ION UPTAKE AND ELECTRICAL POTENTIALS IN PLANTS

By

R.M. SPANSWICK, B.Sc.

A Thesis presented for the degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Science.

May, 1964.

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SYMBOLS AND ABBREVIATIONS

A^- : concentration of indiffusible anions in the D.F.S.

A : area ($cm.^2$).

C_i : internal concentration (moles $cm.^{-3}$, unless specified).

C_o : external concentration (moles $cm.^{-3}$, unless specified).

Ca_c, Cl_c, K_c, Na_c : cytoplasmic concentrations of the respective ions.

Ca_o, Cl_o, K_o, Na_o : external concentrations of the respective ions.

Ca_v, Cl_v, K_v, Na_v : vacuolar concentrations of the respective ions.

E : electrical potential difference.

${}_cE_o$: electrical potential of the cytoplasm w.r.t. the external medium.

${}_vE_c$: electrical potential of the vacuole w.r.t. the cytoplasm.

E_j : Nernst potential for the ion j.

J_{in} : influx (moles $cm.^{-2}sec.^{-1}$).

J_{out} : efflux (moles $cm.^{-2}sec.^{-1}$).

J_j : net flux of the ion j.

P_{Ca} : permeability coefficient for calcium.

P_{Cl} : permeability coefficient for chloride.

P_K : permeability coefficient for potassium.

P_{Na} : permeability coefficient for sodium.

T_j : transport number for the ion j.

V : volume ($cm.^3$).

z_j : charge on the ion j.

α : P_{Na}/P_K.

β : P_{Ca}/P_K.

γ : P_{Cl}/P_K.

ψ : electrical potential.

μ : chemical potential.

$\bar{\mu}$: electrochemical potential.

ATP : adenosine triphosphate.

ATPase : adenosine triphosphatase.

DCMU : 3(3,4-dichlorophenyl)-1,1-dimethylurea.

DNP : dinitrophenol.

EDTA : ethylene-diamine-tetra-acetic-acid.

1.1 INTRODUCTION

In the field of ion transport, animal physiologists have made substantial progress during the last decade and a great deal of information is now available concerning nerve, muscle, and various epithelial tissues. Before the techniques evolved by the animal physiologists were applied to plants a considerable delay occurred which may have been in part due to the morphological complexities of plant cells. Recently, however, these techniques have been applied with considerable success to plant cells, especially to the large internodal cells of the Characeae.

It is now clear that the plant cell may be regarded as a three compartment system consisting of the cell wall, the protoplasm, and the vacuole. The cell wall acts as a physical extension of the environment but the protoplasm is bounded by the plasmalemma and tonoplast membranes which act as the diffusion barriers containing the sites at which 'active transport' takes place. Electrolytes move from one compartment to another by diffusion, mass flow, solvent drag, or active transport but, in systems in which osmotic pressure is constant, diffusion and active transport are the main processes involved. It is now almost universally recognised that the electrochemical potential gradient, not simply the concentration gradient, is the driving force on an ion moving passively across a membrane, and several measurements of the driving forces on particular ions have been made. However, the forces measured are usually between the vacuole and the external medium and not the forces across the individual membranes. When the ionic fluxes have also been measured it has been possible to suggest whether active transport takes place. In a few cases, connections with metabolism have been established.

Several works on the ionic relations of plants have been published recently including books by Briggs, Hope, and Robertson (1961), Sutcliffe (1962), and Jennings (1963). The general approach adopted in this thesis

is that outlined in a review by Dainty (1962). It is not yet possible to treat the problem of ion transport in plants at the molecular level but, by using a suitable member of the Characeae, an attempt has been made to determine more accurately the forces acting on the ions, the membranes at which active transport takes place, and the parameters which control the electrical properties of the membranes. Electrical measurements have also been made on a higher plant organ, the exuding root system, in order to obtain evidence on the controversial problem of transport into the xylem.

In the remaining sections of this chapter the principles involved in this study will be outlined and further information about particular systems will be introduced where appropriate.

1.2 EXTRACELLULAR ION EXCHANGE - THE CELL WALL

Electron microscope studies have shown that the cell wall consists of long cellulose microfibrils of 100 - 450 Å in diameter with interspaces of the order of 100 Å (Preston, 1952). The microfibrils may lie parallel to one another as in Valonia, or unorientated and apparently interwoven as in the higher plants. The wall also contains pectins which seem to be distributed uniformly throughout it. The pectins have ionizable carboxyl groups which are normally balanced by the divalent cations calcium and magnesium. It has been shown that the wall calcium can be completely exchanged for sodium and that the ion distribution obeys the Donnan equations (Dainty and Hope, 1959; Dainty, Hope and Denby, 1960). It may therefore be supposed that the forces between the cations and the exchange sites are purely electrostatic and that there is no chemical selectivity other than that due to electric charge.

The compartment of a cell or tissue which is in direct physical contact with a surrounding physical solution has been termed free space by G.E. Briggs (Briggs and Robertson, 1957) to distinguish it from the osmotic volume into which solutes, but not the solvent, penetrate relatively slowly. Laties (1959) has described free space as "that part of a plant tissue which is in free communication with the environment, without permeation barriers".

The volume of the free space depends upon the method used to measure it. This led to the definition of the term, apparent free space (A.F.S.) (Briggs and Robertson, 1957) as follows:

"If U is the estimated free space uptake of a solute by a tissue originally devoid of this solute from a solution of concentration a_o, then the A.F.S. is U/a_o".

The A.F.S. will obviously depend on the substance used in its estimation and Dainty and Hope (1959) have suggested the use of the terms sodium free space, mannitol free space, chloride free space etc. Briggs and Robertson (1957), however, have divided the A.F.S. into two components and introduced the terms water free space (W.F.S.) and Donnan free space (D.F.S.) to describe these components.

The W.F.S. is that part of the A.F.S. which contains mobile anions and cations in equal concentrations. Uptake into the W.F.S. will therefore be proportional to the external concentration. The D.F.S. is that part of the A.F.S. in which the concentration of an ion of one sign is greater than that of the opposite sign, this being due to the presence of indiffusible ions.

It was originally thought that the D.F.S. was located in the cytoplasm (Briggs and Robertson, 1957; Briggs, Hope and Pitman, 1958). However, Dainty and Hope (1959) and Dainty, Hope and Denby (1960) showed that practically all the quickly exchangeable cations in Chara australis occurred in the cell wall. Pitman (unpublished) has since shown that the D.F.S. exchange capacity of beet tissue can be accounted for by the cell wall material. Jansen, Jang, Albersheim and Bonner (1960) obtained similar results with Avena coleoptiles.

This evidence is in agreement with the kinetic studies on single giant algal cells (MacRobbie and Dainty, 1958; Diamond and Solomon, 1959; Hope and Walker, 1961; MacRobbie, 1962), which suggest there is a permeation barrier between the cell wall and the cytoplasm. It is thus established that the A.F.S. is located in the cell wall.

From considerations of the structure of the cell wall it is obvious that the D.F.S. will not be a homogeneous phase. The indiffusible anions are attached to the surface of the microfibrils between which some of the

spaces will be so large that the electrical double layers will not overlap appreciably. In this case there will be no clear demarcation between the D.F.S. and the W.F.S. In an analysis of this problem Dainty and Hope (1961) derive an expression for the width x_D of the electrical double layer which is equivalent to a Donnan phase as far as the average ionic concentrations are concerned. They showed that

$$x_D = \sqrt{\frac{ERT}{2 \epsilon F^2 C_o}} \qquad (1)$$

where ϵ is the permittivity of the medium and C_o is the concentration in the bulk phase in equiv.cm^{-3}. This means that the apparent volume of the D.F.S. should be proportional to $\frac{1}{\sqrt{C_o}}$ if the indiffusible anions are randomly distributed in large pores.

The D.F.S. in beet tissue appears to have a constant volume for external concentrations of 1 - 20 m.equiv.l^{-1} and this suggests that there is appreciable overlapping of the electrical double layers. Dainty and Hope (1959) found that the W.F.S. in Chara australis was constant over a small range of concentrations. They put forward the suggestion that the D.F.S. was confined to micropores ($<$ 100 Å diameter) and that the W.F.S. was in macropores ($>$ 100 Å diameter) which contained relatively few fixed charges. This view was supported by the kinetics of ion exchange which occurred in two or three phases with very different time constants. The effect was more pronounced with calcium than with sodium and this would be expected if the rate of exchange were slowed down by steric hinderances in small charged pores.

1.3 PASSIVE ION TRANSPORT

Studies on the ultrastructure of the protoplasm have shown that it is a very complex organization of membranes, mitochondria, and other sub-cellular particles. However, from the point of view of ionic studies, it is usually necessary to treat the protoplasm as a homogeneous phase and assume that only the surface membranes act as important diffusion barriers. There is some justification for this in the fact that the electrical potential of the cytoplasm measured with a microelectrode is uniform and therefore there cannot be electrical potential differences across the endoplasmic reticulum. That this does not apply to sub-cellular particles was demonstrated by Loewenstein and Kanno (1963) who found a potential difference of 15 mV across the nuclear membrane of Drosophila salivary gland cells. For the present it is necessary to treat the protoplasm as a uniform phase.

1.31 Passive equilibrium

If there is no net flow of solute across a membrane, an ion will be acted upon by two forces, namely the chemical potential gradient $d\mu/dx$ and the electrical potential gradient $zFd\psi/dx$. The sum of these forces is the electrochemical potential gradient:

$$\frac{d\bar{\mu}}{dx} = \frac{d\mu}{dx} + \frac{zFd\psi}{dx}$$

$$= \frac{d}{dx}\ (\mu + zF\psi) \qquad (2)$$

If an ion j is in equilibrium between two solutions separated by a membrane, there can be no net driving force on the ion, and hence its electrochemical potential in both solutions must be equal:

$$\bar{\mu}_j^i = \bar{\mu}_j^o$$

where i and o refer to the two solutions. From this we can deduce the Nernst Equation:

$$\psi^{i} - \psi^{o} = E_j = \frac{RT}{z_j F} \ln \frac{a_j^o}{a_j^i} \qquad (3)$$

where a_j is the chemical activity of the ion. This equation gives the electrical potential difference across the membrane when the ion is in passive equilibrium. In biological systems it is usually adequate to replace the activities by concentrations:

$$E_j = \frac{RT}{z_j F} \ln \frac{C_j^o}{C_j^i} \qquad (4)$$

The use of this equation as a criterion for active transport will be dealt with later.

1.32 The electrical potential

From the previous section it is clear that it is necessary to be able to measure the electrical potential between two phases separated by a membrane as well as the concentrations on either side. Dainty (1962) considers the membrane potential to be a diffusion potential arising from the unequal transport of charge through the membrane by the various ions. The Donnan potential would be a special case with some of the ions having zero permeability.

A membrane potential is measured by inserting salt bridges, connected to Ag-AgCl or calomel electrodes, into the solutions on either side of the membrane. If one side is cytoplasm, a micro-pipette with a tip diameter of the order of 1μ is used as a salt bridge. The cell membrane seals round the micro-pipette and little damage is done to the system. Each

salt bridge is usually filled with 3N KCl which is said to eliminate the liquid junction potential at the point of contact with the solution into which it is inserted. This is justifiable since the mobilities of the K and Cl ions are nearly equal and the concentration of KCl is much greater than the concentration of the solutions in contact with the salt bridges (MacInnes, 1961, chapter 13). However, as Adrian (1956) has shown, this may not be true for micro-electrodes if the tip becomes charged as it would modify the relative transport numbers of the K and Cl ions and give rise to a 'tip-potential'. Nevertheless, Page (1962a) found that the membrane potential of cat heart muscle measured with electrodes having tip potentials of 0 - 6 mV in Ringer solution was not significantly different from the potential measured with electrodes having tip potentials between -6 and -15 mV.

1.33 The steady state

In general, plant cells will not be in ionic equilibrium with their environment, but over short periods of time they may be considered to be in a steady state. For any ion moving passively and independently across a membrane, the general flux equation (Teorell, 1956) is of the form:

Flux = concentration x mobility x force.

For an ion j which is at a concentration C_j (mole $cm.^{-3}$) at a point x and which has a mobility u_j ($cm.sec.^{-1}joule^{-1}cm.mole^{-1}$), the flux is

$$J_j = \acute{C}_j \cdot \acute{u}_j \left(-\frac{d\acute{\bar{\mu}}_j}{dx}\right)$$

$$= -\acute{u}_j RT \frac{d\acute{C}_j}{dx} - \acute{u}_j \acute{C}_j z_j \frac{F d\acute{\psi}}{dx} \qquad (5)$$

where the primes indicate that the symbols refer to quantities inside the

membrane material and activities have been replaced by concentrations.

Equations similar to (5) can be written for each ion, integrated across the membrane, and solved subject to the restrictions of electrical neutrality and zero current flow through the membrane. To simplify this procedure, Goldman (1943) introduced the assumption of a constant field throughout the membrane. Assuming also that the phase boundary potentials are negligible or equal and opposite, equation (1) can be solved and expressed in terms of observable quantities:

$$J_j = -\frac{z_j \acute{u}_j EF\, k_j}{a} \cdot \frac{C_o - C_i \exp(z_j FE/RT)}{1 - \exp(z_j FE/RT)}$$

$$= P_j z_j \frac{FE}{RT} \cdot \frac{C_o - C_i \exp(z_j FE/RT)}{1 - \exp(z_j FE/RT)} \qquad (6)$$

(Hodgkin and Katz, 1949) where $P_j = \acute{u}_j\, RT\, k_j/a$, $k_j = \acute{C}_j/C_j$ and $\underline{a}$ is the thickness of the membrane. The permeability coefficient P_j must not be regarded as a constant and, since it cannot be determined independently, is defined in terms of equation (6).

The net flux given by equation (6) is the difference of the influx and the efflux which are proportional to C_j^o and $C_j^i \exp(z_j FE/RT)$ respectively. Thus:

$$\frac{J_{in}}{J_{out}} = \frac{C_j^o}{C_j^i \exp(z_j FE/RT)} \qquad (7)$$

This formula was deduced independently by Ussing (1949) and Teorell (1949). It is quite general since it expresses the fact that the independent, passive movement of ions is proportional to the electrochemical ~~potential~~ activity. At equilibrium it reduces to the Nernst equation.

1.34 The membrane potential

If Na, K and Cl ions are present and it is assumed that no current is flowing, i.e., $J_K + J_{Na} = J_{Cl}$, equation (6) for each ion can be combined to give the Goldman equation for the membrane potential:

$$E = \frac{RT}{F} \ln \frac{P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i}{P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o} \qquad (8)$$

Patlak (1960) has shown that an equation similar in form to (8) can be deduced on quite general grounds from equation (7).

Another approach is the electrical model used by nerve physiologists. The individual ion currents are written as the product of a conductance and a driving force, the latter given by the difference between the Nernst potential and the membrane potential (see Dainty, 1960). For the condition of no net current flow, the membrane potential is given by the sum of the products of the Nernst potential times the transport number for each ion:

$$E = E_{Na} T_{Na} + E_K T_K + E_{Cl} T_{Cl} + \text{----------} \qquad (9)$$

In certain cases it is possible to obtain information about cell membranes by varying the external ionic concentrations, measuring the change in membrane potential, and using equation (8) or (9) to interpret the results. Hodgkin and Horowicz (1959) have adopted this approach for muscle and Hope and Walker (1961) have used it in their experiments on Chara australis.

1.4 ION TRANSPORT AND METABOLISM

Intracellular ionic concentrations in plant cells often differ markedly from the equilibrium concentrations calculated from the Nernst equation. This fact is usually explained by postulating the presence of 'ion pumps' which transport ions from one side of a membrane where the electrochemical potential is low to the other side where it is higher. Since this means doing work against the electrochemical potential gradient, the ion pumps require a supply of energy from the metabolism of the cell. This process, known as 'active transport', integrates ion transport into the general metabolic activity of the organism.

Before examining the idea of active transport in more detail, the 'sorptionist' theory must be mentioned. It is opposed to the concept of active transport and to the idea of a membrane providing the rate limiting step to diffusion of ions into the cell. According to Ling (1962) the ions are selectively adsorbed into a three-dimensional protein lattice containing fixed charges. The selectivity is said to result from the different hydrated radii of the ions and the preferential adsorption of potassium compared with sodium is explained in this way. In fact the selectivity exhibited by living cells is much greater than that of any non-living system. Furthermore the theory cannot explain how cells of the Characeae maintain ions in their vacuoles at concentrations far from equilibrium in the absence of proteins, nor can it account for the net transport of ions across epithelial tissues. Further objections to this theory have been assembled by Booij (1963) who concludes that the existence of a selective membrane is a necessity for the living cell.

1.41 Criteria for active transport

The electrochemical potential difference for an ion j, $\Delta\bar{\mu}_j$,

between the solutions on either side of a membrane may be calculated from measurements of the ionic concentrations and the membrane potential. If the ion is in equilibrium $\Delta\bar{\mu}_j$ will be zero. If the ion is not in equilibrium the value of $\Delta\bar{\mu}_j$ will give the magnitude and direction of the driving force on the ion. Before this result can be interpreted it is necessary to know whether there is flux equilibrium since there could be a net influx or efflux due to the growth or running down of the system. If there is flux equilibrium and yet $\Delta\bar{\mu}_j$ is not zero, the portion of the flux not accounted for by the Ussing-Teorell equation (equation 7) may be due to active transport. It is sometimes possible to check this by using specific inhibitors to remove the active part of the flux. If the cell is not in flux equilibrium it is necessary to measure both partial fluxes simultaneously but, as this is very difficult, systems which are approximately in flux equilibrium are usually chosen as experimental material.

If, after making allowance for the active components, the partial fluxes still do not fit the Ussing-Teorell equation, exchange diffusion may be taking place (Ussing, 1960). Exchange diffusion, the process whereby a carrier makes a one-to-one exchange of ions at each surface of the membrane, invalidates the assumption that the ions cross the membrane as ions. This process requires no energy and increases the partial fluxes. It has not yet been demonstrated in plant cells.

Another possible cause of deviation from the Ussing-Teorell equation is the non-independent movement of ions which could be due to a phenomenon such as 'filing' in narrow pores (Hodgkin and Keynes, 1955). However, this particular idea has to be stretched to unrealistic lengths to explain the discrepancy between the measured membrane resistance in plant cells and that calculated from the ion fluxes (Williams, Johnston and Dainty, 1964).

It seems probable that it will be necessary to discard the assumption of independent ion movement and consider the interactions between the ions in the membrane using the formalism of irreversible thermodynamics. Kedem (1961) has used this approach to set up criteria for active transport in terms of a cross-coefficient R_{ir} relating the active transport of a solute i to a chemical reaction r taking place in the membrane. However, this approach is only useful if it is possible to measure the short-circuit current and the influence of ion-flow on energy consumption as well as the membrane potential; usually it is necessary to rely on the Ussing-Teorell equation as the criterion.

1.42 Mechanism of active transport

There is ample evidence that active transport occurs widely in both animal and plant cells although the number of ions which have been studied is somewhat limited (Andersen and Ussing, 1960). Sodium and potassium are the ions most commonly studied. Active excretion of sodium is universal and in most cases it is linked with active transport of potassium into the cell (Glynn, 1959). This has been demonstrated for the Characeae (e.g. MacRobbie, 1962), marine algae (Blount and Levedahl, 1960), and higher plants (Etherton, 1963).

There are few authenticated cases of active chloride transport in animal cells. Keynes (1963) has shown that there is an active influx in squid axon, while Draper, Friebel,and Karzel (1963) and Page (1962b) have demonstrated that frog sartorius and cat heart muscle have a higher intracellular chloride content than would be expected from equilibrium considerations. In plant cells active transport of chloride is well established (MacRobbie and Dainty, 1958; Blount and Levedahl, 1960; MacRobbie, 1962). It is unaffected by cardiac glycosides and must therefore be separate from the sodium potassium system.

