ION FLUXES DURING THE ACTION POTENTIAL IN CHARA

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Cells from the internodes of the fresh-water alga *Nitella* have been subjected to electrophysiological analysis by various investigators (Hormann, 1898; Osterhout & Harris, 1928; Blinks, 1930; Weidmann, 1949) and to a lesser extent studies of this sort have been made on the related genus *Chara* (Osterhout & Hill, 1940). Because the findings in these two forms are in many respects similar, it is convenient to treat much of the information in the literature as applicable to both. The resting membrane potential is rather greater than 100 mV (inside negative), and about equal to the propagated action potential, as measured with external electrodes. The rising phase of the spike is considerably more rapid than the falling phase. Conduction velocities are of the order of 1–2 cm/sec and the duration of the action potential is about 15 sec. The time scale of an action potential is, therefore, about a thousand-fold slower than that of a squid axon at 6.5°C.

Internodal cells of *Nitella* and *Chara* differ in that most species of this latter form possess a cortical layer of numerous small cells covering the cell wall. The wall has a thickness of 10–20 μ and covers the outer cytoplasmic membrane, while underlying this structure is a cytoplasm about 10–20 μ thick, then a vacuolar membrane and below this a large central vacuole. The material used in this investigation has been identified as *Chara globularis*, a corticated species. From a morphological point of view it should be referred to as a tissue. The experimental data to be presented show, however, that the cortical cells outside the cell wall do not act as an important barrier to ion fluxes, while the internodal cell is a physiological unit; we shall use the word ‘cell’ in what follows as more properly descriptive of the behaviour of the organism.

Resting values for Na⁺ and K⁺ transfers into *Nitella* have been measured by Brooks (1939) and by Holm-Jensen, Krogh & Wartiovaara (1944) who found that K⁺ transfer was considerably more rapid than that of Na⁺, in confirmation of the findings of Osterhout & Harris (1928) that K⁺ was in general much more effective in depolarizing the cells than was Na⁺. Our work has been concerned with the measurement of ion flows taking place during
rest and during an action potential in Chara, and with the correlation of such changes with gradients in the electrochemical potential of the ions involved.

METHODS

Cells of corticated Chara were collected from ponds and stored in pond water in a refrigerator at 14°C, under continuous low level illumination from a 14 W fluorescent tube. Some days before use the strands of cells were clipped into individual inter-nodal cells and these were stored in fresh pond water. Cells used for experiments were 0.6-1.0 mm in diameter and about 50 mm long.

Analyses of pond water and cell sap for Na+ and K+ were made with a flame photometer, and Cl− was estimated by titration with Hg(NO3)2 using the method of Schales & Schales (1941). Ion influx measurements were made by immersing a cell in solutions containing either 23Na, 42K, or 36Cl for various periods of time and then washing in distilled water. The cell was then mounted on a slide designed to secure reproducible geometry and counted with a Geiger-Müller tube. At the end of the experiment, one end of the cell was cut off and 10 mm³ of cell sap was removed for counting and analysis in order to determine its specific activity. Ion efflux measurements were made in two ways: by immersing a radioactive cell in 30 ml of inactive experimental solution the loss of radioactivity over various periods of time could be followed by repeatedly counting the cell, while the solutions into which the radioactive ions were lost were concentrated and counted separately. At the end of an efflux experiment, the sap was collected and analysed for specific activity. When ion flux measurements were to be correlated with ion movements during the action potential, the cell was mounted in a lucite trough so arranged that its entire length except for about 3 mm at one end was immersed in the experimental solution. The recording electrodes (Ag–AgCl electrodes filled with the experimental solution) were placed in contact with the cell in the bath; the cathode of the stimulating circuit was placed on the 3 mm end of the cell projecting out from the bath, and initiated excitation at this point. The response at the recording electrodes, although much shunted by the bath, was measurable. Stimulation with 1 msec square-wave pulses was usually carried out at a rate of 1/min, although lower frequencies were sometimes used.

Radioactive ion uptake curves were also measured for cell walls with their cortication. In such cases, one end of the cell was cut off close to the node and the sap gently expressed. The wall was then immersed in distilled water for 24 hr and then transferred to a radioactive solution. Uptake and loss of radioactive ions was followed by counting the wall at various times after its immersion in either radioactive or inactive solutions.

Because of the very high longitudinal resistance of the cell wall in dilute (ca. 0.5 mM) salt solutions, of the order of 1-5 MΩ cm (Blinks, 1930) it seemed likely that resting potential measurements made with external electrodes between a KCl-depolarized and an untreated region were not seriously in error. A micro-electrode study of the action potential did, however, seem important in order to establish with certainty the level of maximum depolarization. Attempts to achieve penetration with electrodes of less than 1 μ tip diameter were entirely unsuccessful because of the structural rigidity of the cell wall. A reduction of the tension on the wall by the external application of hypertonic sucrose solutions did not help micro-electrode penetration but an increase in tip size of the electrodes to 10 μ did allow penetration. The cell was usually penetrated at one end, in a direction along the axis of the cell (Fig. 1). The electrodes were filled with 100 mM-KCl corresponding to the mean Cl− concentration of the sap. It is recognized that higher concentrations of KCl are desirable from the standpoint of the elimination of junction potentials; however, the electrode tip area is so large that diffusion of KCl into the sap can be appreciable. Such electrodes were connected via a cathode follower to an oscilloscope; a cathode-ray oscilloscope was used to follow potential changes while a direct-writing oscillograph was used for recording. For certain purposes it was convenient to use another method of measuring potential. This is shown in Fig. 1 and uses a 1.5 cm gap of flowing distilled water to provide electric insulation between two chambers, one containing 100 mM-KCl and the other an experimental solution. Soft paraffin seals were used to prevent the flow of solutions into the distilled-water chamber. Tests with thread, mounted
across the chambers, showed that the resistance between the two end chambers was 3–4 MΩ when they both contained 100 mM salt solution. With a Chara cell mounted in the chamber, the effective membrane resistance was usually of the order of 0.4 MΩ and Ag–AgCl electrodes with 10 mM-NaCl bridges were used to connect the end chambers to a differential amplifier with 10 MΩ input impedance. For stimulating cells mounted in the flowing distilled water chamber, large Ag–AgCl electrodes were connected between the experimental chamber and the tip of the cell projecting out from the paraffin seal. The electrode in the experimental chamber was usually the cathode, except for some experiments in which anode-break excitations were studied.

