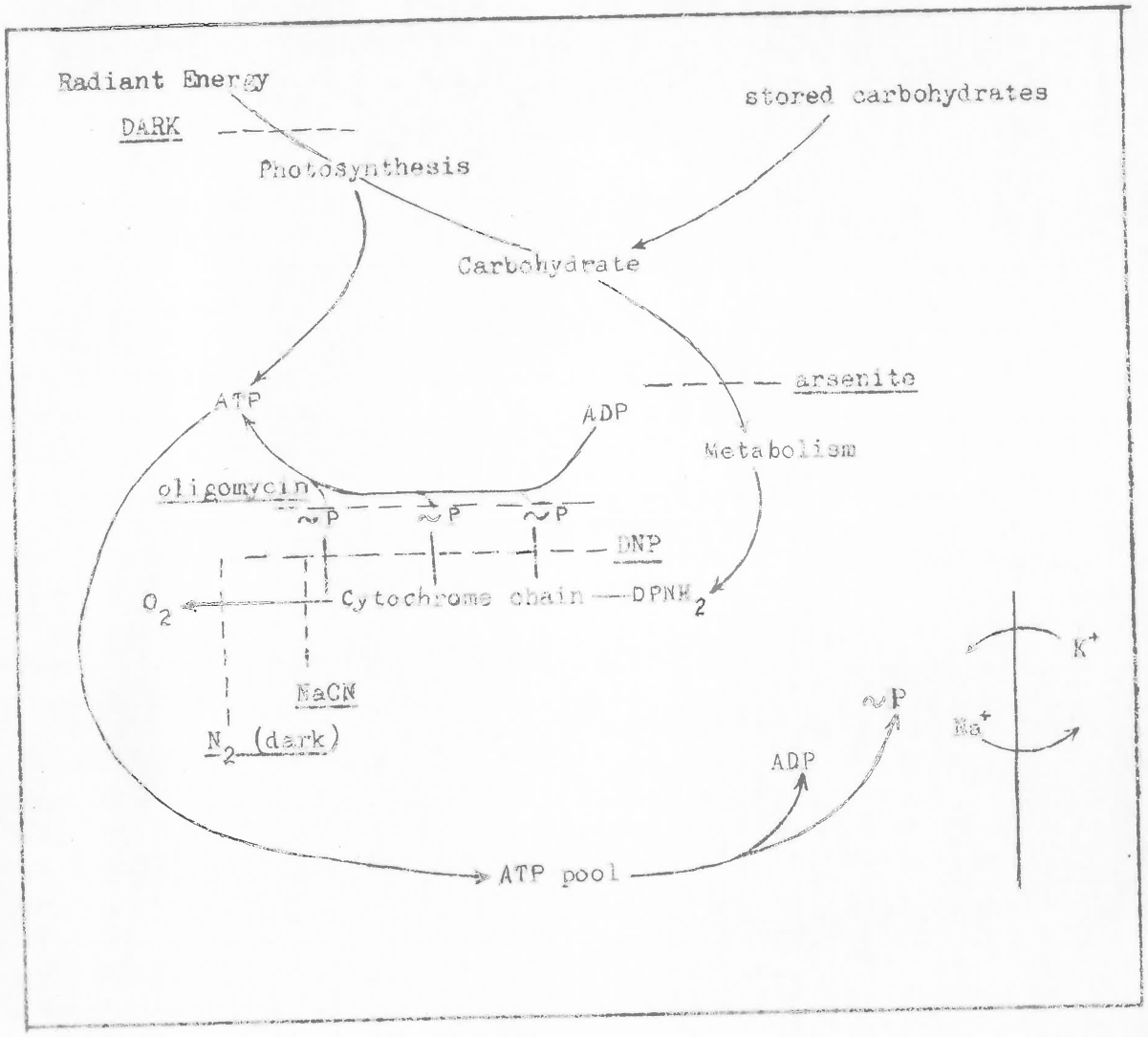

Figure 7.1 Diagrammatic representation of the suggested relationships of respiration photosynthesis and metabolism with ion transport in Chaetomorpha. All inhibitors and metabolic blocking agents are underlined.

MacRobbie (1962) in a study of ionic fluxes in Nitella translucens predicted that there should be a potential difference of about 10 mV across the tonoplast. Spanswick and Williams (1964) have confirmed this prediction and on the basis of the known potential differences across each membrane and the concentrations of major ions in each compartment, were able to draw some conclusions about the direction of the active pumps at each membrane.

With a knowledge of the potential differences across each membrane and ionic concentrations it is possible to test for the active transport of any particular ion, by comparing the observed potential with the potential difference calculated from the concentration ratio, providing that the ion is in flux equilibrium.

In Chaetomorpha evidence points to both potassium and sodium ions being in flux equilibrium, therefore the above method can be used to draw some conclusions about the active pumps at each membrane. The following three tables show the values obtained for potentials across the membranes.

It can be shown that the sodium is pumped out of the cytoplasm across the plasmalemma and potassium is pumped into the cytoplasm across the plasmalemma. There are also active inward pumps for both ions located at the tonoplast. Though these active pumps at the tonoplast have not been estimated it is probable that they are not large since the concentration gradient is small.

Table 7:7 is a summary of measurements made on Chaetomorpha darwinii.

TABLE 7:4

THE CONCENTRATION RATIO OF IONS IN THE COMPARTMENTS OF CHAETOMORPHA

	C_o/C_i plasmalemma	C_o/C_i tonoplast
Na	$^{500}/_{20}$ = 33.3	$^{20}/_{24.6}$ = 0.81
K	$^{13}/_{600}$ = 0.022	$^{600}/_{541}$ = 1.11

TABLE 7:5

THE POTENTIAL (IN mV) ACROSS THE MEMBRANES WHERE

$$E_j = 58 \log {}^{C_o}/_{C_i}$$

	E_j plasmalemma	E_j tonoplast
Na	+ 80	- 5.2
K	-96.6	+ 2.7

TABLE 7:6

VALUES OF E WHERE $E = E_o - E_j$

E_o plasmalemma = -30mV E_o tonoplast = +40mV

	E plasmalemma	E tonoplast
Na	- 110	+ 45
K	+ 67	+ 37

TABLE 7:7

SUMMARY OF RESULTS OF EXPERIMENTS ON ION BALANCE IN CHAETOMORPHA

	Sea Water	Cytoplasmic Phase	Vacuole
Potassium Concentrations	13		540
Content (u.equiv/gm)		15 - 20	
Sodium Concentrations	500		25
Content (u.equiv/gm)		(0.5 - 1.0)	
$^{K}/_{Na}$ ratio	0.026	17 - 25	21

Boundary	Outer	Inner
Potential (inwards)	-35 mV	+45 mV
Passive Fluxes (p.moles/cm^2/sec)		
Inwards - Potassium	7	200
Sodium	85 - 125	4
Outwards - Potassium	65 - 140	200
Sodium	4	4
Active Fluxes		
Potassium inwards	≈ 100	?
Sodium outwards	≈ 100	?

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