

THE ELECTRICAL MEASUREMENT OF CHLORIDE FLUXES IN *NITELLA**

By D. S. MAILMAN†‡§ and L. J. MULLINS†

[Manuscript received November 16, 1965]

Summary

The chloride efflux from cells of *Nitella clavata* at rest and during an action potential was continuously monitored by a Ag/AgCl electrode which measured the concentration of chloride in a thin film of solution flowing over the surface of the cell. Changes in the concentration of chloride subsequent to the passage of an action potential were followed and permitted calculation of the change in the flux of chloride ion across the cell membrane. The cell wall offers appreciable diffusion resistance to chloride ion, the effective diffusion constant being less than 1% of chloride diffusion in free solution. Resting fluxes of chloride were measured in these cells and averaged 3 pmole/cm² sec. The extra efflux of chloride during an action potential averaged 114 pmole/cm² impulse.

I. INTRODUCTION

The bioelectric activity of excitable plant cells of the *Nitella* genus and similar algae has been extensively studied (Blinks 1930; Curtis and Cole 1937). Unlike axons in which an action potential is brought about by an influx of sodium and an efflux of potassium, these algal cells have an efflux of both chloride and potassium during an action potential. Considerations of electrochemical equilibrium and alterations of tracer fluxes during bioelectric activity as compared with those at rest indicate that the resting potential is in large measure a potassium diffusion potential while the action potential is the result of a selective increase in chloride permeability (Gaffey and Mullins 1958; Mullins 1962).

It is widely assumed that for many excitable cells specific changes in the permeability of the membrane to certain ions are intimately related to the genesis of the resting and action potentials. Two methods have been employed to assess permeability changes of excitable membranes. The first involves the electrical measurement of current flow during an extrinsically imposed alteration of the potential across the cell membrane, the voltage-clamp technique. The second method involves the measurement of tracer fluxes across a cell membrane before and during a series of action potentials. An estimation of the flux of ions per impulse is assumed to be the difference of the two fluxes divided by the number of impulses. As informative as these methods are, it would seem desirable to have a direct measure of the instantaneous flux of ions during rest and during the passage of an action potential. While the voltage-clamp technique accurately measures the current which flows

* Aided by a contract [AT(30-1)-2464] from the United States Atomic Energy Commission.

† Department of Biophysics, School of Medicine, University of Maryland, Baltimore.

‡ Present address: Department of Biology-Biophysics, University of Houston, Texas.

§ Supported by a training grant (GM 719) from the National Institute of General Medical Sciences.

during an action potential, the ion which carries the current can be determined only by ion-replacement studies. Conversely, tracer studies effectively identify the specific ion fluxes involved but cannot resolve them with respect to time. An electrode sensitive to the concentration of a specific ion might be expected to resolve both these difficulties if placed on the surface of a cell; it could respond quickly and specifically to the ion provided that it could detect the low concentration of ions involved and be independent of the electrical activity of the cell.

Previous studies (Mullins 1962) have shown that the magnitude of the chloride efflux per impulse is of the order of a few hundred picomoles per square centimetre; if this amount of chloride could be confined within a $10\ \mu$ thick surface layer of a cell, the volume involved per square centimetre of cell surface would be $1\ \mu\text{l}$. Thus the resulting concentration for 100 pmoles released into a volume of $1\ \mu\text{l}$ is $100 \times 10^{-12}/10^{-6} = 100\ \mu\text{M}$. Such a concentration is well within the detection range of an Ag/AgCl electrode initially equilibrated in a chloride-free solution. Experiments were therefore undertaken with a view to making simultaneous measurements comparing the potential of such an Ag/AgCl electrode with that measured by an electrode recording the action potential at the point where such a chloride-sensitive electrode was placed.

II. MATERIALS AND METHODS

(a) Materials

Cells of *Nitella clavata* were employed in these experiments. Strain X033 was used in most of the experiments and proved well suited to the experimental procedure. In a few experiments strain 390 cells were used; although they were larger, they were considerably more fragile and became leaky to chloride when stimulated or if handled extensively. However, strain 390 cells, which were almost flat due to leakage, could still conduct action potentials; these were only slightly distorted as compared to normal.

Both strains have the advantage of large size (approximately $0.7\ \text{mm}$ in diameter and $40\text{--}60\ \text{mm}$ long) and prolonged action potential (approximately $5\ \text{sec}$). They have the disadvantage of a diffusion barrier in the form of a cell wall which is imposed between the cell membrane and the external solution. Going from the outside to the inside, the general morphology of these cells is as follows. There is a cell wall composed of cellulose pectate and approximately $10\ \mu$ thick. The wall acts as a cation-exchange membrane but has little anion-binding properties and chloride can be washed out with distilled water (Gaffey and Mullins 1958). The cell wall abuts the cell membrane; next is a layer of fixed cytoplasm approximately $5\ \mu$ thick which contains the chloroplasts and next to this a layer of streaming cytoplasm also about $5\ \mu$ thick. A tonoplast membrane separates the streaming cytoplasm from a large central vacuole which occupies most of the cell and is composed primarily of an inorganic salt solution of sodium and potassium chlorides.

