CHLORIDE IN CELLS OF CHARA AUSTRALIS

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Summary

A potentiometric method of measuring the vacuolar and cytoplasmic chloride activity in cells of Chara australis is described. It was found that the activity of chloride ions in the vacuole was approximately 100 mN (average for 13 cells), and in the cytoplasm chloride activity was found to be approximately 10 mN (average for 13 cells).

The electrochemical equilibrium potentials, $E_{Cl}$, for chloride across the plasmalemma and tonoplast are far removed from the resting potentials across these membranes. During an action potential the membrane potential shifts towards $E_{Cl}$, as would be expected if the action potential were produced by a transient increase in the permeability of the membrane to chloride ions.

I. Introduction

In cells of Chara australis a fast and large action potential occurs across the plasmalemma, and a small and slow action potential occurs across the tonoplast (Findlay and Hope 1964a). Since it appears that chloride plays a major role in the production of the action potential, it is important to know the activity of chloride ions in the cytoplasm. From this the electrochemical potential for chloride ions across both membranes can be calculated. If in fact chloride ions were in electrochemical equilibrium then one would not expect a transient change in permeability to chloride ions to cause the action potential.

The concentration of $K^+$, $Na^+$, $Cl^-$, and $Ca^{2+}$ ions in the vacuole of cells of C. australis can be fairly easily estimated by analysis of extracted cell sap. The $K^+$, $Na^+$, and $Ca^{2+}$ are readily determined with a flame-photometer while $Cl^-$ can be determined by potentiometric titration. This method does not necessarily give the activity of chloride ions in the vacuole.

Until now no direct measurements of chloride ion concentration or activity in vivo either in the vacuole or in the cytoplasm have been made. Extraction of the cytoplasm is difficult because of its small volume and because of its distribution as a thin layer between the cell wall and the vacuole; this makes direct chemical analysis very difficult. However, MacRobbie (1962, 1964) obtained values of $\sim 130$ mN for $K^+$ and $\sim 40$ mN for $Na^+$ in the cytoplasm of Nitella translucens. Similar results were obtained by Spanswick and Williams (1964), except for a much lower value for $Na^+$ (14 mN). The chloride concentration in the flowing cytoplasm, obtained by these authors, was $\sim 65$ mN.

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Findlay and Hope (1964b), in voltage-clamp experiments with fast voltage-current scans, were able to deduce a value for chloride concentration in the cytoplasm as follows. Curves of current against potential for the plasmalemma at the start of a voltage-clamp run, and also at the peak of the action potential (i.e. when the current flowing across the plasmalemma was at its peak) were plotted. The difference between these two currents was also plotted on the same graph. If the action potential were due to a transient increase in the permeability of the plasmalemma to chloride ions, the point (i.e. potential) at which this difference is zero would give the potential at which chloride is in electrochemical equilibrium across the plasmalemma. From this potential the chloride concentration in the cytoplasm, [Cl]cyt, was obtained from the external chloride concentration, [Cl]ext, and the appropriate Nernst equation, viz:

$$E_{Cl} = -\frac{RT}{F} \ln \left(\frac{[Cl]_{ext}}{[Cl]_{cyt}}\right).$$

The values obtained were between 1 and 10 mV Cl\(^{-}\), but it should be noted that it had to be assumed that all the additional transient current was carried by chloride ions and that the potassium and sodium currents could be calculated from the resting permeabilities.

With the aim of measuring directly the chloride activity in the vacuole and cytoplasm, a technique has now been developed to produce Ag/AgCl microelectrodes that, together with a KNO\(_3\)-filled glass microelectrode, can be inserted into a cell of *C. australis*.

II. Theory

In principle there is no difficulty in producing chloride-sensitive electrodes. These consist of a silver wire or strip, covered with a film of AgCl. The potential of an Ag/AgCl electrode in contact with a solution containing chloride ions is given by:

$$E = E^0 - \frac{RT}{F} \ln a_{Cl},$$

where \(a_{Cl}\) is the activity of the chloride ions in solution. In practice \(E^0\), the standard electrode potential of the system, also contains the reference potential. It follows that if the electrode is immersed alternately in two solutions containing chloride ions of activity \(a_1\) and \(a_2\) (or if identical electrodes are simultaneously immersed in the separate solutions), the difference in potential measured is

$$E_1 - E_2 = \frac{RT}{F} \ln \left(\frac{a_2}{a_1}\right).$$

The activities of the chloride ions may differ considerably from their normalities, owing to the influence of other ions present (the salt effect), but this is important only if the chloride estimates so obtained are compared with values obtained, for instance, by chemical analysis of vacuole contents.

