MEMBRANE FLUXES AND ELECTRIC CONDUCTANCE IN CHARACEAN CELLS

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Summary

The effects of changing the membrane potential difference (p.d.) of *Chara corallina* and *Nitella translucens* cells on the unidirectional fluxes of potassium, sodium, and chloride ions are described. In both species, a net efflux was observed at the resting potential. Changes in net flux of potassium with p.d. amounted to 1–1·5 p-mole cm^{-2} sec^{-1} in 10 mV, contributing a partial ion conductance of about 10–15 μmho cm^{-2} at p.d.'s near the resting potential. The corresponding values for Na^+ and Cl^- were 1 and 1–2 μmho cm^{-2} respectively.

When the partial ion conductances are added, they fall short of the average total conductance measured electrically, which was 21 μmho cm^{-2} in *N. translucens* and 40–70 μmho cm^{-2} in *C. corallina*. The conductances refer to the plasmalemma, with an error of about 10%. The possible causes of this discrepancy are discussed and it is concluded that fluxes, particularly when the ratio of influx to efflux departs appreciably from one, have been grossly underestimated. One of the reasons given relates to the local changes in concentration and specific activity within the cell wall; these quantities are not always equal to those in the external medium, as assumed when calculating the fluxes.

Flux ratios of potassium were found to change more rapidly with p.d. than expected if the ion movements are independent. The flux ratio changed by a factor of 10 for each 20–25 mV instead of each 59 mV as expected from independent diffusion. Models to explain this behaviour are outlined.

The likelihood of other ion fluxes, for example of H^+, contributing to the electric conductance is discussed in detail, prompted partly by evidence presented by Kitasato (1968). Both new experimental evidence and reinterpretation of Kitasato's results lead to the conclusion that proton fluxes across the plasmalemma are low and that potassium conductance is the major contributor to plasmalemma conductance.

I. INTRODUCTION

Recently, Kitasato (1968) gave an account of the effect of H^+ on the potential difference (p.d.) and ion fluxes in *Nitella clavata*, from which it was concluded that fluxes of protons account for most of the electric conductance near the resting potential, though the latter is quite close to the equilibrium (Nernst) potential for potassium ions.

This has prompted us to publish more fully results on the effect of membrane potential on ionic fluxes in *Chara corallina*‡ and *N. translucens*, which were reported briefly some time ago (Hope and Walker 1965). These studies have since been extended.

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There are several features of ionic relations in characean cells which do not find a ready explanation in the simple model proposed by us previously (Hope and Walker 1961), namely that the plasmalemma is passively permeable to potassium and sodium ions with a permeability preference of about 10:1.

(1) Under some circumstances the plasmalemma p.d. does not respond to changes in the concentration of potassium ion in the external solution, $[K_o]$ (Hope and Walker 1961; Oda 1961; Spanswick, Stolarek, and Williams 1967; Kitasato 1968).

(2) The resting p.d. at the plasmalemma is often larger (cytoplasm more negative) than the estimated equilibrium level for K^+, based on likely cytoplasm concentration or activity and external concentration (Oda 1961; Hope 1965).

(3) The measured electric conductance of the plasmalemma bears no simple relation to the conductance calculated from measurements of ionic fluxes of the main ions present (K^+, Na^+, Cl^-) (Hope 1963; Williams, Johnston, and Dainty 1964). The discrepancy is always in the direction of a higher electric conductance than "flux conductance", assuming independent diffusion and a relationship (Hodgkin 1951):

$$g_j = (\mathbf{F}^2/\mathbf{R}T)\phi_j, \tag{1}$$

where g_j is the membrane conductance contributed by the ions j; **F**, **R**, and T have their usual meaning, and ϕ_j is the unidirectional flux of ions j across the membrane. Equation (1) is applicable only to ions in equilibrium, which is sometimes the case for potassium in *C. corallina* in artificial pond waters at pH 6 (Hope and Walker 1960).

The equation predicts a conductance many times lower than that measured electrically, about four times for Cl^- in frog muscle fibres (Hodgkin and Horowicz 1959) and more than 10 times for *C. corallina* and *N. translucens*. In this dilemma, it is suggested that the assumption of independent diffusion should be abandoned, and that permeation through long, narrow pores may give rise to interacting fluxes. In a model based on this idea Hodgkin and Keynes (1955) proposed that g_j should be given by:

$$g_j = n(\mathbf{F}^2/\mathbf{R}T)\phi_j, \tag{2}$$

where n is a factor equal to the number of ions in the pore, or one less (see also Lea 1963).

However, values of n invoked, 4 for Cl^- in muscle (Hodgkin and Horowicz 1959) and 10–30 for K^+ in *N. translucens* (Williams, Johnston, and Dainty 1964) and *C. corallina* (Hope and Walker 1960; Hope 1963), have never been confirmed by independent measurement and are therefore still hypothetical. The value of 4 for Cl^- was not confirmed by Spurway (personal communication).

A more general method of testing for independent ion migration, when the unidirectional passive fluxes are unequal (ions not "at equilibrium"), is through the use of the Teorell–Ussing flux-ratio equation (Teorell 1949; Ussing 1949), which states that, for passive fluxes which do not interact:

$$\ln(\phi_i/\phi_o) = (z\mathbf{F}/\mathbf{R}T)(\Psi_j - \Psi_M). \tag{3}$$

Ψ_j in equation (3) is the Nernst or equilibrium p.d. equal to $(\mathbf{R}T/z\mathbf{F})\ln(a_o/a_i)$ and Ψ_M is the trans-membrane p.d. Equation (3) predicts a 10-fold change in flux ratio

when the p.d. changes by 59 mV at 25°C. Experiments of Hodgkin and Keynes (1955) with *Sepia* axons showed that the flux ratio for K^+ actually changed more rapidly with Ψ_M than in equation (3). The flux ratio obeyed approximately an equation of the form:

$$\ln(\phi_i/\phi_o) = (n\mathbf{F}/\mathbf{R}T)(\Psi_K - \Psi_M), \quad (4)$$

with $n = 2{\cdot}5$ (not 4 as sometimes quoted in the literature).