The cells were grown in a salt solution of the following composition, expressed in $\mu\text{moles/l}$: NaCl 1000; CaCl_2 1000; NaHCO_3 2000; $\text{Mg}(\text{NO}_3)_2$ 100; MgSO_4 900; KH_2PO_4 20; K_2SO_4 100; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ 0.014 ; FeSO_4 3.6 ; MnSO_4 0.91 ; ZnSO_4 0.76 ; CuSO_4 0.047 ; CoCl_2 0.17 ; the micronutrient salts were present as the CDTA

complexes. The cells were exposed to constant artificial light and were kept at a temperature of 22°C. After harvesting the cells were stored in a solution of composition 2 mM NaCl, 1 mM MgSO₄, 1 mM CaCl₂, and 0.05 mM K₂SO₄ (solution A) for at least 2 weeks before the experiments were initiated. Cells could be maintained for several months in this solution. Some cells of the 390 strain were placed in solutions containing 11 mM NaNO₃, 2 mM Ca(NO₃)₂, and varying chloride concentrations (solution B).

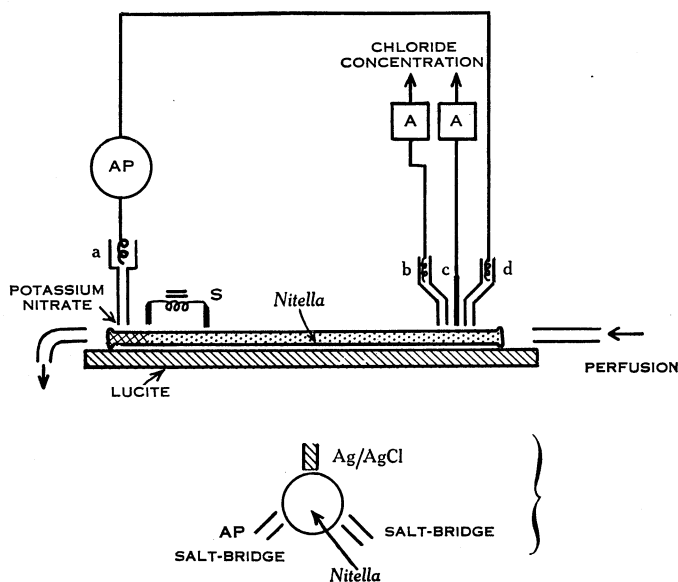


Fig. 1.—Upper: General arrangement for chloride flux measurements. The *Nitella* cell lies on a Lucite plate which is inclined at 65° to permit draining of the perfusion solution. The left end of the cell is depolarized with potassium nitrate, *S* is a stimulus isolation transformer, and an action potential (*AP*) is recorded between electrodes *a* and *d*. Chloride concentrations are recorded by electrodes *b* and *c* at the right end of the cell. The perfusion solution also enters at the right and is drawn off at the left. Lower: Radial distribution of the action potential, recording, and chloride-sensitive electrodes *b*, *c*, and *d*, respectively.

(b) Chloride Flux Measurements

Cells were removed from their storage solution (see above) and placed on a flat Lucite plate as shown in Figure 1. In use, this plate together with the electrode assembly was then tilted to an angle of 65° from horizontal to provide drainage of the solution which continuously flowed over the cell surface. The solution was usually 11 mM NaNO₃ plus 1 mM Ca(NO₃)₂; this was delivered from a motor-driven syringe at a rate of about 10 μ l/min. Cells were fixed in position by bringing the three agar-bridge electrodes shown in Figure 1 firmly in contact with the cell. An Ag/AgCl electrode was then positioned between the agar-bridge electrodes *b* and *d*. Adjustment of the electrode positioning was made with a micromanipulator in order to minimize

the possibility of the electrode picking up a component of the action potential. When properly adjusted, the potential between the Ag/AgCl electrode and one of the adjacent agar-bridge electrodes was stable to 1 mV over tens of minutes, but the electrode gave an easily measurable increment in potential with time when the solution flow over the cell was stopped. Such measurements could be converted into values for the resting efflux of chloride from the cell. When the cell was stimulated, the Ag/AgCl electrode could also be used to follow changes in chloride concentration with time. The solution flow was immediately adjacent to the Ag/AgCl and salt-bridge electrodes and these were positioned at the extreme end of the cell so that the estimation of chloride concentration in the surface film of the cell was obtained from trans-membrane chloride fluxes rather than from possible chloride being washed down the cell from adjacent regions. Any alterations in the observed concentration of chloride must therefore have originated from within the cell.