Apart from the active transport of hydrogen ions in stomach, kidney, and yeast, the only other established case of active cation transport seems to be the excretion of calcium by squid axon (Hodgkin and Keynes, 1957).

A variety of active transport mechanisms have been proposed (Ussing, 1960), but two main categories may be distinguished; those which use metabolic energy directly and those in which ATP is used as an intermediary. The following summary is intended to give some idea of the present status of the theories and does not claim to be comprehensive.

Lundegardh has been one of the main proponents of the theory involving the direct utilization of metabolic energy (see Lundegardh, 1960, for his latest version). He postulates that electrons obtained from metabolites are transported across a membrane via a chain of cytochromes thereby setting up an electrical potential difference which would cause anions to flow in the opposite direction to the electrons. The hydrogen ions left on the inside of the membrane would exchange passively with the cations left behind by the anions. He suggests that in plant cells the cytochromes are situated at the tonoplast and in this case the vacuole should be electrically positive with respect to the external medium, but Walker (1955) and Etherton and Higinbotham (1960) have shown that the potential of the vacuole is negative. The hypothesis also requires the metabolites to be supplied on the vacuolar side of the membrane. This makes it difficult to account for the accumulation of anions by the Characeae which are generally thought not to contain organic molecules in their vacuoles. Further evidence against this hypothesis is given by Sutcliffe (1962).

Robertson (1960) has devised a more plausible form of this hypothesis. A respiratory carrier is oxidized at the outer surface of the membrane, thus losing an electron, and combines with an anion. The uncharged complex diffuses to the inner surface of the membrane where it is reduced by an electron from a metabolic substrate and releases the anion. This pump is neutral whereas the Lundegardh type is electrogenic i.e. contributes to the membrane potential.

Conway (1959) has proposed a complicated adaptation of the redox pump to account for active sodium transport in animal cells but it is difficult to explain the high ratios (> 4) of Na ions transported to oxygen molecules reduced in some systems, notably frog skin. Also it is difficult to

explain how DNP poisoning abolishes Na transport and yet increases oxygen consumption. Robertson (1960) concludes that while the redox pump may take part in anion transport a different mechanism is probably required for active cation transport.

Most of the hypotheses put forward to explain sodium and potassium transport involve a carrier and use ATP as an energy source. It is supposed that the carrier combines specifically with the ion to be actively transported at one boundary of the membrane and diffuses to the opposite side where the specificity of the carrier is somehow changed so that it releases the ion. There is strong evidence for the existence of a sodium potassium exchange pump in animal cells (Glynn, 1959) and most probably in plant cells also (MacRobbie, 1962). The pump is specifically inhibited by the application of cardiac glycosides to the outer surface of the membrane where they compete with potassium for the pump. The experiments of Caldwell et al. (1960) who injected high energy phosphates into squid axon and Hoffman (1960) who incorporated ATP into erythrocytes by reverse haemolysis, leave little doubt that ATP can drive the sodium potassium pump. Dunham (1957) pointed out that if the pump utilises ATP there must be an ATPase associated with it. Skou (1957, 1960) isolated a Na + Mg-activated ATPase from crab nerve and showed that its properties in solution were remarkably similar to the sodium potassium transport system. The existence of enzymes with similar properties has now been demonstrated for most tissues (Kinsolving, Post and Beaver, 1963). Hokin and Hokin (1961) have suggested that the phosphatidic acid cycle is involved in sodium transport but they have not correlated their work with transport studies. Their original model only involved the transport of sodium. Recently (Hokin and Hokin, 1963) they have extended it to include potassium transport but only by making further arbitrary assumptions. Hokin, Hokin, and

Mathison (1963) have shown that phosphatidic acid phosphatase is present in erythrocyte membrane and they suggest that it may be involved in the ATPase system of Skou.

Skou (1963) has reviewed the evidence implicating the participation of his ATPase system in sodium and potassium transport and has put forward a working hypothesis for its action involving the movement of affinity for Na versus K through a number of fixed sites located through the membrane. According to the theory of Eisenman, Rudin and Casby (see Eisenman, 1961), the selectivity of a system to monovalent cations depends on the electrostatic field strength of the negatively charged groups in the system. At low field strengths the affinity for K is higher than for Na and vice versa at high field strengths. Skou suggests that the affinity is shifted by electrons moving from one group of fixed charges to another through the membrane and shows how the movements of an electron outwards could produce an outward movement of Na and an inward movement of K if there are at least three charged sites in a row through the membrane. It has been shown that oxidative phosphorylation is a reversible process (see Klingenberg, 1964, for a review) and that ATP may be used to induce the movement of electrons. In Skou's scheme the electron is moved back to the inside of the membrane by the hydrolysis of ATP and the cycle is ready to start again.

It is interesting to note that Skou uses the same theoretical basis to account for the selectivity of the membrane that Ling uses to explain the selectivity of the protoplasm.

The recent discovery of the 'elementary-particles' containing all the functional enzymatic components of the electron transport chain on the surface of mitochondrial membranes(Fernandez-Moran, 1962), indicates that similar particles corresponding to Skou's enzyme system may soon be found in the plasma membrane.

1.43 Active transport and the membrane potential

If an ion carrier is charged as it crosses the membrane extra terms will be required in an equation for the membrane potential. In principle it should be possible to decide whether a pump is neutral or electrogenic by stopping it with an inhibitor or blocking agent. If the pump is electrogenic there should be a sharp change in the membrane potential but if it is neutral there will only be a slow change as the ions involved equilibrate with the external medium. In practice the situation is more complicated since the inhibitor may also alter the permeability properties of the membrane.

1.44 Some connections between ion uptake and metabolism in higher plants

When a tissue slice is transferred from water to a salt solution there is an increase in its rate of respiration which is sensitive to inhibitors, such as cyanide, which do not affect the ground respiration. Attempts have been made to relate this increased respiration to the number of anions accumulated in order to provide evidence for or against the redox pump theory (Robertson, 1960). However, there is some uncertainty as to the magnitude of the salt respiration, and the possibility that part of the energy may have been used in transporting cations seems to have been overlooked. The results are therefore inconclusive.

An increase in the organic acid content of a plant cell will produce hydrogen ions which can exchange with the cations in the external medium and thus give rise to a net uptake of cations. Ulrich (1942) has shown that the nature of the external ions influences the rate of organic acid synthesis; synthesis is promoted by highly permeable cations and impermeable anions. Briggs, Hope and Robertson (1961) have put forward a hypothesis to explain the phenomenon based on the assumption of competition between

organic and inorganic anions for transport into the vacuole. Anions which could enter the cytoplasm easily would compete with the organic anions for transport into the vacuole, the concentration of organic anions in the cytoplasm would increase, and this would cause the rate of synthesis to fall.

The rate of accumulation of salts in some photosynthetic plants is increased by light. This could be due to a direct effect on the permeability of the membrane or to an indirect effect due to some photosynthetic product. Briggs, Hope and Robertson (1961) discuss experiments of Arisz which suggest that non-cyclic photosynthetic phosphorylation, not inhibited by DNP, may be responsible for light-dependent ion uptake.

C H A P T E R II

ION UPTAKE AND ELECTRICAL POTENTIALS IN NITELLA TRANSLUCENS.

2.1 INTRODUCTION

Ionic relations of the Characeae

The giant internodal cells of the Characeae are an ideal experimental material for the study of ion uptake in plants as they have a simple geometrical shape and uncontaminated samples of sap may easily be obtained. The cells, which are regular cylinders up to 15 cm. in length and 1.5 mm. in diameter, have a cell wall about 10μ thick surrounding a layer of protoplasm of about the same thickness. Chloroplasts are arranged in a regular helical pattern in a layer next to the plasmalemma, while the rest of the protoplasm streams in a direction parallel to the rows of chloroplasts. Between the ascending and descending streams of protoplasm there are neutral lines where the cytoplasm is stationary. Much early work was done on these cells (see for example Blinks, 1955) but is only recently with the development of microelectrode and isotopic tracer techniques that it has been possible to make measurements which can be given a sound theoretical interpretation.

Walker (1955) used microelectrodes to measure the electrical potentials of the cytoplasm and vacuole of an unidentified Nitella species. He showed that, after a shallow insertion, the microelectrode is soon covered by the streaming cytoplasm without a significant change in the measured potential. Eventually a visible seal is formed over the tip of the microelectrode and the potential falls by 30 - 40 mV. In later papers Walker (1957, 1960) showed that the plasmalemma accounted for most of the electrical resistance between the vacuole and the external solution, and he obtained lower values (15 k ohm.cm.^{2}) than those obtained previously by Blinks ($\sim$ 100 k ohm.cm.^{2}). This evidence, although helping to dispose of the hypothesis that the cytoplasm was part of the free space, also showed up the, still unresolved, discrepancy between the measured resistance and that calculated from flux measurements (about 250 k ohm.cm^2).

Kishimoto (1959) also found that the main potential drop in Chara corallina was across the plasmalemma. A fall in the potential of 5 - 20 mV occurred when cytoplasm streamed over the tip of the microelectrode.

Oda (1956, 1961) has measured the resting potential and ionic concentrations in the vacuoles of cells of Chara Braunii. His results suggest that potassium and chloride are pumped into the cell and that sodium is pumped out of the cell. Both Oda (1961) and Kishimoto (1959) have investigated the effect on the resting potential of changing the external ionic concentrations. Oda found that only at high external concentrations of KCl did the membrane potential approach the behaviour expected for a potassium electrode, while the chlorides of Na, Mg, and Ca had a smaller effect on the membrane potential. Kishimoto obtained similar results when he increased the sodium or potassium chloride concentration in his standard solution. However, when the salt solution was made up with distilled water he obtained a much greater change in potential which suggests that the calcium in the standard solution has a buffering effect, although he did find that the effect was seasonal.

MacRobbie and Dainty (1958) have measured the ion fluxes and concentrations in Nitellopsis obtusa, a brackish water species. The Nernst potentials obtained from their concentration measurements are: E_{Na} =-15 mV, E_K = -130 mV, and E_{Cl} = +45 mV. If these are compared with the membrane potential of -120 mV (Spanswick, 1961) for cells in the same solution, it suggests that sodium is pumped out of the cell, chloride pumped inwards, and potassium either in equilibrium or pumped into the cell. The cells were shown to be in flux equilibrium and uptake occurred in three phases which were identified with the cell wall, the protoplasm, and the vacuole, the plasmalemma being more permeable than the tonoplast. The chloride

pump was provisionally placed at the tonoplast and the sodium pump at the plasmalemma.

Diamond and Solomon (1958) measured the potassium fluxes in Nitella axillaris and arrived at a similar picture of the cell divided into three compartments, though, in this case, the flux at the plasmalemma was less than that at the tonoplast. In addition it was shown that the chloroplasts equilibrate very rapidly with the cytoplasm.

Gaffey and Mullins (1958) using Chara globularis, a corticated species, showed that potassium was in equilibrium while sodium was pumped out and chloride pumped into the cell.

The work of Dainty and Hope, in showing that the free space of Chara australis could be fully accounted for by the cell wall, has been covered in Section 1.2.

Hope and Walker (1960) measured the ionic concentrations and sodium fluxes in Chara australis. They found that the efflux was greater than the influx but this was probably because the measurements were made in an artificial pond water with a lower sodium concentration than the field pond water. The fluxes increased with temperature and the influx rose at increased external concentrations. The fluxes were 1 - 5 times greater in the light than in the dark and were unaffected by lack of aeration, which suggests a connection with a photosynthetic, non-oxidative part of the cell metabolism. From measurements of the ionic concentrations in the sap and of the membrane potential, they concluded that potassium is approximately in equilibrium, while sodium is actively pumped out of the cell. The vacuolar concentration of calcium was much lower, while that of chloride was much higher than would be expected if these ions were in equilibrium.

Hope and Walker (1961) investigated the changes in the membrane potential

and resistance of cells of Chara australis when the external ionic concentrations were varied. It was shown that the membrane potential could be described by a Goldman equation of the type:

$$E = \frac{RT}{F} \ln \frac{P_K K_o + P_{Na} Na_o}{P_K K_i + P_{Na} Na_i} = \frac{RT}{F} \ln \frac{K_o + \alpha Na_o}{K_i + \alpha Na_i}$$

By varying the ratio of K_o to Na_o they obtained a value of 0.06 for α. It was necessary to use a Ca-free solution and pre-treat the cells in 5 mM NaCl in order to remove exchangeable calcium, since the presence of calcium prevented the potential changing except at high values of K_o. The measurements of membrane resistance showed current rectification of the type predicted by the Goldman equation.

Hope (1963) has measured the potassium fluxes in Chara australis. His analysis of the efflux kinetics suggests that the fluxes at the tonoplast are larger than those at the plasmalemma by a factor of about 20. Raising the external potassium concentration from 0.1 mN to 1.0 mN increased the fluxes across the plasmalemma from about 0.4 p-equiv.$cm.^{-2}sec.^{-1}$ to 2 - 5 p-equiv.$cm.^{-2}sec.^{-1}$. The omission of calcium from the external medium doubled the influx while bicarbonate ions and dark conditions had no significant effect. Preliminary experiments with ouabain suggested that the potassium influx was largely passive and the conductance calculated from the passive fluxes of sodium and potassium was about ten times less than the measured conductance.

Barr and Broyer (1964) have investigated the effect of light on the sodium influx, membrane potential, and protoplasmic streaming in Nitella clavata. Cells were kept under constant lighting conditions at various intensities until the protoplasmic streaming and Na influx became constant.

Although light had no effect on the membrane potential or the vacuolar concentrations, the Na influx showed a marked increase with light intensity at low intensities but levelled off at higher intensities. Because the electrochemical potential gradient for sodium tends to drive it passively into the cell, it is not possible to distinguish between an increase in active transport down the electrochemical potential gradient and a light-induced increase in permeability. When bicarbonate was in the external medium the Na influx was increased at higher light intensities and the authors suggest this indicates some connection with photosynthesis. It is fairly certain that cells kept under these conditions are in flux equilibrium, in which case the reported vacuolar concentrations of K and Cl indicate an active influx of both these ions.

MacRobbie (1962) has made detailed measurements of the ion fluxes and concentrations in Nitella translucens. The Nernst potentials for the ions in the vacuole were: $E_K = -168$ mV, $E_{Na} = -103$ mV and $E_{Cl} = +120$ mV. Comparison with the value of the membrane potential of -140 mV obtained by Spanswick (1961) shows that sodium is pumped out of the cell while potassium and chloride are pumped inwards. The existence of a sodium potassium exchange pump of the type found in animal cells is probable because ouabain inhibited a large part of the potassium influx. Sodium and potassium were 1.5 times more concentrated in the flowing cytoplasm than in the vacuole and from this MacRobbie predicted that there should be a potential of about 10 mV across the tonoplast with the vacuole positive with respect to the cytoplasm. The chloride pump was provisionally located at the tonoplast. Ionic concentration measurements in the chloroplast layer suggested that the chloroplasts might have some independent mechanism of ionic regulation. The cells were approximately in flux equilibrium, the plasmalemma being the main permeability barrier. The

chloride influx was greatly reduced in the dark but was unaffected by ouabain or lack of aeration, suggesting a direct link with photosynthetic phosphorylation.

The resistance of the membranes of Nitella translucens has been measured by Williams, Johnston and Dainty (1964). It was found that, with this plant also, the measured resistance was about ten times less than that calculated using the figures for the passive Na and K fluxes obtained by MacRobbie (1962). They suggest that the discrepancy could be partly explained on electro-osmotic grounds.

The evidence to date permits the following generalization: Internodal cells of the Characeae may be treated as three compartment systems consisting of the protoplasm, vacuole, and the cell wall which acts as a weak-acid cation exchange resin and accounts for the free space. The ionic fluxes at the tonoplast are usually greater than those at the plasmalemma in agreement with the relative electrical resistances of the two membranes. However, the absolute value of the resistance is about ten times smaller than that calculated from the passive fluxes of Na and K. Sodium is actively pumped out of the cell while chloride and, in the majority of cases, potassium are pumped into the vacuole. In calcium-free media the membrane potential is determined by the permeabilities and concentrations of sodium and potassium.

An attempt to investigate certain aspects of the ionic relations of Nitella translucens in greater detail is described in this chapter.

2.2 THE STEADY STATE : I. MEMBRANE POTENTIALS AND Na, K AND Cl CONCENTRATIONS.

2.21 Introduction

In spite of the considerable amount of work done on the internodal cells of the Characeae, the information which might be considered basic to the study of this three compartment system is not available for one particular species. Such information includes the concentrations of the ions in each compartment, the fluxes across the two membranes, and the electrical potential of each compartment relative to the external medium. Fortunately, interactions between ions in the external medium and the cell wall are purely physical and therefore, given sufficient time to equilibrate, the ions in the cell wall will be at the same electrochemical potential as those in the external medium. This does not apply to the cytoplasm and vacuole as active transport occurs at the plasmalemma. Before the site and direction of the ion pumps can be specified it is necessary to know the electrochemical potential differences across both the tonoplast and the plasmalemma for each ion in addition to the ionic fluxes.

MacRobbie (1962) has made a detailed study of the fluxes and concentrations of Na, K, and Cl in Nitella translucens. The work described in this section was designed to supplement that of MacRobbie by providing measurements of the electrical potentials and ionic concentrations in the cytoplasm and vacuole of cells in the same external medium.

2.22 Methods

Experimental Material.

Supplies of Nitella translucens were collected from a fresh-water loch near Dunkeld, Perthshire, and stored in an artificial pond water (composition: NaCl 1.0 mM, KCl 0.1 mM, $CaCl_2$ 0.1 mM). The cells survived for several months in surroundings which were cool and shaded. As healthy cells have

a high turgor pressure and rapid protoplasmic streaming, only those cells which exhibited these characteristics were used in experiments.

Electrical measurements

The apparatus used for inserting microelectrodes into Nitella cells is shown in Plate 1. The cell lies in a Perspex bath with glass sides and is viewed through a microscope mounted horizontally. Small cells were mounted in a shorter bath of similar design shown in detail in Fig. 1. A maximum magnification of 400X was obtained by using a 10X eyepiece and a Cooke 40X long working distance objective. Two microelectrodes could be inserted into the cell using a Zeiss sliding micromanipulator. The multi-way tap, used in experiments described in later sections of this chapter, made it possible to change the solution in the bath very rapidly. The solution, fed in through the tap from polythene reservoir bottles placed on a rack above the apparatus, was removed from the bath by suction.

The microelectrodes were made from 2 mm. o.d. 'Pyrex' glass tubing using a microelectrode puller. The tip diameters were 0.5 - 1μ and, with sharply tapered shanks, the electrodes were able to penetrate the cell wall quite easily. They were filled by boiling in 3 N KCl under reduced pressure for about 15 minutes and those which had high tip potentials or gave unsteady readings were discarded.

A block diagram of the electrical apparatus is shown in Fig. 2. Each microelectrode was attached to a 'Pye' calomel reference electrode by means of a piece of rubber tubing filled with 3N KCl. The electrode in the external solution was also a 'Pye' calomel electrode. These electrodes are very stable and are more convenient in use than Ag-AgCl electrodes. Measurements were made between:

a) A microelectrode inserted into the vacuole of the cell and the reference electrode in the bathing solution.