Cell walls were studied electrically in two ways: if both ends of a cell were cut off, a Ag–AgCl wire 0.3 mm in diameter could be threaded through the central vacuole and the two ends of such a wire fastened together, thus forming the cell wall into a loop. This assembly could be lowered into an experimental solution such that the central half of the wall was in contact with the solution.

Fig. 1. On the left is shown (from above) the arrangement for recording action potentials with a micro-electrode. The cell is in a lucite trough and held between two V-shaped grooves. One end projects out of the bath through a paraffin seal and the micro-electrode is inserted here.

On the right is the arrangement for recording action potentials with a flowing distilled-water gap. The water flows through the centre compartment, and recording is between the KCl-depolarized region and the experimental, Ex.

When the ends of the wall had dried out somewhat, paraffin was applied to the junction between the Ag wire and the ends of the wall and measurements between the loop of cell wall and a Ag–AgCl reference electrode in the solution could be made. Because the small silver wire inside the wall showed signs of polarization with d.c. currents greater than about 100 μA, another method for electrical measurements with walls was used. Each end of the wall was cannulated by a small glass pipette and the junction sealed with soft paraffin. As before, the central half of the wall was held in experimental solutions. Ag–AgCl electrodes connected the inside of the glass pipettes and the bath solution to a source of current. The internal environment of the wall could be changed by applying gentle suction to one of the glass pipettes and thus causing the filling solution to move through the wall. Potential measurements across the wall were made with a vacuum tube voltmeter of 10^14 Ω input impedance and potentials could be read to 1 mV. Resistance measurements were made by measuring the current flow at a series of applied potentials in the presence and in the absence of the cell wall.

RESULTS

Properties of the cell wall

Electrical measurements of the resistance of a number of cell walls equilibrated and measured in 0.1 M-KCl solution gave a value of 4 Ω cm², which, though very low when compared with membrane resistances, is appreciable for a structure about 10 μ thick, as this corresponds to a specific resistivity of
4000 Ω cm. This value is similar to that found for *Nitella* (1000 Ω cm) by Curtis & Cole (1937). Freshly prepared walls occasionally showed a rectification such that when the inside electrode was an anode the resistance was about 12 Ω cm², whereas it was 4 Ω cm² for currents in the reverse direction. A very occasional wall showed a transient to current flow, suggesting that the membrane was still capable of responding to current flow with a conductance change. Both effects disappeared if the walls were left during the night in distilled water or NaCl solution.

The wall gave no potential when the following 100 mm solutions were placed respectively on the inside and the outside: KCl/NaCl, K₂SO₄/NaCl, KCl/K₂SO₄. It was originally thought that there might be a potential between sulphate and chloride because we did not maintain osmotic balance between the solutions. If this is not done, the rapid movement of water across the wall appears to give a potential. We have never observed a stable potential across the wall.

If cell walls are immersed in solutions of ⁴²K or ⁴⁶Na, a rather large and rapid uptake of these ions takes place, with a half-time of about 5 min. Radioactive walls can be washed repeatedly with distilled water and yet lose very little of the radioactivity they have taken up, but all counts can be removed by immersion in inactive Na⁺ or K⁺ solutions. The absolute amount of material taken up is 0.5 mm/kg wet wall, and no distinction is made between Na⁺ and K⁺. Similar experiments with ³⁶Cl show that only a very small amount of this ion is taken up and it can all be removed with a brief wash in distilled water. Clearly the wall behaves as a cation exchange system.

The role of the cortical cells outside the cell wall in affecting the ion fluxes to be described later would appear to be small for the following reasons. The specific resistivity of the *Chara* wall with cortication is not greatly different from that for *Nitella* which has a wall without cortical cells. The wall resistance of 4 Ω cm² is to be compared with *Rₘ*, the membrane resistance of 200,000 Ω cm², as these two resistances are in series. The cortical cells might be expected to take up small amounts of radioactive ions which might confuse ion flux analyses; the evidence is, however, that owing to the large cation exchange capacity of the wall such uptake is unmeasurable. Further, the wall does not distinguish between Na⁺ and K⁺, whereas the intact cell does by a considerable factor. A series resistance of a magnitude similar to that of the wall occurs in the squid giant axon and is presumably due to some small impedance to ion flow brought about by the Schwann cell layer (Frankenhaeuser & Hodgkin, 1956).

**Resting and action potentials**

Analyses have been made of *Chara* sap and of pond water, with which the ions in the sap are presumably in a steady state. The equilibrium potentials for Na⁺, K⁺ and Cl⁻ have been computed from equations (1)–(3) where brackets
ION FLUXES DURING ACTION POTENTIAL

Indicate ion concentrations, and \( o \) and \( i \) refer to outside and inside, and are shown together with the analytical values for the ions in Table 1.

\[
E_{Na} = \frac{RT}{nF} \ln \frac{[Na^+]_o}{[Na^+]_i};
\]
\[
E_{K} = \frac{RT}{nF} \ln \frac{[K^+]_o}{[K^+]_i};
\]
\[
E_{Cl} = \frac{RT}{nF} \ln \frac{[Cl^-]_o}{[Cl^-]_i}.
\]

No comprehensive analyses of the cytoplasm for ion content have as yet been made. This is a reflection not of the small amounts of such material available in a cell, but of the difficulty in separating it from the much greater volume of cell sap. Published analyses of the cytoplasm (Holm-Jensen et al. 1944; Krogh, 1946) have shown no difference in [K\(^+\)] when compared with sap. Such analyses as we have agree with this finding.