To investigate the possible effect of other ions capable of forming sparingly soluble silver salts (e.g. thiocyanate or orthophosphate), it is necessary to examine in more detail the potential developed at the silver wire. In the absence of interfering ions, the potential is that of a silver wire in equilibrium with Ag\(^+\) ions in solution. These arise from the dissociation of the AgCl coating on the wire. For the reason already stated, unit activity coefficients will be assumed in the following exposition, and the activity of Ag\(^+\), for instance, will be equated with the concentration, [Ag\(^+\)].
For any anion, X⁻, whose silver salt is only sparingly soluble, an upper limit to [X⁻] is imposed by the corresponding solubility product \( K_{AgX} \), the constant product of [Ag⁺] and [X⁻]. Also, since for the Ag/AgCl wire:

\[
AgCl \rightleftharpoons Ag^+ + Cl^-
\]

with a solubility product \( K_{AgCl} \), it follows that

\[
E = E^0_{Ag} + \frac{RT}{F} \ln [Ag^+],
\]  

(3)

and by substitution

\[
E = E^0 + \frac{RT}{F} \ln \left\{ \frac{K_{AgCl}}{[Cl^-]} \right\}.
\]  

(4)

Clearly, if its concentration were sufficiently high, any other anion with a low solubility product could limit the Ag⁺ concentration to a value below that appropriate to the chloride system alone. In such an event the electrode would not be an efficient sensor for chloride ions, and the potential would be largely governed by [X⁻] through an equation analogous to equation (4). However, for such anions it is possible from their solubility products, \( K_s \), to set limiting concentrations below which, for given values of [Cl⁻], these anions do not significantly influence [Ag⁺] and hence the chloride response of the Ag/AgCl electrode.

**Table 1**

<table>
<thead>
<tr>
<th>Anion Type</th>
<th>( X^- ) Ion Concentration (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>([Cl^-] = 10 \text{ min})</td>
</tr>
<tr>
<td>CNS⁻</td>
<td>(1 \times 10^{-4})</td>
</tr>
<tr>
<td>PO₄⁻</td>
<td>(1 \times 10^4)</td>
</tr>
<tr>
<td>CO₃⁻</td>
<td>(5 \times 10^4)</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>(1 \times 10^{11})</td>
</tr>
<tr>
<td>C₂O₄⁻</td>
<td>(1 \times 10^7)</td>
</tr>
<tr>
<td>O²⁻</td>
<td>(1 \times 10^8)</td>
</tr>
</tbody>
</table>

Table 1 shows calculated values of [X⁻] above which [Ag⁺] is no longer determined by [Cl⁻] alone, these data being derived for chloride concentrations of 10⁷ and 10⁸ min, which are respectively the apparent concentrations in the cytoplasm and vacuole.

For compounds of the type \( Ag_2X \) and \( Ag_3X \) expressions for [Ag⁺] derived from the corresponding solubility products \( K_{Ag_2X} \) and \( K_{Ag_3X} \), respectively, are:

\[
[Ag^+] = \frac{K_{Ag_2X}}{[X^-]}^{\frac{1}{4}},
\]

and

\[
[Ag^+] = \frac{K_{Ag_3X}}{[X^-]}^{\frac{1}{4}}.
\]
Of the ions listed in Table I, CNS\(^{-}\), SO\(_4\)\(^{2-}\), C\(_6\)O\(_3\)\(^{-}\), and PO\(_4\)\(^{3-}\) could occur to some extent in the vacuolar sap or the cytoplasm. However, it is immediately obvious that only CNS\(^{-}\) ions will interfere in concentrations which conceivably may be present in either the vacuole or the cytoplasm.

A spectrophotometric analysis was made for CNS\(^{-}\) in extracted cell sap (cytoplasm plus vacuole), using as standards reference solutions of NaCNS treated with FeCl\(_3\). Cell sap extracts were centrifuged to remove solid matter, and aliquots then treated with FeCl\(_3\). To obtain a sufficient volume about 100 cells were used. In setting an upper limit for the concentration of CNS\(^{-}\) in the cytoplasm it was assumed that all of the measured CNS\(^{-}\) in the total cell sap was originally concentrated in the cytoplasm.

Although colorimetric analysis at these very low concentrations is highly inaccurate, it was possible to set an upper limit of 10\(^{-5}\)–10\(^{-6}\) for CNS\(^{-}\) present in the cytoplasm. At this concentration range, interference with the chloride estimations made with the aid of Ag/AgCl electrodes is unlikely, as the upper limit of CNS\(^{-}\) concentration at which the electrode ceases to respond quantitatively to chloride ions is 10\(^{-4}\)N.