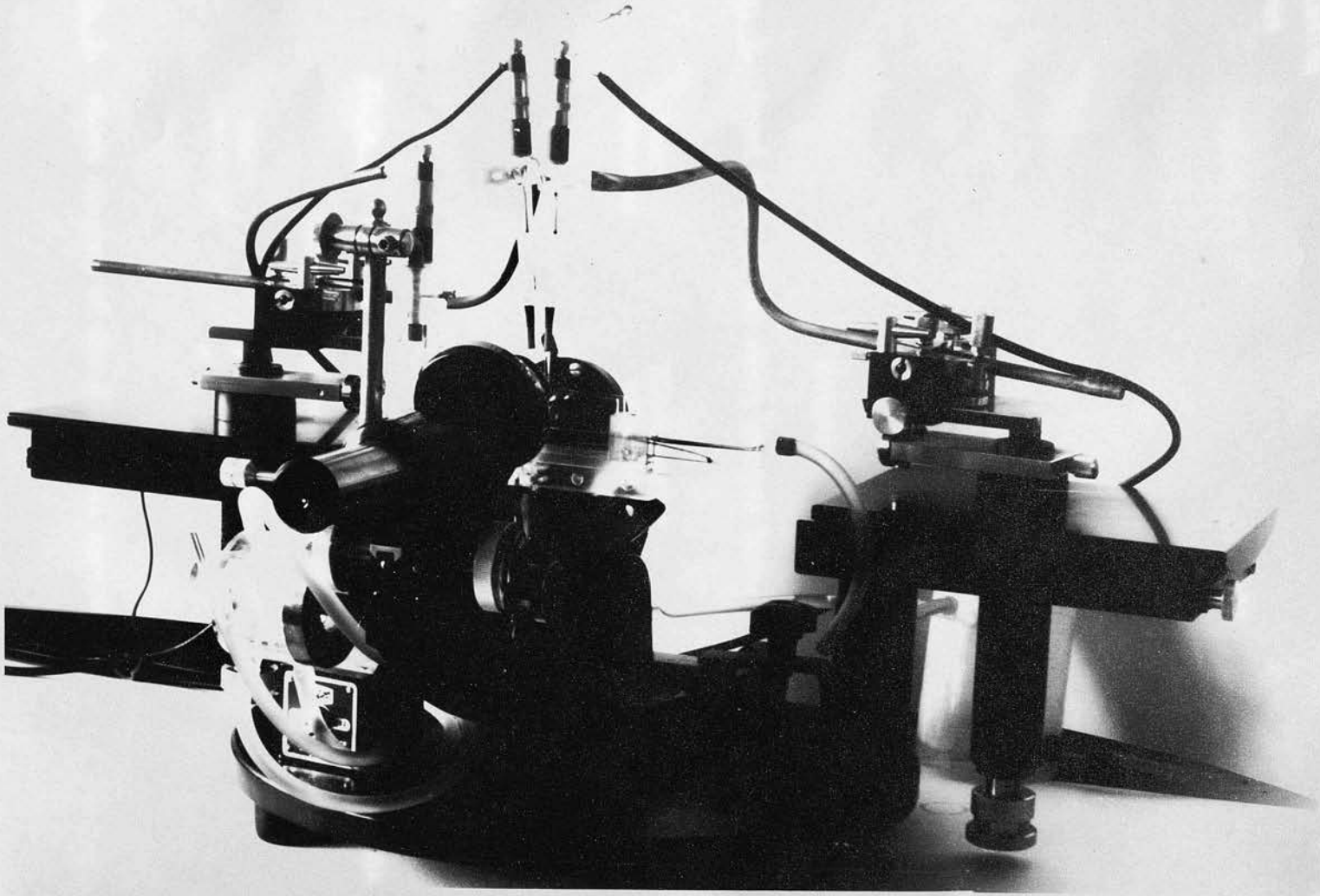


Plate 1. General view of apparatus for inserting microelectrodes.

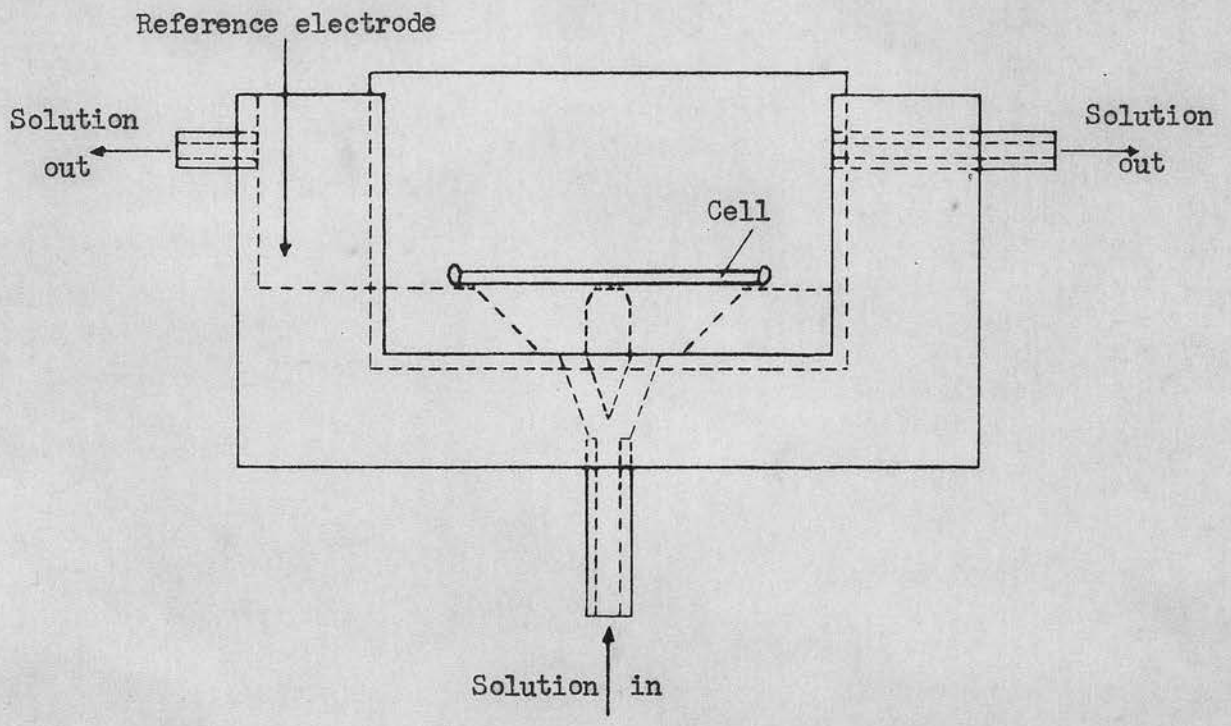


Fig. 1. Front view of the short perspex bath.

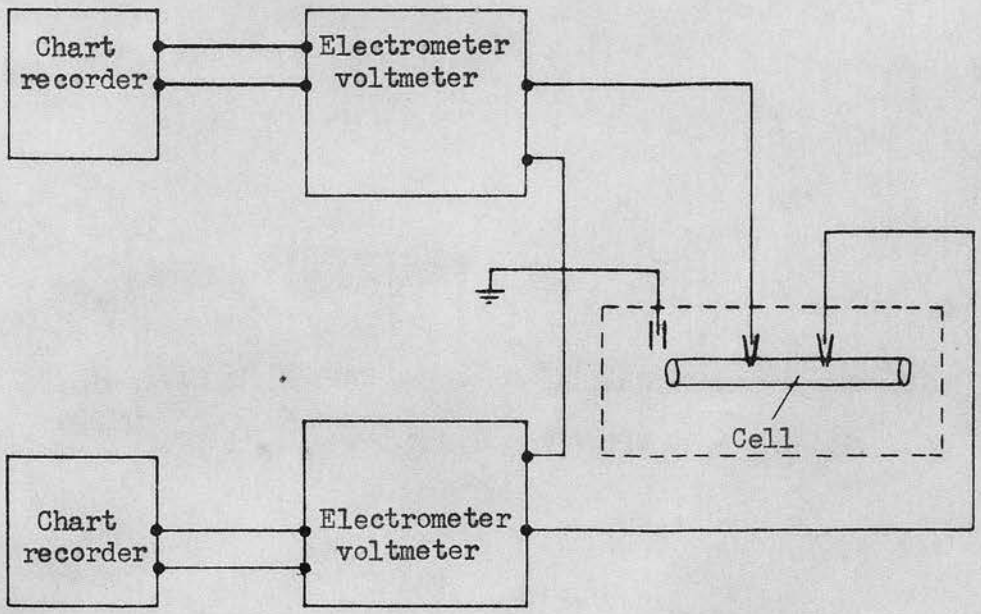


Fig. 2. Block diagram of the electrical apparatus.

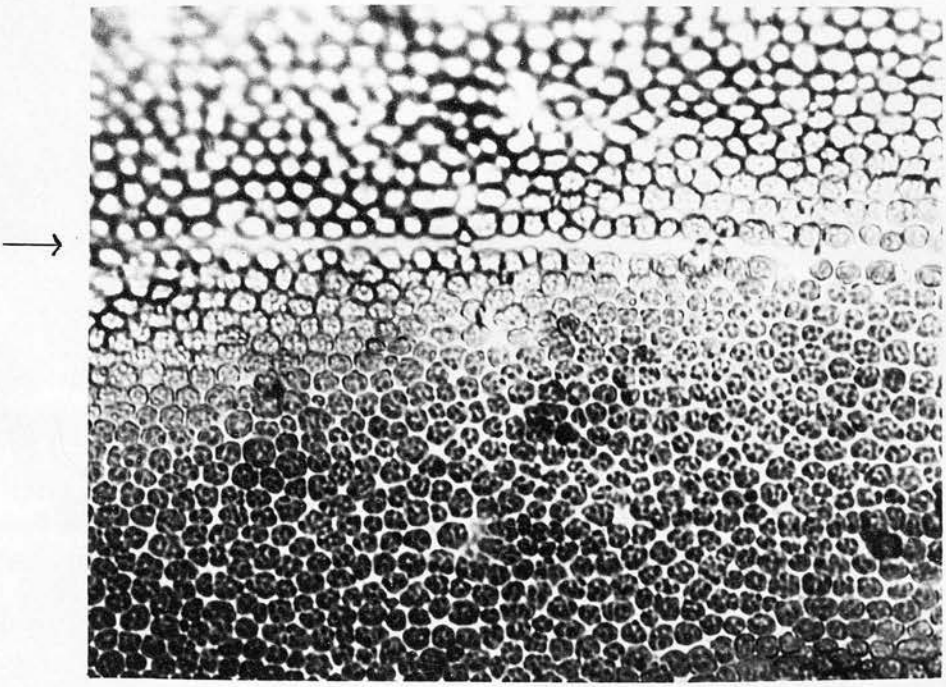


Plate 2. Part of the surface of a Nitella cell showing the neutral line between the streams of cytoplasm.

b) A microelectrode inserted into the flowing cytoplasm and the same reference electrode.

Each potential difference was recorded simultaneously by separate high impedance Vibron 33B millivoltmeters which were connected to chart recorders to give a continuous record of the potential differences.

The insertion of microelectrodes into the cell, particularly into the cytoplasm, requires precise manipulation. It is also very important to be sure of the location of the microelectrode tip in the cell. One of the difficulties is that when an electrode is inserted into the vacuole the streaming cytoplasm will flow over the electrode tip within a few minutes (see Walker, 1955) and it is then in the cytoplasm. In the cells of the Characeae the cytoplasm flows in a helical path in one direction along the the cell and back along the adjacent helix in the opposite direction (Plate 2). Thus if a microelectrode is inserted about 100 μ into the cell at the junction of the two streams the opposing movement of these streams prevents the cytoplasm from flowing over the electrode tip; this electrode is now in the vacuole and will remain there. A second microelectrode inserted a few microns into the cell, and at some distance from the streaming junction, will be in the cytoplasm; if it accidentally enters the vacuole it will soon be covered with flowing cytoplasm. The potential recorded by the electrode in the cytoplasm remains steady for about 15 minutes after which the cytoplasmic sealing process described by Walker causes it to drop to about -70 mV. At the end of each experiment the 'cytoplasmic' electrode was inserted into the vacuole to check for any electrical asymmetry between the electrodes. The value recorded by the 'vacuolar' electrode was assumed to be correct and the value given by the 'cytoplasmic' electrode adjusted accordingly. The asymmetry between the electrodes when in the vacuole was rarely more than 2 mV even when the

asymmetry in A.P.W. was much greater. For this reason it seems unjustifiable and unnecessary to use the tip potentials measured in A.P.W. to correct the value of the resting potential. A similar conclusion was reached by Page (1962).

The experiments were carried out at temperatures between 18°C and 21°C. It is important to carry out the experiments at fairly constant temperatures since Hogg and Johnston (private communication) have demonstrated that the resting potential of Nitella translucens has a temperature coefficient of 1.5 mV per °C.

Chemical analyses

By centrifuging cells at about 300 g for a few minutes it is possible to collect the flowing cytoplasm at one end and yet leave the chloroplast layer intact (MacRobbie, 1962). The junction between the sap and cytoplasm was clearly visible under a dissecting microscope. The two fractions were removed as follows: the cell was pinched at the junction between the fractions with a pair of fine forceps and the end containing the sap cut off. 'Microcap' pipettes were than used to remove known volumes of sap and cytoplasm from the separated fractions. If a cell is at least 9 cm. long about one μl of cytoplasm may be collected in this way.

A Unicam SP 900 Flame Spectrophotometer was used to measure the concentrations of sodium and potassium. Each unknown was compared with standard solutions and the result obtained by proportion. Previous calibration had shown that the galvanometer reading varied linearly with the concentration of the sample at the dilutions used (1 μl of sample per 5 ml. H_2O).

Chloride was determined by electromatic titration. A 1 μl sample was placed in a drop of 5% nitric acid and N/100 silver nitrate was added from an Agla syringe driven by the calibrated screw from a screw gauge. The

tip of a glass tube which was fitted to the syringe was in contact with the drop and electrical connections were made between a platinum wire sealed inside the glass tube, a Vibron 33B millivoltmeter, and a silver wire dipping in the drop. The samples were compared with standard solutions and the end point was taken as the point where the potential dropped most rapidly.

2.23 Results

Electrical measurements on young cells

The first measurements were made on young transparent cells about 3 cm. long because the position of the microelectrode tips could be seen more easily than in older cells. One electrode was inserted into the vacuole and left for between 30 minutes and one hour until the potential became steady before another electrode was inserted into the cytoplasm. The values obtained for the potentials of the cytoplasm and vacuole with respect to the external solution in 10 cells are given in Table 2.1. The mean potential across the tonoplast is 17 $\pm$ 1 mV, the cytoplasm being more negative than the vacuole.

Ionic concentrations and electrical potentials in the cytoplasm and vacuole.

The vacuolar and cytoplasmic ionic concentrations were determined using cells with a length of 9 - 15 cm., the membrane potentials being measured on the same cells before analysis. The results for sodium and potassium are given in Table 2.2 and the results for chloride in Table 2.3. The electrical measurements for the last ten cells in each table are common because the concentrations of Na, K, and Cl were all determined for each cell. The mean potential difference across the tonoplast for the 30 cells is 18 $\pm$ 0.8 mV, the cytoplasm always being more negative than the vacuole.

Table 2.1 Electrical potential differences (mV) in young cells of *Nitella translucens*.

	E_{vo}	E_{co}	E_{vc}
	-115	-131	16
	-122	-135	13
	-122	-138	16
	-107	-124	17
	-109	-126	17
	-102	-122	20
	-110	-129	19
	-110	-128	18
	-108	-124	16
	-127	-142	15
Mean ± S.E.M.	-113 ± 2.5	-130 ± 2	17 ± 1

A comparison of the concentrations obtained here with those obtained by MacRobbie (1962) shows good agreement except for the concentrations of sodium in the cytoplasm. MacRobbie gives the mean of eight measurements as 54 mM compared with 37 mM in the vacuole while the corresponding values obtained here are 14 mM and 65 mM. As the plants were obtained from the same source at different times of the year this may be evidence for a seasonal variation or it may be due to a difference in technique. However, a further ten concentration measurements gave mean values of 15 mM and 62 mM for the cytoplasmic and vacuolar concentrations respectively. Also the measurements in Section 2.4 which were made a year later show a lower concentration of sodium in the cytoplasm than in the vacuole.

Table 2.2 The concentrations of sodium and potassium (mM) in the cytoplasm and vacuole and the electrical potential differences $_vE_o$ and $_cE_o$ (mV).

$_vE_o$	$_cE_o$	Na_c	Na_v	K_c	K_v
-106	-124	5	71	120	73
-112	-136	16	83	116	60
-119	-132	18	83	104	61
-116	-133	11	71	128	70
-120	-133	38	71	119	73
-121	-138	4	65	140	64
-119	-140	12	67	135	74
-124	-135	8	75	124	74
-122	-136	5	68	130	72
-121	-142	30	49	89	69
-129	-142	7	59	126	77
-117	-137	16	59	114	71
-124	-146	16	60	116	76
-131	-150	10	52	128	104
-116	-134	26	55	112	76
-138	-160	11	56	117	73
-124	-141	10	53	112	89
-124	-141	14	62	109	90
-127	-143	16	86	109	63
-110	-124	11	56	122	86
± Mean S.E.M. -121 ± 1.6	-138 ± 1.8	14 ± 1.9	65 ± 2.4	118.5 ± 2.6	75 ± 2.4

Table 2.3 The concentration of chloride (mM) in the cytoplasm and vacuole and the electrical potentials (mV) relative to the external solution.

$_{v}E_{o}$	$_{c}E_{o}$	Cl_{c}	Cl_{v}
-126	-142	89	155
-100	-125	74	157
-107	-133	73	131
-108	-135	56	140
- 96	-111	62	168
-108	-130	93	167
-112	-128	56	145
-118	-144	53	174
-126	-140	61	163
-120	-134	67	167
-129	-142	44	140
-117	-137	69	153
-124	-146	70	157
-131	-150	51	174
-116	-134	96	177
-138	-160	68	162
-124	-141	60	177
-124	-141	60	171
-127	-143	48	169
-110	-124	58	162
Mean ± S.E.M. -118 ± 2.4	-137 ± 2.3	65 ± 3.2	160 ± 2.9

MacRobbie (1962) suggested tentatively that the chloride pump was at the tonoplast in which case there would be a very low chloride concentration in the ~~vacuole~~ cytoplasm. The high value obtained here could obviously be due to contamination of the cytoplasm by the sap which has a much higher concentration. However, the low cytoplasmic concentration of sodium measured in the same cells sets a limit to the amount of chloride that could be present due to contamination if all the sodium were present as a result of contamination. This limit, about 35 mM, means that the measured concentration of 65 mM cannot possibly be due to contamination by the sap.

2.24 Discussion

The potential difference across the tonoplast membrane has been measured for different cells by a number of workers (Walker, 1955, and Findlay, 1959, on an unidentified species of Nitella; Blount and Levedahl, 1960, on Halicystis ovalis; Etherton and Higinbotham, 1960, on Avena root hairs). The conclusion from all these experiments was that the vacuolar potential is practically identical with the potential difference across the plasmalemma and that the potential difference across the tonoplast is very small. These measurements were carried out by inserting a single microelectrode firstly into the cytoplasm and then into the vacuole and by observing any change in the potential difference during the process.

MacRobbie (1962) measured the Na and K concentrations in the cytoplasm and vacuole of Nitella translucens and predicted that the potential difference across the tonoplast should be about 10 mV with the cytoplasm negative with respect to the vacuole if the ions were in passive equilibrium. The present experiments have substantiated this prediction. Recently Findlay and Hope (1964) have shown that in Chara australis the potential difference across the tonoplast is between 5 and 20 mV; once more the cytoplasm was

negative with respect to the vacuole.

With this knowledge of the potential difference across the membrane and the ionic concentrations on either side of the membrane one can test for active transport of any particular ion simply by comparing the observed potential difference with that calculated from the Nernst equation, providing the ion is in flux equilibrium. MacRobbie has demonstrated that both Na and K are in flux equilibrium and for the present it will be assumed that Cl is also in flux equilibrium. Tables 2.4 and 2.5 give the observed potential differences and the Nernst potentials for Na, K, and Cl for both membranes. This information is presented in a slightly different form in Table 2.6 which gives the difference between the observed p.d. and the Nernst potential (ΔE, mV) for each ion across each membrane.