**Table 1. Steady state concentrations of ions in sap**

<table>
<thead>
<tr>
<th>Ion</th>
<th>Pond water</th>
<th>Sap</th>
<th>Equilibrium potential of ion (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(^+)</td>
<td>0-046</td>
<td>65±1-9</td>
<td>-184</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>0-15</td>
<td>66±1-9</td>
<td>-155</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>0-04</td>
<td>112±1-5</td>
<td>+202</td>
</tr>
</tbody>
</table>

The mean value of the resting potential of cells in pond water is \(-181\) mV, which suggests that potassium ions in the sap are in electrochemical equilibrium with those in the environment. Sodium ion appears to be somewhat less concentrated than required by conditions of equilibrium; this cannot be attributed to a permeability difference, as we are presumably dealing with a steady-state situation. Chloride ion, in marked contrast to Na\(^+\) and K\(^+\), is concentrated in sap against a very large gradient in electrochemical potential.

Little is known regarding the permeabilities of the outer and inner cytoplasmic membranes in Chara; certain characteristics can be inferred, however, from purely osmotic considerations. The cell wall is the only structure capable of resisting the osmotic pressure under which the cells normally exist. It would appear, therefore, that cytoplasm and vacuole must be in osmotic equilibrium. Since the total osmotic pressure of the cell appears to be equivalent to 300 mM sucrose and the contribution from cytoplasmic proteins must be small, most of the osmotic pressure must be exerted by ions both in the cytoplasm and in the vacuole. One might suppose, by analogy with animal cells, that a Donnan distribution exists between the cytoplasm and the sap, with a non-diffusible anion in the cytoplasm. Such a distribution would effectively raise the cytoplasmic concentrations of Na\(^+\) and K\(^+\) and lower that of Cl\(^-\). The osmotic
imbalance inherent in a Donann distribution would have to be adjusted by the continuous pumping of some ion from the cytoplasm.

Attempts have been made (Walker, 1955) to demonstrate that a potential exists between the cytoplasm and vacuole in *Nitella* cells which were relatively free of chloroplasts and thus permitted visual control of the micro-electrode tip. No potential difference was observed, however. Our cells were so opaque that it was not possible to follow the micro-electrode tip visually. In several thousand punctures, however, there was never an incident of a two-step change in potential. Our conclusion is, therefore, in agreement with the measurements cited above, that the potential appears when the electrode enters the cytoplasm and is unchanged when it enters the vacuole. Some care is necessary with respect to the micro-electrode puncture of *Chara* because a ‘seal’ of the electrode to the cell does not develop immediately. This is suggested by the observation that whereas the membrane potential measured with the outside electrode remote from the site of insertion of the micro-electrode gives normal values, if the outside electrode is placed just at the site of insertion of the micro-electrode the measured membrane potential is often somewhat low. After some hours, a ‘seal’ is apparently made and the depolarized region disappears. Electrodes have been introduced that give stable potentials for 24 hr or more and, with 1 mM-NaCl solution outside, an external recording and an internal electrode give identical traces for the action potential. A further difficulty with micro-electrodes is that they tend to ‘unseal’ when the cell is subjected to relatively high NaCl concentrations. This has been noticed when a cell, punctured while in 1 mM-NaCl, was transferred to 100 mM-NaCl. Experimental treatments with high external osmotic pressures are better carried out with a cell mounted in a flowing distilled-water gap.

When action potentials are recorded in *Chara*, either with a micro-electrode or with a flowing-water gap, they rise from a mean resting value of $-181 \text{ mV}$ to an average value of +7 mV; the time constant of the rise is of the order of 0.1 sec, while that of the fall is about 5 sec. The rate of rise of the action potential cannot be measured properly in the flow chamber because the non-uniform excitation leads to some conduction. The electric time constant of the membrane for small voltage displacements is 0.2 sec, the cell shows sub-threshold local responses very similar to those observed for squid axon, and has a sharp threshold for excitation at a depolarization 40–60 mV below that of the resting potential.

One of the characteristics of the cell is the great variety of wave forms and amplitudes that the action potential can assume; it has been possible to find cells with resting potentials as great as $-240 \text{ mV}$ and action potentials of 200 mV. The action potential has been observed to overshoot zero by some 30 mV and various types of oscillatory behaviour of the falling phase have been noted. One experimental method of altering the wave form of the action
potential measured at the cathode is simply that of stimulating with supra-threshold shocks of 1 msec duration. The essentially instantaneous displacement of the membrane potential to lower and lower absolute values serves to slow markedly the falling phase of the ensuing action potential without affecting either the rate of rise or the amplitude of the spike. The results are shown in Fig. 2.