In order to test the behaviour of the Ag/AgCl electrode under conditions where there was appreciable current flow in its surroundings, a cotton wick was substituted for the *Nitella* cell in the experimental chamber and external potentials of 1.3 V were applied between stimulating electrode and upper ground reference. Such a voltage application gave a measured potential difference of 225 mV between the ends of the wick, which is a potential larger than any physiological potential likely to result from bioelectric activity in the system. The potential difference between the chloride electrode and its reference salt-bridge electrode under these conditions was only 0.5 mV.

Stimulation of the cell was effected by square wave pulses from a pulse generator and a stimulus isolation unit; the cathode was approximately 2 mm from the depolarized region of the cell and an action potential was initiated there. The action potential was then conducted upwards to the very end of the cell where both the recording and Ag/AgCl electrodes were located. The action potential was thus propagated in a direction opposite to the flow of perfusion solution and conduction velocities were of the order of 2 cm/sec.

(c) *Electrode Preparation and Connection*

The Ag/AgCl electrode was prepared electrolytically: a 20-mm length of silver wire 0.9 mm in diameter immersed in 0.1N HCl was subjected to the current from a 1.3 V battery for 30 sec. This electrode had the purple colour characteristic of the more stable types of these electrodes (Janz and Taniguchi 1953) and proved to be stable, reproducible, and sensitive to the low concentrations of chloride found on the surface of the *Nitella*. Over a 12-hr period the potential of an Ag/AgCl electrode was found to have changed by only 4 mV as compared to its potential at the beginning of the period.

The three agar electrodes were prepared from a 3% agar solution containing 11 mM NaNO₃ and 1 or 2 mM Ca(NO₃)₂; this solution had the same basic composition as the solution used to perfuse the cells. The agar electrodes were each connected to a series of salt-bridges interposed between the cell and the oscilloscope to prevent chloride from diffusing to the region of the Ag/AgCl electrode. The final beaker contained saturated potassium chloride, and a calomel or Ag/AgCl reference electrode

completed the circuit to the oscilloscope. The resistance of each of the salt-bridges was 650 k Ω . The observed action potentials were corrected for the voltage drop occurring in the salt-bridges.

The salt-bridge at the lower end of the cell and one of the ones at the upper end were displayed on the oscilloscope differentially and employed to measure the injury potential of the cell. The lower end of the cell was maintained in a more or less depolarized state by the application of 100 mM KNO₃ supplied via a wick connected

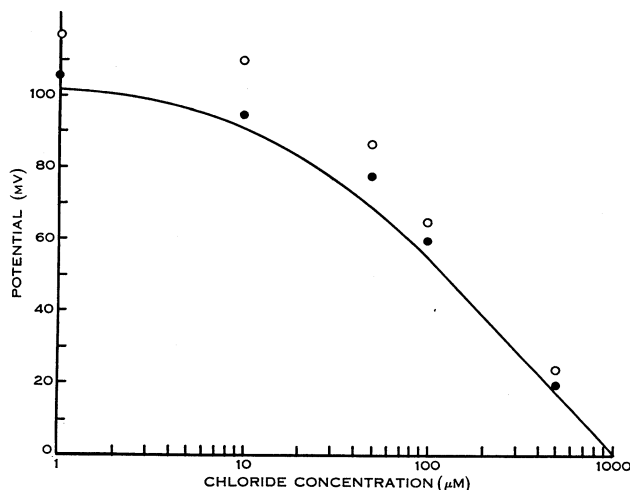


Fig. 2.—Potential of an Ag/AgCl electrode (compared with a potassium nitrate salt-bridge reference) is plotted as a function of chloride concentration. The solid line is the function

$$E = 58 \log\{1000/[Cl] + 17\},$$

where the chloride concentration is micromolar. The value 17 μ M is derived from the solubility product of silver chloride and represents the chloride concentration around the electrode when its bathing solution is nominally chloride-free. ● Measured potentials for an electrode with a solution flow rate of 5 μ l/min. ○ Measured potentials for an electrode with a solution flow rate of 10 μ l/min.

to a reservoir (so as to maintain a constant supply of KNO₃). The end of the wick was placed just above the lower salt-bridge. Depolarization was effected so that the recorded response would be monophasic. The other salt-bridge at the upper end of the cell was used as a reference for the Ag/AgCl electrode in the measurement of the chloride concentration. In order to measure the relatively small potential due solely to the chloride concentration, both the large electrode potentials of the Ag/AgCl electrode and the action potential had to be nullified. The electrode potential was balanced by means of a potential source placed in series between the electrode and the oscilloscope. Immediately prior to each stimulus the voltage was again brought to zero and the potentiometer setting noted.