The general treatment of the interaction of ions, via the thermodynamics of the steady state, indicates that deviations from the simple flux-ratio equation (3) would be caused by interactions between (i) influx and efflux of the ion in question, (ii) fluxes of water and the ions, and (iii) fluxes of other ions and the ions in question (Meares 1959; Kedem and Essig 1965; Coster and George 1968).

The work described here is an attempt to measure the separate ion conductances in the plasmalemma of cells of *N. translucens* and *C. corallina*. The experiments yielded a preliminary value for n [cf. equation (4)] and values for g_K, g_{Na}, and g_{Cl} to compare with g_M, the total conductance measured electrically.

An alternative explanation of the results of Kitasato (1968) is offered. It is believed that the resting p.d. in the Characeae is indeed determined mainly by potassium ion fluxes and that the large fluxes of protons postulated by Kitasato are unlikely to exist.

II. Material and methods

(a) *Material*

N. translucens was obtained from a loch near Dunkeld, Perthshire. The plants were kept for up to 4 months in dim light in artificial pond water (APW) containing: KCl, 0·1 mM; NaCl, 1·0 mM; and $CaCl_2$, 0·1 mM in double-glass-distilled water at pH about 5·8. This was also the bathing medium in many experiments. The cells actually used were often not those collected, but secondary growth from the original plants. This was necessary because we required the cells to be short compared with their characteristic length (see below). The original plants had by the time we received them lost most of their short "leaf" cells, and consisted largely of chains of very long, mature internodal cells. Our cells were not, therefore, very like those used for flux studies by MacRobbie (1962, 1964). Selected cells, about 2 cm in length, were freed from neighbouring internodal cells—and in later experiments, from most of the small, adhering nodal cells—and kept in fresh APW for 1–3 days before use.

C. corallina was grown in a laboratory culture of APW and river mud (Norwich) or of nutrient solution and river mud (Flinders) at about 20°C in dim light. The experimental solution was either APW or Flinders pond water (FPW) comprising KCl, 0·2 mM; NaCl, 2·0 mM; $CaCl_2$, 0·05 mM.

(b) *Experimental Methods*

The method used for the measurement of potassium fluxes will be described in some detail; for sodium influx and chloride efflux the same methods were used with slight modifications.

The cell was held horizontally in a Perspex cell-holder, milled from a block and polished. The top, bottom, and back of the cell-holder was less than 1 mm thick, and the volume of the solution it contained was less than 1 ml. Provision was made for a continuous flow of fresh solution when required. Around the cell-holder was a scintillation counter consisting of an EMI 9524 photomultiplier tube and a piece of plastic phosphor NE 102A with a thin aluminium cover. The shape of the latter was such as to fit around the cell-holder on three sides for the purpose of detecting as much cell radioactivity as possible. The scintillation detector was connected to a scaler or a ratemeter with a chart recorder.

The electrical apparatus was arranged for the measurement of the membrane potential of the cell (vacuolar potential) and the total membrane conductance (plasmalemma in series with tonoplast), and for the automatic control (voltage clamping) of the vacuolar potential.

Two microelectrodes of tip diameter up to 10 μm were inserted into the vacuole of the cell. One served for potential measurements and the other for the injection of current. The electrode tips were about 1 mm apart, near the central axis of the cell, and about in the middle of its length. In the solution bathing the cell was a long silver chloride–silver wire, which earthed the bath through a 10-kΩ resistor and served as the indifferent current electrode. A second, short electrode of silver chloride–silver wire was used as the indifferent potential electrode. A backing-off potentiometer was placed in series with this electrode. It was set, with reference to a flowing-KCl microelectrode and calomel half-cell, so that the p.d. measured between the inserted microelectrode and the external AgCl wire was equal to the membrane potential of the cell.

This membrane potential could be measured with a Keithley model 603 electrometer and recorded on a chart recorder. Using the equipment in this way, the membrane conductance was measured by determining the change in membrane p.d. with a pulse of current of about 0·5 sec duration, the amplitude of which was measured with a second Keithley 603 electrometer. A typical measurement involved a change in membrane p.d. of $\pm$5–10 mV.

In the voltage-clamping mode the first electrometer measured the difference between the actual membrane p.d. and the command potential set on a calibrated potentiometer. The amplified difference signal (gain of up to -2000) went through a low-pass filter to the current microelectrode. The second electrometer was then used for measuring and recording the membrane current as the p.d. across a 10-kΩ resistor between the bath and earth. Membrane conductance was measured by applying a further 10-mV step to the clamped potential for about 0·5 sec; the change in membrane current was recorded. In a few experiments the membrane potential was not clamped, but was kept approximately constant by manual control of the membrane current.

In order to measure potassium influx, the solution bathing the cell was removed* and replaced by a similar solution in which the K^+ was labelled with ^{42}K of high specific activity. The ^{42}K was obtained by the irradiation of "specpure" K_2CO_3 at high pile factor (Harwell), or by irradiation of ^{41}K-enriched K_2SO_4 at moderate pile factor (Lucas Heights, N.S.W.). The specific activity on delivery was 1–2 mCi/mg salt. After a minute or so the solution was removed and replaced by fresh radioactive solution. At the end of 10–40 min the labelling solution was removed and the cell rinsed for about 30 min in flowing, inactive solution. In some later experiments a "high-salt" solution containing NaCl, 10 mM; KCl, 1·0 mM; and $CaCl_2$, 1·25 mM was used in order to elute cell wall radioactivity more quickly. Its application lasted 1 min, and after reversion to the usual APW the resting potential was normal. During these elutions, the radioactivity of the cell was continually measured. The radioactivity of the whole cell at the end of the 30-min rinse was taken as equal to the radioactivity within the protoplast (Hope 1963), the cell wall activity having been mostly removed.