![Records show the response of a Chara cell in pond water recorded at the cathode in a flow chamber. The level of zero p.d. is shown as a horizontal bar 4 sec long; the vertical bar on the right is 50 mV. a, response to 1 msec stimuli of 3.3, 3.4, and 3.5 V; these yielded two local responses and an action potential. b, response to a stimulus of 5 V, c, 10 V, d, 20 V, e, 50 V and f, 3.5 V.](image)

**Alteration of the ionic composition of the environment.** In so far as experiments over about 12 hr are concerned, bioelectric activity in *Chara* does not appear to require the presence of an ion in the external environment; the immersion of a cell for many hours in distilled water or 200 mM sucrose does not affect its ability to give an action potential. More prolonged immersion of the cell in a variety of 10 mM salt solutions does, however, lead to a reversible loss of excitability. In particular, treatment with any salt solution not containing Cl results in a loss of excitability; this can be restored by immersion of the cell in solutions of any chloride; choline chloride is quite satisfactory for this purpose. Table 2 shows the results obtained using twenty-four cells in each solution. Control cells in 10 mM-NaCl showed no loss of excitability after 22 days immersion. The time required for loss of excitability is much shorter than the time for half exchange of radioactive chloride with that of the sap (14 days); hence we suppose that the externally added anion has exchanged
with a considerable fraction of the cytoplasmic Cl\textsuperscript{−}, thereby inducing a local depletion of this ion. The experiment indicates that Cl\textsuperscript{−} has some vital role in excitation that cannot be taken by an ion as chemically similar as I\textsuperscript{−}.

It seemed likely that the depolarization observed during the action potential in Chara resulted from a sudden increase in the permeability of the membrane to Cl\textsuperscript{−}. To test this hypothesis properly it would be desirable to raise the concentration of Cl\textsuperscript{−} present externally to a level where the gradient in electrochemical potential of this ion across the membrane was zero. To do this would require the external Cl\textsuperscript{−} to be 1400 times the internal concentration or 168,000 mM, whereas osmotic considerations limit the external NaCl concentration to about 150 mM. Further difficulties are encountered in going to external NaCl concentrations higher than 10–20 mM, as the membrane has a measurable permeability to cations other than K\textsuperscript{+}. At external NaCl concentrations of less than 10 mM the membrane potential appears to be controlled by the distribution of K\textsuperscript{+} inside and out, while with 100 mM-NaCl outside there is a depolarization of about 75 mV. Such a level of depolarization does not prevent the initiation of an action potential but does make less simple the interpretation of records obtained to show that the increased [Cl\textsuperscript{−}]\textsubscript{o} decreases the level of depolarization which the action potential reaches during its peak. In this connexion it appears that \textquote{inactivation} of the system responsible for the sudden increase of membrane chloride permeability (P\textsubscript{Cl}) is not sensitive to decreases in membrane potential from resting levels of −180 to about −60 mV, because an action potential can be obtained in response to a further depolarization. Below −60 mV the membrane is largely unresponsive to stimulation.

**Table 2. Excitability changes in chloride-free solutions**

<table>
<thead>
<tr>
<th>Solution (10 mM)</th>
<th>NaI</th>
<th>NaNO\textsubscript{3}</th>
<th>Na\textsubscript{2}SO\textsubscript{4}</th>
<th>K\textsubscript{2}SO\textsubscript{4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours of immersion needed for loss of excitability in 90% of cells</td>
<td>48</td>
<td>48</td>
<td>42</td>
<td>24</td>
</tr>
<tr>
<td>Hours of immersion needed for recovery of excitability in 10 mM/l. choline chloride, for 90% of cells</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

Solutions of choline chloride were found to produce less depolarization at a given concentration than did NaCl, and MgCl\textsubscript{2} appeared even more satisfactory in this respect. Cells were mounted in an insulating-gap chamber, depolarized with KCl at one end and treated with 100 mM-MgCl\textsubscript{2} at the other end. The cell was then stimulated once every 5 min and the responses recorded, and after the action potential had declined to a steady value, the cell was transferred to 150 mM-MgSO\textsubscript{4} and the action potential again recorded after a recovery period. The records from such an experiment are shown in Fig. 3. Because the resting potential declines considerably in any concentrated salt
solution, the quantitative relationship between the height of the action potential and $[\text{Cl}^-]_o$ is best seen with dilute solutions, and a plot of the changes in the resting potential and in the action potential, measured as the peak level of depolarization is reached, is shown for choline chloride in Fig. 4. Increases in $[\text{Cl}^-]_o$ lead to both a diminution in the peak level of depolarization reached during an action potential, and decreases in the rate of rise of the action potential with time which, for the $\text{MgCl}_2$ records, is from 200 to 10 mV/sec. The interpretation of these curves is complicated by our lack of information regarding the diffusion delay for $\text{Cl}^-$ that is made by the cell wall. It is also not clear to what extent the cytoplasmic $[\text{Cl}^-]$ is raised by treatment with solutions of high $[\text{Cl}^-]$. At any rate, it is usual for a high $[\text{Cl}^-]_o$ to require

Fig. 3. The effect of 100 mM-$\text{MgCl}_2$ on the action potential. Horizontal bar shows the level of zero p.d. and is 4 sec long; vertical bar is 50 mV. The left-hand column shows, at top, the action potential of a cell in 100 mM-$\text{MgCl}_2$ after 5 min. The next three lower traces show the changes in the action potential after 10, 20 and 30 min continued immersion in $\text{MgCl}_2$. At the top of the right-hand column is the action potential of a cell after 35 min immersion in $\text{MgCl}_2$, and the three traces below show the changes in action potential after the cell had been transferred to 150 mM-$\text{MgSO}_4$ for 5, 10 and 15 min. All recording was at the cathode with the cell in a distilled-water gap chamber.
from 15 to 30 min to reduce the level of depolarization during an action potential to a steady value.

In the range of concentrations 1–10 mM none of the following ions (as chlorides) significantly affected the resting potential of Chara: Li⁺, Na⁺, Ca²⁺, Mg²⁺, or choline⁺, whereas K⁺ reduced the membrane potential an average of 55 mV. Concentrations of K⁺ lower than 1 mM were very much less effective in depolarizing the membrane, a change from 0–1–1 mM gave about a 25mV decrease, while from 10–100 mM-K⁺ the average change of the membrane potential was 46 mV. This figure is not highly significant, because an enhanced influx of Cl⁻ is to be expected and this would tend to diminish the depolarizing action of K⁺. The foregoing observations would argue against there being a large Donnan potential contribution from the cell wall in series with the membrane potential, since the wall ion exchange system takes up Na⁺ or K⁺ without distinction but only the latter ion has any influence on the over-all potential of the cell. The membrane potential of a cell in 1–10 mM-Na₂SO₄ is the same as that for the cell in an equivalent concentration of NaCl; hence it cannot be assumed that the active uptake of Cl⁻ is a necessary condition for a stable resting potential, when this is measured in terms of hours.