Two methods were used to connect the Ag/AgCl electrode and its reference salt-bridge electrode to the oscilloscope. In the first a variable resistance was used to

equalize the resistance in both leads to the input of the differential amplifier of the oscilloscope and in the second a high impedance amplifier was introduced into both input circuits to minimize the effect of differences in the resistance of the electrodes. In both systems, a potential source was used to zero the voltage. Both these systems yielded essentially similar results.

(d) *Electrode Calibration*

The potential of the Ag/AgCl electrode was determined by placing a wick of the approximate size of the *Nitella* in the chamber and perfusing it with a solution of known chloride concentration while the Ag/AgCl electrode was positioned against the wick. A curve for chloride concentration *v.* potential was thereby obtained and is shown in Figure 2. It had previously been determined that at a flow rate of either 5.1 or 10.3 $\mu\text{l}/\text{min}$ the potentials were relatively fixed for a change in flow rate at a constant concentration or for a change in concentration at a given flow rate. At a flow rate of 2.1 $\mu\text{l}/\text{min}$ the curve of concentration against potential was significantly different, especially at the lower concentrations. A flow rate of 10.3 $\mu\text{l}/\text{min}$ was chosen since it was low enough to give a relatively high concentration for a given amount of chloride diffusing from the cell and yet high enough to give good electrode response. The stability of the electrode system was determined during the course of the experiment by perfusing the cell with a solution of high chloride concentration (500–1000 μM) and determining the potential. The total amount of chloride released during an action potential was determined by plotting concentration against time from the first noticeable increase in chloride to the point at which the concentration again became stable. The area under the curve was cut out and weighed. If one assumes that the same concentration of chloride was present at all points on the surface of the cell and that the rate of perfusion was constant over the cell surface, it follows that the product of the flow rate and the total concentration as determined from the graph would give the net amount of chloride released by a unit area of the cell either during an action potential or at rest over the total time period of the experiment. Since the average area of the cells was 1 cm^2 , the total amount of chloride released would be the average flux per impulse per 1 cm^2 or per cell.

(e) *Extracellular Space*

In order to determine the volume of the film of solution on the surface of the cell, *Nitella* cells were repeatedly dipped in perfusion solution containing dye, drained against a glass surface, and redipped into a known volume of perfusion solution without dye. The latter solution was read in a spectrophotometer at 550 $\text{m}\mu$ against known dilutions of the dye. The cells were then gently flattened and their length and width determined under a dissecting microscope by means of calipers accurate to 0.1 mm. The volume of adsorbed solution was then calculated from the total volume of adsorbed dye and the surface area of the cell (Table 1).

During the course of the experiment on several cells the flow of the perfusion solution was halted and the increase of the chloride concentration in the surface film followed. The rate of the increase in chloride concentration was fast enough that there

would be little likelihood of electrode drift occurring in that interval and the effects of flow would also be eliminated. The initial rate of increase, as well as the average concentration, was determined and the average resting flux during the interval was calculated. The effects of evaporation and electrode drying were accounted for by employing a similar stoppage of the perfusion on the wick and following the potential change. The potential changes due to drying at the same time intervals were subtracted from the potential changes observed and this derived potential difference was employed in calculating the actual change in concentration.

TABLE 1
LENGTH AND CIRCUMFERENCE OF CELLS AND CALCULATED
VOLUME OF ADSORBED SOLUTION

Length of Cell (cm)	Circumference of Cell (mm)	Adsorbed Volume ($\mu\text{l}/\text{cm}^2$)
6.0	2.4	1.3
4.6	2.4	1.0
4.0	1.8	0.8
5.6	2.0	0.9
4.0	2.2	1.1
4.1	2.2	1.2
		0.8
4.7 \pm 0.9†	2.2 \pm 0.3†	1.0 \pm 0.2†

* Indicates volumes determined from two sets of "dips".

† Mean \pm S.D.

(f) *Diffusion through Cell Wall*

In order to determine the diffusion properties of the cell walls, an internodal cell was cut at both ends. Under a binocular microscope cannulae were inserted in both ends and were tied with threads. The cell and cannulae were then returned to the chamber and the cell was fixed in its former position. All electrodes were returned to their former position. The perfusion solution was forced through the inside of the cell against 150 cm of water pressure with a manually operated syringe in order to simulate, at least qualitatively, the hydrostatic pressure normally forcing the cell wall outwards. This treatment effectively destroyed the cell membrane, leaving only the cell wall. The internal perfusion tubing was then attached to another syringe containing a solution of known chloride concentration in such a manner that a small air bubble was introduced between the two solutions. The new solution was forced rapidly through the cell and the potential change of the Ag/AgCl electrode on the surface was followed. Since chloride ions must diffuse through the wall before they can be detected by the chloride electrode, the method measures the diffusion delay involved. The proportion of the released chloride which can cross the cell wall in a given time can be calculated from the diffusion constant of the wall, but a direct measure would be desirable since the perfusion could wash away chloride while it was still diffusing

through the wall and before it reached the chloride electrode. The diffusion delay was directly determined by using the internally perfused cell and either visually determining the point at which the solution containing the chloride passed under the electrode and marking it by changing the zero setting of the second beam of the oscilloscope, or by noting the point at which the noise level of the chloride potential decreased. The point at which the chloride passed under the electrode was found to precede the first visible increase in chloride concentration by 0.5–1.0 sec. The solutions used to perfuse the inside of the cell membrane were coloured with a small amount of dye in order to determine if the wall had developed leaks during the treatment. No leaks were observed and indeed the dye did not appear to penetrate the cell wall or to be taken up by it.