This routine of loading and elution was carried out in each cell at various membrane potentials; the cell was then sometimes sufficiently active for the subsequent detection of potassium efflux. Since the efflux of radioactivity continues to fall for some hours (cf. Hope 1963, fig. 1), the standard procedure was to elute a loaded cell for 8 hr in flowing inactive APW. Mean effluxes during 20–40-min periods were then determined as follows. The solution was allowed to remain stagnant around the cell and was then removed by a pipette, the cell-holder refilled, re-emptied, and the combined solutions dried on a planchet for counting. A single action potential during a 30-min efflux was sufficient nearly to double the radioactivity in the sample. Such samples have not been included in the results. At the end of a number of efflux periods at different membrane potentials the cell was counted for the last time with the scintillation counter. It was then removed from the apparatus and its dimensions measured. The end was cut off and a drop of clear sap expressed for potassium determination by flame-photometry, and radioactivity determination by counting a dried sample on a planchet. Alternatively the whole cell was cut into 1 or 2 ml of distilled water and aliquots of this used for radioactivity and potassium analysis.

* A film of water remained over the cell surface and electrodes and enabled the voltage clamping to be maintained although most of the solution bathing the cell was removed.

All counts were corrected for decay to a standard time, using a half-life of 12·45 hr for ^{42}K. Samples were checked for half-life. Much of the counting was done with the aid of an automatic, gas-flow, low-background counter, Nuclear Chicago type 1043, or with an I.D.L. low-background counter.

These procedures gave all the information required for calculation of influx and efflux in absolute units. Efflux calculations were straightforward; influx calculations required the use of a conversion factor from the whole-cell scintillation counter to the planchet counter. The justification of these methods will be discussed in the next section, where errors will also be estimated.

Thus, for potassium and *N. translucens* cells, the influx and efflux were determined on a single cell during the course of 24 hr while the cell was under constant conditions except for the membrane potential and membrane current. The microelectrodes did not seal during this time (cf. Walker 1955), so the only electrode insertions occurred an hour or so before the first measurement. With *C. corallina* influx and efflux were measured on different, but comparable cells.

The influx of sodium (labelled with ^{22}Na) was determined for two other *N. translucens* cells in a similar way, but the cells did not take up enough tracer for efflux measurements to be possible. A few measurements of sodium fluxes have been made, as a function of membrane potential, with *C. corallina* cells.

Cells of *C. corallina* were loaded with ^{36}Cl for chloride efflux measurements by soaking for up to 4 weeks in sealed chambers in a solution of: NaCl, 10 mM; KCl, 0·1 mM; $CaCl_2$, 0·1 mM. The solution was changed weekly. At the end of the soaking period the cell was mounted in the apparatus, the membrane potential controlled, and the efflux measured as before. As it has been found (Hope, Simpson, and Walker 1966) that the chloride efflux in *N. translucens* is strongly light-dependent, the efflux was measured in the light and again on the same cell in the dark after 10 hr darkness.

Unfortunately no cells of *N. translucens* were available for the determination of chloride efflux against potential.

The experiments were carried out in a room without temperature control, at temperatures between 23 and 30°C. The cells were illuminated continuously during the experiment with a tungsten lamp fitted with a heat filter.

(c) *Assumptions and Errors in the Methods*

(i) *Electrical*

The electrical apparatus used was calibrated against a standard cell and resistance boxes and was capable of an accuracy of better than ± 1 mV. However, the long cylindrical cell acts as a "leaky cable", and if current is passed in at its centre to control the membrane p.d. at the centre, the control at the ends will be less effective. A conventional treatment of the problem shows that, if an applied current produces a change in membrane potential of v_0 at the centre and v_1 at the ends,

$$v_1/v_0 = \cosh(l/\lambda)^{-1}, \tag{5}$$

where l is the half-length of the cell and λ is the characteristic length, i.e.

$$\lambda = \tfrac{1}{2}(rd/\rho)^{\frac{1}{2}}, \tag{6}$$

where r is the membrane resistance (Ω cm^2), d the cell diameter (cm), and ρ the resistivity of the cell sap (Ω cm). The external resistance has been neglected in comparison with the longitudinal resistance of the vacuole. Equation (5) can be used to predict the error in potential at the end of a cell voltage-clamped at the centre. For one of the cells, $l = 1{\cdot}05$ cm, $\lambda = 3{\cdot}7$ cm and $v_1/v_0 = 0{\cdot}96$; this means a 4% error in the change of membrane potential. Changes of membrane potential did not exceed 50 mV in this cell, with an error of 2 mV at the cell end. The average error is perhaps 1 mV, at extreme excursions from the membrane resting potential. This calculation has been performed for all cells and the correction applied where necessary, though barely significant. ρ is taken as 55 Ω cm, corresponding to a mixture of KCl and NaCl at a total concentration of 0·15N, approximately that of the sap in these cells. No attempt was made to measure or clamp the potential of the cytoplasm in these cells because of the danger of the

electrodes sealing during the long time of the experiment. Nor was the conductance of the plasmalemma measured directly but that of tonoplast and plasmalemma in series was measured. This will lead to an underestimate of the plasmalemma conductance of about 10% (Findlay, personal communication). The plasmalemma p.d. is underestimated by about 10–20 mV by taking vacuolar readings (Findlay and Hope 1964; Spanswick and Williams 1964), but it is simpler not to apply this correction—all results therefore are quoted in terms of the p.d. between the vacuole and the bathing medium. The systematic error in voltage-clamping of the plasmalemma p.d. due to the change in p.d. across the tonoplast will be of the order of $r_t/(r_p+r_t)$, i.e. about 10%, where r_t and r_p are the resistances of the tonoplast and plasmalemma respectively. This means that the changes in influx and efflux across the plasmalemma with Ψ_M are greater than the values show.