**Ion fluxes at rest and during activity**

Because the concentration ratios of K⁺, Cl⁻, and Na⁺ between sap and external solution are quite different from those found in animal cells, it

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**Fig. 4.** The magnitude of the resting and action potentials in various concentrations of choline chloride-sucrose solutions is shown. The sucrose concentration at any point is 200 mM–2 × choline chloride concentration. The broken line has a slope of 58 mV for a tenfold change in Cl⁻ concentration. Recording is with a micro-electrode in the sap. Semilog. scale.
appears useful to calculate the flux ratios to be expected in Chara, on the basis that the ions move passively along their electrochemical gradients. The equation (Ussing, 1949; Teorell, 1949) for flux ratios can be written as in (4)

$$M_{out}: M_{in} = \exp(E_m - E_e) \frac{nF}{RT},$$

(4)

where $M$ is the ion flux, $E_m$ is the membrane potential, and $E_e$ the equilibrium potential of the ion (equations 1–3). The flux ratios for K$^+$ and Cl$^-$ for a cell in pond water are shown in Fig. 5, together with the change to be expected in Cl$^-$ flux ratios when its outside concentration is changed from that of pond water to 100 mM.

![Graph showing flux ratios for K$^+$ and Cl$^-$ as a function of membrane potential.](image)

**Fig. 5.** The computed flux ratios for Cl$^-$ and K$^+$ are shown as a function of membrane potential for a cell in pond water. The dotted line shows the computed flux ratio for Cl$^-$ when $[\text{Cl}]_o$ is 100 mM, and the sap concentration is unchanged. Semilog. scale. Inset shows a diagrammatic representation of an action potential with a resting potential of −180 mV, a threshold of 40 mV, a peak of +7 mV, and a half-time of the falling phase of 4 sec.

Measurement of the kinetics of uptake of $^{22}\text{Na}$, $^{42}\text{K}$, and $^{36}\text{Cl}$ by Chara gave the curves shown in Fig. 6. As the surface:volume ratio is rather small in this cell, a long time, of the order of several days, is required for equilibration of the radioactive ions with those of the sap, and uptake curves can be expected
to be linear with times of the order of hours. The initial rapid uptake of K⁺ or Na⁺ has been shown, by other experiments, to be due to the ion exchange properties of the cell wall. Measured values for influx were, therefore, computed from the slope of the essentially linear part of the uptake curve and are shown in Table 3. As the cell wall does not have anion exchange properties,

![Graph](image)

**Fig. 6.** The uptake of radioactivity (counts/min) by a Chara cell plotted against time for ²²Na, ⁴²K and ³⁶Cl.

**Table 3.** Resting ion fluxes in Chara

<table>
<thead>
<tr>
<th>Ion</th>
<th>External concn. (mM)</th>
<th>Influx (pmole/cm².sec)</th>
<th>Influx/µM external concn. (pmole/cm².sec)</th>
<th>Efflux (pmole/cm².sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>1.4</td>
<td>2.8 ± 0.36* (30)</td>
<td>2.0</td>
<td>6.7 ± 1.2 (66)</td>
</tr>
<tr>
<td>Na⁺</td>
<td>10.0</td>
<td>0.7 ± 0.05 (35)</td>
<td>0.07</td>
<td>6.2 ± 1.1 (17)</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>35.0</td>
<td>1.9 ± 0.12 (21)</td>
<td>0.05</td>
<td>5.8 ± 1.0 (20)</td>
</tr>
</tbody>
</table>

* S.E. of mean; number of cells used is shown in parenthesis.

the Cl⁻ uptake is entirely linear. The specific activity of the samples of ⁴²K available to us was not sufficiently high to enable work at concentrations of 0.04 mM, which might be regarded as physiological; the actual concentration used was 1.4 mM-KCl and the depolarization produced was of the order of 30 mV. The efflux of ⁴²K previously accumulated in the cell was followed into an external solution containing 1.4 mM-KCl, and efflux values computed from the slope of the linear part of the efflux curve (see Fig. 7). After several points on such a curve had been determined, the sap was removed from the cell for a determination of its specific activity and, with appropriate corrections for the loss of radioactivity during the experiment, the K⁺ efflux rate could be computed. These values, together with those for ²²Na and ³⁶Cl, are given in Table 3.

The values for the resting ion fluxes shown in Table 3 suggest that K⁺ is relatively close to the steady-state condition. The K⁺ concentration used was
some thirty times that of the normal environment and the resting potential was diminished 30 mV. The observed flux ratio \( M_{\text{out}}:M_{\text{in}} \) of 2.5 can be compared with that calculated from equation (4). The calculated flux ratio is about 0.1, in agreement with the finding that \( K^+ \) depolarizes the membrane relatively slightly when \([K^+]_o\) is small.

The \( Na^+ \) influx, when corrected for concentration, is about 3.5% that of \( K^+ \), while the \( Na^+ \) efflux is large and suggests, as indeed does the analysis of sap, that an active process keeps this ion from assuming a purely passive distribution in the cell. The observed \( M_{\text{out}}:M_{\text{in}} \) is 9, while purely passive behaviour would demand a value of 0.006.