A large value of ΔE for a particular ion indicates that there is a big driving force acting on the ion. The sign of ΔE, taking into account the charge on the ion, gives the direction of the driving force. The values of ΔE for Na, K, and Cl at the plasmalemma show that each ion is subject to large driving forces: for Na the force is directed from the external medium to the cytoplasm while the reverse is true for K and Cl. It may therefore be concluded that Na is pumped out of the cytoplasm across the plasmalemma and that K and Cl are pumped into the cytoplasm from the external medium. Similarly it can be argued that the driving force on Na at the tonoplast is relatively large and in such a direction that Na must be pumped from the cytoplasm into the vacuole in order to maintain the observed concentration gradient. The driving forces on K and Cl at the tonoplast are small and show that these ions are near electrochemical equilibrium.

The conclusions regarding active transport of Na and K at the plasmalemma are in agreement with those of MacRobbie who found that ouabain inhibited the

Table 2.4 The observed potential difference and the Nernst potentials for Na, K, and Cl for the plasmalemma membrane. All potentials are expressed in mV.

$_cE_o$	E_{Na}	E_K	$_cE_o$	E_{Cl}
-138	-66	-178	-137	+99

Table 2.5 The observed potential difference and the Nernst potentials for Na, K, and Cl for the tonoplast membrane. The potential of the cytoplasm is taken as zero. The S.E.M. for the observed potentials is $\pm$ 1 mV. All potentials are in mV.

$_vE_c$	E_{Na}	E_K	$_vE_c$	E_{Cl}
+ 17	-39	+ 12	+ 19	+23

Table 2.6 The calculated values of ΔE ($= E_{obs} - E_j$, mV) for Na, K, and Cl for the plasmalemma and tonoplast membranes; E_{obs} is the observed potential difference across a particular membrane, E_j is the Nernst potential for the ion j for the same membrane.

ION	ΔE	
	Plasmalemma	Tonoplast
Na	- 72	-56
K	+ 40	+ 5
Cl	-236	- 4

K influx and suggested that this was evidence in favour of the existence of a linked Na-K pump. The results of MacRobbie's experiments indicated that both Na and K are in equilibrium across the tonoplast but it is now evident that Na is by no means in equilibrium though K is close to equilibrium.

MacRobbie and Dainty (1958) and MacRobbie (1962) suggested that the chloride pump was situated at the tonoplast but it is now clear that it must be at the plasmalemma. One of the reasons given for placing it at the tonoplast was that it was necessary to maintain the osmotic pressure of the vacuole above that of the protoplasm to prevent the protoplast swelling. It now seems possible that the sodium pump at the tonoplast may perform this function.

2.3 THE STEADY STATE : II. MEMBRANE POTENTIALS AND Ca CONCENTRATIONS AND VACUOLAR FLUXES.

2.31 Introduction

Apart from one or two measurements of the vacuolar concentration, there is practically no information in the literature concerning the uptake of calcium by cells of the Characeae. Walker (1957, 1958) has calculated that if Ca were in equilibrium in Nitella the cytoplasmic concentration would be approximately 20 M. Such a high value is obviously absurd but he considers that the maintenance of a low value is probably due to the impermeability of the plasmalemma rather than to a 'calcium pump'. The flux measurements necessary to decide this question have not previously been attempted.

The work of Moore, Jacobson, and Overstreet (1961) is representative of the information available for the higher plants. Their measurements of the uptake of calcium by excised barley roots suggest that it is only taken up by the free space. The amount, if any, that penetrated the cells was so small that it was not detectable by the methods used. Thus there are no figures available for the calcium fluxes across plant cell membranes.

The movement of calcium in muscle and nerve has recently been reviewed by Shanes (1963). Flux measurements in muscle tissue are complicated by the fact that the calcium in the intercellular spaces exchanges very slowly with calcium in the external medium. This difficulty was overcome by Hodgkin and Keynes (1957) in their experiments on squid axons. They were able to obtain unambiguous results for the influx by measuring the activity in the extruded axoplasm, and for the efflux by washing out labelled calcium which had previously been injected into the axon. Measurements of the mobility of the calcium inside the axon showed that the ratio of ionised to

total calcium could not be greater than 0.02, which means that the internal concentration of ionised calcium was less than 10^{-5} times the equilibrium value for a resting potential of -60 mV. Since the efflux was approximately equal to the influx, it was concluded that calcium must be actively extruded from the axon even though DNP had no effect on the efflux. The permeability of the membrane to calcium was very low compared with that for potassium; on the basis of the constant field equation they estimated that $P_K : P_{Ca} = 1 : 0.001$.

Nitella is a particularly suitable material for the measurement of calcium fluxes because it is possible to obtain uncontaminated samples of vacuolar sap (albeit by a special method), and also because it is possible to carry on efflux experiments over a considerable period of time with the cell in its normal state.

2.32 Methods

Electrical measurements and chemical analyses.

The methods used in measuring the electrical potentials and in separating the samples of sap and cytoplasm were identical with those described in Section 2.22.

The concentrations of calcium in the sap and the flowing cytoplasm were determined by flame photometry. Anion interference seriously diminishes the amount of light emitted by calcium but, by making up the solutions to contain $^{N}/_{25}$ EDTA (Greweling, 1961), good agreement with the results of a colorimetric titration of a sample of sap from the same cell was obtained.

Labelled solutions and estimation of activity.

^{45}Ca was obtained from the Radiochemical Centre, Amersham, as a solution of $CaCl_2$ with a specific activity of 4.5 c. per gm. Ca. In preparing labelled A.P.W. the proportion of unlabelled calcium was decreased so as to obtain a final concentration of 0.1 mM Ca. In some cases the activity of

the solutions was increased by addition of extra labelled calcium but the total increment in the calcium concentration was never more than 3%. Samples were counted using an Ekco scintillation counter type N 664A in conjunction with an Ekco automatic scaler type N 610A. 5 ml. of Nuclear Enterprises NE 220 liquid scintillator was added to 0.5 ml aqueous solution for each count. The background was about 0.8 c.p.s.

While water has a quenching effect when it is added to liquid scintillator containing a 5 µl. sample of radioactive A.P.W., it increases the number of counts obtained when the sample is radioactive sap. It is thought that, since the sap has a high salt concentration, precipitation may occur when it is added to the phosphor and addition of water may bring the calcium back into solution, thus overiding the quenching effect. For this reason, the water content of samples was always made up to 0.5 ml. when added to the scintillator.

Influx experiments.

Influxes were measured by placing cells of measured volume and surface area in A.P.W. having an activity of about 0.5 $\mu c.ml.^{-1}$. After 200 - 300 minutes the cells were transferred to an inactive solution and given washings of 5, 10 and 15 mins. to remove surface activity, then the sap was removed and 5 µl. samples taken for counting. In Section 2.43 it is shown that the method of obtaining the sap from the cell is very important since contact between the cell wall and the sap is accompanied by a rapid exchange of calcium. The most satisfactory results are obtained when the sap is ejected from the cell by its own turgor pressure. This may be done by rapidly cutting off one end of the cell with a razor blade in such a way that the main body of the cell is propelled away from the blade but leaves behind an appreciable quantity of sap. This method worked in only 60% of the experiments in which it was used but it was the only method which gave consistent results.

The activity of the labelled A.P.W. was decreased by 20 - 40% during the experiment. However, the decrease in activity was approximately linear with time and the average of the activities at the beginning and end of the experiment was used in calculating the influx.

Efflux experiments.

Cells to be used in washing-out experiments were loaded up in solutions having an activity of 0.5 - 1.0 $\mu c.ml.^{-1}$ for 4 - 8 weeks. Most of the labelled calcium was absorbed by the cell wall and this reduced the specific activity of A.P.W. so much that it was necessary to transfer the cells to fresh solutions of labelled A.P.W. periodically. The dimensions of each cell were measured before putting the cell in the loading-up solution and at the end of the experiment. Usually there was no significant change in the dimensions.

During the washing-out experiments the cells were continuously illuminated and were at room temperature (19° - $21^{\circ}C$, thermostatically controlled). In the early stages of the experiment the cell was suspended in a glass tube and a syringe was used to draw up the solution. In the later stages of the experiment separating funnels were found to be convenient containers since the solutions could easily be renewed.

The large amount of calcium in the cell wall was the main complication in the efflux as well as the influx experimemts. About 90% of the labelled calcium in the cell wall was removed by using 10 mM $CaCl_2$ in place of A.P.W. for the first four aliquots of washing out solution. Even with this treatment, initial experiments lasting 2 - 3 days gave a value for the efflux which was about 100 times greater than the influx. Experiments were therefore continued for longer times and at the end of the experiments the cell wall was opened out, all traces of cytoplasm removed, and then loaded up to approximately the same specific activity as in the intact cell

at the beginning of the experiment. The contribution of the cell wall to the efflux was found by repeating the washing out experiment with the isolated cell wall.

At the end of the experiment the total activity remaining in the vacuole was found from sap isolated by the turgor method used for the influx experiments. The value obtained by this method was checked by extracting the remaining activity with 10 mM $CaCl_2$. Good agreement between the two values was always obtained.

2.33 Results

Concentration and potential measurements.

The results of measurements of Ca concentrations and electrical potentials are given in Table 2.17. The potential across the tonoplast is about 7 mV greater than in the cells used in the previous section even though they came from the same batch. It is difficult to obtain steady readings on the flame photometer when EDTA is present and, because the volume of cytoplasm available for analysis was 1 µl. or less, the concentration was only measured to an accuracy of 10 - 20%. Far more vacuolar fluid was available and the concentration could easily be measured to within 5%.

Since there was a large exchange of labelled calcium when the sap was in contact with the cell wall a control experiment was performed to see if there was a net transfer of calcium. Sap from six cells was extracted by the turgor pressure method and the concentration of calcium compared with that obtained from the remaining sap squeezed out of the cell. The ratios of the concentrations obtained by the two methods were: 1.05, 1.03, 1.08, 0.98, 1.00 and 0.99. It thus appears that there is no significant difference between the concentrations obtained by the two methods.

Table 2.7 The concentrations of calcium (mM) in the cytoplasm and vacuole and the electrical potential differences $_{v}E_{o}$ and $_{c}E_{o}$ (mV).

$_{v}E_{o}$	$_{c}E_{o}$	Ca_{c}	Ca_{v}
-100	-130	11	15
-113	-141	5	11
-121	-140	10	13
-103	-127	7	12.5
- 99	-125	8	11
-124	-144	12	13.5
-104	-125	11	13.5
-105	-132	8	9
-114	-131	5	13
-121	-147	6	12
Mean ± S.E.M. -110 ± 3	-134 ± 2.6	8.3 ± 0.8	12.3 ± 0.6

Theoretical basis for flux experiments.

Since the exchange of calcium with the cell wall is so large and slow it would obscure any cytoplasmic component of the efflux curve. It is therefore impossible to distinguish between the fluxes at the plasmalemma and the tonoplast, and only the accumulation or loss of ions from the vacuole will be considered. This may be described by the equation:

$$\frac{d(VC_i^*)}{dt} = J_{in} A \frac{C_o^*}{C_o} - J_{out} A \frac{C_i^*}{C_i} \qquad (10)$$

where V is the volume and A the surface area of cell; J_{in} and J_{out} are the influx and efflux respectively; C_o^* is the concentration of labelled ion in the external solution and the total concentration of the ion is C_o; C_i^* and C_i are the labelled and total concentrations of the ion in the sap.

During an influx experiment the cell is in contact with radioactive solution for a short time and the specific activity of the sap, (C_i^*/C_i), attains only a very low value compared with the specific activity of the external solution, (C_o^*/C_o). Thus the second term on the right hand side of equation (10) may be neglected and the equation reduces to:

$$\frac{dC_i^*}{dt} = J_{in} \frac{A}{V} \frac{C_o^*}{C_o} \qquad (11)$$

In a washing-out experiment the specific activity of the external solution is maintained at a very low value compared with the specific activity of the sap. Under these conditions equation (10) reduces to:

$$-\frac{dC_i^*}{dt} = J_{out} \frac{A}{V} \frac{C_i^*}{C_i} \qquad (12)$$

The solution of this equation is:

$$C_i^* = C_{io}^* \exp\left(- J_{out} \frac{A}{VC_i} t \right) \qquad (13)$$

where C_{io}^* is the concentration of radioactive ions in the cell at the beginning of the experiment. A plot of $\log_{10} C_i^*$ against time yields a straight line with a slope of $0.434 \frac{AJ_{out}}{VC_i}$ from which the efflux may be calculated.

Influx experiments.

It has been mentioned in Section 2.32 that the apparent value of the influx depends on the manner in which sap is removed from the cell. The method first used was to cut off one end of the cell and squeeze out the sap. This allows the sap to come into contact with the cell wall for a few seconds and during this time exchange of ions may take place. That this is so, is evident from a comparison of the values of the influx obtained by this method with cells which had been washed with A.P.W. (Table 2.8, first column) and 10 mM $CaCl_2$ (Table 2.9, first column) after removal from the radioactive solution. Washing with 10 mM $CaCl_2$ causes a large reduction in the apparent influx. These results can be explained only if the cell wall contains calcium at a higher specific activity than the sap after washing with A.P.W., and at a lower specific activity than the sap after washing with $CaCl_2$. Exchange of calcium between the sap and the cell wall would then give the observed results.

Other methods were used to remove the sap in an effort to avoid contact with the cell wall. One method (Column 2 in Tables 2.8 and 2.9) involved inserting a fine capillary tube into the cut end of a cell, securing it in position by tying a thread around the cell wall and the tube, and squeezing the cell so that the sap was ejected through the tube. However

Table 2.8 Values of the calcium influx ($pM.cm.^{-2}sec.^{-1}$) obtained in cells washed with A.P.W. after removal from the radioactive solution.

Method by which sap sample was obtained:							
Squeezed out		Through tube		Blown out		By turgor	
Expt.	Influx	Expt.	Influx	Expt.	Influx	Expt.	Influx
F 2	0.25	F27	0.21	F39	0.084	F29	0.083
F 3	0.81	F29	0.20	F41	0.73	F35	0.045
F 4	0.15	F32	0.30	F45	0.14	F36	0.052
F 5	0.32	F38	0.031	F49	a.0.045 b.0.028	F40	0.048
F 6	0.36			F52	0.22	F45	0.034
F 7	0.53			F58	0.31	F47	0.048
F 8	0.29			F61	0.38	F52	0.020
F 9	0.22			F62	0.48	F58	0.012
F10	0.27					F61	0.081
F11	0.40					F62	0.054
F12	0.24						
F13	0.26						
F14	0.15						
F15	0.25						
F16	0.27						
F17	0.65						
F18	0.22						
F19	0.20						
F20	0.25						
F21	0.25						
F33	0.73						
F40	0.29						
F47	0.15						
Mean ± S.E.M.	0.33 ± .04		0.19 ± .06		0.27 ± .07		0.048 ± .007

Table 2.9 Values of the calcium influx ($pM.cm.^{-2}sec.^{-1}$) obtained in cells washed with 10 mM $CaCl_2$ after removal from the radioactive solution.

Method by which sap sample was obtained:							
Squeezed out		Through tube		Blown out		By turgor	
Expt.	Influx	Expt.	Influx	Expt.	Influx	Expt.	Influx
F23	0.049	F31	0.035	F43 a.	0.024	F28	0.055
F25	0.019	F37 a.	0.012	b.	0.0034	F30	0.026
F42 a.	0.032	b.	0.0032	F44	0.0076	F31	0.094
b.	0.0082			F46	0.0089	F37	0.023
F55 a.	0.0082			F48	0.0097	F44	0.072
b.	0.0105			F51	0.012	F46	0.028
F59	0.0017			F53 a.	0.034	F48	0.031
				b.	0.0078	F55	0.035
				F57	0.0017	F59	0.022
				F60 a.	0.014	F60	0.053
				b.	0.032		
Mean ± S.E.M.	0.018 ± .006		0.017		0.014 ± .003		0.044 ± .008

the results show that this method had little effect on the apparent influx, probably because the sap was exposed to the cell wall due to damage of the membranes during the manipulations. Another method (results given in 3rd column of the tables) was to insert a capillary tube into the cut end of the cell, cut off the other end, and blow the sap out. This method does not appear to be successful either, though in most cases the cell was left lying for about two minutes with one end cut off while sap samples obtained by the method described next were taken. In experiments F39 and F49, F51 and F53 the sap was blown out immediately and the results are more consistent, though this is not true for F57 in which the sap was also blown out immediately.

The other method, described in Section 2.32 is to use sap ejected by the turgor pressure when the cell is cut. Since this process takes place very rapidly there is no time for ion exchange between the sap and the cell wall to take place. That this is so may be seen from a comparison of the figures in the fourth columns of Tables 2.8 and 2.9 where there is no significant difference between the mean values of the influx obtained after removing surface activity with A.P.W. or 10 mM $CaCl_2$. This being so, it seems justifiable to accept these results as giving the best value for the calcium influx.

After sap had been obtained by the turgor method, the sap remaining in the cell was removed by one of the other methods and, by reference to the experiment numbers, it may be seen that the results vary in the direction predicted by the hypothesis of rapid ion exchange between the sap and the cell wall. Where more than one value is given, samples have been taken from successive drops of sap obtained from the cell. The variation in the magnitude of these results for the same cell demonstrates the importance of exchange with the cell wall in these experiments.

Efflux experiments.

Preliminary experiments showed that at the end of 60 hours the apparent efflux from the cell was about 5 pM $cm.^{-2} sec.^{-1}$, i.e., one hundred times the value expected if the cell were in flux equilibrium. Clearly most of the activity was coming from the cell wall. However, it was possible to estimate the efflux from the vacuole by continuing the experiments for longer periods and removing most of the calcium from the cell wall with 10 mM $CaCl_2$ at the beginning of the experiment. At the end of the experiment the isolated cell wall was reloaded and a separate experiment carried out to determine its contribution to the efflux. As it is difficult to reload the isolated cell wall to its original specific activity, it is

necessary to apply a correction factor to the efflux. The comparatively large amount of activity washed out from both the intact cell and the isolated cell wall during the first 24 hours was taken as an index of the specific activity of the cell wall in each case, and the amount of activity from the isolated cell wall in each period was multiplied by the ratio:

$$\frac{\text{Activity obtained from intact cell during first 24 hours}}{\text{Activity obtained from isolated cell wall during first 24 hrs.}}$$

The importance of the cell wall is illustrated by the figures in Table 2.10 which show (a) the variation in the amount of labelled calcium in the cell with time, and (b) the corresponding amounts of labelled calcium in the isolated cell wall plus the amount remaining in the vacuole at the end of the experiment with the intact cell. Since the two sets of figures are almost identical it is not possible to suggest that there is a component corresponding to the efflux from the vacuole. The similarity in the time courses of the efflux from the isolated cell wall and the intact cell is seen clearly in Fig. 3 which is a semi-logarithmic plot of the rate of loss of activity against time. The parallel course of the curves suggests that the efflux from the vacuole may be very small compared with the influx. The uncertainty arising in applying the correction to the efflux from the isolated cell wall made it necessary to continue the experiments for many days until the efflux from the intact cell was smaller than the influx before this suggestion could be verified. The third curve in Fig. 3 illustrates the manner in which the curve for the intact cell would be expected to flatten off if the efflux from the vacuole were 0.046 $pM.cm.^{-2}sec.^{-1}$. The values of the efflux during the last 2 - 4 days of each experiment are given in Table 2.11. In the third column the efflux has been calculated assuming that all the activity lost from the cell came from the vacuole.