(ii) *Fluxes*

As a simplification the cell has been treated as a two-phase system of cell wall and interior; the errors due to this are in part self-cancelling, but they must be separately considered. For K^+ and Cl^- fluxes the justification is provided by the very high fluxes at the tonoplast found by MacRobbie (1962, 1964) and Coster and Hope (1968). For both ions these are at least 30 times the plasmalemma fluxes, often much more; our determinations of sap specific activity can therefore be applied to the cytoplasm with an error of 3–5% at most.

The presence of the cell wall introduces two types of error. First, the diffusion delay in the cell wall renders the precise influx and efflux periods difficult to determine. For this reason we were inclined to favour 20–40-min periods (cf. 2-min periods used by Kitasato 1968). Secondly, the radioactive ions retained by the Donnan phase of the cell wall (Dainty and Hope 1959) will increase the apparent influx; however, with a standard washing time this will affect only the determination of the influx in the first influx period for each cell. Each influx depends on the difference of two radioactivity determinations, accurate to 2–3%, but the error in this difference may be quite large, because small differences were involved. In efflux measurements, counting was normally carried out to a standard deviation of $\pm 2\%$, but errors due to irregular drying on the planchet may be $\pm 10\%$. During each influx period and wash period there will be an efflux of radioactive ions from the interior of the cell. The efflux is larger at smaller membrane potentials, when the influx is small, which means a systematic error in the low influx points, tending to reduce the apparent influx.

In some experiments, errors of the above sort, and others due to contamination on the slides holding the cells, have led to a large scatter in the results from a single cell. To show trends, mean fluxes have been grouped in ranges of p.d. for this purpose.

III. Results

(a) N. translucens

The mean influxes and effluxes of potassium in seven cells, grouped in 20-mV ranges of potential, are plotted in Figure 1. The semi-logarithmic plot was adopted because, for each cell, the fluxes then tended to fall on a straight line.

Some of the influx may have been active (MacRobbie 1962, 1964, 1965). The fraction of the influx which was active in these cells was, however, low, as suggested by the very small reduction in the influx caused by DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], and by experiments on the same batch done by MacRobbie (personal communication). Most of the measured influx is therefore believed to be passive, which is confirmed by the alteration of the influx by the membrane potential.

From the lines fitted to the points in Figure 1, the slope or chord potassium conductance, G_K, can be directly obtained, for example between the potentials Ψ_1 and Ψ_2:

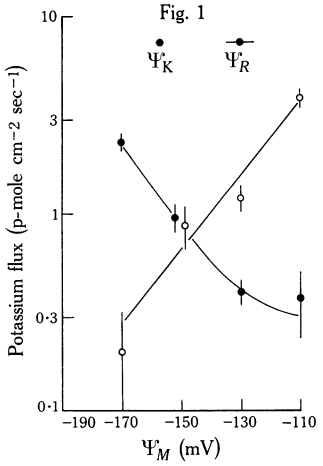
$$[G_K]_{\psi 1,\psi 2} = \mathbf{F}[(\phi_i-\phi_o)_{\psi 1}-(\phi_i-\phi_o)_{\psi 2}]/(\Psi_2-\Psi_1). \quad (7)$$

If the interval $\Psi_2 - \Psi_1$ is small enough, this will be equal to the differential conductance:

$$[g_K]_{\Psi 1} = (\partial J_K / \partial \Psi_M)_{\Psi 1}, \quad (8)$$

where J_K is the current density due to potassium. Taking the interval -125 to -135 mV as the range of p.d. used in many of the determinations of g_M, the corresponding value of g_K, just over 7 μmho cm^{-2}, can be compared with $g_M = 21 \pm 2$ μmho cm^{-2} in the seven cells.

When the flux ratios for potassium are plotted against potential, to test whether equation (3) is obeyed, the relationship shown in Figure 2 is revealed. The deviations from "independent" influx and efflux are considerable. The slope of $\log_{10}$(flux ratio) *v.* Ψ_M is 2–3 times that of equation (3). The intercept, where the flux ratio is unity, differs somewhat from Ψ_K.



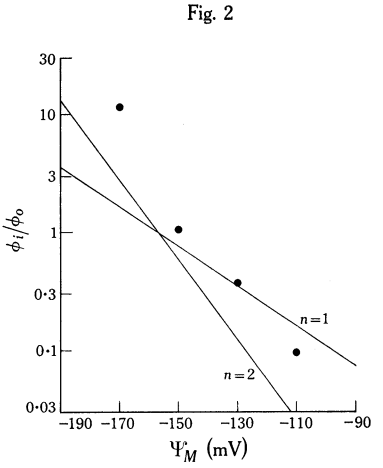


Fig. 1.—Mean influx (●) and efflux (○) of potassium in seven cells of *N. translucens* in APW, plotted on a logarithmic scale against the membrane potential (Ψ_M) recorded from the vacuole. Standard errors of the means are also shown. The fluxes were grouped into ranges of p.d. such as -100 to -119 mV (plotted at -110 mV), etc. The mean resting potential (Ψ_R) and Nernst potential for K^+ (Ψ_K) are also indicated. The lines were fitted visually.

Fig. 2.—Flux ratios for potassium in *N. translucens*, plotted on a logarithmic scale against vacuolar p.d. (Ψ_M). The points are the ratios of the means from the results plotted in Figure 1. The lines are theoretical relationships from equation (4) in the text, with $n = 1$ or 2, and Ψ_K, the Nernst p.d. for K^+, taken as -157 mV.