![Fig. 7. The loss of radioactivity from a Chara cell for \( {\text{^{42}K}} \) and \( {\text{^{22}Na}} \). Vertical bars denote periods of stimulation. These measurements were made by repeatedly counting the intact cell. The \( {\text{^{36}Cl}} \) efflux shown represents the radioactivity lost to the immersion solution during periods of rest and stimulation.](image)

The computed \( Cl^- \) flux ratio \( M_{\text{out}}:M_{\text{in}} \) is 4000, whereas the observed value is about 3 and is presumably 1 under physiological conditions. \( Chara \) cells are very sensitive to mechanical stimulation and, in spite of considerable care taken in handling them, \( Cl^- \) efflux always exceeded influx. We suppose that the handling incidental to flux measurements initiated a \( Cl^- \) ‘leak’ in the membrane. That active transport of \( Cl^- \) is involved appears certain because it is easy to demonstrate net increases in \([Cl^-]\) in cell sap with the cell in 10 mm-NaCl, although to increase the \([Cl^-]\) of the sap will require an experiment of several days duration.

The effect of stimulation is to increase by a factor of 20–30 times the resting \( K^+ \) and \( Cl^- \) effluxes. The \( Na^+ \) influx and efflux as well as the \( Cl^- \) influx are entirely unaffected by this stimulation. The \( K^+ \) influx is reduced to an immeasurable value, but this is likely to be an artifact of the experimental
arrangement. If the K⁺ and Cl⁻ effluxes are expressed as quantity of ion leaving the cell in an impulse, the value is of the order of 10,000 pmole/cm².impulse. The results obtained are shown in Table 4.

In the absence of any information regarding the specific activities of K⁺, Na⁺, or Cl⁻ in the cytoplasm, we have assumed that these values were the same as sap specific activities. The validity of this assumption rests entirely on the assignment of a very low ion permeability to the outer, cytoplasmic, membrane and a very high ion permeability to the inner, vacuolar, membrane. No such assumption would be necessary if the specific activity of the sap were allowed to reach 1·0, but this requires an inconveniently long pre-loading of the cells. The actual values of specific activity of sap were about 0·3 and if the cytoplasmic specific activities were 1·0 at the time of the experiment, then the efflux values given in Table 4 are three times too high.

<table>
<thead>
<tr>
<th>Ion fluxes during stimulation</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Cl⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Influx (pmole/cm².sec)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At rest</td>
<td>0·7±0·05</td>
<td>2·8±0·36</td>
<td>1·9±0·12</td>
</tr>
<tr>
<td>During stimulation</td>
<td>0·7±0·05 (14)</td>
<td>0 (31)</td>
<td>1·9±0·20 (24)</td>
</tr>
<tr>
<td>After stimulation</td>
<td>0·7±0·007</td>
<td>3·1±0·45</td>
<td>2·1±0·15</td>
</tr>
<tr>
<td>Total no. of impulses</td>
<td>30</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td><strong>Efflux (pmole/cm².sec)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At rest</td>
<td>6·2±1·1</td>
<td>6·7±1·2</td>
<td>5·8±1·0</td>
</tr>
<tr>
<td>During stimulation</td>
<td>6·2±1·1</td>
<td>211±48</td>
<td>131±4·9</td>
</tr>
<tr>
<td>After stimulation</td>
<td></td>
<td>7·0±1·6</td>
<td></td>
</tr>
<tr>
<td>Total no. of impulses</td>
<td>30</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td><strong>Efflux (pmole/cm².impulse)</strong></td>
<td>0 (23)</td>
<td>3·1±1·1×10⁴ (26)</td>
<td>0·9±0·7×10⁴ (24)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the total number of cells used.

**DISCUSSION**

The sequence of events in the generation of an action potential in Chara would appear to be as follows. A depolarization of the resting membrane leads to an increase in its permeability to Cl⁻: because of the steep gradient in electrochemical potential of this ion across the membrane, there is a net outward flow of negative charge which, if it exceeds the net efflux of positive ions, will depolarize the membrane further. Though $E_{Cl}$, which equals +202 mV, is the limit that the membrane should approach were it exclusively permeable to Cl⁻, the peak depolarization is +7 mV, indicating that some other ion is contributing to the equivalent electric circuit. A decline in $P_{Cl}$ permits the efflux of cations to return the membrane potential to its resting level.

Since Na⁺ fluxes do not appear to change during bioelectric activity, attention is drawn to the movements of K⁺, as they may affect the membrane potential. It is necessary to calculate whether the observed efflux of K⁺ can be explained on the basis of a purely passive increase with depolarization, or whether $P_{K}$ of the membrane also changes. We assume that the total mem-
brane current $I_m$ at the peak of the action potential (where $dI_m/dt=0$) is equal to $i_K + i_{Cl} = 0$, where $i$ is the ionic current, and equals $nF(M_{out} - M_{in})$. Now, the efflux of a univalent cation, on constant-field assumptions (Goldman, 1943), can be written as equation (5), where $P$ is the permeability and $C_i$ the inside concentration of the ion.

$$M_{out} = \frac{PC_iFE_m \exp(-E_m F/RT)}{RT - 1 - \exp(-E_m F/RT)} \tag{5}$$

A numerical solution of (5) for $E_m = -181$ and $+7$ mV gives 220 as the factor by which the resting efflux will be increased by the depolarization. If we take 5 pmole/cm² sec as the resting efflux, then at $+7$ mV the calculated efflux is 1100 pmole/cm² sec. The idealized action potential shown in the inset in Fig. 5 has been subjected to integration by summing the effluxes calculated at half-second intervals; the computed $K^+$ efflux is 3400 pmole/cm² sec. The net $K^+$ loss is practically equal to this figure because at large depolarizations the $K^+$ influx is a negligible fraction of the efflux. The calculated $K^+$ loss per impulse is only 10% of the value given in Table 4, or 30% of the Cl⁻ efflux per impulse; but if, as has been suggested, the Cl⁻ efflux is three times too high, then the $K^+$ loss calculated on the basis of purely passive movement agrees with the observed loss. The efflux of $Na^+$ should also increase during an action potential by a factor similar to that for $K^+$, but this is not observed. It is, however, difficult to determine what fraction of the $Na^+$ efflux is passive. If the passive fraction were small enough it might still be unmeasurable when increased by a factor of 200. The conclusion that $K^+$ loss results solely from the effects of membrane depolarization on $K^+$ efflux must be a tentative one. Reasons are given below for supposing that $M_{out}$ increases beyond values given by equation (5).