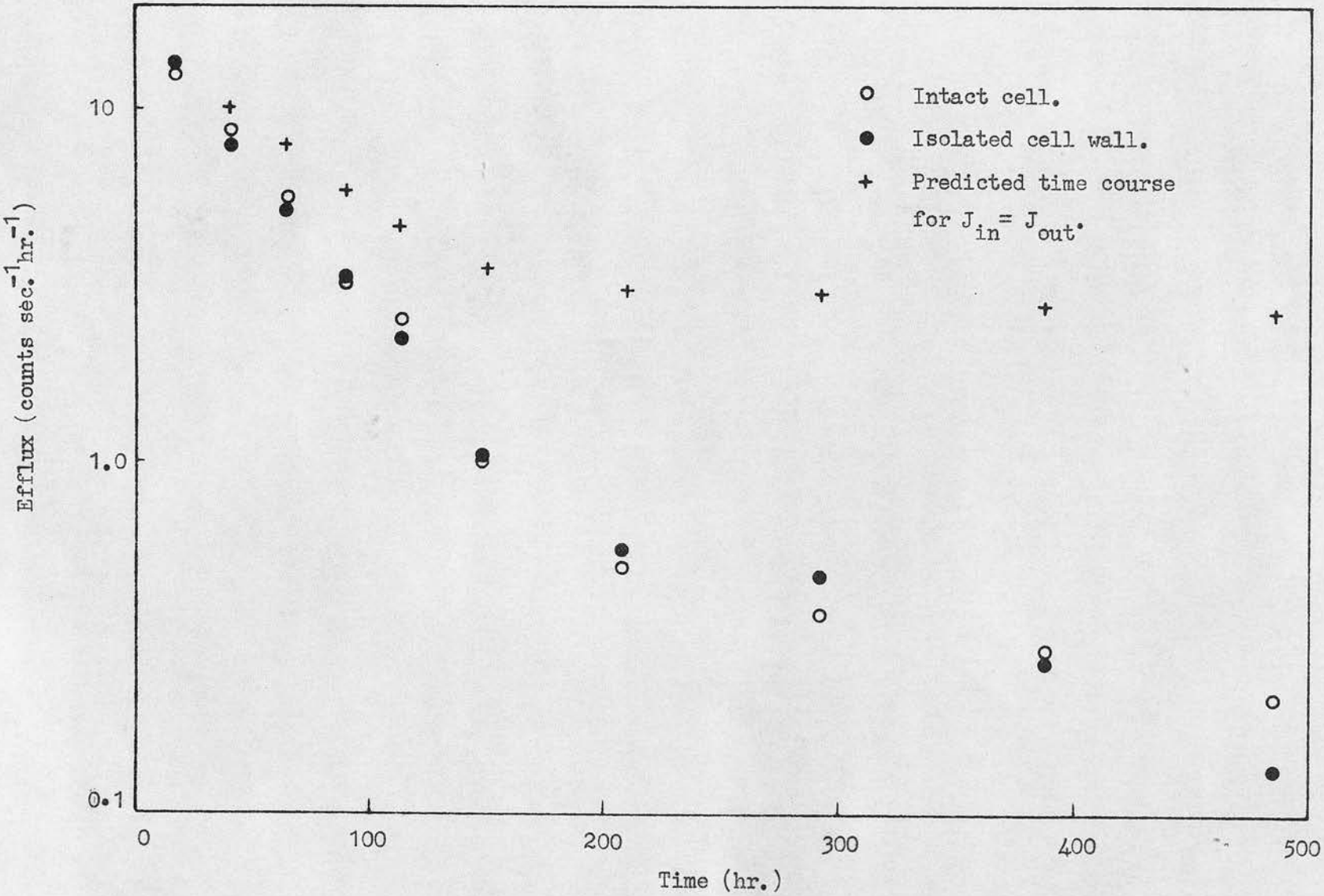


Fig. 3. Time course of the calcium efflux from the intact cell and the isolated cell wall(expt. E38)

Table 2.10 Variation with time of (a) the amount of labelled calcium in the intact cell and (b) the amount of labelled calcium in the isolated cell wall plus the amount in the vacuole at the end of the experiment with the intact cell (Expt. E38).

Time (hrs.)	a (c.p.s.)	b (c.p.s.)
0	47,721	47,725
6	4,656	4,645
29	4,362	4,333
53	4,148	4,114
77	4,012	4,016
101	3,931	3,933
125	3,869	3,879
172	3,821	3,829
244	3,785	3,785
340	3,749	3,739
436	3,721	3,713
532	3,700	3,700

The fourth column gives the efflux from the vacuole obtained by subtracting the corrected efflux from the cell wall from the total efflux. The fact that some of these values are negative means that the correction factor applied to the efflux from the cell wall is not very reliable. The last three results are the most important since the efflux from the intact cell is as much as an order of magnitude smaller than the influx, and even less when allowance is made for the contribution of the cell wall. The magnitude of the passive efflux calculated from the Ussing-Teorell equation is

50 times smaller than the influx. In the present circumstances it is not possible to make an accurate measurement of such a small flux. However, the fact that the efflux is much smaller than the influx suggests that the efflux may be wholly passive.

Table 2.11 The calcium efflux from the vacuole ($pM.cm.^{-2}sec.^{-1}$). The third column gives the apparent efflux from the intact cell during the final 2 - 4 days of the experiment. The fourth column gives the value of the efflux corrected for the contribution of the cell wall. The effluxes are calculated assuming the vacuolar calcium concentration is 12 mM.

Expt.	Duration (days)	Efflux from intact cell	Corrected Efflux
E24	7	0.074	-0.015
E25	10	0.039	-0.008
E27	10	0.097	0.027
E33	17	0.035	-0.037
E35	16	0.056	0.021
E36	18	0.0060	-0.0064
E38	22	0.0040	0.0015
E41	15	0.013	0.0090

The concentration of indiffusible anions in the D.F.S.

The information obtained during the measurement of the effluxes from the cell walls gives the amount of calcium in the cell wall. This may be used to make a rough estimate of the indiffusible anion concentration in the D.F.S. and provide a check on the value obtained by Dainty and Hope (1959).

The cell walls used in the efflux experiments were blotted and weighed,

heated at 100°C for several hours, and reweighed to find the total volume of wall water. Using the figure of 0.46 µl. per µl. of wall water obtained by Dainty and Hope (1959) for the iodine free space in Chara australis, the concentration of calcium in the D.F.S. may be calculated. Since A.P.W. also contains sodium and potassium, the concentration of fixed anions, A^-, is given by:

$$A^- = Ca_i + Na_i + K_i \tag{14}$$

where the subscript i refers to the D.F.S.

The Donnan distribution equation is

$$\frac{Ca_i}{Ca_o} = \left(\frac{Na_i}{Na_o}\right)^2 \tag{15}$$

Hence
$$Na_i = Na_o\sqrt{\frac{Ca_i}{Ca_o}} \tag{16}$$

and similarly,
$$K_i = K_o\sqrt{\frac{Ca_i}{Ca_o}} \tag{17}$$

Substituting Na_o = .001 equiv./l. and K_o = .0001 equiv./l. we get

$$A^- = Ca_i + 0.0011\sqrt{\frac{Ca_i}{Ca_o}} \tag{18}$$

Using this equation the concentration of indiffusible anions in the D.F.S. may be calculated (Table 2.12).

2.34 Discussion

From the measurements of the vacuolar calcium concentration and the electrical p.d. it is possible to show, using the Nernst equation, that there is a net potential of -50 mV driving calcium into the vacuole. Combined with the fact that the influx is much greater than the efflux this suggests

Table 2.12 The concentration of indiffusible anions in the D.F.S. calculated assuming the D.F.S. occupies 54% of the water in the cell wall.

Expt.	Wet wt. (mg.)	Dry wt. (mg.)	c.p.s. in cell wall	Specific activity of ext. soln. (c.p.s. $mole^{-1} \times 10^{-10}$)	A (equiv.l^{-1})
E22	13.45	1.45	343,000	15.2	0.76
E23	7.10	1.40	244,000	19.6	0.88
E24	4.45	1.05	122,000	16.8	0.85
E25	5.50	1.00	148,000	19.4	0.74
E27	7.70	1.40	103,000	8.4	0.79
E29	5.60	1.40	184,000	19.7	0.91
E33	11.05	1.75	312,000	19.3	0.70
E35	9.15	1.25	221,000	17.6	0.59
E36	4.65	0.45	53,000	8.9	0.52
E38	5.70	0.90	93,000	10.5	0.68
				Mean $\pm$ S.E.M.:	0.74 $\pm$ 0.04

that it is not necessary to postulate the existence of a calcium pump maintaining a low vacuolar concentration; the equilibrium concentration would be 625 mM.

Assuming that the cell maintains its original size and the membrane potential remains constant, the concentration in the vacuole of a cell with a volume of 50 µl. and surface area 2 $cm.^2$ doubles in about 80 days. The life expectancy of a cell is not known but is probably short enough for a calcium pump to be unnecessary.

In these circumstances it is difficult to explain the low concentration of calcium in the cytoplasm especially as most of it is probably bound

(98% is bound in squid axon). Assuming that none of the cytoplasmic calcium is bound, the values of ΔE ($= E_{obs} - E_j$) for the plasmalemma and tonoplast are: $E_{Pl} = -79$ mV and $E_{To} = +33$ mV, the sap being positive relative to the cytoplasm. Thus, following the reasoning for sodium in Section 2.34, calcium will tend to diffuse passively into the cytoplasm from both the external solution and the vacuole. If most of the calcium in the cytoplasm is bound the driving forces on the calcium will be increased. The existence of a calcium pump directed towards the vacuole could be postulated with some conviction if the fluxes in each direction between the cytoplasm and vacuole were equal. However, the measurement of the tonoplast fluxes is impracticable at present and the existence of a calcium pump at the tonoplast must be regarded as a speculation.

There is also the possibility that the calcium in the sap is almost wholly bound but, as suggested by its rapid rate of exchange with the cell wall and later confirmed by electrophoresis, it is completely ionised.

Assuming that the influx is wholly passive, it is possible to calculate the value of the permeability coefficient, P_{Ca}, from the constant field equation:

$$J_{OP}^{Ca} = \frac{zFE}{RT} \cdot P_{Ca} \cdot Ca_o \qquad (19)$$

where J_{OP}^{Ca} is the passive flux from the external solution to the cytoplasm ($= 0.046$ pM $cm.^{-2} sec.^{-1}$). Substituting $E = -136$ mV, $P_{Ca} = 4.3 \times 10^{-8}$ $cm.sec.^{-1}$ and hence the ratio $P_K : P_{Ca} = 1 : 0.078$. This is much smaller than the ratio of 1 : 0.001 for squid axon and suggests that the plasmalemma membrane in Nitella discriminates less between potassium and other cations.

The information obtained on the exchange of calcium with the cell wall is in agreement with the picture presented by Dainty and Hope (1959).

There, the fast and slow components in the exchange of calcium were explained by postulating pores of differing sizes in the cell wall. In wide pores the rate of exchange will be limited only by diffusion whereas in long narrow pores the filing effect discussed by Harris (1956), steric hinderances, and possible electrostatic effects will decrease the rate of exchange.

In the present experiments, the slow component in A.P.W. had a half time of approximately 2000 minutes. This represents about 9% of the total activity in the wall and as might be expected, is between the 10% with a half time of 800 minutes in 1 m.equiv.$l.^{-1}$ $CaCl_2$ and the 74% with a half time of 15 days in 1 m.equiv.$l.^{-1}$ NaCl found by Dainty and Hope (1959).

The best value for the concentration of indiffusible anions is 0.8 equiv.$l.^{-1}$ obtained by Dainty, Hope, and Denby (1960). This compares well with the value of 0.74 equiv.$l.^{-1}$ obtained here. It has been shown how exchange with the cell wall makes the measurement of calcium fluxes very difficult. Although these difficulties are especially acute in the case of calcium (a) because of the high concentration in the D.F.S. and (b) because a larger fraction exchanges slowly than in the case of other cations, it is nevertheless pertinent to suggest that these phenomena may give rise to errors in the measurement of the fluxes of other cations. It may be necessary, for instance, to perform efflux experiments with the isolated cell wall before interpreting the middle component of a three phase washing out curve as being due to the cytoplasmic compartment. The method of isolating the vacuolar sap may also be of importance.

2.4 STUDIES ON THE ISOLATED CELL WALL

2.41 Introduction

In Section 2.3 it was shown how the cell wall can be a very important factor in studying the ionic relations of plant cells. In that case it was because the indiffusible anions in the cell wall were mainly associated with calcium ions and this seriously complicated the measurement of the calcium fluxes. The cell wall can give rise to two further effects, both of which are important in experiments in which the effect on the membrane potential of changing the external ionic concentrations is measured, as in Section 2.5. Firstly, the cell wall supports a layer of solution next to the plasmalemma which cannot be stirred. Diffusion through this layer determines the rate at which the membrane potential will reach its new value after a change in the ionic concentrations of the external solution. Secondly, a diffusion potential may be set up in the cell wall on changing the external solution and it is desirable to eliminate it or determine its magnitude and duration.

With Nitella it is possible to investigate both these effects by using isolated sleeves of cell wall.

2.42 Methods

Preparation of isolated cell walls.

The diameter of the cell wall was measured while it was intact so that the surface area of any length of cell wall could easily be calculated. The cell was plasmolysed in M KCl to detach the cytoplasm from the cell wall, then the ends were cut off and the cytoplasm washed out with water using a hypodermic syringe.

Physical parameters of the cell wall.

The thickness was measured by cutting off a very short cylinder from the

cell wall and standing it on end in a pool of water on a microscope slide to ensure that the cross-section of the cell wall was perpendicular to the objective. Using a calibrated eyepiece graticule and 400 X magnification the accuracy was about $\pm$ 0.5 μ.

The length of cell wall used in each diffusion experiment was heated at 100°C over P_2O_5 to remove the water and weighed. The volume of solid material in the cell wall was calculated by assuming it had density of 1.4 gm.cm.$^{-3}$ as did Dainty, Hope and Denby (1960). The total volume of the wet cell wall was calculated from the measurements of thickness and surface area.

Electrical and diffusion experiments.

The apparatus is shown in Fig. 4. Fine glass tubes with a layer of grease on the outside are inserted in either end of the cell and firmly tied in place with thread. The distance between the end of the capillary tubes was measured using a travelling microscope. The cell wall was surrounded by one solution in chamber A and a second solution flowed from a reservoir through the tube of cell wall into chamber B. The solution in chamber A was continuously stirred by a stream of water-saturated air.

The electrical potential difference across the cell wall was measured by inserting a Pye calomel electrode in each chamber and recorded with a Vibron 33B millivoltmeter and a chart recorder.

The rate of diffusion of salts across the cell wall was investigated by flowing the concentrated solution inside the cell wall and measuring the concentration in chamber A with a Mullard conductivity bridge and conductivity cell which had previously been calibrated with solutions of known concentration.

2.43 Results

Diffusion potentials in the cell wall.

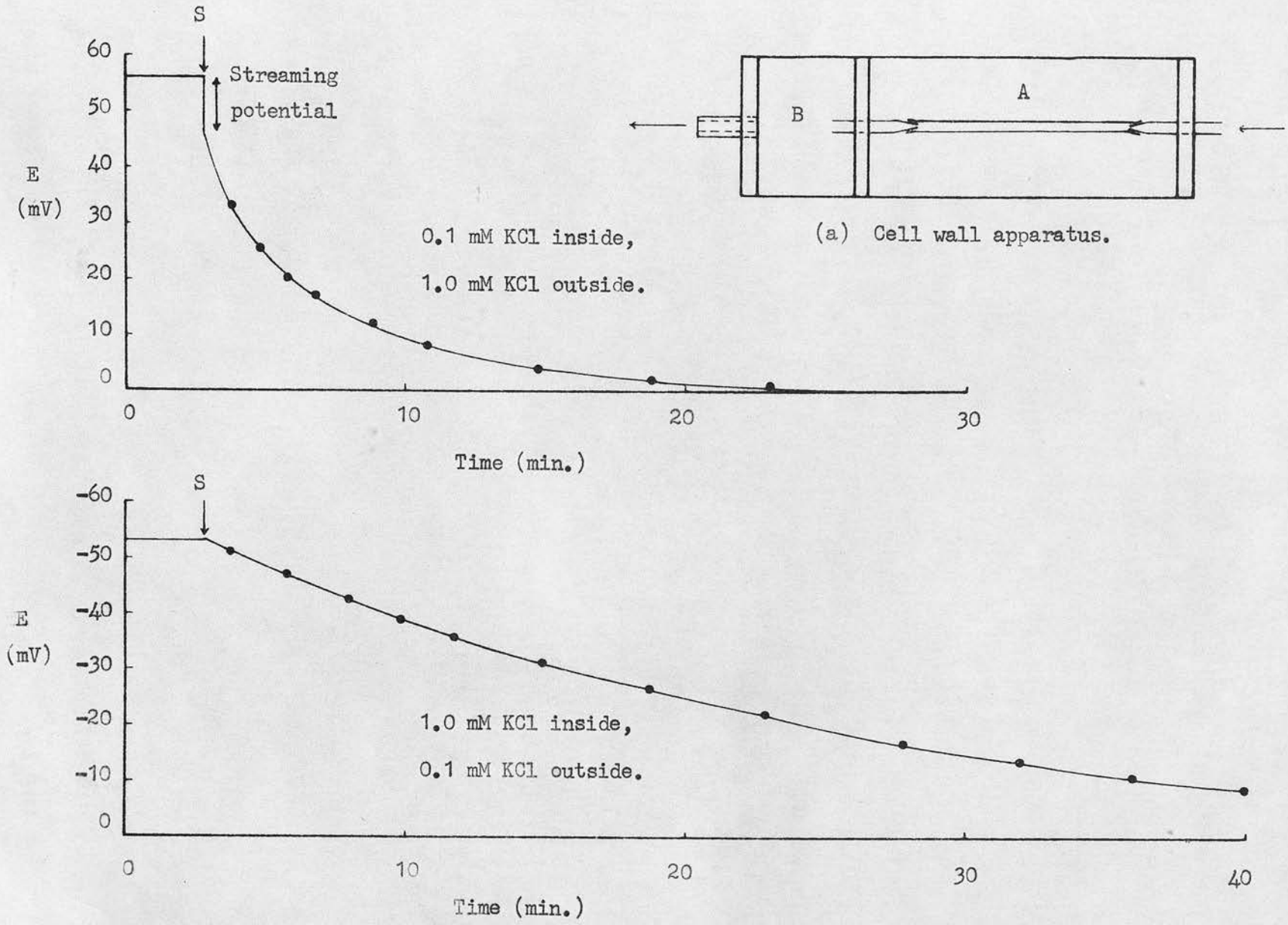


Fig. 4. Time course of the p.d. when the flow of solution through the cell wall is halted at S.

With a 0.1 mM KCl solution on one side of the cell wall and a 1 mM KCl solution on the other side, a p.d. of about 50 mV is set up. The sign of the potential is that expected for a membrane containing fixed negative charges, i.e., the concentrated solution is negative with respect to the dilute solution. If the dilute solution is on the inside there is a streaming potential added to the diffusion potential, the cell wall acting as a single charged pore. The effect of stopping the flow of solution through the cell wall is illustrated in Fig. 4. With the dilute solution inside there is a streaming potential which is abolished immediately the flow of solution is halted. Thereafter the potential difference changes smoothly as the solutions on either side of the membrane equilibrate. With the 1 mM KCl solution on the inside the streaming potential is negligible and the potential difference decreases more slowly when the flow is halted and the solutions equilibrate. This difference in the rate of change of the potentials is to be expected in a situation where two compartments of unequal size equilibrate and the potential difference depends on the ratio of the concentrations.

If more concentrated solutions are used the potential across the cell wall is decreased even though the ratio of the concentrations is the same. This is because the increased conductivity of the solutions has a 'shorting' effect in such a leaky membrane.