Figure 3 gives the results obtained for the sodium influx as a function of vacuole potential on two cells of *N. translucens*. The passive efflux has not been measured in these experiments, but MacRobbie (1962) records 0·1 p-mole cm^{-2} sec^{-1} at an unknown vacuolar potential, probably in the range -140 to -110 mV. For the cells in Figure 3, we may estimate the passive efflux as follows: Ψ_{Na} is calculated to be near -105 mV from typical concentrations of Na^+ in the sap; if the influx and efflux are equal at -105 mV and have similar slopes, the passive effluxes at -135 and -125 mV are approximately 0·04 and 0·06 p-mole cm^{-2} sec^{-1} respectively,

whilst the influxes are 0·33 and 0·24 p-mole cm^{-2} sec^{-1} respectively. The conductance equivalent to these changes in flux, by equation (7), is 1 μmho cm^{-2}.

(*b*) C. corallina

Similar measurements of influx and efflux to those described above were made with *C. corallina* cells in APW. Usually it was not feasible to make observations of influx and efflux on the same cell; the microelectrodes sealed more rapidly than in *N. translucens*. Once again, the scatter was large when results from a single cell were considered, but Figure 4 shows an almost linear relation between $\log_{10}$ (influx or efflux) against vacuolar potential, over a range of the latter. Some residual efflux might have been expected from nodal cells not under control of the p.d.; this may have raised the efflux values with more relative effect at the more negative p.d. values.

The crossover point is near the average Ψ'_K (*c.* −170 mV) and at the resting potential there is a net efflux (as with *N. translucens*) of about 1·5 p-mole cm^{-2} sec^{-1}. The potassium conductance about the resting potential averaged 15 μmho cm^{-2}, whilst the electric conductance was about 70 μmho cm^{-2}.

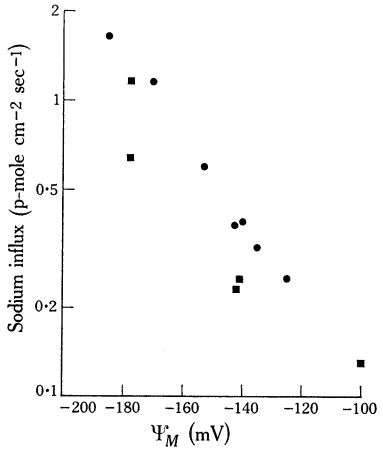


Fig. 3.—The influx of Na^+ into two cells of *N. translucens*, plotted on a logarithmic scale against the vacuolar p.d., Ψ'_M.

The mean fluxes in Figure 4 were used to calculate the flux ratios at the appropriate p.d. values. These ratios are plotted in Figure 5. Even allowing for the large standard errors involved, the departures from independent ion fluxes are enormous, the flux ratio changing by a factor of 10 in 25 mV or so, instead of the 59 mV expected from equation (4).

Further experiments were attempted with the external medium FPW, designed to increase the transport number for K^+ in the cell wall [see Section IV(*b*)]. The results were very variable but with one cell a potassium conductance of 16 μmho cm^{-2} was found, out of a total of 42 μmho cm^{-2}. This is the highest fraction of the conductance, attributable to K^+, which has been found with *C. corallina*.

Sodium influx was found to be very similar in its relation to vacuolar p.d. to that in *N. translucens*. Two further cells were used to measure the efflux of sodium, at various vacuolar potentials. No discernible trend with p.d. was found, the efflux being approximately constant at 0·75 p-mole cm^{-2} sec^{-1} between −130 and −200 mV. Conductance due to Na^+ in *C. corallina* in APW did not exceed 1 μmho cm^{-2}.

The chloride conductance has been discussed previously by Hope, Simpson, and Walker (1966, fig. 4), who used results from the present series of experiments. Also, Coster and Hope (1968, fig. 2) described the influence of p.d. on chloride efflux with particular reference to the "punch-through" phenomenon which occurs at plasmalemma p.d. values of about −300 mV. In both these sets of experiments, the conductance due to change of chloride efflux with potential, at the resting potential, amounted to 1–2 μmho cm^{-2}. The passive chloride influx is expected to be very small, and not to contribute to the conductance.

Fig. 4

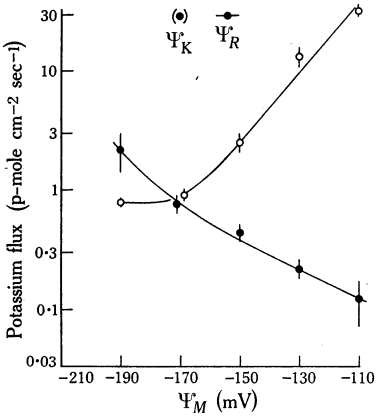


Fig. 5

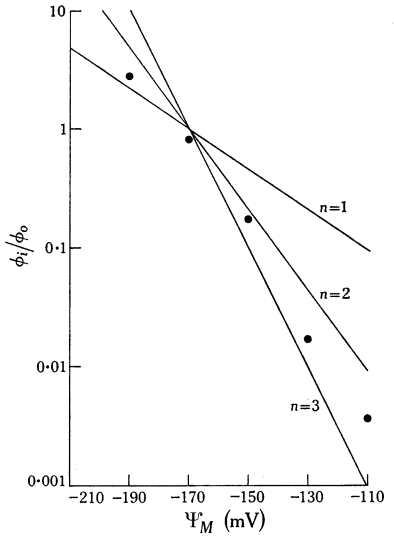


Fig. 4.—Mean influx (●, 10 cells) and efflux (○, 7 cells) of K^+ in *C. corallina* in APW, plotted on a logarithmic scale against vacuolar p.d., Ψ'_M. Standard errors of the means (SEM) are shown by bars. The fluxes were grouped as in Figure 1. The mean resting p.d. (Ψ'_R) with SEM is indicated. The mean Nernst p.d. was −170 mV in the only three cells for which data is available.