It should be noted that the presence of bromide ions in the cytoplasm and vacuole in concentrations of 2\times10\(^{-5}\)N and 2\times10\(^{-4}\)N, respectively, would interfere with the chloride estimations made with Ag/AgCl electrodes. For iodide ions the concentrations for interference are approximately 10\(^{-6}\) and 10\(^{-5}\)N, respectively.

III. METHODS

The unusually large dimensions of the cells of *Chara australis* are of great advantage in experiments of this nature, though even with these cells considerable difficulty was encountered in developing Ag/AgCl electrodes of sufficiently small dimensions. Streaming of the cytoplasm is very easily seen in the cells of this species.

The cells used were usually whorl cells, 1–1.5 mm in diameter and 10–15 mm long, and reasonably transparent ones were selected for the experiments, so as to facilitate observation of cytoplasmic streaming and hence to observe whether a probe was covered with cytoplasm.

The Ag/AgCl electrodes were made from silver-cored wire (G.D. Microwire made by Applied Research Inc., England). Silver-in-glass wire with a silver core of 5 \(\mu\) and an outside diameter of 30\(\mu\) was available. About 5 cm of this wire was glued into a drawn-out glass rod by means of Perspex dissolved in chloroform (Fig. 1). The microwire was polished at the tip to about 10 \(\mu\) with hydrofluoric acid and about 10 \(\mu\)
of the silver wire was left exposed. Electrical contact was made with mercury and the electrode was inserted through an ordinary glass microelectrode, filled with 3N KNO₃, which served as the reference electrode. Both the Ag/AgCl and the reference electrode were thus in the same region and consequently were simultaneously covered by the cytoplasm as it crept up over the electrodes. The KNO₃-filled probe was also used to measure the membrane potential difference by using another microelectrode, filled with KNO₃-in-agar, just outside the cell.

A thin coating of AgCl was deposited on the silver electrode by passing a direct current through a dilute KCl solution in which the silver electrode served as the anode. This operation was done under the microscope so that the growth of the AgCl layer could be observed.

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Fig. 2.—Diagram of the measuring circuits. The membrane potential is measured with the KNO₃-filled internal microelectrode (I.P.) against the KNO₃-filled external reference electrode (R.P.). The KNO₃-filled internal microelectrode also acts as reference for the Ag/AgCl electrode (S.P.). Electrical connection with the KNO₃-filled probes is made via KCl-agar bridges connected to calomel half-cells (C). E.S., external solution (1·0 mN NaCl, 0·1 mN KCl, 0·5 mN CaCl₂).

The membrane potential and the potential between the Ag/AgCl electrode and the reference electrode were measured with Keithley electrometer millivoltmeters (Keithley Instruments Ltd.). Electrical contact with the KNO₃-filled microelectrodes was made with KCl-in-agar bridges connected to calomel half-cells, thereby eliminating most of the liquid-junction potentials. A schematic diagram of the experimental set-up is shown in Figure 2.

Probes were calibrated by plotting observed potentials against activity of Cl⁻ ions for a number of different standard solutions (usually 1, 10, and 100 mN KCl), the chloride ion activities of which were obtained by applying the Debye–Hückel theory of solutions to the known concentrations. The calibration curve of E against the logarithm of Cl⁻ activity at 18°C was a straight line of slope approximately —58 mV, as predicted by equation (1). Figure 3 shows that the correction to the activity due to the salt effect is considerable even at 100 mN.
Fig. 3.—A typical calibration curve for one of the Ag/AgCl electrodes, using as a reference a KNO₃-filled glass micro-electrode connected via a KCl–agar bridge to a calomel half-cell. The dashed curve represents concentration and the full curve calculated activity, each plotted on a logarithmic scale against potential difference.