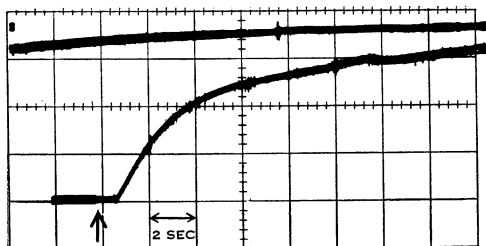


Fig. 3.—Oscilloscope tracing of the response of the Ag/AgCl electrode to a change in chloride concentration flowing through a perfused cell wall. At the point shown by the arrow, internal chloride concentration was changed from zero to 1 mM. Two sweeps are shown although the equilibration process is largely over in the first sweep.

Because the experimental results showed that the diffusion constant for Cl^- in the cell wall was very much less than 1% of the diffusion constant for Cl^- in aqueous solution, no attempt was made to correct for diffusion delays in the water film covering the cell surface (and the Ag/AgCl electrode). The wall was thus treated as the only diffusion barrier for purposes of kinetic analysis. This assumption appears justified because diffusion in the water film was faster than the response time of the Ag/AgCl electrodes. The entire analysis depends on the further assumption that $[\text{Cl}]_s$ is the same at all points on the cell surface. The water space of the cell wall was taken as 100% of its volume, an assumption that is estimated to make the fluxes about 5% in error.

(g) Membrane Potential Measurements

Since the absolute value of the transmembrane potential could not be determined with the apparatus described above, several cells were placed in a microelectrode chamber and their resting and action potentials were measured in this manner. This method was not equivalent to the one employed in the actual experiment since here the cells were completely immersed in the basic perfusion solution (solution B, chloride-free). The resting potential of the cells after sealing of the microelectrode was -110 mV which declined to -100 mV within 1 hr. The action potential was approximately 80 mV.

(h) Analytical

The internal vacuolar concentration of sodium, potassium, and chloride during the period of storage as well as after a few experiments was determined by puncturing the cell with a needle and extruding the vacuolar sap onto a glass coverslip. A 5- μl precalibrated capillary pipette was used to sample the sap and the sample was

diluted with 3 ml of doubly de-ionized water. Chloride was analysed by Cotlove chloridometer, sodium and potassium by flame-photometry.

III. RESULTS

The technique described in Section II(f) was employed to evaluate the diffusion resistance offered by the cell wall. The response of the Ag/AgCl electrode to a step change in chloride concentration made inside the wall of a cell subjected to internal perfusion is shown in Figure 3, where the arrow indicates the time at which the chloride concentration front reached the Ag/AgCl electrode. It is clear that the response of the electrode to the step change made is slow (in contrast with a time constant of a few hundred milliseconds for the equilibration of the Ag/AgCl electrode with an aqueous solution containing chloride) and suggests that the diffusion constant for chloride in the wall is low.

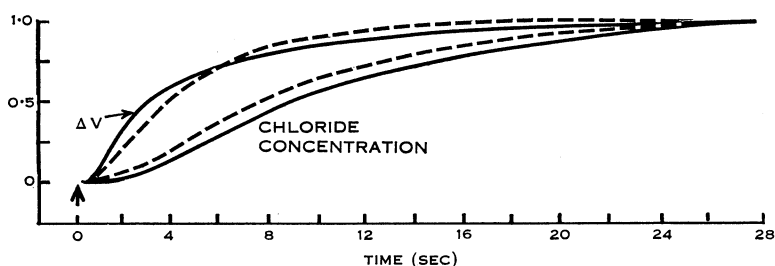


Fig. 4.—Voltage-time tracing of Figure 3 is redrawn and labelled ΔV on this graph. The chloride concentration calculated from the voltage changes is also shown, while the dashed lines show solutions of the diffusion equation given in the text (see p. 393) for $D = 10^{-7}$ (upper) and $D = 5 \times 10^{-8}$ (lower). The ordinate is the fraction of the infinite time value a particular variable shows, while the abscissa is time in seconds.