Fig. 5.—Flux ratios for K^+ in *C. corallina* plotted on a logarithmic scale against Ψ'_M. The points were obtained from the ratios of the mean influxes and effluxes in Figure 4. The lines are the expected relationships using text equation (4) with $\Psi'_K = -170$ mV and $n = 1$, 2, or 3.

IV. Discussion

(a) *Present Results*

It has been found that the unidirectional fluxes of potassium can be altered by clamping the potential between the vacuole and external medium at various levels. The influx was increased by making this p.d. more negative; the efflux was increased if this p.d. were made more positive. The change in net flux (influx minus efflux) with change in potential is equivalent to a partial conductance. We might expect that, since K^+, Na^+, and Cl^- are by far the most abundant ions in these cells,

$$g_M = g_K + g_{Na} + g_{Cl}. \quad (9)$$

Assembling these estimates of these quantities from the results given above leads to

the following tabulation, for cells in APW:

Conductance (μmho cm^{-2})	g_K	g_{Na}	g_{Cl}	$\Sigma_j g_j$	g_M
N. translucens	7	1	(1)	9	21
C. corallina	15	1	2	18	70

The discrepancy between g_M and Σg_j is considerable. We are led to ask whether the flux determinations or electric conductance measurements are in error, or whether an unidentified ion species carries much of the current. Errors in flux determinations and the possibility of other current carriers are discussed in Sections IV(b) and IV(c). Part of the discrepancy has its origin in the methods of estimating membrane conductance.

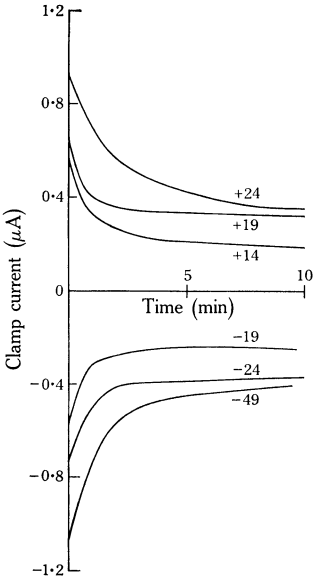


Fig. 6.—The currents needed to maintain a cell of *C. corallina* clamped at p.d. values differing from the resting level by the amounts (mV) indicated, over a period of time. The traces were taken from continuous chart recordings.

When the current flowing during a voltage clamp was plotted against time, it was generally found that it decreased, whether the cell was depolarized or hyperpolarized. The cause of the decrease is discussed in more detail in the following section; the decrease is thought to relate to changes in ionic concentration just outside the plasmalemma, caused by the current flow. Figure 6 shows examples of the net current during several voltage clamps.

The usual method of estimating the membrane conductance is to pass a small current for a few seconds and divide the current density by the change in p.d.; provided there is no cable decrease of current density along the length of the cell this is reasonable. Over longer times it might be more appropriate to think of the mean conductance during a voltage clamp as $\int_0^{t_1} J(t)\mathrm{d}t/(t_1 \Delta\Psi_M)$ where $J(t)$ is the current density, a function of time. Plainly this would yield a lower conductance than that calculated from a short pulse of current.

It can be assumed that a decreasing clamp current reflects a decreasing net flux of some ion species.

The fact that the membrane conductance and the partial ion conductance are compared for rather differing conditions is one reason for the discrepancy.

The results summarized in Figures 2 and 5 provide a measure of a factor n in the flux-ratio equation (4). If n were unity, then the changes in flux ratio with potential would be a factor of 10 for each 59 mV change in Ψ_M, and the fluxes could be called passive and independent, i.e. non-interacting. Our results approximate $n = 2$–3, similar to a value of $2{\cdot}5$ found for *Sepia* axons by Hodgkin and Keynes (1955). Possibly some similarity exists in the passive diffusion mechanism in the axolemma of *Sepia* and the plasmalemma of characean cells.

It can be deduced from the results that in the species examined, potassium ions do not migrate independently. Non-independence has been postulated in the past (Hope and Walker 1960; MacRobbie 1964; Williams, Johnston, and Dainty 1964) on the basis of failure of equation (1) to yield the electric conductance when the unidirectional fluxes of K^+ and Na^+ are inserted.

With this established, it follows that the Goldman equation (Goldman 1943) is not applicable to passive movements of potassium in these cells. This equation was used by Hope and Walker (1961) to interpret potential and resistance measurements on cells of *C. corallina* in calcium-free solutions, and its agreement with the resistance data may now be suspected of being fortuitous, unless experiments similar to the present ones confirm the independence relation for those conditions. It is also likely that the use of the same equation by Hogg, Williams, and Johnston (1968) in interpreting data on the effect of temperature on membrane p.d. and resistance is not justified. It is equally hazardous to calculate an "ion permeability" from a flux measurement using this equation (Smith 1967; Raven 1968).

The mechanism which produces a factor of 2 or more in the flux-ratio equation is still unknown; the model of Hodgkin and Keynes (1955) is possible, and would necessitate a narrow pore with several ions in its length. Alternatively, the electro-osmotic interaction proposed by Dainty, Croghan, and Fensom (1963) is a likely explanation of a value of n greater than 1. It is, of course, possible that both mechanisms operate—steric interaction between potassium ions and ion–water–ion interaction—in pores of moderate width.

Further interactions between other fluxes (for example Na^+ and Cl^-) and K^+ fluxes may be important. Such interactions can be taken into account in the flux-ratio equation (see, for example, Meares 1959).