This system is very useful for investigating the diffusion potentials set up in the cell wall when the solution outside the (intact) cell is changed as in the experiments of Hope and Walker (1961). When the external solution was changed from 0.1 mM KCl to 1 mM KCl they obtained a sharp transient change in the membrane potential which they ascribed to a diffusion potential in the cell wall. To get over this difficulty they designed

their solutions so that the total concentration of KCl and NaCl was constant but the ratio of Na to K varied. It was claimed that this would eliminate the diffusion potential in the cell wall and it did give a smooth change in the membrane potential with no transient change. Their claim has been tested by using the isolated cell wall. When a solution containing 0.1 mM KCl and 1 mM NaCl is on both sides of the cell wall the potential difference is zero. When the external solution was changed to 1 mM KCl + 0.1 mM NaCl the change in potential was never more than 5 mV and usually much less. Hence their claim is justified.

The results of testing the effects of other solutions on the p.d. across the cell wall will be included in the sections describing the effect on the membrane potential of the intact cell.

Diffusion of salts across the cell wall.

It is possible to deduce a value for the permeability of the cell wall to a salt from the time course of the potential change when the flow of solution on the inside is halted. The value obtained for P_{KCl} is about 10^{-4} cm.sec.$^{-1}$. However, there is a considerable spread in the results and the uncertainties in some of the parameters reduce the usefulness of the method.

The second method used to determine P_{KCl} involved the measurement of the salt concentration in the external solution at regular intervals starting with distilled water outside and flowing 10 mM KCl through the tube of cell wall. The rate at which the salt passed across the cell wall became almost constant after the first 30 minutes. The concentration of the external solution, C_o, was plotted against time and the value of P obtained from the Fick equation,

$$\frac{dC_o}{dt} = P \frac{A}{V_o} (C_i - C_o), \qquad (20)$$

where A is the area of the cell wall and V_o the volume of solution in chamber A (10 mls.). Values of P_{KCl} for two cell walls after various overnight pretreatments are given in Table 2.13.

Table 2.13 Values of P_{KCl} obtained after pretreatment of the cell wall with various salt solutions.

Pretreatment:		A.P.W.	5 mM NaCl	100 mM KCl	100 mM $CaCl_2$
P_{KCl} x 10^4 (cm.sec.$^{-1}$)	1	1.75	1.82	1.86	2.35
	2	1.10	1.15	1.10	1.50

Pretreatment with $CaCl_2$ appears to increase the permeability of the cell wall, otherwise the values for each cell wall are almost constant.

In a further series of experiments the permeability to both KCl and $CaCl_2$ was measured and also the thickness and dry weight of the cell wall. The results are given in Table 2.14. The significance of these results will be discussed in the next section.

Table 2.14 Values of P_{KCl} and P_{CaCl_2} and the physical parameters of the cell walls.

Experiment:	1	2	3	4
P_{KCl} x 10^4 (cm.sec.$^{-1}$)	1.27	1.39	1.10	1.04
P_{CaCl_2} x 10^4 (cm.sec.$^{-1}$)	4.20	3.68	5.05	3.32
Thickness (μ)	12	15	14	20
Surface area (cm.^{2})	1.063	0.835 ~~0.863~~	0.864	0.998
Dry weight (mg.)	0.55	0.48	0.53	0.82

2.44 Discussion

The results of the potential measurements have shown that Hope and Walker (1961) designed their solutions correctly to eliminate interference from transient diffusion potentials in the cell wall. The time course for the equilibration of the internal and external solutions (Fig. 4) shows that the cell wall is a fairly 'leaky' membrane and suggests that diffusion potentials in the cell wall should not be long lived in the intact cell where it is only the volume of the cell wall itself which is equilibrating with the external solution. However, more information on this point may be obtained from the results of the diffusion experiments.

Diffusion across the cell wall will be considered in terms of a theoretical model which takes into account the increase in the length of the diffusion path when only a fraction of the volume of the cell wall is available for diffusion.

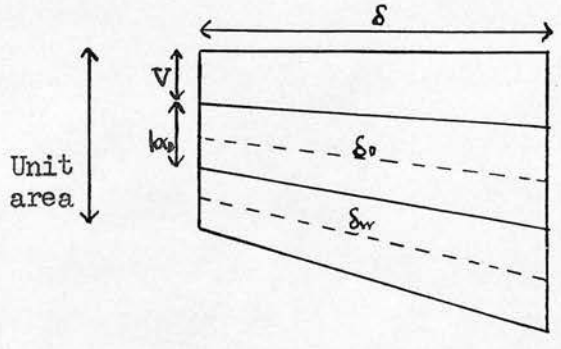
The cell wall may be considered as a three phase system:

(a) Solid phase - vol. V per unit vol. cell wall.

(b) D.F.S. - vol. kx_D per unit vol. cell wall (see later).

(c) W.F.S.

If the cell wall has a thickness δ , the situation may be represented as follows:



δ_D = mean diffusion path through D.F.S.

δ_W = mean diffusion path through W.F.S.

Mackie and Meares (1955) have given an approximate treatment for the mean diffusion path through an ion exchange resin. They show that, if v is the volume per unit volume of membrane not accessible to the diffusing

particle, the average diffusion path is given by

$$\delta' = \frac{1 + v}{(1 - v)} \tag{21}$$

Also, the effective area available for diffusion = $A_g (1 - v)$, where A_g is the geometrical area of the membrane.

Dainty and Hope (1961) have derived an expression for the thickness of electrical double layer which is formally equivalent to a Donnan phase:

$$x_D = \sqrt{\frac{ERT}{2\epsilon F^2 C_o}}$$

$$\text{i.e.} \quad x_D \propto \sqrt{\frac{1}{C_o}} \tag{22}$$

where C_o is the concentration in the bulk phase in equiv.$cm.^{-3}$. This means that the thickness of a double layer in a solution containing divalent ions will be compressed by a factor $1/\sqrt{2}$ compared with the thickness in a solution containing univalent ions at the same molar concentration.

If this theory holds for the cell wall, the volume of the D.F.S. may be written as kx_D or $K/\sqrt{C_o}$, since $x_D \propto 1/\sqrt{C_o}$.

The cell wall acts as a cation exchange resin and hence the only volume available for the diffusion of anions is the W.F.S. This means that for a salt crossing the cell wall the diffusion of the anions will be rate limiting, i.e., $P_{KCl} \doteqdot P_{Cl}$.

Thus for a salt

$$v = V + kx_D \tag{23}$$

Therefore the effective area of the cell wall, $A = A_g (1 - V - kx_D)$

and

$$\delta_W = \frac{(1 + V + kx_D)}{(1 - V - kx_D)} \tag{24}$$

This means that the effective diffusion coefficient $\acute{D}$ in the Fick equation is given by:

$$\acute{D} = D \cdot \frac{\delta}{\delta_W} \cdot \frac{A}{A_g} = D \frac{(1 - V - kx_D)^2}{(1 + V + kx_D)}$$

or,
$$P = \frac{\acute{D}}{\delta} = \frac{D}{\delta} \frac{(1 - V - kx_D)^2}{(1 + V + kx_D)} \qquad (25)$$

Using the information contained in Table 2.14 this expression may be used to estimate the proportion of the water in the cell wall occupied by the D.F.S. The estimated values of kx_D when KCl is present and the corresponding values of P_{KCl} and P_{CaCl_2} are given in Table 2.15.

Table 2.15 Estimates of the proportion of wall water occupied by the D.F.S. and the corresponding permeability coefficients using $D_{KCl} = 1.7 \times 10^{-5} cm.^2 sec.^{-1}$, $D_{CaCl_2} = 1.2 \times 10^{-5} cm.^2 sec.^{-1}$. Δ_{KCl} and Δ_{CaCl_2} are the thicknesses of the unstirred layers required to reduce the larger estimates of the permeability coefficients to the observed values.

Experiment:	1	2	3	4
$P_{KCl} \times 10^4$ (cm.sec.$^{-1}$)	1.27	1.39	1.10	1.04
$P_{CaCl_2} \times 10^4$ (cm.sec.$^{-1}$)	4.20	3.68	5.05	3.32
kx_D (μl.(μl.H_2O)$^{-1}$)	0.82	0.79	0.81	0.79
$P_{KCl} \times 10^4$ (cm.sec.$^{-1}$)	1.18	1.42	1.13	1.04
$P_{CaCl_2} \times 10^4$ (cm.sec.$^{-1}$)	4.88	4.87	4.47	3.47
kx_D (μl.(μl.H_2O)$^{-1}$	0.81	0.78	-	0.78
$P_{KCl} \times 10^4$ (cm.sec.$^{-1}$)	1.34	1.58	-	1.11
$P_{CaCl_2} \times 10^4$ (cm.sec.$^{-1}$)	5.17	5.15	-	4.73
Δ_{KCl} (μ)	70	147	-	103
Δ_{CaCl_2} (μ)	54	108	-	108

The observed values of the permeability coefficients are included in the first two lines to facilitate comparison with the calculated values. The value of kx_D for the first set of estimated coefficients was chosen to give good numerical agreement between the observed and calculated coefficients. Good agreement for P_{KCl} is obtained when the D.F.S. occupies about 80% of the wall water. By dividing kx_D by $\sqrt{2}$ the equation also predicts the permeability coefficient for $CaCl_2$ reasonably well.

In practice the observed permeability coefficients are decreased by the presence of unstirred layers at both surfaces of the cell wall. The last five rows in Table 2.15 give the values of the permeability coefficients obtained by assuming slightly smaller values for the volume of the D.F.S. and also the total thickness of the unstirred layers (Δ_{KCl} and Δ_{CaCl_2}) required to give the observed values of the permeability coefficients. With the exception of experiment 3, when kx_D for KCl is about 79% of the wall water the predicted value for the thickness of the unstirred layers is about 100 μ, i.e., about 50 μ on either side of the cell wall. This seems to be a reasonable figure (c.f. Dainty and Hope, 1959). The observed increase in the permeability with $CaCl_2$ in experiment 3 is too large to permit the thickness of the unstirred layers to be estimated in this way.

From measurements of the volume of the W.F.S., Dainty and Hope (1959) estimated that the D.F.S. occupied 54% of the wall water when the external medium was an artificial pond water with the composition: NaCl, 1.0 m.equiv.l^{-1}; $CaCl_2$, 0.5 m.equiv.l^{-1}; KCl, 0.1 m.equiv.l^{-1}. This figure may be compared with the estimate obtained from equation (25) by assuming a linear concentration gradient across the W.F.S. of the cell wall and calculating the mean value of $1/\sqrt{C_o}$. For the A.P.W. of Dainty and Hope $1/\sqrt{C_o} = 800$, and in the above case $1/\sqrt{C_o} < 600$ for KCl and < 430 for $CaCl_2$. Since the volume of the D.F.S. is proportional to $1/\sqrt{C_o}$, this

means that the volume of the D.F.S. in the experiments presented here should be < 0.75 times that found by Dainty and Hope. In fact, the ratio is about 1.5. This discrepancy may be due to a real difference between the cell walls of Nitella translucens and Chara australis or the assumptions underlying equation (25) may be false. A further possibility is that the blotted weight of the cell walls in Dainty and Hope's experiment may have been an overestimate due to the presence of extra water on the surface. The comparison of the two sets of results would have been more satisfactory if the permeability coefficients for the anion had been measured using radioactive tracers with A.P.W. on both sides of the cell wall.

Taking the value of P_{KCl} as 1.2×10^{-4} cm.sec.$^{-1}$ it may be shown that the cell wall is equivalent to un unstirred layer of water about 1,400 μ thick. This means that it will take the solution at the surface of the plasmalemma about 20 minutes to equilibrate with the external solution when the ionic concentrations in the latter are changed.

2.5 THE EFFECT OF CHANGES IN THE EXTERNAL IONIC CONCENTRATIONS ON THE MEMBRANE POTENTIAL.

2.51 Introduction

Studies on the variation of the membrane potential with changes in the external ionic concentrations have been carried out successfully by animal physiologists, notably Hodgkin and Horowicz (1959). Hope and Walker (1961) have applied the method to plant cells in their work on Chara australis.

In plant cells the method is complicated by the presence of the cell wall. The experiments in Section 2.4 illustrate how the cell wall may be expected to slow down equilibration with the external solution and how diffusion potentials may arise during the process.

The changes in potential obtained when the external concentrations are changed may be used to evaluate the coefficients, or ratios of the coefficients, in the equations for the membrane potential described in Section 1.34. Hope and Walker (1961) found that a Goldman equation containing only terms for sodium and potassium could be used to describe the changes in membrane potential, but only in Ca-free solution.

Some of Hope and Walker's experiments have been repeated on Nitella translucens and the effect of other ions on the membrane potential has also been investigated.

2.52 Methods and Results

The apparatus used for measuring the membrane potentials has been fully described in Section 2.2. The multi-way tap made it possible to change the bathing solution in a few seconds.

Variations of K_o, Na_o, and Ca_o.

The relative effects of sodium, potassium, and calcium ions on the

resting potential were investigated using a basic solution in which they were present at equal molar concentrations and solutions in which the concentration of each ion was in turn increased by a factor of ten. The ionic concentrations of the solutions are given in Table 2.16.

Table 2.16 Composition of solutions. Concentrations in mM.

Ref.	Na	K	Ca	Cl
A.P.W.	1.0	0.1	0.1	1.3
D1	0.1	0.1	0.1	0.3
D2	0.1	0.1	1.0	2.2
D3	0.1	1.0	0.1	1.3

The solutions were changed in the following order: D1, A.P.W., D3, D1, D2, D1. The steady values of the potential given in Table 2.17 were recorded 20 - 30 minutes after the solution was changed.

The transient diffusion potential in the cell wall on changing from D1 to A.P.W. was about 22 mV which is in agreement with the value obtained from an experiment using the isolated cell wall. For the change from D1 to D2 the diffusion potential in the cell wall was about 12 mV. The experiment with the isolated cell wall indicated that the cell wall should equilibrate with A.P.W. in about 5 minutes and with D2 in about one minute. The more rapid equilibration with D2 is due to the high concentration of calcium which decreases the volume of D.F.S. by a factor of $1/\sqrt{2}$ compared with the same molar concentration of potassium. Thus the comparatively large effect of calcium ions on the membrane potential cannot be due to a sustained diffusion potential in the cell wall.

Table 2.17 The effect of variations in K_o, Na_o, and Ca_o on the membrane potential (mV).

Expt.	D1	A.P.W.	D3	D1	D2	D1
1	-155	-137	-138	-141	-131	-141
2	-124	-120	-121	-124	-117	-134
3	-125	-108	-120	-118	- 92	-116
4	-124	-109	-108	-122	-106	-117
5	-121	-104	-103	-108	- 85	-104
6	-116	-114	-110	-119	-100	-117
7	-124	-116	-107	-117	- 94	-120
8	-122	-111	-108	-113	- 96	-116

Variation of Cl_o.

The effect of increasing the external chloride concentration was determined by adding choline chloride to solution D1 to give solution D4 (0.1 mM NaCl, 0.1 mM KCl, 0.1 mM $CaCl_2$, 3 mM choline chloride). Choline is often used by animal physiologists as an inert cation and any effect on the membrane potential should therefore be due to the chloride ion. The results in Table 2.18 indicate that the external chloride concentration is not important since the potential would be expected to go more negative if the membrane were chloride permeable and instead it goes slightly more positive.

The effect of reducing the chloride concentration was tested by substituting sodium and potassium benzenesulphonate for the respective chlorides in A.P.W. As can be seen from Table 2.19 the membrane potential is increased. This is again in the opposite direction to that expected if the membrane were permeable to chloride ions.

Table 2.18 The effect of an increase in Cl_o on the membrane potential (mV).

Expt.	D1	D4	D1
1	-109	-103	-110
2	-110	-100	-104
3	-117	-118	-118
4	-126	-118	-124
5	-120	-117	-122
6	-118	-114	-123

Table 2.19 The effect of a decrease in Cl_o on the membrane potential (mV). Solution D7 has the composition: 0.1 mM potassium benzenesulphonate, 1 mM sodium benzenesulphonate, 0.1 mM $CaCl_2$.

Expt.	A.P.W.	D7	A.P.W.
1	-113	-117	-115
2	-123	-122	-122
3	-137	-140	-133
4	-152	-162	-151
5	-117	-121	-116

The effect of bicarbonate ions on the membrane potential.

Hope (unpublished) has found that the substitution of bicarbonate ions for chloride produces a hyperpolarization of 40 - 80 mV at the plasmalemma in Chara australis. The effect on Nitella translucens was tested by replacing 1 mM NaCl by 1 mM $NaHCO_3$ in A.P.W. There was a large initial increase in potential during the first four minutes followed by a slow decline during the next hour to a steady value. Table 2.20 gives both the maximum and steady values of the membrane potential.

By using two microelectrodes, one in the cytoplasm and one in the vacuole, it was shown that the change in potential was at the plasmalemma. This means that the maximum potential difference across the plasmalemma is about -200 mV. This is about 20 mV more negative than the Nernst potential for potassium which is the ion with the most negative Nernst potential. The effect cannot therefore be explained in terms of an increased permeability to one of the ions in A.P.W.

Table 2.20 The effect of bicarbonate ions on the membrane potential (mV).

Expt.	A.P.W.	Bicarbonate Max. value	Bicarbonate Steady value	A.P.W.
1	-109	-165	-159	-122
2	-112	-180	-131	-115
3	-116	-184	-138	-122
4	-122	-188	-137	-132
5	-128	-179	-144	-132
6	-125	-173	-127	-127
7	-151	-210	-154	-155
8	-116	-169	-126	-119

The transient nature of the effect is typical of a diffusion potential in the cell wall. However, the potential difference with the bicarbonate solution on one side of an isolated cell wall and A.P.W. on the other side was negligible, and thus the increase in the membrane potential is not due to a diffusion potential in the cell wall.

The pH of the bicarbonate solution was found to be 7.8 as opposed to 5.5 for A.P.W. The effect of an increase in pH was tested by adding small amounts of NaOH to A.P.W. just before the solution was to be used so as to avoid absorption of CO_2. At a pH of about 7.8 there was a slow increase in the membrane potential of 10 - 35 mV. Thus the effect of pH could not possibly explain the rapid initial rise in potential of 50 - 70 mV when bicarbonate is applied, but it could account for the difference, if any, between the steady value and the value in A.P.W.

Experiments with cell pretreated with 5 mM NaCl.

Comparison of the membrane potentials in A.P.W. and solution D3 in Table 2.17 shows that changing the ratio of the sodium to potassium con-

centrations by a factor of one hundred has little effect on the potential. Hope and Walker (1961) found that it was necessary to pretreat their cells for several hours in 5 mM NaCl before they would respond to changes in the ratio of the sodium to potassium concentrations in calcium-free solution. This is also the case with Nitella translucens.

Large cells were used in the present experiments so that the ionic concentrations in the cytoplasm and vacuole could be measured. The solutions were similar to those used by Hope and Walker, the total concentration of (KCl + NaCl) being 1.1 mM. The sequence of external potassium concentrations was: 0.05, 0.1, 0.4, 0.7, 0.85, 1.0, 1.05, 1.0, 0.85, 0.7, 0.4, 0.4, 0.1, 0.05 mM. The 0.05 and 1.05 mM potassium solutions were incorporated to compensate for the hysteresis effect which makes the potential more negative when the potassium concentration is being increased than when it is being decreased. Without these extra solutions the average membrane potential in the 1.0 mM potassium solution would be more positive, and the potential in the 0.1 mM potassium solution more negative. The average potential in each solution was added to the measured potential difference across the tonoplast, to give the potential difference across the plasmalemma. The potential difference across the tonoplast was found to be independent of the external ionic concentrations.

The result of one experiment is illustrated in Fig. 5, curve A. Curve B is derived from the Goldman equation:

$$E = \frac{RT}{F} \ln. \frac{K_o + \alpha Na_o}{K_i + \alpha Na_i} \qquad (26)$$

using the values of E in the 1.0 and 0.1 mM potassium solutions to calculate the parameters α and $(K_i + \alpha Na_i)$. The curve predicted by the Goldman equation is flatter than that obtained experimentally and in most experiments the discrepancy is even greater.