For a step change in Cl^- concentration inside the cell wall from $C_i = 0$ to $C_i = 500 \mu\text{M}$, the expected concentration at the outside of the cell wall as a function of time, C_t , is given by Carslaw and Jaeger (1959) as

$$\frac{C_t}{C_m} = 2 \sum_{n=0}^{n=\infty} (-1)^n \frac{\{\text{erfc}(2n+1)h\}}{2(Dt)^{\frac{1}{2}}},$$

where C_m is the infinite time value of membrane concentration, h is the wall thickness, D is the diffusion constant for Cl^- in the wall, and t is time. For the first 5 sec or so, the equation gives satisfactory accuracy with $n = 0, 1$, while for longer times, it is necessary to take terms for $n = 0, 1, 2, 3$.

In order to evaluate the apparent diffusion constant of the cell wall for Cl^- , the curve shown in Figure 3 was transformed into a graph of chloride concentration as a function of time; trial values of D , the diffusion constant for Cl^- in the wall, were then selected and solutions of the diffusion equation were obtained. The results are shown in Figure 4 where a value of $5 \times 10^{-8} \text{ cm}^2/\text{sec}$ comes close to fitting the

experimental data. While it is clear that the experimental measurements do not fit the calculated curve especially well during the first few seconds, it is just in this region of the curve that there is the greatest uncertainty with respect to chloride concentration. The measurements taken after about 5 sec are the most reliable.

(a) *Resting Chloride Flux*

Measurements of chloride flux under resting conditions in intact cells could be made in two ways. In the first, the external perfusion flow could be interrupted and the rate of change of chloride concentration with respect to time could be related to chloride efflux. In the second method, the steady-state chloride concentration at a fixed flow rate could be related to the chloride efflux necessary to sustain the observed concentration. This latter method was especially suitable for obtaining chloride fluxes immediately following an action potential because these values were high.

TABLE 2
CHLORIDE FLUXES FROM *NITELLA*

Expt. No.	Resting Fluxes (pmole/cm ² sec);			Extra Efflux from an Action Potential (pmole/cm ² impulse)
	During Stopped Perfusion	Determined from Surface Concentration	2 Min after Stimulation	
1	2.7	4.3	8.8	116
2	3.2	0.6	4.5	189
3	2.8	1.7	6.6	106
4	3.9	0.7	1.7	13
5	2.6	3.4	6.8	58
6	2.5	3.8	14.1	362
7		9.7	18.2	222
8		1.7	11.8	44
9				11
10				30
11				168
12				54
Mean \pm S.D.:	3.0 \pm 0.5	3.2 \pm 3.0	9.1 \pm 5.4	114 \pm 100

Table 2 gives values for the initial, stable resting efflux from a number of cells both prior to and after they had been stimulated, as well as values for the excess chloride flux during an action potential. The mean value of resting efflux is 3.2 pmole/cm² sec. The excitation of a cell apparently had an effect on the transient efflux during an action potential in addition to a prolonged effect on the resting efflux.

(b) *Chloride Efflux during the Action Potential*

The results obtained with the cell wall diffusion delay shown in Figure 4 make it clear that the chloride release occurring during an action potential will not be recorded synchronously with an action potential which lasts, as in the cells studied,

something of the order of 4 sec because at this time an instantaneous release of chloride at $t = 0$ would result in not more than 20% of the chloride being present at the surface of the wall. A typical record of chloride release during an action potential is shown in the two traces of Figure 5(a), where the upper curve is the record of the Ag/AgCl electrode and the lower is the action potential. The upper trace reaches a maximum about 15 sec after the action potential and declines quite slowly with time. Since the

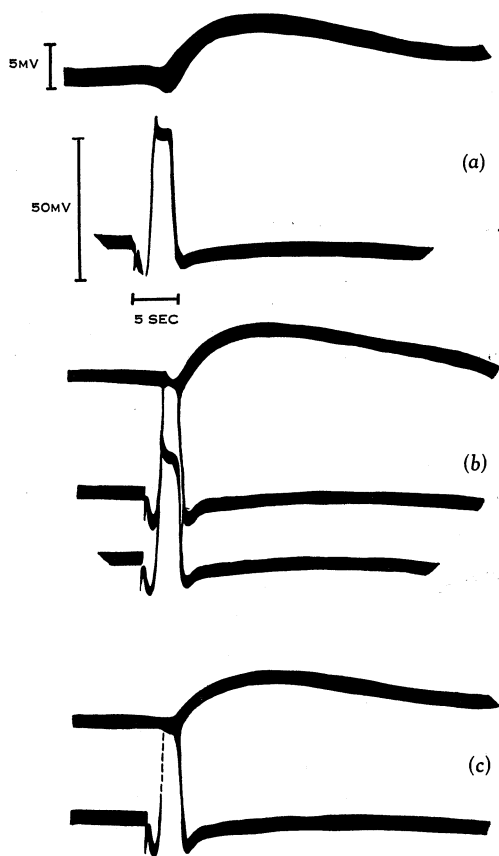


Fig. 5.—Each of the three double-beam cathode ray traces (a), (b), and (c) shows, at the top, the record of the Ag/AgCl electrode, and below, the recorded action potential. In (a) the action potential is followed by a prolonged displacement of the chloride trace. In (b) two action potentials were initiated with a 2-min recovery period between them. The base line for the action potential trace was displaced between the two stimuli but the record shows that the two traces may be exactly superimposed. The trace in (c) was taken at the end of the experiment.