The slope of the graph of sodium influx against potential suggests that sodium, too, does not diffuse independently, and that the sodium flux-ratio equation may contain a factor of the order of 2. This remains to be determined. Certainly the influx, like the K^+ influx, is far more sharply dependent on membrane potential than would be predicted by the Goldman equation. Thus, if the Goldman equation for the sodium influx,

$$\phi_{i,\mathrm{Na}} = P_{\mathrm{Na}} \cdot \frac{\mathbf{F}\Psi_{\mathrm{M}}}{\mathbf{R}T} \cdot \frac{a_{o,\mathrm{Na}}}{1-\exp(\mathbf{F}\Psi_M/\mathbf{R}T)}, \qquad (10)$$

is fitted to the observations at -118 mV, it predicts an influx at -177 mV of 0·26 p-mole cm^{-2} sec^{-1} while the observed value is 1·2 p-mole cm^{-2} sec^{-1}. It seems unlikely that any equation based on passive independent movement would predict so steeply sloping an influx curve.

(b) Sources of Error in Determination of Fluxes

When an inward current of positive ions flows, it is carried mainly by calcium ions within the wall but by potassium ions through the membrane (according to our hypothesis). It is of interest to make an estimate of the relative abundance of these two ions just outside the plasmalemma, by assuming in the resting cell a simple Donnan equilibrium between the wall and APW, or between the wall and the normal artificial pond water used by Kitasato (1968) when investigating the effect of membrane potential on potassium flux in *N. clavata*. The following concentrations (m-equiv/l) of ions within the cell wall are to be expected if the wall contains 500 m-equiv. of indiffusible anions per litre of wall water (Dainty and Hope 1959):

	External Medium			Cell Wall		
	K^+	Na^+	Ca^{2+}	K^+	Na^+	Ca^{2+}
Cell in APW	0·1	1·0	0·2	4·7	47·3	448
From Kitasato (1968)	0·1	2·0	4·0	1·2	21·7	477

Though it is to be expected that Ca^{2+} may have a much lowered mobility in an ion-exchange system such as the cell wall it is clear that the transport number for Ca^{2+} and Na^+ together will be much greater than that for K^+. For inward currents this would lead to depletion of K^+ outside the plasmalemma and an enhancement of K^+ for outward currents. This effect has been dealt with theoretically by Barry and Hope (1969). The effect is magnified by a slower than normal exchange between external K^+ and wall Ca^{2+} (or vice versa), a phenomenon clearly shown in results of Dainty and Hope (1959). The profile changes have important implications for flux measurements.

Changes with time which are postulated to occur during prolonged voltage clamps include the following. Their expected effects on the fluxes, estimated with tracers, are shown in the final two columns. $[K_W]$ is the concentration of K^+ just outside the plasmalemma and S_W the corresponding specific activity.

Depolarizing clamp	$[K_W]\uparrow$	$S_W\downarrow$ (influx) $S_W\uparrow$ (efflux)	$\phi_i\downarrow$ $\phi_o\downarrow$
Hyperpolarizing clamp	$[K_W]\downarrow$	$S_W\sim$	$\phi_i\downarrow$ $\phi_o\sim$

When a constant current is passed for some time the membrane p.d. changes in ways entirely consistent with the postulated effect, namely a positive outward current causes a drift of p.d. in the depolarizing direction, due to $[K_W]$ increasing. Conversely a constant, positive, inward current causes a drift in the hyperpolarized direction, due to $[K_W]$ decreasing. These drifts reverse until the normal resting p.d. is attained, when profile disturbance is eliminated by diffusion within the cell wall. The half-time for the recovery is several minutes.

The feasibility of one of these effects can be judged from the following calculation. The small amount of K^+ within the wall [*c.* 4·7 n-equiv. cm^{-2} in equilibrium

with APW; *c.* 1·2 n-equiv. cm^{-2} in equilibrium with Kitasato's medium (1968)] means that the specific activity in the wall may rise, during current-assisted effluxes, to an appreciable fraction of the internal specific activity. For example, in several of the *C. corallina* cells used for efflux measurements, the internal specific activity was about 6×10^{10} counts min^{-1} (equiv. $K^+)^{-1}$. Hence merely 300 counts min^{-1} in the cell wall gives a specific activity there *equal* to that inside the cell. In half-hour periods, radioactivities of this magnitude were collected outside the cell.

Clearly, the extent of underestimation due to back-flux when the specific activity builds up in this way is difficult to calculate without knowing the mobility of ions within the cell wall. The latter varies with the ionic strength of the medium (Dainty and Hope 1959). Furthermore, the transport number for K^+ will vary with pH, since the wall contains anions which are the result of ionization of weak acids such as polyuronic acid (Dainty, Hope, and Denby 1960).

The underestimations of influx and efflux would be expected to be greater in proportion to the deviation from the resting potential. This means that the slopes of influx and efflux *v.* Ψ_M are too low in our experiments, and therefore g_K is also underestimated.

We see no way at present to prevent these errors, since attempts to increase the transport number for K^+ within the wall, by lowering $[Ca_o^{2+}]$ for example, cause the cells to have a shortened life, and increase the radioactivity within the cell wall relative to the rest of the cell, making influx measurements more difficult.

Simons (personal communication) has made an analysis of particle flow through membranes. According to this, one of the assumptions usually made when calculating fluxes, namely that "the back-flux of tracer through the membrane is negligible" implies rather stringent requirements for the ratio of specific activities between the "labelled" side and the side into which the tracer is flowing. Simons proposes that the observed tracer influx is given by:

$$_{obs}\phi_i^* = {}_{true}\phi_i^* \, [1-(S_i/S_o)(\phi_o/\phi_i)],$$

where S_o and S_i are the specific activities outside and inside. The usual assumption is that S_i/S_o should be $\ll 1$ for influx, and $S_o/S_i \ll 1$ for efflux.