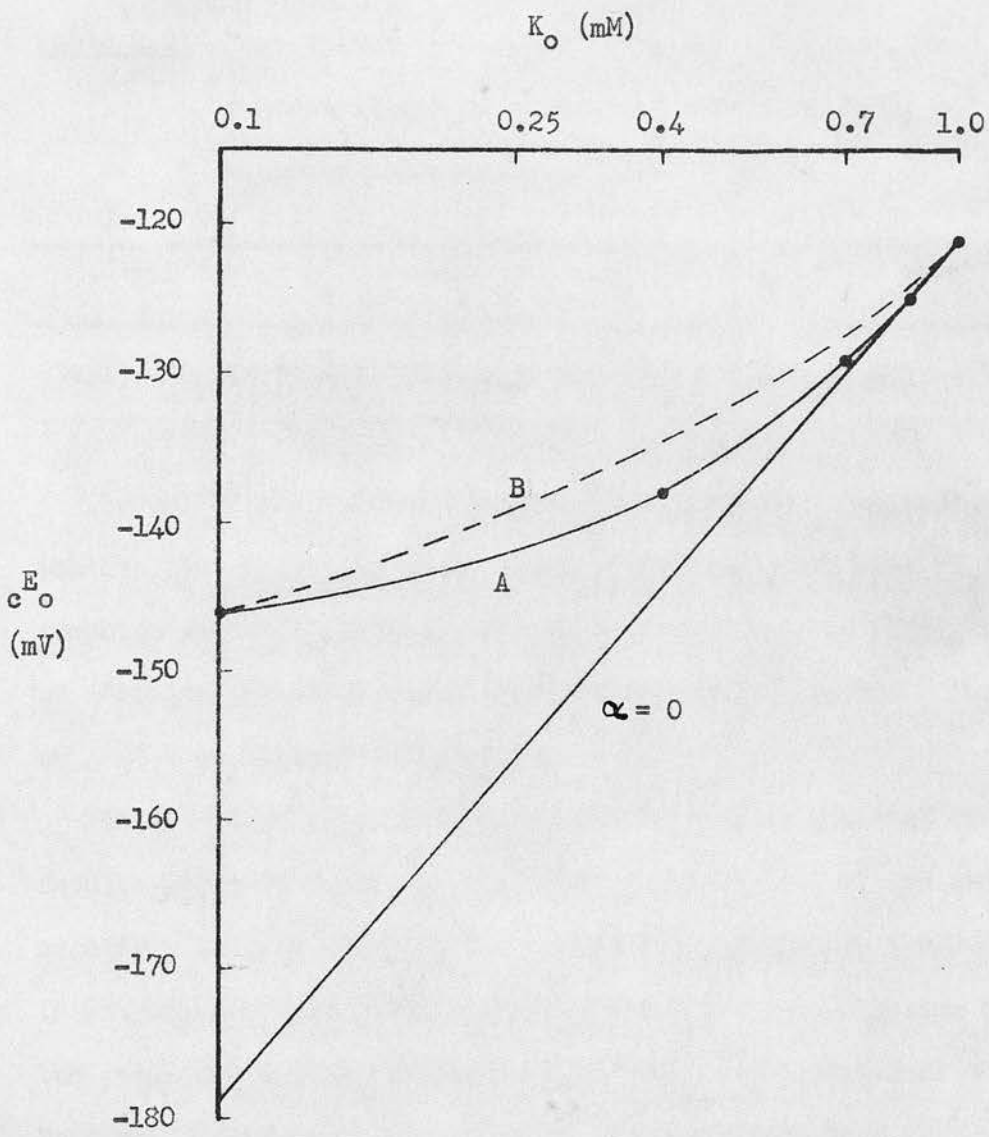


Fig. 5. Potential difference between the cytoplasm and the external medium as a function of K_o, keeping $K_o + Na_o = 1.1$ mM (curve A). Curve B is derived from equation (26) with $\alpha = 0.28$ and $K_i + \alpha Na_i = 125$ mM.

The average values of α, the membrane potentials, and the measured concentrations for 27 cells are given in Table 2.21.

Table 2.21 Mean values ($\pm$ S.E.M.) of α, the cytoplasmic and vacuolar concentrations of sodium and potassium (mM), and the membrane potentials (mV) for 27 cells.

α	K_c	Na_c	K_v	Na_v	$_cE_o$	$_vE_c$
0.25 ± 0.01	93 ± 4	37 ± 2.5	67 ± 3.5	73 ± 2.2	-141 ± 1.5	$+15 \pm 1$

The use of the Goldman equation with terms for only sodium and potassium implies that $P_{Cl}Cl_o$ is small compared with P_KK_i and that $P_{Cl}Cl_i$ is small compared with P_KK_o. It is possible to test whether $P_{Cl}Cl_o$ is important by substituting other anions for chloride (Table 2.22). The small effects suggest that it is not important.

The effect of substituting choline for sodium was also examined. The results given in Table 2.2 show that the potential always becomes more negative, but, on changing the solution to 1.0 mM KCl + 0.1 mM NaCl, which is practically equivalent to a ten-fold change in potassium concentration, the potential usually changes by less than the theoretical 58 mV (line iv). This could mean that the term $\delta\ Cl_i$ is comparable to K_i.

2.53 Discussion

The experiments described in this section can be interpreted meaningfully only in terms of one of the equations for the membrane potential described in Section 1.34.

The results of the experiments on unpretreated cells will first be considered in terms of the electrical analogue. If we consider all the

Table 2.22 The effect of anion substitutions in the membrane potential (mV).

Chloride	Benzenesulphonate	Chloride
-103	-104	- 98
-112	-118	-111
-109	-116	-109
-111	-112	-108
-129	-132	-125
-119	-120	-118
-127	-131	-127
-115	-122	-116
-132	-143	-137
Chloride	**Nitrate**	**Chloride**
-106	-106	-106
-111	-111	-111
-109	-110	-108
-108	-107	-106
-117	-113	-113
-118	-115	-115
Chloride	**Sulphate**	**Chloride**
-106	-106	-106
-111	-108	-110
-106	-102	-104
-113	-107	-107
-107	-110	-109
-106	-106	-106
-117	-113	-113

Table 2.23 Changes in the membrane potential (mV) on substituting choline for sodium.

External medium	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
(i) 0.1 mM potassium, 1.0 mM sodium	-117	-125	-145	-149	-122
(ii) 0.1 mM potassium, 1.0 mM choline	-126	-140	-156	-162	-135
(iii) 1.0 mM potassium, 0.1 mM sodium	- 90	-100	-105	-106	- 96
(iv) (ii) - (iii).	- 36	- 40	- 51	- 56	- 39

ions in the external solution we may write:

$$E = T_K E_K + T_{Na} E_{Na} + T_{Ca} E_{Ca} + T_{Cl} E_{Cl} \qquad (27)$$

Since changes in the external chloride concentration have little or no effect on the membrane potential (Tables 2.18 and 2.19), T_{Cl} must be negligibly small and the equation reduces to:

$$E = T_K E_K + T_{Na} E_{Na} + T_{Ca} E_{Ca} \qquad (28)$$

The effect of the ten-fold increases in cation concentration given in Table 2.17 may be simply interpreted in terms of this equation since such increases will make E_K and E_{Na} more positive by 58 mV and E_{Ca} more positive by 29 mV. The transport numbers T_K etc. may then be calculated from the change in membrane potential, δE, as $\delta E / \delta E_K$ etc. The values of the transport numbers are given in Table 2.24.

The transport numbers represent the proportion of the current carried across the membrane by each ion and therefore $\leq T_j$ should be unity. Taking into account the small number of experiments and the random variations in potential during the course of an experiment, the value of 0.92 for $\leq T_j$

in Table 2.24 suggests that the electrical analogue describes the membrane potential fairly well. However, the value of 0.62 for T_{Ca} means that the membrane potential is governed by the concentrations of calcium and this is in contradiction to the information obtained from measurements of the internal calcium concentration which predict a low value of E_{Ca}. Moreover, the calcium fluxes are so much smaller than the fluxes of sodium and potassium measured by MacRobbie (1962) that it is impossible to account for such a large value of T_{Ca}.

Table 2.24 Values of the transport numbers for Na, K, and Ca calculated from the information in Table 2.17.

Expt.	T_{Na}	T_K	T_{Ca}	$\leq T_j$
1	0.31	0.05	0.35	0.71
2	0.07	0.05	0.41	0.53
3	0.29	-	0.86	1.15
4	0.26	0.24	0.47	0.97
5	0.29	0.09	0.72	1.10
6	0.03	0.16	0.62	0.81
7	0.14	0.18	0.85	1.17
8	0.19	0.09	0.64	0.92

It is possible to overcome this objection by assuming that the concentration of calcium in the external solution controls the selectivity of the membrane, i.e., controls the ratio $T_{Na} : T_K$. From the values of the Nernst potential in Table 2.4 it is clear that $_cE_o$ could vary from -66 to -178 as T_{Na}/T_K varied from infinity to zero if the membrane potential is given by the equation:

$$E = E_K T_K + E_{Na} T_{Na} \qquad (29)$$

However, from Table 2.24 $T_{Na} + T_K = 0.31$ and this low value is not consistent with sodium and potassium ions carrying all the current across the membrane.

There remains the possibility that there is some other carrier of electrical charge across the membrane but as this seems unlikely we are left with a contradiction between the results of electrical and flux measurements.

Objections to the use of the Goldman equation have been put forward by Hogg and Johnston (personal communication). The original derivation of the equation by Gibbs (1899) makes it clear that it only holds if $\leq |z_j| C_j$, the summation being for all diffusible ions, is equal on either side of the membrane. Hogg and Johnston have shown that when $\leq |z_j| C_j$ is not equal on both sides of the membrane the condition of a linear field is incompatible with electrical neutrality throughout the membrane. This is the situation with Nitella. However, Hope and Walker (1961) found that they were able to explain their results in terms of a Goldman equation and a similar analysis will be attempted here.

The equation generally used by animal physiologists is:

$$E = \frac{RT}{F} \ln. \frac{P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i}{P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o} \qquad (30)$$

The experiments in Tables 2.17 and 2.18 show that $P_{Cl} Cl_o$ is negligible in comparison with $(P_K K_i + P_{Na} Na_i)$ and the term may therefore be ignored. It is more difficult to investigate the importance of the term $P_{Cl} Cl_i$ since it is not possible to vary Cl_i. The measurements of the cytoplasmic chloride concentration in Section 2.23 show that Cl_i is much greater than K_o or Na_o and hence the term $P_{Cl} Cl_i$ may be important even if P_{Cl} is much smaller than P_K. MacRobbie and Dainty (1958) showed that the chloride efflux in Nitellopsis obtusa was approximately equal to the influx. By

assuming that the chloride efflux in Nitella translucens is equal to the influx measured by MacRobbie (1962), it can be shown that $\gamma = \frac{P_{Cl}}{P_K} = 0.005$. With Cl_i = 65 mM, the value of γCl_i is approximately 0.3. Since there is very little difference in the membrane potential when the cell is in A.P.W. or solution D3, it may be assumed that $\alpha = 1$. Taking $\gamma Cl_i = 0.3$ and $\alpha = 1$, the predicted change in potential on transferring from solution D1 to A.P.W. or solution D3 is 25 mV. This is large compared with the experimental value of about 10 mV but smaller than the 43 mV predicted by assuming γCl_i is negligible. The predicted change could be made smaller by assuming a larger value of γCl_i. MacRobbie (unpublished) has shown that the chloride influx is greater in cells collected in a later season, so this is a possibility.

The change in potential when the calcium concentration is increased could be explained in terms of an increase in the value of γ or α. α is apparently already larger than the value obtained from flux measurements and an increase in α contrasts with the situation in animal cells where α is decreased by increasing Ca_o (Draper and Karzel, unpublished; Kimizuka and Koketsu, 1963). An increase in γ seems more plausible but obviously flux experiments would be required to settle this point.

The results in Table 2.17 may also be examined in terms of the Goldman equation containing terms for calcium:

$$E = \frac{RT}{F} \ln \left\{ (K_o - K_i) + \alpha (Na_o - Na_i) + \sqrt{\left[(K_o - K_i) + \alpha (Na_o - Na_i) \right]^2 + 4 (K_i + \alpha Na_i + 2\beta Ca_i)(K_o + \alpha Na_o + 2\beta Ca_o)} \right\} / 2 (K_i + \alpha Na_i + 2\beta Ca_i) \qquad (31)$$

It may be assumed that βCa_i is very small compared with K_i because β is small and Ca_i is probably much less than the measured cytoplasmic concentration due to adsorption. The values of K_i and Na_i from Section 2.23 will be used in evaluating this equation.

Table 2.25 The changes in potential (mV) predicted by the Goldman equation containing terms for calcium, and the observed changes in potential. The values are calculated assuming $\beta = 0.078$.

Soln. change	$\alpha = 1$	$\alpha = 0.18$	Observed
D1, D2	13	18	18
D1, D3	41	54	9
D1, A.P.W.	41	19	9
A.P.W., D3	0	34	0

From the electrical and flux measurements in Section 2.3 and the flux measurements of MacRobbie (1962), $\beta = 0.078$. This value has been used in calculating the potential changes tabulated in Table 2.25. In general there is poor agreement between the predicted and experimental values but with the values of α and β calculated from flux measurements the effect of increasing Ca_o is accurately predicted. Except for the change from A.P.W. to D3 with $\alpha = 1$, the predicted effect of changing K_o or Na_o is always too large. The inclusion of terms for Cl_i reduces the predicted changes with increased Na_o and K_o to about the same extent as with equation (30), but it also decreases the predicted change in potential for increased Ca_o to 6.5 mV when $\alpha = 0.18$.

Hope and Walker (1961) found that the membrane potential responded to changes in K_o/Na_o in Ca-free solutions after the cells had been pretreated in 5 mM NaCl. This is also the case with Nitella translucens and the average value of $\alpha (= 0.25)$ agrees fairly well with the value of 0.18 calculated from flux measurements (MacRobbie, 1962). Hope and Walker found that their results could often be described by the Goldman equation containing terms for sodium and potassium only. However, the

experimental curve obtained here (Fig. 5) is not as straight as that predicted theoretically. The anion substitutions in Table 2.22 show that γCl_o is not important but, by assuming γCl_i has a value of 0.18 mM, the average value of α can be reduced to 0.18 to agree with flux measurements. This makes little difference to the shape of curve B in Fig. 5. but it could explain why the figures in line (iv) of Table 2.23 are less than the theoretical 58 mV, since the equation then predicts 37 mV for line (iv). The only way to fit the Goldman equation to the experimental curve is to assume that α decreases as K_o increases. The value of $(K_i + \alpha Na_i)$ calculated using the average values of α and the membrane potential is 94.5 mM which agrees fairly well with the value of 102 mM calculated from the average values of α and the cytoplasmic concentrations.

It is clear that the Goldman equation is of limited value at present. Many of the unsettled points in this discussion could be checked by electrical or flux measurements but lack of time has prevented this.

The brief investigation of the effect of bicarbonate ions has raised a point of disagreement with the results of Hope (unpublished) obtained with Chara australis. The maximum increase in the p.d. in Nitella is approximately the same as that found by Hope but it decreases to a steady value which is not very different from the value in A.P.W. (Table 2.20). Apparently Hope obtained a steady increase in the membrane potential as long as the bicarbonate solution was present. He attempts to explain his results in terms either of an active influx of anions via an electrogenic pump or by the production of acid in the chloroplasts, giving a large H^+ term in the Goldman equation. The postulate of an electrogenic pump seems incompatible with the transient nature of the increase in the membrane potential. The membrane potential reaches its maximum value after four minutes in the bicarbonate solution. It seems most unlikely that a high

concentration of hydrogen ions could be produced in the protoplasm during this short period as the rate of change is more representative of a reaction at the membrane surface only rate limited by diffusion through the cell wall. Although the effect of an increase in pH is not sufficient to account for the effect of the bicarbonate it does suggest that the bicarbonate ion may be affecting the membrane surface in a similar manner. Blinks (1955) has suggested that changes in the acidity of the medium affect the surface properties of the plasmalemma. If this is the case, it is not surprising that the effect of bicarbonate is transient since the membrane potential becomes more negative than the Nernst potential for potassium for a short time.

2.6 ACTIVE TRANSPORT AND THE MEMBRANE POTENTIAL.

2.61 Introduction

The linkage between metabolism and ion transport processes is commonly studied by observing the effects of metabolic inhibitors, light intensity and quality etc. on these processes. It is difficult to obtain unambiguous evidence since these agents may have a direct effect on the permeability of the cell membrane. However, by studying the effect of light and inhibitors on the membrane potential it is possible to clarify their action to some extent.

There are three possible causes of a change in the membrane potential: (a) a change in the permeability of the cell membrane, (b) the blocking of an electrogenic pump, and (c) changes in the internal ionic concentrations. One would expect (a) and (b) to have half-times of the order of minutes while a change due to (c) alone would have a half-time of the order of days in Nitella since the cell is very large. If an ionised inhibitor is used there could also be a transient change in the membrane potential due to diffusion in the cell wall.

Three types of inhibitor will be considered: (i) ouabain, a specific inhibitor of sodium and potassium transport, (ii) photosynthetic inhibitors and dark conditions, and (iii) general respiratory inhibitors.

2.62 Methods and Results

The electrical apparatus used in these experiments was the same as that described in Section 2.22.

Control experiments.

The membrane potentials of cells continuously bathed in A.P.W. and under normal laboratory lighting conditions were recorded for several hours. With the electrode inserted deep into the cell at the junction of the two cyto-

plasmic streams sealing did not occur. The results are summarized in Table 2.26.

Table 2.26 The resting potential (mV) of cells bathed in A.P.W. under normal lighting conditions.

Expt.	Time (hours)						
	0	1	3	6	9	12	24
1	-114	-116	-155	-155	-	-	-120
2	-106	-104	- 93	-106	-107	-110	-
3	-106	-	-	-	-	-108	-
4	-114	-122	-121	-124	-	-	-
5	-110	-118	-110	- 96	-	-	-
6	-120	-108	-100	-114	-112	-131	-
7	-109	-104	-103	-130	-138	-135	-
8	-109	- 99	-113	-117	-114	-119	-

Ouabain.

MacRobbie (1962) found that 5×10^{-5}M ouabain was sufficient to produce maximum inhibition of the potassium influx in Nitella translucens.

Ouabain was obtained from B.D.H. Ltd. and the activity of each batch was confirmed by observing its effect on the electrical potential difference across the isolated frog skin. Fresh solutions were always used since ouabain is not stable in aqueous solution.

The effect on the membrane potential of 5×10^{-5}M ouabain in A.P.W. is shown in Table 2.27.

DCMU and light.

MacRobbie (unpublished) has shown that the chloride influx is reduced by a factor of eight by dark conditions or 5×10^{-6}M DCMU.

The effect of DCMU on the resting potential was tested by flowing a

solution of DCMU in A.P.W. past the cell for periods up to 12 hours (Table 2.28).

Table 2.27 The effect of ouabain on the resting potential (mV).

Expt.	Time (hours)								
	0	0.1	0.5	1	2	3	4	7	12
1	-130	-116	-110	-104	-130	-110	-	-	-
2	-102	-102	-103	-106	-114	-124	-130	-	-
3	-122	-126	-129	-129	-136	-	-	-	-
4	-138	-120	-112	-123	-133	-	-	-	-
5	-108	- 93	- 96	- 96	- 96	-	-	-	-
6	-131	-130	-124	-127	-119	-101	-101	-111	-126
7	-114	-115	-113	-107	- 97	- 94	- 99	-102	-117
8	-116	-136	-127	-127	-130	-132	-	-	-
9	-143	-148	-130	-126	-118	-	-	-	-
10	-138	-138	-110	-115	-116	-	-	-	-

Table 2.28 The effect of 5×10^{-6} M DCMU on the resting potential (mV).