From the values of $K^+$ influx and efflux during rest and the depolarization, (30 mV), we can calculate that at $-180$ mV the fluxes should be equal to 3·7 pmole/cm² sec. Using the relationship $g_K = F^2(M)/RT$ gives $g_K = 15 \mu$hmho/cm²; this may be compared with a value of 5 $\mu$hmho/cm² from the membrane time constant in pond water. Since the Cl⁻ fluxes are clearly not in a steady state, we should differentiate equation (5) with respect to potential in order to obtain an expression for conductance. The extra labour of using such an equation is not justified by the accuracy of the data and we may write instead that $g_{Cl} = i_{Cl}/(E_m - E_{Cl})$. The Cl⁻ efflux is probably never greater than 1 pmole/cm² sec, even though we measure a value several times this. If we use such an efflux multiplied by $nF$ as $i_{Cl}$, then

$$g_{Cl} = -1(96500)(1 \times 10^{-12})/(-0.383) = 0.25 \mu$hmho/cm².

At the peak of the action potential, where $i_K = -i_{Cl}$,

$$g_{Cl} = -1(96500)(1100 \times 10^{-12})/(-0.195) = 545 \mu$hmho/cm²,

about a 2000-fold change.
The net efflux of Cl\(^-\) necessary to depolarize the membrane from \(-181\) to \(+7\) mV is 20 pmole/cm\(^2\), or about 1/5000th of the observed efflux per impulse. As the shortest time for such a depolarization is about 0.1 sec, and this corresponds to a net efflux of 20 pmole/cm\(^2\).sec, we conclude that the capacitative current is also a negligible fraction of the total ion efflux. If the efflux of K\(^+\) is solely the result of passive movements brought about by changes in membrane potential, then the shape of the action potential describes the time course of \(g_{Cl}\). This is unlikely to be precisely true, but it is a useful first approximation.

In interpreting the experimental data it is necessary to take into account two complications in Chara. The first is the presence of outer and inner cell membranes, and the second is the presence of the cell wall. We have made the assumption that we are dealing with the outer cytoplasmic membrane both because micro-electrode studies are difficult to interpret except on this basis, and because our findings of loss of excitability upon short-time immersion of cells in solutions not containing Cl\(^-\) are hard to understand if the vacuolar membrane were the one involved, since the half-time for Cl\(^-\) loss from the vacuole is about 2 weeks. That the vacuolar membrane offers some resistance to ion flow would seem to be indicated by these experiments. The loss of excitability that is observed is understandable only if the larger reservoir of Cl\(^-\) in the vacuole cannot readily enough mix with the anions of the cytoplasm to prevent it from becoming loaded with, for example, nitrate. A ready access to ions in the sap might appear necessary, as we calculate that 1 cm length of cell, with a 10 \(\mu\) thick layer of cytoplasm, has \(3 \times 10^{-7}\) l. of cytoplasm, and if the [K\(^+]\] is 50 mM then there is \(1.5 \times 10^{-8}\) mole of K, corresponding to \(5 \times 10^{-8}\) mole K/cm\(^2\). This is only about 2-5 times the K\(^+\) efflux during a single impulse, yet the cell will give a second impulse, only slightly smaller than the first, at an interval of time as short as 45 sec, and thirty impulses in as many minutes are readily obtained. As, however, the measured K\(^+\) and Cl\(^-\) effluxes are at least 5000 times greater than those necessary to produce the observed potential change, during repetitive stimulation the cell may economize on the number of ions lost per impulse. A further reason for suspecting that it is the outer membrane that is of importance in these studies is that, for osmotic reasons, the cytoplasm must contain considerable electrolyte, and the extreme values of the measured potential across the membrane (\(>200\) mV) would be very difficult to explain if they were developed between two regions of a cell both of which were rich in electrolyte. A second structure that complicates the analysis of both the bioelectric and ion flux measurements is the cell wall. Operationally this behaves as an unstirred aqueous layer (ca. 10 \(\mu\) thick) and as a barrier of considerable variability to ion flow. Our present experiments have not characterized the wall sufficiently well, hence only tentative conclusions can be offered. Because the wall, when imbibed with 100 mM-KCl,
ION FLUXES DURING ACTION POTENTIAL

has a conductance only about 2.5% that of an equivalent thickness of KCl solution, we suppose that the resistance offered to the diffusion of K+ is similarly affected. An action potential releases of the order of $10^{-8}$ mole of K+ and the diffusion of this away from the membrane surface and into the cell wall may be treated by reference to the following model. As the radius of a cell (500 μ) is very large compared with the thickness of the wall (10 μ), we can consider the membrane and the inner surface of the wall as planes in contact with each other; at zero time $t$, the membrane liberates $Q$ moles/cm² of K+ and this diffuses into the wall where we assume the diffusion constant of K+, $D = 4 \times 10^{-7}$ cm²/sec, or about 2.5% of the rate in free solution. Carslaw & Jaeger (1947) give equation (6), which relates the concentration at any distance $x$ from the plane at $x'$ with time, after the instantaneous release of a given amount of material.