Fig. 4.—(a) Vacuole chloride concentration measured in a number of cells using the Ag/AgCl microelectrodes. The external solution was artificial pond water (1·0 mN NaCl, 0·1 mN KCl, 0·5 mN CaCl₂). (b) Cytoplasm chloride activity (concentration) measured with the Ag/AgCl microelectrodes. The distribution has a maximum around 10 mN. Chloride concentration and activity are equal at 10 mN for the standard KCl calibrating solutions. However, in the cytoplasm the total ionic strength is probably much higher than in the calibrating solution, and hence chloride concentration and activity may not be the same. The external solution was artificial pond water.
IV. Results

(a) Measurement of Chloride Concentration in the Vacuole

Thirteen such measurements were made with the Ag/AgCl microelectrodes. All resting levels of [Cl\(^-\)] were of the order of 80-140 mM, when the external solution was 1.0 mM NaCl, 0.1 mM KCl, 0.5 mM CaCl\(_2\). The chloride concentration was also calculated by assuming that it was equal to the sum of the sodium and potassium concentrations. The latter were determined from extracted cell sap using a flame-photometer. The values for [K\(^+\)] + [Na\(^+\)] were in the range 100-150 mM. In general, the flame-photometer analysis and measurements with the Ag/AgCl probes yielded substantially the same values. Figure 4(a) shows a histogram of the vacuolar chloride concentration for a number of cells.

(b) Cytoplasm Chloride Activity

Measurements made showed considerable variation. Figure 4(b) is a histogram of the chloride activity in the cytoplasm for 13 cells. The vacular chloride concentration is shown for comparison in Figure 4(a). The mode of the distribution occurs at 10 mM for Cl\(^-\) activity. This is comparable with the value estimated by Findlay and Hope (1964b).

In all these measurements the Ag/AgCl electrode first recorded the potential corresponding to the vacuole chloride concentration (approx. -55 mV). As the cytoplasm crept up over the electrodes the potential would suddenly drop to about -110
mV, corresponding to a Cl− activity of about 10 mN. To check whether this was not due to a malfunctioning of the electrode, the latter was sometimes pushed in a little further, i.e. back into the vacuole; in every case the potential returned to its original value of about −55 mV, corresponding to a Cl− concentration of about 100 mN for the vacuole. Figure 5 shows the potential, and hence Cl− activity, recorded on the chart recorder, for one of the cells. The activity of the Cl− in the cytoplasm cannot be related to the concentration by using the calibration curve, since the ionic strength of the cytoplasm is much higher than 10 mN. The external solution used in these experiments was 1·0 mN NaCl, 0·1 mN KCl, 0·5 mN CaCl2.

V. Discussion

The results described give the chloride activity in the vacuole and the cytoplasm as approximately 100 and 10 mN, respectively.

The electrochemical equilibrium potentials $E_{Cl}$ for chloride ions across the tonoplast and plasmalemma are respectively given by

$$E_{Cl}^{pl} = -(RT/F)\ln(\frac{[\text{Cl}^-]_{cyt}/[\text{Cl}^-]_{vac}}{1})$$

and

$$E_{Cl}^{pt} = -(RT/F)\ln(\frac{[\text{Cl}^-]_{ext}/[\text{Cl}^-]_{cyt}}{1})$$

The activity of chloride in the external solution is 1·6 mN (at this level, activity = concentration), hence $E_{Cl}^{pl} = 58$ mV and $E_{Cl}^{pt} = 46$ mV.

Since the resting potentials across the tonoplast and plasmalemma are approximately +10 mV and −180 mV, respectively, it is clear that chloride ions are not in electrochemical equilibrium across either membrane.

For the plasmalemma, the difference between $E_{Cl}$ and the membrane potential is approximately 230 mV, directed outwards, while for the tonoplast it is approximately 50 mV, directed from the vacuole to the cytoplasm.

If it is assumed that the concentrations of K+ and Na+ ions in the cytoplasm in Chara australis are of the same order as that in Nitella translucens, i.e. about 140 mN K+ and 40 mN Na+ (MacRobbie 1962), then, since the activity of chloride is only 10 mN, there must be approximately 170 mN of some other anion (or anions) present to maintain electrical neutrality in the cytoplasm. These are assumed to be fixed charges in a Donnan-type system.

Assuming the above approximate concentrations of K+ and Na+, the electrochemical equilibrium potentials for these ions across the tonoplast and the plasmalemma can be calculated. This is shown in Figure 6, which also shows a record of the separate action potentials across both membranes (after Findlay and Hope 1964a).

If the action potential is caused by a transient change in the permeability of the plasmalemma to chloride ions (Gaffey and Mullins 1958; Mullins 1962; Findlay and Hope 1964b), we would expect the potential during the active state to shift towards $E_{Cl}$. From Figure 6 it can be seen that this is the case.

It should be noted that the shift in the membrane potential during the active state causes a large efflux of potassium, since these become further removed from
electrochemical equilibrium. This would account for the fact that the plasmalemma potential never reaches $E_{Cl}$.

When the transient chloride permeability returns to the resting level the potential also returns to that of the resting state, since the latter is dominated by the chemical gradient of potassium ions (Hope and Walker 1961).

VI. REFERENCES