Expt.	Time (hours)							
	0	0.5	1	2	3	6	9	12
1	-114	-114	-118	-112	-100	-	-	-
2	-144	-145	-144	-103	-110	-131	-138	-150
3	-102	- 84	- 85	- 84	- 82	- 89	-106	-107
4	-114	-113	-105	-103	-100	- 92	- 84	- 66
5	- 90	- 88	- 87	- 89	- 88	- 84	- 86	- 82

Table 2.29 The effect of dark conditions on the resting potential (mV)

Expt.	Time (hours)						
	0	0.5	1	2	3	6	8
1	- 87	- 85	- 85	- 84	- 84	- 84	- 94
2	-114	-132	-117	-108	-106	-103	-108
3	-114	-110	- 92	- 90	- 92	-110	-110
4	-110	-108	-115	-109	-107	-123	-124
5	-113	-104	- 86	- 84	- 89	-102	-103

For another group of cells the membrane potential was recorded continuously for several hours in the dark (Table 2.29). Neither treatment appears to have any marked systematic effect.

Dinitrophenol.

Etherton and Higinbotham (1960) investigated the effect of 0.2 mM DNP on the resting potential of Avena coleoptile cells. They found that the potential decreased sharply and did not always recover when DNP-free solution was restored.

The effect of 0.2 mM DNP on Nitella was similar except that the potential rose about 20 mV before falling off with a half-time of about 22 minutes. The fall in potential was accompanied by the cessation of protoplasmic streaming. The change was reversible if the exposure to DNP was for a short time but the cell was usually dead by the following day.

The effect of 10^{-5}M DNP was similar but less drastic. The potential fell with a half-time of about 80 minutes and the rate of protoplasmic streaming declined. Again the treatment resulted in the death of the cell.

2.63 Discussion

The experiments in this section must be interpreted with caution since the control experiments indicate that the membrane potential may vary by several millivolts in quite short periods. It is possible that the membrane potential is more stable at lower temperatures. The temperature of the loch where the plants grow is about 10°C in the Spring and Autumn when the Nitella is most abundant. The experiments were carried out at 20°C but it is hoped that future experiments will be done at lower temperatures. Another possible source of error is a variation in the pH and bicarbonate levels of the solutions. In spite of these reservations it should be possible to interpret consistent changes in potential of more than 20 mV.

The experiments with ouabain indicate that there is no significant consistent change in the membrane potential. This supports the idea that the sodium potassium pump is a neutral non-electrogenic pump. The absence of any marked response of the membrane potential to DCMU or dark conditions means that the chloride pump may also be neutral.

The rapid action of 0.2 mM DNP and the subsequent death of the cell suggests that DNP affects the membrane directly. It would be instructive to observe the effect of DNP on the membrane resistance since a large decrease in resistance would indicate that the integrity of the membrane had been destroyed. The action of 10^{-5}M DNP is probably similar. These experiments suggest that some of the work on tissue slices in which DNP was used may need a critical reappraisal.

2.7 GENERAL DISCUSSION

The experiments described in this chapter give support to the concept of the plant cell acting as a three compartment system with regard to the movement of ions.

The cell wall acts as a cation exchange resin membrane both in its ability to support diffusion potentials and to exchange ions with the external medium. The concentration of indiffusible anions in the D.F.S. is 0.74 equiv.$l.^{-1}$ in close agreement with the value obtained by Dainty, Hope, and Denby (1960). This high concentration of indiffusible anions gives rise to considerable difficulties in the measurement of the calcium fluxes and it is possible that it may have given rise to errors in previous measurements of cation fluxes.

Experiments with the isolated cell wall indicate that it is equivalent to an unstirred layer of water about 1,400μ thick for the diffusion of anions, though this value is dependent on the cation concentration in the medium. This means that, when the total ionic concentration in the bathing medium is changed, it should take about 20 minutes for the membrane potential to reach a steady value. Experiments with intact cells indicate that equilibration may be complete within five minutes.

The flux measurements of MacRobbie (1962) and the electrical measurements in Section 2.2 may be used to calculate the permeability coefficients for the plasmalemma using the constant field equation: $P_K = 5.5 \times 10^{-7}$cm.$sec.^{-1}$, $P_{Na} = 1 \times 10^{-7}$cm.$sec.^{-1}$. On the assumption that the calcium influx was wholly passive it was shown in Section 2.3 that $P_{Ca} = 4.3 \times 10^{-8}$cm.$sec.^{-1}$. These values and the measured cytoplasmic concentrations may be inserted into the Goldman equation containing terms for calcium (p. 77). This yields a value of -152 mV for the potential of the cytoplasm with respect to A.P.W. whether $\beta\ Ca_i$ is negligible or not, i.e., 14 mV more negative than the observed value. The equation containing only terms for sodium and potassium predicts a membrane potential of -153 mV.

Better agreement is obtained with the cells pretreated with 5 mM NaCl.

(Section 2.5), where the combination of the measured concentrations with the value of 0.25 for α obtained from the electrical experiments, predicts a value of -143 mV for the membrane potential which is close to the measured value of -141 mV. The value of 0.18 for α gives a membrane potential of -148 mV. Thus not only does pretreatment with 5 mM NaCl make the cells sensitive to changes in the ratio of the sodium and potassium concentrations but it also gives better agreement between the measured and predicted values of the membrane potential. The latter fact may, however, be an incidental consequence of the higher sodium and lower potassium concentrations in the batch of cells which were pretreated. Control experiments have shown that the different concentrations were not due to the pretreatment. The mechanism by which pretreatment produces the response to changes in the sodium and potassium concentrations is not known. Future measurements of the fluxes after pretreatment should show whether α is changed, or whether the constancy of the potential depends in some way on the intimate relationship between the cell wall and the plasmalemma. It seems strange that when calcium is present the Goldman equation accurately predicts the response of the potential to a change in the external calcium concentration, but fails completely for changes in the sodium or potassium concentrations. It is unlikely that a satisfactory equation for the membrane potential will be obtained until the role of calcium is more fully understood.

Very little can be said about the electrical properties of the tonoplast although comparison of the observed potential difference with the Nernst potentials for sodium, potassium, and chloride suggest that the potential may be determined by the potassium and chloride permeabilities and concentrations.

Sufficient information is now available for it to be possible to state definitely which ions are actively transported at the plasmalemma although the situation at the tonoplast is not so clear due to the difficulty of measuring the fluxes. By combining the flux measurements of MacRobbie (1962) with the present electrical and concentration measurements it is clear that at the plasmalemma sodium is pumped out of the cell while potassium and chloride are pumped inwards. The influx of calcium is probably wholly passive while the efflux is so small as to defy accurate measurement.

The experiments designed to determine whether the pumps are electrogenic or not (Section 2.6) are not very accurate, but it is clear that neither the sodium potassium pump nor the chloride pump contribute a large electrogenic component to the potential difference across the plasmalemma.

At the tonoplast, potassium and chloride are nearly in electrochemical equilibrium though this conclusion would not hold if the ions were at a higher concentration in the sub-cellular particles than in the remainder of the flowing cytoplasm. MacRobbie (1962) has shown that the sodium fluxes at the tonoplast are much higher than those at the plasmalemma and, since there is flux equilibrium at the plasmalemma, there must be flux equilibrium at the tonoplast. Therefore the low concentration of sodium in the cytoplasm can only be explained if there is a sodium pump at the tonoplast directed towards the vacuole in addition to the pump at the plasmalemma. It seems probable that calcium is also pumped from the cytoplasm into the vacuole, especially since most of the calcium in the cytoplasm is probably bound. However, it is not possible to be definite about the existence of a calcium pump until the difficult problem of measuring the fluxes at the tonoplast has been overcome.

The picture of the ionic state of Nitella translucens derived from the

present experiments is shown diagrammatically in Fig. 6. The conclusions about the active transport of sodium, potassium and chloride are based on the flux measurements of MacRobbie (1962).

Fig. 6. A schematic presentation of the ionic state of Nitella translucens.

Medium	Plasmalemma: Wall	Plasmalemma: Flowing cytoplasm	Tonoplast: Vacuole
K_o = 0.1 mM	70% H_2O	K_c = 118.5 mM	K_v = 75 mM
Na_o = 1.0 mM	A^- = 0.74 equiv.l^{-1}	Na_c = 14 mM	Na_v = 65 mM
Ca_o = 0.1 mM		Ca_c = 8 mM	Ca_v = 12 mM
Cl_o = 1.3 mM		Cl_c = 65 mM	Cl_v = 160 mM
		$_cE_o$ = -137 mV	$_vE_o$ = -119 mV
	Active K →		
	Active Na ←	Active Na →	
	Active Cl →		
	Passive Ca →	0.046 pM $cm.^{-2}sec.^{-1}$	
		Active Ca? →	

C H A P T E R III

ACTIVE TRANSPORT OF IONS ACROSS THE ROOT OF RICINUS COMMUNIS.

3.1 INTRODUCTION

During the past decade there has been much disagreement about the processes involved in the transport of ions across the root into the xylem. Hylmo (1953, 1958) thinks that ions are carried passively across the root by the transpiration stream, while Brouwer (1956) considers that ions enter the xylem mainly by a process dependent upon metabolism.

However, it is now generally agreed that both active and passive processes are involved in some degree (Bollard, 1960; Lopushinsky and Kramer, 1961; Jackson and Weatherley, 1962; Jensen, 1962), and it is clear that active transport must occur during sap exudation from a detopped plant since the salt concentration in the sap is usually greater than that in the solution surrounding the roots. The question then arises as to whether the anion, the cation, or both ions of the salt are actively transported. In principle it is possible to give an answer for an exuding root system, since the electrochemical potential difference between the sap and the external medium can be determined for each ion by measuring the electrical potential difference between the sap and the external medium and the concentrations of each ion in the two media. In this chapter an attempt to do this is described.

3.2 METHODS

Castor oil plants (Ricinus communis) were grown for about six weeks in aerated culture solution made up after Stout and Arnon (1939) except that 1/10th strength solution was used and iron given as chelate. Five days before detopping, the plants were transferred to a 1 mM KCl solution which was changed daily. KCl was used to avoid the complications encountered when using ions which become incorporated into organic compounds. Seven

hours before the measurements were made the plants were detopped below the leaves, and the stem cortex containing the phloem was cut back about 2 cm. Pieces of polythene tubing were pushed over the stumps to act as reservoirs for the sap.

The electrical potential difference between the sap and the external solution was measured using a high impedance millivoltmeter with the following electrode system:

| Calomel electrode | 3M KCl | 3M KCl-agar salt bridge | Sap | Root system | External solution | 3M KCl-agar salt bridge | 3M KCl | Calomel electrode |

The loss of KCl into the sap and the external solution from the KCl-agar salt bridges was minimized by using glass micropipettes with a tip diameter of only 100μ.

The methods of chemical analysis were as follows:

a) Sodium and potassium were determined using a flame photometer.

b) Calcium and magnesium were determined by complexometric titration using an EEL micro-titrator.

c) The concentration of chloride was found by measuring the potential difference between a Ag/AgCl wire and a calomel electrode, after calibration with standards of known concentration.

d) Nitrate was determined colorimetrically by the phenoldisulphonic acid method.

e) Sulphate was precipitated with barium chloride and estimated turbidometrically using a colorimeter.

f) Phosphate was determined colorimetrically by the ammonium molybdate method.

3.3 RESULTS

The results of measurements of electrical potential difference, K and

Cl concentrations, and pH, made on 16 plants are given in Table 3.1.

Table 3.1 Measurements of the electrical potential difference, E (mV), between the sap and the external solution, the concentrations (m.equiv.l^{-1}) of potassium and chloride in the sap (i) and the external solutions (o), and the pH values for the two solutions.

Plant	E	K_o	Cl_o	K_i	Cl_i	pH External Solution	pH Sap
1	-54	1.1	1.0	7.5	1.9	7.5	7.3
2	-62	1.1	1.0	8.4	2.4	7.5	7.0
3	-54	1.1	1.0	8.3	3.6	7.6	7.4
4	-56	1.1	1.0	8.1	3.6	7.6	7.1
5	-56	1.1	1.0	9.3	5.0	7.6	6.9
6	-61	1.1	1.0	8.6	3.3	7.9	6.7
7	-56	1.1	1.0	6.3	1.9	7.9	6.7
8	-53	1.1	1.0	9.2	2.6	7.9	6.7
9	-55	1.1	1.0	5.8	1.6	7.9	6.5
10	-69	1.1	1.1	8.8	2.1	7.6	6.9
11	-58	1.1	1.1	7.8	2.3	7.6	6.5
12	-55	1.1	1.1	6.1	3.1	7.6	6.6
13	-58	1.2	1.2	10.0	4.7	7.5	6.6
14	-58	1.2	1.2	9.0	3.4	7.5	6.4
15	-54	1.2	1.2	11.8	3.0	7.5	6.5
16	-49	1.2	1.2	9.6	2.3	7.5	6.2

It will be seen that the chloride concentration in the sap is much lower than the potassium concentration even though the concentrations of the two ions in the external solution are equal. This suggested that

exchange was taking place with ions previously accumulated from the culture solution and so the sap was analysed to determine the concentrations of the other ions present (Table 3.2). There was insufficient sap available from each plant to make a complete analysis and therefore analyses were made on samples of sap pooled from the groups of plants indicated in Table 3.2.

Table 3.2 The concentrations (m.equiv.$l.^{-1}$) of the ions in sap pooled from the indicated groups of plants. The concentration of magnesium was not determined for plants 1 - 5. The equivalent concentration of phosphate was calculated from the concentration in mM using the fact that the pK value for the second hydrogen ion is 7.2.

Plants	Concentration in sap								Total cation conc.	Total anion conc.
	K	Na	Ca	Mg	Cl	SO_4	Phosphate	NO_3		
1 - 5	8.2	0.4	1.5	-	3.3	3.4	1.0	0.2	12.1*	7.9
6 - 9	7.5	0.2	0.5	1.4	2.3	3.5	0.4	0.6	9.6	6.8
10 - 12	7.5	0.1	0.8	1.4	2.5	3.4	0.6	0.3	9.8	6.8
13 - 16	10.1	0.3	1.5	2.0	3.3	7.4	0.3	0.4	13.9	11.4

*assuming a magnesium concentration of 2.0 m.equiv./l.

There remains a discrepancy of about 3 m.equiv.$l.^{-1}$ between the total anion and cation concentrations in Table 3.2. Dr. Poole, of this department, found that citrate was present in the sap at a concentration of 0.7 m.equiv.$l.^{-1}$ and it is possible that other organic anions make up the discrepancy.

3.4 DISCUSSION

An exuding root may be considered simply as a membrane separating the sap from the external medium. From the measurements of concentration and electrical potential in Table 3.1 the values of $E_{obs} - E_K$ and $E_{obs} - E_{Cl}$ may be calculated for each plant (Table 3.3).

These calculations show that, for the potassium ion, the electrochemical potential of the sap relative to the external solution is small or negative, and since the ion is positively charged this means it is moving passively into the sap. The value of $E_{obs} - E_{Cl}$ is also negative, but large, and since the chloride ion is negatively charged this potential is acting so as to drive it into the external solution. But it is known that there is a net influx of KCl into the sap from the external solution because, on placing the root system in distilled water, the sap concentration of potassium is reduced to as little as 0.1 mM; if the plant is later returned to the original solution, the sap concentration rises to its former level (Bowling, 1961). Thus it follows that the chloride ion must be actively transported into the sap against the electrochemical potential gradient while the potassium ion follows passively to maintain electrical neutrality. The difference in pH of one unit indicates that the hydrogen and hydroxide ions are approximately in equilibrium.

So far the inequality between the potassium and chloride concentrations and the presence of other ions in the sap has been ignored. These data can only be usefully interpreted by employing a specific model of ion transport across the root. Bowling (1961) investigated the relationship between salt uptake and transpiration in Ricinus roots and found it necessary to postulate the existence of two ion 'stores', one inside and one outside a diffusion barrier. These ion stores may be incorporated

Table 3.3 The electrochemical potential difference (mV) between the sap and the external solution for potassium and chloride calculated from the data in Table 3.1 using equation 4.

Plant	$E_{obs} - E_K$	$E_{obs} - E_{Cl}$
1	- 6	-70
2	-11	-84
3	- 3	-86
4	- 6	-88
5	- 2	-97
6	-10	-91
7	-12	-72
8	+ 1	-77
9	-13	-67
10	-17	-85
11	- 9	-77
12	-12	-81
13	- 5	-92
14	- 7	-84
15	+ 4	-77
16	+ 3	-65

into a model which assumes that the endodermis acts as the diffusion barrier for the movement of ions. In this case the external ion store would be the cortical cells and the internal store would be the cells inside the endodermal layer. Ions in the external solution would diffuse

through the free space of the cortex as far as the endodermis; there, active transport into the internal store would take place followed by diffusion into the xylem.

The plants had been grown on KCl for five days before the experiments so the cortical cells should have been approaching equilibrium with the external solution. Furthermore, the cortex free space attains equilibrium with the external medium very quickly and it may therefore be assumed that the solution in the free space outside the endodermis contained only potassium and chloride ions in electrochemical equilibrium with the external solution. It is suggested that, before the KCl appears in the sap exuding from the cut stem, exchange with the internal store takes place. If there is no net gain or loss of ions by the internal store, the total concentration of ions in m.equiv.$l.^{-1}$ in the exuding sap should be equal to the concentration of KCl at the internal surface of the endodermis, though entry of water above the region of accumulation may cause some dilution.

Table 3.4 Calculation of the Nernst potentials for potassium and chloride assuming that their concentrations are equal to the total concentration of the sap, the mean value of the electrical potential difference for each group of plants, and the resulting values $E_{obs} - E_K$ and $E_{obs} - E_{Cl}$. All quantities are expressed in mV.

Plants	Mean E	E_K	E_{Cl}	$E_{obs} - E_K$	$E_{obs} - E_{Cl}$
1 - 5	-56	-60	+52	+4	-108
6 - 9	-56	-55	+48	-1	-104
10 - 12	-61	-55	+46	-6	-107
13 - 16	-55	-62	+57	+7	-112

The values of the Nernst potential based on this assumption are given

in Table 3.4 together with the corresponding values of $E_{obs} - E_K$ and $E_{obs} - E_{Cl}$. The value of $E_{obs} - E_K$ is small in each case, but there is a 'force' of about 110 mV or 2500 cal.mole^{-1} driving chloride towards the external solution. Hence, if this model of ion transport across the root is correct, it may be concluded that chloride is actively transported into the xylem while potassium is approximately in passive equilibrium. A detailed test of this model calls for experiments nearer the level of single cells.

Recently Lopushinsky (1964) has measured the amount of labelled calcium in the exudate of detopped tomato plants growing on quarter-strength Hoagland's solution containing ^{45}Ca. He found that the concentration of ^{45}Ca was about twice as great in the exudate as in the external solution, and concluded that calcium was actively transported into the xylem. However, since he did not measure the electrical potential difference between the sap and the external solution and the concentrations of the other ions in the sap, this conclusion is certainly not justified. If, as seems probable, the electrical potential of the sap in tomato roots is negative with respect to the external solution, the movement of calcium could be wholly passive.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. E.J. Williams, for his encouragement and advice during the course of this work. I would also like to thank Professor J. Dainty, formerly Director of the Biophysics Department, and all other members of the Biophysics Department for their advice.

The work described in Chapter 3 was carried out in collaboration with Dr. D.J.F. Bowling of the Botany Department, Aberdeen University. The author, however, accepts full responsibility for the interpretation of the results. I am indebted to Professor Weatherley for his hospitality.

Mr. J. Stolarek, on leave from Lublin University, Poland, gave valuable assistance with the experiments described in Section 2.5.

I would like to thank Mr. J. Tait for his skilful construction of various essential pieces of apparatus, and Mrs. Gilbertson for technical assistance.

During the year 1961-2 the author held a D.S.I.R. Research Studentship.

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