stability of the Ag/AgCl electrode and the reproducibility of its response are critical in any attempt to analyse the response to an action potential, the record shown in Figure 5(b) was made. Here two action potentials were initiated with a 2-min recovery period between them. Between the two stimuli, the base line of the action potential recording beam was displaced. Both action potentials gave a chloride response and the two traces superpose exactly. After another 2-min period the record shown in Figure 5(c) was obtained. From these and many other records, it is clear that there is an easily measurable response of an Ag/AgCl electrode to an action potential and such records have a remarkably similar time course which is quite distinct from the electrical disturbance which gave rise to the record. When such records are analysed

quantitatively as described in Section II, they yield an extra efflux with activity that is in close agreement with values obtained from isotopic measurements.

A further requirement for an electrode that is to respond solely to chloride concentration is that if the concentration of chloride in the external bathing solution is increased, the electrode should no longer show a potential deflection when an action potential is initiated in the cell. Figure 6 shows the results of an experiment in which a cell was excited and the tracing of potential *v.* time for the Ag/AgCl electrode obtained.

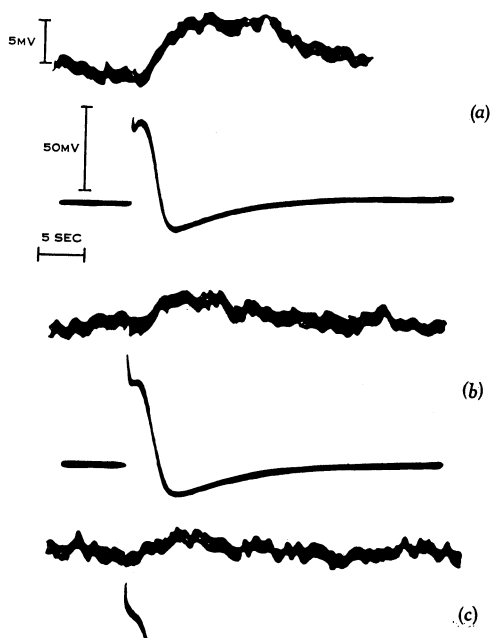


Fig. 6.—(a) Records of action potential and Ag/AgCl electrode response taken at zero chloride concentration and show the usual pattern following an action potential. (b) Records taken in a solution containing $100\ \mu\text{M}$ chloride (substituted for nitrate in the perfusion solution)—the potential due to chloride is very much diminished. (c) Record taken in a solution with a chloride concentration of $1000\ \mu\text{M}$ (only the peak of the action potential trace is visible).

The solution flowing over the cell was then changed to one containing $100\ \mu\text{moles/l}$ Cl^- and a second photograph was obtained. Then the solution was changed to one containing $1000\ \mu\text{moles/l}$ and a further measurement was obtained. It is clear that an increase in chloride concentration diminished the response of the Ag/AgCl electrode and a large increase in chloride concentration abolished the response. This is understandable because of the relationship between increment in chloride concentration and potential; an increment in chloride concentration from 17 to $100\ \mu\text{M}$ gives a large potential change while an increment from 1000 to $1100\ \mu\text{M}$ gives only a negligible potential change.

In a series of measurements of the response of the Ag/AgCl electrode to action potentials initiated in cells, the average release of chloride during an action potential was found to be $114\ \text{pmole/cm}^2$ impulse (cf. Table 2). These measurements were made on cells that had been in the experimental chamber only a few minutes as well as on cells that had spent many hours in the chamber. As might have been expected, the cells showed quite variable concentrations of chloride at their surface, reflecting large differences in the resting efflux of chloride from the cell. Nonetheless, when such

measurements of chloride concentration at the surface are plotted against the excess chloride released during an action potential, there is no obvious relationship between chloride leak and the extra efflux of activity as shown in Figure 7.