The effect of failure to meet this new criterion, i.e. $(S_i/S_o)(\phi_o/\phi_i) \ll 1$ during influx, would be to cause an underestimation of influxes on the right of the crossover point (where $\phi_o/\phi_i > 1$), even if the specific activity ratio were apparently satisfactorily small. By similar reasoning the effluxes on the left of the crossover point may be underestimated if the inequality $(S_o/S_i)(\phi_i/\phi_o) \ll 1$ is not satisfied. Plainly more attention should be paid to the possibility of back-flux in tracer experiments. We are unable to say to what extent failure to observe the above restrictions caused errors in our estimates. This is because the relevant S_o is that in the cell wall, and this is rather inaccessible to measurement.

(c) *Possible Contribution of Other Ions to Membrane Conductance*

Kitasato (1968) has claimed that hydrogen ions account for most of the conductance of the plasmalemma in *N. clavata*. The main evidence, most of which is of necessity indirect since no tracer technique for hydrogen ions is yet available, was as follows:

(i) The p.d. did not change with changes in external potassium concentration in the range 0·1–1·0 mM, and depolarized only about 15 mV in the range 1–10 mM; the pH was 5·3. This non-responsiveness was also reported by Hope and Walker (1961), but disappears (the plasmalemma becomes K^+-responsive) when the cell wall is brought into equilibrium with a lowered- or zero-calcium pond water (see also Spanswick, Stolarek, and Williams 1967). This probably corresponds to increased exchangeability (K^+ for Na^+, or K^+ for Ca^{2+}) as monovalent cations replace divalent ones in the wall Donnan system. The exchange can be extremely slow, and mere immersion in Ca^{2+}-free pond water does not produce a zero-calcium cell wall! It is also possible that Ca^{2+} is adsorbed to the plasmalemma in such a way that K^+ within conduction regions of the membrane are screened and hence the p.d. and resistance prevented from changing.

(ii) The p.d. in *N. clavata* changed with external pH of an artificial pond water (see first tabulation, p. 1190), but only in the pH range 6–4. Between pH 6 and 8 there was virtually no response. Walker (unpublished data) found temporary hyperpolarization by slightly alkaline solutions but depolarization of a similar magnitude to that reported by Kitasato (1968) in slightly acid media.

(iii) The threshold of the action potential, and its peak, did not change markedly with pH. From this it was concluded that a change in phase-boundary p.d. (between the medium and edge of the cell wall) was not involved, otherwise it would have been expected that the threshold and peak would have changed by the same amount as the pH-induced change in overall p.d.

Though it is to be expected that the cell-wall-indiffusible anions would become steadily deionized in solutions of pH 6 (see fig. 1 in Dainty, Hope, and Denby 1960), in *N. clavata* it appears that it was indeed the trans-membrane p.d. which was affected by pH. Besides an explanation in terms of a specifically proton-permeable plasmalemma, it is possible to offer the following alternatives:

(1) The potassium permeability was decreased as the pH was lowered, or
(2) the sodium permeability was increased as the pH was lowered. In either instance the plasmalemma p.d. would be expected to depolarize and approach the equilibrium level for Na^+, i.e. about -50 mV, which it does.
(3) A third possibility is an increased resting permeability to chloride, occasioned by lowered pH.

(iv) Yet another piece of evidence was the finding that the potassium conductance (more accurately $F(\partial\phi_o/\partial\Psi_M)$ was only a small fraction of the membrane conductance in the medium used by Kitasato (1968) (see first tabulation, p. 1190), or only about 20% of the membrane conductance in Ca^{2+}-free solution.

This is entirely consistent with our ideas on the effect of transport number for K^+ in the cell wall on the concentration of K^+ during influx and efflux [discussed in Section IV(*b*)]. It is noted also that Kitasato (1968) collected efflux radioactivity in 2-min intervals. This may have been a further cause of loss of K^+ conductance since wall residues of radioactivity would carry over into samples for different p.d.

The difficulties encountered in accepting that the membrane conductance is due to a proton flux are several; the most serious seems to be that there must be a

passive influx (matched by an active efflux) of protons amounting to *c.* 40 p-mole cm^{-2} sec^{-1}. This arises because the internal pH is expected to be about 5 and H^+ ions are about 100 mV away from their equilibrium potential. According to Kitasato the resting potential, which "happens" to be close to the Nernst potential for K^+, is set by an electrogenic H^+ pump. Evidence from use of dinitrophenol is inconclusive unless it can be shown that this inhibitor has no effect on P_K, P_{Na}, and P_{Cl}.

Coster and Hope (unpublished data) looked for hydrogen ion fluxes in *C. corallina* by monitoring the external pH in a small, stagnant volume of APW, during the passage of hyperpolarizing and depolarizing currents. Only very small changes in pH were observed, which may in any case have been due to adjustments within the cell wall. It was concluded that g_H was a maximum of 0·5 μmho cm^{-2}, the contribution of hydrogen ion fluxes to the total current being 0·1–0·3%.

(*d*) *Conclusions*

The membrane conductance of *N. translucens* and *C. corallina* has been analysed into partial conductance due to K^+, Na^+, and Cl^- by means of determinations of unidirectional fluxes at various set membrane p.d. values. Less than half the conductance has been accounted for in this way. However, since many circumstances combine to cause underestimation of the fluxes, using radioactive tracers, and for the reasons detailed above for rejecting conductance due to protons, it is believed that the plasmalemma in these cells conducts electricity by means of fluxes of K^+ and Na^+, the transport numbers being about 0·9 and 0·1 respectively.

The flux ratio, ϕ_i/ϕ_o, was found in the experiments reported here to vary more rapidly with p.d. than expected from independent behaviour. The slope of the relation between $\log_{10}(\phi_i/\phi_o)$ and Ψ_M for independent passive fluxes (1/58) has to be multiplied by a factor n to match the experimental results. The factor n is about 2. If the partial conductance of K^+ is underestimated, as claimed, then n is also underestimated and may be 5 or more.

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