$$C = \frac{Q}{2(Dt)^{\frac{1}{4}}} \exp \left[-\frac{(x-x')^2}{4Dt}\right].$$

(6)

For flow of matter in only one direction, as in the case here, the factor 2 in the denominator is to be struck out. A plot of this function for surface concentration (where the second term in the equation drops out) is shown in Fig. 8. We also assume that the cell continues to pump Cl⁻ inward at a rate of 2 pmole/cm² sec, that this rate is not affected by the external concentration of Cl⁻, and that K+ is likely to accompany the Cl⁻. The subtraction of this influx from the amount of material in an element of wall volume (unit area

Fig. 8. The surface [K⁺] has been computed from the equation in the text and is shown by the dotted line. When the K⁺ influx accompanying Cl⁻ transport is subtracted, the surface [K⁺] is given by the lower solid line. The potential, $E_m = \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_t}$, given by the computed values of [K⁺]₀, is shown by the line marked p.d.

$C = \frac{Q}{2(Dt)^{\frac{1}{4}}} \exp \left[-\frac{(x-x')^2}{4Dt}\right].$
and 0.1 μ thick), gives the solid curve shown in Fig. 8. The concentration of K⁺ in the surface film next to the membrane can then be used to calculate the membrane potential change and such values are also shown in Fig. 8. The calculation is not likely to be very accurate at small values of time, but serves to demonstrate that it is plausible to attribute the greatly delayed repolarization time in the Chara action potential to the persistence of K⁺ at the outer surface of the membrane.

The falling phase of a normal action potential (Fig. 2a) consists of a rapid and a slow phase. The slow phase disappears and the rapid phase has a shorter time constant if the action potential is recorded in a solution where [K⁺]₀ is 5 mM. This suggests that the slow phase is entirely the result of a diffusion delay for K⁺ imposed outside the membrane, while the rapid phase is partly affected. The other traces in Fig. 2 are most easily explained on the basis that suprathreshold stimulation greatly increases the K⁺ efflux during an impulse. To obtain a greater efflux of K⁺, however, it is necessary to discard the suggestion made earlier that the K⁺ efflux results from the effects of Eₘ on K⁺ fluxes, and to suppose that both gₐ and gₜ reach very much higher values than those previously calculated, when the membrane potential is suddenly displaced 100 mV instead of 40 mV. A non-specific increase in ion permeability of the membrane appears to be ruled out by observations that there is no change in Na⁺ fluxes during activity.

The rise of depolarization after a threshold stimulus has a time constant of about 0.1 sec or one half the membrane time constant. A similar observation has been made with Nitella (Weidmann, 1949). This is compatible with the notion that the permeability of the membrane to Cl⁻ has increased. The usual method of testing for such a permeability change is to find that both the influx and the efflux of an ion have increased. Under the conditions obtained in our experiments, the Cl⁻ efflux is 6 pmole/cm². sec and if this is assumed to be all passive, from the steady-state flux ratio M_out:M_in of 3 x 10⁻⁷, the influx is 2 x 10⁻⁶ pmole/cm². sec. Even if the permeability to Cl⁻ were increased by a factor of 10⁵, an increased influx over the influx we ascribe to transport would be difficult to measure. A further complication is the possibility of a 'long pore' effect (Hodgkin & Keynes, 1955). Even at the peak of the action potential, the computed Cl⁻ flux ratio is about 1500, a value too great to make measurable an increase in influx.

The apparent K⁺ influx during activity drops to zero, while the efflux is enormously increased. The change in the influx may be an artifact of the method used for measurement, because unless the cell wall ion exchanger is equilibrated with radioactive solution before any measurements are made, the rapid uptake of K⁺ by ion exchange obscures any uptake into the cell. Such an equilibration takes about 20 min, and at this time the cell has only a small intracellular ⁴²K specific activity. Stimulation therefore results in a large
efflux of inactive K+, and this exchanges with some of the ⁴²K on the wall; the net radioactivity of the cell decreases. The result obtained may also be a consequence of 'long pores'. Measurement of the ⁴²K efflux can be made by allowing the specific activity of cell K+ to arise to large values, but influx measurements are necessarily inaccurate in the presence of large K+ effluxes.

**SUMMARY**

1. Sodium, potassium, and chloride ions are concentrated in the sap of *Chara* so that the equilibrium potentials for these ions are −155, −184, and +202 mV, while the resting potential is −181 mV. The resting membrane resistance of 200,000 Ω cm² agrees with the measured K+ flux in the steady state of about 3 pmole/cm² sec.

2. Threshold stimulation leads to an increased efflux of K+ and Cl− with no change in their influx and no change in the flux of Na+ in either direction. The efflux of K+ and Cl− reach a value of about 10,000 pmole/cm² sec. impulse. The membrane resistance at the peak of the action potential, computed from Cl− efflux, is 2,000 Ω cm².

3. The maintenance of excitability requires no ion in the bathing solution for periods of about 12 hr. With longer immersion times Cl− is required externally in low concentrations if the cell is to be excitable. High concentrations of Cl− externally first depress the height of the action potential and may ultimately abolish excitability.

4. It is supposed that bioelectric activity involves a transient increase in the permeability of the membrane to Cl− and that the depolarization produced by Cl− efflux is followed by an increased K+ efflux which returns the membrane potential to resting levels.

This investigation has been aided by a grant (B-139) from the National Institute for Neurological Diseases and Blindness, Bethesda, Md. Our material was originally identified as *C. contraria* and we are greatly indebted to Professor R. D. Wood of the Department of Botany, University of Rhode Island, for his identification (*C. globularis* Thuill.) and for pointing out that physiological work done on this species is likely to be recorded under the name *C. fragilis* Desv. We also wish to thank Professor J. S. Karling for advising us regarding the cortication of this species.

**REFERENCES**