IV. DISCUSSION

An attractive possibility for analysing the records of chloride release during an action potential is that one might be able to calculate the time course of chloride release at the cell membrane from the measured changes in chloride concentration at the surface of the wall. The very low diffusion constant for chloride (and presumably for other anions), however, makes the calculations only approximate, because it is likely that slight inhomogeneities in the wall could make large differences in the behaviour of the chloride fluxes. It does seem important to note that if D_{Cl} in the wall is 5×10^{-8} cm²/sec and the wall thickness d is 10^{-3} cm then P_{Cl} is 5×10^{-5} cm/sec or a value of the same order of magnitude (Findlay and Hope 1964a) as that reached by

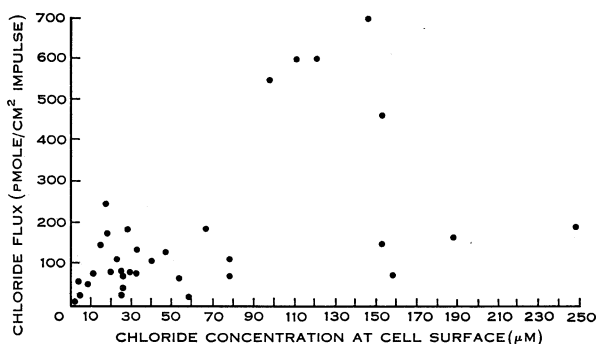


Fig. 7.—Extra efflux of chloride associated with an action potential is plotted as ordinate against the measured cell surface concentration of chloride at the time an action potential was initiated. This latter is a measure of resting chloride efflux from the cell. The points show that there is no correlation between resting chloride efflux and extra efflux from the cell during activity.

the excitable membrane at the peak of its activity. This, in turn, suggests that during an action potential, chloride concentration just outside the excitable membrane may rise to levels equal to those in the cytoplasm or that E_{Cl} may vary greatly during the course of bioelectric activity. Quantitative treatment of this point is impossible without values for chloride concentration in the cytoplasm; estimates for this range from 1–100 mM (for a vacuolar chloride concentration of 100 mM). Obviously, the lower the chloride concentration in the cytoplasm, the more E_{Cl} will shift during an action potential, given the same efflux of chloride. As there is substantial agreement (Gaffey and Mullins 1958; Hope and Findlay 1964) that the efflux of Cl^- is responsible for the depolarizing phase of the action potential, suggestions that chloride concentration in the cytoplasm is as low as 1 mM can be refuted by the observation that action potentials going from -100 to -10 mV can be obtained from *Nitella* in 10 mM NaCl and 1 mM $CaCl_2$. A 1 mM chloride concentration in the cytoplasm would not allow a

peak potential greater than -58 mV and even this would require perfect selectivity of the membrane for chloride.

Such a small fraction of the chloride efflux (less than 1%) is used to discharge the membrane capacity during an action potential that to a good approximation the time course for the release of K^+ and Cl^- must be identical. During the entire 5 sec action potential one can expect largely a paired release of potassium and chloride, with an excess chloride release at the start of the action potential and an excess potassium release at its end. These excess releases are each less than 1 pmole as compared with 100–300 pmole released during the action potential. The maximum rate of rise of an action potential is about 10 V/sec, which, for a $1 \mu F/cm^2$ membrane capacity corresponds to a peak inward current of $10 \mu A/cm^2$. This is about the peak current observed for voltage-clamp depolarizations to potentials corresponding to about half the peak voltage of the action potential.

The release of equivalent amounts of potassium and chloride during an action potential leads to the loading of the cell wall with this material; K^+ can move readily through the wall ion-exchange system while Cl^- cannot. Thus, a diffusion potential can be expected with the inside of the wall negative with respect to the outside. This potential has the same sign as the intracellular potential and will sum with the action potential. Much of the late negativity found in some *Nitella* and *Chara* action potentials can be ascribed to this potential arising from the slow diffusion of potassium chloride from the wall. The situation is not as simple as this, however, because the recent findings of Findlay and Hope (1964) show that in *Chara* it is possible to observe electrical activity in both the cytoplasmic and vacuolar cell membranes.

V. REFERENCES

- BLINKS, L. R. (1930).—The direct current resistance of *Nitella*. *J. Gen. Physiol.* **13**: 495.
- CARSLAW, H. S., and JAEGER, J. C. (1959).—"Conduction of Heat in Solids." p. 308. (Oxford Univ. Press: London.)
- CURTIS, H. J., and COLE, K. S. (1937).—Transverse electric impedance of *Nitella*. *J. Gen. Physiol.* **21**: 189.
- FINDLAY, G. P., and HOPE, A. B. (1964a).—Ionic relations of cells of *Chara australis*. *Aust. J. Biol. Sci.* **17**: 400.
- FINDLAY, G. P., and HOPE, A. B. (1964b).—Ionic relations of cells of *Chara australis*. *Aust. J. Biol. Sci.* **17**: 62.
- GAFFEY, C. T., and MULLINS, L. J. (1958).—Ion fluxes during the action potential in *Chara*. *J. Physiol.* **144**: 505.
- HOPE, A. B., and FINDLAY, G. P. (1964).—The action potential in *Chara*. *Plant & Cell Physiol.* **5**: 377.
- JANZ, J. G., and TANIGUCHI, H. (1953).—Silver-silver halide electrodes. *Chem. Rev.* **53**: 397.
- MULLINS, L. J. (1962).—Efflux of chloride ions during the action potential of *Nitella*. *Nature, Lond.* **196**: 986.